Ratio of Pro-Resolving and Pro-Inflammatory Lipid Mediator Precursors as Potential Markers for Aggressive Periodontitis

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Abstract

Aggressive periodontitis (AgP) is a rapidly progressing type of periodontal disease in otherwise healthy individuals which causes destruction of the supporting tissues of the teeth. The disease is initiated by pathogenic bacteria in the dental biofilm, and the severity of inflammation and attachment loss varies with the host response. Recently, there has been an increased interest in determining the role of lipid mediators in inflammatory events and the concept of pro-inflammatory and pro-resolution lipid mediators has been brought into focus also in periodontal disease. The present study aimed to determine the profile of omega-3 or n3- as well as omega-6 or n6- polyunsaturated fatty acids (PUFAs) and PUFA-metabolites of linoleic acid, arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in gingival crevicular fluid (GCF), saliva and serum in AgP patients and healthy controls.

Introduction

Aggressive periodontitis (AgP) is a rapidly progressive form of inflammatory periodontal disease. If not treated, AgP may result in tooth loss even in young people [1]. The initiating factors of periodontal disease are bacteria and bacterial products which form a biofilm covering the tooth surface in the subgingival area. In susceptible individuals, the host-mediated response to the pathogenic flora is subsequently responsible for the periodontal tissue destruction [2,3]. The course of the disease is modified by different environmental factors, and the patient's genetic makeup contributes to patient susceptibility, in particular in the case of aggressive forms of periodontitis [4–6].

Persistence of inflammation or the failure of tissue to return to the normal state favours tissue destruction [7]. Thus, resolution of inflammation is essential to the process of regaining tissue homeostasis after infection, and it has become clear that this is not a passively, but an actively coordinated process involving several biochemical pathways, enzymes and mediators [8,9]. Over recent years, there has been an increased interest in determining the role of lipid mediators in anti-inflammatory events, and the concept of pro-inflammatory and pro-resolution lipid mediators has also been brought into focus in periodontal disease pathogenesis [10].

Host factors, including both immune components and resident cells, contribute to tissue destruction via synthesis and release of different pro-inflammatory molecules [11]. The pathogens trigger the cell membrane of the immune cells, leading to the release of free fatty acid, mainly arachidonic acid (AA). Pro-inflammatory lipid mediators such as thromboxanes (TX), prostaglandins (PG) and leukotrienes (LT), as well as anti-inflammatory lipid mediators such as lipoxins (LX), are among the products generated, mainly via AA-metabolism.

Arachidonic acid is metabolized to biologically active eicosanoids through the cyclooxygenase pathway (COX-1 and COX-2),
which results in production of various PG-like PGE$_2$ and PGD$_2$
[12,13]. Other mediators such as LT, particularly LTB$_4$, arise
through the conversion of hydroxyeicosatetraenoic acid (HETE)
by 5-lipoxygenase (5-LOX). The AA-derived prostanoids and LT
are associated with the destruction of collagen and bone resorption
occurring in periodontal disease [14,15]. Lipoxygenases involved
in AA, EPA and DHA metabolism are the 5-LOX, 12-LOX and
15-LOX. The human 12-LOX can be classified into two types, the
platelet type and the epidermis type [16,17] while the human type
can be classified into the reticulocyte type [15-LOX-1] [18],
leukocyte type [19], and the epidermis type [15-LOX-2] [20,21].
Prostaglandins are biological mediators involved in inflammation
and have been implicated as stimulators of bone loss [8]. Levels of
PGE$_2$ are significantly elevated in the gingival crevicular fluid
(GCF) of patients with periodontal disease. High levels of PGE$_2$
are associated with disease aggressiveness and constitute a reliable
indicator of current clinical periodontal destruction [14,15]. High
levels of LTB$_4$ have also been found in periodontal inflamed
tissues and are considered to be an indicator of periodontal
inflammation [14].

Omega-6 (n6) and omega-3 (n3) polyunsaturated fatty acids
(PUFAs) are substrates for the production of various eicosanoids
and docosanoids (Figure 1). The mono-hydroxylated PUFAs
metabolites of AA, 15-HETE and 5-HETE are direct precursors
for LXs, which, in contrast to PGs and LTs, attenuate the
inflammatory effect [22,23]. The major n3-PUFAs are eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which
are the precursors of various novel pro-resolution lipid mediators
like resolvins (Rv), protectins (PD) and maresins (MaR) which are
responsible for the active resolution of inflammation [24–27]. The
PUFAs EPA and DHA are substrates for various classes of Rv of the
E (originating from EPA) and D (originating from DHA) series,
respectively [28]. Interestingly, local application of RvE1, which is
one of the bioactive pro-resolution mediators, reduced inflamma-
tion and promoted regeneration of pathologically lost tissues in a
rabbit model of periodontal disease [29]. In humans, the daily
dietary supplementation of n3-PUFAs in combination with aspirin
was reported to reduce the frequency of deep periodontal pockets
and inflammatory lipid mediators in saliva [30].

Little is known about the presence and the amount of different
bioactive lipid mediators in oral fluids during periodontal
inflammation and its resolution. In particular, a comparison
between their levels locally near the inflamed site (in GCF and
saliva) and systemically (in serum) has, to our knowledge, not been
done. In this report, we analysed eicosanoids and docosanoids in
GCF, saliva and serum in a group of AgP patients and healthy
controls in an attempt to understand better the roles and
relationships of these lipid mediators.

Results

In the present study the levels of various PUFAs and mono-
hydroxylated PUFAs metabolites and pro-resolution and pro-
inflammatory lipid mediators in GCF, saliva and serum samples
from AgP patients and healthy controls were measured by high
performance liquid chromatography – electrospray ionization
Table 1. Concentration of lipid mediators in GCF samples.

| Pathway                        | Mediator         | AgP (n = 16) ng/ml | HC (n = 12) ng/ml | P value |
|--------------------------------|------------------|--------------------|------------------|---------|
| **PUFAs**                      |                  |                    |                  |         |
|                               | AA               | 15.5 ± 15.5        | 2.7 ± 2.3        | 0.001*  |
|                               | EPA              | 0.2 ± 0.2          | 0.1 ± 0.0        | 0.012*  |
|                               | DHA              | 1.0 ± 0.7          | 0.4 ± 0.2        | 0.046*  |
|                               | LA               | 17.2 ± 8.6         | 9.7 ± 4.7        | 0.007*  |
| **5-lipoxygenase pathway**     | 5-HETE          | 0.1 ± 0.1          | 0.1 ± 0.0        | 0.179   |
|                               | LTB4             | 0.1 ± 0.1          | 0.1 ± 0.0        | 0.113   |
|                               | 20-OH-LTB4       | 0.4 ± 0.5          | 0.2 ± 0.2        | 0.690   |
|                               | 20-COOH-LTB4     | 3.1 ± 2.7          | 2.1 ± 4.5        | 0.054   |
|                               | LTC4             | 0.1 ± 0.1          | 0.1 ± 0.1        | 0.780   |
|                               | 5-oxoETE**       | 0.6 ± 0.6          | 0.3 ± 0.3        | 0.055   |
|                               | LXB4             | 0.1 ± 0.1          | 0.1 ± 0.1        | 0.960   |
|                               | LXA4             | 0.2 ± 0.4          | 0.1 ± 0.1        | 0.146   |
|                               | 5-HEPE           | 0.1 ± 0.1          | 0.1 ± 0.0        | 0.120   |
|                               | LTB5             | 0.2 ± 0.2          | 0.1 ± 0.1        | 0.646   |
|                               | LXA5             | 0.2 ± 0.3          | 0.3 ± 0.3        | 0.129   |
|                               | 4-HDHA           | 0.1 ± 0.0          | 0.1 ± 0.0        | 0.120   |
| **8-hydroxylation pathway**    | 8-HETE           | 0.1 ± 0.1          | 0.1 ± 0.0        | 0.037*  |
|                               | 8-HEPE           | 0.1 ± 0.1          | 0.1 ± 0.1        | 0.258   |
|                               | 10-HDHA          | 0.1 ± 0.1          | 0.1 ± 0.1        | 0.356   |
|                               | PD1              | 0.1 ± 0.1          | 0.1 ± 0.0        | 0.903   |
| **12-lipoxygenase pathway**    | 12-HETE          | 1.1 ± 0.9          | 0.6 ± 0.7        | 0.039*  |
|                               | 12-oxoETE**      | 0.8 ± 1.3          | 0.9 ± 0.7        | 0.200   |
|                               | HXA2             | 0.7 ± 0.7          | 0.8 ± 1.1        | 0.815   |
|                               | HXB2             | 0.9 ± 1.1          | 1.0 ± 0.9        | 0.655   |
|                               | 12-HEPE          | 0.1 ± 0.2          | 0.1 ± 0.0        | 0.020*  |
|                               | 14-HDHA          | 0.1 ± 0.0          | 0.1 ± 0.1        | 0.331   |
|                               | RvD1             | 0.3 ± 0.4          | 0.2 ± 0.1        | 0.848   |
|                               | RvD2             | 0.8 ± 0.7          | 0.7 ± 0.4        | 0.422   |
|                               | MaR              | 0.1 ± 0.1          | 0.1 ± 0.1        | 0.533   |
|                               | 9-HODE           | 6.3 ± 6.4          | 2.6 ± 1.6        | 0.029*  |
| **15-lipoxygenase pathway**    | 15-HETE          | 1.5 ± 2.0          | 0.1 ± 0.1        | 0.001*  |
|                               | 15-oxoETE**      | 1.6 ± 1.8          | 0.2 ± 0.2        | 0.050   |
|                               | 15-HEPE          | 0.2 ± 0.2          | 0.1 ± 0.0        | 0.042*  |
|                               | 17-HDHA          | 0.2 ± 0.3          | 0.1 ± 0.0        | 0.010*  |
|                               | 13-HODE          | 14.6 ± 13.0        | 4.9 ± 3.1        | 0.009*  |
|                               | 13-oxoODE**      | 14.4 ± 17.4        | 14.6 ± 9.2       | 0.486   |
| **Cyclooxygenase pathway**     | PGD2             | 0.9 ± 0.8          | 1.3 ± 1.0        | 0.429   |
|                               | PGJ2             | 2.2 ± 1.6          | 0.7 ± 0.5        | 0.008*  |
|                               | d15d12PGD2       | 0.1 ± 0.1          | 0.1 ± 0.2        | 0.823   |
|                               | PGJ2             | 0.1 ± 0.1          | 0.1 ± 0.1        | 0.785   |
|                               | d15d12PGJ2       | 0.1 ± 0.0          | 0.1 ± 0.0        | 0.212   |
|                               | TXB2             | 0.1 ± 0.1          | 0.1 ± 0.1        | 0.674   |
|                               | PGF2             | 0.3 ± 0.3          | 0.2 ± 0.2        | 0.571   |
|                               | PGE2             | 1.8 ± 1.9          | 1.3 ± 1.3        | 0.632   |
| **Alternative pathways**       | 20-HETE          | 0.3 ± 0.2          | 0.2 ± 0.2        | 0.056   |
|                               | 20-COOH-AA       | 0.8 ± 0.7          | 0.3 ± 0.4        | 0.044*  |
|                               | 18-HEPE          | 0.9 ± 1.9          | 1.2 ± 1.7        | 0.118   |
|                               | 20-HDHA          | 0.2 ± 0.2          | 0.1 ± 0.1        | 0.269   |
|                               | 11-HETE          | 0.1 ± 0.0          | 0.1 ± 0.0        | 0.020*  |
combined with tandem mass spectrometry (HPLC-ESI-MS-MS) and enzyme-linked immunoassorbent assay (ELISA). Not all samples could be analysed, and the final number of individuals analysed for each sample type are specified in Tables 1, 2 and 3. In total for each individual, 60 lipids were measured using the HPLC-ESI-MS-MS method and 51 could be quantified in the biological liquids. The concentrations of these lipids were lower in saliva samples (Table 2) compared with both serum (Table 3) and GCF samples (Table 1). Levels of these lipids were increased in most biological samples of AgP patients compared with healthy controls, although not all results were statistically significant.

GCF analysis

Both n6-PUFAs (linoleic acid (LA) and AA) and n3-PUFAs (EPA and DHA) were detected at significantly higher concentrations in the GCF of AgP patients than in healthy controls (Table 1). Furthermore, the 12-LOX pathway metabolites like 12-HETE, 12-hydroxyicosapentaenoic acid (12-HEPE), 9-hydroxyoctadecanoic acid (9-HODE) and 14-hydroxyicosahexaenoic acid (14-HDHA); the 15-LOX metabolites 15-HETE, 13-HODE, 15-HEPE and 17-HDHA and various other PUFA metabolites like 11-HETE, 20-COOH-AA and the oxidative stress marker 8iPGF2 were all present at enhanced concentrations (significantly different) in AgP patient samples (Table 1). Finally and interestingly, the concentration of the COX-pathway metabolite PGE2 was significantly higher in GCF samples of AgP patients (2.2±0.6 ng/ml) compared with healthy controls (0.7±0.5 ng/ml) (P = 0.008) (Table 1).

The GCF samples were additionally analysed by ELISA for PGE2 and LXA4. The levels of PGE2 were significantly increased in GCF of AgP compared with healthy controls (Table 1). In contrast, 12-oxoETE was present in significantly lower concentrations in the patient group. The concentrations of PGE2 in saliva and healthy controls were not significantly different.

Saliva analysis

From the PUFA levels of LA, AA, EPA and DHA were found at slightly higher concentration in saliva samples of AgP patients compared with controls (Table 2). Lipid derivatives originating from the 5-LOX pathways, in particular LTB4 (P = 0.002), 20-OH-LTB4 (P = 0.048), 5-HETE (P = 0.017), 5-oxoETE and from the 15-LOX pathways, 15-HETE (P = 0.004), 15-oxoETE and 17-HDHA, were found in higher concentrations in AgP patients (Table 2). In contrast, 12-oxoETE was present in significantly lower concentrations in the patient group. The concentrations of PGE2 in saliva in AgP patients and healthy controls were not significantly different.

Serum analysis

As with GCF, the levels of the fatty acids in serum including AA, EPA, DHA and LA, were higher in AgP patients (Table 3). Several PUFA metabolites originating from various LOX pathways, in particular 5-HETE, 8-HETE, 12-HETE, 15-HETE, 13-HODE, 13-oxoODE, 5-oxoETE, 15-oxoETE, LXA4, 5-HEPE, 12-HEPE, 8-HEPE, 4-HDHA, 10-HDHA, 14-HDHA and 17-HDHA were increased in AgP patient samples compared with healthy controls (Table 3). The concentration of the lipid mediator d15d12PGD2, a COX metabolite, was increased in the serum samples of AgP patients, as well as 20-COOH-AA, 20-HDHA and 11-HETE (Table 3). In general, concentrations of various PUFA metabolites were much higher in serum than in saliva and GCF.

Ratios of precursors of pro-resolution/pro-inflammatory lipid mediators and LOX/COX-pathway analysis

There were considerable variations of the concentrations of DHA, EPA and AA between groups in GCF, saliva and serum analysed in this study (Table 1, 2 and 3). The ratio of the n3-PUFAs (DHA and EPA) concentration vs. the concentration the n6-PUFAs (LA and AA) in GCF was significantly lower in AgP compared with the control group (P < 0.004) (Figure 3A). By contrast, there was no significant difference in these ratios in saliva and serum (Figure 3B and C).

In addition the ratio of the direct precursors of pro-resolution lipid mediators (14-HDHA, 17-HDHA and 18-HEPE), which are precursors of resolvins and protectins, was calculated against various precursors of the AA-derived and mainly pro-inflammatory derivatives (5-HETE, 12-HETE and 15-HETE) as well as ratios of sum of HEPEs and sum of HDHAs vs. the sum of HETEs were calculated (Table 4). The ratio of precursors of pro-resolution to pro-inflammatory lipid markers (HEPE/HETE and HDHA/HETE) were mainly reduced in the GCF, saliva and serum of AgP patients in comparison with healthy controls but was just significant in GCF samples (P < 0.05) (Table 4).

The sum of 5-, 12-, 15-LOX and COX pathway originating metabolites were mainly upregulated in serum, GCF and saliva samples from AgP compared with healthy controls (Table 4).

Discussion

To draw a general picture of the contribution of bioactive lipid mediators in periodontal health and disease, lipid mediators were profiled using the HPLC-ESI-MS-MS methodology for serum, saliva and GCF samples from AgP patients and healthy controls. The precise concentration of PUFAs, PUFAs metabolites and further lipid mediators determined using lipidomic technology leads to a deeper understanding of the role of lipid mediators in the mechanism of periodontal disease [31]. In this study, we report
### Table 2. Concentration of lipid mediators in saliva samples.

| lipid mediators | AgP (n = 16) ng/ml | HC (n = 12) ng/ml | P value |
|-----------------|-------------------|-----------------|---------|
| 6-Lipoxygenase pathway |                   |                 |         |
| 11-HETE         | 0.23 ± 0.27       | 0.06 ± 0.08†    | 0.017*  |
| LTB4            | 0.47 ± 0.41       | 0.12 ± 0.15†    | 0.002*  |
| 20-OH-LTB4      | 0.57 ± 0.93       | 0.13 ± 0.13†    | 0.048*  |
| 20-COOH-LTB4    | 0.29 ± 0.30       | 0.32 ± 0.35†    | 0.832   |
| LTC4            | 0.02 ± 0.00       | 0.02 ± 0.00†    | –       |
| 5-oxoETE**      | 0.06 ± 0.05       | 0.03 ± 0.02†    | 0.012*  |
| LXB4            | 0.02 ± 0.00       | 0.02 ± 0.00†    | –       |
| LXA4            | 0.05 ± 0.09       | 0.02 ± 0.01†    | 0.302   |
| 5-HEPE          | 0.02 ± 0.00       | 0.02 ± 0.00†    | 0.737   |
| LTB5            | 0.02 ± 0.00       | 0.02 ± 0.00†    | 0.364   |
| LXA5            | 0.02 ± 0.01       | 0.02 ± 0.00†    | 0.264   |
| 4-HDHA          | 0.02 ± 0.00       | 0.02 ± 0.00†    | 0.224   |
| 8-hydroxylation pathway |         |                 |         |
| 8-HETE          | 0.17 ± 0.21       | 0.16 ± 0.32‡    | 0.913   |
| 8-HEPE          | 0.04 ± 0.06       | 0.02 ± 0.01†    | 0.269   |
| 10-HDHA         | 0.03 ± 0.01       | 0.02 ± 0.00†    | 0.047*  |
| PD1             | 0.03 ± 0.02       | 0.02 ± 0.00†    | 0.085   |
| 12-Lipoxygenase pathway |       |                 |         |
| 12-HETE         | 3.05 ± 2.49       | 2.41 ± 3.09†    | 0.488   |
| 12-oxoETE**     | 1.11 ± 1.57       | 1.57 ± 1.38†    | 0.362   |
| HXA3            | 0.73 ± 1.14       | 0.25 ± 0.54†    | 0.120   |
| HXB3            | 0.69 ± 2.09       | 0.28 ± 0.50†    | 0.436   |
| 12-HEPE         | 0.12 ± 0.14       | 0.31 ± 0.95†    | 0.365   |
| 14-HDHA         | 0.13 ± 0.13       | 0.13 ± 0.15†    | 0.915   |
| RvD1            | 0.02 ± 0.00       | 0.02 ± 0.01†    | 0.295   |
| RvD2            | 0.03 ± 0.02       | 0.03 ± 0.03†    | 0.391   |
| MvR             | 0.02 ± 0.00       | 0.02 ± 0.00†    | 0.284   |
| 9-HODE          | 0.31 ± 0.35       | 0.26 ± 0.49     | 0.759   |
| 15-Lipoxygenase pathway |          |                 |         |
| 15-HETE         | 0.22 ± 0.43       | 0.03 ± 0.02†    | 0.004*  |
| 15-oxoETE**     | 0.12 ± 0.21       | 0.02 ± 0.02†    | 0.068   |
| 15-HEPE         | 0.02 ± 0.00       | 0.02 ± 0.00†    | 0.203   |
| 17-HDHA         | 0.04 ± 0.03       | 0.02 ± 0.01†    | 0.036*  |
| 13-HODE         | 0.43 ± 0.45       | 0.44 ± 0.72     | 0.965   |
| 13-oxoODE**     | 11.65 ± 11.82     | 14.66 ± 19.76   | 0.570   |
| Cyclooxygenase pathway |       |                 |         |
| PGD2            | 0.11 ± 0.09       | 0.08 ± 0.10†    | 0.333   |
| PGE2            | 0.17 ± 0.26       | 0.08 ± 0.06†    | 0.142   |
| d15d12PGD2      | 0.02 ± 0.00       | 0.02 ± 0.01†    | 0.327   |
| PGJ2            | 0.02 ± 0.01       | 0.02 ± 0.00†    | 0.571   |
| d15d12PGJ2      | 0.03 ± 0.03       | 0.02 ± 0.00†    | 0.364   |
| TXB2            | 0.08 ± 0.10       | 0.05 ± 0.06†    | 0.334   |
| PGF2            | 0.03 ± 0.02       | 0.02 ± 0.01†    | 0.032*  |
| PGE3            | 0.09 ± 0.11       | 0.08 ± 0.12†    | 0.764   |
| Alternative pathways |            |                 |         |
| 20-HETE         | 0.02 ± 0.00       | 0.02 ± 0.00†    | 0.252   |
| 20-COOH-AA      | 1.76 ± 1.95       | 0.91 ± 0.65†    | 0.093   |
| 18-HETE         | 0.03 ± 0.02       | 0.02 ± 0.00†    | 0.138   |
| 20-HDHA         | 0.04 ± 0.04       | 0.02 ± 0.01†    | 0.261   |
| 11-HETE         | 0.09 ± 0.15       | 0.02 ± 0.01†    | 0.083   |
mainly elevated levels of n6- and n3-PUFAs as well as various eicosanoids and docosanoids in serum, GCF and saliva of AgP patients compared with the healthy controls. On the other hand, the ratio of the concentrations of precursors of pro-inflammatory or pro-resolving lipid mediators from n3-PUFAs vs. concentrations of n6-PUFAs (DHA and/or EPA)/AA were lower in GCF samples of AgP patients compared with healthy controls, which was the main novel finding in the present study.

EPA and DHA concentrations were significantly higher in AgP patients when compared with the healthy controls. Figueredo et al. [32] also reported of significantly higher levels of EPA and DHA in serum of patients with generalized chronic periodontitis compared with that of patients with gingivitis. Whereas there were significantly lower EPA/AA and DHA/AA ratios in GCF of the AgP patients compared to healthy controls, this was not the case in serum and saliva samples in which approximately similar ratios in AgP patients and controls were demonstrated. In a previous study it was reported that in periodontitis patients with bone loss, the serum levels of fatty acids from the n6-PUFA pathways, which were obtained after saponification, were higher than in healthy individuals, whereas the opposite was observed with fatty acids of the n3-PUFA pathway [22]. The bone loss was linked to an imbalance between the two pathways with mainly an increase in AA and a decrease in EPA and DHA.

Recently the active resolution of inflammation was recognized as a process driven by lipoxins originating from AA and novel compounds from EPA and DHA known as resolvins, which have a potential to dampen or resolve the damaging aspects of the inflammatory response. LXA₄ is an endogenous lipid mediator which plays an important role in the local resolution processes [7,33]. In the present study, we found LXA₄ concentration to be increased in patients compared with healthy controls when measured by HPLC-ESI-MS-MS, but this was significant only in serum samples. LXA₄ in GCF was also determined by ELISA. Using this technique, LXA₄ was identified at slightly higher concentrations in healthy controls (P = 0.049), but the difference was minor. The pro-resolution lipid mediators such as RvD1, MaR and PD1 were detected either at very low concentrations or were below the detection limit. This can be explained either by their instability during sample collection and storage or simply by very low and undetectable endogenous concentrations with current analytical technologies [34,35]. Recently, the “Serum Metabolome Project” identified 65 lipids by LC-MS [36]. RvE1 and RvD1 were among the lipid mediators identified in plasma of systemically healthy individuals with unknown periodontal status. Whereas these molecules have regulator functions in inflammatory response, as well as in regulation of bone formation and bone resorption, PGE2 has been proposed as a measure of disease status in GCF [32]. In our study, PGE2 was present at higher concentrations in GCF samples of AgP patients than in controls, which is in line with a previous study in which PGE2 concentration was significantly increased in GCF during the active phase of the disease [14]. Relatively small differences in PGE₂ levels [ng/ml] were observed when comparing AgP patients with healthy controls in the current analysis. The mean PGE₂ concentration in GCF was 2.2 ng/ml in AgP patients and 0.7 ng/ml in healthy controls as determined by LC-MS.

In the present study, the concentrations of the mono-hydroxylated PUFA-metabolites; 5- and 12- HETE were mainly found in higher levels in GCF and serum, while 15-HETE was found in significantly higher levels in all samples of the AgP patients. A positive association with high levels of HETEs has also been reported in other types of disease with an inflammatory component [38,39]. Here we report that LTB₄ was markedly increased in saliva samples of AgP patients, and its potential as an important marker for diagnosing periodontal disease activity should be further investigated. High concentrations of PGs, LTB₄ and cysteinyl-leukotrienes have been reported to promote the accumulation of inflammatory cells such as polymorphonuclear leukocytes (PMNs) by increasing the blood flow and vascular permeability [40].

While initial fluid accumulation in the periodontal pocket seems to represent a transudate of interstitial fluid produced by an osmotic gradient leading to similar concentrations of for instance proteins in GCF and interstitial fluid, GCF is regarded as a serum exudate during inflammatory processes, with similar protein concentrations as in serum. This may also apply to the similarities in concentrations of lipid molecules in GCF and serum. Based on these findings, and with the techniques used in this study, GCF seems to be the most reliable biological fluid to reflect the local destruction [reviewed by Griffiths [41]].

The PMNs underlying the junctional epithelium in the periodontium represent the first line of defense against periodontal pathogens [42]. Transmigration of PMNs across epithelial surfaces represents a shared phenomenon among inflammatory mucosal

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**Table 2.**

| Oxidative stress marker | AgP (n = 16) ng/ml | HC (n = 12) ng/ml | P value |
|-------------------------|-------------------|------------------|---------|
| 8iPGF₂                  | 0.03 ± 0.04       | 0.02 ± 0.00†     | 0.165   |
| 8iPGF₃                  | 3.91 ± 10.35      | 0.91 ± 1.58†     | 0.242   |

The concentration of lipid mediators in saliva of aggressive periodontitis (AgP) patients and healthy controls (HC) was measured by HPLC-MS-MS. Results in ng/ml are expressed as mean ± SD. * Significantly different (P<0.05, Mann Whitney test). † Various values below the quantification limit set to 0.02 ng/ml. ** These derivatives may partly origin from autooxidation.

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| Pathway                              | Compound   | AgP (n = 16) ng/ml | HC (n = 12) ng/ml | P value |
|-------------------------------------|------------|-------------------|------------------|---------|
| **PUFAs**                           | AA         | 855.4 ± 325.0     | 472.6 ± 261.2    | 0.002*  |
|                                     | EPA        | 33.9 ± 33.4       | 8.2 ± 6.9        | 0.001*  |
|                                     | DHA        | 175.7 ± 112.7     | 82.2 ± 61.0      | 0.003*  |
|                                     | LA         | 318.9 ± 210.1     | 225.4 ± 148.7    | 0.129   |
| **5-lipoxygenase pathway**          | 5-HETE     | 24.1 ± 36.1       | 0.4 ± 0.2        | 0.000*  |
|                                     | LTB4       | 0.3 ± 0.3         | 0.2 ± 0.2        | 0.500   |
|                                     | 20-OH-LTB4 | 0.3 ± 0.6         | 0.3 ± 0.3        | 0.186   |
|                                     | 20-COOH-LTB4| 1.6 ± 2.2       | 2.5 ± 2.0        | 0.111   |
|                                     | LTC4       | 0.1 ± 0.1         | 0.1 ± 0.1        | 0.926   |
|                                     | 5-oxoETE** | 0.5 ± 0.4         | 0.2 ± 0.2        | 0.028*  |
|                                     | LXB4       | 0.2 ± 0.2         | 0.5 ± 0.1        | 0.550   |
|                                     | LX4        | 1.1 ± 1.3         | 0.1 ± 0.2        | 0.030*  |
|                                     | 5-HEPE     | 1.0 ± 1.4         | 0.1 ± 0.1        | 0.007*  |
|                                     | LTB5       | 0.1 ± 0.0         | 0.1 ± 0.1        | 0.391   |
|                                     | LXA2       | 0.1 ± 0.1         | 0.2 ± 0.4        | 0.938   |
|                                     | 4-HDHA     | 6.3 ± 9.2         | 0.1 ± 0.0        | 0.002*  |
| **8-hydroxylation pathway**         | 8-HETE     | 1.6 ± 1.2         | 0.4 ± 0.3        | 0.001*  |
|                                     | 8-HEPE     | 0.7 ± 0.6         | 0.3 ± 0.4        | 0.047*  |
|                                     | 10-HDHA    | 0.4 ± 0.3         | 0.1 ± 0.1        | 0.019*  |
|                                     | PD1        | 0.4 ± 0.5         | 0.1 ± 0.1        | 0.074   |
| **12-lipoxygenase pathway**         | 12-HETE    | 259.8 ± 212.7     | 77.3 ± 76.2      | 0.005*  |
|                                     | 12-oxoETE**| 2.8 ± 2.2         | 2.3 ± 3.0        | 0.100   |
|                                     | HXA2       | 3.1 ± 2.8         | 0.9 ± 0.8        | 0.005*  |
|                                     | HXB2       | 0.7 ± 1.7         | 0.7 ± 0.7        | 0.053   |
|                                     | 12-HEPE    | 3.2 ± 3.2         | 1.4 ± 2.0        | 0.011*  |
|                                     | 14-HDHA    | 5.0 ± 5.3         | 1.7 ± 1.6        | 0.017*  |
|                                     | RvD1       | 0.1 ± 0.2         | 0.2 ± 0.3        | 0.856   |
|                                     | RvD2       | 62.2 ± 22.8       | 0.9 ± 1.9        | 0.383   |
|                                     | MaR        | 0.2 ± 0.1         | 0.1 ± 0.1        | 0.134   |
|                                     | 9-HODE     | 4.6 ± 4.1         | 2.6 ± 4.1        | 0.008*  |
| **15-lipoxygenase pathway**         | 15-HETE    | 5.5 ± 4.4         | 1.3 ± 1.3        | 0.001*  |
|                                     | 15-oxoETE**| 0.9 ± 1.3         | 0.1 ± 0.1        | 0.037*  |
|                                     | 15-HEPE    | 0.3 ± 0.4         | 0.2 ± 0.1        | 0.174   |
|                                     | 17-HDHA    | 0.8 ± 0.8         | 0.2 ± 0.1        | 0.003*  |
|                                     | 13-HODE    | 4.5 ± 4.0         | 1.6 ± 1.0        | 0.005*  |
|                                     | 13-oxoODE**| 11.2 ± 9.0        | 5.0 ± 3.6        | 0.038*  |
| **Cyclooxygenase pathway**          | PGD2       | 200.5 ± 151.7     | 138.9 ± 191.9    | 0.058   |
|                                     | PGF2       | 0.4 ± 0.3         | 0.3 ± 0.3        | 0.207   |
|                                     | PGE2       | 0.4 ± 0.6         | 0.4 ± 0.3        | 0.756   |
|                                     | d15d12PGD2 | 2.5 ± 2.1         | 0.9 ± 0.6        | 0.002*  |
|                                     | PGJ2       | 0.2 ± 0.2         | 0.1 ± 0.1        | 0.163   |
|                                     | d15d12PGJ2 | 0.1 ± 0.0         | 0.1 ± 0.0        | 0.975   |
| **Alternative pathways**            | 20-HETE    | 0.4 ± 0.6         | 0.4 ± 0.3        | 0.756   |
|                                     | 20-COOH-AA | 18.4 ± 4.7        | 1.8 ± 1.8        | 0.001*  |
|                                     | 18-HEPE    | 0.5 ± 0.3         | 0.3 ± 0.4        | 0.003*  |
|                                     | 20-HDHA    | 0.6 ± 0.6         | 0.1 ± 0.1        | 0.001*  |
conditions. In order to be recruited to the site of the battle, the cells depend on chemotactic signals [43]. Hepoxilin A3 (HXA3) has been shown to be a potent chemoattractant for PMNs. In our study, the concentration of HXA3 in GCF was not significantly different between patients and controls. In serum, however, the concentration of HXA3 was higher in patients compared to controls ($P = 0.005$).

A balanced ratio between n3- and n6-PUFAs is suggested by World Health Organization (WHO) to decrease risk of coronary heart disease (WHO 1995). Recently, resolution actions in periodontal disease have been credited to products from the n3-PUFAs [30]. The finding in the present study further demonstrated a possible effect of n3- to n6-PUFAs ratios in AgP patients. Future studies are needed to confirm the effect of pro-resolution vs. pro-inflammatory imbalance in different types of periodontal diseases.

Conclusions

In the present study, we report on generally elevated levels of eicosanoids and docosanoids and various n6- and n3-PUFAs in the GCF, saliva and serum of AgP patients compared with healthy controls. In addition, there were significantly increased concentrations of PGE2 in GCF of AgP patients compared with healthy controls determined via two independent methodological approaches. We demonstrated a lower ratio of precursors of pro-resolution to pro-inflammatory lipid mediators in the GCF of AgP patients compared with healthy controls. GCF seemed to be the most reliable biological fluid for the assessment of the periodontal condition. Findings from this study suggest that the ratio of the precursor of pro-resolution vs. pro-inflammatory derivatives might be appreciated as markers of local destruction in aggressive periodontitis. Further studies focussing on various markers for diseases severity in bigger cohorts of patients and correlating these values with various lipid markers and ratios are in progress.

Materials and Methods

Study population

Nineteen AgP patients (4 males and 15 females) and 19 controls (7 males and 12 females) were recruited consecutively from patients seeking treatment at the University of Science and Technology (UST), Omdurman, Khartoum, Sudan, from December 2008 to July 2009. To be included in the study, bleeding on probing (BOP), probing pocket depth (PPD) and clinical attachment level (CAL) $\leq 5$ mm had to be present at least at one central incisor and one first molar. The patients were all <35 years (13–35 years; mean 23.4 ± 6.4, median 23.5). The
number of teeth in each patient was from 21 and 28. The diagnosis was confirmed by radiographs in addition to a full-mouth clinical examination performed by the same dentist. Before the examinations, intra-individual calibration of the examiner was undertaken. Medical history was recorded for each patient, and subjects who had received antibiotics or periodontal treatment within the last three months before the examination, or were pregnant or had any systemic disease that could affect the progression of periodontal disease, were excluded. The 19 control subjects were systemically healthy employees and students at UST, with PPD ≤3 mm and CAL ≤2 mm for all teeth, and in most cases all teeth were present.

Ethics Statement
Ethical approval was obtained from the Research Ethics Committee at UST, Omdurman, Sudan, and the Regional Committee for Medical Research Ethics (REK) Western Norway (REK 177.04) (Bio bank no. 2355). Each subject signed an informed consent written in their native language (Arabic) prior to participation. On behalf of the young participants involved in the study, the informed consents were assigned by their guardians, all in accordance with the policy of REK, University of Bergen, and UST.

Collection of biological materials
Saliva. Patients and controls were asked to rinse the mouth with water before saliva was collected in a 10 ml sterile plastic tube. The unstimulated whole saliva (UWS) samples were processed the same day by centrifuging for 10 min at 3000×g (SUPERFIT centrifuge, Mumbai, India). Each supernatant was dispersed into 4 tubes and stored in liquid nitrogen.

Blood. Peripheral blood was collected in vacutainer tubes; for serum. The whole tubes were preserved at bench-side for not more than two hours, and centrifuged for 10 min at 3000×g (SUPERFIT centrifuge, Mumbai), India. Each supernatant was dispensed into 4 tubes and stored in liquid nitrogen.

Gingival crevicular fluid. A perio-paper (PERIOPAPER® Gingival Fluid Collection Strips, OralFlow Inc, New York, USA) was used for collection of GCF from the mesio-buccal site of posterior teeth (mean pocket depth of sampled teeth was 5.9 mm). Prior to the GCF collection, the area was isolated with cotton rolls and exposed to a gentle air stream for five seconds. Then the perio-paper was placed subgingivally for 10 seconds, before being removed and stored in an empty tube in liquid nitrogen.

HPLC-ESI-MS-MS Analysis of free fatty acids, eicosanoids and docosanoids in GCF, saliva and serum
The previously described procedure for the HPLC-ESI-MS-MS method was followed with minor modifications [44], but including complete quantification of the detected lipid derivatives.

Sample preparation. The whole analytical sample preparation procedure is based on an established method used for retinoid quantification. Saliva samples were much lower in analyte concentration and were dried in the Eppendorf concentrator 5301 (Eppendorf, Germany) at 45°C from 1.5 ml to 50 μl prior to extraction. In summary, 50 μl of serum, saliva extract or GCF and 150 μl acetonitrile were shaken for 3 min, the precipitated protein was centrifuged at 13,000 rpm, 4°C for 6 min, 130 μL of the resulting supernatant was spiked with 10 μl isotope supernatant mix, evaporated in Eppendorf reaction vials with an Eppendorf concentrator at 30°C for ~10 μl. The Eppendorf concentrator was vented with argon to prevent degradation of eicosanoids and docosanoids. The dried extract was resuspended with approximately 25 μl of HPLC solvent A [64.3% water (water, Chromasolv Plus from Sigma-Aldrich, Hungary) and 35.5% acetonitrile (Merk KGaA, Germany) and 0.2% formic acid (Fluka, Hungary)] to yield 35 μl, then vortexed (15 sec), shaken (3 min) and transferred into micro injection inserts vials (Waters, Hungary). These glass vials with the 35 μl extract were transferred into brown screw top vials with PTFE/silicone septa sample (Waters, Hungary) and placed in the pre-cooled (15°C) autosampler of the Waters 2695XE separation module.

Figure 3. Ratio of n3- to n6-PUFAs in gingival crevicular fluid (GCF) (A), saliva (B) and serum (C) samples. The figure shows the ratios of concentration of (EPA plus DHA) vs. AA in respective fluids of aggressive periodontitis patients (AgP, black bar) and healthy controls (HC, grey bar). * = P<0.05 by use of Mann-Whitney test.
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Table 4. Ratios and sums of lipid mediators from GCF – gingival crevicular fluid, SAL – saliva, SER – serum of aggressive periodontitis (AgP) patients and healthy controls (HC).

|                                | AgP (n = 16) | HC (n = 12) | P value |
|--------------------------------|--------------|-------------|---------|
| **AA-derived metabolite ratios** |              |             |         |
| 5-HETE/AA GCF                  | 0.01±0.01    | 0.04±0.03   | 0.002*  |
|                               | **0.04±0.07**| 0.02±0.03   | 0.199   |
|                               | **0.02±0.03**| 0.001±0.001 | 0.010*  |
| 12-HETE/AA GCF                 | 0.14±0.16    | 0.22±0.13   | 0.086   |
|                               | **0.83±1.75**| 1.46±4.25   | 0.544   |
|                               | **0.29±0.20**| 0.15±0.09   | 0.062   |
| 15-HETE/AA GCF                 | 0.10±0.12    | 0.04±0.04   | 0.178   |
|                               | **0.04±0.09**| 0.02±0.05   | 0.303   |
|                               | **0.01±0.00**| 0.003±0.002 | 0.009*  |
| **EPA-derived metabolite ratios** |              |             |         |
| 18-HEPE/EPA GCF                | **19.67±31.59**| 13.90±36.80 | 0.024*  |
|                               | **0.42±0.52**| 0.45±0.31   | 0.802   |
|                               | **0.03±0.03**| 0.06±0.12   | 0.918   |
| **DHA-derived metabolite ratios** |              |             |         |
| 14-HDHA/DHA GCF                | 0.13±0.11    | 0.20±0.10   | 0.046*  |
|                               | **0.38±0.63**| 0.58±1.58   | 0.613   |
|                               | **0.03±0.02**| 0.02±0.02   | 0.654   |
| 17-HDHA/DHA GCF                | **0.37±0.54**| 0.17±0.08   | 0.754   |
|                               | **0.18±0.27**| 0.10±0.22   | 0.345   |
|                               | **0.004±0.003**| 0.003±0.003 | 0.227   |
| **PUFA-ratios**                |              |             |         |
| EPA/AA GCF                     | 0.02±0.01    | 0.03±0.02   | 0.016*  |
|                               | **0.02±0.02**| 0.02±0.03   | 0.805   |
|                               | **0.03±0.03**| 0.02±0.02   | 0.512   |
| DHA/AA GCF                     | **0.09±0.06**| 0.18±0.10   | 0.008*  |
|                               | **0.13±0.17**| 0.13±0.04   | 0.982   |
|                               | **0.21±0.90**| 0.19±0.09   | 0.629   |
| **Sum of PUFA-originating metabolites** |              |             |         |
| SUM HETEs GCF                  | **3.13±2.56**| 1.00±0.82   | 0.003*  |
|                               | **3.76±2.85**| 2.69±3.20   | 0.288   |
|                               | **260±236**  | 195±208     | 0.002*  |
| SUM HEPEs GCF                  | **1.38±1.91**| 1.41±1.66   | 0.816   |
|                               | **0.30±0.29**| 0.69±1.89   | 0.374   |
|                               | **5.70±5.12**| 4.04±4.37   | 0.006*  |
| SUM HDHAs GCF                  | **0.71±0.45**| 0.36±0.16   | 0.020*  |
|                               | **0.23±0.18**| 0.20±0.15   | 0.481   |
|                               | **13.0±14.8**| 2.22±1.81   | 0.002*  |
| **HEPE or HDHA/HETE ratios**   |              |             |         |
| HEPE/HETE ratio GCF            | **0.83±1.16**| 1.54±1.35   | 0.029*  |
|                               | **0.13±0.18**| 0.45±0.90   | 0.128   |
|                               | **0.03±0.01**| 0.04±0.03   | 0.202   |
| HDHA/HETE ratio GCF            | **0.28±0.12**| 0.45±0.17   | 0.005*  |
|                               | **0.10±0.13**| 0.16±0.18   | 0.309   |
|                               | **0.05±0.04**| 0.04±0.02   | 0.809   |
| **LOX/COX pathways**           |              |             |         |
| 5-LOX pathway GCF              | **5.3±3.1**  | 3.6±4.5     | 0.267   |
|                               | **1.8±1.4**  | 0.8±0.5     | 0.010*  |
|                               | **35.6±46.7**| 4.3±2.4     | 0.009*  |
| 12-LOX pathway GCF             | **11.2±9.1** | 6.9±3.3     | 0.129   |
|                               | **6.2±5.2**  | 5.3±4.9     | 0.582   |
|                               | **286±225**  | 82.9±81.4   | 0.001*  |
| 15-LOX pathway GCF             | **32.6±28.9**| 19.9±11.2   | 0.166   |
|                               | **12.5±12.2**| 15.2±20.3   | 0.619   |

PUFAs in Aggressive Periodontitis
Chromatographic system. The HPLC system consisted of a Waters 2695XE separation module (Waters, Hungary) including a gradient pump, autosampler, degasser and a heated column compartment. A MS-MS detector with an ESI ionizing option was used (Micromass Quattro Ultima PT from Waters, UK; a gift from Biosystems Int., France) as a detector. The system was controlled via the MassLynx software (Waters, Hungary).

HPLC conditions. The eluents were degassed in the Waters 2695XE separation module prior to mixing, then passed through an in-line filter (1–2 μm; Knauer, Germany) before reaching the analytical column (LiChroCART, 125 X 2 mm; Superspher 100, RP-18, endcapped) from Merck KGaA (Germany) embedded in the column compartment. A multilinear gradient was formed from solvent A (methanol; Merck KGaA Germany). The gradient consisted of the following steps: 0.0 min 20% B, 3.0 min 20% B, 5.0 min 60% B, 15.0 min 100% B, 15.9 min 100% B and 16.0 min 5% B. The flow rate was adjusted to 0.4 ml/min and the column was heated to 40°C. From the same biological extract, 10 μl for each HPLC analysis was used. This step was performed twice using the same HPLC conditions and two different MS-MS analysis options for better resolution and quantification of the various analytes.

MS options. The Micromass Quattro Ultima PT was controlled via the MassLynx software. Argon with an inlet pressure of 0.8 bar was used. ESI (electrospray ionization source, Waters, Hungary) was vented by nitrogen continuously produced by the nitrogen generator (Peak Scientific NM30 Nitrogen Generator) including compressor (Waters, Hungary) with the inlet flow set at 3.6 e-3 mbar.

Multiple reaction monitoring settings for ESI: with a negative ESI – setting, was performed with the HPLC eluent following the ion source temperature of 85°C. The desolvation gas flow was 780 l/h, the desolvation temperature was 400°C, the cone gas flow was 10 l/h, the capillary current was 3 μA and the cone voltage was 50 V. Aperture voltage was set at 0 V and the RF lens voltage was 50 V. Aperture voltage was set at 0 V and the RF lens voltage was 50 V.

Multiple reaction monitoring settings for PUFA, eicosanoids/ docosanoids semi-quantification: Method A from 0.0 – 9.0 min for PGF2 349.0 ->192.7, collision energy 22 eV; PD1 and PD1 isomers like PDX 359.0 ->153.3, collision energy 17 eV; TXB2 369.0 ->195.0, collision energy 13 eV; PGE3 349.0 ->233.0, collision energy 17 eV, 195.0, collision energy 13 eV; RvD1, RvD2 375.0 ->141.3, collision energy 13 eV; RvE1 375.1 ->141.3, collision energy 13 eV from 9.0–12.5 min for 13-HODE 294.7 ->170.7, collision energy 16 eV; 9-HODE 294.7 ->194.7, collision energy 16 eV; 5-HEPE 317.0 ->115.0, collision energy 17 eV; 12-HEPE 317.0 ->179.0, collision energy 17 eV; 15-HEPE 317.0 ->219.0, collision energy 17 eV; LTC4 629.3 ->272.0, collision energy 14 eV from 12.5–16.0 min for LA 279.3 ->279.0, collision energy 10 eV; 8-HEPE 317.0 ->255.0, collision energy 17 eV; 18-HEPE 317.0 ->259.0, collision energy 17 eV; 5-oxoETE, 12-oxoETE and 15-oxoETE 317.0 ->273.0, collision energy 17 eV, 20-HETE 319.0 ->245.0, collision energy 10 eV; LTC4 629.3 ->272.0, collision energy 14 eV; LTA4 438.0 ->333.0, collision energy 13 eV from 12.5–16.0 min for LA 279.3 ->59.2, collision energy 25 eV; EPA 310.1 ->203.2, collision energy 12 eV; AA 303.0 ->259.3, collision energy 11 eV, DHA 327.1 ->29.3, collision energy 14 eV. Method B from 0.0–9.8 min for PGE2, d15d12PGD2, PGD2, d15d12PGJ2, PGJ2, 315.0 ->271.3, collision energy 13 eV; LTB4 333.0 ->195.0, collision energy 15 eV; LTB4 335.0 ->195.0, collision energy 15 eV; RvE1 349.1 ->195.3, collision energy 13 eV; HXAs, HXBs, 20-COOH-AAA 355.0 ->273.3, collision energy 15 eV; LXA4 349.0 ->115.0, collision energy 12 eV; 20-OH-LTB4 351.0 ->195.0, collision energy 13 eV; MaR 359.0 ->250.0, collision energy 13 eV from 9.8–12.5 min for 5-HETE 318.7 ->115.0, collision energy 14 eV; 8-HETE 319.0 ->155.0, collision energy 14 eV; 11-HETE 319.0 ->167.0, collision energy 14 eV; 12-HETE 319.0 ->179.0, collision energy 14 eV; 15-HETE 319.0 ->218.9, collision energy 11 eV; 4-HDHA 343.0 ->101.0, collision energy 10 eV; 10-HDHA 343.0 ->181.0, collision energy 10 eV; 14-HDHA 343.0 ->205.0, collision energy 10 eV; 17-HDHA 343.0 ->245.0, collision energy 14 eV; 20-HDHA 343.0 ->285.0, collision energy 10 eV; 13-oxoODE 293.0 ->249.0, collision energy 17 eV and from 12.5–16.0 min for LA 279.3 ->59.2, collision energy 25 eV; EPA 310.1 ->203.2, collision energy 12 eV; AA 303.0 ->259.3, collision energy 11 eV, DHA 327.1 ->29.3, collision energy 14 eV.

Standard solutions. Stock solutions of the PUFAs, eicosanoids and docosanoids were prepared by dissolving the solutions obtained from Cayman-Chemicals (Estonia), BioMol International (Kastel-Med KFT, Budapest, H), Sigma-Aldrich (Budapest H), Larodan Lipids (Malmo¨, Sweden) and Dr. Charles Serhan (Harvard, USA) with methanol to yield a final concentration of 10 μg/ml. All stock solutions were stored in darkness at −80°C. The reference PUFAs, eicosanoids and docosanoids were used for the assay validation.

Quantification. Individual eicosanoids and docosanoids were quantified based on the determination of the “Area Under
ELISA
Levels of PGE$_2$ and LXA$_4$ in GCF. Perio-papers saturated with GCF from AgP patients and controls were immersed in 12 mM (pH 7.6) Tris buffer. The lipids recovered from the GCF samples were diluted in the assay buffer of the ELISA kit; 10 times for the analysis of PGE$_2$ and 5 times for the LXA$_4$. The PGE$_2$ and LXA$_4$ were quantified by the competitive ELISA (Neogen with GCF from AgP patients and controls were immersed in standards provided by the manufacturer. All GCF samples were run in duplicate and measured by FLUOstar OPTIMA, and data were reported in ng/ml.

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