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International Journal of Dentistry and Oral Health

ISSN 2378-7090 | Open Access

RESEARCH ARTICLE

Volume 10 - Issue 4

Exploring the Role of Inflammation and Fibrosis-Associated Gene Expression in Drug-Induced Gingival Enlargement and Periodontitis

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Received: 23 Nov, 2024 | **Accepted:** 03 Dec, 2024 | **Published:** 09 Dec, 2024

Citation: Kuo JH, Lin LW (2024) Exploring the Role of Inflammation and Fibrosis-Associated Gene Expression in Drug-Induced Gingival Enlargement and Periodontitis. Int J Dent Oral Health 10(4): dx.doi.org/10.16966/2378-7090.428

Abstract

Aim: This study aimed to analyse the gene expression profile of inflammation- and fibrosis-related targets in drug-induced gingival enlargement (DIGE) in subjects with periodontitis.

Materials and Methods: Forty-four patients were divided into four groups: N (control), P (periodontitis), A (periodontitis undertaken amlodipine), and G (DIGE with periodontitis). Periodontal flap surgery was performed at least 6 months after therapeutic intervention and gingival tissue samples were subjected to histomorphometric analysis.

Results: None had DIGE grades 2-3 after initial therapy. Compared to controls, inflammatory and fibrosis markers cluster of differentiation 68, matrix metalloproteinase (MMP) 12, A disintegrin and metalloprotease (ADAM) 17, connective tissue growth factor, and cathepsin L were pronounced in DIGE patients; SIRT1 was higher in non-responders.

Conclusion: The importance of proper dental plaque management for the treatment of DIGE with periodontitis Elevated SIRT1 may indicate antifibrotic properties.

Keywords: Gingival overgrowth; Periodontitis; Fibrosis; Sirtuin 1; Connective tissue growth factor

Introduction

Drug Induced Gingival Enlargement (DIGE): DIGE is seen as a result of the excessive production of the extracellular matrix, therefore, leading to an abnormal increase in gingival tissue secondary to certain pharmacological agents causing cellular hyperplasia and hypertrophy [1]. This can be seen after the use of many drugs including phenytoin and sodium valproate (antiepileptic drugs), cyclosporine (immunosuppressive agents) as well as calcium channel blockers (CCBs) (nifedipine, verapamil, diltiazem, amlodipine and felodipine) [2-5]. DIGE can lead to aesthetic and functional complications, hindering oral hygiene maintenance and impacting an individual's ability to eat and communicate effectively [5]. However, despite a specific drug class being implicated in these changes in some patients only, other patients on the same medications do not develop gingival enlargement; thus there is a responder/non-responder phenomenon with large variability of gingival phenotype change severity and extent among affected individuals [6]. Additionally, patients diagnosed with DIGE do not exhibit fibrosis in other organs, suggesting a tissue-specific response [7].

The mechanisms underlying DIGE are categorized into two primary pathways: non-inflammatory (biochemical) and inflammatory [8,9]. Consequently, our research was divided into two distinct sections. The first section focused on the expression of inflammation-related proteins, including a cluster of differentiation (CD) 68, [10,11] matrix metalloproteinase 12 (MMP12), [12,13] and a disintegrin and metalloproteinase 17 (ADAM17) [14]. The second section examined fibrosis-related proteins, such as connective tissue growth factor (CTGF), [7] and cathepsin L [15]. Each gene chosen for this investigation is associated with specific pathways relevant to DIGE and periodontitis. As far as we are aware, some of these genes have been studied in relation to DIGE and periodontitis before but not in non-responder patients with periodontitis-this refers to patients diagnosed with periodontitis and treated at the same time with amlodipine for hypertension who did not display clinical signs of gingival overgrowth.

We explored the potential for variable responses to amlodipine in nonresponders by characterization of the antifibrotic properties of this agent. Moreover, the expression of sirtuin1 (SIRT1), which is a member of the mammalian silent information regulator 2 (Sir2) gene family, which functions as a nicotinamide adenine dinucleotide (NAD)+-dependent deacetylase in the nucleus, was analyzed [16,17]. The NAD+dependency of the deacetylation activity of Sir2 might influence its biological functions [18,19]. SIRT1 is implicated in

various physiological processes [20-22] and has been shown to alleviate fibrosis by inhibiting the transforming growth factor-beta 1 (TGF-β)/ suppressor of mothers against decapentaplegic signaling pathway in tissues such as [23,24] the kidney [25] and heart [26]. While TGF-β is known to induce DIGE [27], the precise role of SIRT1 in the context of DIGE remains unclear. To date, no research has evaluated the potential impact of DIGE on SIRT1 expression.

DIGE is a chronic inflammatory disease of fibrotic gingival tissue, while periodontitis is related to collagen breakdown. Though identifiable DIGE patients are able to observe symptoms of periodontitis while taking amlodipine for hypertension management, the correlation between DIGE and well-documented pathologies like those of periodontitis is poorly examined. The goal of this study is to compare cross-sectional data from patients with periodontitis, healthy controls, and patients with a diagnosis of periodontitis and have DIGE while on amlodipine therapy. The main aims of this study were to (1) explore candidate gene expression in relation to DIGE data for amlodipine treatment efforts guided by published literature and preliminary results and (2) comparison the expression of SIRT1 in treatment responders *versus* non-responders.

Material and Methods

Patients and gingival samples

The study examined 44 biopsies from both male and female patients. This study collected data on age, sex, type of medication therapy, the duration of drug therapy when they come to follow-up and baseline DIGE grade [28]. In the initial phase of periodontal treatment, comprehensive supragingival and subgingival scaling and root planning were performed under local anesthesia using manual and ultrasonic instrumentation with either three to four appointments per arch or one quadrant scheduled per appointment. Furthermore, for all participants diagnosed with periodontitis oral hygiene instructions were performed, but periodontally healthy participants received scaling and oral hygiene education.

Four separate groups of 11 patients (N, P, A, G) N served as the control group, with no clinical signs of gingival inflammation. Patients diagnosed with periodontitis according to the criteria of the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions were assigned to the P group [29]. Group A consisted of periodontitis non-responders receiving amlodipine for hypertension but with no clinical evidence of gingival enlargement. In contrast, responders in Group G with periodontal disease who were receiving amlodipine presented with clinical signs of gingival enlargement, as determined by the baseline DIGE grade [28].

Standard human gingival biopsy specimens were obtained from patients undergoing flap surgeries during routine practice to stop the clinically progressive form of periodontitis as defined by periodontal pockets of ≥4 mm with bleeding on probing or deep pockets of 6 mm together with minor plaque scores (≤20-25%) [30]. Moreover, during the crown lengthening procedure for restorative purposes at E-Da Hospital (institutional review board number: EMRP-112–049), samples had also been taken. The specimens were fixed in 4% phosphate-buffered formalin (pH 7.4) and processed for routine laboratory paraffin embedding. Pathological analyses were blinded and independently performed by two experienced oral pathologists from Kaohsiung Medical University Chung-Ho Memorial Hospital.

Hematoxylin-eosin (H&E) staining

H&E staining was done according to the manufacturer's protocol (Gill II Hematoxylin, Leica, 3801522; Eosin Y, Leica, 3801600). The tissue sections were dewaxed firstly in xylene and then gradually hydrated using a series of percentages of alcohol solution. Afterwards, sections were stained with hematoxylin for 5 minutes at room temperature and distilled water was used for rinsing followed by eosin staining which was done for 30 seconds at room temperature. The sections were dehydrated with gradients of alcohol and xylene for 5 minutes after staining. Light microscope at 400× (This image was taken on a light microscope Classzi, Leitz lamps in Germany) Physiology changes on samples were determined.

Immunohistochemistry

Tissue sections were heated in the microwave oven with citrate buffer (0.1 mol/L, pH 6.0) for three 5-min at 650 W to expose antigens and subsequently various monoclonal and polyclonal antibodies including anti-SIRT1 (Abcam, 1:200), anti-CD68 (Abclonal, 1:100), anti-CTGF (GeneTex, 1:100), anti-cathepsin L (1:50), anti-ADAM17 (1:100) and anti-MMP12(1:300). The sections were rehydrated in phosphate-buffered saline (PBS, pH 7.2) and incubated with one of the primary antibodies, biotinylated anti-mouse and anti-rabbit immunoglobulins and subsequently with a streptavidin peroxidase conjugate. All incubations were performed at room temperature for 60 min and washed five times with PBS (10 min each) between steps. Peroxidase activity was visualized using 3,3-diaminobenzidine tetrahydrochloride with hydrogen peroxide in a buffered solution for 12 min and the sections were further counterstained by hematoxylin.

Quantitative analysis

Ten randomly selected, non-contiguous, and non-overlapping squares were used to determine the percentage of positive surface area using ImageJ software. Measurements were performed by a single examiner after a calibration process.

Statistical analysis

An independent two-sample t-test was conducted to compare the mean percentage area between the two groups, with statistical significance set at $p<0.05$.

Results

Study group

44 biopsy samples from 44 patients (22 males and 22 females), with a mean age of 55.3 years (age range: 44–77 years) as shown in table 1. A total of 28 of the patients underwent further treatment for narcissism through internal medicine, and none had their medication regimens altered during that time. Moreover, no participant displayed DIGE grade 2-3 at the initial stage of periodontal therapy.

Inflammation-associated gene expression

All experimental groups demonstrated significantly higher CD68 expression *versus* Group N ($p <0.001$) (Figure 1A), but no statistically significant differences between the other three groups (Figure 1B). In all groups, MMP12 expression levels were higher than in Group N ($p <0.001$), with Group G having the highest expression (Group A: $p = 0.031$). There was no difference between Groups A and P ($p=0.194$) (Figure 1C). Likewise, ADAM 17 protein expression levels were also found to be significantly up regulated in all experimental groups compared with Group N ($p <0.001$). Both sets were confirmed by an additional group study to express higher in Group A than Group P ($p=0.076$) (Figure 1D).

Fibrosis-associated gene expression

All experimental groups had significantly higher expression levels

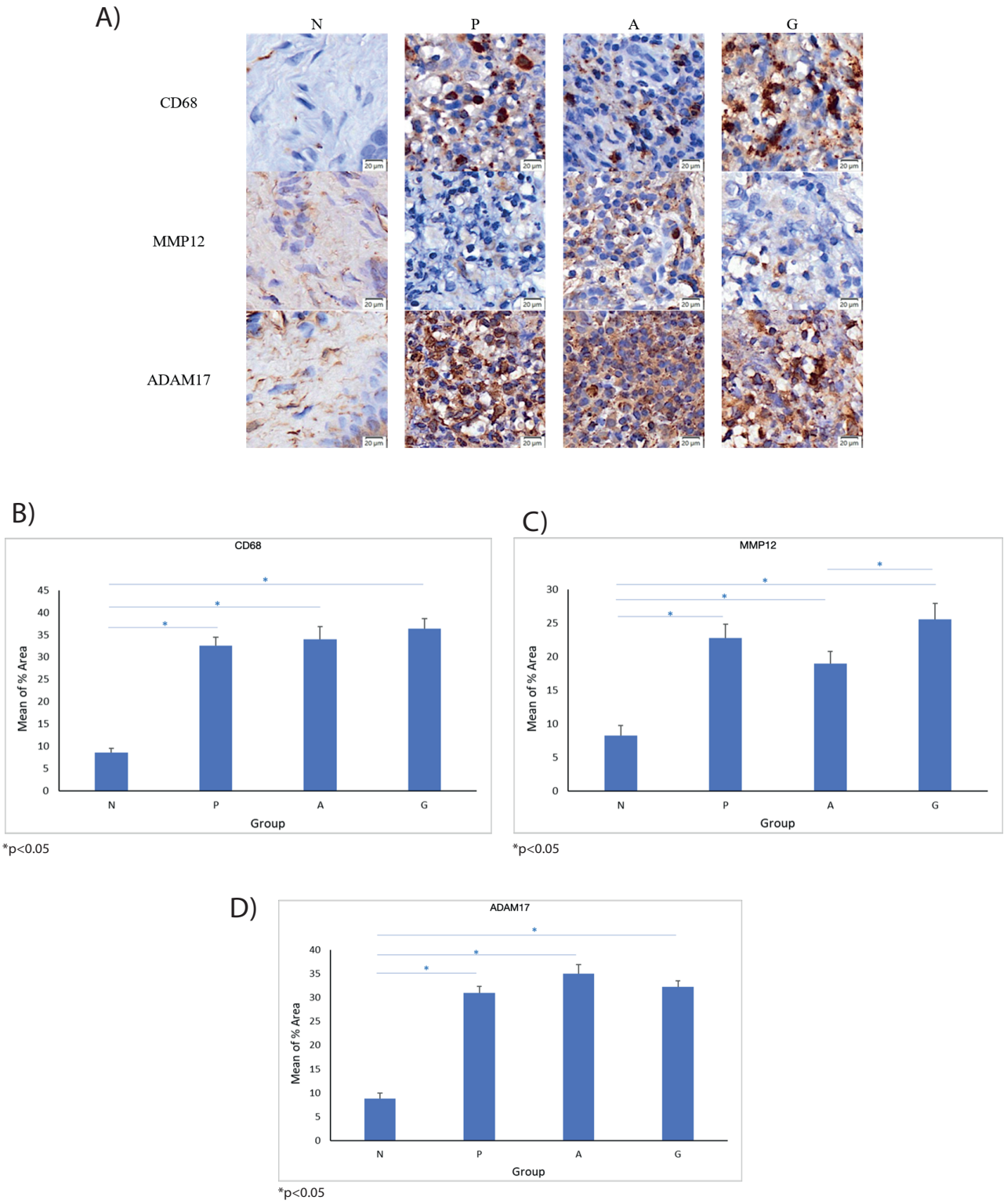

Figure 1: Immunohistochemical analysis of markers associated with inflammation.
(A) Immunostaining was conducted utilizing anti-CD68 antibodies (Abclonal, diluted 1:100), anti-MMP12 antibodies (diluted 1:300), and anti-ADAM17 antibodies (diluted 1:100) at a magnification of ×400.
(B) The intensity of immunohistochemical staining for CD68.
(C) The intensity of immunohistochemical staining for MMP12.
(D) The intensity of immunohistochemical staining for ADAM17.

Table 1: Patient characteristics.

	N	P	A	G
Age	51.9 ± 12.6 y/o	53 ± 5.8 y/o	61 ± 13.1 y/o	55.3 ± 9.6 y/o
Gender(F:M)	5:6	7:4	6:5	4:7
Drug			Amlodipine 5mg+Vasartan 160mg/F.C. tab	
Duration of drug use T0			7.1 ± 2.8 months	9.1 ± 2.9 months
Mean PPD T0	2.6 ± 0.2mm	6.3 ± 0.5mm	6.4 ± 0.4mm	6.9 ± 0.4mm
Mean PPD T1	2.5 ± 0.8mm	4.6 ± 0.7mm	4.3 ± 0.5mm	4.6 ± 0.4mm
Mean BOP T0	14.1 ± 3.1%	64.6 ± 7.6%	64.3 ± 8.5%	65.8 ± 7.3%
Mean BOP T1	13.2 ± 5.1%	14 ± 2.9%	12.8 ± 1.9%	14.1 ± 3.5%
DGO grade 2-3 at T0	0%	0%	0%	65 ± 4% sites
DGO grade 2-3 at T1	0%	0%	0%	0%

Values are means ± SD.
PPD = Periodontal pocket depth, BOP% = Sites of bleeding on probing/total site, T0 = Baseline measurement, T1 = the measurement taken immediately prior to periodontal surgery.

of CTGF compared to the control group (Group N) ($p < 0.001$) with Group G having even higher expression levels than Groups A and P (Group A: $p=0.038$, Group P: $p < 0.001$) (Figure 2A). CTGF expression in Group A was also significantly higher than that subgroup (Group P > Group A; $p=0.005$) (Figure 2B). The levels of Cathepsin L expression were also significantly higher in all experimental groups when compared with Group N ($p < 0.001$), but there were no statistically significant differences detected among the three experimental groups (Figure 2C). All experimental groups had significantly higher SIRT1 expression compared to group N ($p < 0.001$) with the highest being in group A (group G: $p < 0.001$, group P: $p=0.012$). Moreover, lower SIRT1 expression levels also reached a significative statistical difference between Group G and Group P ($p=0.023$) (Figure 2D).

Discussion

DIGE typically presents within the first three months after initiating treatment with associated medications [9,31], and its severity is often exacerbated by the accumulation of local bacterial plaque [5]. The primary treatment approach includes initial periodontal management combined with either the modification or cessation of the implicated medication, requiring approximately 1 to 8 weeks for gingival lesion resolution [9,32-34]. After drug substitution initiation, surgery is considered only after 6 to 12 months [35-38]. The participants in the present study did not have any adjustment of medication during the acute treatment phase, as confirmed by an internal medicine physician. Furthermore, following initial periodontal therapy none of the participants displayed DIGE grade 2-3. The periodontal surgery in the present study was performed on all patients with clinically diagnosed periodontitis to stop the progression of periodontal disease. The present results point up the necessity of strict supragingival and subgingival plaque control in controlling DIGE along with periodontitis.

Inflammation-associated gene expression

The pathogenic mechanisms of DIGE fall into two categories: non-inflammatory (biochemical) and inflammatory. Non-inflammatory mechanisms may include inhibition of sodium and calcium ion influx [8], while possible inflammatory pathways may be associated with a perturbation in the homeostasis of gingival connective tissues, as a result, drug interactions with innate and acquired immune responses [9]. DIGE has been associated with modified immune responses [11], with studies indicating significantly elevated levels of CD68-labeled cells specifically in the nifedipine group within the connective tissue beneath the oral epithelium compared to the control and cyclosporine A groups [11]. In the present study, patients with periodontitis, as well as responders and non-responders to amlodipine-DIGE showed significantly greater percentage of CD68-positive cell area compared to those from the control group while no significant differences were found among these three groups. However, the identified ratio between DIGE and periodontitis may not detect differences in CD68 expression in patients with periodontitis treated with amlodipine but free of clinical signs of gingival enlargement.

Macrophage elastase (MMP12) is an enzyme primarily found in mature tissue macrophages [39]. Not only is MMP12 well known for its role in cleavage of nonmatrix proteins, but it also may play a role in enhancing macrophage antimicrobial functions [40]. MMP12, when elevated in proinflammatory monocyte-derived cell types, is associated with tissue degradation in periodontitis [13,41]. Furthermore, elevated MMP12 expression by mRNA levels has been proven in DIGE-patients with periodontal disease [12]. In our findings, MMP12 expression in the control group was statistically lower than all other groups, with the highest levels seen in Group G (Group A: $p=0.031$). Groups A and P were comparable ($p=0.194$). These findings are consistent with the established literature demonstrating a higher expression of MMP12 in periodontitis-induced tissues. In addition, the expression pattern observed in DIGE and periodontitis indicates that MMP12 may participate as an essential molecule in the interaction of DIGE and periodontitis.

ADAM17, or tumor necrosis factor-alpha converting enzyme (TACE), is a member of the ADAM family, which is evolutionarily related to the MMP family [42]. Significantly higher levels of ADAM17 mRNA expression are observed in DIGE-related inflammatory gingival tissue than in normal gingival tissue.14Our data are in accordance with this report, showing by DIGE that ADAM17 positive cell area was found significantly larger in patients with periodontitis compared to controls ($p < 0.001$). Furthermore, ADAM17 was elevated in the periodontitis non-responders when compared to the cohort with periodontitis alone, however this difference did not reach significance ($p=0.076$). Our results show that ADAM17 expression is

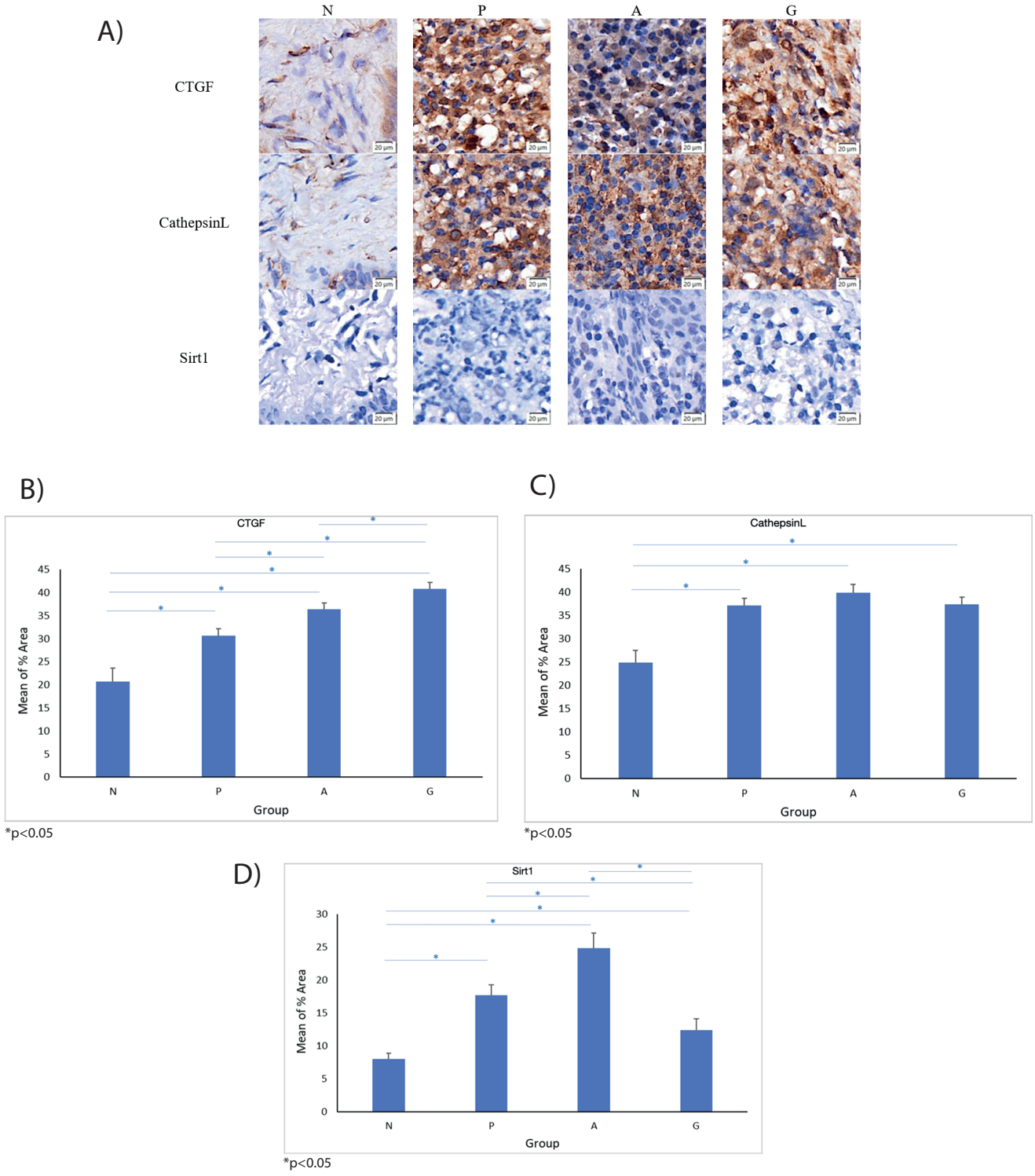

Figure 2: Immunohistochemical analysis of markers associated with fibrosis.
(A) Immunostaining was conducted utilizing anti-CTGF antibodies (GeneTex, diluted 1:100), anti-Cathepsin L antibodies (diluted 1:50), and anti-SIRT1 antibodies (Abcam, diluted 1:200), with observations made at a magnification of ×400.
(B) The intensity of immunohistochemical staining for CTGF.
(C) The intensity of immunohistochemical staining for Cathepsin L.
(D) The intensity of immunohistochemical staining for Sirt1.

significantly upregulated in periodontitis, and further in periodontitis with DIGE. It is necessary to investigate how amlodipine exposure affects ADAM17 expression in gingival tissue.

Fibrosis-associated gene expression

Expression of Genes Associated with Fibrosis CTGF is increased in a number of organ-specific fibrotic diseases, including the skin [43-45], kidney [46], lungs [47], and liver [48]. In periodontitis, CTGF might be crucial in promoting osteoclastogenesis, potentially leading to alveolar bone loss [49]. The difference in CTGF expression between different forms of DIGE was highest in phenytoin-affected gingival tissues, intermediate for CCBs and lowest or undetectable with cyclosporin A-induced overgrowth [50]. Upregulated by TGF-β1, CTGF enhances extracellular matrix synthesis, contributing to fibrosis persistence in gingival tissues [7]. In the present study. CTGF was statistically greater in the G group than in any of the other groups. Moreover, CTGF expression was significantly higher in the A group compared to the P group (p=0.005). Notably, the responder group continued to show elevated CTGF levels after 6 months of periodontal treatment although clinical signs of gingival overgrowth were significantly reduced. These sustained levels can be interpreted into two patterns, either the CTGF expression in periodontal tissues impacted by amlodipine or persistent CTGF which explains DIGE recurrence rates documented to range from 27% to 43%.35

Cathepsin is a protease influenced by the mild acidic status of the presynaptic environment originating from the Greek word káthepsein (to digest) [51]. The protease is implicated in inflammatory and tissue-destructive events in periodontitis [52,53]. It is reported that the activity of cathepsin in gingival tissue is approximately 1.6 to 2.8 times higher than in granulomatous tissue [54]. Downregulation of cathepsin L activity is associated with accumulation of ECM in fibrotic diseases [55,56]. Notably, nifedipine, cyclosporine, and phenytoin selectively inhibit cathepsin L activity and mRNA in cultured gingival fibroblasts by nifedipine, cyclosporine and phenytoin further support this view [57], while histological changes seen in the connective tissue such as elongated rete pegs and thickened connective tissue due to altered epithelium in cathepsin L-deficient mice are also characteristic features observed during gingival overgrowth [15]. Evidence of increased mRNA levels in DIGE tissues of biopsies extracted from patients with periodontitis *vs* healthy gingival tissues [12]. In the present study, cathepsin L expression was significantly increased across all experimental groups compared to the N group, with no significant differences among Groups A, P, and G. These findings suggest increased cathepsin L expression in periodontal tissues affected by periodontitis. Furthermore, the effect of amlodipine on periodontal tissue cathepsin L concentrations could also be heterogeneous, consistent with the reported reduction of clinical swelling in the gingiva after initial therapy for periodontitis.

SIRT1 mediates pathways involved in a range of physiological processes such as aging, metabolism and cancer [20-22]. SIRT1 over expression has been shown to assist in the suppression of pro-inflammatory markers activated by inflammation, contributing to the initiation and development of periodontal disease [58]. Recent studies also indicate that SIRT1 expression is also decreased in systemic sclerosis-associated pulmonary fibrosis [23,59]. Additionally, 6-gingerol has been shown to improve pulmonary fibrosis *via* activation of SIRT1 [60]. SIRT1 expression was highest in non-responders, with a statistically significant increase compared to other groups reflected in our study. In particular, the proportion of SIRT1 expressing positive was significantly lower in the DIGE group compared with that of non-responders (p <0.001) and Group P (p=0.023). These intergroup differences in SIRT1 expression might indicate possible antifibrotic roles or other mechanisms of action of SIRT1 on the amlodipine response among periodontitis non-responders and further investigations are warranted.

Conclusion

This cross-sectional study examines DIGE in individuals with periodontitis, addressing selection biases in biopsy cases and challenges associated with validating these biases. Results indicate that participants remained on their medications without significant gingival enlargement after treatment.

Histological assessments revealed elevated inflammatory marker (CD68, MMP12, and ADAM17) levels in patients with DIGE, suggesting their potential role in disease progression. Meanwhile, higher CTGF levels could be associated with fibrosis or recurrent gingival overgrowth, and increased cathepsin L levels were found to have consistent expression in the study groups. Interestingly, SIRT1 expression was increased in non-responders indicating a potentially antifibrotic role.

Our results underline the relevance of plaque control on DIGE in periodontitis and suggest that further research is needed to elucidate molecular mechanisms underlying DIGE. To our knowledge this is the first to examine SIRT1 expression in this setting and future studies examining SIRT1 substrates will help to modulate DIGE through its action on relevant effectors.

Acknowledgments

The authors acknowledge the technical support provided by Kaohsiung Medical University Chung-Ho Memorial Hospital.

Funding Information

This research was funded by E-Da Hospital, grant number EDAHP113032.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Data Availability Statement

Data supporting the findings of this study are available from the corresponding author upon reasonable request.

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