

Daily Smoking Intensity and Hypoxia-Related Biomarkers in Periodontally Healthy Individuals: A Cross-Sectional Clinical Study

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Research Article

Keywords: Gingival crevicular fluid, Hypoxia-inducible factor-1, Periodontal health, Smoking, Vascular endothelial growth factor

Posted Date: September 10th, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10613216/v1>

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Additional Declarations: No competing interests reported.

Abstract

Background

Smoking can disturb periodontal tissue homeostasis through several interconnected mechanisms, including vascular dysfunction, inflammation, oxidative stress, and hypoxia-related responses. Whether these molecular changes differ according to daily smoking intensity in clinically periodontally healthy individuals remains unclear. Therefore, this study evaluated GCF HIF-1 α and HIF-1 β levels together with gingival HIF-1 α and VEGF mRNA expression in periodontally healthy individuals stratified by daily smoking intensity.

Methods

This cross-sectional clinical study was conducted in 100 periodontally healthy individuals. Participants were grouped according to smoking status and self-reported daily cigarette consumption: Group 1, non-smokers; Group 2, ≤ 5 cigarettes/day; Group 3, 6–10 cigarettes/day; Group 4, 11–19 cigarettes/day; and Group 5, ≥ 20 cigarettes/day. Clinical periodontal parameters were recorded. GCF HIF-1 α and HIF-1 β levels were measured using enzyme-linked immunosorbent assay. Gingival HIF-1 α and VEGF mRNA expression levels were evaluated using RT-qPCR, with Group 1 used as the calibrator group. Depending on data distribution, intergroup comparisons were performed using one-way analysis of variance or the Kruskal–Wallis test.

Results

Age and clinical periodontal parameters were comparable among the groups, whereas daily cigarette consumption differed significantly. GCF HIF-1 α levels differed significantly among groups; the highest mean value was observed in Group 4, and the lowest in Group 5. This difference, however, did not follow a progressive dose-dependent pattern. No significant intergroup difference was observed in GCF HIF-1 β levels. Gingival HIF-1 α mRNA expression was increased 1.58-, 2.08-, 1.62-, and 1.68-fold in Groups 2, 3, 4, and 5, respectively, compared with Group 1. VEGF mRNA expression was also increased in smoking groups, with the highest expression observed in Group 5.

Conclusions

In periodontally healthy individuals, smoking was associated with changes in hypoxia-related biomarkers. The higher gingival HIF-1 α and VEGF mRNA expression observed in the smoking groups suggests a possible tissue-level hypoxia-related transcriptional response. Since these changes were not progressively related to daily smoking intensity, the number of cigarettes smoked per day may be an incomplete indicator of the biological impact of smoking on periodontal tissues.

Background

Smoking is one of the most important environmental modifiers of periodontal tissue homeostasis. It has been associated with increased susceptibility to periodontal destruction, altered host response, impaired wound healing, and reduced predictability of periodontal treatment outcomes [1]. More recent reviews also emphasize that smoking cessation may reduce the risk of periodontal deterioration and improve periodontal treatment response [2, 3].

The adverse periodontal effects of smoking are not limited to clinically evident tissue destruction. Smoking may also affect vascular function, oxidative stress, immune regulation, subgingival ecology, and tissue repair mechanisms before clear periodontal breakdown becomes clinically detectable [4]. For this reason, periodontally healthy individuals may still be informative for studying early smoking-related molecular changes that are not apparent in routine periodontal measurements.

Periodontal health is generally established using clinical and radiographic parameters, including bleeding on probing, probing depth, attachment status, and radiographic bone loss. The 2017 World Workshop classification provides a widely accepted framework for distinguishing periodontal health from gingival inflammation and periodontitis [5]. However, clinically healthy periodontal tissues may still differ biologically, particularly in response to environmental modifiers such as smoking.

Smoking-related changes in periodontal tissues are likely to involve local vascular and metabolic mechanisms. By affecting gingival microcirculation and vascular regulation, chronic smoking may impair local oxygen delivery and has been linked with microvascular dysfunction in oral soft tissues [4]. Oxidative stress and altered cellular metabolism may further modify periodontal tissues' response to local stress, even before clear periodontal breakdown is clinically detected [4, 6, 7].

Another point that should be considered is that the number of cigarettes smoked per day is an imperfect measure of actual exposure. Two individuals reporting a similar daily cigarette count may still differ in their exposure to nicotine, tar, carbon monoxide, and other smoke constituents, depending on cigarette characteristics and smoking behavior. Puff volume, puff frequency, inhalation depth, and overall smoking topography may also influence the biological dose delivered to oral tissues. In the study by Hammond et al., cigarettes smoked per day and nicotine yield showed only weak associations with salivary cotinine, whereas an estimate incorporating cigarette consumption, brand elasticity, and puffing behavior was more closely related to nicotine uptake [8]. Biomarker-based evidence has also shown that cigarettes per day may be an imprecise marker of tobacco toxin exposure [9].

Hypoxia-inducible factor-1 (HIF-1) is a central transcriptional regulator of cellular adaptation to reduced oxygen availability. HIF-1 consists of an oxygen-sensitive HIF-1 α subunit and a more constitutively expressed HIF-1 β subunit, also known as aryl hydrocarbon receptor nuclear translocator [10]. Under hypoxic or stress-related conditions, HIF-1 α stabilization enables dimerization with HIF-1 β and transcriptional regulation of hypoxia-responsive genes [10].

Among these downstream targets, vascular endothelial growth factor (VEGF) is particularly relevant to periodontal tissues due to its roles in angiogenesis, vascular permeability, endothelial cell behavior, and tissue remodeling. VEGF has been discussed as a translational biomarker in oral non-communicable diseases, including periodontal conditions [11]. Accordingly, the HIF-1 α /VEGF axis offers a relevant framework for examining hypoxia-related and angiogenic responses in periodontal soft tissues.

Recent studies have placed increasing emphasis on HIF-1 α in periodontal biology. Shan et al. reviewed HIF-1 α as a regulator linking host–environment interactions to periodontal phenotypes, including angiogenesis, immune regulation, alveolar bone metabolism, and tissue remodeling [6]. More recently, Fadl and Leask highlighted the contribution of the HIF-1 α axis to periodontitis and emphasized the need for studies evaluating HIF-related responses in human periodontal samples [12].

Gingival crevicular fluid (GCF) is a site-specific oral biofluid located in close proximity to gingival tissues and has been widely used to assess local periodontal biomarker profiles [13]. Systematic reviews have also emphasized the diagnostic potential of oral-fluid and GCF-derived molecular biomarkers in periodontal research [14, 15]. Previous studies have evaluated hypoxia-related biomarkers in GCF and saliva. Afacan et al. contributed to the periodontal hypoxia-biomarker literature by evaluating GCF and salivary levels of HIF-1 α , VEGF, and TNF- α in periodontal health and disease, as well as treatment-related changes in GCF HIF-1 α , VEGF, and TNF- α following non-surgical periodontal therapy [16, 17]. More recently, Bozyel et al. evaluated GCF levels of HIF isoforms and VEGF in relation to smoking and periodontitis [7].

Although hypoxia-related biomarkers have been studied in periodontal health–disease comparisons, treatment response, and smoking–periodontitis interactions, their behavior across daily smoking-intensity groups in clinically periodontally healthy individuals remains less well defined [7, 16, 17]. Therefore, it remains unclear whether different levels of daily smoking are associated with GCF HIF-1 α /HIF-1 β levels and gingival HIF-1 α /VEGF mRNA expression before measurable periodontal deterioration becomes clinically evident.

Assessing HIF-1 α in both GCF and gingival tissue may capture different aspects of the local hypoxia-related response. GCF HIF-1 α reflects the protein-level status of the sulcular microenvironment, whereas gingival HIF-1 α mRNA expression reflects the transcriptional response of periodontal soft tissues. In this context, GCF HIF-1 β may help characterize the HIF complex, while gingival VEGF mRNA expression may represent a downstream angiogenic response related to HIF-associated signaling.

Based on this rationale, the present study evaluated GCF HIF-1 α and HIF-1 β levels and gingival HIF-1 α and VEGF mRNA expression in periodontally healthy individuals with varying levels of daily smoking.

Materials and Methods

Study Design and Participants

This cross-sectional clinical study was conducted at the Department of Periodontology, Faculty of Dentistry, Tokat Gaziosmanpaşa University. The study protocol was approved by the Clinical Research Ethics Committee of Tokat Gaziosmanpaşa University (approval no: TOGÜ KAEK 2019/07; date: 16 April 2019). All participants were informed about the study procedures, and written informed consent was obtained before enrollment.

A total of 100 periodontally healthy individuals were included in the study. Participants were classified as periodontally healthy according to the 2017 classification of periodontal and peri-implant diseases and conditions [18]. Participants were allocated into five groups according to their smoking status and daily cigarette consumption, with 20 individuals in each group: Group 1, non-smokers who had never smoked during their lifetime; Group 2, individuals smoking ≤ 5 cigarettes/day; Group 3, individuals smoking 6–10 cigarettes/day; Group 4, individuals smoking 11–19 cigarettes/day; and Group 5, individuals smoking ≥ 20 cigarettes/day.

Individuals aged 18–45 years who were systemically healthy, had at least 20 teeth, had not received periodontal treatment or antibiotic therapy within the previous 6 months, and had an indication for gingivectomy or crown-lengthening surgery as part of routine periodontal treatment were considered eligible. Participants were required to be periodontally healthy based on routine clinical and radiographic examinations. Individuals who did not meet these criteria were excluded.

Clinical Periodontal Examination

Clinical periodontal measurements were performed using a periodontal probe with a 0.5-mm tip diameter (Hu-Friedy, Chicago, IL, USA). Plaque index (PI), gingival index (GI), gingival sulcus depth, and bleeding on probing were recorded. PI was assessed according to the criteria described by Silness and Løe [19], and GI was assessed according to the criteria described by Løe and Silness [20]. Gingival sulcus depth was measured by inserting the periodontal probe parallel to the long axis of the tooth without applying excessive force.

Gingival Crevicular Fluid Collection and ELISA Analysis

Before gingival crevicular fluid (GCF) collection, supragingival plaque and food debris were removed from the tooth surfaces. The area was then gently air-dried and isolated with cotton rolls. GCF samples were collected using paper strips, which were gently inserted into the gingival crevice and left in place for 30 seconds. Care was taken to avoid mechanical trauma during sampling. The strips were then placed in Eppendorf tubes and stored at -80°C until biochemical analysis.

GCF levels of HIF-1 α and HIF-1 β were evaluated using the sandwich enzyme-linked immunosorbent assay (ELISA) method. Commercial 96-well ELISA kits were used according to the manufacturer's instructions (YL Biont, Shanghai YL Biotech Co., Ltd., Shanghai, China). On the day of analysis, samples were brought to room temperature, phosphate-buffered saline was added to each tube, and the tubes were vortexed to extract the GCF samples from the paper strips. The optical density of the samples was

measured at 450 nm using a microplate reader. Concentrations were calculated using the standard absorbance–concentration curve.

Gingival Tissue Sampling and RT-qPCR Analysis

Gingival tissue samples were obtained during routine gingivectomy or crown-lengthening procedures. Tissue biopsies of approximately 2×2 mm were collected and stored at – 80°C until analysis, as described previously [21].

For gene expression analysis, gingival tissues were frozen in liquid nitrogen, homogenized, ultrasonicated, and centrifuged at 25,000 g for 16 minutes. Homogenates were used for real-time quantitative polymerase chain reaction (RT-qPCR) analysis of HIF-1 α and VEGF mRNA expression.

mRNA isolation was performed using Trizol RNA extraction reagent. The amount of isolated mRNA was determined using a NanoDrop spectrophotometer. One-step RT-qPCR was performed using a commercial qRT-PCR kit. The following primers were used:

HIF-1 α forward: 5'-TGC TCA TCA GTT GCC ACT TC-3'

HIF-1 α reverse: 5'-CCA AGC AGG TCA TAG GTG GT-3'

VEGF forward: 5'-AAG GCT GAG CTG GAG GAA-3'

VEGF reverse: 5'-GGA GCA TGA TTG AGA CTC GC-3'

GAPDH forward: 5'-GTCTCCTCTGACTTCAACAGCG-3'

GAPDH reverse: 5'-ACCACCCTGTTGCTGTAGCCAA-3'

The cycling conditions were as follows: reverse transcription at 50°C for 30 minutes, initial denaturation at 95°C for 10 minutes, followed by amplification cycles of 95°C for 15 seconds, 55°C for 30 seconds, and 72°C for 35 seconds. GAPDH was used as the reference gene, and relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method. Group 1, the non-smoker group, was used as the calibrator group.

Statistical Analysis

Sample size was determined based on power analysis using Minitab 16 software (Minitab Inc., State College, PA, USA). Statistical analyses were performed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). The normality of data distribution was evaluated using the one-sample Kolmogorov–Smirnov test.

Age, daily cigarette consumption, PI, GI, gingival sulcus depth, and bleeding on probing were compared among groups using one-way analysis of variance followed by Tukey's post hoc test when appropriate. GCF HIF-1 α and HIF-1 β levels were compared among groups using the Kruskal–Wallis test, followed by pairwise Mann–Whitney U tests for post hoc comparisons. Statistical significance was set at $p < 0.05$.

Results

Demographic and Clinical Characteristics

The study population consisted of 100 periodontally healthy individuals, equally distributed across the five groups. The mean age was 24.00 ± 2.07 years in Group 1, 23.66 ± 4.40 years in Group 2, 23.35 ± 4.21 years in Group 3, 23.12 ± 2.80 years in Group 4, and 24.50 ± 3.70 years in Group 5. No statistically significant difference was observed among the groups in terms of age ($p > 0.05$; Table 1).

Clinical periodontal parameters were comparable among groups. PI, GI, vestibular, and lingual gingival sulcus depth, and bleeding on probing did not differ significantly among the study groups ($p > 0.05$). As expected, daily cigarette consumption differed significantly among groups ($p < 0.05$; Table 1).

Table 1

Demographic and clinical characteristics of the study groups. Values are presented as mean $\pm$ standard deviation. Group 1: non-smokers; Group 2: ≤ 5 cigarettes/day; Group 3: 6–10 cigarettes/day; Group 4: 11–19 cigarettes/day; Group 5: ≥ 20 cigarettes/day. PI: plaque index; GI: gingival index.

Parameter	Group 1	Group 2	Group 3	Group 4	Group 5
Age, years	24.00 ± 2.07	23.66 ± 4.40	23.35 ± 4.21	23.12 ± 2.80	24.50 ± 3.70
Daily cigarette consumption	0.00 ± 0.00	2.88 ± 1.18	7.05 ± 0.96	12.00 ± 1.96	25.50 ± 3.50
Plaque index	0.86 ± 0.35	1.22 ± 0.42	1.11 ± 0.48	1.12 ± 0.50	1.16 ± 0.38
Gingival index	1.06 ± 0.25	1.22 ± 0.42	1.29 ± 0.46	1.43 ± 0.51	1.16 ± 0.38
Gingival sulcus depth, vestibular	2.15 ± 0.46	2.32 ± 0.42	2.19 ± 0.55	2.30 ± 0.53	2.38 ± 0.46
Gingival sulcus depth, lingual	1.92 ± 0.28	2.08 ± 0.41	1.85 ± 0.42	2.01 ± 0.53	1.96 ± 0.55
Bleeding on probing	0.64 ± 0.76	0.68 ± 0.52	0.55 ± 0.34	0.78 ± 0.45	0.97 ± 0.56
GCF HIF-1 α and HIF-1 β Levels					

GCF HIF-1 α levels differed significantly among the groups ($p < 0.05$). Group 4 showed the highest mean HIF-1 α value, whereas Group 5 showed the lowest value. Pairwise comparisons showed that Group 4 had significantly higher HIF-1 α levels than Groups 2, 3, and 5. Group 1 also showed significantly higher HIF-1 α levels than Groups 2, 3, and 5, but significantly lower levels than Group 4. Despite these intergroup differences, HIF-1 α levels did not demonstrate a progressive dose-dependent pattern according to daily cigarette consumption (Table 2; Fig. 1).

No statistically significant difference was observed among the groups in terms of GCF HIF-1 β levels ($p > 0.05$). The mean HIF-1 β level was 15.05 ± 8.69 in Group 1, 11.81 ± 3.50 in Group 2, 11.09 ± 3.27 in Group 3, 12.54 ± 5.22 in Group 4, and 10.68 ± 2.00 in Group 5 (Table 2; Fig. 1).

Table 2

GCF HIF-1 α and HIF-1 β levels according to smoking status. Values are presented as mean $\pm$ standard deviation. Group 1: non-smokers; Group 2: $\leq$ 5 cigarettes/day; Group 3: 6–10 cigarettes/day; Group 4: 11–19 cigarettes/day; Group 5: $\geq$ 20 cigarettes/day. GCF: gingival crevicular fluid; HIF: hypoxia-inducible factor; ELISA: enzyme-linked immunosorbent assay.

Biomarker	Group 1	Group 2	Group 3	Group 4	Group 5	p value
HIF-1 α ELISA	1.30 $\pm$ 0.45	1.17 $\pm$ 0.18	1.03 $\pm$ 0.16	1.52 $\pm$ 1.46	0.73 $\pm$ 0.42	0.004
HIF-1 β ELISA	15.05 $\pm$ 8.69	11.81 $\pm$ 3.50	11.09 $\pm$ 3.27	12.54 $\pm$ 5.22	10.68 $\pm$ 2.00	0.101
Gingival HIF-1 α and VEGF mRNA Expression						

All smoking groups showed higher gingival HIF-1 α mRNA expression than Group 1. Relative to Group 1, HIF-1 α mRNA expression increased 1.58-fold in Group 2, 2.08-fold in Group 3, 1.62-fold in Group 4, and 1.68-fold in Group 5. Although HIF-1 α expression was higher in smoking groups than in Group 1, the increase did not show a progressive dose-dependent pattern (Table 3; Fig. 2).

A similar pattern was observed for VEGF mRNA expression, which was higher in the smoking groups than in Group 1. Relative to Group 1, VEGF mRNA expression increased 2.13-fold in Group 2, 2.15-fold in Group 3, 1.79-fold in Group 4, and 3.16-fold in Group 5. The most pronounced VEGF upregulation was observed in Group 5 (Table 3; Fig. 2).

Table 3

Relative gingival HIF-1 α and VEGF mRNA expression levels. Relative expression levels were normalized to GAPDH and calculated using the $2^{-\Delta\Delta Ct}$ method. Group 1 was used as the calibrator. Group 1: non-smokers; Group 2: $\leq$ 5 cigarettes/day; Group 3: 6–10 cigarettes/day; Group 4: 11–19 cigarettes/day; Group 5: $\geq$ 20 cigarettes/day. HIF: hypoxia-inducible factor; VEGF: vascular endothelial growth factor.

Gene expression	Group 1	Group 2	Group 3	Group 4	Group 5
HIF-1 α mRNA expression	1.00	1.58	2.08	1.62	1.68
VEGF mRNA expression	1.00	2.13	2.15	1.79	3.16

Discussion

The present study evaluated hypoxia-related biomarkers at both GCF protein and gingival tissue mRNA levels in periodontally healthy individuals with different levels of daily smoking. The main findings were that clinical periodontal parameters were comparable among the groups, whereas GCF HIF-1 α levels showed significant intergroup differences. In contrast, GCF HIF-1 β levels did not differ significantly among the groups. In gingival tissue samples, HIF-1 α and VEGF mRNA expression levels were increased in smoking groups compared with non-smokers, although these increases did not follow a progressive dose-dependent pattern according to daily smoking intensity.

The absence of significant differences in clinical periodontal parameters is important for interpreting the molecular findings of this study. Smoking is a well-established modifier of periodontal tissue homeostasis and treatment response, but its biological effects may not always be immediately reflected in conventional periodontal measurements [1, 22]. In the present study, plaque index, gingival index, gingival sulcus depth, and bleeding on probing were similar among groups, suggesting that the observed molecular differences were not simply secondary to clinically detectable periodontal inflammation. This supports the concept that smoking-related biological alterations may occur in clinically healthy periodontal tissues before overt periodontal destruction becomes measurable.

This interpretation is consistent with current views on the effects of smoking on the periodontal microenvironment. Smoking may influence gingival perfusion, oxygen delivery, oxidative stress, immune regulation, and microbial–host interactions, all of which may contribute to changes in periodontal tissue biology [4, 23]. Therefore, the evaluation of molecular biomarkers in periodontally healthy individuals may provide additional information beyond routine clinical periodontal examination. However, the findings should not be interpreted as evidence of established periodontal disease; rather, they may reflect early or subclinical alterations in the local tissue response.

GCF HIF-1 α levels differed significantly among the groups in the present study. The highest mean HIF-1 α level was observed in Group 4, whereas the lowest mean value was observed in Group 5. Pairwise comparisons showed that Group 4 had significantly higher HIF-1 α levels than Groups 2, 3, and 5, and Group 1 also showed higher levels than Groups 2, 3, and 5 but lower levels than Group 4. This pattern indicates that GCF HIF-1 α was sensitive to intergroup variation, but it did not show a simple progressive increase with daily smoking intensity.

Earlier clinical studies have examined HIF-related markers in periodontal fluids and tissues from different perspectives. Afacan et al. measured HIF-1 α , VEGF, and TNF- α in GCF and saliva across periodontal health and disease, and later evaluated treatment-related changes in these GCF markers after non-surgical periodontal therapy [16, 17]. Bozyel et al. subsequently investigated GCF HIF isoforms and VEGF in the context of smoking and periodontitis [7]. These studies provide an important basis for the current work; however, they mainly address disease status, treatment response, or smoking–periodontitis interactions. Our study differs by restricting the sample to clinically periodontally healthy individuals and by comparing groups according to daily smoking intensity.

The GCF HIF-1 α findings did not indicate a simple stepwise response to smoking. Although GCF HIF-1 α differed among groups, the highest value was observed in Group 4 rather than in the highest smoking category. This pattern may reflect the nature of GCF as a local fluid compartment influenced by several biological inputs, including gingival tissue activity, immune-cell contribution, vascular changes, oxygen availability, cellular stress, and low-grade inflammatory activity within clinically healthy limits [13, 14]. Thus, GCF HIF-1 α protein levels may not depend on daily cigarette number alone. This could explain why intergroup differences were detected without a linear relationship with smoking intensity.

In contrast to GCF HIF-1 α , gingival HIF-1 α mRNA expression was increased in all smoking groups compared with non-smokers. This finding suggests that smoking may be associated with a tissue-level transcriptional response related to hypoxia signaling, even when conventional periodontal parameters remain comparable among groups. Previous gingival tissue studies have shown increased HIF-1 α expression in periodontal disease and have linked HIF-1 α with VEGF expression, inflammation, angiogenesis, and tissue remodeling [24–26]. The present study adds to this literature by evaluating HIF-1 α mRNA expression in periodontally healthy individuals with different smoking intensities.

The partial discrepancy between GCF HIF-1 α protein levels and gingival HIF-1 α mRNA expression is biologically plausible. These two measurements are complementary but not interchangeable. Gingival HIF-1 α mRNA reflects transcriptional activity in the sampled tissue, whereas GCF HIF-1 α reflects the protein-level status of the sulcular microenvironment. Moreover, HIF-1 α is regulated not only at the transcriptional level but also through oxygen-dependent protein stabilization and degradation mechanisms [10, 27]. Therefore, the lack of complete parallelism between GCF HIF-1 α protein levels and gingival HIF-1 α mRNA expression should be considered a reflection of different biological compartments rather than a methodological inconsistency.

GCF HIF-1 β levels did not differ significantly among the groups. This finding is biologically plausible. HIF-1 is composed of an oxygen-sensitive HIF-1 α subunit and a more constitutively expressed HIF-1 β subunit, also known as aryl hydrocarbon receptor nuclear translocator [10]. Because HIF-1 β is generally regarded as a relatively stable binding partner rather than the primary oxygen-sensitive component of the HIF-1 complex, the absence of significant intergroup differences in GCF HIF-1 β does not weaken the interpretation of HIF-1 α -related changes [10]. Instead, it supports the idea that HIF-1 α may be the more dynamic component of the local hypoxia-related response.

VEGF mRNA expression was also increased in all smoking groups compared with non-smokers, with the highest expression observed in Group 5. VEGF is a key angiogenic mediator and one of the important downstream targets associated with hypoxia-related transcriptional responses. It has been implicated in vascular permeability, endothelial cell behavior, tissue remodeling, and periodontal inflammatory processes [11, 28]. The concomitant increase in gingival HIF-1 α and VEGF mRNA expression in smoking groups may indicate a hypoxia-related transcriptional response involving angiogenic adaptation. However, because VEGF expression also did not show a strictly progressive pattern across smoking-intensity groups, the findings should not be interpreted as evidence of a simple dose-dependent mechanism.

One possible explanation for the non-linear pattern observed in both GCF HIF-1 α and gingival gene expression findings is that daily cigarette consumption may not accurately reflect the actual biological dose of smoking exposure. Although cigarettes per day is a practical and widely used clinical grouping variable, it does not account for differences in cigarette type, nicotine yield, tar content, carbon monoxide exposure, puff volume, puff frequency, inhalation depth, or individual smoking topography. Hammond et al. reported that cigarettes smoked per day and nicotine yield were only weakly associated with salivary

cotinine, whereas a composite measure including cigarette consumption, brand elasticity, and puffing behavior was more strongly associated with nicotine uptake [8]. Similarly, Joseph et al. showed that cigarettes per day may not be a reliable measure of tobacco toxin exposure across all smoking levels [9].

This issue is particularly relevant to the present study. An individual reporting low daily smoking may still have relatively high biological exposure if they consume higher-yield cigarettes, inhale more deeply, or smoke with greater puff intensity. Conversely, an individual reporting higher daily smoking may have lower exposure per cigarette depending on cigarette composition and smoking behavior. This may partly explain why Group 5 did not show the highest values for all hypoxia-related biomarkers despite representing the highest daily smoking category. Therefore, the absence of a linear dose-response pattern does not necessarily negate a smoking-related biological effect; rather, it highlights the complexity of translating self-reported daily smoking into actual tissue-level exposure.

The combined evaluation of GCF protein levels and gingival tissue mRNA expression provides a broader view of hypoxia-related responses in clinically healthy periodontal tissues. GCF analysis allowed the assessment of HIF-1 α and HIF-1 β at the sulcular protein level, whereas gingival tissue RT-qPCR provided information on HIF-1 α and VEGF gene expression. This dual-level approach is useful because the periodontal response to smoking is likely mediated through several biological compartments, including vascular, epithelial, connective tissue, immune, and crevicular fluid components. Nevertheless, the results should be interpreted within the limits of the study design.

This study has some limitations. First, the cross-sectional design does not allow causal inference regarding the relationship between smoking and hypoxia-related biomarker alterations. Second, smoking status and daily cigarette consumption were based on self-report. Therefore, variations in cigarette type, nicotine and tar yield, carbon monoxide exposure, puffing behavior, inhalation depth, and individual smoking topography could not be fully controlled. Third, objective biomarkers of smoking exposure, such as serum or salivary cotinine, total nicotine equivalents, or exhaled carbon monoxide, were not assessed. Current tobacco research guidelines emphasize the value of biochemical verification methods for characterizing tobacco exposure and abstinence, particularly when self-report may be insufficient [29]. These limitations should be considered when interpreting the absence of a strictly dose-dependent pattern according to daily smoking intensity.

Future studies should include larger cohorts, objective biomarkers of tobacco exposure, and more detailed assessment of smoking behavior. In addition to daily cigarette consumption, variables such as smoking duration, pack-years, cigarette type, inhalation pattern, cotinine levels, and exhaled carbon monoxide may help clarify the relationship between smoking exposure and periodontal hypoxia-related responses. Longitudinal studies would also be useful to determine whether hypoxia-related molecular alterations in clinically healthy periodontal tissues precede measurable periodontal deterioration.

Taken together, our findings indicate that smoking-related molecular differences may be present in periodontally healthy individuals despite comparable clinical periodontal parameters. However, these alterations do not appear to be explained solely by the number of cigarettes smoked per day. These

findings support the view that daily smoking intensity is a useful but incomplete indicator of the biological impact of smoking on clinically healthy periodontal tissues.

Conclusion

Within the limitations of this cross-sectional study, smoking was associated with changes in hypoxia-related biomarkers in periodontally healthy individuals. GCF HIF-1 α varied among groups, whereas GCF HIF-1 β remained similar. At the tissue level, smoking groups showed higher HIF-1 α and VEGF mRNA expression than non-smokers, supporting a possible hypoxia-related transcriptional response.

These changes were not progressively related to daily smoking intensity. This may reflect differences in cigarette composition, inhalation behavior, and smoking topography. Future studies using objective exposure markers are needed to clarify how smoking intensity is linked to hypoxia-related periodontal tissue responses.

Abbreviations

ELISA: Enzyme-linked immunosorbent assay

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

GCF: Gingival crevicular fluid

GI: Gingival index

HIF: Hypoxia-inducible factor

PI: Plaque index

RT-qPCR: Real-time quantitative polymerase chain reaction

VEGF: Vascular endothelial growth factor

Declarations

Ethics approval and consent to participate

The study protocol was approved by the Clinical Research Ethics Committee of Tokat Gaziosmanpaşa University (approval no: TOGÜ KAEK 2019/07; date: 16 April 2019). All participants were informed about the study procedures, and written informed consent was obtained before enrollment.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was supported by Tokat Gaziosmanpaşa University Scientific Research Projects Coordination Unit.

Authors' contributions

OY contributed to patient selection, clinical data collection, sample collection, laboratory analyses, and preparation of data. MMT contributed to the conception and design of the study, supervision of the research process, interpretation of the data, manuscript writing, and critical revision of the manuscript. EC contributed to laboratory analyses and processing of laboratory data. OK contributed to the conception and design of the study, statistical analysis, interpretation of the data, manuscript writing, and critical revision of the manuscript. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

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Figures

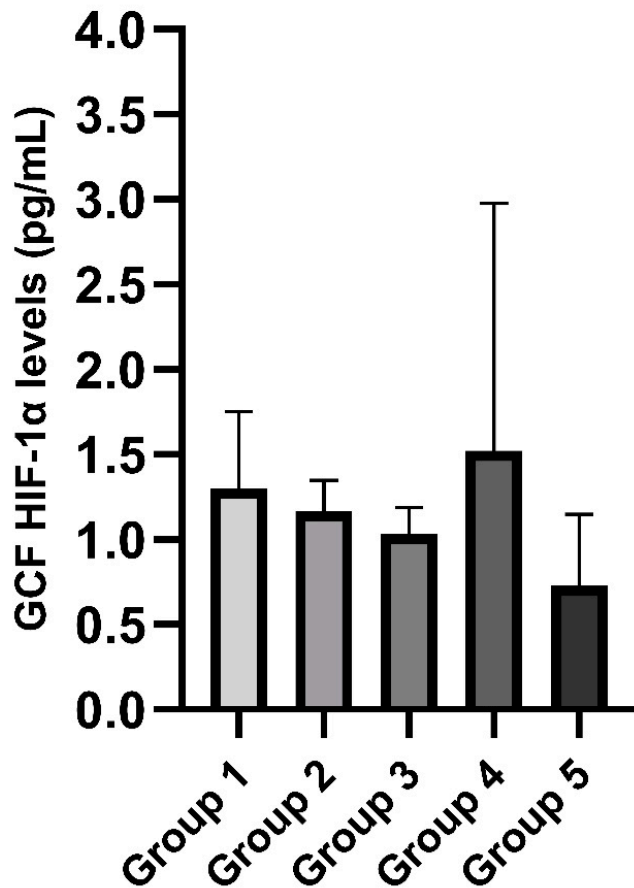
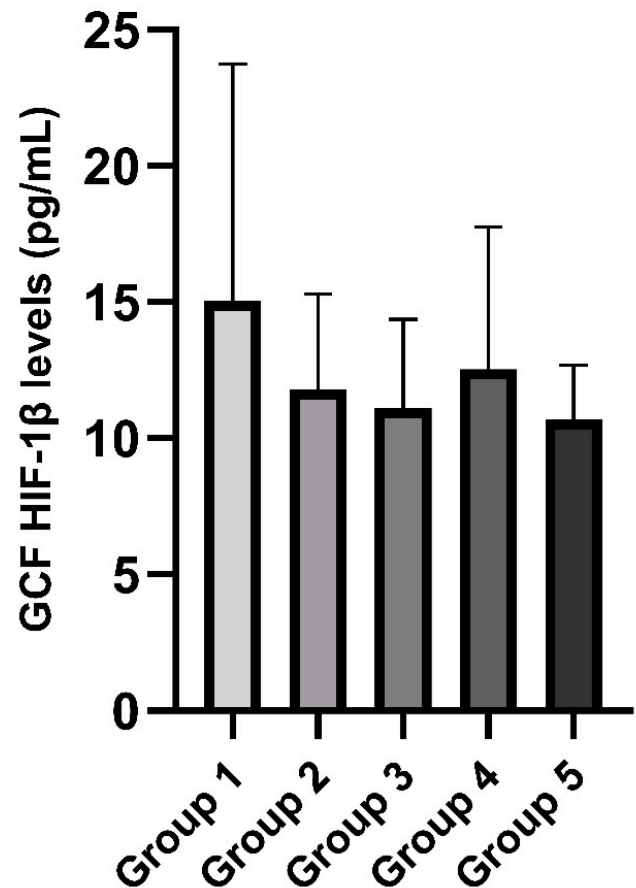
A**B**

Figure 1

Gingival crevicular fluid levels of HIF-1 α and HIF-1 β according to study groups. (A) GCF HIF-1 α levels differed among the groups, but no clear dose-dependent pattern was observed according to daily cigarette consumption. (B) GCF HIF-1 β levels did not differ significantly among the groups. Data are presented as mean $\pm$ SD. Group 1: non-smokers; Group 2: ≤ 5 cigarettes/day; Group 3: 6–10 cigarettes/day; Group 4: 11–19 cigarettes/day; Group 5: ≥ 20 cigarettes/day.

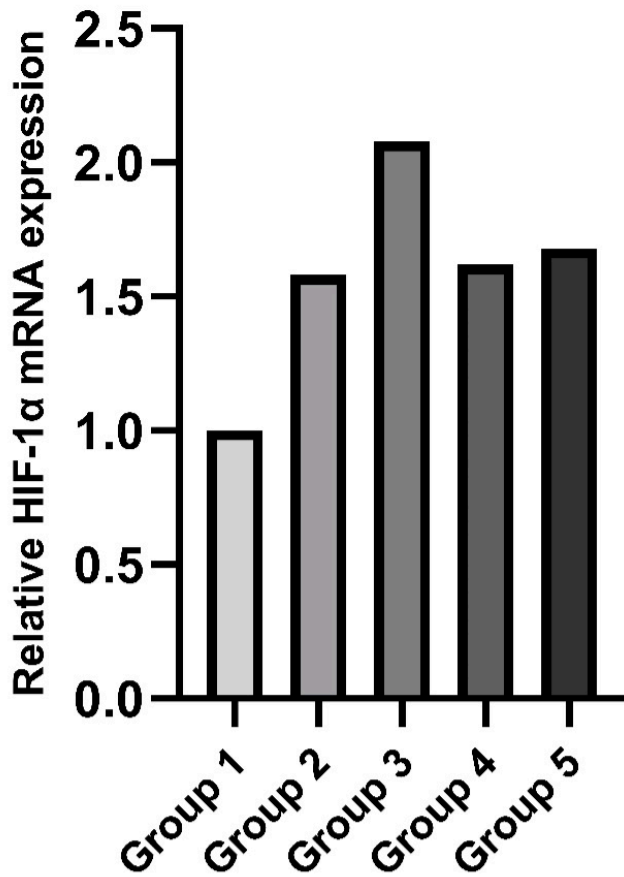
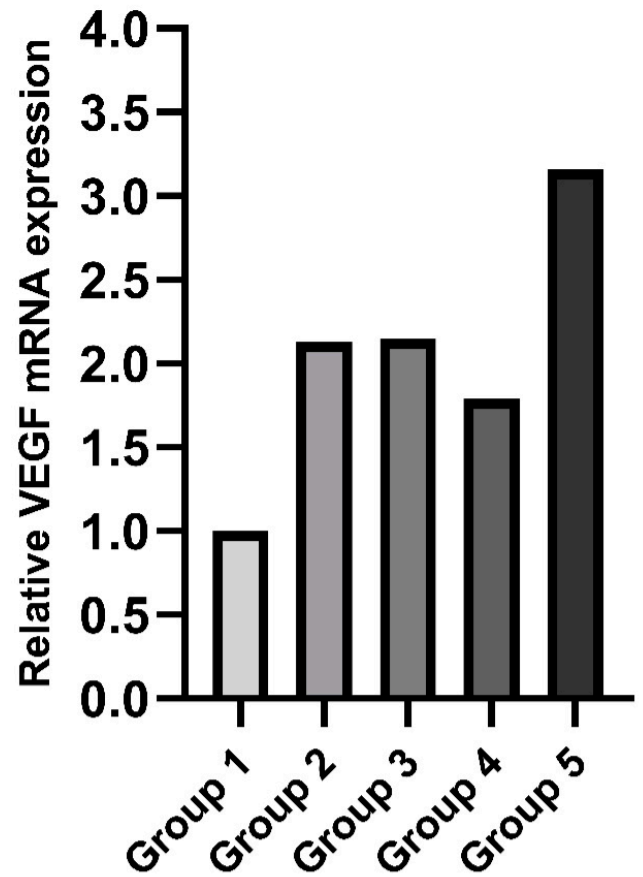
A**B**

Figure 2

Relative gingival mRNA expression of HIF-1 α and VEGF according to study groups. (A) HIF-1 α mRNA expression was increased in smoking groups compared with Group 1. (B) VEGF mRNA expression was increased in smoking groups, with the highest expression observed in Group 5. Expression levels were normalized to GAPDH and calculated using the $2^{-\Delta\Delta C_t}$ method, with Group 1 used as the calibrator. Group 1: non-smokers; Group 2: ≤ 5 cigarettes/day; Group 3: 6–10 cigarettes/day; Group 4: 11–19 cigarettes/day; Group 5: ≥ 20 cigarettes/day.