

UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA
DOCTORAL SCHOOL



CONTRIBUTIONS TO THE POSSIBLE
COMMON PATHOGENIC PATHWAYS OF THE
PERIODONTAL DISEASE – CHRONIC HEPATITIS C
ASSOCIATION

PhD THESIS

ABSTRACT

PhD COORDINATOR:

PROF. UNIV. DR. PETRA ȘURLIN

PhD STUDENT:

DORIN-NICOLAE GHEORGHE

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KEY WORDS

Periodontal disease

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Pathogenic

Connections

Physiopathogenic mechanisms

INTRODUCTION

In 2012, the first official joint meeting of the European Federation of Periodontology and the American Academy of Periodontology on “Periodontitis and Systemic Health” took place. The meeting aimed to increase the awareness among general dental practitioners, periodontists and other general medicine specialists on the proven scientific correlations between the periodontal disease and systemic conditions such as diabetes mellitus, cardiovascular disease or even non-pathologic situations as pregnancy and child-birth. These connections have formed the concept of “periodontal medicine” (Chapple & Genco, 2013).

Although the scientific data is rich in research projects and results on the bidirectional connections between periodontal disease and systemic conditions, the subject has been scarcely approached from this standpoint on the association of periodontal disease and chronic hepatitis C. The common chronic inflammatory character of the two conditions and the important changes of the normal function of the metabolic and immunological processes caused by hepatitis C viral infection were the initial motivation and justification of the research project’s design. In addition, Romania has one of the highest hepatitis C prevalence rates in Europe, with an estimated over half a million infected individuals (Gheorghe L. Et al., 2010). Regarding the periodontal disease’s prevalence in the country, no national epidemiological studies have been conducted so far, but it is estimated that more than half of the population manifests signs of periodontal inflammation such as frequent gingival bleeding (Mârțu et al., 2013). Therefore, given the high national and global prevalence rates of the two conditions, the research into possible common pathogenic connections could help upgrade the type of dental and periodontal care that chronic hepatitis C receive and, thus, to improve their life quality. In addition, when needed, periodontal therapy could also be included as part of the general treatment plan of these patients.

The personal contributions on the research subject consisted of complementary studies that allowed concluding on the initial scientific hypothesis: the existence of common pathogenic pathways between periodontal disease and chronic hepatitis C, which need to be identified and tested, thus proving the bidirectional nature of this association.

To prove the scientific hypothesis, general and specific research objectives were established:

A. General objective: the testing of the work hypothesis that common pathogenic connections exist between periodontal disease and chronic hepatitis C of bidirectional nature, by conducting clinical and fundamental studies.

B. Specific objectives:

1. Testing the given hypothesis that chronic hepatitis C can act as a negative factor on the clinical aspects of periodontal disease, by conducting preliminary clinical, immunological, imagistic and histological studies, presented in Chapter III.

2. Testing the given hypothesis that periodontal disease can have a negative impact on the clinical, metabolic and immunological status of chronic hepatitis C patients, by conducting clinical and fundamental studies, presented in Chapter IV.

STATE OF CURRENT KNOWLEDGE

I. PERIODONTAL DISEASES

The most common etiological factor of the majority of periodontal diseases is the bacterial plaque or the so-called bacterial biofilm (Schaudinn et al., 2009). The first signs of gingival inflammation tend to appear after more than 48 hours of bacterial biofilm deposition beneath the gingival sulcus (Ji, Choi & Choi, 2015). Besides the bacterial determinant factor, which is in a close connection with the type of immuno-inflammatory response of the host, some other local and systemic favouring factors are involved in the aetiology of periodontal disease (Genco & Borgnakke, 2013). There are two possibilities for the gingival inflammation to evolve, one of chronicity and another one of altering the profound tissues towards the profound periodontal system, for the susceptible patients, according to the intensity and the quality of immuno-inflammatory response of the host, but also to the presence of the risk factors mentioned above (Chapple et al., 2015). Chronic periodontitis has an oscillatory type of evolution, when the balance periods and apparent healing alternate with some active episodes of the disease, when the tissular destruction is actually produced (Holtfreter et al., 2015). Towards the last decades, scientific researchers have stated some results that allowed the constitution of a new concept of periodontal medicine, which implies relationships between periodontal disease and some systemic conditions such as diabetes mellitus, cardiovascular diseases and rheumatoid arthritis (Linden, Lyons & Scannapieco, 2013).

II. CHRONIC HEPATITIS C AND ITS IMPACT ON THE GENERAL AND ORAL HEALTH

The infection with the hepatitis C virus (HCV) represents one of the most important problems for the global public health and it may also be considered one of the most geographical extensive epidemic, affecting a large number of patients and a important amount of time since mankind is trying to find a cure for it. Therefore, at a global level, there are some estimated figures: 130-150 millions of people, 2.8% of total population of the planet are infected with hepatitis C virus (Vos et al., 2016). The main way of transferring the altered blood cells from one infected patient to a healthy one is represented by their subcutaneous or percutaneous penetration using some sharp or cutting objects that may cross the epidermal or mucosal barriers (Wilkins et al., 2010). The penetration of the hepatitis C virus in the organism is a key moment for the activation of physio-pathological processes that may lead to the developing of an infectious condition, which will produce the hepatic tissues' disintegration by means of chronicity (Poynard et al., 2003). The incomplete elimination of the HCV from the human body will lead to a chronic infection, as it is the case with 80% of infected patients (Osburn et al., 2010).

While the dental and periodontal implications in patients with chronic hepatitis C (CHC) may be an indirect response of the viral infection, the extra-hepatic manifestations in the oral cavity (such as lichen planus, Sjögren syndrome and oral squamous cell carcinoma) are a direct cause of the immunologic deficiency of this type of patients (Alavian, Mahboobi & Karayiannis, 2013). Testing the saliva for identifying

the antigens and antibodies has proved similar or superior grades of specificity when compared to blood serum, by using some modern methods of diagnosis and detection of CHC, such as rapid oral tests, which can be improved (Açikgöz et al., 2009). Considering some internal factors such as cellular insulin resistance, a dysfunctional immune system or chronic inflammation, with the external ones such as lack of a proper dental assistance, all these may increase the periodontal disease rate of appearance in patients with Chronic Hepatitis C.

PERSONAL CONTRIBUTIONS

III. RESEARCH ON THE POSSIBLE PHYSIOPATHOGENIC MECHANISMS OF PERIODONTAL DISEASE AND CHRONIC HEPATITIS C ASSOCIATION

In order to test the given hypothesis that chronic hepatitis C can as a negative factor on the evolution of periodontal disease, we set up a preliminary study aimed to identify connections between the periodontal and hepatic conditions, from a clinical, metabolical, immunological, imagistic and histological standpoint. The existence of such connections could motivate the extent of study on a wider group of patients in order to assess pro-inflammatory markers in samples of gingival crevicular fluid from these patients.

For the preliminary study, four research directions stemming from the assessment of the pathogenic mechanisms were established, on clinical, metabolical, immunological, imagistic and histological levels.

For the development of the preliminary study, following the four research directions, three groups of patients were set:

- PH group: periodontal patients with asymptomatic/inactive chronic hepatitis C, addressing for periodontal examination, diagnosis and treatment,
- P group: periodontal patients with no systemic illness, addressing for periodontal examination, diagnosis and treatment,
- H group: control, systemically and periodontal healthy patients, addressing for periodontal evaluation prior to orthodontic treatment, some of whom required extraction of upper bicuspids for orthodontic reasons

III.1. CLINICAL ASSESSMENT OF PERIODONTAL AND SOME BIOCHEMICAL HEPATIC PARAMETERS IN PERIODONTAL PATIENTS WITH CHRONIC HEPATITIS C

The aim of this study was to investigate whether chronic hepatitis C can act as a negative factor on periodontal disease, by identifying some correlations between the assessed parameters of periodontal disease and some biochemical liver parameters in periodontal patients with chronic hepatitis C, in comparison to those of systemically healthy periodontal patients.

The periodontal status of the patients was evaluated by assessing the periodontal pocket depth, the number of teeth with periodontal pockets deeper than 4mms, the Silness and Loe gingival index, the maximum periodontal probing depth and

the number of existing teeth. The level of serum hepatic enzymes (transaminases) was also assessed, as well as the age of the chronic hepatitis C diagnosis.

The periodontal status of the PH patients was less favourable than that of the P group, statistically significant differences being found between the two groups regarding the number of existing teeth, the number of teeth with periodontal pockets deeper than 4mms and the maximum periodontal probing depth. Such differences were also found between the transaminases levels for the two groups, as well as for all the assessed parameters between the periodontal patients and the control group.

Given the limits of this study, it can be stated that chronic hepatitis C could act as a negative factor on periodontal disease's evolution. Periodontal patients with chronic hepatitis C can express a less favourable oral, periodontal and hepatic status than the systemically healthy periodontal patients, in some accordance with the age of the chronic hepatitis C diagnosis. These results motivate the conduction of complementary studies aimed to assess in quantity and quality the existence of some biological markers within gingival tissue and fluid samples of hepatitis C patients that would confirm the initial findings of this study.

III. 2. IMMUNOLOGICAL ASSESSMENT OF GINGIVAL CREVICULAR FLUID INTERLEUKIN 1-ALPHA AND INTERLEUKIN 1-BETA LEVELS IN PERIODONTAL PATIENTS WITH CHRONIC HEPATITIS C

Increased Interleukin-1beta levels have been found in gingival fluid samples originating from active periodontal disease sites, in close connection to the disease's severity (Duarte et al., 2014). Interleukin-1beta is a particular member of the interleukin-1 family, being an important indicator of the inflammatory disease's evolution and intensity (Graves & Cochran, 2003). The hypothesis of the study was that the IL-1 β and IL-1 α gingival fluid levels could be more elevated in periodontal patients with chronic hepatitis C, compared to periodontal patients with no systemic disease. This fact could imply that chronic hepatitis C could act as an additional negative factor for periodontal disease manifestations.

The gingival crevicular samples were collected from the participating patients by using absorbent paper strips (PerioPaper, Oraflow Inc., Smithtown, NY, SUA). For the quantitative assessment of the two markers the Enzyme-Linked Immunosorbent Assay – ELISA was used. Special micro-quantity detection kits were used for the cytokine panel, according to the manufacturer's indications and prescribed method (Quantikine, R & D Systems, Minneapolis, USA). Statistically significant differences were found between the IL-1 α levels of the PH and P groups (the average value for the PH group being 1.8 times higher than that of the P group). The IL-1 β gingival crevicular levels also recorded statistically significant differences between the PH and P groups (the average value of the PH group being 2.2 times higher than that of the P group).

The increased gingival fluid levels of IL-1 β and IL-1 α can offer some explanations about the physiopathologic mechanisms that lay between periodontal disease and chronic hepatitis C. By changing the general homeostasis, the chronic inflammatory reaction that the two conditions trigger can lead on a modified host immune response. Given the limitations of the study due to the low number of

participating patients, it can be suggested that periodontal patients with chronic hepatitis C could express more elevated profile of some cytokines, as a result of the added effect that periodontal and hepatic chronic inflammation have on one another.

III.3. IMAGISTICAL ASSESSMENT OF GINGIVAL EPITHELIUM'S STRUCTURAL CHANGES BY OPTICAL COHERENCE TOMOGRAPHY IN PERIODONTAL PATIENTS WITH CHRONIC HEPATITIS C

Optical coherence tomography (OCT) is an imagistic method which can deliver optical sections of an object's surface or shallow depth, in black/gray and white images (Srinivasan et al., 2017). This *in vitro* study uses OCT to assess the possible influence that chronic hepatitis C could have on the inflammatory changes in periodontal patients. In addition, the changes occurring with the structure of the gingival epithelial tissue could also be observed and the density of the tissue could be measured on the resulted images of the OCT analysis. Some associations between the gingival inflammatory status and the clinical status could be found, including in periodontal patients with chronic hepatitis C.

For this study, only the patients requiring surgical periodontal therapy were included (for the PH and P groups), as well as those with indication for tooth extraction for orthodontic reasons (for the H group). The gingival tissue samples were collected from periodontal pockets during standard periodontal surgical therapy for the periodontal patients and during periodontally sound teeth extraction for orthodontic purposes for the control patients. The gingival tissues samples were OCT-assessed and for each the median resulting picture was used for pixel density analysis in the gingival epithelium area, using ImageJ software.

The highest pixel density average value was found for the OCT gingival tissue images of the H group, followed by that of the P group (1.35 times lower) and that of the PH group (1.79 lower), the differences between the groups being statistically significant.

In what concerns the periodontal disease, the chronic inflammatory reaction caused by hepatitis C virus infection can have a negative impact on the density of the gingival epithelium. The structural changes found in the tissular architecture of the gingival epithelium could be interpreted as an indicator of the inflammatory status in periodontal patients, in comparison to the healthy control group. Given the limitations of the study caused by the small number of participating patients, it can be stated that in the future, OCT assessment could become a helpful diagnosis periodontal tool, if further technical developments will be made. In what concerns the structural changes of the shallow gingival epithelium, analyzed via OCT, chronic hepatitis C could be considered as a negative factor when associated with periodontal pathology.

III.4. HISTOPATHOLOGICAL AND IMMUNOHISTOCHEMISTRY STUDY ON THE GINGIVAL TISSUE'S CHANGES IN PERIODONTAL PATIENTS WITH CHRONIC HEPATITIS C

Matrix-metalloproteinases (MMPs) are a class of proteolytic enzymes that act on the protein components of the extracellular matrix (Dahan et al., 2001). In periodontal

disease, MMP-9 has been found in high concentrations, especially in chronic forms of gingivitis and periodontitis (Marcaccini et al., 2010).

The aim of this study was to make a qualitative assessment of the histopathological changes that occur within the gingival tissue of the periodontal patients with chronic hepatitis C and to observe any structural particularities, given the gingival inflammatory reaction. Moreover, the level of MMP-9 tissular expression in the inflamed gingival tissues could also be assessed by immunohistochemistry analysis.

The histopathological assessment was performed by standard hematoxylin-eosin staining. The immunohistochemistry analysis aimed to detect the existence of tissular MMP-9 by LSAB technique, using rabbit anti-MMP-9 antibodies (Abcam ab38898, Cambridge, UK). Upon staining and immunohistochemistry analysis, the samples were microscopically assessed with Nikon Eclipse 90i (Tokyo, Japan) and photographed NIS Elements (Nikon, Tokyo, Japan). The level of MMP-9 positive immunohistochemistry reaction was measured on each image with the help of the integrated optical density (IOD) technique.

The average IOD value was lowest for the H group, while the P group's value was 8.1 times higher and PH group's one 13.6 times. The average IOD value of the PH group was 1.6 times higher than that of the P group.

From a histological standpoint, the inflammatory periodontal reaction is better represented within the tissue samples of the periodontal patients with chronic hepatitis C than within those of the systemically healthy periodontal patients. The immunohistochemistry reaction showed that the gingival tissue samples of the PH group expressed higher levels of MMP-9 than those of the P group. Taking into account the low number of participating patients, MMP-9 could signal a more intense inflammatory reaction for the periodontal patients with chronic hepatitis C in comparison to the systemically healthy periodontal patients, at least from a histopathological perspective. Thus, MMP-9 could be an important marker for inflammation assessment in both periodontal disease and chronic hepatitis C.

IV. RESEARCH ON THE PERIODONTAL CHANGES OCCURING IN CHRONIC HEPATITIS C PATIENTS AND THE POSSIBLE INVOLVED MECHANISMS

In order to test the hypothesis that periodontal disease can have a negative impact on the clinical, metabolic and immune status of the chronic hepatitis C patients, some patients of the Gastroenterology Ward of the Emergency County Hospital in Craiova, who had previously been diagnosed with chronic hepatitis C in an inactive/asymptomatic stage of evolution, were periodontally examined.

This study included four groups of participating patients:

- HCV + BP group: patients with chronic hepatitis C who were also diagnosed with periodontal disease upon oral examination,
- HCV group: patients with chronic hepatitis C, with no periodontal condition
- BP group: periodontal patients with no systemic disease, addressing for periodontal diagnosis and treatment,
- H group: control patients, periodontally and systemically healthy, addressing for oral preventive examination.

For the participating patients, two main research directions were set: the clinical and the immunological one. There is only a couple of studies in the existing scientific literature that addressed this research idea, which gives the proposed method novelty. By identifying some correlations between the dental/periodontal status and the metabolic and immunologic aimed parameters, some physiopathogenic mechanisms, that would connect common pathways between periodontal disease and chronic hepatitis C, could be suggested

IV.1. CLINICAL AND STATISTICAL ASSESSMENT OF THE PERIODONTAL, METABOLIC AND HEPATIC STATUS IN CHRONIC HEPATITIS C PATIENTS

The purpose of the study was to analyze, from a clinical and statistical point of view, the periodontal, hepatic and metabolic status of patients having chronic hepatitis C in order to identify some particularities between periodontal and hepatic diseases expressions, by comparing groups of patients such as chronic hepatitis C without periodontal disease patients, periodontal patients without systemic disorders and healthy control patients, also aiming to identify some correlations between clinical and metabolic parameters.

The study participants were periodontally examined for establishing the clinical level of periodontal disease; they were also submitted to some laboratory blood tests for the metabolic and hepatic evaluations and a hepatic elastography test was performed in patients having chronic hepatitis C in order to determine the level of hepatic fibrosis.

Keeping in mind the existing chronic hepatic pathology, periodontal patients presented worsened metabolic, hepatic and immunological status compared with the group of patients with chronic hepatitis C but without any periodontal disease, which may suggest the negative impact of the periodontal disease added over chronic hepatitis C manifestations. When there is no periodontal disease, maybe because of the social and behavioural particularities and the restraint towards a dental treatment both for patients and for medical staff, patients having chronic hepatitis C presented a less favourable dental status than the control group, which emphasizes the negative impact this hepatic disorder might have over the oral health state. Due to the low number of patients that did not allow the proper division in groups of different periodontal disease stages, a future research project should expand this area of interest, in order to find some possible correlations between the dental status, the presence of chronic hepatitis C and the level of severity for periodontal disease. Identified correlations between clinical and metabolic parameters support the systemic implications that periodontal disease involves in patients with chronic hepatitis C. The homeostasis disruption could offer some arguments for establishing some associations between periodontal and hepatic inflammation, occurring in chronic hepatitis C and periodontal disease when combined.

IV.2. IMMUNOLOGICAL STUDY ON THE GINGIVAL CREVICULAR FLUID NLRP3 INFLAMMASOME, CASPASE-1 AND INTERLEUKIN 18 LEVELS IN CHRONIC HEPATITIS C PATIENTS WITH/WITHOUT PERIODONTAL DISEASE

Caspase-1 is an enzyme having a determinant role in starting the immuno-inflammatory reaction, because it makes the transformation of cytokine precursors such as interleukin-1 β (IL-1 β) and interleukin 18 (IL-18) in adult, functional molecules (Lu et al., 2016). The activation of NLRP3 inflammasome is triggered by bacterial stimuli such as lipopolysaccharide and bacterial ARN, by endogenous stimuli, for example extracellular ATP, oxygen reactive products, uric acid and cholesterol crystals or by DAMPs stimuli (Ulland & Sutterwala, 2011). NLRP3 has been associated with cellular insulin resistance in hepatitis C patients, which can also occur in periodontal patients (Gurav, 2012; Rheinheimer et al., 2017). Alongside IL-1 β , IL-18 is the first cytokine disposed in the inflammatory reaction cascade, being activated by NLRP3 inflammasome (Dinarello, Novick & Kaplanski, 2013). IL-18 has important implications towards the cellular mechanisms of immunity, its proper stimulation of natural killer cells and other T lymphocytes causing the release of other cytokines from the interferon family, mostly the gamma interferon (IFN- γ) (Novick et al., 2013).

The purpose of this study is to measure the levels of NLRP3, CASP1 and IL-18 in the gingival fluid samples of patients having chronic hepatitis C with/without periodontal disease, for an eventually evaluation of significant differences between these elements that plays an important role in the triggering of the inflammatory reaction, but also to verify the possible correlations between the levels of NLRP3, CASP1 and IL-18 and the oral and periodontal status of these patients, which was exhibited in the previous study.

After the clinical, oral and periodontal evaluations of all patients, the appropriate collection of the gingival fluid was performed individually by using absorbing paper strips (PerioPaper, Oraflow Inc., Smithtown, NY, SUA), which were gently introduced in the gingival sulcus or the periodontal pocket. The ELISA method was used (enzyme-linked immunosorbent assay) for quantity dosing of targeted molecular compounds in the gingival fluid. The existing correlations between the immunological parameters values and the clinical, metabolic data of the patient batch, stated in the previous study, were analyzed.

The highest average value of NLRP3 in the gingival fluid was registered in the HCV + BP group, being 1.69 times higher than the similar value of the HCV group, 1.26 times higher than that of the BP group and 2.66 times higher than that of the H group. The lowest average value of the CASP1 in the gingival fluid was found for H group, while the medium value was 4.77 times higher for the HCV group, 7.11 times higher for the BP group and 9.43 times higher for the HCV+BP group. The highest average value of IL-18 in the gingival fluid samples was found in the HCV+BP group, the medium values of IL-18 for the HCV group being 1.8 times lower, for the BP group 1.31 times lower and for the H group 3.36 times lower. All the obtained differences were statistically significant.

Patients with periodontal disease and chronic hepatitis C presented higher values for NLRP3, CASP1 and IL-18 in the gingival fluid samples than periodontal

patients without hepatitis, which may prove the possible implications between the association of two types of inflammatory conditions over the general inflammatory status of a patients and the synergy of pathogenic mechanisms that occurs in such patients. Patients having chronic hepatitis C, but without any periodontal disease, presented higher significant values for NLRP3, CASP1 and IL-18 in their gingival fluid than the control group, which may suggest the important impact that chronic hepatic inflammation has on the general homeostasis, even if there is no associated periodontal disease. The proper measurement of NLRP3, CASP1 and IL-18 within the gingival fluid may constitute a viable and precise marker for measuring the inflammation activity of periodontal disease, these specific levels of the mentioned parameters offering variations that are related to the patients' periodontal status.

V. FINAL CONCLUSIONS

1. From a clinical point of view, the dental and periodontal status of periodontal patients with chronic hepatitis C was less favourable than that of periodontal patients without chronic hepatitis C, probably because of some socio-behavioural, metabolic, immunologic and histological particularities of these patients.

2. Inflammation changes were identified at an imagistic and also at a histological level in the gingival tissue samples collected from periodontal patients having chronic hepatitis C, which may suggest the possibility that this kind of patients have a higher periodontal inflammation reaction than the periodontal patients without hepatitis.

3. From an immunological point of view, higher levels of some markers with pro-inflammatory role were found in the gingival fluid of periodontal patients with chronic hepatitis C than in those without hepatic disorder, which demonstrate the synergism and reciprocal augmentation that might exist between periodontal and hepatic inflammation, but also offer a common physiopathogenic substrate of the two diseases.

4. The over-adding of periodontal inflammation in patients with chronic hepatitis C may cause some imbalance in their inflammatory status, which may have some important implications over the general homeostasis and metabolic mechanisms.

5. As it was previously suggested by other scientific researches, the cellular insulin resistance may constitute a pathogenic mechanism responsible for the appearance of chronic inflammation disorders in chronic hepatitis C and periodontal disease, assumption sustained by the actual presence of some pro-inflammatory markers such as the inflammasome NLRP3.

6. The extension of further scientific research, concerning both cellular and molecular levels, may offer a physiopathogenic fundamental concept common between the inflammatory reactions occurring in periodontal disease and chronic hepatitis C and the way they might interact.

7. The proper evaluation of dental and periodontal status should be included in the monitoring strategy of patients having chronic hepatitis C, with double purpose: improving their life quality by means of functional adjusting their dento-maxilar apparatus and reducing the intensity of the systemic inflammatory reaction, so that some metabolic and immunological imbalances, that may cause the worsening of both disorders or even the triggering of some new conditions, could be prevented

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