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Association between Non-Alcoholic Steatohepatitis-Related Hepatocellular Carcinoma and Periodontopathic Bacteria: A Cross-Sectional Pilot Study

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Abstract: The incidence of non-alcoholic steatohepatitis (NASH)-related hepatocellular carcinoma (HCC) is increasing annually as the metabolic syndrome factors increase. This study aimed to analyze the involvement of periodontopathic bacteria in NASH-related HCC (NASH-HCC). Questionnaire investigation, periodontal examination, medical examination, and specimen collection (saliva, mouth-rinsed water, and peripheral blood) were performed in 40 patients with NASH and in 20 patients with NASH-HCC. Immunoglobulin (Ig) G antibody titers against *Porphyromonas gingivalis* ($p = 0.031$) and *Fusobacterium nucleatum* ($p = 0.003$) were significantly higher in the NASH-HCC group than in the NASH group. *P. gingivalis* and *F. nucleatum* ratios were higher in the NASH-HCC group than in the NASH group; however, only *F. nucleatum* ratio was significant ($p = 0.009$). The Shannon index of salivary bacterial flora was significantly lower in the NASH-HCC group than in the NASH group ($p < 0.001$). The NASH-HCC group had a significantly lower salivary IgA concentration ($p = 0.007$) and a slower salivary IgA flow rate ($p = 0.003$). In all participants, the salivary IgA flow rate and the *F. nucleatum* ratio showed a significant negative correlation ($p = 0.02$). Oral *P. gingivalis* and *F. nucleatum* were possibly associated with NASH-HCC pathogenesis, and salivary IgA levels were correlated with *F. nucleatum*.

Keywords: hepatocellular cancer; non-alcoholic steatohepatitis; periodontitis; *Porphyromonas gingivalis*; *Fusobacterium nucleatum*; immunoglobulin A; saliva

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease, classified into non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis

(NASH) [1,2]. The former hardly progresses; however, the latter is progressive and may lead to cirrhosis or hepatocellular carcinoma (HCC) [3]. Most cases of HCC are caused by viral hepatitis; however, recently, the incidence has gradually decreased due to the direct-acting antiviral drug therapy for hepatitis C and nucleic acid analog therapy for hepatitis B [4,5]. Conversely, the incidence of NASH-related HCC (NASH-HCC) is increasing annually as metabolic syndrome factors, such as obesity and diabetes, increase [6]. The multiple parallel hit theory has been hypothesized to cause NASH [7]. However, the pathogenic mechanism of NASH-HCC has not been elucidated.

Periodontal disease is an independent risk factor for NAFLD [8] and is significantly associated with liver fibrosis in NAFLD [9]. Qin et al. [10] analyzed the intestinal microbiota of patients with cirrhosis. They reported that oral cavity-derived bacteria were detected at a high frequency in correlation with the severity of the disease, leading to the later mouth–gut–liver axis concept [11]. The detection rate of *Porphyromonas gingivalis*—a major causative bacterium of periodontitis —in the saliva of patients with NAFLD was significantly higher than that of control participants and increased with the aggravation of NAFL and NASH disease [12]. Furthermore, Sato et al. [13] recently demonstrated that the positive rate of salivary *P. gingivalis* (>0.01%) in patients with NAFLD was significantly correlated with liver cirrhosis. Additionally, Furusho et al. [14] reported the presence of *P. gingivalis* in the immunohistochemical staining of liver tissues from patients with NASH. It was presumed that this occurred because *P. gingivalis* entered the blood from the periodontitis lesion and reached the liver.

Epidemiological studies have reported that periodontopathic bacteria increase the risk of developing various cancers and cancer-related deaths [15]. Notably, *P. gingivalis* level is high in the esophageal flora of patients with esophageal cancer [16] and correlates with malignancy [17]. In addition, *Fusobacterium nucleatum*—a periodontal pathogen—has been detected in digestive organs, including the esophagus, stomach, and large intestine, and in breast cancer tissues [18–21]. Recently, Komiya et al. [22] revealed that *F. nucleatum* strain in colorectal cancer tissue is similar to that of saliva and originates in the oral cavity. However, few studies exist regarding HCC; one reported high blood levels of reactive oxygen species in HCC patients with periodontitis [23]. Moreover, little is known about the relationship between NASH-HCC and periodontopathic bacteria.

We hypothesized that *P. gingivalis* and *F. nucleatum* are associated with the pathogenesis of NASH-HCC. This study aimed to analyze the oral microbiota data of patients with NASH and NASH-HCC and examine the association of periodontopathic bacteria in NASH-HCC.

2. Materials and Methods

2.1. Participants

This study was a cross-sectional pilot study. We examined patients with NASH and NASH-HCC aged $\geq$ 20 years who visited or were admitted to the Department of Gastroenterology and Hepatology, Yokohama City University Hospital, between November 2020 and April 2022. The study was conducted at the Department of Dentistry, Oral and Maxillofacial Surgery and Orthodontics of the same hospital. Additionally, the study protocol was approved by the Ethics Committee of Kanagawa Dental University and Yokohama City University and was registered on a clinical trial database (UMIN000042754). Before beginning the study, the participants provided consent after they were briefed on the purpose of the study, its summary, and the confidentiality of personal information. Furthermore, all procedures were performed per the policies of the Declaration of Helsinki.

We excluded patients who had ingested antimicrobial agents within 1 month before enrollment and those with an edentulous jaw from the study. Of the 69 patients initially registered, five were excluded because of the use of antimicrobial agents between obtaining consent and the date of testing, three failed to comply with visits on the day of the test and two were excluded for missing data in multiple tests. Eventually, the tests including periodontal, salivary, and medical examinations were performed on a total of 60 patients (40 with NASH and 20 with NASH-HCC), and data were used for analysis.

2.2. Background Information

Questionnaires were used to collect information about the participants, including their age, gender, smoking history, number of meals per day, number of snacks per day, frequency of brushing teeth per day, duration of brushing teeth each time (min), and the number of dentist visits per year. In addition, height and body weight information were extracted from the participants' medical charts to calculate body mass index (BMI).

2.3. Clinical Assessment

Two dentists affiliated with the Department of Periodontology, Kanagawa Dental University conducted periodontal examinations. Probing depth and bleeding on probing were recorded at six sites per tooth (buccal mesial, buccal central, buccal distal, lingual mesial, lingual central, and lingual distal). Moreover, the plaque index was recorded at four sites per tooth (mesial, buccal, distal, lingual), and the degree of tooth mobility was evaluated for all teeth. Probing was performed with a periodontal probe (Contact-Probe; Nihon Shiken Co., Ltd., Tokyo, Japan) with a force of 0.2 N. The examiner calibrated the measurement in advance using a periodontal disease model (P15FE-500HPRO-S2A1-GSF; NISSIN, Kyoto, Japan).

2.4. Saliva Sampling and Bacterial Flora Analysis

Saliva samples were collected in two ways. The saliva used for measuring flow rate and IgA concentration was collected with Salivette® (SARSTEDT, Nümbrect, Germany). Briefly, a set of polypropylene/polyethylene polymer sponges were placed under the participant's tongue for 2 min, after which the saliva-filled sponge was placed in a tube. The tube was immediately ice-cooled, centrifuged (1200× g, 20 min, 4 °C), and stored at −80 °C until analysis.

The saliva used for bacterial flora analysis was collected by having the participant gargle 7.5 mL of physiological saline for 10 s to mix with the saliva, and collecting the fluid spat out by the participant in a tube. The tubes were immediately ice-cooled and stored at −80 °C until analysis. Bacterial DNA extraction from the collected saliva samples, amplification of the 16S rRNA gene, decoding of the base sequences by the MiSeq sequencer (Illumina, Inc., San Diego, CA, USA), and analysis of the bacterial flora were performed at a medical laboratory (Cykinso Inc., Tokyo, Japan) [24,25].

2.5. Blood Sampling and Medical Examination

Peripheral blood sampling was conducted at the Department of Gastroenterology and Hepatology, Yokohama City University Hospital. Through medical examinations comprising biochemical and liver function tests, we analyzed the levels of endotoxins, C-reactive protein (CRP), casual blood glucose (CBG), low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides (TG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transferase (GGT), and total bilirubin (T-Bil). A portion of the serum was stored at −80 °C until analysis by the enzyme-linked immunosorbent assay (ELISA).

2.6. Measurement of Salivary IgA Concentration

Salivary IgA concentration was measured by ELISA using the Human IgA ELISA kit (Bethyl Laboratories, Montgomery, AL, USA) following the manufacturer's protocol. The saliva flow rate (mL/min) was determined by dividing the saliva weight (mg) by the collection time. The specific gravity of saliva was assumed to be 1.00 g/mL. Lastly, the salivary IgA flow rate (μg/min) was calculated by multiplying the absolute IgA concentration (μg/mL) by the saliva flow rate (mL/min).

2.7. Measurement of Serum Antibody Titers against Periodontal Pathogens

Serum immunoglobulin G (IgG) antibody titers against *P. gingivalis* and *F. nucleatum* were measured using ELISA, following a previous protocol [26]. Briefly, bacterial solutions

of *P. gingivalis* ATCC33277 and *F. nucleatum* ATCC25586 adjusted to an optical density of 1.0 were fixed in a 96-well microplate. After allowing it to stand overnight, a blocking solution (50 mM Tris, 0.14 M NaCl, 1% bovine serum albumin, pH 8.0) was added to each well and allowed to stand at room temperature for 30 min. Thereafter, the cells were washed thrice with a washing solution (50 mM Tris, 0.14 M NaCl, 0.05% polysorbate 20, pH 8.0), and a 5000-fold diluted serum was added to each well. After standing at room temperature for 1 h, the cells were washed five times with the washing solution, and Goat F(ab')2 anti-Human IgG(Fab')2(HRP) (Abcam, Cambridge, UK) diluted 10,000-fold was added to each well. After standing at room temperature for 1 h, the cells were washed with the washing solution five times, and 3,3′,5,5′-tetramethyl-benzidine was added to each well. Lastly, the cells were left to stand at room temperature and away from light for 15 min before adding the stopping solution (0.18 M H_2SO_4). Absorbance was measured at a wavelength of 450 nm with a microplate reader (Bio-Rad, Hercules, CA, USA).

2.8. Statistical Analysis

Statistical analysis was performed using the SPSS Statistics Ver 27.0 (IBM, Tokyo, Japan). Differences for gender and smoking were verified by χ^2 test. In the other items, Mann–Whitney's U test was used for comparisons between the groups. *P*-values were calculated by Monte Carlo method with 99% confidence intervals. Spearman's rank correlation coefficient was used for correlation analysis. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Participant Information, Lifestyle Habits, and Periodontal Examination

Table 1 shows the participants' information and lifestyle habits. The NASH-HCC group was significantly older ($p < 0.001$) than the NASH group. Additionally, the NASH-HCC group had a significantly higher number of dentist visits per year ($p = 0.005$) than the NASH group. However, both groups showed similar results in all seven items of the periodontal examination (Table 2).

Table 1. Participant information and lifestyle habits.

Parameter	NASH Group (N = 40)	NASH-HCC Group (N = 20)	*p*-Value (99% CI)
Gender (Female/Male)	18/22	6/14	0.26
Smoking (non-smoker/smoker)	9/31	4/16	0.376 (0.363–0.388)
Age (years)	60.5 (55–70)	78.5 (65–81.8)	<0.001 *
BMI	27.5 (25.8–31.1)	25.8 (22.2–31.5)	0.278 (0.266–0.289)
Number of meals per day	3 (3–3)	3 (3–3)	0.218 (0.208–0.229)
Number of snacks per day	1 (0–2)	1.5 (1–2.8)	0.386 (0.373–0.398)
Frequency of brushing teeth per day	2 (1–2)	2 (2–2)	0.799 (0.789–0.809)
Duration of brushing teeth each time (min)	3 (3–5)	4 (3–5)	0.210 (0.199–0.220)
Number of dentist visits (times/year)	0 (0–2)	2 (0.7–4)	0.005 * (0.003–0.007)

Abbreviations: BMI, body mass index; CI, confidence intervals. The figures for gender and smoking indicate the number of individuals, and statistical analysis was performed using the χ^2 test. The presented values for the other items are medians (first quartile–third quartile), and the Mann–Whitney's U test was used for statistical analysis (* $p < 0.05$).

Table 2. Periodontal examinations.

Parameter	NASH Group (N = 40)	NASH-HCC Group (N = 20)	*p*-Value (99% CI)
Number of teeth	26 (21–28)	25 (19.5–26.8)	0.324 (0.312–0.336)
PD (mm)	2.9 (2.6–3.2)	2.9 (2.6–3.2)	0.855 (0.846–0.864)
BOP (%)	15.0 (10.7–30.9)	15.2 (7.1–30.6)	0.456 (0.443–0.468)
Tooth mobility	0 (0–0.6)	0 (0–0.1)	0.495 (0.482–0.507)
PlI	0.9 (0.7–1.4)	0.9 (0.7–1.4)	0.927 (0.921–0.934)
PESA (mm^2)	1355 (1174–1654)	1306 (938–1494)	0.241 (0.230–0.252)
PISA (mm^2)	234 (141–380)	194 (86–506)	0.408 (0.395–0.420)

Abbreviations: PD, Probing depth; BOP, Bleeding on probing; PlI, Plaque index; PESA, periodontal epithelial surface area; PISA, periodontal inflamed surface area; CI, confidence intervals. BOP (+) = 1; BOP (−) = 0. The presented values are medians (first quartile–third quartile), and the Mann–Whitney's U test was used for statistical analysis.

3.2. Bacterial Examination

Table 3 shows IgG antibody titer measurements and the ratios of periodontal pathogens in the saliva. IgG titers were significantly higher for *P. gingivalis* ($p = 0.031$) and *F. nucleatum* ($p = 0.003$) in the NASH-HCC group than in the NASH group.

P. gingivalis and *F. nucleatum* ratios to the total number of bacteria in saliva were higher in the NASH-HCC group than in the NASH group; however, only *F. nucleatum* ratio was significant ($p = 0.009$). Conversely, the *Treponema denticola* ratio was significantly higher in the NASH group ($p = 0.013$) than in the NASH-HCC group. Additionally, the Shannon index of bacterial flora in saliva was significantly lower in the NASH-HCC group ($p < 0.001$) than in the NASH group.

Table 3. Bacterial examinations.

Parameter	NASH Group (N = 40)	NASH-HCC Group (N = 20)	*p*-Value (99% CI)
Serum antibody titer			
P. gingivalis IgG titer (EU)	0.09 (0.05–0.18)	0.18 (0.11–0.26)	0.031 * (0.026–0.035)
F. nucleatum IgG titer (EU)	0.01 (0–0.03)	0.04 (0.02–0.09)	0.003 * (0.002–0.005)
The ratio of periodontopathogenic bacteria in saliva			
P. gingivalis ratio (%)	0.03 (0–1.25)	0.46 (0–0.69)	0.381 (0.369–0.394)
T. forsythia ratio (%)	0.14 (0.06–0.35)	0.07 (0.02–0.31)	0.400 (0.387–0.412)
T. denticola ratio (%)	0.05 (0–0.2)	0 (0–0.03)	0.013 * (0.010–0.016)
F. nucleatum ratio (%)	0.14 (0–0.35)	0.35 (0–0.61)	0.009 * (0.007–0.012)
P. intermedia ratio (%)	0 (0–0.33)	0 (0–0.23)	0.752 (0.741–0.763)
Diversity of bacterial flora			
Shannon index	6.75 (6.51–7.14)	6.42 (6.04–6.64)	<0.001 *

CI, confidence intervals. The presented values are medians (first quartile–third quartile), and the Mann–Whitney's U test was used for statistical analysis (* $p < 0.05$).

3.3. Medical Examination

Table 4 shows the results of medical examinations. Endotoxin ($p < 0.001$) and CBG levels ($p = 0.04$) were significantly higher in the NASH-HCC group than in the NASH group. In addition, the T-Bil level of the NASH-HCC group was significantly higher than that of the NASH group ($p = 0.014$).

Table 4. Medical examinations.

Parameter	NASH Group (N = 40)	NASH-HCC Group (N = 20)	*p*-Value (99% CI)
Endotoxin (EU)	0.13 (0.08–0.2)	0.21 (0.16–0.28)	<0.001 *
CRP (mg/dL)	0.14 (0.09–0.44)	0.15 (0.07–0.5)	0.734 (0.722–0.745)
CBG (mg/dL)	118 (101–153)	140 (122–163)	0.04 * (0.035–0.045)
LDL-cholesterol (mg/dL)	109 (93–131)	104 (90–135)	0.782 (0.772–0.793)
HDL-cholesterol (mg/dL)	56 (45–60)	53.5 (50.3–60.8)	0.925 (0.918–0.931)
TG (mg/dL)	154 (103–267)	122 (71–283)	0.271 (0.260–0.283)
AST (U/L)	50.5 (26.3–62)	38 (29–65.5)	0.831 (0.821–0.841)
ALT (U/L)	51.5 (25.3–71.8)	32 (24–54.5)	0.167 (0.157–0.176)
GGT (U/L)	65 (36.3–126)	66.5 (49.5–111.3)	0.684 (0.672–0.696)
T-Bil (mg/dL)	0.8 (0.6–1)	1.3 (0.75–1.7)	0.014 * (0.011–0.017)

Abbreviations: CRP, C-reactive protein; CBG, casual blood glucose; LDL, low-density lipoprotein; HDL, high-density lipoprotein; TG, triglycerides; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, γ-glutamyltransferase; T-Bil, total bilirubin; CI, confidence intervals. The presented values are medians (first quartile–third quartile), and the Mann–Whitney's U test was used for statistical analysis (* $p < 0.05$).

3.4. Correlation between Salivary IgA Levels and Bacterial Ratio in Saliva

The saliva flow rate was similar between the NASH and NASH-HCC groups (Figure 1a). However, the salivary IgA concentration ($p = 0.007$) and flow rate ($p = 0.003$) in the NASH-HCC group were significantly lower and slower than in the NASH group, respectively (Figure 1b,c).

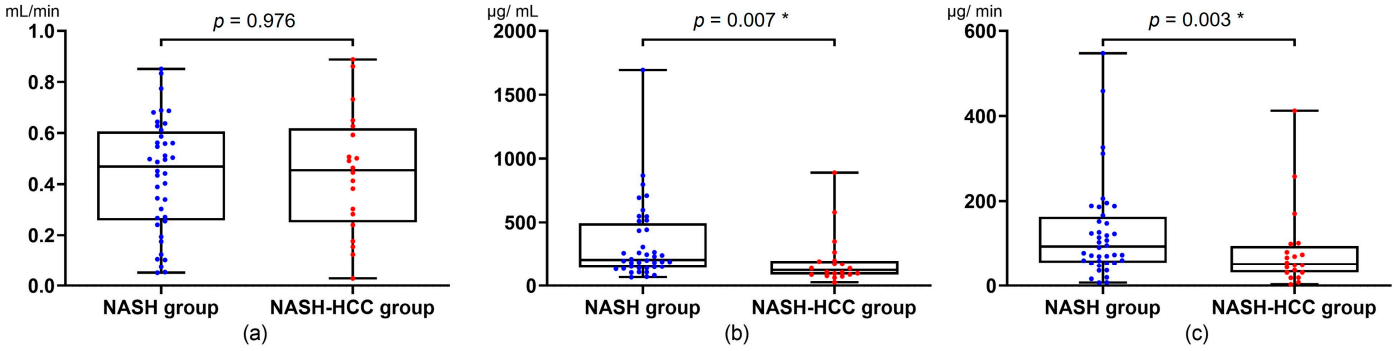

Figure 1. Salivary IgA levels: (**a**) saliva flow rate; (**b**) salivary IgA concentration; (**c**) salivary IgA flow rate. The presented values are medians (first quartile–third quartile), and the Mann–Whitney's U test was used for statistical analysis (* $p < 0.05$). Each 99% confidence intervals were 0.972–0.980: (**a**), 0.005–0.010: (**b**) and 0.001–0.004: (**c**).

Table 5 shows the correlations between the salivary IgA flow rate, the bacterial ratio in saliva, and the Shannon index in all participants. The *F. nucleatum* ratio was significantly correlated with the salivary IgA flow rate ($p = 0.02$).

Table 5. Correlation between salivary IgA flow rate and oral bacterial ratio.

	IgA Flow Rate in Saliva (μg/min)		
	Correlation Coefficient	*p*-Value	N
P. gingivalis ratio (%)	0.07	0.6	60
T. forsythia ratio (%)	0.03	0.8	60
T. denticola ratio (%)	0.03	0.8	60
F. nucleatum ratio (%)	−0.30	0.02 *	60
P. intermedia ratio (%)	0.13	0.3	60
Shannon index	0.003	0.98	60

Spearman's rank correlation coefficient was used for statistical analysis (* $p < 0.05$).

4. Discussion

This is the first report of an association between NASH-HCC and periodontopathogenic bacteria. Although the periodontal conditions of both groups were similar, the salivary *P. gingivalis* and *F. nucleatum* ratios and the serum antibody titers cross-reacting with them were higher in the NASH-HCC group than in the NASH group. Additionally, *F. nucleatum* ratio in the saliva and the salivary IgA flow rate showed a negative correlation.

Clinical test items used in the medical examinations were evaluated based on the Japanese common reference interval [27,28]. Moreover, CRP levels in the NASH-HCC group and TG, AST, ALT, and GGT in both groups were higher than the reference interval. Furthermore, endotoxin and CBG levels in the NASH-HCC group were higher than those in the NASH group. Ohara-Nemoto et al. [29] reported that *P.gingivalis* expresses dipeptidyl peptidase 4, which is involved in regulation of blood glucose levels, possibly explaining the higher CBG level in the NASH-HCC group. Since T-Bil increases in liver cirrhosis and liver cancer [30], the NASH-HCC group had higher levels than the NASH group. In addition, ALT varied more in the NASH group, possibly because this group included patients who were undergoing treatment. Overall, the above findings suggest that although some differences existed between groups NASH and NASH-HCC, there was no profound pathological difference in the state of infection and inflammation.

As mentioned earlier, the NASH-HCC group participants were older and had higher endotoxin and CBG levels than those in the NASH group. However, the results of the periodontal examination were similar for both groups. Notably, the periodontal condition is reportedly maintained by regular supportive periodontal therapy [31]. The NASH-HCC group had more dentist visits than the NASH group; therefore, they received more periodontal care. Hence, even if microbial shift occurred in the periodontal pockets of the NASH-HCC group, severe periodontal disease could have been prevented.

We analyzed the ratio of periodontopathogenic bacteria in saliva using a next-generation sequencer. *P. gingivalis* and *F. nucleatum* ratios were higher in the saliva of the NASH-HCC group than those in the NASH group. This indicates that these bacteria may be involved in the progression from NASH to HCC. The detection rate of *P. gingivalis* in saliva reportedly increases as the disease severity increases in healthy participants, NAFLD, and NASH [12]; we observed a similar association in NASH-HCC. Omura et al. [32] reported that *P. gingivalis* was detected in venous blood and hepatocytes of patients with NASH who died of sepsis. This suggests the role of oral *P. gingivalis* in NASH progression to cirrhosis or HCC. Notably, of the two periodontopathogenic bacteria associated with NASH-HCC, *F. nucleatum* has recently been associated with other systemic diseases. It is involved in the development and progression of various cancers, including colorectal cancer, by evading immune mechanisms, affecting oncogenes and tumor suppressor genes, promoting cell proliferation, and by adhesion mechanisms with cancer cells [33–35]. Moreover, *F. nucleatum* was detected in liver abscesses with extensive necrosis and fibrosis [36]. In the present study, *T. denticola* ratio was low in the NASH-HCC group. Compared with the 57% detection in the NASH group, *T. denticola* was detected in 35% of the participants in the NASH-HCC group; this ratio might have decreased with the increase in other bacterial species ratios.

We measured serum antibody titers crossing *P. gingivalis* and *F. nucleatum*. *P. gingivalis* is the major pathogenic bacterium associated with the development and progression of periodontitis [37]. According to a report, *P. gingivalis* infection in mice on a high-fat diet increases blood LPS levels, causes hepatic fibrosis, and progresses NASH [14]. Additionally, Ahn et al. [38] reported extensive lobular inflammation in the liver of *P. gingivalis*-infected obese mice and elevated levels of metabolic regulators, including peroxisome proliferator-activated receptor γ and fatty acid transporter CD36. *F. nucleatum* is involved in various periodontal diseases, such as mild reversible gingivitis and advanced irreversible gingivitis [39]. Until now, this bacterium was known to be ubiquitously distributed in the oral cavity but hardly detected in other body parts [40]. However, following reports indicating its involvement in cancer, it is now considered a significant bacterium [18–21,33]. Therefore,

P. gingivalis and *F. nucleatum* can affect systemic disease, justifying why we selected and measured the antibody titer levels of both bacteria.

The serum antibody titers crossing *P. gingivalis* and *F. nucleatum* were higher in the NASH-HCC group than in the NASH group. Our previous report has suggested that the combination of salivary *P. gingivalis* ratio and serum *P. gingivalis* IgG titers was related to the progression of periodontitis [41]. In addition, the serum antibody titer that crosses periodontopathogenic bacteria in patients with periodontitis remains high for >30 months, reflecting an infection history [42]. Moreover, *F. nucleatum* ratio was high in the NASH-HCC group in this study. We have previously reported that when the periodontopathogenic bacterial ratio in the saliva is high, the serum antibody titer that crosses the bacteria increases [41]. Based on these observations, we speculated that the serum antibody titers against *P. gingivalis* and *F. nucleatum* became high due to the history of continuous infection and the current composition of the bacterial flora.

The NASH-HCC group had a lower salivary IgA concentration and flow rate than the NASH group. Additionally, the median age of the study participants was 60.8 years in the NASH group and 78.5 years in the NASH-HCC group; thus, the NASH-HCC group was older. However, Miletic et al. [43] reported no difference in salivary IgA flow rates for participants in their 60s and 80s. Therefore, the effect of aging on salivary IgA flow rates in the NASH-HCC group may have been small. Interestingly, salivary IgA concentrations in patients with periodontal disease are higher than those in healthy individuals [44]. Conversely, the salivary IgA flow rate is reportedly lower in patients with breast cancer before and during chemotherapy than in healthy individuals [45]. In addition, patients with pediatric cancer have decreased salivary IgA concentration regardless of whether they are receiving anticancer drug treatments, and salivary IgA concentration decreases in patients with cancer even before treatment initiation [46]. This is attributed to metabolic, hormonal, and immunological changes that lead to immune dysfunction in patients with cancer [46]. Furthermore, patients with acquired immunodeficiency syndrome (AIDS)—a disease that causes systemic immune dysfunction—have a reduced salivary IgA concentration [47]. Moreover, T-cell exhaustion is common in immune dysfunction in cancer and AIDS [48]. In cancer, CD8+ T cell reduction causes immunosuppression, and injury of CD4+ T cells in AIDS leads to immunosuppression [47]. Additionally, experiments in mice have reported that decreasing CD4+ T and CD8+ T cells reduce the number of IgA+ B cells [49]. Therefore, we speculated that immunosuppression with a decrease in IgA+ B cell count occurred in patients with HCC. For these reasons, the salivary IgA flow rate was possibly lower in the NASH-HCC group than in the NASH group, although the periodontal condition was similar in both groups.

Analysis of the salivary IgA flow rate and periodontopathogenic bacterial ratio in the saliva revealed a negative correlation for *F. nucleatum*. Salivary IgA specifically binds to oral bacteria and determines the resident bacteria [50]. In addition, the Gram-positive bacteria *Streptococcus* spp. was reportedly decreased in the oral cavity of IgA knockout mice, and Gram-negative periodontal pathogens *Aggregatibacter*, *Actinobacillus*, and *Prevotella* spp. were increased, resulting in alveolar bone loss and osteoclast activation [51]. Therefore, salivary IgA controls pathogenicity and maintains normal metabolic activity by binding to highly pathogenic oral bacteria [51]. *F. nucleatum* is a Gram-negative periodontal pathogen, similar to *Prevotella*; hence, salivary IgA may regulate its ratio. Consequently, the high salivary *F. nucleatum* ratio in the NASH-HCC group is possibly associated with the low salivary IgA flow rate, meaning that a decrease in salivary IgA flow rate increases the *F. nucleatum* ratio in the oral cavity, facilitating NASH progression to HCC. This may lead to a vicious cycle of further decline in salivary IgA levels. Although *P. gingivalis* had higher salivary ratios and serum antibody titers as well as *F. nucleatum*, it did not correlate with the salivary IgA flow rate. A possible reason may be that *P. gingivalis* is a late colonizer which grows in a deeper subgingival pocket compared to *F. nucleatum*, and is therefore more likely to avoid the host immune response in the oral cavity [52].

The Shannon index—which indicates bacterial flora diversity—was low in the NASH-HCC group. The Shannon index of salivary bacterial flora is lower in participants with non-severe periodontitis than in those with severe periodontitis [53]. Additionally, the Shannon index of salivary bacterial flora in patients with lung cancers is reportedly low [54], and that of subgingival plaque is reduced by radiotherapy [55]. Therefore, the low Shannon index of the bacterial flora in saliva in the NASH-HCC group—despite the similar periodontal conditions between both groups— might be related to cancer and its treatment.

Immune dysfunction and decreased salivary IgA levels occur in patients with cancer [46]. Hence, they may promote periodontal infection. There are still many unclear points regarding the association between cancer treatments and periodontal disease. Further investigation is necessary in this area.

Limitations

The number of participants was small, and there were differences between groups, possibly affecting the results of the statistical analyses. Additionally, the treatment stages of the participants in each group were not aligned, which may have affected the test values. Furthermore, a limited number of bacterial species was analyzed. Data on a greater variety of bacteria will contribute to a more comprehensive understanding of the interbacterial network and other factors involved.

5. Conclusions

Oral *P. gingivalis* and *F. nucleatum* were possibly associated with the pathogenesis of NASH-HCC. Moreover, *F. nucleatum* was negatively correlated with salivary IgA levels.

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