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ORIGINAL ARTICLE

Systemic immune response against the oral pathogens *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* is associated with the formation and rupture of intracranial aneurysms

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Abstract

Background and purpose: Periodontal infections are associated with the formation and rupture of intracranial aneurysms (IAs). This study investigated the role of two key periodontal pathogens, *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans*.

Methods: Immunoglobulin A (IgA) and IgG antibodies against *P. gingivalis* and *A. actinomycetemcomitans* were measured with enzyme immune assay from the serum of 227 IA patients, of whom 64 also underwent clinical oral examination. As a control group, 1096 participants in a cross-sectional health survey, Health 2000, underwent serological studies and oral examination. Logistic regression was used for multivariate analysis. Immunohistochemistry was performed to demonstrate bacteria-derived epitopes in the IA wall.

Results: Widespread gingivitis and severe periodontitis were more common in IA patients than in controls (2× and 1.5×, respectively). IgA antibodies against *P. gingivalis* and *A. actinomycetemcomitans* were 1.5× and 3–3.4× higher, respectively, in both unruptured and ruptured IA patients compared to controls ($p \leq 0.003$). IgG antibodies against *P. gingivalis* were 1.8× lower in unruptured IA patients ($p < 0.001$). In multivariate analysis, high IgA, but low IgG, antibody levels against *P. gingivalis* (odds ratio [OR] = 1.4, 95% confidence interval [CI] = 1.1–1.8 and OR = 1.5, 95% CI = 1.1–1.9; OR = 0.6, 95% CI = 0.4–0.7 and OR = 0.5, 95% CI = 0.4–0.7) and against *A. actinomycetemcomitans* (OR = 2.3, 95% CI = 1.7–3.1 and OR = 2.1, 95% CI = 1.5–2.9; OR = 0.6, 95% CI = 0.4–0.8 and OR = 0.6, 95% CI = 0.5–0.9) were associated with the risk of IA formation and rupture. Immunohistochemistry showed *P. gingivalis* epitopes in the IA wall.

Conclusions: Exposure to the periodontal pathogens *P. gingivalis* and *A. actinomycetemcomitans* and dysfunctional acquired immune response against them may increase the risk of IA formation and IA rupture.

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KEYWORDS
aneurysms, infections, oral disease, periodontitis, subarachnoid hemorrhage

INTRODUCTION

Intracranial aneurysms (IAs) are dilations of large cerebral arteries that may rupture, causing aneurysmal subarachnoid hemorrhage (aSAH). The prevalence of IAs is 2%–3% in the adult population [1] but only approximately one third of them rupture during a lifetime [2]. Because aSAH has devastating consequences, with up to 40% mortality, many of the unruptured IAs (uIAs) are treated before rupture by endovascular or microsurgical interventions. These treatment options are not risk-free; serious neurological complications occur in 5%–7% of the treated patients, including a small risk of death [3,4]. Interventions should therefore be performed on only those uIAs with a high risk of rupture. Because clinical parameters are not sufficient to discriminate uIAs with rupture risk [5], novel biomarkers predicting increased risk of rupture are needed.

Accumulating evidence associates periodontal diseases and bacteria with uIAs and aSAH [6–9]. Especially gingivitis, a reversible inflammation in gingival tissue, and periodontitis, irreversible chronic inflammatory disease of tooth-supporting structures, are associated with the risk of IA formation and rupture [7]. In previous studies, oral bacterial DNA has been found in 58%–71% of the wall samples of unruptured and ruptured IAs, as well as expression of toll-like receptors that trigger an inflammatory response when activated by bacteria-derived molecules [8,10]. Inflammation of the uIA wall has been shown to mediate the formation, progression, and eventual rupture of uIAs [11–13]. Although the multiple factors that trigger and sustain the inflammatory reaction promoting uIA formation and progression remain to be fully elucidated, the association of periodontal diseases with uIA formation and risk of aSAH has been demonstrated in two independent clinical series [7,9]. Furthermore, increased IgA and IgG antibody levels against periodontal pathogens are associated with other forms of stroke [14,15]. These observations, combined with the two other observations that both oral bacteria-derived DNA and toll-like receptors are present in the uIA wall, strongly suggest that exposure to periodontal infections or other oral bacteria plays an important role in the progression of uIA disease. This could be used as part of the diagnostic workup to identify rupture-prone uIAs.

Although clinical oral examination is the most sensitive and specific method to detect periodontal diseases, it requires consultation with a dental professional, and might not always be performed in a uniform standardized way. This complicates its use as part of the rupture risk assessment of uIAs. Moreover, periodontal disease status is dynamic and may evolve as a response to the patient's health-related habits, and thus the clinical oral examination might miss prior exposure that still contributes to rupture risk through modulation of the individual immunological memory and response.

In this study, we investigated whether serological measurements of prior exposure to relevant periodontal pathogens reflect the clinical oral status in uIA patients, and whether these serological measurements could potentially be useful in the assessment of the risk for uIAs or aSAH.

MATERIALS AND METHODS

Study subjects

This was a case–control type study comparing antibody levels of uIA patients with a control population without aneurysms, as well as aSAH survivors with a control population without diagnosed aneurysms. Patients referred to Kuopio University Hospital (KUH) for the treatment of an uIA (n = 130) or after aSAH (n = 97) were recruited into the study. Controls were obtained from participants of the cross-sectional Finnish Health 2000 health survey study (n = 1096) [16]. A flow chart describing how the study cohorts were formed is presented in the online data supplement (Figure S1a,b).

For the KUH patients, medical and smoking history was obtained from a questionnaire performed during patient recruitment and complemented with data collected from the patient's medical records. For the Health 2000 participants, a detailed questionnaire of medical history and health-related habits (including smoking) was performed during the study [16]. In addition, for the participants in the Health 2000 survey, the Finnish national registry for hospital discharge diagnosis (HILMO or Care Register for Health Care) was screened for diagnosis codes signifying aSAH (International Classification of Diseases, 10th Revision [ICD-10] codes I60.0–I60.9) or uIA (ICD-10 code I67.1) as well as for aneurysm-related surgical or endovascular procedures according to the Nordic Classification for Surgical Procedures.

This study was reviewed and approved by the ethical review boards of the Hospital District of Northern Savo as well as by the Ethics Committee for Epidemiology and Public Health of the Hospital District of Helsinki and Uusimaa, Finland. All study participants gave written informed consent.

Oral examination

Of the 227 recruited KUH uIA and aSAH patients, 64 (26%) underwent oral examination as previously described [7]. In brief, the periodontal status was evaluated; depth of periodontal pockets was measured with a probe from four to six sites per tooth from all permanent teeth excluding wisdom teeth. Patients with 4–6-mm probing

depths together with bleeding on probing were diagnosed with periodontitis, and those with greater than 6-mm probing depths were diagnosed with severe periodontitis. Gingival status was assessed by bleeding on probing test. In this study, gingivitis was diagnosed if more than two tooth sextants had gingival bleeding on probing. The health survey participants who served as controls underwent a similar oral examination.

Quantitation of IgA and IgG antibodies against *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* in serum samples

Blood samples were collected by peripheral venipuncture into EDTA tubes, followed by separation of the serum by centrifugation. The collected serum samples were stored at −80°C, and analyzed for immunoglobulin A (IgA) and IgG antibodies against *P. gingivalis* and *A. actinomycetemcomitans* with enzyme immunoassay multiserotype enzyme-linked immunosorbent assay (ELISA) as previously described [14,17]. In brief, the antigens used to coat the ELISA plates were composed of killed whole bacteria. *P. gingivalis* antigen included three and *A. actinomycetemcomitans* six reference strains representing different serotypes. Two dilutions of each sample were measured in duplicates, and binding of both IgG- and IgA-class antibodies was detected on separate plates. The mean absorbance values from four wells were calculated, normalized per reference serum samples on each plate, and used as a continuous variable of ELISA units (EU).

Immunohistochemistry for *P. gingivalis* in aneurysm wall tissue samples

We performed immunohistochemical stainings to search for *P. gingivalis*-derived proteins and bacteria-derived lipopolysaccharide (LPS) in tissue samples taken from fundus of two uIAs that were surgically treated. A detailed description of the immunostaining protocols is given in the data supplement.

Statistical analysis

The primary outcome variable in this study was IA formation, defined as the patient being diagnosed with a UIA. The secondary outcome was aSAH caused by rupture of a formed IA, defined as the patient being diagnosed with a ruptured IA. Although aSAH also requires prior IA formation, we studied the unruptured and ruptured IA groups separately, not including ruptured IAs in the analysis of IA formation, as possible association of the studied variables with IA rupture but not formation could have confounded the results.

Fisher exact test or chi-squared tests were used to compare categorical variables and the nonparametric Mann–Whitney *U* test for continuous variables due to skewed distributions that did not follow normal distribution in histograms. Spearman rank correlation coefficient was used for correlation. Logistic regression was used for multivariate logistic regression analysis adjusted for established risk factor of uIA formation and aSAH, namely, age, gender, smoking, and hypertension. Statistical analysis was performed with SPSS 22.0 statistical software (IBM), and $p < 0.05$ was considered significant.

RESULTS

Serum IgA antibodies against *P. gingivalis* and *A. actinomycetemcomitans*

Median levels of IgA antibodies against *P. gingivalis* were significantly higher in both the uIA (1.83 [0.43–24.33] vs. 1.2 [0.12–24.17] EU, $p = 0.003$) and aSAH (1.84 [0.24–22.43] vs. 1.2 [0.12–24.17] EU, $p < 0.001$) groups when compared to controls (Tables 1 and 2; Figure 1). Levels of IgA antibodies against *A. actinomycetemcomitans* were also significantly higher in the uIA (4.72 [0.26–16.03] vs. 1.47 [0.03–12.35] EU, $p < 0.001$) and aSAH (5.02 [0.62–15.23] vs. 1.47 [0.03–12.35] EU, $p < 0.001$) groups (Tables 1 and 2; Figure 1).

Serum IgG antibodies against *P. gingivalis* and *A. actinomycetemcomitans*

Median levels of IgG antibodies against *P. gingivalis* were significantly lower in the uIA group (2.92 [0.86–16.81] vs. 5.23 [1.17–21.40] EU, $p < 0.001$), whereas a nonsignificant difference was found in the aSAH group (3.14 [0.82–19.05] vs. 5.23 [1.17–21.40] EU, $p = 0.599$) when compared to controls (Tables 1 and 2; Figure 1). Median levels of IgG antibodies against *A. actinomycetemcomitans* were, however, nearly the same between the uIA and SAH groups and their control groups (Tables 1 and 2; Figure 1).

High prevalence of gingivitis and periodontitis in the uIA patients and aSAH survivors

Gingivitis and periodontitis were clearly more prevalent in the studied uIA patients (Table 1) and in aSAH survivors (Table 2) than in the control group, which is in line with our prior report comparing uIA and aSAH patients with age- and sex-matched controls from the same region [7]. The presence of generalized gingivitis in at least four tooth sextants was 2× higher in uIA (69.7% vs. 35.4%, $p < 0.001$) and 2.2× higher in aSAH (79.1% vs. 35.4%, $p < 0.001$) patients than in controls. The prevalence of severe periodontitis was 1.5× higher in the uIA group (34.3% vs. 23.3%, $p = 0.320$; Table 1) and 2.3× higher in the aSAH group (54.2% vs. 23.3%, $p = 0.001$; Table 2) compared to controls. Clinical periodontitis and gingivitis status had weak correlation or no correlation with antibody levels in statistical analyses (Figure S2 in the data supplement).

TABLE 1 Demographics, risk factors, and examined gingivitis and periodontitis of uIA patients

Variable	uIA patients, *n* = 130	Controls, *n* = 1096	*p*
Age, years	58.0 (19.0–77.0)	47.5 (30.0–93.0)	<0.001[a]
Gender, females, *n*	79/130 (60.8%)	546/986 (55.4%)	0.260
Hypertension, diagnosed	80/130 (61.5%)	353/982 (38.5%)	<0.001[a]
Diabetes, Type I or II	10/130 (7.7%)	2/1037 (0.2%)	<0.001[a]
Current smoking	55/129 (42.6%)	295/981 (30.1%)	0.005[a]
No smoking at the time of IA diagnosis	74/129 (57.4%)	686/981 (69.9%)	0.005[a]
No periodontitis	6/35 (17.1%)	184/986 (18.7%)	0.320
Periodontitis	17/35 (48.6%)	572/986 (58.0%)	
Severe periodontitis	12/35 (34.3%)	230/986 (23.3%)	
Bleeding gingival sextants, *n*			
0–1	0/33 (0.0%)	379/1004 (37.7%)	<0.001[a]
2–3	10/33 (30.3%)	270/1004 (26.9%)	
4–6	23/33 (69.7%)	355/1004 (35.4%)	
A. actinomycetemcomitans IgA, EU	4.72 (0.26–16.03)	1.47 (0.03–12.35)	<0.001[a]
A. actinomycetemcomitans IgG, EU	2.95 (0.56–16.40)	2.56 (0.27–12.18)	0.271
P. gingivalis IgA, EU	1.83 (0.43–24.33)	1.20 (0.12–24.17)	0.003[a]
P. gingivalis IgG, EU	2.92 (0.86–16.81)	5.23 (1.17–21.40)	<0.001[a]

Note: Data are presented as median and range or as proportions. Fisher exact test (two-sided) was used for categorical variables and nonparametric Mann–Whitney *U* test for continuous variables.

Abbreviations: EU, enzyme-linked immunosorbent assay units; Ig, immunoglobulin; uIA, unruptured intracranial aneurysm.

[a]Statistically significant.

Serology of *P. gingivalis* and *A. actinomycetemcomitans* as risk factors for uIA formation

In logistic regression models adjusted for age, gender, current smoking, hypertension, gingivitis, and periodontitis, the serum levels of IgA antibodies against both pathogens were significantly associated with higher risk of uIAs (odds ratio [OR] = 1.4, 95% confidence interval [CI] = 1.1–1.8, $p = 0.004$ and OR = 2.3, 95% CI = 1.7–3.1 per EU, $p < 0.001$, respectively; Table 3). The higher levels of serum IgG antibodies against both bacteria were associated with a lower risk of uIA formation (OR = 0.6, 95% CI = 0.4–0.8, $p = 0.001$ and OR = 0.6, 95% CI = 0.4–0.7 per EU, $p < 0.001$, respectively; Table 3).

Serology of *P. gingivalis* and *A. actinomycetemcomitans* as risk factors for aSAH

In a logistic regression model adjusted for age, gender, current smoking, hypertension, gingivitis, and periodontitis, levels of serum IgA antibodies against *P. gingivalis* or *A. actinomycetemcomitans* were significantly associated with risk of aSAH (OR = 1.5, 95% CI = 1.1–1.9, $p = 0.006$ and OR = 2.1, 95% CI = 1.5–2.9 per EU, $p < 0.001$, respectively; Table 4). The levels of serum IgG antibodies against *P. gingivalis* and *A. actinomycetemcomitans* were significantly associated with a lower risk of aSAH (OR = 0.5, 95% CI = 0.4–0.7, $p = 0.001$ and OR = 0.6, 95% CI = 0.5–0.9 per EU, $p = 0.007$, respectively; Table 4).

Histological confirmation of the presence of *P. gingivalis* in the IA pathology

Tissue samples from two uIA patients treated microsurgically were studied with immunostainings against epitopes of *P. gingivalis*, as well as for LPS derived from the outer cell surface of gram-negative bacteria. Both LPS and *P. gingivalis* gingipain were detected in both aneurysms (Figure 2; Figure S3 in the data supplement). Staining for LPS was more widespread than for gingipain or other *P. gingivalis*-derived epitopes studied.

DISCUSSION

In this study, we report the association of periodontal bacterial antibodies with IA formation and rupture. *P. gingivalis* and *A. actinomycetemcomitans* were selected as the studied pathogens because of their key role in the development and progression of periodontitis [18–22] as well as their prior association with other vascular diseases [15,23,24], as well as their known capability to invade the vascular wall [25–27]. Both are among the most studied oral pathogens in the context of systemic illnesses. Interestingly, high IgA but low IgG antibody levels for these bacteria were associated in this study with IA formation, suggesting increased exposure but insufficient systemic immunological response to these bacteria in persons who develop uIAs. This could at least in part explain why oral bacterial DNA has been found in IA walls [8,10] and furthermore could support the

TABLE 2 Demographics, risk factors, and examined gingivitis and periodontitis of aSAH patients

Variable	aSAH patients, *n* = 97	Controls, *n* = 1096	*p*
Age, years	53.0 (27.0–80.0)	47.5 (30.0–93.0)	0.008[a]
Gender, females, *n*	50/97 (51.5%)	546/986 (55.4%)	0.521
Hypertension, diagnosed	59/96 (61.5%)	353/982 (35.9%)	<0.001[a]
Diabetes, Type I or II	2/97 (2.1%)	2/1083 (0.2%)	0.036[a]
Current smoking	44/96 (45.8%)	295/981 (30.1%)	0.003[a]
No smoking at the time of IA diagnosis	52/96 (54.2%)	686/981 (69.9%)	
No periodontitis	0/24	184/986 (18.7%)	0.001[a]
Periodontitis	11/24 (45.8%)	572/986 (58.0%)	
Severe periodontitis	13/24 (54.2%)	230/986 (23.3%)	
Bleeding gingival sextants, *n*			
0–1	1/24 (4.2%)	379/1004 (37.7%)	<0.001[a]
2–3	4/24 (16.7%)	270/1004 (26.9%)	
4–6	19/24 (79.2%)	355/1004 (35.4%)	
A. actinomycetemcomitans IgA, EU	5.02 (0.62–15.23)	1.47 (0.03–12.35)	<0.001[a]
A. actinomycetemcomitans IgG, EU	2.92 (0.65–16.86)	2.56 (0.27–12.18)	0.810
P. gingivalis IgA, EU	1.84 (0.24–22.43)	1.20 (0.12–24.17)	<0.001[a]
P. gingivalis IgG, EU	3.14 (0.82–19.05)	5.23 (1.17–21.40)	0.599

Note: Data are presented as median and range or as proportions. Fisher exact test (two-sided) was used for categorical variables and nonparametric Mann–Whitney *U* test for continuous variables.

Abbreviations: aSAH, aneurysmal subarachnoid hemorrhage; EU, enzyme-linked immunosorbent assay units; IA, intracranial aneurysm; Ig, intracranial aneurysm.

[a]Statistically significant.

previously reported association between periodontitis and IA formation and rupture [7]. Also of great interest is our finding that the risk of aSAH was associated with low levels of IgG antibodies against *P. gingivalis* and *A. actinomycetemcomitans*. This suggests that lack of acquired immune response against periodontal pathogens, especially *P. gingivalis*, whose virulence factor was present in the uIA wall, could well maintain IA wall inflammation and degenerative remodeling predisposing ultimately to IA rupture [6].

Periodontitis, *P. gingivalis*, and *A. actinomycetemcomitans* in cardiovascular diseases including aneurysms

Periodontitis and the key pathogens *P. gingivalis* and *A. actinomycetemcomitans* have been associated with atherosclerosis and strokes [15,23,24]. *P. gingivalis* is considered to be one of the key pathogens in dysbiosis in chronic periodontitis [18,19]. Its presence in human gingival pocket microbiota increases with age and severity of disease [28]. The mean age of the IA study population was 52.8 years, which increases the probability of *P. gingivalis* carriage of an individual. *A. actinomycetemcomitans*, on the other hand, is linked to aggressive periodontitis, especially in the younger population [22]. We chose to focus on these two pathogens because of both their significance in the pathogenesis of periodontitis and their known association with other vascular diseases. Although we did not specifically screen the oral microbiome of our patients for these pathogens, we interpret the high titers of *P. gingivalis*- and *A. actinomycetemcomitans*-reactive IgA antibodies in IA patients as a marker suggesting prior or current exposure to these pathogens.

P. gingivalis actively invades the vascular wall and induces atherosclerotic changes in $ApoE^{null}$ mice models [29,30]. Viable *P. gingivalis* and *A. actinomycetemcomitans* have been found in artery wall atheromas also in humans [27,31]. These periodontal pathogens not only cause atherosclerotic changes but also are associated with development of abdominal aortic aneurysm (AAA) [32–35]. In an animal model of AAA, *P. gingivalis* injections caused neutrophil activation, leading to proteolytic changes via myeloperoxidase production and enlargement of AAA wall, ultimately increasing the risk of AAA rupture [34]. Neutrophil activation plays an important role also in the degenerative remodeling of the IA wall [36] probably through similar mechanisms as in AAAs.

Mechanisms for how periodontal *A. actinomycetemcomitans* and *P. gingivalis* may cause aneurysms

Periodontal and other oral bacteria may enter circulation via inflamed gingival tissue, causing transient bacteremia. This bacteremia occurs in surgical procedures including tooth extractions and root scaling but may also occur in daily activities such as chewing

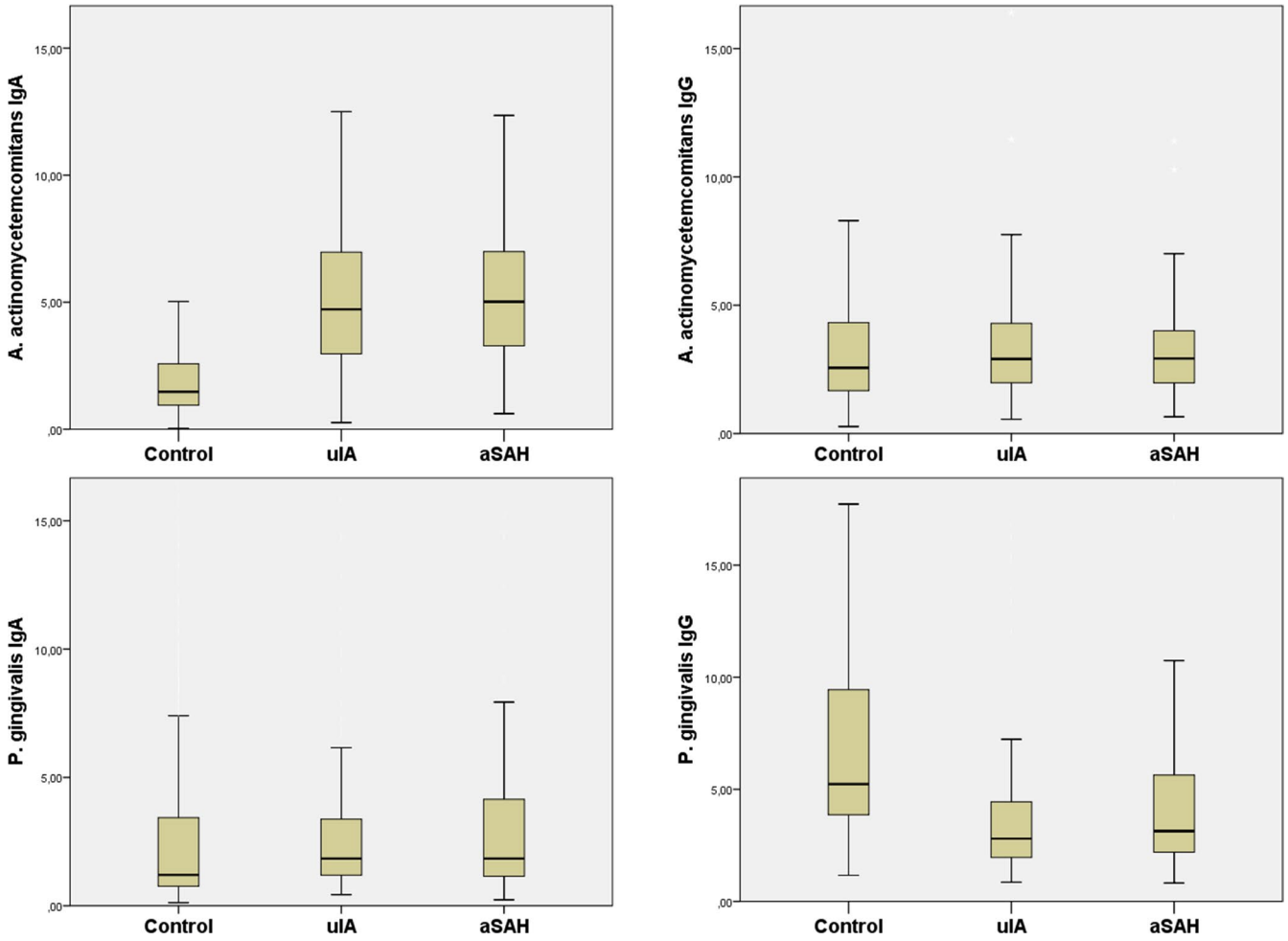

FIGURE 1 Median immunoglobulin A (IgA) and IgG class antibody levels in serum in the unruptured intracranial aneurysm (uIA), aneurysmal subarachnoid hemorrhage (aSAH), and control groups. Horizontal black lines in the bars indicate medians, and vertical lines indicate lowest and highest IgA or IgG antibody levels. The boxes indicate the lower and upper quartiles

and tooth brushing [37–39]. The magnitude of this bacteremia increases with the extent and severity of the gingival inflammation [40]. Transient bacteremia could explain why periodontal and other oral bacteria transmigrate to extraoral sites such as atheromas, AAAs, and uIAs.

Whereas acquired IgG antibodies in the circulation are the pivotal factors of humoral immunology against invading pathogenic bacteria, dimeric or polymeric secretory IgA antibodies are found mainly in the mucosa, where they act as a first-line immunological response against bacterial colonization [41,42]. In the serum, the monomeric IgA binds to Fc alpha receptor (FcαR1 or CD89), inducing an inflammatory reaction including both cytokine production of neutrophils as well as macrophage activity [41]. In turn, IgG in the serum acts as a host-protecting antibody through opsonization of invading pathogens and activation of the complement system [43]. In the context of our study, IgA levels primarily reflect recent or repeated exposure to *P. gingivalis* and *A. actinomycetemcomitans*, whereas IgG levels reflect the acquisition or activation of an acquired immune response against these pathogens.

There are several possible explanations for low IgG levels in IA patients, one of which could be the ability of *P. gingivalis* and *A. actinomycetemcomitans* to dysregulate complement activation and evade complement-mediated killing [44–47]. Another possible explanation is immunization to endured pathogen burden without elevation in IgG when the gingival and periodontal condition remains unhealthy. Also, the number of bacteria or bacterial metabolites/fragments in circulation could be insufficient to trigger the IgG level to rise, while still being sufficient to induce an inflammatory response in the cerebral artery wall. Moreover, the opsonization and complement activation triggered by IgG decrease with age [43].

Regarding other known risk factors for uIA and aSAH, it is worth noting that smoking is among the most important behavioral risk factors for gingivitis and periodontitis [48]. Moreover, recent studies support the causal relationship between periodontitis and hypertension [49,50]. Of great interest is also the observation that both systolic and diastolic blood pressure can be lowered by treating periodontitis [49], suggesting that improved oral hygiene might reduce the risk of uIA formation and aSAH not only through direct

TABLE 3 Association of antibody levels with IA formation

Variable	Odds ratio	95% CI	*p*
IA formation, Model 1, 1010 cases in analysis			
Age, years	1.023	0.977–1.071	0.339
Female gender	1.449	0.554–3.788	0.449
Hypertension, diagnosed	0.582	0.199–1.752	0.336
Current smoking	1.427	0.536–3.803	0.477
No periodontitis	1		
Periodontitis	5.745	0.908–36.349	0.063
Severe periodontitis	2.801	0.776–10.112	0.116
Bleeding gingival sextants, *n*			
0–1	1		
2–3	Insufficient statistical power		
4–6	Insufficient statistical power		
A. actinomycetemcomitans IgA, EU	2.325	1.739–3.109	<0.001[a]
A. actinomycetemcomitans IgG, EU	0.575	0.413–0.801	0.001[a]
P. gingivalis IgA, EU	1.434	1.119–1.838	0.004[a]
P. gingivalis IgG, EU	0.556	0.416–0.742	<0.001[a]

Note: Periodontal probing depth was categorized according to the presence of at least one tooth with ≥6-mm probing depth (severe periodontitis), 4–5-mm probing depth (periodontitis), or no teeth having ≥4-mm probing depth (no periodontitis). Gingival bleeding was defined as a number of tooth sextants in which bleeding occurred from the gingival margin on probing. Periodontal probing depth, gingival bleeding on probing, and antibody counts were included in the models simultaneously despite intervariable correlation.

Abbreviations: CI, confidence interval; EU, enzyme-linked immunosorbent assay units; IA, intracranial aneurysm; Ig, immunoglobulin.

[a]Statistically significant.

immunological mechanisms, but by affecting the classical risk factors indirectly.

Potential clinical applications

Periodontitis is often asymptomatic until endured inflammatory reaction degrades the tooth-supporting tissues to the point that the tooth becomes loose. Because of this, a thorough clinical assessment by a dental professional is necessary for proper diagnosis. Clinical oral assessment can be laborious to perform for every IA patient, as demonstrated by our managing to coordinate dental examination concomitant with other clinical visits for only 26% of the study participants. Moreover, our study showed a weak or no correlation between clinical oral status and antibody levels among uIA and aSAH patients, suggesting unidentified mechanisms or individual vulnerabilities, as in the control group correlation was stronger. It is worth noting that clinical oral status can change or fluctuate over time. Elevated IgA and IgG levels seem to correlate to bacteria carriage rather than to clinical periodontal status [14] and this could be the key finding considering the risk of aSAH. Our study suggests that through measuring the levels of circulating IgA and IgG antibody levels, one can detect oral pathogen exposure relevant to the uIA disease through a simple blood test without the need for more complex clinical oral examinations. Moreover, our results suggest that persons at higher risk for uIA formation and aSAH could be identified through measurement of acquired antibodies against relevant oral pathogens. These serological measurements could potentially be used to identify persons at risk of uIA formation and aSAH, and possibly to counsel uIA patients for their risk of aSAH as well as for their risk of developing de novo uIAs. Further studies on the predictive value of oral pathogen serology to discriminate unstable uIAs from those remaining stable in long-term follow-up are warranted.

Limitations of the study

Our study has some limitations concerning diagnostics of the periodontitis. Because our control data were obtained from Health 2000 data, we used the same methods to diagnose periodontitis in the KUH cohort. There is, however, a more recent classification of periodontitis [51] which could not be used in this study setting, as the Health 2000 survey used a different method to diagnose periodontitis. This could have led to over- or underestimation of the prevalence of periodontitis and could also explain, in part, why periodontal status did not correlate to IgA and IgG levels in this study.

For the total studied population of 1323 IA patients and controls, sample size analysis with a CI of 95%, margin of error of 5%, and population proportion of 35% that reflects the prevalence of gingivitis or periodontitis in the general Finnish population [7] gives a sample size

TABLE 4 Association of antibody levels with aSAH

Variable	Odds ratio	95% CI	*p*
aSAH, Model 2, 1001 cases included in analysis			
Age, years	0.984	0.937–1.033	0.508
Female gender	1.395	0.456–4.270	0.560
Hypertension, diagnosed	0.991	0.303–3.238	0.988
Current smoking	1.480	0.495–4.427	0.483
No periodontitis	1		
Periodontitis	Insufficient statistical power		
Severe periodontitis	Insufficient statistical power		
Bleeding gingival sextants, *n*			
0–1	1		
2–3	3.211	0.332–31.065	0.314
4–6	5.525	0.644–47.437	0.119
A. actinomycetemcomitans IgA, EU	2.097	1.526–2.883	<0.001[a]
A. actinomycetemcomitans IgG, EU	0.636	0.457–0.885	0.007[a]
P. gingivalis IgA, EU	1.473	1.115–1.946	0.006[a]
P. gingivalis IgG, EU	0.514	0.361–0.731	0.001[a]

Note: Periodontal probing depth was categorized according to the presence of at least one tooth with ≥6-mm probing depth (severe periodontitis), 4–5-mm probing depth (periodontitis), or no teeth having ≥4-mm probing depth (no periodontitis). Gingival bleeding was defined as a number of tooth sextants in which bleeding occurred from the gingival margin on probing. Periodontal probing depth, gingival bleeding on probing, and antibody counts were included in the models simultaneously despite intervariable correlation.

Abbreviations: aSAH, aneurysmal subarachnoid hemorrhage; CI, confidence interval; EU, enzyme-linked immunosorbent assay units; Ig, immunoglobulin.

[a]Statistically significant.

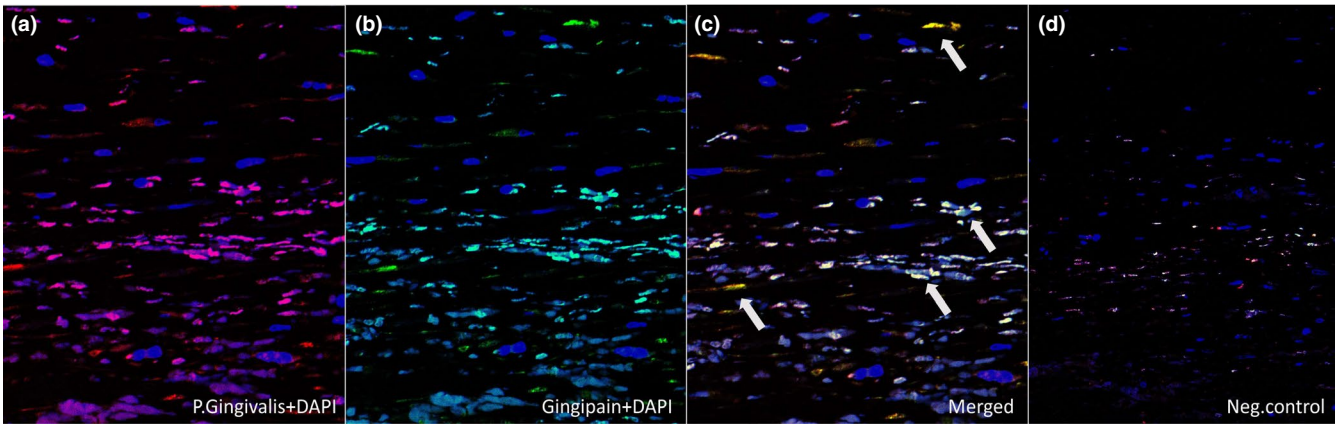

FIGURE 2 Immunofluorescence staining of the unruptured intracranial aneurysm wall for *P. gingivalis* epitopes (a; in red and cell nuclei in blue) and for gingipain, a specific protein derived from *P. gingivalis* (b; in green and cell nuclei in blue). (c) When merged, multiple aneurysm wall cells show double positivity for both lipopolysaccharide and gingipain (in yellow or white depending on the intensity of the signal, examples marked with white arrows). (d)The negative (Neg.) control provided is a merged image of both the red and green fluorescence channels taken from the corresponding negative control section (primary antibody omitted) with the same microscope settings as the positive signal (nuclei counterstained in blue). DAPI, 4,6-diamidino-2-phenylindole

of 277. The same calculation using population proportions of from 70% to 80% reflecting the prevalence of gingivitis or periodontitis in the Finnish IA patient population [7] gives sample sizes from 260 to 208. Thus, with the 223 IA patients included in our study, our study sample was just about sufficient to detect an association with overall IA formation, but the sample size remains somewhat limited when comparing unruptured IA and ruptured IA groups separately. This may also explain the lack of statistically significant associations in univariate analysis of IgG antibodies.

A third limitation is the lack of identification of *P. gingivalis* and *A. actinomycetemcomitans* in the oral cavity of the studied patients. However, from previous studies we know that these

bacteria cumulate in the oral cavity if the individual has gingivitis or periodontitis [52,53]. Moreover, the composition of the oral microbiome of the patients may have changed over time, and thus even if neither *P. gingivalis* nor *A. actinomycetemcomitans* would have been detected at the time of IA diagnosis, it does not preclude their involvement in the pathogenesis of the disease. Unlike cross-sectional detection of a pathogen at a specific point of time, antibody titers used in this study reflect both current and prior exposure to the pathogens.

CONCLUSIONS

We report high levels of IgA but low levels of IgG antibodies against oral pathogens *P. gingivalis* and *A. actinomycetemcomitans* in the serum of uIA patients and aSAH survivors compared to a control population. The results of this study add to the scientific evidence for the important role of oral infection and pathogens in the pathogenesis of uIAs and aSAH.

CONFLICT OF INTEREST

The authors declare no conflict of interest in this study.

AUTHOR CONTRIBUTIONS

Joona Hallikainen: Data curation (equal), formal analysis (equal), investigation (equal), methodology (equal), writing–original draft (equal), writing–review & editing (equal). **Mikko Pyysalo:** Writing–review & editing (equal). **Sara Keränen:** Data curation (equal), writing–review & editing (supporting). **Jari Kellokoski:** Conceptualization (equal), supervision (equal), writing–review & editing (equal). **Timo Koivisto:** Writing–review & editing (equal). **Anna Liisa Suominen:** Data curation (equal), methodology (equal), supervision (equal), writing–review & editing (equal). **Pirkko Pussinen:** Investigation (supporting), writing–review & editing (supporting). **Tanja Pessi:** Writing–review & editing (supporting). **Juhana Frösen:** Conceptualization (equal), data curation (equal), formal analysis (equal), funding acquisition (equal), supervision (equal), writing–review & editing (equal).

DATA AVAILABILITY STATEMENT

Due to the nature of this research and General Data Protection Regulation policy, participants of this study did not agree for their data to be shared publicly, so supporting data are not available.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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