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Difference in the expression of inflammatory mediators in gingival crevicular fluid in postmenopausal patients with chronic periodontitis with and without menopausal hormone therapy



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ABSTRACT

Objective: Hormonal changes experience by women produce significant changes in the periodontium. The aim of this study is to assess whether menopausal hormone therapy, in patients diagnosed with moderate chronic periodontitis and menopause presents a beneficial effect, in terms of clinical and immunological outcomes.

Study design: Thirty subjects with moderate chronic periodontitis and menopause were selected and assigned to two groups in accordance to the presence of menopausal hormone therapy. Periodontal clinical parameters, microbiological samples and immunological variables were assessed in both groups. Inter-group differences were evaluated using non-paired Student t-tests and chi square tests. Also, Pearson coefficient correlation was performed to determine the correlation between variables.

Results: There were statistically significant differences between groups for clinical attachment level, probing pocket depth, interleukin 1 β and interleukin 6. Smoking habit, deeper PPD and higher IL-6 levels in non-menopausal hormone therapy users group, tend to increase the interleukin 1 β GCF levels. These findings were supported by serum estrogen levels. The variables levels were higher in the menopausal hormone therapy users group.

Conclusion: Within the limitations of the present study, the hypothesis that menopausal hormone therapy user's women will show better periodontal status and differences in immunological variables respect to those being non-menopausal hormone therapy users was supported.

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1. Introduction

Aging process is complex due to its interaction among cultural, social, physiological and economic factors, so it would be inaccurate to restrict it just to age. In Spain, life expectancy presents an ascendant pattern that nowadays is greater in women than in men, a mean of seven years, establishing an aging population profile characterized not only by low birth but also low mortality rates, so there is a reduced natural growth of the population.

It should be emphasized that in Spain there are approximately six million women aged over 50 years old [1]. About 40% of them will exhibit symptoms due to menopause that will significantly affect their quality of life. So, a therapeutic approach could be a public health priority in the next years, with a great medical, social and economic impact due to the increasing number of women who will live a post-menopausal period. Some aspect such diet and lifestyle should be take into account in order to improve the quality of women's life during this period of their life [2]. Hence the importance of adult female population in the context of health cares.

Menopause is defined as the permanent cessation of menstruation which induces physiological changes due to the decline in estrogen secretion with loss of follicular function [3]. Generally, it tends to appear at the age of 45–55 years. The overall mean onset is 50 years old, with differences between industrialized and developing countries. Spanish Society of Gynecology and Obstetrics (SEGO) established that the onset age in this cohort is 51.4 years old, ranging from 48 to 54 years [4].

Hormonal changes experience by women in physiological and non-physiological situation (i.e. hormone replacement therapy) produce significant changes in the periodontium, especially in plaque-induced gingivitis [5]. Gingiva is a target tissue for steroid hormones action. During hormonal fluctuations periods, clinical modifications have been identified in the periodontal tissue [6]. In particular, estrogens may influence cytodifferentiation of the stratified squamous epithelium and also in the synthesis and maintenance of collagen fibers [7]. It has been demonstrated that estrogen receptors are present in osteoblasts related to bone metabolism [8], in fibroblasts from periosteum, in lamina propria [9] and, in periodontal ligament [10].

Estrogen-progesterone interaction with inflammation mediators may explain the higher inflammation observed during hormonal fluctuation periods. Thus, a reduction in the levels of interleukin 1 β (IL-1 β) and interleukin 6 (IL-6) induced by progesterone may lead reductions of the tissue inhibitor of metalloproteinases and increase the activity of proteolytic enzymes and high levels of tumor necrosis factor (TNF), resulting in inflammation and clinical manifestations [5,11].

Therefore, normal estrogen levels in plasma may be necessary for periodontal protection. In fact, the amount of circulating estradiol appears to be inversely correlated with the prevalence of periodontal diseases [12,13]. In vitro studies suggest that reduced levels of female sex hormones (estradiol and progesterone) alter the local and systemic production of IL-1 β and IL-6 and, postulating

that these changes may be responsible for an increased bone loss associated to menopause. [14,15].

Currently there are no clinical studies assessing the effect of menopausal hormone therapy (MHT) in post-menopausal women with periodontitis. The specific hypothesis of this investigation was that moderate chronic periodontitis women under MHT would present differences in immunological patient-based variables compared to those without MHT.

Therefore, the purpose of the present study was to compare the effect of MHT on clinical and immunological variables in moderate chronic periodontitis patients with and without MHT.

2. Material and methods

In this cross-sectional, pilot study, a consecutive sample of 83 menopausal women with moderate chronic periodontitis from the Menopause Program of the Gynecology and Obstetric Department at Madrid Centro Médico was screened for participation in the study for their posterior evaluation in the Graduate Periodontology Clinic of the Complutense University of Madrid (Spain). The protocol was submitted and approved by the Research Ethics Committee of the Hospital Clínico San Carlos (Madrid).

The following inclusion criteria were considered: systemically healthy women aged between 48–64 years with physiologic menopause and established amenorrhea for more than 12 months, presenting at least 16 teeth and with a diagnosis of moderate to advance chronic periodontitis, characterized with clinical attachment loss (CAL) ≥ 5 mm in ≥ 4 sites and probing pocket depth (PPD) ≥ 6 mm in ≥ 8 sites in, at least, two different quadrants [16]. Patients were excluded if they had taken antibiotics in the previous 3 months or those presenting systemic conditions or medications that may affect the periodontal status.

After signing an IRB-approved informed consent and having explained the study aims, procedures and the voluntary character of their participation, medical and socio-demographic data were collected. Socio-demographic data included the following: age, gender, tobacco usage, employment status, marital status, schooling, systemic diseases, allergies and medications. Subsequently, all patients were examined by one calibrated examiner, for periodontal parameters, microbiological and gingival crevicular fluid (GCF) sampling.

2.1. Clinical evaluation

One calibrated examiner assessed plaque index [17] (PI), gingival index [18] (GI), PPD, gingival recession (GR) and CAL, recorded at six sites per tooth (mesiobuccal, buccal, distobuccal, distolingual, lingual and mesiolingual), at all teeth excluding third molars. PPD, GR and CAL were recorded using a periodontal probe (North Caroline Probe, Hu-Friedy, Leinmen, Germany). A calibration trial for periodontal clinical parameters was performed with the examiner involved in 10 randomly selected patients with chronic periodontitis, not related with the study, where CAL and PPD full-mouth measures were assessed in a duplicate manner within 2 weeks.

2.2. Microbiological evaluation

Four sites, one per quadrant, being the most accessible site with the deepest pocket and bleeding, were selected. Clinical variables were specifically recorded at these sites (presence of plaque, bleeding on probing [BOP], PPD and GR). Samples were taken with two consecutive medium size paper points (Paper Point, Maillefer, Ballaigues, Switzerland) in each site. All paper points were pooled in a vial with reduced transport fluid, and transported to the laboratory, where they were processed within the next 2 h. At the laboratory, the samples were vortexed (30 s), serially diluted and plated in different media: blood agar medium (No. 2 of Oxoid; Oxoid Ltd, Basingstoke, UK), with 5% horse blood, and haemin (5 mg/l) and menadione (1 mg/l); and a selective medium for *Aggregatibacter actinomycetemcomitans*. The blood agar plates were studied after 7 and 14 days of anaerobic incubation (80% N₂; 10% H₂; 10% CO₂ at 37 °C), and the selective plates after 3–5 days of 37 °C incubation in air with 5% CO₂. Plates were carefully examined for the identification of *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia/nigrescens*, *Tannerella forsythia*, *Parvimonas micra*, *Capnocytophaga* spp., *Eikenella corrodens* and *Fusobacterium* spp., based on the morphology of the colony and using different standard biochemical tests to confirm the initial identification (RapID ANA II, Thermo Scientific, Waltham, MA, USA). Other relevant colonies (those representing an important proportion of the microbiota) were also isolated for further characterization. The total number of colonies, as well as the number of each bacterial species, was counted in a representative plate (containing between 30 and 300 colonies). Counts of *A. actinomycetemcomitans* were performed on the selective plates, based on its typical colony morphology, a catalase reaction and a set of specific enzymes.

Total counts of anaerobes in colony forming units (CFU) per sample and the frequency of detection, counts and proportions of periodontal pathogens were calculated.

2.3. Gingival crevicular fluid samples

Gingival crevicular fluid was collected from the mesiobuccal sulcus of upper first molars by means of filter paper strips (Periopaper, Harco, Irvine, CA, USA). All samples were measured for gingival fluid volume with a calibrated gingival fluid meter

(Periotrom 8000, Harco, Irvine, CA, USA) and placed in a sterile eppendorf tube [11,19]. GCF was used to measure IL-1 β and IL-6 using enzyme-linked immunosorbent assays (BLK Diagnostic International, Badalona, Barcelona, Spain). Analyses were performed according to the manufacturer's protocol. Results were calculated using the standard curves created. Concentrations were corrected for GCF volume and defined as nanograms per milliliter. The total amount of IL-1 β and IL-6 was expressed in picograms.

2.4. Data analysis

Two groups of patients were determined at baseline examination: a) the MHT users group, defined as subjects with menopausal hormone therapy (MHT); b) the non-MHT users group, defined as those without menopausal hormone therapy.

2.5. Statistical analysis

The patient was considered the unit of the analysis. Socio-demographic data were computed for each participant from data provided and compared between groups by means of χ^2 tests. Clinical parameters were registered and averaged, first for each individual patient, and then for each group. Clinical variables were expressed as mean and standard deviation (SD), including the mean percentage of sites in each PPD category: 1–3 mm (shallow), 4–6 mm (moderate) and >6 mm (deep). Clinical variables of plaque index and gingival index were transformed into quantitative variables. Inter-group differences were evaluated using non-paired Student t-tests. All variables were first evaluated to confirm a normal distribution, by means of Kolmogorov–Smirnov tests.

Data were analyzed (Statistical Package for Social Sciences for MAC, SPSS Inc., Chicago, IL, USA) and the statistical significance level was set at 5% ($p \leq 0.05$) for all analysis.

3. Results

Out of the 83-screened subjects, 30 postmenopausal women (aged 50–64 years) were included in the study. The average age of menopause onset was established in 52.33 years. Forty-one subjects did not fulfill the inclusion-exclusion criteria, and twelve refused to participate in the study (Fig. 1).

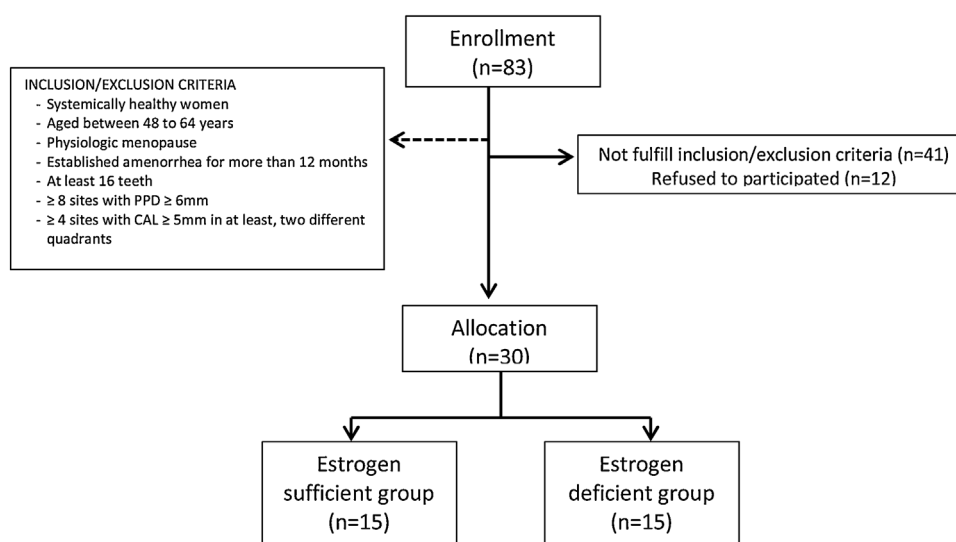


Fig. 1. Flow-chart of the study, with number of patients. MHT = Menopausal hormone therapy.

Table 1
Socio-demographic characteristics of participants.

Variable		MHT users (n = 15)	Non-MHT users (n = 15)	p
Age	Mean ± standard deviation	58.2 ± 4.1	58.2 ± 3.7	0.772
Menopause age onset	Mean ± standard deviation	52.8 ± 3.8	51.86 ± 2.7	0.541
Marital status	Unmarried	4	3	0.648
	Married	9	11	
	Divorced	0	0	
	Widowed	2	1	
Tobacco	Smokers	8	9	0.068
	Non-smokers	7	6	
Drugs	Yes	2	1	0.271
	No	13	14	
Systemic disease	Yes	1	0	0.585
	No	14	15	
Allergies	Familiar	1	2	0.749
	No	14	13	
Schooling	Middle school	1	3	0.164
	High school	10	7	
	Professional training	0	0	
	Degree	4	5	
Employment Status	Hired hand	2	2	0.978
	Self-employment	4	3	
	Student	0	0	
	Housewife	7	5	
	Retired	1	1	
	Unemployed	1	4	
	Other	0	0	

MHT = Menopausal hormone therapy.

3.1. Socio-demographic data

The socio-demographic data of all subjects are summarized in Table 1 and it shows the population distribution according to the socio-demographic data. No statistically significant differences ($p > 0.05$) were detected between groups for any of the variables collected.

3.2. Clinical parameters

3.2.1. Plaque and gingival index

There were no statistically significant differences between groups, being the average plaque value of 1.06 ± 0.44 . The highest value corresponded to proximal sites (1.20 ± 0.46) while the minimum value to free surfaces (0.78 ± 0.42) (Table 2).

3.2.2. Gingival recession

Mean gingival recession was 0.82 ± 0.10 mm and there were no statistically significant differences between groups ($p > 0.05$).

Table 2

Mean percentages (\pm standard deviation) of periodontal clinical parameters in the two groups.

Variables	MHT users	Non-MHT users	p
Plaque Index	1.04 ± 0.38	10.8 ± 0.51	0.813
Gingival Index	0.60 ± 0.29	0.80 ± 0.38	0.353
CAL (mm)	3.7 ± 0.46	4.6 ± 0.39	0.002
PPD (mm)	2.93 ± 0.14	3.71 ± 0.16	0.001
REC (mm)	0.77 ± 0.78	0.87 ± 0.63	0.699

MHT = Menopausal hormone therapy; CAL = clinical attachment level; PPD = probing pocket depth; REC = recession.

There were also no differences when data was analyzed based on the type of site: proximal and buccal/lingual/palatal sites ($p > 0.05$).

3.2.3. Probing pocket depth

Mean PPD of the whole sample was 3.32 ± 0.70 mm. In the subgroup analysis, the non-MHT users group showed statistically significant higher values than the MHT users group: 3.71 ± 0.16 mm vs 2.93 ± 0.14 mm ($p < 0.05$).

Deeper PPD were localized at the proximal sites (3.70 ± 0.80 mm) and shallower PPD were localized at buccal and lingual/palatal sites (2.55 ± 0.59 mm), there were statistically significant differences between groups for PPD in both type of sites, showing deeper values, approximately 1 mm, in the MHT users group.

3.2.4. Clinical attachment level

There were statistically significant differences between groups for CAL, showing greater levels in the MHT users group (4.6 ± 0.20 mm).

3.3. Immunological variables

Mean GCF volume obtained from the patients was 0.67 ± 0.21 μ L. There were statistically significant differences between groups: more CGF volume was obtained from patients without menopausal hormone therapy (0.81 μ L) than patients with the therapy (0.53 μ L) ($p = 0.026$).

The whole sample mean concentration of IL-1 β was 53.24 ± 30.22 pg/mL. For this variable, there were statistically significant differences between groups ($p < 0.001$), being the group without menopausal hormone therapy the one showing higher levels.

For the IL-6, whole sample mean concentration was 51.37 ± 30.13 pg/ml and there were also statistically significant differences between groups ($p < 0.001$), being the group without menopausal hormone therapy the one showing higher levels (80.16 ± 9.44 pg/mL) (Table 3).

3.4. Correlation analysis between immunological and periodontal clinical parameters

IL-1 β GCF levels showed to be statistically related to the study group ($p < 0.001$), the smoking habit ($p = 0.013$), mean PPD ($p = 0.005$), PPD in posterior teeth ($p < 0.001$), PPD at proximal sites ($p = 0.003$) and IL-6 levels ($p < 0.001$).

Both the study group and IL-6 levels obtained the highest correlation with the IL-1 β concentration ($r = 0.943$ and $r = 0.940$ respectively). PPD showed also high correlation while the smoking habit showed moderate correlation ($r = 0.448$). Then the smoking habit, deeper PPD and higher IL-6 levels in non-MHT users group, tend to increase the IL-1 β GCF levels.

IL-6 GCF levels showed to be statistically related to the study group ($p < 0.001$), mean PPD ($p < 0.001$) at both tooth level: anterior teeth ($p = 0.160$) and posterior teeth ($p < 0.001$), and site level: proximal sites ($p < 0.001$) and non-proximal ($p = 0.010$); and IL-1 β levels ($p < 0.001$). Both the study group and IL-1 β levels obtained the highest correlation with the IL-6 ($r = 0.966$ and $r = 0.940$ respectively). High correlation was obtained for PPD at posterior teeth and proximal sites. Then the increase of the value of any of these variables would tend to increase also IL-6 GCF levels (Table 4).

3.5. Microbiological variables

The percentage of sites positive for *Aggregatibacter actinomycetemcomitans*, *Tannerella forsythia*, *Porphyromonas gingivalis*, *Prevotella intermedia*, *Parvimonas micra*, *Fusobacterium nucleatum* and *Camphylobacter rectus* over the course of the study is shown in Table 5. There were no statistically significant differences between groups.

4. Comment

The results of the present pilot study show that the patients undergoing menopausal hormone therapy (MHT), test group, had

Table 3
Mean percentages (\pm standard deviation) of immunological parameters in the two groups.

Variables	MHT users	Non-MHT users	<i>p</i>
GCF volume (μ L)	0.53 ± 0.38	0.81 ± 0.51	0.026
IL-1 β concentration (pg/mL)	25.24 ± 8.55	81.25 ± 11.74	<0.001
IL-6 concentration (pg/mL)	22.58 ± 6.19	80.16 ± 9.44	<0.001

MHT = Menopausal hormone therapy; GCF = gingival crevicular fluid; IL-1 β = interleukin 1 beta; IL6 = interleukin 6.

Table 4
Correlation analysis between immunological and periodontal clinical parameters.

Variables	IL-1 β concentration (pg/mL)	IL-6 concentration (pg/mL)
Group	0.943	0.966
Tobacco	0.448	0.284
Mean PPD	0.501	0.601
Mean anterior PPD	0.353	0.436
Mean posterior PPD	0.556	0.660
Mean interproximal PPD	0.524	0.616
Mean free PPD	0.355	0.461
IL-1 β concentration (pg/mL)	1	0.940
IL-6 concentration (pg/mL)	0.940	1

PPD = probing pocket depth; IL-1 β = interleukin 1 beta; IL-6 = interleukin 6.

Table 5
Percentages of sites positive for each bacterial species in the two groups.

Variables	MHT users	Non-MHT users
Aa	4.6	4.7
Pg	49.56	53.4
Tf	8.16	6.65
Pi	19.3	23.5
Pm	30.4	25.0
Fn	25.5	30.6
Cr	2.9	1.0

MHT = Menopausal hormone therapy; A.a. = *Aggregatibacter actinomycetemcomitans*; P.g. = *Porphyromonas gingivalis*; T.f. = *Tannerella forsythia*; P.i. = *Prevotella intermedia*; P.m. = *Parvimonas micra*; F.n. = *Fusobacterium nucleatum*; C.r. = *Camphylobacter rectus*.

shallower PPD compared to the control group, those who didn't receive the therapy. This difference was statistically significant ($p < 0.001$). For the rest of the clinical variables, the control group showed greater values but these differences didn't reach a statistically significance ($p > 0.05$).

Risks associated to the MHT generate controversy [20,21] although it is being prescribed to avoid hot flashes and systemic menopause consequences as osseous fractures due to osteoporosis. Prospective studies [22,23] showed a 50% reduction in hip fractures in women undergoing MHT compared to those who didn't received the therapy. This effect of the MHT in bone metabolism has been proposed to influence alveolar bone loss [12,13] and periodontal status.

Previous research has shown that during menopause the risk of tooth loss is reduced in women receiving MHT [13,23]. Plasmatic estrogen levels influence alveolar bone density and lower levels have shown to be related to a more advanced periodontal affection [24]. In this pilot study women in the test group showed presented better periodontal situation after receiving MHT. These results are correlated to those obtained in a study with 190 Spanish patients that showed better CAL in those receiving MHT for at least one year [25]. However, a randomized clinical trial by Pilgram and coworkers [26] didn't find statistically significant differences for CAL in 135 women under MHT. For gingival recession, the results of the present study showed a tendency for more recession in women without MHT ($p = 0.669$), these results are in agreement with those obtained by Ronderos and coworkers [27] who did get statistically significant differences for this parameter.

Reindhart and coworkers [5] reported that women under MHT had less gingival inflammation and that having serum levels of estradiol > 40 pg/ml was associated to less loss of attachment. In the current study blood samples were not taken to report hormonal serum levels, being one of the study's limitation.

When analyzing other studies, the effect of the MHT on the periodontal status is similar between them. Studies in Brazilian and German patients established that in those aged between 50–69 years old the prevalence of periodontal disease is higher in menopause women who are not under MHT compared to those who are this statement is in agreement to the results of the present study [28,29]. However, the American population analyzed in the NHANES III study observed a lower prevalence in patients without MHT [30] and for the Japanese woman differences between both study groups could not be found [31].

Sexual hormones have shown to influence periodontal tissues and periodontal disease progression [32–34] then it could explain the results obtained in the present study that seem to justify that MHT plays a roll in chronic periodontitis. However there is little information about the effect of feminine sexual hormones on oral microbiota in menopause women.

In the present study it was observed that patients of the non-MHT users group showed more oral manifestations than the MHT

users group but didn't reach statistically significant differences, this has been also observed in previous similar studies [25,35].

Reinhardt and coworkers [36] showed that GCF levels of IL-1 β y IL-6 were increased in menopause patients with refractory periodontitis who didn't received MHT compared to those who received it, so local inflammation would not be the only cause for this increase but also the level of estrogens. Another study performed by the same authors determined that patients under MHT showed lower levels of IL-1 β compared to patients without MHT; this data suggests that low levels of estrogens are associated to gingival production of IL-1 β influencing the progression of periodontal disease. In the present study the results are in agreement with previous data: test group showed lower levels of inflammatory mediators and a better periodontal status. The level of estrogens and PPD explains these differences. According to the variable PPD, when it increases 0.2 mm, there is an increment of 8.01 pg/ml of IL-1 β and 7.73 pg/ml of IL-6.

To our knowledge, the present study is the first to analyze in detail the association of the immunological, microbiological and periodontal status in menopause women comparing two groups based on the presence of MHT. However, one of the main limitations of this study is that there is no information about the estrogens levels to established future detection thresholds and relate them with other variables. The preliminary results of this pilot study should be interpreted with caution due to the small sample size and the cross-sectional design. In this type of study it can be established an association between variables but not a temporary consistency or casualty due to the lack of a follow-up of the patients. The type of MHT was not assessed in the present study, and despite there are no systematic reviews that evaluate the effect of different type of MHT on the microbiological, immunological and periodontal status, it should be considered as an important factor affecting the estrogen levels. Finally, another limitation of the present study is the lack of stratification by estrogen levels, PPD or number of cigarettes in smoker patients.

Within the limitations of the present study, the hypothesis that moderate chronic periodontitis women under MHT would present differences in immunological variables, based on patient, compared to those without MHT was supported.

Conflict of interest

The authors declare that they have no conflicts of interest in this study. The study was designed, conducted and analyzed by researchers belonging to the Host Response in Oral Pathology (HROP) Research Group (Complutense University, Madrid, Spain).

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