

**Expression of growth mediators in the gingival crevicular fluid of patients with aggressive periodontitis undergoing periodontal surgery.**

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Concise title

Gingival crevicular fluid in patients with aggressive periodontitis

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**Abstract**

**Objectives:** To describe changes in growth factor mediators in the gingival crevicular fluid (GCF) of patients with aggressive periodontitis (AgP) undergoing regenerative (GTR) and access flap (AF) surgery.

**Materials and Methods:** This was a 12-month, single-blind, split-mouth RCT involving 18 AgP patients with a bilateral intrabony defect which was treated with GTR or AF. GCF was collected prior to surgery and at subsequent follow-up visits from 3 days to 12 months post-operatively and the levels

of Ang-1, VEGF, bFGF, BMP-2, OPG, TIMP-1, KGF and PDGF-AB were measured. At baseline, 6 and 12 months post-surgery periodontal clinical parameters were evaluated. ANOVA was applied to test for differences in the amount of mediators ( $p < 0.05$ ).

**Results:** Higher amounts of BMP-2 and OPG and a higher area under the curve (AUC) of KGF at the GTR versus AF sites were observed. The maximum change in the amount of KGF correlated significantly with periodontal clinical parameters at the GTR sites at 6 and 12 months. The AUC over 30 days of the amount of Ang-1, VEGF and KGF significantly correlated with periodontal clinical parameters at the AF sites at 6 months.

**Conclusions:** AF and GTR differentially affected the profile of the growth mediators in GCF, and significant correlations between certain GCF mediators and periodontal clinical outcomes were identified.

**Clinical Relevance:** GCF components represent attractive prognostic markers for periodontal tissues undergoing repair or regeneration. However, the available evidence is not robust enough to suggest the use of a specific marker and future adequately powered studies are warranted to identify the most relevant mediators that could be applied in clinical practice.

**Keywords:** gingival crevicular fluid, aggressive periodontitis, randomised controlled trial, periodontal surgery

## Introduction

Periodontal therapy aims to restore healthy and normal periodontal functions. It consists of an initial non-surgical treatment that, apart from establishing an effective oral hygiene programme, focuses on the removal of the root surface deposits causing inflammation, and on controlling bacterial infection [1]. When the initial phase of periodontal therapy does not resolve residual periodontal pockets, a surgical treatment is often recommended. Although several types of periodontal surgery have been described, the surgical approaches can be categorised into two main groups according to the healing processes, which are repair and regeneration.

With a periodontal access flap (AF) surgery, a flap is raised to provide access for root debridement and promote healing mainly through the down growth of the gingival epithelium (repair with long junctional epithelium) [2]. Alternatively, guided tissue regeneration (GTR) aims to restore the original architecture of the lost periodontal tissue through a *restitutio ad integrum* (regeneration). Both techniques have shown positive results in terms of improvements of clinical and radiographic parameters [3] and histological outcomes [4, 5].

Different growth factors and mediators regulate the cascade of events taking place during periodontal wound healing, although the exact sequence of events and mediators involved in this complex biological process is still not completely understood [6].

Gingival crevicular fluid (GCF) is the fluid that is present in the space between the gingivae and the tooth surface. It has been suggested that GCF is an osmotic capillary transudate, or an inflammatory exudate in the presence of tissue inflammation [7]. GCF contains a wide range of biochemical components arising from serum, leukocytes, structural cells of the periodontium and oral bacteria [8], including antibodies, cytokines, enzymes and tissue degradation products [9, 10]. Therefore, the molecular components of GCF have been considered to be of potential diagnostic or prognostic value as markers of changes in periodontal disease [11, 12].

Amongst the different mediators that have been suggested to have a potential role in the resolution/progression of periodontitis are mediators associated with angiogenesis, bone formation, differentiation of osteoblast precursors, whose concentration in GCF has shown to change after different types of periodontal treatments (such as VEGF, FGF, BMP-2, OPG, TIMP-1 and PDGF-AB) [15-21]. However, the available studies are quite heterogeneous in terms of mediators analysed, technique used to quantify the marker, population selection and periodontal treatment considered. Hence, there is the need for future studies to better clarify the role of different GCF mediators as diagnostic and/or prognostic tools for different forms of periodontitis. Moreover, it would be important to identify if tissue regeneration and tissue repair are associated with a different expression pattern of growth factors and mediators, which could then potentially be used in the future to predict for the healing processes.

The aim of the present study was to identify and measure the levels of 8 key wound healing mediators (Ang-1, VEGF, bFGF, BMP-2, OPG, TIMP-1, KGF and PDGF-AB) in the GCF of patients with

aggressive periodontitis undergoing AF and GTR and to correlate periodontal clinical parameters with the underlying molecular changes in this fluid.

## **Materials and Methods**

### *Study design and subject population*

This was a 12-month, single-blind, split-mouth randomised controlled clinical trial (RCT) involving 18 patients. The study was approved by the National Hospital for Neurology and Neuro-surgery/Institute of Neurology and the Eastman Joint Research Ethics Committee, London, UK (study reference: 04/Q0512/93). The CONSORT 2010 checklist for reporting a RCT was followed. The study population was recruited among patients with AgP attending the new patient clinic at the Periodontology Department of the Eastman Dental Hospital. The subjects were confirmed with the diagnosis of AgP according to the periodontal disease classification of the International Classification Workshop 1999 [22], based on several 'common criteria' including high occurrence in young adults, rapid periodontal attachment loss and bone destruction, familial aggregation and the patient being otherwise clinically healthy. Another key clinical feature was a level of plaque deposit which was not consistent with the severity of periodontal tissue destruction.

Each subject also had to meet the following inclusion criteria:

- medically healthy,
- minimum age of 15 years old and over,
- presence of similar (in terms of number of walls and depth) bilateral vertical intrabony defects,
- the periodontal defects exhibited a probing pocket depth (PPD) of at least 5 mm, with radiographic evidence of alveolar bone loss of at least 3 mm. Any tooth (single and multi-rooted) was considered, with the exception of third molars and distal to second molars

Subjects were excluded from the study for the following reasons:

- diagnosed with other forms of periodontal disease, such as CP,
- oral hygiene was not improved after the initial therapy,
- furcation involvement of the teeth considered for the intrabony defect
- pregnant or lactating,
- presenting with any chronic illness,
- contraindication to the surgical treatment,
- smoking > 10 cigarettes/day.

The potential participants were allocated for initial treatment with a staff hygienist, who performed scaling and root debridement and gave oral hygiene instructions. After 6 weeks, the subjects were re-examined and, at this stage, patients with residual periodontal pockets that fulfilled the criteria of the

study were recruited after signing an informed consent form. The participants were then scheduled for a total of 10 visits over a 12-month period, which included the 'baseline' visit, the surgical-intervention visit and eight post-surgery follow-up appointments (day 3-5, day 7 $\pm$ 1, day 14 $\pm$ 1, day 28 $\pm$ 3, day 42 $\pm$ 3, day 84 $\pm$ 3, 6 months  $\pm$ 7 days and 12 months  $\pm$  7 days). Clinical parameters (PPD; clinical attachment level, CAL) were recorded at baseline, 6- and 12- months follow-up by one blinded investigator that previously completed a calibration exercise in 5 patients, where measurements were repeated twice after one week of interval. Standardised radiographs were taken at baseline and at 6- and 12- months post-surgery, as previously described [23, 24].

### *Sample size*

Sample size estimation was based on the results of a study comparing changes in CAL between conventional access flap surgery (AF) and non-surgical treatment in Juvenile Periodontitis (JP) [25]. The standard deviation (SD) of the differences between both treatments were calculated and then used for estimation of the sample size in the present study. A sample size of 16 subjects was found to be necessary in order to have an 80% power to detect a difference in means of 0.75 mm of probing measurement (CAL), assuming a SD of CAL differences between treatments of 0.838, using a paired t-test with a 0.050 two-sided significance level. Two additional patients (a total of 18 patients) were enrolled to account for possible drop-outs.

### *Randomisation and allocation concealment*

The sites receiving AF or GTR were randomly allocated using a balanced random permuted block approach (4-unit block size). Allocation to treatment intervention (AF or GTR) was concealed in an opaque envelope and revealed to the surgeon only on the day of the treatment, after flap elevation and debridement of the defect.

### *Surgical procedures*

The details of the surgical procedure and pre/post-operative regime have been previously described [24]. Briefly, at both sides of the mouth, a simplified papilla preservation flap (SPPF) was performed according to Cortellini et al. [26]. After both defects were completely debrided and treated to the same standard, the envelope was opened and the treatment intervention was then assigned as either repositioning and suturing back the flap without (AF) or with the placement of a resorbable membrane (GTR/test) (RESOLUT XT®, WL Gore & Associates Ltd., Flagstaff, Arizona, USA). The surgical procedures were performed by the same experienced surgeon.

At the GTR site, the intrabony defect was covered by the membrane overlapping the margins of the defect by 2–3 mm. The membrane was adapted and stabilised to the root surface by a sling suture (GORE-TEX® suture, WL Gore & Associates Ltd., Flagstaff, Arizona, USA) around the root trunk.

The periosteum was then released at the base of the buccal flap to allow a tension-free coronally repositioning of the flap.

Following surgery, the subjects were instructed to rinse with 0.2% chlorhexidine (Corsodyl®, GSK, Brentford, UK) twice daily and avoid brushing or flossing at the surgical areas for a period of 6 weeks. The sutures were removed 2 weeks post-surgery. Professional tooth cleaning consisting of supragingival prophylaxis with a rubber cup and 1% chlorhexidine gel were scheduled at 3, 7, 14, 28 and 42 days post-surgery. All patients were maintained in periodontal supportive therapy and received professional prophylaxis and calculus removal at 3, 6 and 12 months.

#### *Periodontal clinical measurements*

Details on the pre- and post-surgery clinical measurements carried out by a blind, calibrated examiner have been reported somewhere else [24]. Briefly, PPD and CAL were recorded at pre-surgical baseline, 6 and 12 months post-surgery with a UNC-15 periodontal probe in 6 points per tooth. Full-mouth plaque scores (FMPS) were recorded at baseline prior to surgery at 1, 1½, 3, 6 and 12 months postoperatively, while full-mouth bleeding scores (FMBS) were recorded at the baseline, 6 and 12 months post-operatively.

#### *GCF sample collection*

GCF samples were collected immediately prior to surgery (visit 2) and at all follow-up visits (day 3-5, day 7±1, day 14±1, day 28±3, day 42±3, day 84±3, 6 months ±7 days and 12 months ± 7 days).

For each subject, three areas of sampling were identified: AF, GTR and 3 healthy control sites (HC). The AF and GTR areas included two GCF collection sites, located at the buccal and lingual sides closest to the intrabony defects (e.g. in an intrabony defect distal to 45, the collection points would be disto-lingual and disto-vestibular to 45). Unaffected adjacent sites were selected for GCF sampling in the control group, representing a normal healthy periodontium. This control group comprised 4 GCF sites per subject, 2 buccal and 2 lingual, which were at least one complete unit away from the AF and the GTR treated sites but that were included in the flap elevation.

The selected sites were isolated with cotton rolls, the saliva was removed using a fine-bore high-power suction tip and supragingival plaque, if present, was removed using a curette [27]. Care was taken to avoid any mechanical injury to the gingival tissues. A pre-cut paper strip (2 x 10 mm) (Whatman Lab. Sales Ltd.; Maidstone, UK) [28] was carefully placed at the entrance of the crevice and left in position for 2 minutes to collect GCF, as previously described [27]. The weight of the fluid was converted to volume by assuming that the density of the GCF was 1.0 µg, which is equivalent to 1.0 µl. [29]. The volume of GCF collected was measured, the strips within each group (AF, GTR and HC sites) were pooled, transferred to plastic micro-centrifuge tubes (0.4 ml; Alpha laboratories, Hampshire, UK) and stored at -70 °C until elution was performed as previously described technique [28].

### *Multiplex beads assay (MBA) using 8 CX beads*

A multiplex bead assay protocol using carboxylated-modified-surface fluorescent beads (CX beads) (7.6  $\mu\text{m}$ ; excitation 488 nm, emission 653 nm) together with the Tyramide Signal Amplification (TSA) procedure was previously developed and validated to simultaneously measure 8 growth mediators (angiopoietin-1, Ang-1; vascular-endothelial growth-factor, VEGF; bone morphogenetic protein-2, BMP-2; osteoprotegerin, OPG; tissue-inhibitor of matrix metalloproteinase-1, TIMP-1; basic-fibroblast growth-factor, bFGF; keratinocyte growth-factor, KGF; and platelet-derived growth-factor-AB, PDGF-AB) in GCF samples [30]. Briefly, the MBA is a bead-based immunoassay which utilises the principal of the sandwich ELISA, in which polystyrene beads are used as a solid support for the capture of specific antigen (Ag). The level of the Ag is then detected by a second antibody (Ab), which is tagged with the 'reporter' fluorescence and analysed by single-laser flow cytometry (FCM).

A 'capture' mouse monoclonal antibody (mAb) specific for each of the 8 mediators (Ang-1, VEGF, BMP-2, OPG, TIMP-1, bFGF, KGF and PDGF-AB) was chemically coupled to the CX beads using a two-step protocol [31] and the amount of Ab binding was determined by incubating for 1 hour with goat anti-mouse antibody conjugated with Alexa488 Ab. The beads were then washed and analysed by FCM. In preliminary experiments (data not shown), it was found that 100  $\mu\text{g}$  of capture mAb yielded the highest levels of Ab-bead binding for the VEGF, BMP-2, TIMP-1 and KGF mAbs, whereas 50  $\mu\text{g}$  of capture Ab was found to give optimal conjugation of the Ang-1, OPG, bFGF and PDGF mAbs. Using these concentrations, further incubation with goat anti-mouse Alexa488 Ab resulted in median fluorescence intensity (MFI) values for Ang-1, VEGF, BMP-2, OPG, TIMP-1, bFGF, KGF, and PDGF-AB of 4698, 2267, 6321, 5328, 4179, 4826, 7915 and 3978, respectively.

The separately-conjugated beads were next mixed together, and the MBA was carried out using increasing concentrations of a mixture of the corresponding 8 recombinant human (rh) proteins as standards, as previously described [30]. The absolute amount of the analytes (ng) was determined using the standard curve obtained for each antigen, carried out at the same time. A 4-parameter logistic model of the concentration-response relationship was used for fitting the curve. Because each GCF was eluted in a total volume of 0.1 mL, the absolute amount was calculated by dividing the outcome from the standard curve (ng/L) by  $10^{-4}$  and expressed as ng/site.

### *Statistical analysis*

Statistical analyses were carried out using SPSS data analysis software (Ver.14.0; SPSS Inc.). A p-value  $< 0.05$  was considered statistically significant.

To summarise GCF data obtained from GTR and AF surgery, medians with 25<sup>th</sup> and 75<sup>th</sup> percentiles were used, as the distribution of the data was skewed. The data were analysed by a repeated measures analysis of variance (ANOVA) using the log values of the variable followed by Bonferroni's post hoc comparisons at post-surgery compared with the pre-surgical baseline values. The log values of the

variable were used in order for ANOVA assumptions (normality of the residuals and constant variance) to be satisfied.

The total availability of the growth mediators in GCF was calculated by the area under the curve (AUC) of the total amount of the growth mediators using Stata software (version 10.0, Stata corp., Texas, USA). The AUC analysis estimates the total availability of the mediator amount over the course of the study rather than at specific time points [32]. The AUC analysis was calculated at 2 different time points: baseline to 30 days and baseline to 180 days. The first 30 days corresponded to the time frame in which initial or soft-tissue healing was complete, whereas the 180 days corresponded to the period of bone repair and maturation [32]. The Wilcoxon Sign Rank Test was applied to test differences in AUC for the different growth mediators in the three groups.

The relationship between growth mediator levels in the GCF and changes in the clinical outcomes (CAL gain and PPD reduction) following AF and GTR surgeries at 6 and 12 months was determined using Spearman's coefficient for rank data. For this analysis, the clinical changes (CAL gain and PPD reduction) at the buccal and lingual sites of the intrabony defect were averaged for each subject in each group (AF and GTR) at 6 and 12 months.

## **Results**

Eighteen subjects were enrolled, with sixteen completing the full requirements of the study. For 2 of the subjects it was not possible to obtain GCF samples at certain time points.

For each subject, 2 GCF samples for each GTR site and AF site and 4 from healthy unaffected sites (GCF control) were pooled according to the GCF group category.

Data on early wound healing observations, complications, clinical and radiographic outcomes have already been published [23, 24]. Briefly, compared to the baseline levels, the clinical outcomes at 6 and 12 months showed that both therapies resulted in a significant gain in CAL and a decrease in PPD following surgery, although no significant differences between the treatments could be demonstrated.

### *Changes in GCF volume*

Based on the total of 403 GCF samples collected from the test and control sites (134 GCF samples from the GTR and 134 from the AF sites, and 135 GCF from the healthy control sites), the results in Figure 1 show that the average GCF volume of all of the three groups increased significantly 3-5 days after surgery ( $p < 0.05$ ), and this level was maintained at  $7 \pm 1$  days. However, by  $14 \pm 1$  days and throughout the remaining sampling period of  $180 \pm 7$  days, the GCF volumes at the AF and healthy control sites decreased to levels that were not significantly different from their respective baseline volumes. Conversely, the average GCF volume at the GTR sites remained significantly elevated during the first  $42 \pm 3$  days ( $p < 0.05$ ), and then gradually declined to the same volume as baseline.

### *Growth mediators in GCF*



Each of the 403 GCF samples was eluted from paper strips and the total amounts of 8 growth mediators (Ang-1, VEGF, BMP-2, OPG, TIMP-1, bFGF, KGF and PDGF-AB) was obtained using the validated MBA. A calibration curve using the rh proteins as standards was carried out at the same time the GCF samples were analysed, and the total amounts of the analytes (in pg) were calculated from the median fluorescent intensity (MFI) units using these standard curves. For the purpose of statistical analysis, the limit of detection (LOD) values of each of the mediators obtained from the MBA was used instead of a value of zero when the mediator could not be detected in the GCF. VEGF and TIMP-1 could be detected in all of the GCF samples and Ang-1, OPG, and PDGF-AB could be detected in 94%, 99% and 92%, of the samples, respectively. BMP-2, bFGF and KGF could be detected in the GCF of only 87%, 88% and 73% of the samples, respectively.

- ***Angiopoietin-1 (Ang-1)***

At the GTR sites, the average amount (pg) of Ang-1 increased significantly at 3-5 days and peaked at  $7\pm 1$  days post-surgery ( $p < 0.05$ ), but thereafter declined to levels that were not significantly different from the baseline values. At the AF sites and the healthy control sites, the average amount of Ang-1 was also found to be maximum at  $7\pm 1$  days post-surgery but thereafter declined to levels that were similar to the baseline values over the remainder of the study (Table 1).

ANOVA showed that the GTR sites contained significantly greater amounts of Ang-1 than those of the healthy control sites ( $p < 0.05$ ). There were no significant differences either between the GTR and AF sites or between the AF and healthy control sites.

- ***Vascular-endothelial growth factor (VEGF)***

The average amount (pg) of VEGF at the GTR and AF sites nearly doubled at 3-5 days post-surgery and remained at relatively high levels until  $28\pm 3$  days post-surgery, thereafter declining to the same levels as the baseline values. The average amount of VEGF at the healthy control sites peaked at day  $7\pm 1$ , but thereafter declined to levels that were lower than the baseline values. However, the changes in the amounts of VEGF post-surgery within these 3 GCF groups were not statistically significant (Table 1).

ANOVA showed that the healthy control sites expressed significantly lower amounts of VEGF than the GTR and AF sites ( $p < 0.05$ ). No significant difference was observed between the GTR and AF sites.

- ***Bone morphogenetic protein-2 (BMP-2)***

The results in Table 1 show that at the GTR sites the average amount of BMP-2 progressively increased from baseline to 3-5 days post-surgery and reached a statistical significant elevation at  $7\pm 1$  days post-surgery ( $p < 0.05$ ). BMP-2 then declined to levels that were not significantly different from the baseline

values. Similar changes were observed at the AF and control sites, although these did not reach a statistically significant level (Table 1).

Following ANOVA, overall comparison between the groups showed that the GTR sites contained significantly greater amounts of BMP-2 than the healthy control sites ( $p < 0.05$ ). There were no significant differences either between the GTR and the AF sites or between the AF and the healthy control sites.

- ***Osteoprotegerin (OPG)***

There was a significant increase ( $p < 0.05$ ) (approximately 2-fold) in the amounts of OPG at 3-5 days post-surgery only at the GTR sites, which thereafter declined (Table 1). At the AF and healthy control sites, no statistically significant increase was observed 3-5 days post-surgery, and thereafter BMP-2 expression gradually declined to levels that were similar to baseline.

ANOVA showed that the GTR and AF sites contained similar amounts of OPG, and that the amounts of OPG in these two sites were significantly greater than those in the healthy control sites ( $p < 0.05$ ).

- ***Tissue inhibitor of matrix metalloprotease-1 (TIMP-1)***

At 3-5 days post-surgery, in all groups the average amounts of TIMP-1 were found to significantly increase ( $p < 0.05$ ) compared with the baseline values. However, at  $7 \pm 1$  days post-surgery, the amount of TIMP-1 at the GTR sites declined to levels that were not significantly different from the baseline, whereas the amount of TIMP-1 in the AF and the control sites remained significantly higher than baseline ( $p < 0.05$ ) and declined starting from day  $14 \pm 1$  (Table 1).

ANOVA showed that although the amounts of TIMP-1 in the GTR sites were not significantly different from those in the control and the AF sites, the AF sites expressed significantly greater levels of TIMP-1 than the control sites ( $p < 0.05$ ).

- ***Basic fibroblast growth factor (bFGF)***

Although not statistically significant, a trend towards an increase in the amount (pg) of bFGF was observed in the GTR and AF sites at day 3-5 post-surgery, which peaked at day  $7 \pm 1$ . Thereafter the amounts of bFGF in both the GTR and AF groups decreased to the baseline levels (Table 1). The healthy control sites showed only very small changes in the amounts of bFGF throughout the study (Table 1).

ANOVA revealed no significant differences between any of the 3 GCF sites

- ***Keratinocyte growth factor (KGF)***

In all groups, there was an increase (approximately 2-3 fold) in the amount (pg) of KGF over the first  $7 \pm 1$  days post-surgery. This change was not statistically significant and the levels of KGF decreased thereafter decreased to levels that were similar to the baseline values (Table 1).

As observed for bFGF, the overall comparison between the groups performed with ANOVA showed no significant differences between any of the 3 GCF sites in terms of KGF levels.

- ***Platelet derived growth factor-AB (PDGF-AB)***

In the GTR and AF sites, the average amount (pg) of PDGF-AB was found to peak at day 7±1 post-surgery and to remain at a relatively high level until 42±3 days, thereafter gradually decreasing to the same levels as the baseline values (Table 1). At the healthy control sites, the average amount of PDGF-AB was found to increase (approximately 2-fold) at 3-5 days and 7±1 days post-surgery, but thereafter declined to levels that were similar to the baseline (Table 1). However, the changes in the amounts of PDGF-AB were not statistically significant for any of the groups.

Moreover, ANOVA showed that there were no significant differences between the 3 GCF groups.

In summary, the comparison between the three groups showed that there were no significant differences between the GTR and the AF sites for any of the mediators. However, the GTR sites expressed significantly higher amounts of Ang-1, VEGF, BMP-2 and OPG compared with the control sites, and the AF sites expressed significantly higher amounts of VEGF, OPG and TIMP-1 compared with the control sites.

*Total availability of the amount of the growth factor*

Over the first 30 days, in the GTR group the AUC of the amount of Ang-1, BMP-2 and OPG was significantly higher than in the control group ( $p < 0.05$ ), but not significantly different from the AF group (Table 2). The AUC of the amount of OPG was also significantly higher in the AF compared with the control group. Only the AUC of the amount of KGF showed a significantly higher value in the GTR compared with the AF group (Table 2).

Over the first 30 days the AUC of the amount of VEGF, TIMP-1, bFGF and PDGF-AB was similar in all the groups (Table 2). However, over 180 days the AUC of the amount of VEGF and OPG showed significantly higher values at both the GTR and AF sites compared with the control sites ( $p < 0.05$ ) (Table 2). Notably, none of the growth mediators showed differences in terms of AUC from baseline to 180 days between the GTR and AF groups.

*Correlation between biochemical changes in GCF and clinical outcomes*

The results in Table 3 show significant correlation coefficients ( $r$ ) between the growth mediators and the clinical outcomes (CAL gain,  $\Delta$ CAL and PPD reduction,  $\Delta$ PPD) at 6 and 12 months post-surgery. The maximum change in the amount of KGF correlated significantly with both the  $\Delta$ CAL and the  $\Delta$ PPD at the GTR sites at 6 months ( $p < 0.05$ ;  $r = 0.506$  and  $r = 0.574$ , respectively) (Table 3). A significant correlation was also obtained for KGF and the changes in  $\Delta$ CAL and  $\Delta$ PPD at 12 months ( $p < 0.05$ ;  $r$

= 0.038 and  $r = 0.028$ , respectively) (Table 3). In contrast, there was no significant correlation at the AF sites for KGF or any other mediators at either 6 nor 12 months (Table 3).

The AUC over 30 days of the amount of Ang-1 significantly correlated with both the  $\Delta$ CAL and  $\Delta$ PPD at the AF sites at 6 months ( $r = 0.570$  and  $r = 0.573$ , respectively), but not at 12 months (Table 3). A positive correlation was also observed in the AF sites for the AUC of the amount of VEGF and  $\Delta$ CAL at 6 as well as at 12 months. In addition, at the AF sites there was a significant correlation between the AUC of the amount of KGF over 30 days and the  $\Delta$ PPD at 6 months ( $r = 0.560$ ). No significant correlation was found between the AUC of the total amount of any mediators over 30 days and the clinical parameters in the GTR sites.

A negative correlation between the AUC over 180 days of the amount of BMP-2 ( $r = -0.521$ ), bFGF ( $r = -0.515$ ) and KGF ( $r = -0.585$ ) and the  $\Delta$ PPD at 6 months post-surgery was observed at the AF sites (Table 3). There was no significant correlation for any other growth mediators.

During the course of this study it was observed that a few subjects had extremely high or low levels of certain growth mediators in the GCF, at one or more times after surgery. For these subjects, the amounts of each of the growth mediators in GCF, collected over 6 months, were plotted in a box plot diagram using a logarithmic scale to distinguish the values outside a 95% confidence interval (CI) (data not shown). However, no significant association was found between the 'extreme' values of the total amount of the growth mediators and the resulting clinical parameters. Likewise, no significant association was observed between the very high and very low values of the AUC of the amount of the mediators and the resulting clinical parameters (data not shown).

## **Discussion**

Successful restoration of damaged periodontal tissues involves several processes including inflammation, cell migration and differentiation, which will ultimately result in the regeneration of intact functional PDL, cementum, gingival connective tissue and alveolar bone [33]. These complex processes are controlled at least partly by growth mediators and the factors that regulate their expression during wound healing.

GCF can be considered amongst the most non-traumatic investigational methods available for providing information about periodontal tissue, including the status of the connective tissue and the degree of hard tissue destruction [34]. Constituents in GCF have been shown to reflect the ongoing processes surrounding periodontal tissues including, inflammation, connective tissue turnover/breakdown, alveolar bone resorption and periodontal wound healing [29, 35-37]. It has therefore been hypothesised that mediators of the healing process, such as Ang-1, VEGF, BMP-2, OPG, TIMP-1, bFGF, KGF and PDGF-AB are likely to be present in GCF and to play an important part in periodontal repair and regeneration. The present study, for the first time, simultaneously quantified the profile of these mediators in periodontal wound fluids at different time intervals following periodontal surgery.

The baseline GCF volumes of the periodontitis-affected sites of the present study (the GTR and the AF sites) were found to be not significantly different from the GCF volumes obtained from periodontally healthy control sites. In contrast, some previous studies have reported a relationship between an increased volume of GCF and active periodontal disease [37, 38], with an increase in GCF volume considered an important indicator of gingival inflammation and periodontitis [39]. However, it is important to note that in the present study GCF was collected from sites that had already received initial periodontal treatment, possibly accounting for the lack of significant difference observed.

The volume of GCF obtained from sites treated by periodontal surgery increased significantly 3-5 days post-surgery. While the GCF volumes of the AF and healthy control sites decreased to levels that were similar to the baseline after 14 days, the GCF volumes of the GTR sites took 42 days to decrease to levels that were similar to the baseline. The initial increase in GCF volume may reflect the inflammatory stage of periodontal wound healing, as it has been shown that this process is accompanied by enhanced permeability of gingival blood vessels, resulting in an increased amount of fluid passing through the vascular walls into the extravascular space [38]. Our findings suggest that GTR treatment might have caused an additional prolonged initial inflammation of the surrounding periodontal tissues, probably due to the membrane implantation itself and to the possible reservoirs of inflammatory exudate and saliva created in cases of membrane exposure, which occurred in the majority of GTR sites (13 out of 18) during the first 4 post-operative weeks.

In the present study, the early increase (over the first  $7\pm 1$  days post-surgery) in the levels of Ang-1, VEGF, bFGF and PDGF-AB suggests that these factors are likely to contribute to regulate and promote angiogenesis and vascularisation during the initial stage of periodontal healing. Ang-1 and its receptor (Tie-2) are known to play a key part in the formation and stabilisation of new blood vessels [40], whilst a number of studies have demonstrated the role of VEGF as a potent inducer of vascular permeability and angiogenesis in oral biology, including its association with periodontitis [41-47], periapical lesions [48] and gingival overgrowth [49].

Our data shows that the absolute amount of bFGF also increased during the first  $7\pm 1$  days after surgery. Previous studies on surgical skin wounds [50-52] showed that bFGF peaked within hours of injury and suggested that angiogenesis was likely to have been initiated by bFGF and progressed by VEGF. Since the earliest collection point in the present study was 3 days post-surgery, it is possible that the highest levels of bFGF might have been expressed even earlier than 3 days. Although overall only very low amounts of PDGF-AB were found to be present in the GCF, a previous study suggested that even a small amount of PDGF-AB is sufficient to provide paracrine regulation between endothelial cells (that secrete this factor) and the PDGF receptors (which is expressed in cells forming the blood vessel wall, i.e. pericytes and vascular smooth muscle cells) [53].

Notably, the GTR sites expressed significantly greater amounts of BMP-2 than the healthy control sites. Over the first 30 days, the AUC of the amount of this mediator at GTR sites was significantly greater than at the healthy control sites, suggesting that BMP-2 may be actively involved in periodontal

regeneration. The amount of another important mediator involved in bone deposition and remodelling, OPG, also significantly increased in the GTR (and AF) sites compared to the healthy control sites. The high levels of TIMP-1 identified in the GCF of all groups strongly suggests that this mediator has a fundamental role during the healing of the periodontium, in agreement with other studies that showed the importance of matrix metallo-proteinases (MMPs) and their inhibitors (TIMPs) in collagenous and non-collagenous extracellular matrix (ECM) accumulation and remodelling [54]. Finally, the total availability of the amount of KGF at the GTR sites within the first 30 days was found to be significantly higher than at the AF sites, thus suggesting that KGF may be another key player during early periodontal wound healing, possibly via facilitating the proliferation of gingival epithelial cells.

A primary objective of the present study was to determine whether there is an association between changes in GCF growth mediators and periodontal clinical parameters, which might possibly be predictive of ultimate clinical outcomes and thus the success of therapeutic intervention. Our data show that the total amount of KGF in the GTR-treated sites was significantly correlated with the clinical outcomes ( $\Delta$ CAL and  $\Delta$ PPD for KGF and  $\Delta$ CAL for PDGF-AB) at 6 and 12 months post-surgery (Table 3). Hence, measuring KGF in GCF might be a valuable prognostic indicator of the clinical value of GTR therapy and would deserve further investigation.

Moreover, the total availability (AUC) of the amount of Ang-1 and VEGF over the first 30 days post-surgery was found to be correlated with the clinical changes post AF surgery, and therefore might be tested in future studies as reliable indicators of the success of AF periodontal treatment.

Unexpectedly the study showed a negative correlation between the total availability of BMP-2 and the post-surgery clinical parameters (AF and GTR). Although the reasons for these negative correlations are not clear, the finding indicates that the presence of BMP-2 at a consistently high level in the periodontal environment over a prolonged period of time may be detrimental to the final wound healing outcome. Despite the well-known osteoinductive activity of BMP-2 [55], this mediator has previously been reported to be either anti-mitogenic [56-58] or mitogenic [59, 60], depending on the dose. In addition, a high concentration of rhBMP-2 (100 ng/ml) has been reported to inhibit collagen accumulation and calcium deposition in cultured osteoblasts *in vitro* [61].

Likewise, a negative correlation between the total availability of bFGF and KGF and the clinical parameters post AF surgery was observed, again possibly due to dose-dependent effects on the healing process of both hard and soft tissues.

The clinical relevance of measuring OPG in the GCF as a prognostic indicator of periodontal clinical outcomes remains unclear, as no significant association between the biochemical parameters of OPG and any of the changes in clinical outcome was detected during the present study.

A potential source of bias of this study was the relatively high exposure rate of the membranes placed at the GTR treated sites (13 out of 18), which is known to increase the risk of bacterial contamination

and negatively affect clinical outcomes [62, 63], resulting in a reparative rather than regenerative process. This might have masked more significant differences between the two test groups and between the GTR and healthy control group in terms of growth mediator levels and of correlation with the periodontal clinical parameters. It may also be relevant to highlight that the sample size was based on the possibility of detecting a significant difference in the periodontal clinical outcomes, which might have not been adequate for detecting a significant difference between the GCF groups in the level of the growth mediators at each time point post-surgery. For the same reason, it is possible that the lack of significance between the extremely high or low levels of any of the growth mediators in GCF and the clinical parameters could be ascribed to the sample size.

In summary, this study establishes the potential of GCF as non-invasive and potentially valuable tool for prognostic use after periodontal surgical treatment. However, future adequately powered studies are warranted to better identify the most important mediators involved in effective clinical therapy.

#### **Compliance with ethical standards**

**Conflict of interest:** Dr Rakmanee, Dr Calciolari, Professor Olsen, Dr Darbar, Professor Griffiths, Dr Petrie and Professor Donos declare they do not have any conflict of interest related to this study

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**Ethical approval:** All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent:** Informed consent was obtained from all individual participants included in the study.

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### Figure legend

**Figure 1** The box plot shows the distribution of the GCF data, and the bold horizontal lines indicate the median levels of GCF volume ( $\mu\text{l}$ ) obtained from the GTR sites (■), AF sites (■) and healthy control sites (□). The vertical lines correspond to the interquartile range of the data. The data are the pooled results of all the subjects in each group. \* Shows statistically significant differences ( $p < 0.05$ ) compared with the baseline volume within the same group. § Shows statistically significant differences ( $p < 0.05$ ) between the GCF groups at each time point.

### Table legend

**Table 1** Total amounts (pg) of growth mediators measured in GCF at different time points. The values shown are the medians of the total amounts (pg) and the 25<sup>th</sup> and 75<sup>th</sup> percentiles of the raw data (in brackets). For TIMP-1, the total amounts were measured as  $\text{pg} \times 10^3$ . \* Shows statistically significant differences ( $p < 0.05$ ) between the values at the pre-surgical baseline and at time intervals post-surgery within the same group, using Bonferroni's analysis.

### Table 2

The table shows the total availability of the absolute amount of the mediators expressed as  $(\text{pg})^2\text{days}$ . The values are presented as medians of the area under the curve (AUC)  $\times 10^3$  ( $(\text{pg})^2\text{days}$ ) and the 25<sup>th</sup> and 75<sup>th</sup> percentiles of the raw data (in brackets). AUC analysis of total amount of the mediators was calculated from baseline (Day 0) to 30 days and baseline to 180 days post-surgery. § Indicates significant differences ( $p < 0.05$ ) between the affected sites (either GTR or AF) and the GCF healthy control sites. \* Indicates significant differences ( $p < 0.05$ ) between the GTR and the AF group.

### Table 3

The table summarises the significant correlations ( $p < 0.05$ ) observed between the growth mediators and the clinical parameters ( $\Delta$  CAL and  $\Delta$  PPD) at 6 and 12 months. The correlation coefficients ( $r$ ) are reported, together with the  $p$  values (in brackets).

Group	Growth mediator	Time of GCF collection (days)							
		0	3-5	7±1	14±1	28±3	42±3	84±3	180±7
		Amount of growth mediators (Median) [25 <sup>th</sup> - 75 <sup>th</sup> percentile]							
GTR	Ang-1	76.7 [48.8,127.5]	<b>356.3*</b> [132.9, 651.2]	<b>403.2*</b> [183.3, 902.9]	132.1 [89.5, 327.8]	204.3 [139.3, 357.3]	200.1 [89.85, 389.95]	108 [54.3, 278.8]	110.6 [63.5, 175.6]
	VEGF	118.1 [88.5, 201.4]	296.6 [129.6, 348.8]	187.3 [128.5, 376.1]	142.1 [94.2, 199.0]	162.9 [119.8, 273.0]	113.9 [74.7, 340.7]	128.3 [66.8, 252.8]	91.8 [64.4, 244.6]
	BMP-2	36.5 [24.1, 63.7]	104.4 [55.9, 135.5]	<b>187.3*</b> [73.3, 254.2]	142.1 [33.0, 119.2]	162.9 [65.1, 138.3]	113.9 [28.7, 140.4]	128.3 [31.3, 169.9]	91.8 [31.0, 101.2]
	OPG	28.8 [22.2, 50.8]	<b>78.7*</b> [55.8, 133.5]	65.9 [37.5, 87.7]	38.6 [25.7, 62.1]	52.2 [39.4, 124.2]	49.9 [22.4, 80.2]	42.4 [26.7, 79.2]	47.4 [22.6, 75.7]
	TIMP-1	3.4 [1.9, 9.2]	<b>47.1*</b> [18.8, 109.8]	28.3 [8.5, 107.8]	22.6 [5.2, 51.0]	15.4 [7.3, 51.1]	8.2 [4.7, 38.5]	5.2 [2.2, 15.6]	6.0 [2.4, 16.6]
	bFGF	157.3 [58.3, 224.4]	356.5 [228.4, 526.0]	375.6 [264.7, 892.9]	292.1 [139.3, 457.7]	268.2 [213.9, 561.0]	225.5 [79.7, 550.8]	191.4 [71.0, 653.2]	184.4 [77.7, 265.4]
	KGF	53.7 [12.1, 96.4]	158.1 [11.5, 304.2]	191.7 [47.3, 377.1]	96.9 [34.2, 244.6]	105.4 [54.9, 401.1]	59.5 [28.8, 140.4]	54.6 [13.9, 157.6]	48.4 [9.1, 74.7]
	PDGF-AB	38.5 [22.5, 67.8]	71.9 [37.9, 127.7]	87.2 [59.3, 150.9]	66.3 [30.4, 80.8]	74.8 [48.6, 123.0]	73.5 [27.3, 115.9]	50.2 [22.2, 121.6]	38.2 [17.2, 64.9]
	AF	Ang-1	87.7 [54.0, 126.6]	211.4 [172.4, 512.4]	310.5 [122.0, 364.7]	152.1 [42.9, 261.3]	142.6 [92.6, 200.9]	141.8 [67.3, 276.3]	106.7 [76.8, 177.6]
VEGF		110.3 [67.9, 218.2]	220.3 [120.2, 305.5]	178.8 [137.1, 315.1]	125.7 [77.9, 292.7]	187.4 [102.4, 258.9]	142.2 [100.2, 204.9]	126.4 [90.8, 259.0]	109.4 [77.1, 194.4]
BMP-2		41.8 [24.4, 107.5]	98.2 [59.0, 151.5]	102.5 [50.0, 123.3]	92.3 [21.0, 164.8]	77.4 [41.1, 105.3]	66.9 [35.6, 119.1]	77.9 [27.2, 96.7]	73.2 [18.9, 130.9]
OPG		38.6 [26.4, 38.6]	63.1 [43.1, 101.3]	55.8 [32.1, 67.8]	42.1 [26.3, 88.2]	54.1 [37.0, 78.9]	55.1 [37.2, 107.8]	47.8 [29.8, 65.7]	45.3 [24.8, 82.9]
TIMP-1		4.7 [1.6, 18.2]	<b>55.7*</b> [30.5, 106.5]	<b>51.0*</b> [22.8, 76.8]	37.5 [9.8, 60.9]	13.4 [6.5, 21.4]	12.3 [6.5, 21.4]	9.3 [4.5, 25.4]	8.4 [2.8, 21.3]
bFGF		166.9 [90.9, 255.6]	328.0 [200.4, 605.5]	428.9 [215.0, 516.3]	395.3 [69.9, 565.5]	239.6 [142.4, 410.8]	267.1 [100.8, 458.4]	219.6 [96.5, 280.8]	175.1 [57.8, 421.3]
KGF		48.4	97.9	100.5	51.6	48.5	34.2	43.7	30.6

	PDGF-AB	[9.1, 112.9] 47.4 [29.4, 76.0]	[9.2, 252.5] 73.2 [57.2, 135.2]	[26.4, 196.1] 91.1 [49.6, 114.2]	[9.1, 185.9] 72.5 [23.6, 183.7]	[10.1, 136.4] 63.7 [41.3, 85.9]	[9.1, 110.5] 71.8 [28.5, 112.2]	[9.6, 104.9] 56.5 [27.1, 81.5]	[9.6, 91.7] 41.6 [17.4, 120.9]
Control	Ang-1	76.7 [37.7, 127.5]	171 [119.7, 314.1]	201.1 [121.7, 333.1]	124.1 [70.9, 145.3]	91.6 [72.7, 211.6]	78.1 [60.2, 147.3]	72.8 [48.5, 239.9]	79.0 [28.9, 151.0]
	VEGF	109.5 [68.4, 141.0]	126.7 [87.2, 250.2]	163.9 [100.5, 224.2]	126.3 [57.7, 163.4]	83.2 [60.4, 167.8]	80.1 [59.7, 137.5]	99.5 [66.6, 154.2]	89.7 [51.6, 152.3]
	BMP-2	36.5 [24.1, 63.7]	74.2 [64.7, 110.8]	83.3 [46.6, 158.5]	49.7 [25.8, 77.4]	35.9 [29.8, 83.3]	35.2 [22.7, 83.6]	34.8 [14.3, 73.7]	32.5 [11.3, 85.5]
	OPG	27.8 [21.4, 47.7]	41.8 [33.5, 52.1]	31.7 [25.4, 73.8]	29.4 [18.2, 42.3]	30.8 [21.3, 81.9]	25.4 [21.7, 49.2]	30.8 [21.7, 63.6]	39.9 [17.9, 54.6]
	TIMP-1	2.8 [1.8, 5.9]	<b>51.2*</b> [20.3, 105.4]	<b>50.5*</b> [18.0, 86.4]	17.1 [8.4, 32.6]	13.1 [2.7, 25.4]	3.9 [1.6, 6.2]	3.9 [2.2, 9.4]	5.5 [1.0, 9.0]
	bFGF	117.3 [30.1, 224.4]	143.2 [191.4, 388.8]	195.4 [162.1, 594.1]	165.4 [84.6, 317.1]	146.4 [72.1, 331.9]	171.7 [84.5, 330.9]	154.1 [34.4, 272.8]	139.9 [31.1, 259.0]
	KGF	59.1 [16.9, 67.8]	116.2 [43.9, 84.8]	113.4 [44.0, 116.9]	35.1 [30.9, 87.4]	90.1 [20.8, 73.7]	28.9 [31.5, 84.5]	37.1 [20.1, 97.2]	31.7 [17.1, 68.1]
	PDGF-AB	38.5 [16.9, 67.8]	57.7 [43.9, 84.8]	63.9 [44.0, 116.9]	50.4 [30.9, 87.4]	31.4 [20.8, 73.7]	40.9 [31.5, 84.5]	34.8 [20.1, 97.2]	29.8 [17.1, 68.1]

Table 1

Growth mediators	AUC from baseline to 30 days (pg) <sup>2</sup> days (x 10 <sup>3</sup> )			AUC from baseline to 180 days (pg) <sup>2</sup> days (x 10 <sup>3</sup> )		
	GTR	AF	Control	GTR	AF	Control
<b>Ang-1</b>	<b>8.2 §</b> [5.4, 15.7]	5.7 [2.8, 8.6]	3.9 [3.0, 6.3]	26.9 [19.3, 56.2]	28.1 [13.3, 43.1]	20.8 [9.8, 29.7]
<b>VEGF</b>	5.1 [4.2, 7.6]	4.0 [3.5, 9.4]	4.1 [2.4, 5.8]	<b>24.7 §</b> [16.9, 52.1]	<b>26.9 §</b> [15.5, 54.8]	14.9 [12.6, 32.9]
<b>BMP-2</b>	<b>2.9 §</b> [2.0, 4.4]	2.5 [1.1 4.9]	2.0 [0.9, 3.7]	11.6 [7.6, 27.4]	16.7 [4.3, 24.5]	7.4 [4.1, 18.0]
<b>OPG</b>	<b>1.6 §</b> [1.1, 3.6]	<b>1.7 §</b> [1.0, 2.6]	1.1 [0.6, 2.2]	<b>8.4 §</b> [5.4, 15.5]	<b>12.0 §</b> [4.9, 15.2]	6.9 [3.4, 11.8]
<b>TIMP-1</b>	1061 [583, 1787]	1109 [487, 1778]	846 [450, 1505]	2765 [1362, 4756]	4295 [1695, 4754]	2007 [927, 3959]
<b>bFGF</b>	8.93 [8.0, 13.9]	10.2 [4.9, 14.9]	6.9 [3.0, 13.4]	40.1 [17.7, 81.2]	47.0 [21.6, 69.1]	26.6 [14.2, 65.4]
<b>KGF</b>	<b>4.4 *</b> [2.5, 11.0]	2.8 [0.7, 5.1]	3.2 [1.6, 5.0]	17.9 [6.6, 27.5]	11.1 [2.0, 22.2]	12.3 [4.9, 23.0]
<b>PDGF-AB</b>	2.1 [1.5, 3.2]	2.9 [1.2, 3.4]	1.5 [1.1, 2.5]	11.2 [5.0, 18.8]	13.1 [5.6, 17.7]	8.8 [3.9, 17.0]

Table 2



Biochemical parameters	Mediators	Surgical site	Clinical parameters	Correlation coefficient (p-value) 6 months	Correlation coefficient (p-value) 12 months
Maximum change in the amount of the mediator	KGF	GTR	$\Delta$ CAL	0.506 (0.041)	0.641 (0.038)
			$\Delta$ PPD	0.574 (0.044)	0.547 (0.028)
AUC over 30 days of the amount of the mediator	Ang-1	AF	$\Delta$ CAL	0.570 (0.021)	-
	VEGF	AF	$\Delta$ PPD	0.573 (0.020)	-
			$\Delta$ CAL	0.539 (0.031)	0.517 (0.040)
KGF	AF	$\Delta$ PPD	-	-	
AUC over 180 days of the amount of the mediator	BMP-2	AF	$\Delta$ CAL	-	-
	bFGF	AF	$\Delta$ PPD	-0.521 (0.038)	-
			$\Delta$ CAL	-	-
KGF	AF	$\Delta$ PPD	-0.515 (0.041)	-	
			$\Delta$ CAL	-	-
			$\Delta$ PPD	-0.585 (0.027)	-

Table 3

