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The periodontal pathogen *Fusobacterium nucleatum* is associated with disease severity in multiple sclerosis

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Increasing evidence suggests that periodontitis may contribute to central nervous system disorders through chronic inflammation, but its role in multiple sclerosis (MS) remains unclear. This exploratory, cross-sectional study investigated the associations between the relative abundance of periodontal pathogens in the oral cavity and the clinical characteristics of MS. We enrolled 98 patients with MS, neuromyelitis optica spectrum disorder, or myelin oligodendrocyte glycoprotein antibody-associated disease. Tongue coating samples were analyzed using quantitative polymerase chain reaction targeting four periodontal species. High relative abundance was defined as an abundance exceeding the third quartile in proportion to the total abundance of bacteria. Associations between clinical and MRI features were assessed. Among the 56 patients with MS, only a high relative abundance of *Fusobacterium nucleatum* was associated with disease severity, as measured by the Expanded Disability Status Scale (EDSS) ($p = 0.009$). No associations were observed for the other three pathogens or in the non-MS groups. In a multivariate analysis, a high relative abundance of *Fusobacterium nucleatum* remained independently associated with the EDSS score. These findings suggest a potential association between the relative abundance of *Fusobacterium nucleatum* in the oral cavity and disease severity in MS.

Keywords Fusobacterium nucleatum, Multiple sclerosis, Periodontal pathogens, Expanded disability status scale, Disease severity

Multiple sclerosis (MS) is a central inflammatory demyelinating disease classified as an autoimmune disorder that targets the myelin sheath. While the specific etiology of MS remains unknown, it is considered multifactorial and involves environmental factors such as viral infections, smoking, vitamin deficiencies, and genetic predispositions. The prevalence of MS has been steadily increasing in Japan since the 1980s[1]. This rapid increase is thought to be influenced by environmental changes, with a particular focus on alterations in the gut microbiome, which have been extensively studied. In patients with MS, a reduction in the abundance of gut bacteria that produce short-chain fatty acids has been reported[2], suggesting a potential association with MS pathology. Additionally, increased oxidative stress in the gut has been observed in individuals with secondary progressive MS, and this increase may contribute to disease progression[3].

Recently, attention has expanded to include the role of the oral microbiota, alongside the gut microbiota, in central nervous system diseases. Periodontopathic bacteria, which constitute part of the oral microbiota, are closely linked to the brain through the so-called "oral–brain axis", with inflammation and immune mechanisms believed to mediate this connection[4].

Periodontal disease is a chronic bacterial infection that triggers persistent inflammation in periodontal tissues, leading to the destruction of connective tissues and alveolar bone and ultimately resulting in tooth loss[5]. With a global prevalence of 40–60%[6], periodontal disease is a common condition, and the presence of periodontal disease has been shown to increase the risk of developing systemic diseases such as atherosclerosis, diabetes, and

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rheumatoid arthritis. Chronic periodontitis is a multifactorial disease initiated by pathogenic bacteria in dental plaque that induces an immune response in susceptible hosts. The systemic effects of periodontal disease include bacteremia from periodontal lesions and the release of inflammatory mediators into the systemic circulation[7]. Additionally, periodontal bacteria have been linked to neurodegenerative diseases such as Alzheimer's disease[8]. In collaboration with dental professionals, we previously reported that poor oral health and serum levels of periodontal antibodies are associated with poor outcomes in stroke patients[9–11]. An association between periodontal disease and autoimmune diseases, such as rheumatoid arthritis, is also well established[12].

Certain oral microbiota species are more prevalent in MS patients than in healthy controls[13]. A case–control study in Taiwan revealed an association between chronic periodontitis and MS in women but not in men[14]. A meta-analysis further suggested that patients with chronic periodontitis have nearly twice the risk of developing MS[15], underscoring the importance of the relationship between MS and the oral environment. However, how periodontal disease influences clinical factors, relapse and progression in MS and whether the effects vary depending on the specific bacterial species involved remain unclear.

In this study, we aimed to quantify the periodontal bacterial load in tongue coating samples obtained from patients with central inflammatory demyelinating diseases (MS, neuromyelitis optica spectrum disorder [NMOSD], and myelin oligodendrocyte glycoprotein antibody-associated disease [MOGAD]) and to investigate the relationships between the periodontal bacterial load and clinical factors and the differential effects of various bacterial species.

Results

Patient enrollment and disease classification

Among the 112 eligible patients who visited our hospital during the study period, 98 patients (76 females [77.6%], mean age 48.6 ± 14.5 years) were enrolled after 11 patients who did not provide consent and 3 patients with missing data were excluded. Among the 98 patients, 56 were diagnosed with MS, 31 with NMOSD, and 11 with MOGAD. The baseline characteristics of all the patients are presented in Table 1. The treatment profiles of patients with MS, NMOSD, and MOGAD are summarized separately in Supplementary Table S1. In the MS cohort, 36 patients received high-efficacy (HE) treatments, and 20 patients received low-efficacy (LE) treatments or were drug free. In the NMOSD group, all patients were treated with either biologics or steroids/immunosuppressants. In the MOGAD group, 3 patients were drug free, and 8 patients were maintained on steroid and/or immunosuppressive therapy. We compared the proportion of patients with a high relative abundance of periodontal pathogens across the MS, NMOSD, and MOGAD groups. No significant differences were detected in the proportion of patients with a high relative abundance of *F. nucleatum*, *P. gingivalis*, *P. intermedia*, or *T. denticola* among the three groups (Table 2). Furthermore, within the MS group, we compared the proportion of patients who had a high relative abundance of each pathogen between patients and received HE treatments and the proportion of patients who had a high relative abundance of each pathogen and received LE treatments or were drug free. In the NMOSD cohort, no significant differences in the proportion of patients with a high relative abundance of any of the four periodontal pathogens were observed (*F. nucleatum*: 22.2% vs. 25%, $p = 0.814$; *P. gingivalis*: 33.3% vs. 25%, $p = 0.516$; *P. intermedia*: 25% vs. 30%, $p = 0.686$; *T. denticola*: 20.0% vs. 14.3%, $p = 0.647$). Similarly, in the MOGAD cohort, no significant differences in the proportions of patients with a high relative abundance of pathogens were detected between those in the immunological treatment group and those in the drug-free group (*F. nucleatum*: 33.3% vs. 37.5%, $p = 0.814$; *P. gingivalis*: 0% vs. 0%; *P. intermedia*: 0% vs. 12.5%, $p = 0.521$; *T. denticola*: 0% vs. 50.0%, $p = 0.206$). In addition to pathogen abundance, we also examined oral hygiene-related variables across disease groups and EDSS score categories. Most variables, including brushing frequency and duration and the use of fluoride toothpaste, mouthwash, and interdental devices, did not differ significantly between the MS, NMOSD, and MOGAD groups or between patients with an EDSS score ≥ 4 and those with an EDSS score < 4. However, patients with an EDSS score ≥ 4 were significantly more likely to report ongoing dental treatment than those with an EDSS score < 4 were (67.7% vs. 38.5%, $p = 0.007$). Notably, this difference did not remain significant when the patients were stratified into individual disease groups.

Comparison of periodontal pathogen abundance and clinical characteristics

Univariate analysis of the relationship between each periodontal bacterium and the EDSS score revealed that compared with MS patients with a low relative abundance of *F. nucleatum*, MS patients with a high relative abundance of *F. nucleatum* had significantly higher EDSS scores, with a greater proportion of these patients

	Total (*N* = 98)	MS group (*N* = 56)	NMOSD group (*N* = 31)	MOGAD group (*N* = 11)	*p* value
Age, years	48.6 ± 14.5	46.9 ± 13.2	55.9 ± 13.6	37.0 ± 13.8	< 0.001*
Sex (female), n (%)	76 (77.6)	43 (76.8)	25 (80.7)	8 (72.7)	0.845
Disease duration, years	12.2 ± 9.4	13.4 ± 9.4	11.8 ± 10.0	6.5 ± 5.3	0.035*
EDSS score	1.8 [0–9]	1.8 [0–9]	3.5 [0–7.5]	0 [0–6]	0.040*
EDSS score ≥ 4.0, n (%)	31 (31.6)	16 (28.6)	14 (45.2)	1 (9.1)	0.066

Table 1. Patient characteristics. The data are presented as the means ± standard deviations or medians (minimum values, maximum values) for continuous variables and as frequencies and percentages for discrete variables. *$p < 0.05$. MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; MOGAD, myelin oligodendrocyte glycoprotein antibody-associated disease; EDSS, Expanded Disability Status Scale.

		Total (*N* = 98)	MS group (*N* = 56)	NMOSD group (*N* = 31)	MOGAD group (*N* = 11)	*p* value
Fusobacterium nucleatum	High	24 (24.5%)	13 (23.2%)	7 (22.6%)	4 (36.4%)	0.644
	Low	74 (75.5%)	43 (76.8%)	24 (77.4%)	7 (63.6%)	
Porphyromonas gingivalis	High	23 (23.5%)	17 (30.4%)	6 (19.4%)	0	0.076
	Low	75 (76.5%)	39 (69.6%)	25 (80.6%)	11 (100%)	
Prevotella intermedia	High	22 (22.4%)	15 (26.8%)	6 (19.4%)	1 (9.1%)	0.386
	Low	76 (77.6%)	41 (73.2%)	25 (80.6%)	10 (90.9%)	
Treponema denticola (※)	High	18 (23.1%)	8 (18.2%)	7 (26.9%)	3 (37.5%)	0.417
	Low	60 (76.9%)	36 (81.8%)	19 (73.1%)	5 (62.5%)	

Table 2. Proportion of patients with high and low relative abundances of periodontal pathogens stratified by central inflammatory demyelinating disease. MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorder; MOGAD, myelin oligodendrocyte glycoprotein antibody-associated disease. High and low abundances were defined using the third quartile (Q3) cutoff for the ΔΔCt-calculated relative abundance. High = top 25%, low = below Q3. (※) For *Treponema denticola*, the total number of evaluated patients was lower than the total number of patients in each disease group because several samples did not yield valid Ct values by qPCR and were thus considered nondetectable. As a result, the total number of patients evaluated for this pathogen was 78 (MS patients 44; NMOSD patients 26; MOGAD patients 8).

	Fusobacterium nucleatum			Porphyromonas gingivalis			Prevotella intermedia			Treponema denticola (※)		
MS group (*N* = 56)	**High** (*N* = 13)	**Low** (*N* = 43)	***p*value**	**High** (*N* = 17)	**Low** (*N* = 39)	***p*value**	**High** (*N* = 15)	**Low** (*N* = 41)	***p*value**	**High** (*N* = 8)	**Low** (*N* = 36)	***p*value**
EDSS ≥ 4.0, n (%)	8 (61.5)	8 (18.6)	0.003*	4 (23.5)	12 (30.8)	0.581	4 (26.7)	12 (29.3)	0.849	2 (25.0)	8 (22.2)	0.865
NMOSD group (*N* = 31)	**High (*N* = 7)**	**Low (*N* = 24)**	***p* value**	**High (*N* = 6)**	**Low (*N* = 25)**	***p* value**	**High (*N* = 6)**	**Low (*N* = 25)**	***p* value**	**High (*N* = 7)**	**Low (*N* = 19)**	***p* value**
EDSS ≥ 4.0, n (%)	3 (42.9)	11 (45.8)	0.889	3 (50.0)	11 (44.0)	0.791	3 (50.0)	11 (44.0)	0.791	3 (42.9)	9 (47.4)	0.838
MOGAD group (*N* = 11)	**High (*N* = 4)**	**Low (*N* = 7)**	***p* value**	**High (*N* = 0)**	**Low (*N* = 11)**	***p* value**	**High (*N* = 1)**	**Low (*N* = 10)**	***p* value**	**High (*N* = 3)**	**Low (*N* = 5)**	***p* value**
EDSS ≥ 4.0, n (%)	1 (25.0)	0	0.165	–	1 (9.1)	–	0	1 (10.0)	0.740	0	0	–

Table 3. Relationships between EDSS scores and bacterial species abundance according to univariate analysis. The data are presented as the medians (minimum values, maximum values) for continuous variables and as frequencies and percentages for discrete variables. *$p < 0.05$. High and low relative abundances are based on the third quartile (Q3) cutoff values for the ΔΔCt-calculated relative abundance of each pathogen. "High" indicates a relative abundance above Q3. "Low" indicates a relative abundance below Q3. Adjusted p values were calculated using the Benjamini–Hochberg procedure for 12 comparisons. Only the association between *F. nucleatum* abundance and an EDSS score ≥ 4 in MS patients remained statistically significant after correction (adjusted $p = 0.036$). Abbreviations: MS, multiple sclerosis; NMOSD, neuromyelitis optica spectrum disorders; MOGAD, myelin oligodendrocyte glycoprotein-associated disease; EDSS, Expanded Disability Status Scale. (※) For *Treponema denticola*, the total number of evaluated patients was lower than the total number of patients in each disease group because several samples did not yield valid Ct values by qPCR and were thus considered nondetectable. As a result, the total number of patients evaluated for this pathogen was 78 (MS patients 44; NMOSD patients 26; MOGAD patients 8).

exhibiting EDSS scores ≥ 4 (EDSS scores ≥ 4.0: 61.5% vs. 18.6%, $p = 0.003$) (Table 3). We used the Benjamini–Hochberg procedure to control the false discovery rate and correct for multiple comparisons, and we applied the method to 12 univariate comparisons across three disease groups and four periodontal pathogens. Only the association between *F. nucleatum* abundance and an EDSS score ≥ 4 in MS patients remained significant after adjustment ($p = 0.036$). In contrast, the relative abundances of *P. gingivalis*, *P. intermedia*, and *T. denticola* were not significantly associated with the proportion of patients with an EDSS score ≥ 4. Furthermore, no significant associations were found between the relative abundance of any periodontal pathogen and patient age, disease duration, number of relapses, treatment details, or lesion location. In the univariate analysis of the NMOSD and MOGAD groups, no correlations were observed between the EDSS score and the proportions of patients with high abundance of any of the periodontal bacterial species.

Associations between *F. nucleatum* and disease severity

Analysis of the association between high relative abundance of *F. nucleatum* and baseline characteristics revealed no significant correlations with variables other than the EDSS score (Supplementary Table S2). However, the proportion of patients with a high relative abundance of *P. gingivalis* was significantly greater in the *F. nucleatum*-

high group (53.9%) than in the *F. nucleatum*-low group (23.3%, $p = 0.036$), suggesting a potential co-occurrence pattern between these two pathogens (Supplementary Table S2). Table 4 summarizes the results of the univariate analysis of baseline characteristics and their associations with EDSS scores. The group with an EDSS score ≥ 4.0 was older, had a longer disease duration, and comprised a greater percentage of patients with secondary progressive MS (SPMS). The optimal cutoff of the EDSS score for identifying a high relative abundance of *F. nucleatum* in patients with MS was ≥ 4, with a sensitivity of 61.5%, a specificity of 81.4%, and an area under the receiver operating characteristic (ROC) curve of 0.690 (95% CI: 0.517–0.863), as illustrated in Supplementary Figure S1. Additionally, no significant differences were observed between the EDSS score and oral care habits or dental treatment history. As illustrated in Supplementary Figure S2, the proportion of MS patients with high relative abundances of both *F. nucleatum* and at least one other periodontal pathogen (*P. gingivalis*, *P. intermedia*, or *T. denticola*) was significantly greater in the EDSS ≥ 4 group (37.5%) than in the EDSS < 4 group (10.0%; $p = 0.015$). In contrast, no significant differences were observed in the NMOSD group (14.3% vs. 11.8%, $p = 0.835$), and no patients with high abundance of two pathogens were identified in either EDSS score category in the MOGAD group (0%). In the MS, NMOSD, and MOGAD groups, the proportions of patients with combinations of multiple periodontal pathogens, excluding *F. nucleatum*, were not significantly associated with the EDSS score. No significant differences were observed in EDSS scores or the proportion of patients with EDSS scores ≥ 4 between the group with MS and elevated abundance of *F. nucleatum* alone and the other groups. However, the EDSS score was significantly higher (6 [0–7] vs. 1.5 [0–9], $p = 0.048$), and the proportion of patients with an EDSS score ≥ 4 was significantly greater (37.5% vs. 10%, $p = 0.015$) in the group with elevated levels of both *F. nucleatum* and other periodontal pathogens than in the remaining groups. Variables with a p value < 0.05 in the univariate analysis were included in the multivariate logistic regression analysis. A high relative abundance of *F. nucleatum* (odds ratio, 10.0; 95% CI, 1.45–69.4; $p = 0.020$) was independently associated with disease severity in patients with MS (Table 5).

Discussion

We demonstrated that a high relative abundance of *F. nucleatum* in tongue coating samples was significantly associated with the EDSS score in patients with MS but not other autoimmune inflammatory demyelinating diseases of the central nervous system. Furthermore, the combination of high *F. nucleatum* abundance with high abundance of other periodontal pathogens was significantly associated with disease severity in patients with MS.

F. nucleatum is a gram-negative anaerobic bacterium that plays a key role in dental plaque formation and is a major contributor to periodontitis. *F. nucleatum* promotes the production of proinflammatory cytokines and biofilm formation, leading to periodontal disease and gingivitis[16]. In addition to oral health, *F. nucleatum* has

	EDSS score ≥ 4.0 (*N* = 16)	EDSS score < 4.0 (*N* = 40)	*p* value
Age, years	56 ± 11.2	43.3 ± 12.3	< 0.001*
Sex (female), n (%)	12 (75)	31 (77.5)	0.841
Disease duration, years	19.0 ± 8.8	11.2 ± 8.7	0.004*
Current smoking, n (%)	2 (12.5)	3 (7.7)	0.573
Current dental visiting, n (%)	9 (56.3)	12 (30.8)	0.077
High relative abundance of *F. nucleatum*, n (%)	8 (50.0)	5 (12.5)	0.003*
MS subtype			< 0.001*
RRMS, n (%)	8 (50.0)	38 (95.0)	
SPMS, n (%)	8 (50.0)	2 (5.0)	
Number of attacks	4.2 ± 4.8	2.2 ± 2.6	0.046
Relapse within one year, n (%)	3 (18.8)	5 (12.5)	0.546
MRI findings			
Periventricular, n (%)	16 (33.3)	32 (80.0)	0.053
Cortical or juxtacortical, n (%)	11 (68.8)	30 (75.0)	0.633
Infratentorial, n (%)	13 (81.3)	28 (70.0)	0.390
Spinal cord, n (%)	13 (81.3)	29 (72.5)	0.495
DMD			0.427
Low-efficacy treatment or drug free, n (%)	7 (43.8)	13 (32.5)	
High-efficacy treatment, n (%)	9 (56.3)	27 (67.5)	

Table 4. Univariate analysis of baseline characteristics to determine associations with disease severity in patients with multiple sclerosis. The data are presented as the means ± standard deviations or medians (minimum values, maximum values) for continuous variables and as frequencies and percentages for discrete variables. Low-efficacy treatments include beta-interferon, glatiramer acetate, and dimethyl fumarate; high-efficacy treatments include natalizumab, ofatumumab, and fingolimod. *$p < 0.05$. Abbreviations: EDSS, Expanded Disability Status Scale; *F. nucleatum*, *Fusobacterium nucleatum*; MS, multiple sclerosis; RRMS, relapsing–remitting multiple sclerosis; SPMS, secondary progressive multiple sclerosis; MRI, magnetic resonance imaging; DMD, disease-modifying drug.

	Model 1		Model 2		Model 3	
	Odds ratio (95% CI)	*p* value	Odds ratio (95% CI)	*p* value	Odds ratio (95% CI)	*p* value
High relative abundance of *F. nucleatum*	6.05 (1.35–27.2)	0.019	9.15 (2.16–38.7)	0.003	10.0 (1.45–69.4)	0.020
Age	1.09 (1.02–1.16)	0.008	–	–	1.12 (1.00–1.25)	0.054
Number of attacks	–	–	1.23 (1.01–1.51)	0.043	1.11(0.78–1.58)	0.571
Disease duration	–	–	–	–	1.00 (0.78–1.57)	0.966

Table 5. Multivariate analyses to determine associations with disease severity in patients with multiple sclerosis. A high relative abundance of *F. nucleatum*, MS subtypes, and disease duration were identified as factors with $p < 0.05$ in univariate analyses of disease severity (Table 2). Multivariate analyses were performed using the high relative abundance of *F. nucleatum* and the following basic factors: age (Model 1), number of attacks (Model 2), and the factors listed above with $p < 0.05$ in the univariate analyses of severity (Model 3). Abbreviations: CI, confidence interval; *F. nucleatum*, *Fusobacterium nucleatum*; MS, multiple sclerosis.

been implicated in oncogenesis, particularly in colorectal cancer[17], and is linked to systemic diseases such as atherosclerosis[18] and rheumatoid arthritis[19]. Recently, the association between chronic periodontitis and central nervous system diseases was proposed to occur through two pathways: chronic inflammation via inflammatory mediators in the bloodstream and direct damage to the central nervous system due to the infiltration of gram-negative bacteria or their toxins[15]. *F. nucleatum* lipopolysaccharide has been linked to endothelial dysfunction, blood-brain barrier disruption, and neuroinflammation[20]. In previous studies, elevated titers of serum antibodies against *F. nucleatum* were found to be correlated with poor outcomes in stroke patients[9,10]. Compared with healthy controls, patients with MS present higher relative oral abundance of *F. nucleatum*[13], suggesting a potential link between *F. nucleatum* and MS.

In this study, we observed a strong correlation between the relative abundance of *F. nucleatum* and the EDSS score in patients with MS. The EDSS score, which is widely used to assess disability severity in patients with MS in clinical trials, reflects their ability to walk, with scores of 4.0 or higher indicating gait impairment[21]. Rostein et al.[22]. identified a high EDSS score as a poor prognostic factor, with a study reporting significantly greater cognitive decline in MS patients with an EDSS score ≥ 4 than in those with an EDSS score < 4[23]. A previous systematic review[24] investigating the relationship between the gut microbiome and MS disease activity or disability progression revealed that a higher abundance of short-chain fatty acid-producing bacteria, such as *Bacteroidota*, is associated with a lower EDSS score, indicating a potential link between the gut microbiota and MS severity. In the present study, ROC curve analysis revealed that the optimal threshold at which the EDSS score was associated with a high relative abundance of *F. nucleatum* in MS patients was ≥ 4. Furthermore, no significant associations were detected between oral hygiene habits, dental treatment history, or the EDSS score at the time of sample collection. These findings suggest that *F. nucleatum* may contribute to the severity of MS. Although this analysis was not designed to predict MS progression, we performed ROC curve analysis to explore whether a clinically meaningful EDSS score threshold—specifically, an EDSS score ≥ 4—corresponds to a greater likelihood of high *F. nucleatum* abundance. This threshold aligns with established definitions of moderate disability in MS patients and supports the interpretation that shifts in oral microbiota may reflect disease severity, even though the AUC value (0.690) indicates only modest discrimination.

F. nucleatum is present not only in the oral cavity but also in the gut. While research on the gut microbiota in MS has progressed, a recent study in which the oral and fecal microbiotas of MS patients were examined[25] revealed an increase in the abundance of inflammation-associated bacteria (including *Fusobacterium* and *Leptotrichia* in the saliva and *Enterobacteriaceae* and *Actinomyces* in the feces) in patients with MS. The abundance of *F. nucleatum* was also increased in the gut, indicating dysbiosis in both the oral and the gut environments and suggesting a possible oral–gut microbiome axis. In this study, we identified the associations between periodontal bacteria and the clinical profiles of not only patients with MS but also patients with NMOSD and MOGAD. Unlike NMOSD or MOGAD, which are characterized by acute inflammatory attacks mediated by disease-specific antibodies, MS is characterized by the persistent activation of T and B cells, leading to chronic inflammation through the release of cytokines such as IL-17 and IFN-γ[26]. Brennan CA et al.. reported that the inoculation of *F. nucleatum* strains in germ-free mice activated an IL-17-dependent immune response, enhancing colorectal cancer tumorigenesis, thus suggesting the involvement of *F. nucleatum*-induced IL-17-mediated chronic inflammation[27]. The proinflammatory characteristics of *F. nucleatum*, which promotes MS pathogenesis, are considered factors that are correlated with disease severity in MS patients.

F. nucleatum not only contributes to biofilm formation by aggregating with other periodontal pathogens but also acts as a proinflammatory agent. *F. nucleatum* supports biofilm formation and accelerates the lifecycle of *P. gingivalis* by producing putrescine from arginine via ornithine[28]. In our study, a high relative abundance of *F. nucleatum* was associated with a significant increase in the relative abundance of *P. gingivalis*, which has been shown to exacerbate MS in mouse models[29]. The proportion of individuals with high abundance of *F. nucleatum* alone did not differ significantly among patients with more severe cases of MS (EDSS score ≥ 4) than among those with less severe cases. However, the proportion of patients with high abundance of both *F. nucleatum* and other periodontal pathogens was significantly greater among those with more severe disease than among those with less severe disease. These findings suggest that patients with MS who exhibit high relative abundances of both *F. nucleatum* and other periodontal pathogens may present with greater disease severity than those who do not. Nonetheless, due to the limited number of patients with high abundances of multiple

pathogens, we did not conduct formal statistical interaction analyses (e.g., by including interaction terms in multivariate models). As such, this observation should be regarded as exploratory and hypothesis-generating rather than conclusive. Further studies with larger cohorts are needed to determine whether such co-occurrence synergistically contributes to MS progression.

This study has several limitations. First, we did not perform clinical assessments of periodontal status, such as the probing depth, attachment loss, or the number of remaining teeth, and thus residual confounding from unmeasured oral conditions cannot be excluded. Second, recent antibiotic use was not systematically documented. Although no participant reported systemic antibiotic use within three months prior to sampling, it remains a potential unmeasured factor. Third, the timing of sample collection in relation to oral activities (e.g., eating or brushing) was not recorded. While the samples were collected under a standardized outpatient protocol and the time was recorded, we cannot rule out transient effects from recent oral behaviors. Fourth, this study employed a single-center, cross-sectional observational design, limiting generalizability and preventing causal inference. Therefore, the associations reported in this study should be interpreted with caution as exploratory and noncausal. Fifth, the overall sample size was modest, especially in the NMOSD and MOGAD groups. In the MS group, only 13 patients were positive for *F. nucleatum*, resulting in wide confidence intervals and limited statistical power. Sixth, although we adjusted for demographic and clinical variables, the number of events was limited, possibly restricting the control of confounding. Finally, the levels of immunological markers such as cytokines were not measured and immune profiles were not assessed; thus the biological mechanisms linking *F. nucleatum* to MS severity remain speculative. Although we included participants across a wide age range to reflect the natural spectrum of neuroinflammatory diseases, the low prevalence and mild presentation of periodontitis in individuals under 20 years of age may have influenced the interpretation of microbiological findings in this subgroup.

In conclusion, this study demonstrated that the relative abundance of *F. nucleatum* in the oral cavity was strongly associated with the EDSS score in patients with MS. Although the results cannot establish causality, the observed association between the *F. nucleatum* abundance and disease severity in MS patients highlights the need for further investigation. Investigating the related immunological mechanisms, such as cytokine-related mechanisms, as well as the effects of interventions such as oral care, is a future research direction.

Methods

Participants and study design

This cross-sectional observational study of prospectively collected samples was conducted at Hiroshima University Hospital between May 2023 and November 2023 and focused on patients with autoimmune inflammatory demyelinating disease aged 15 years or older, including those with MS, NMOSD, and MOGAD.

Baseline clinical characteristics, including age, sex, disease duration, smoking habits, oral care routines (such as the frequency of tooth brushing; duration of tooth brushing; and use of interdental brushes and floss, fluoride toothpaste, and mouthwash), history of dental visits, Kurtzke Expanded Disability Status Scale (EDSS) score[21], number of clinical attacks, and medication details, were collected from all participants. MS was diagnosed based on the 2017 revised McDonald criteria[30], NMOSD was diagnosed based on the criteria proposed by the International Panel for NMO Diagnosis[31], and MOGAD was diagnosed based on the criteria proposed by the International MOGAD panel[32]. SPMS was diagnosed using previously reported criteria[33] as follows: an MS patient experienced, without any relapse, a worsening in the EDSS score over the past 6 months, indicated by an increase of + 1.0 points if the previous EDSS score was ≤ 5.0; an increase of + 0.5 points if the previous EDSS score was ≤ 5.5; a pyramidal or cerebellar score of ≥ 2, with no minimum EDSS requirement; or worsening of functional scores if the EDSS score was stable at ≥ 4.0.

The severity of MS was assessed using the EDSS score. An EDSS score below 4 is determined based on the functional system (FS) scores, whereas a score of 4 or above is evaluated based on both gait ability and FS scores[21]. MRI was performed immediately before sample collection, and lesions were evaluated on the basis of the 2017 revised McDonald criteria, with a focus on periventricular, cortical or juxtacortical, infratentorial, and spinal cord regions[30]. Using previously published classifications[34], we categorized disease-modifying therapies (DMTs) as high-efficacy (HE) or low-efficacy (LE) treatments. HE DMTs included fingolimod, siponimod, natalizumab, and ofatumumab. LE treatments included interferon-β and dimethyl fumarate or no drug treatment.

Ethics statement

This study was approved by the ethics committees of Hiroshima University Hospital (E2022-0289), and the study protocol adhered to federal government directives and the ethical principles outlined in the 1964 Declaration of Helsinki. Written informed consent specific to this study was obtained from all participants prior to enrollment. Full written informed consent was obtained from all participants prior to their inclusion in the study. For participants under 20 years of age, written informed consent was obtained from both the participant and their legal guardian after an appropriate explanation was provided.

Sample collection and bacterial DNA extraction

Tongue coating samples were collected by swabbing the surface of the tongue three times with a sterile sponge brush under consistent pressure (approximately 200 g). The brushes were then suspended in PBS. The bacterial cells were mechanically disrupted using tungsten carbide beads (3 mm; Qiagen) and 250 mg of zirconia/silica beads (0.5 mm; Biospec Products) in a Micro Smash device (Tommy, Japan) for 1 min at 5500 rpm. DNA was extracted with the MasterPure Complete DNA/RNA Purification Kit (Biosearch Technologies) according to the manufacturer's protocol, and the DNA samples were stored at −20 °C for future analysis.

Quantitative polymerase chain reaction analysis of periodontal pathogens

Quantitative polymerase chain reaction (qPCR) was performed with a LightCycler 96 System (Life Sciences) to calculate the percentage of each periodontal pathogen relative to the total bacterial load. The periodontal pathogens assessed were *Porphyromonas gingivalis*, *Prevotella intermedia*, *Fusobacterium nucleatum*, and *Treponema denticola*. The primers used for qPCR are listed in Supplementary Table S3. The percentage of each periodontal pathogen was calculated using the ΔΔCt method, with a universal 16 S rRNA primer used as a control in accordance with a previous report[35]. For *F. nucleatum*, we used primers targeting the *rpoB* gene rather than the *16 S rRNA* gene because of its high sequence similarity to that of related species such as *F. periodonticum*. The *rpoB*-targeted approach offers higher species-level specificity and has been previously validated[36]. For statistical analysis, the common logarithm of the percentage of each periodontal pathogen was used. For each periodontal pathogen, a high relative abundance was defined as a relative proportion exceeding the third quartile (Q3) of the distribution among all participants, in accordance with previous studies investigating associations between periodontal pathogens and neurological outcomes[36]. This threshold was selected to highlight patients with particularly high bacterial loads relative to the population.

Statistical analysis

Categorical variables are presented as numbers and percentages, whereas continuous variables are presented as the means with standard deviations or medians (minimum values, maximum values). Intergroup differences were assessed with unpaired *t* tests or Mann–Whitney *U* tests for continuous variables and Fisher's exact test or the χ^2 test for categorical variables. ROC curves were constructed to determine the optimal EDSS score threshold for association with the high relative abundance of each periodontal bacterial species in each disease group. The optimal cutoff value was determined using the Youden index, which maximizes the sum of sensitivity and specificity. To control for multiple testing in the univariate analyses of the association between severity based on the EDSS score (≥ 4) and the relative abundances of four periodontal pathogens (*F. nucleatum*, *P. gingivalis*, *P. intermedia*, and *T. denticola*) across the three disease groups (MS, NMOSD, and MOGAD), we applied the Benjamini–Hochberg procedure to control the false discovery rate at 0.05. This correction was applied across a total of 12 comparisons. The baseline data from patients were analyzed using a two-step strategy to evaluate the relative importance of variables associated with an EDSS score ≥ 4.0. First, a univariate analysis was performed, followed by a multivariate analysis including factors with a p value < 0.05 in the univariate analysis. Because SPMS was defined in this study using criteria that include sustained disability and typically an EDSS score ≥ 4.0, the MS subtype was excluded from the multivariate models to avoid potential collinearity with the dependent variable (EDSS score ≥ 4.0). We therefore evaluated the associations between the EDSS score (≥ 4.0) and various clinical and microbial factors. A p value less than 0.05 was considered to indicate statistical significance. All analyses were conducted with JMP 18.0 software (SAS Institute, Inc., Cary, NC).

Data availability

The datasets used and/or analyzed during the current study are available from the first author upon reasonable request.

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HN: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, validation, writing—original draft. MN: Conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, validation, writing—original draft. HN: Conceptualization, data curation, investigation, methodology, writing—original draft. TS: Conceptualization, methodology, writing—original draft. YY: Conceptualization, supervision, writing—review and editing. KO: Conceptualization, supervision, writing—review and editing. HM: Conceptualization, supervision, validation, writing—review and editing.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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