Journal of Inflammation Research

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ORIGINAL RESEARCH

Expression of Vascular Adhesion Protein-1 and Thrombospondin-1 in Gingival Crevicular Fluid of Patients with Periodontitis and Non-Alcoholic Fatty Liver Disease

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Purpose: Non-alcoholic fatty liver disease (NAFLD) represents a heterogeneous spectrum of liver diseases that encompass simple steatosis, non-alcoholic steatohepatitis (NASH), and advanced fibrosis or cirrhosis. Periodontitis is a chronic infectious disease with multiple causal factors that presents a complex interaction between the microbial biofilm and the host's immune response. The aim of this study was to investigate the concentrations of Vascular Adhesion Protein-1 (VAP-1) and Thrombospondin-1 (TSP-1) in patients with coexisting periodontitis and NAFLD.

Patients and Methods: This study included 48 patients, who were dental and periodontal assessed. Of these patients, 25 were diagnosed with NAFLD. After performing the periodontal clinical examination, gingival crevicular fluid (GCF) samples were collected. Enzyme-linked immunosorbent assay (ELISA) dedicated kits tests were used for the detection and quantitative determination of VAP-1 and TSP-1 in GCF samples. Statistical methods were applied for the comparison and correlation of data.

Results: VAP-1 and TSP-1 levels showed significant differences between all test and control groups ($p<0.0001$). Statistically significant correlations ($p<0.05$) between VAP-1 and periodontal and liver parameters were found in patients with NAFLD and periodontitis.

Conclusion: Periodontal inflammation is more marked in patients with periodontitis-NAFLD association. Vascular adhesion and angiogenesis could be affected in patients with periodontitis and NAFLD. These findings could suggest that addressing periodontal inflammation in individuals with the periodontitis-NAFLD association may have a broader impact on vascular adhesion and angiogenesis, highlighting the interplay between oral health and liver conditions for comprehensive patient care.

Keywords: non-alcoholic fatty liver disease, periodontitis, VAP-1, TSP-1, gingival crevicular fluid, periodontal disease

Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by the substantial accumulation of fat within the hepatocytes of individuals who do not display signs of excessive alcohol consumption.[1] NAFLD comprises a cluster of liver disorders that encompass simple steatosis and non-alcoholic steatohepatitis (NASH),[2] the latter of which can progress to liver cirrhosis and give rise to complications such as hepatocellular carcinoma (HCC).[2,3] It is the most prevalent hepatic

Received: 16 November 2023
Accepted: 1 May 2024
Published: 15 August 2024

condition in Western nations, affecting a quarter of the global population,[4] and has been linked with various components of the metabolic syndrome, including diabetes mellitus (DM), obesity, dyslipidemia, insulin resistance (IR), and arterial hypertension.[5]

Periodontitis is a multifactorial chronic infectious disease that manifests as a complex interplay between the microbial biofilm and the host's immune response.[6] It perturbs the homeostasis of the periodontal tissues, leading to the destruction of the periodontal ligament and alveolar bone, which ultimately results in tooth loss.[7] In systemically affected individuals, the onset of periodontitis is often precipitated by a perturbation in the subgingival microbiota, which triggers an exaggerated host immune response.[8]

To improve the host's immune response in the setting of periodontal inflammation, the immune system may produce elevated levels of pro-inflammatory mediators, such as interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)-α, matrix-metalloproteinases (MMP), prostaglandin-2, and collagenases, particularly in the presence of local or systemic risk factors.[9,10] Measuring the levels of these pro-inflammatory cytokines can serve as a valuable tool for assessing the progression of periodontitis.[11] The pro-inflammatory infiltrate originating from the connective tissue level promotes the production of gingival crevicular fluid (GCF), a fluid that is enriched with leukocytes.[12] GCF is known to release reactive oxygen species, such as hydrogen peroxide and superoxide, which, in combination with an altered antioxidant defense, can lead to oxidative stress and apoptosis of the connective tissue.[13]

Systemic disorders that are associated with periodontitis, such as diabetes mellitus, obesity, and cardiovascular disease, are characterized by the presence of oxidative stress and endothelial dysfunction.[14–16] Endothelial dysfunction is marked by the adhesion of leukocytes to the vessel wall, which is facilitated by adhesion molecules and endothelial cells.[17] To track the progression of periodontitis, analyzing the levels of cytokines and leukocytes in GCF is considered an essential tool.[18]

Vascular Adhesion Protein (VAP-1) is a sialoglycoprotein homodimer located on endothelial cells that plays a crucial role in liver's inflammation, as it increases leukocyte adhesion and transendothelial migration.[19,20] This protein is also a copper-containing amine oxidase 3 that facilitates the oxidative deamination of primary amines.[21,22] The soluble form of VAP-1, sVAP-1, which is found in circulating serum, is involved in nearly all amino oxidase activity.[23] Elevated levels of sVAP-1 have been observed in various inflammatory conditions, including chronic inflammatory liver diseases.[19] Studies have indicated that sVAP-1 levels are significantly elevated in patients with NAFLD, DM, obesity, and atherosclerosis.[24,25]

Thrombospondin-1 (TSP-1) is a multifunctional extracellular matrix protein produced by various cell types, including endothelial cells, fibroblasts, neutrophils, monocytes, and macrophages.[26] TSP-1 plays a significant role in modulating the inflammatory response by stimulating macrophage migration, promoting neutrophil phagocytosis, and binding CD47 to regulate immune responses in T cells.[27] Furthermore, TSP-1 is associated with bone resorption[28] and is involved in numerous cellular functions, including angiogenesis, inflammation, cell adhesion, and cell migration.[29]

The objective of this study was to determine the levels of VAP-1 and TSP-1 in GCF of patients with periodontitis and NAFLD and to test the possible correlations between these levels and clinical periodontal features or liver biochemical parameters.

Materials and Methods

Study Design

This study was designed according to the STROBE guidelines.[30] The STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines outline essential principles for reporting observational studies. These include specifying study design and rationale, clearly defining variables and measurements, addressing potential biases, justifying sample sizes, transparently reporting statistical methods and results, including participant characteristics and subgroup analyses. In the discussion, findings were contextualized, limitations acknowledged, and comparisons made with existing literature to ensure transparency and facilitate critical evaluation. Full adherence to these principles enhanced the overall quality and interpretability of the study.

The Declaration of Helsinki principles were followed and the approval of the Ethics Committee of the University of Medicine and Pharmacy in Craiova was obtained, as well as the written consent of all patients.

Patient Selection

In this study, from October 2021 to April 2022, patients were included after they presented themselves in the Periodontology Clinic of the Faculty of Dental Medicine in Craiova, where they were dental and periodontal assessed, and in the Gastroenterology Clinic of the County Emergency Clinical Hospital Craiova, University of Medicine and Pharmacy of Craiova, Romania, where they were examined in vue to diagnose them with NAFLD.

Inclusion criteria: i) adult patients >18 years regardless of sex, ii) systemically and periodontally healthy patients, iii) patients who received diagnosis of periodontitis, iv) patients with NAFLD, v) patients with both pathologies.

Exclusion criteria: i) smoking, ii) pregnancy, iii) administration of antibiotics and anti-inflammatories in the 30 days before participation in the study, iv) existence of other associated systemic diseases-diabetes, rheumatoid arthritis, v) treatments for systemic diseases.

The periodontal clinical examination was performed by the same well-trained periodontologist (C.C.A.), for all patients. Periodontal probing depth (PD) was examined in 6 sites (mesio-buccal, centro-buccal, disto-buccal, mesio-oral, centro-oral, disto-oral) for every tooth present and was recorded in the periodontal chart of the patient, in millimeters. We obtained a PD for each individual by summing those values and dividing them by the number of examined sites. The Gingival Index (GI) was determined to evaluate inflammation at the gingival level, on all four surfaces of each tooth, expressed as scores from 0 to 3, as follows: i) 0 = normal gingiva; ii) 1 = mild inflammation, the gum shows a slight color change, slight edema, no bleeding on probing; iii) 2 = moderate inflammation, red gums, edema and bleeding on probing; iv) 3 = severe inflammation, the gum is red, edema and spontaneous bleeding. The gingival index was obtained by summing the values of all teeth, divided by the number of sites examined.

Following the establishment of the diagnosis of periodontitis[6] or NAFLD, after applying the exclusion criteria, 48 patients, aged between 36–68 years, were admitted to the study, of which 25 women, 23 men distributed in one of the four groups: 12 systemically and periodontally healthy patients - control group - C, 11 patients with periodontitis - P, 12 patients with NAFLD - NA and 13 with periodontitis and NAFLD - P+NA (Table 1).

For statistical analysis, data were selected from the medical records: PD, GI, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (GGT) and body mass index (BMI).

Collection of GCF Samples

After performing the periodontal clinical examination, GCF samples were collected from the tooth with the deepest periodontal pocket for patients diagnosed with periodontitis and from the gingival sulcus of periodontally healthy patients. Two samples were obtained one minute apart. To avoid the risk of sample contamination, each tooth was isolated with cotton rolls, then air-dried. With another cotton roll, the supragingival plaque was removed. The GCF samples were collected by the intrasulcular method, by absorption technique, using paper strips (Periopaper, Oraflow, USA). For the test group, the paper strip used for GCF collection was inserted in the site with the deepest periodontal pocket depth, as indicated by the periodontal probing chart. For the control group, homologous teeth and sites were chosen for GCF collection or in its absence, the nearest existing tooth They were inserted into the gingival sulcus until slight resistance was encountered, maintained for 30 seconds, after which they were placed in polyethylene microtubes with 50 µL buffer solution (PBS) and stored at −20 Celsius until use.

Table 1 Demographics

Group	C	P	NA	P+NA
Age (mean ± SD)	38.58 ± 1.92	40.27 ± 2.1	55.5 ± 8.15	58.38 ± 6.04
Gender (female/male) (n)	5/7	6/5	7/5	7/6

Notes: C - control group, P - group of patients with periodontitis, NA - group of patients with NAFLD, P+NA - group of patients with periodontitis and NAFLD, n - number of patients.

Immunological Analysis of VAP-1 and TSP-1

Protein extraction from GCF samples for enzyme-linked immunosorbent assay (ELISA) involves meticulous procedures to ensure the accurate quantification of the targeted proteins (TSP-1 and VAP-1). GCF samples are collected, followed by centrifugation to obtain the soluble fraction. Protein concentration is determined, and samples are then applied to ELISA plates, where the target proteins adhere. Blocking agents minimize nonspecific binding, and subsequent incubation with primary antibodies specific to the target proteins is followed by washing steps. Secondary antibodies, conjugated with enzymes, are introduced, and after additional washing, a substrate is added to induce an enzymatic reaction. The resultant measurable signal, often colorimetric, is directly proportional to the concentration of the target protein, facilitating precise quantification.

The immunological analysis of the GCF samples was carried out at the Immunology Laboratory of the University of Medicine and Pharmacy in Craiova, using the ELISA dedicated kits test for the detection and quantitative determination of VAP-1 (AVIVA Systems Biology, USA) and TSP-1 (RandD Systems, USA). Commercial kits were used according to the manufacturer's instructions and recommended methodology. During the procedure, a common optical analyzer with a wavelength of 450 nm was used, to which a correction was applied up to 540 nm.

Statistical Analysis

After a power computation (G*Power 3.0, University of Dusseldorf, Germany), that returns an achieved power between 84% and 99% given $\alpha = 0.05$, sample size, and effect size after calculated post hoc SD within each group, the data was statistically analyzed using the GraphPad 9.5.0 data analysis tool (GraphPad Prism, USA), applying the Mann–Whitney *U*-test for comparisons between groups of VAP-1 and TSP-1 levels, and the Pearson test ($-1<r<1$) for correlations between the levels of the substances evaluated and parameters of periodontal or liver status. $p<0.05$ was set for statistical significance.

Results

Group Comparisons of VAP-1 Levels

Comparisons of VAP-1 levels showed significant differences between all test and control groups ($p<0.0001$). The highest levels were in the P+NA group (1.13 times higher than in P and 1.34 times higher than in NA), followed by P (1.18 times higher than in NA) and NA with differences statistically significant between P and NA ($p<0.0001$), and between NA and P+NA ($p<0.0001$) (Figures 1–3).

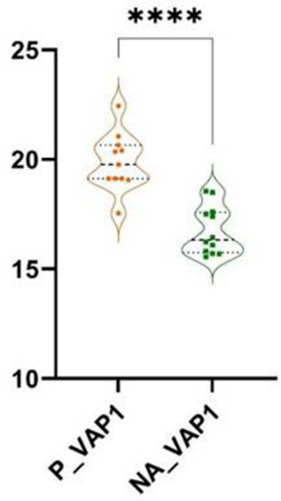

Figure 1 Differences in VAP-1 levels, between P and NA groups, P – group of patients with periodontitis, NA – group of patients with NAFLD. p**** (p-value < 0.0001).

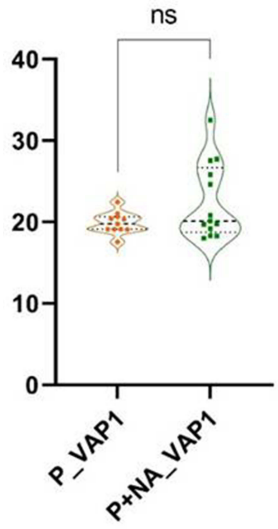

Figure 2 Differences in VAP-1 levels, between P and P+NA groups. P – periodontitis patient group, P+NA – periodontitis and NAFLD patient group, ns-statistically not significant differences, p-value = 0.3307.

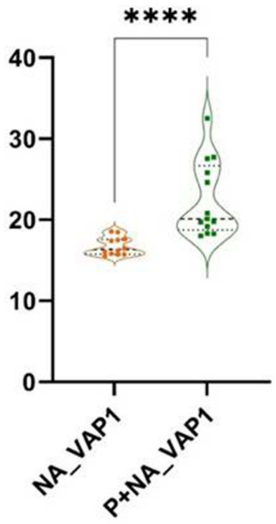

Figure 3 Differences in VAP-1 levels, between NA and P+NA groups, NA – group of patients with NAFLD, P+NA – group of patients with periodontitis and NAFLD. p**** (p-value < 0.0001).

Group Comparisons of TSP-1 Levels

Comparisons between groups regarding TSP-1 levels showed statistically significant differences between all test and control groups ($p<0.0001$). The highest values were in the P+NA group (1.29 times higher than in P and 1.9 times higher than in NA), followed by P (1.6 times higher than in NA) and NA with statistically significant differences between P and NA ($p<0.0001$), P and P+NA, NA and P+NA ($p<0.0001$) (Figures 4–6).

Correlations Between VAP-1 and Periodontal/Liver Parameters in P+NA Group

Statistically significant correlations ($p<0.05$) were found between VAP-1 and periodontal and liver parameters in the P +NA group. A weak direct correlation was found between VAP-1 levels and PD ($r=0.318$, $p=0.034$) but also a moderate correlation between VAP-1 and GI ($r=0.579$, $p=0.015$). A moderate direct correlation between VAP-1 and GGT ($r=0.546$, $p=0.008$) was identified, but also between VAP-1 and BMI ($r=0.565$, $p=0.0053$).

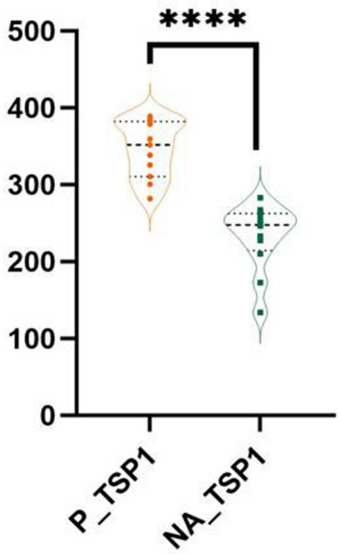

Figure 4 Differences in TSP-1 levels, between P and NA groups, P – group of patients with periodontitis, NA – group of patients with NAFLD, p**** (p-value < 0.0001).

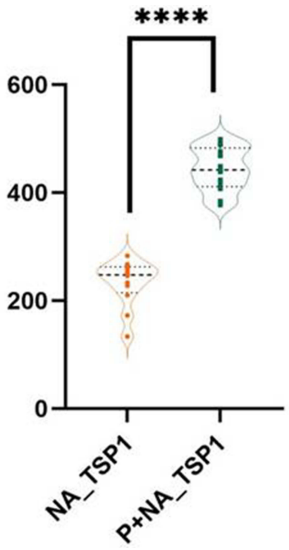

Figure 5 Differences in TSP-1 levels, between NA and P+NA groups, NA – group of patients with NAFLD, P+NA – group of patients with periodontitis and NAFLD, p**** (p-value < 0.0001).

Correlations Between TSP-1 and Periodontal/Liver Parameters in P+NA Group

Statistically significant correlations ($p<0.05$) between TSP-1 and some periodontal and liver parameters in the P+NA group were detected: a moderate direct correlation was generated between TSP-1 and GI ($r=0.423$, $p=0.0063$) and a weak one between TSP-1 and GGT ($r=0.388$, $p=0.005$).

Discussion

The group comparison for VAP-1 levels in GCF, showed that the highest levels were in the P+NA group, followed by P and NA with statistically significant differences between P and NA, but also between NA and P+NA, suggesting an increased impairment of vascular adhesion in patients suffering from both diseases, the mechanism possibly being negatively influenced by the presence of periodontal inflammation.

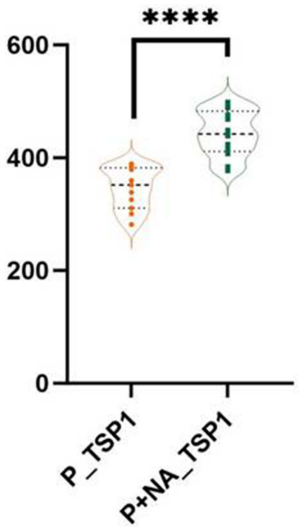

Figure 6 Differences in TSP-1 levels, between P and P+NA groups, P – periodontitis patient group, P+NA – periodontitis and NAFLD patient group, p**** (p-value < 0.0001).

In liver disease cases, increased levels of circulating soluble VAP-1 are observed in patients with chronic liver disease, particularly alcoholic hepatitis, but not in those with acute liver injury,[31,32] suggesting that increased circulating levels of soluble VAP-1 are associated with chronic liver inflammation rather than acute liver injury.[33]

In chronic hepatitis C, VAP-1 concentration was strongly correlated with liver stiffness and was the second strongest influencing variable, after GGT levels, for liver stiffness in the regression analysis. The concentration of VAP-1 increased with advancing stages of fibrosis and the highest concentrations were found in cirrhotic patients.[34]

Weston et al conducted a study which found that patients with chronic liver disease had increased hepatic expression of VAP-1, and that serum levels of sVAP-1 were elevated in patients with NAFLD as compared to controls.[19] Their research also demonstrated that the levels of sVAP-1 were significantly higher in the NAFLD group when compared with age and metabolic type-matched controls.[19] Moreover, the concentration of sVAP-1 was significantly higher in patients who subsequently passed away as compared to those who remained alive.[19]

Levels of sVAP-1 in synovial fluid were found to be increased in patients with osteoarthritis and were significantly correlated with the radiographic severity of the disease.[35] However, the levels of sVAP-1 in serum were lower in patients with symptomatic knee osteoarthritis compared to healthy individuals and were inversely correlated with markers of pain and inflammation. Furthermore, the serum sVAP-1 levels were found to be higher at baseline compared to those in patients with advanced symptomatic knee osteoarthritis, indicating a potential role of sVAP-1 as a biomarker for early detection of osteoarthritis.[35]

The correlations obtained in our study, between the levels in GCF of VAP-1 and the depth of periodontal pockets and also with GI, show the involvement of VAP-1 in periodontal inflammation, but also its role in metabolic syndrome and NAFLD, given the correlations with GGT and BMI.

In the liver, stromal cells express VAP-1, which is capable of promoting leukocyte migration through the generation of reactive oxygen species, which is also dependent of VAP-1 enzyme's activity.[19] It enhances stromal cell proliferation, wound healing, and modulates profibrotic gene expression. The study's findings suggest that VAP-1 amino oxidase activity is linked to liver inflammation and fibrosis, and that VAP-1 may have therapeutic potential for treating chronic fibrotic liver diseases, including NAFLD.[19]

VAP-1 amine oxidase activity promotes steatohepatitis progression.[36] VAP-1 SSAO activity has also been shown to be involved in lymphocyte chemotaxis and transendothelial migration.[36] Other authors stated that the concentration of VAP-1, rather than its amino oxidase activity, may represent a tool for monitoring fibrogenesis in the follow-up of patients with chronic liver diseases.[34]

Our literature research did not return scientific articles that determined VAP-1 in GCF, either in patients with periodontitis or systemic diseases, nor in periodontally or systemically healthy patients. There are articles that have measured VAP-1 in blood using ELISA detection kits with a different detection range than the kit used in the present study. Thus, the novelty of this research brings more knowledge to the understanding of the mechanisms that could be involved in the association of the two diseases, but, at the same time, it does not allow making comparisons with other values from the literature. To get a better understanding of the function of this marker, further research in this direction is needed as well as the identification of VAP-1 in other associations of periodontitis with systemic disorders.

In our study, TSP-1 is increased in patients with periodontitis-NAFLD association, with statistical differences compared to patients suffering from only one of the diseases or to those in the control group, suggesting the cumulative effect of the two pathologies in exacerbating vascularization mechanisms, angiogenesis and inflammation.

After corroborating the results of two studies in obese mice to examine the impact of TSP-1 on NAFLD, it was observed that TSP-1 significantly attenuated the mRNA expression of the inflammatory markers TNF-α and TGF-β1 in adipose tissue from mice obese with a high-fat diet. TNF-α overexpression in adipose tissues correlates with insulin resistance in animals, and insulin resistance correlates with the onset of NAFLD/NASH.[29] TSP-1 deficiency was also observed to attenuate liver fibrosis and other NAFLD symptoms such as serum lipid levels and markers of inflammation.[29] Although in our study we did not determine TSP-1 in serum, given the fact that GCF is a serous exudate and patients in the NA group had higher values of TSP-1 in GCF than those in the control group without liver disease and periodontitis, this may suggest that this difference can highlight the involvement of TSP-1 in NAFLD. Also, our results show an involvement of TSP-1 in patients with both diseases given the correlation between TSP-1 and GGT levels, but also between TSP-1 and GI.

According to another study, TSP-1 is involved in the pathogenesis of NAFLD/NASH by regulating the Peroxisome Proliferator Activated Receptor alpha (PPARα) pathway. Recently, PPARα has emerged as a potential target for the treatment of NAFLD/NASH. Activation of PPARα, together with PPARβ/δ, has been shown to impact steatosis, inflammation, and fibrosis in preclinical models of nonalcoholic liver disease.[37]

A disintegrin and metalloproteinase with thrombospondin motifs-1 (ADAMTS-1) is a type of proteinase that belongs to a family similar in structure to the MMP family, and is involved in the process of extracellular matrix damage and repair.[38] Its functions are closely linked to inflammation, hypoxia, and vascularization. The study conducted by Tayman et al, focused on analyzing ADAMTS-1 levels in GCF and found that patients with periodontitis had higher levels of ADAMTS-1 compared to healthy individuals, which may be attributed to local tissue inflammation, impaired vascularity, and angiogenesis.[38] Our findings confirm the earlier provided literature findings by demonstrating that patients with periodontitis had considerably greater TSP-1 levels in GCF compared to those in the control group.

Elevated levels of ADAMTS-1 in periodontitis may be due to the extensive infiltration of inflammatory cells and migration of keratinocytes. Increased synthesis of ADAMTS-1 in periodontal tissue may be attributed to keratinocytes and fibroblasts.[39] This increased production of ADAMTS-1 may facilitate wound repair through keratinocyte differentiation and fibroblast migration. Fibroblasts are crucial in the healing process of periodontal wounds, and TSP-1, through ADAMTS-1, may play a role in the advanced pathogenesis of periodontitis, in association with tissue hypoxia and vascularization.[39]

TSP-1 and Nuclear factor kappa-light-chain-enhancer of activated B cells pathways could be implicated in the development of inflammation and fibrosis in NAFLD. These pathways could serve as useful non-invasive markers and potential therapeutic targets for NAFLD and non-alcoholic steatohepatitis (NASH) treatment.[29,40]

Due to the reduced number of patients in each group, we did not subdivide them into subgroups regarding sex and age. As the literature is aware, even if less than in the case of other diseases, an approach depending on the age-related sex differences could be more appropriate in terms of major risk factors.[41,42] This could be a limitation of our study.

The study's findings hold significant clinical implications, indicating elevated VAP-1 levels in GCF and highlighting a potential association between periodontitis and NAFLD. This suggests an increased impairment of vascular adhesion in patients with both conditions, emphasizing the role of periodontal inflammation. Correlations between VAP-1 levels and liver parameters or BMI, underscore the broader involvement of VAP-1 in metabolic syndrome and NAFLD. The study contributes novel insights into the molecular pathways linking periodontitis and systemic disorders. Additionally, TSP-1 levels in GCF demonstrate a cumulative effect in patients with periodontitis-NAFLD, with potential implications for

vascularization mechanisms and inflammation. These results, together with the investigation of A disintegrin and metalloproteinase with ADAMTS-1 by similar studies add insights into its role in periodontitis and wound repair [38, 39]. Overall, the study generated results with potential diagnostic and therapeutic implications in dental and hepatological fields, acknowledging limitations and suggesting areas for future research refinement.

Conclusion

Within the limits of this study, levels of VAP-1 and TSP-1 in GCF were higher in patients with periodontitis and NAFLD. These individuals exhibit greater periodontal inflammation, and vascular adhesion and angiogenesis processes could be affected. These findings could suggest that therapeutic interventions targeting periodontal inflammation in individuals with the coexistence of periodontitis and NAFLD may yield broader positive effects on vascular adhesion and angiogenesis. This underscores the intricate interplay between oral health and hepatic conditions, advocating for a comprehensive approach to patient care that addresses both dental and liver health considerations.

Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of UNIVERSITY OF MEDICINE AND PHARMACY OF CRAIOVA (49/20.04.2018). Informed consent was obtained from all subjects involved in the study.

Data Sharing Statement

The data used to support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

Funding

The Article Processing Charges were funding by the University of Medicine and Pharmacy of Craiova, Romania.

Disclosure

The author reports no conflicts of interest in this work.

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