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Proteomic evaluation of GCF in the development of pregnancy related periodontal disease: a pilot clinical study

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Abstract. – OBJECTIVE: This pilot study analyzed the possible changes of periodontal disease status in female patients during the period following pregnancy. Both clinical and laboratory data were collected and analyzed.

PATIENTS AND METHODS: A non-randomized controlled clinical trial was conducted by the Periodontal Department of the Dental Clinic in collaboration with the Pediatrics Department, at Fondazione Policlinico Universitario A. Gemelli, Rome, Italy. Ten female patients, who completed the pregnancy without complications, were enrolled in this research protocol forming the experimental group. During the first post-partum days, gingival crevicular fluid (GCF) samples were collected and analyzed with high-performance liquid chromatography associated with high-resolution mass spectrometry (HPLC ESI MS); periodontal parameters as pocket depth (PD), full mouth plaque score (FMPS) and full mouth bleeding score (FMBS) were recorded, and a professional oral hygiene session was performed. The same protocol was applied after three months with the same patients forming the recall group. A control group was created in order to compare the results with GCF samples from 10 not pregnant fertile women.

RESULTS: Student's *t*-test has been used to evaluate the statistical significance of the collected data. Mean levels of PD decreased from 3.75 mm ± 1.2 mm after pregnancy to 2.88 mm ± 0.85 mm at three months post-partum ($p<0.01$). Mean value of FMPS and FMBS decreased from 21.8% ± 1.35% and 34.27% ± 1.5% after pregnancy to 13% ± 2.81% and 17.55% ± 2.84% at three months post-partum, respectively ($p<0.05$). The concentration of each analyzed peptide has changed in relation to the general improvement of the periodontal status at three months post-partum.

CONCLUSIONS: Pregnancy may be associated with an increased risk of periodontal disease. Both clinical and laboratory data have demonstrated that a professional oral hygiene session can affect the course of pregnancy inducing periodontal diseases allowing a faster healing and *restitutio ab integrum*.

Key Words:
Periodontitis, Pregnancy, Gingival crevicular fluid, Oral Hygiene.

Introduction

The understanding of the etiology and pathogenesis of oral diseases and conditions is continually changing with increased scientific knowledge. The last classification scheme for periodontal and peri-implant diseases and conditions has been updated in June 2018 and it highlights three major categories[1-5]:

a) Periodontal health and gingival disease;
b) Periodontitis;
c) Other conditions affecting periodontium.

Periodontitis is a common chronic inflammatory condition of bacterial etiology that results in the breakdown of the periodontium[6,39,40]. When the inflammation status is only bordered on the soft tis-

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sues and associated teeth, it shows no attachment loss and it is called gingivitis. It is caused by an inadequate oral hygiene and dental plaque formation, but it is reversible with appropriate oral home care[7]. Untreated gingivitis may develop into periodontitis, which results in loss of connective tissue and bone around the teeth[8]. Pregnancy cannot be considered as a risk factor for periodontal disease, but plaque control should be performed by pregnant women accurately[9]. There is evidence suggesting an association between periodontal disease and adverse pregnancy outcomes, such as, preterm birth and low birth weight[10-12]. However, the effect of pregnancy itself on periodontal disease remains unclear[13,14]. It is suggested that pregnancy, which is a stressful state of increased inflammatory activity, and pregnancy-associated hormone changes, can influence periodontal tissues in a negative way. Increased levels of estrogen and progesterone during pregnancy lead to higher vascular permeability of gingival tissues[15]. Gestational gingivitis has been found to be very common among pregnant females, and its prevalence ranges from 35% to 100%[16]. Gingival crevicular fluid (GCF) is a biological fluid, physiologically produced, containing proteins, diverse population of cells, and bacteria from adjacent plaque. Due to their non-invasive sampling, GFC have attracted proteomic research as diagnostic fluid for periodontal, oral, and systemic disease[17]. The composition of gingival crevicular fluid (GCF) is considered as a specific indicator situs of inflammatory status in periodontal tissues[18]. The goal of this non-randomized controlled pilot study is to examine whether there is any change of periodontal disease status in female patients during the first period immediately after partum and its evolution after three months, evaluating the effectiveness of a professional oral hygiene session, identifying and quantifying the amount of proteins in the GCF as inflammatory agents.

Patients and Methods

Study Design

The authors of this research designed and implemented a protocol carried out on a non-randomized controlled clinical trial design, between October 2017 and June 2018, at Fondazione Policlinico Universitario Agostino Gemelli IRCCS, Catholic University of the Sacred Heart, Rome, Italy, with the collaboration between the Periodontal Department of the Dental Clinic and the Institute of Pediatrics, with the support of the Biochemistry Institute. The study started at T0 with the enrollment of ten female patients, who completed pregnancy without complications, forming the experimental group. The average age of the patients was 26 ± 7 years. All patients provided of information, signed a written informed consent for the scientific use of their data according to the 1964 World Medical Association's Declaration of Helsinki and a complete dental examination was scheduled. Patients who received systemic antibiotic therapy in the previous 3 months and who agreed to participate more than 15 days after partum were excluded from the research protocol. The participants received no financial grant or gifts. All the procedures and the research protocol mentioned in this study were in accordance with the ethical standards of the responsible Committee of Fondazione Universitaria Policlinico Agostino Gemelli.

Gingival Crevicular Fluid Samples Collection

After the dental visit was completed, every pathological condition was registered, such as: caries, edentulous zone, root residues or soft tissue lesions. Subsequently GCF samples were collected following the previous standardized scientific research protocol[10]. The tools used for the collection of data were three paper cones 0.20 (Dentsply-Sirona, York, PA, USA): two for the central incisors and one for the left lateral incisor. Cotton rolls were used for relative isolation and sampling sites dried with air; the weight of the cones was registered before use. The cones were inserted in the gingival sulcus at the base of the interdental papilla and removed after 2 minutes and 30 seconds. Cones with visible blood contamination were excluded. GCF samples were collected and stored at -80°C.

Periodontal Status Measurements and Definition

Immediately after the GCF sample collection, all the participants received a full-mouth periodontal examination. To eliminate intra-examiner variability, all the periodontal examinations were performed by the same operator that has been calibrated by an experienced periodontist. The calibration process of the examiner was done by using a probe that exerted pressure on a precision scale (Rs-Kern 440.35A, Milan, Italy); the pressure value for a correct periodontal probing was 30 g[19,39]. At 3 months post-partum the periodontal examination was performed again by the same operator who did the first oral examination.

Measurements were taken at six sites per tooth: mesio-buccal, mesio-lingual, disto-buccal, disto-lingual, mid-buccal, and mid-lingual, using a millimetric probe UNC15 (HU-friedy, Frankfurt, Deutschland). The clinical periodontal parameters were probing depth, PD, full mouth plaque score, FMPS, and full mouth bleeding score, FMBS.

Non-Surgical Periodontal Therapy

After completing the periodontal status measurements, every patient received a professional oral hygiene session, with an ultrasonic scaler and a periodontal tip (HU-friedy, Frankfurt, Deutschland). Each procedure started from the lingual surface of the lower incisors, which was often the most compromised area of the mouth, and was completed on the same day. The periodontal tip allowed to remove the subgingival plaque without damaging the periodontal tissues. No anesthesia was performed during the procedures.

3-Months Recall Program

After 3 months, all the patients enrolled in this study have been recalled to receive the same treatment, including a second GCF sample, forming the recall group. A control group was created in order to match the laboratory data from the experimental and recall group with GCF samples from 10 not pregnant female patients in fertile age, between 20 and 25.

Reverse-Phase High-Performance Liquid Chromatography (RP-HPLC) Coupled to Tandem Mass Spectrometry

HPLC-ESI-high-resolution MS-MS experiments were carried out by an Ultimate 3000 Micro HPLC coupled to an Orbitrap Elite apparatus. High resolution MS and MS data were elaborated by the Proteome Discoverer 1.4 software (version 1.4.1.14, Thermo Fisher Scientific, Wlatham, MA, USA), based on SEQUEST HT cluster as a search engine (University of Washington, licensed to Thermo Electron Corp., San Jose, CA, USA) against Swiss-Prot Homo Sapiens proteome (UniProtKb, Swissprot, released on march 2018) and the characterizations carried out by the software were also manually checked.

Results

Figure 1 represents the variation of periodontal clinical parameters between the case group and the recall group. The mean value of PD decreased from 3.75 ± 1.2 mm in the case group to 2.88 ± 0.85 mm in the recall group. FMPS and FMBS before the oral hygiene session were respectively 21.8 ± 10.3% and 34 ± 14% in the case group and 13 ± 2.81% 17.2 ± 2% in the recall group. Variation of FMPS and FMBS was statistically significant, FMPS (p=0.01) and FMBS (p=0.003). After the

Figure 1. Graphic showing the change of mean PD, FMPS, and FMBS in the experimental group and after 3 months at the recall appointment.

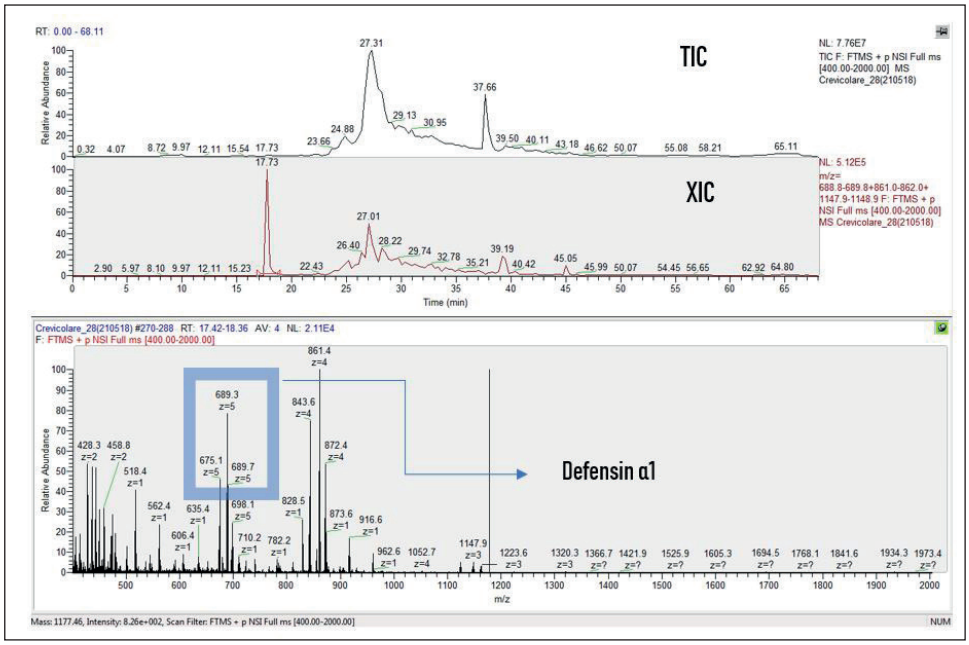

Figure 2. Graphic representation of the total ion current profile TIC, peak ion current extracted XIC and m/z values in a GCF sample with layout for Defensin α1.

analysis of the GCF samples, the results obtained confirmed the presence of α-defensins, β-thymosin Tβ4, Tβ10, and its fragments, fibrinopeptide A and B. The determination of the relative abundances of the various peptides was done by means of the measurement of the chromatographic peak area in the XIC profile as shown in Figure 2 and using the m/z values reported in Table I. The peak area of each peptide is directly proportional to its concentration and it can be used to study and evaluate the relative abundance of the same peptide in different samples, working in constant analytical condition. Data have been analyzed with the Student's *t*-test to clarify the presence of statistically significant variations. A p-value <0.05 was considered statistically significant[20,38].

Table I. Quantified peptides in GCF, elution time and average value of the experimental and theoretical protein masses.

Proteins	Elution Time (min)	Expected Average mass (Theoretical average mass)
α-defensin 1	26.1-26.7	3.442±0.4 (3.442)
α-defensin 2	26.1-26.7	3.371±0.4 (3.371)
α-defensin 3	26.1-26.7	3.486±0.4 (3.486)
α-defensin 4	29.0-29.3	3.707±0.4 (3.707)
Thymosin β4	20.0-20.5	4.963±0.4 (4.963)
Thymosin β10	21.0-21.5	4.935±0.4 (4.936)
Thymosin β4 fragment (21-44)	20.0-20.5	4.744±0.4 (4.744)
Fibrinopeptide A	20.0-20.3	1.536±0.4 (1.536)
Fibrinopeptide A fragment (21-35)	20.0-20.3	1.465±0.4 (1.465)
Fibrinopeptide A fragment (22-35)	19.0-19.3	1.350±0.4 (1.350)
Fibrinopeptide B	22.7-23.2	1.551±0.4 (1.552)

Table II. Analysis of experimental data of peptides and proteins recognized in GCF samples. Extracted ion current peak areas of proteins and peptides deriving from gingival crevicular fluid (GCF) (mean values ± SD x106) and results of the comparison between the recall and the control group.

Proteins	Experimental group	3 months recall group	Control group	*p*-value Sp-R	*p*-value Sp-Cr	*p*-value R-Cr
α-defensin 1	40.5	9.35	6.06	0.01	0.01	Ns
α-defensin 2	105.3	19.9	6.61	0.02	0.01	Ns
α-defensin 3	63.9	7.28	5.33	0.01	0.01	Ns
α-defensin 4	22	8.19	9.49	0.05	Ns	Ns
Thymosin β4	1.15	21.4	4.64	0.01	0.01	0.02
Thymosin β10	1.99	10.5	3.72	0.01	0.01	Ns
Thymosin β4 fragment (21-44)	4.24	2.34	0.87	0.03	0.01	Ns
Fibrinopeptide A	15.8	2.00	0.39	0.01	0.01	0.02
Fibrinopeptide A fragment (21-35)	11.7	1.38	1.05	0.02	0.01	Ns
Fibrinopeptide A fragment (22-35)	16.7	1.12	0.90	0.01	0.01	Ns
Fibrinopeptide B	22.7	2.48	0.91	0.01	0.01	0.01

Proteins have been manually sequenced through the interpretation of the mass spectrum. Elution time, which is defined as the time that a compound takes to cross the chromatographic column, is shown in Table I. Analysis of laboratory data related to the individual peptides recognized within the GCF samples is summarized in Table II.

As shown in Figure 3, from the analysis of laboratory data related to the individual peptides recognized within the GCF samples, levels of all the defensin are always higher in the experimental group, compared to the 3 months recall group and the control group. Although α4 defensin has a low expression in all the three groups, its value in the experimental group is higher than in the other groups. Statistically significant differences emerged: for the α1 and α3 defensin from the comparison between experimental and control group (p=0.01), experimental and recall group (p=0.01); for the α2 defensin from the comparison between

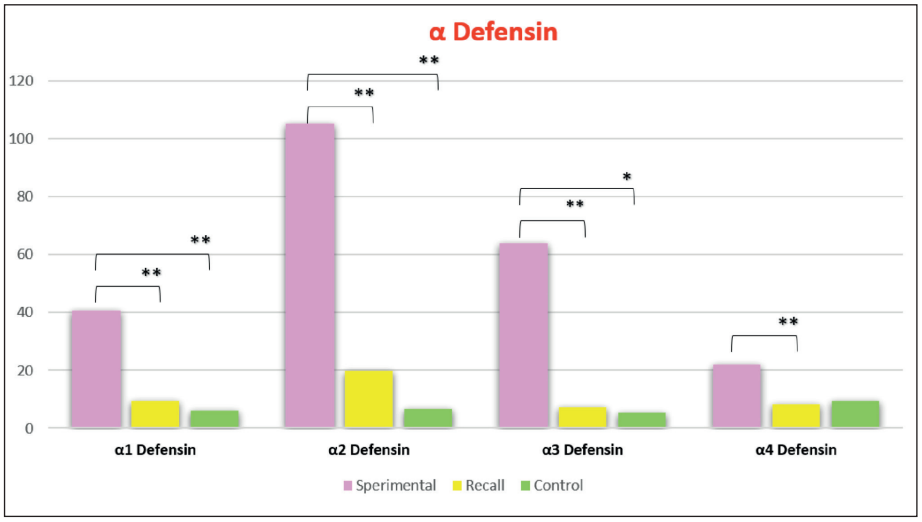

Figure 3. Graphical representation of the average values of XIC peak areas related to defensin α1, α2, α3, and α4 in the three group. Statistical significance is shown with *for p<0.05, **for p<0.01 and ***for p<0.001.

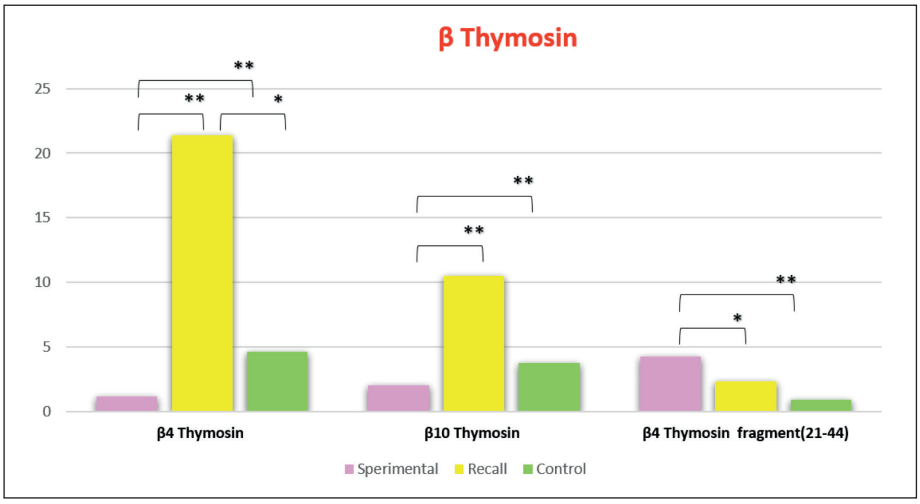

Figure 4. Graphical representation of the average values of XIC peak areas thymosin β4 and β10 in the three groups. Statistical significance is shown with *for $p<0.05$, **for $p<0.01$ and ***for $p<0.001$.

experimental and control group (p=0.02), experimental and recall group (p=0.01); also for the α4 defensin statistical significance has emerged in the variations between the experimental and the recall group (p=0.05).

Figure 4 shows levels of thymosin β4, β10 and one of its fragments (21-44). Thymosin β4 is present in all groups with a peak in the recall group. Thymosin β10 is represented in the same way but with lower peak value in the recall group. Although slightly represented, the thymosin β4 fragment is present in all the three groups, with a peak in the recall group. For these peptides statistically significant differences emerged: for the thymosin 4, the variations between the experimental and the recall group (p=0.01), between the experimental and the control group (p=0.01) and between the recall and the control group (p=0.03). For the thymosin 10, statistical significance has emerged in the variations between the experimental and the recall group (p=0.01) and between the experimental group and the control group (p=0.01). The β4 fragment showed statistically significant values between the experimental group and the recall group (p=0.03)

An analysis of the graph in Figure 5 shows the high concentration of the different fibrinopeptides in the experimental group, with a drastic reduction in the recall group, which is normalized approaching the values observed in the control group.

The fibrinopeptide B recorded the highest value in the experimental group compared to all the others peptides. The differences between the samples analyzed are statistically significant about the fibrinopeptide A in the comparison between the recall and the control group (p=0.03); about the fragment 22-35 of the fibrinopeptide A between the recall and the control group (p=0.04); about the fibrinopeptide B between the experimental group and the recall group (p=0.04), between the experimental and the control group (p=0.02) and between the recall and the control (p=0.01).

Discussion

Although the relationship between periodontal disease and adverse pregnancy outcomes has been debated and documented, the effects of pregnancy on the progression of the periodontal disease itself remains uncertain[19,22,38,40]. In 2013, Xie et al[10] analyzed the changes in periodontal status during pregnancy and in the following two years in an observational study conducted on 39 patients. A gradual regression of periodontal disease has emerged, without any type of etiological therapy, with the incidence rate that has varied from 66.7% in pregnancy to 33.3% postpartum (p<0.01), with decrease in bleeding on probing, BOP, depth of pocket, PD, and clinical attach level, CAL[10,24,39,40]. Tilakaratne et al[23] in 2000 observed the effects of pregnancy on the periodontium in a rural population of female patients, residents of Sri Lanka. From this research emerged an increase in the gingival index (GI) from 1.15% in the first trimester, to 1.28% and 1.43%, respectively in the second and third tri-

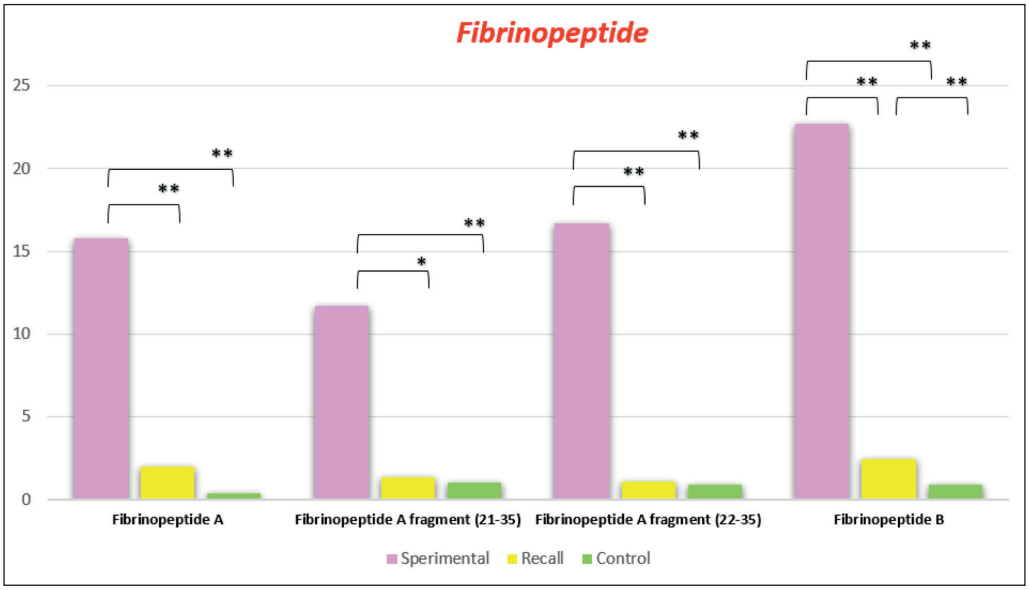

Figure 5. Graphical representation of the average values of XIC peak areas related to fibrinopeptide A, its fragments (21-35), (22-35) and the fibrinopeptide B in the three groups. Statistical significance is shown with *for $p<0.05$, **for $p<0.01$ and ***for $p<0.001$.

mesters, with drastic reduction three months after the birth; no alterations were found regarding the loss of clinical attachment levels. Considering the limitations of the study, the authors were able to affirm the exclusive interest of the marginal periodontal in the periodontitis. Lieff et al[16] in 2004 did not show any change in the altered periodontal indexes present in pregnant women at the twenty-seventh week of pregnancy and 48 hours post-partum. The results of the present study highlight the improvements obtained in the treatment of gingivitis and periodontitis in female patients, in the period immediately post-partum, with clinical data, supported by laboratory analysis. From the medical history questionnaire provided to patients and from the complete periodontal chart, there was a common feature to all patients enrolled in this study; none of them had been followed by a dentist during the period of pregnancy, preferring to postpone any type of treatment, even in the presence of a very evident need for care. Many patients, at the time of the first visit to the Dental Clinic, in addition to a clinical scenario of gingivitis and periodontitis, presented often infectious outbreaks, related to teeth compromised by caries and endodontic lesions, which in some cases undermined the integrity of the tooth. No differences, in terms of clinical data or laboratory results, was found related to the age of patients. In this work, periodontal index changes were analyzed and compared in patients during the postpartum period and after three months, with the therapeutic support of a professional oral hygiene session performed during the first visit. This protocol could be considered innovative compared to the one of Xie et al[10], which did not provide any type of treatment, as it was only an observational study. From the interpretation of the data obtained from periodontal charts, the presence of acute gingivitis and localized periodontitis can be confirmed, in particular in the posterior teeth. The level of oral hygiene at home was found not adequate due to not using interdental cleaning devices, such as dental floss or interdental brush. Similar results were found in 2010 in a study of Villa et al[25] in which, although 99.3% of patients stated that they cleaned their teeth at least twice a day, only 12% of them confirmed that they use interdental cleaning devices[38,39]. For this reason, in the present investigation, at the end of the first visit, all the patients received a practical demonstration about the correct habits of home oral hygiene, such as the brushing technique and the use of dental floss. The ultrasonic scaling of the calculus allowed the removal of supragingival and subgingival plaque deposits, with minimal discomfort for the patients, without the need to perform local anesthesia[26,27]. The gingival tissues were edematous, hyperplastic and bloody, if stimulated by probing or by blowing air. This aspect must be taken into consideration during the interpretation of the ana-

lyzed periodontal indexes, since the increase in the pocket depth, PD, could be linked to the formation of a pseudo-pocket by gingival hypertrophy, rather than to a real loss of periodontal attachment and underlying bone support[28]. The same conclusions were reached in 2017 by Janaray et al[9] who conducted a cohort study on a population of 100 pregnant patients, monitoring the evolution of the periodontal disease; this research concludes that periodontal status deteriorates during pregnancy, with a greater tendency to increase the depth of the pocket compared to the loss of clinical attach, but tends to recovery starting from the sixth month post-partum, in absence of therapeutic treatments. The area that has been found to be most affected by plaque accumulation is the lingual portion of the fifth sextant. Because of the presence of an abundant salivary flow, which originates from the ducts of the major salivary glands, submandibular and sublingual, and of the minor, near the lingual surface of the lower incisors there were important accumulations of calculus. During the three-months recall appointment, it was possible to appreciate a general improvement of the periodontal status, through a marked reduction of bacterial plaque and calculus. Also, pocket depth and bleeding on probing have improved considerably from the baseline, recorded at the time of the visit; this result is due to the synergy obtained between several factors: the improvement of patients' oral hygiene habits, the non-surgical manual etiological therapy performed by the dentist and the gradual regressive progression of the pregnancy related periodontitis. From general oral health point of view, also the identification of pathological foci, endodontic lesions, and their solution allowed the re-establishment of a new equilibrium within the oral cavity of the patients. In the present study, data obtained from clinical research were supported by data derived from laboratory analysis of gingival crevicular fluid samples, with the aim of researching the molecules of protein nature present within it, to understand its function and potential for periodontal diagnosis. The crevicular fluid has been preferred over saliva because of its close correlation with the tissues involved in pathological process related to periodontal disease: the sulcular and junctional epithelium, which constitute the gingival sulcus. The research focused on assessing the presence of peptides that play a role in the inflammatory process and in defense mechanisms by the host. The proteins analyzed were: α-defensins, thymosin, fibrinopeptides. Figure 3 shows the distribution of the recognized α-defensins within the crevicular fluid. The defensins derive from the cytoplasmic granules of neutrophils and macrophages and clinically have an inflammatory and immune function: they manage to penetrate into the cytoplasmic membranes and induce the formation of pores with consequent death by cell lysis[29]. The α defensins analyzed in this study are produced by neutrophils, consequently their increase is understandable when the body is forced to respond to the inflammatory insult caused by the periodontitis in place, as in postpartum gingivitis. These proteins are involved in the innate immune response. It can be noticed a marked increase in the study group and the reduction in the recall group which tends to normalize by approaching the values observed in the control group[18]. The presence of thymosin β_4 and β_{10} in the crevicular fluid was described by the Inzitari et al report[17]; thymosin is present not only inside the cell but also in extracellular fluids, such as in serum, wound exudate, and crevicular fluid[9]. In the samples analyzed in this study, thymosin β4 was recognized, a peptide with important functions related to wound healing processes[10]. The intracellular release mechanisms of Tβ4 are unknown and its exact function within the nucleus remains unclear. The extracellular Tβ_4 instead has a series of important functions, such as the ability to create a covalent bond with the fibrin clot, supporting the subsequent process of tissue repair and remodeling, to down-regulate different chemokines and inflammatory cytokines, such as TNF-α and to inhibit the migration of macrophages and neutrophils. Its action at the level of the oral cavity is related to the ability to suppress the production of interleukin-8 after stimulation by TNF-α and acts as an antimicrobial, anti-inflammatory and antiapoptotic peptide on gingival fibroblasts. While Tβ_4 is a powerful stimulator of angiogenesis, Tβ_{10} inhibits it and changes in the relationship between these two peptides can exert both positive and negative control[9]. From these considerations, it is possible to understand the trend of the concentration of thymosin. The value of Tβ4 is decreased in the group of patients with periodontitis, highlighting the presence of an established inflammatory pathological scenario, while it is significantly increased in the recall group. This trend reflects the organism's response to the harmful stimulus, with a reduction in the inflammatory infiltrate and the subsequent attempt to repair tissues, with angiogenesis increase. The poor representation of Tβ10 does not allow to for-

mulate a conclusion regarding its role in the periodontitis; however, it can be noticed a decrease of its value in the study group and its relative increase in the control group. The fibrinopeptides analyzed in this research are four and they were all found in the experimental group. The reason may be due to the fact that fibrinogen and fibrinopeptides are involved in the healing process and revascularization of the area affected by periodontal disease. Revascularization after vessel injury requires cell proliferation and *in vitro* studies have investigated this aspect, measuring the proliferation of cultured human endothelial cells and fibroblasts on fibrin surfaces[12]. The breakdown of fibrinopeptide A and the exposure of terminal fragments increase the proliferation of cells on fibrin and it has been demonstrated, in the same study, that specific structural features of the temporary fibrin matrix, formed in lesion sites, can modulate the proliferative response of vascular cells.

Conclusions

The collection of crevicular fluid is a rapid, economic, non-invasive procedure that allows to broaden the knowledge about certain molecular mechanisms that underlie pathophysiological situations[30]. From the comparison between clinical and laboratory data in the present study, it has been showed that GCF and the amount of proteins contained can be predictable indicators of periodontal health. It has been necessary to obtain GCF samples without contaminating the papers point with blood cell or salivary fluid, although the presence of edematous and bloody tissues. The period of pregnancy is a delicate moment in the lives of patients and the dentist should offer his or her support in maintaining a healthy oral status and in identifying the need for dental treatment, avoiding, if possible, long stays in the chair during the first trimester of pregnancy. A professional oral hygiene session in the immediate postpartum period allowed to mitigate established inflammatory pathological scenario that could have caused further damage in the oral cavity of affected patients. This procedure has in no way interfered with breastfeeding and allowed to reduce the risk of transmission of periodontal-pathogenic bacteria from mother to child. The evolution of this study will be to increase the number of patients enrolled, increase the follow-up to six months, to monitor the progression of periodontal disease.

Conflict of Interests

The Authors declare that they have no conflict of interests. No support in the form of grants was taken for this study.

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