RESEARCH PAPER

The connection between ruptured cerebral aneurysms and odontogenic bacteria

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▶ Additional material is published online only. To view please visit the journal online (http://dx.doi.org/10.1136/jnnp-2012-304635).

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Received 20 November 2012
Revised 14 April 2013
Accepted 5 May 2013
Published Online First 12 June 2013

To cite: Pyysalo MJ, Pyysalo LM, Pessi T, *et al*. *J Neurol Neurosurg Psychiatry* 2013;**84**: 1214–1218.

ABSTRACT

Background Patients with ruptured saccular intracranial aneurysms have excess long-term mortality due to cerebrovascular and cardiovascular diseases compared with general population. Chronic inflammation is detected in ruptured intracranial aneurysms, abdominal aortic aneurysms and coronary artery plaques. Bacterial infections have been suggested to have a role in the aetiology of atherosclerosis. Bacteria have been detected both in abdominal and coronary arteries but their presence in intracranial aneurysms has not yet been properly studied.

Objective The aim of this preliminary study was to assess the presence of oral and pharyngeal bacterial genome in ruptured intracranial aneurysms and to ascertain if dental infection is a previously unknown risk factor for subarachnoid haemorrhage.

Methods A total of 36 ruptured aneurysm specimens were obtained perioperatively in aneurysm clipping operations (n=29) and by autopsy (n=7). Aneurysmal sac tissue was analysed by real time quantitative PCR with specific primers and probes to detect bacterial DNA from several oral species. Immunohistochemical staining for bacterial receptors (CD14 and toll-like receptor-2 (TLR-2)) was performed from four autopsy cases.

Results Bacterial DNA was detected in 21/36 (58%) of specimens. A third of the positive samples contained DNA from both endodontic and periodontal bacteria. DNA from endodontic bacteria were detected in 20/36 (56%) and from periodontal bacteria in 17/36 (47%) of samples. Bacterial DNA of the *Streptococcus mitis* group was found to be most common. *Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum* and *Treponema denticola* were the three most common periodontal pathogens. The highly intensive staining of CD14 and TLR-2 in ruptured aneurysms was observed.

Conclusions This is the first report showing evidence that dental infection could be a part of pathophysiology in intracranial aneurysm disease.

INTRODUCTION

Subarachnoid haemorrhage (SAH) from a ruptured intracranial aneurysm causes approximately 50% mortality among working-aged patients.[1–4] SAH patients are younger and financial costs are higher than in other strokes.[5] The mechanisms behind aneurysm development, structural weakening and rupture are poorly understood.

Known risk factors for aneurysm development and rupture include ageing, female gender, smoking, high blood pressure, excessive alcohol consumption and a family history for SAH.[6–8] Risk factors are generally the same as in other cardio- and cerebrovascular diseases. In long-term follow-up studies, SAH patients have also been reported to die of cerebrovascular and cardiovascular diseases twice as often as general population.[9 10] Both ruptured cerebral and aortic aneurysms have typical atherosclerotic changes in pathological studies supporting the speculation that cardio- and cerebrovascular diseases are related.[11 12]

Oral infections seem to play an important role in the pathogenesis of cardiovascular diseases. Oral pathogens have been detected in coronary artery plaques.[13–17] The most common chronic oral bacterial disease is periodontitis and the incidence of severe periodontitis is approximately 20% in Finnish population.[18] Poor dental hygiene, dental operations and bacterial endocarditis have been found to be predisposing factors in the formation of rare mycotic intracranial aneurysms.[19 20]

Before intracranial aneurysm ruptures, the wall undergoes inflammatory changes such as apoptosis, T cell and macrophage infiltration and complement activation but the aetiology of these inflammatory changes remains unknown.[11 12 21] We therefore hypothesise that bacterial driven inflammation could be behind the rupture of intracranial aneurysms and test our hypothesis by analysing oral bacterial DNA from samples from SAH patients and autopsy cases and staining bacterial receptors from autopsy specimens.

METHODS

The study group consisted of 36 patients with SAH. Seven specimens from the aneurysm wall were obtained at medicolegal autopsy and 29 specimens were obtained perioperatively after prompt microsurgical clipping of the saccular aneurysm under sterile conditions. Specimens were collected between June 2010 and January 2013. Ruptured aneurysms clipped by experienced neurosurgeons were considered suitable for this study if a specimen could be taken with microscissors after the aneurysm was clipped without any risk to the patient. Inclusion criteria for patients were: age over 18 years, saccular aneurysm wall that was clipped and sample was technically possible to take. SAH patients who had aneurysms that were not treated by clipping and whose aneurysm wall was not safely excised were excluded. Inclusion criteria for autopsy cases were: out-of-hospital death due to the aneurysmal SAH, age over 18 years, time elapsed postmortem under 5 days, time interval between death and storage of the body in the

mortuary less than 24 h, intact middle torso and bowel, no use of antibiotics for 2 weeks before death, no signs of bacterial infections or drug addiction and no visible wounds or necrosis. All bodies in the mortuary were kept refrigerated at around +4° C. The bodies were not generally cooled during transportation to the mortuary. Samples from ruptured aneurysms were collected using sterile techniques. Arterial blood sample via arterial cannula was obtained from each patient during the procedure and contralateral cerebral artery tissue samples from autopsy cases were taken to be used as negative controls (ie, reference sample) for bacterial DNA analysis. The specimens were frozen at −70°C after collection.

All patients gave their informed consent to the study. The study was approved by the Hospital Ethics Committee and the National Supervisory Authority for Welfare and Health.

Detection of bacteria

Total DNA was extracted from the aneurysm wall using standard methods. Presence of candidate bacterial DNA for periapical abscess bacteria (*Streptococcus* sp. mainly *Str. mitis*-group, *Str. mitis, Str. oralis, Str. sangui* and *Str. gorgonii, Streptococcus anginosus*-group, *Staphylococcus aureus, Staphylococcus epidermidis*) and periodontal bacteria (*Porphyromonas gingivalis, Aggregatibacter (néé Actinobacillus) actinomycetemcomitans, Fusobacterium nucleatum, Prevotella intermedia, Dialister pneumosintes, Parvimonas micra* and *Treponema denticola*) were identified using real time quantitative PCR and the ABI PRISM 7900 sequence detection system (Applied Biosystems, Foster City, California, USA). Total amount of bacterial DNA in the samples was determined by using universal bacterial primers and probes as well as measurement for human housekeeping gene, RNaseP (Applied Biosystems). Details of the measurement are presented in online supplement 1. Briefly, the relative amounts of these organisms in specimens were calculated by the comparative critical threshold cycle (Ct) method (ΔΔCt, $\Delta Ct_{sample} - \Delta Ct_{reference\ sample}$),[22] with a simplification. Reference sample was either arterial blood (patients) or contralateral cerebral artery tissue samples (autopsy cases). Sample was marked to be positive if $2^{-\Delta\Delta Ct} \geq 2$*SD of the sample gene copies.

Immunohistochemical studies on staining of bacteria recognising receptors

The presence of bacteria recognising receptors in formalin-fixed histological sections from four autopsy cases was studied using CD14 (Novocastra) and toll-like receptor (TLR)-2 (Abcam) antibodies diluted in Dako REAL antibody diluent (S2022), pipetted on slides for 50 min and washed in TBS-Tween for 2×5 min. Secondary staining was performed with Dako REAL EnVision Detection System (K5007) and visualised with diaminobenzidine (DAB) according to the kit protocol. Confirmatory staining was done with primary antibody replaced with dilute as well as with DAB only to exclude the possibility of erroneous staining result due to endogenous perioxidase activity or necrotic cells.

RESULTS

Patient characteristics

Characteristics of 36 SAH subjects (clinical patients and autopsy cases) and their aneurysms are shown in table 1. A total of 13 patients were previously healthy without any regular medication. Nine patients had hypertension, three had hypercholesterolaemia, three had hypothyreosis, one patient had diabetes, and others had diseases like allergy, rheumatoid arthritis and previous head trauma. Six patients had a suspicion of excessive alcohol consumption and a total of 16 out of 29 patients were smokers. Four patients had a positive family history for SAH.

Table 1 Patient and aneurysm characteristics

	Autopsy specimen	Clipping specimen
Number of patients	7	29
Mean age	53	57
Female/male	4/3	16/13
Location		
ICA	1	1
MCA	5	23
ACoA	0	4
VBA	1	0
ACA	0	1
Mean fundus size (mm)	7	10

ACA, anterior cerebral artery; ACoA, anterior communicating artery; ICA, internal carotid artery; MCA, middle cerebral artery; VBA, vertebro-basilar aneurysms.

Microbiological and immunohistochemical findings in aneurysm walls

Using real time quantitative PCR, bacteria were detected in 21/36 (58%) of aneurysms (table 2). The prevalence of bacteria was 62% in surgical samples and 43% in autopsy samples. In the surgical group, endodontic and periodontal pathogens were identified in 15/29 (52%) and 13/29 (45%) of aneurysms, respectively. Figure 1 shows the frequencies of bacterial DNA positive findings in all cases (29 patients and seven autopsy cases). A third of the positive samples contained bacterial DNA from both periodontal and endodontic bacteria. Bacterial DNA from the *Streptococcus mitis* group was found to be the most common. *A actinomycetemcomitans*, *F nucleatum* and *T denticola* were the three most common periodontal pathogens. Within the patients, the total amount of bacterial DNA in the aneurysm tissue samples was 44.5 times higher than that found in their control blood samples (mean; SD 44.5; 62.90). Similar results were observed in autopsy cases. Highly intensive staining of CD14 and TLR-2 in ruptured aneurysms of all studied four autopsy samples was observed (figure 2).

DISCUSSION

Chronic oral infections are common; the prevalence of severe periodontitis is around 20% in Finnish population.[18] This causes systemic inflammation in an otherwise healthy population.[23] Periapical lesion at the tooth apex has a prevalence as high as 27% in Finnish population.[18] Clinically relevant oral pathogens have previously been detected in atherosclerotic coronary plaques and abdominal aorta aneurysms.[15 17 24] Our study is the first to identify oral bacteria from intracranial aneurysm wall. *Streptococcus mitis*-group and *P gingivalis* were the most commonly found pathogens in our study and these are

Table 2 The prevalence of bacterial DNA in the samples

	Negative	Positive
Surgical patients	11 (38%)	18 (62%)
Autopsy group	4 (57%)	3 (43%)
Total	15 (42%)	21 (58%)

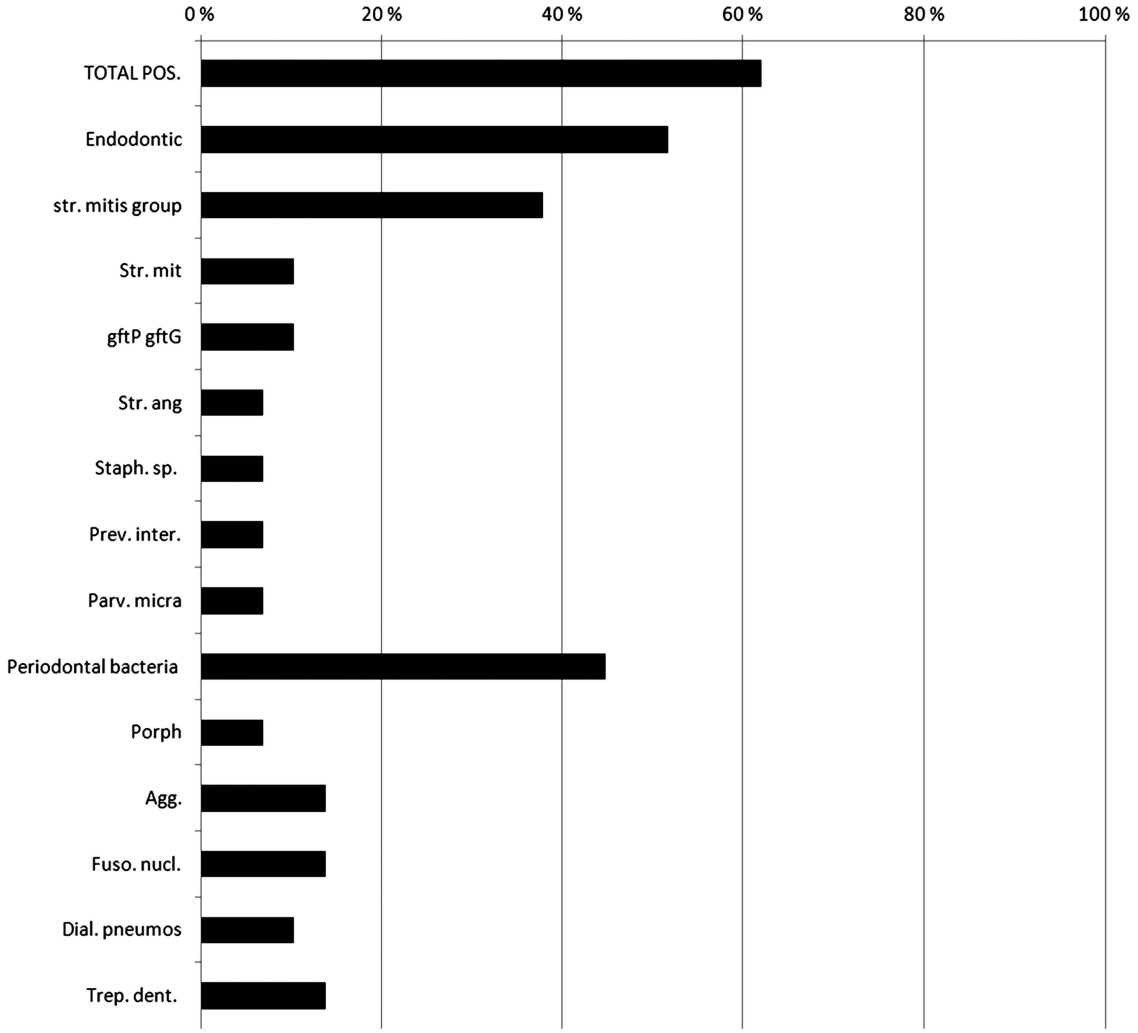

Figure 1 Distribution of bacterial DNA findings in all subjects (29 patients and seven autopsy cases) using specific primers and probes in real time quantitative PCR. Blood sample obtained from the arterial sheath before the procedure (patients) and contralateral cerebral artery tissue samples (autopsy cases) were used as inner controls. TOTAL POS: positive result from one or more measurements; Endodontic: positive result from one or more measurements of Streptococcus sp. (mainly Str. mitis-group), Str. mitis, Str. oralis, gftP and gftG-streptococcal virulence factor, Str. anginosus-group, Staphylococcus aureus or S epidermidis; str. Mitis-group: Streptococcus mitis-group (Str. mitis, Str. salivarius, Str. gordonii, Str. sangui, Str. pneumoniae, Str. oralis), Str. thermophilus, Lactobacillus lactis; Str. mit: Streptococcus mitis; gftP gftG: virulence factors of gftP and gftG, that is, recognition of Str. sangui and Str. Gordonii; Str. ang: Str. Anginosus-group (Str. anginosus, Str. milleri, Str. constellatus, Str. intermedius); Staph. Sp.: Staphylococcus aureus, S. epidermidis; Prev. inter: Prevotella intermedia; Parv. micra: Parvimonas micra Periodontal bacteria: positive result from one or more measurements of Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Prevotella intermedia, Dialister pneumosintes, Parvimonas micra or Treponema denticola; Porph: Porphyromonas gingivalis; Agg. Aggregatibacter actinomycetemcomitans; Fuso. nucl: Fusobacterium nucleatum; Dial. pneumos: Dialister pneumosintes; Trep. dent: Treponema denticola.

also clinically important and common bacteria in dental infections.

To the best of our knowledge, there are no other studies analysing dental bacteria among ruptured intracranial aneurysms. The mechanisms of SAH have been widely studied but the relation between infection and aneurysm wall inflammation should be studied further.

TLRs detect conserved microbial patterns and endogenous ligands, and play a key role in innate immune signalling and initiating inflammatory responses. CD14 functions as a comolecule for both TLR-2 and TLR-4.[25] It has been shown that *P gingivalis* and oral streptococci induce pro-inflammatory cytokine release and accumulation of macrophages through activation of the CD14–TLR-2 complex.[26 27] The detection of DNA specific for oral pathogens together with co-stimulation of TLR-2–CD14 in ruptured aneurysm samples suggests that these pathogens disseminate into the systemic circulation, localise in aneurysm and cause TLR-mediated inflammation. This study is the first one showing the presence of the bacteria in the ruptured aneurysm tissue and the activation of bacterial receptors, CD14 and TLR-2. To exclude the possibility of false staining result due to endogenous debris or necrotic cells, chromogenic reporter molecule DAB was used. No staining of DAB was seen. Although sample size is small, these findings suggest that these bacteria may have a role in the formation or rupturing of intracranial aneurysms.

The major limitations of our study are small sample size and the lack of separate control group. In this study, peripheral arterial blood during the procedure (patient) as well as healthy piece of cranial artery (autopsy) has served as inner negative controls

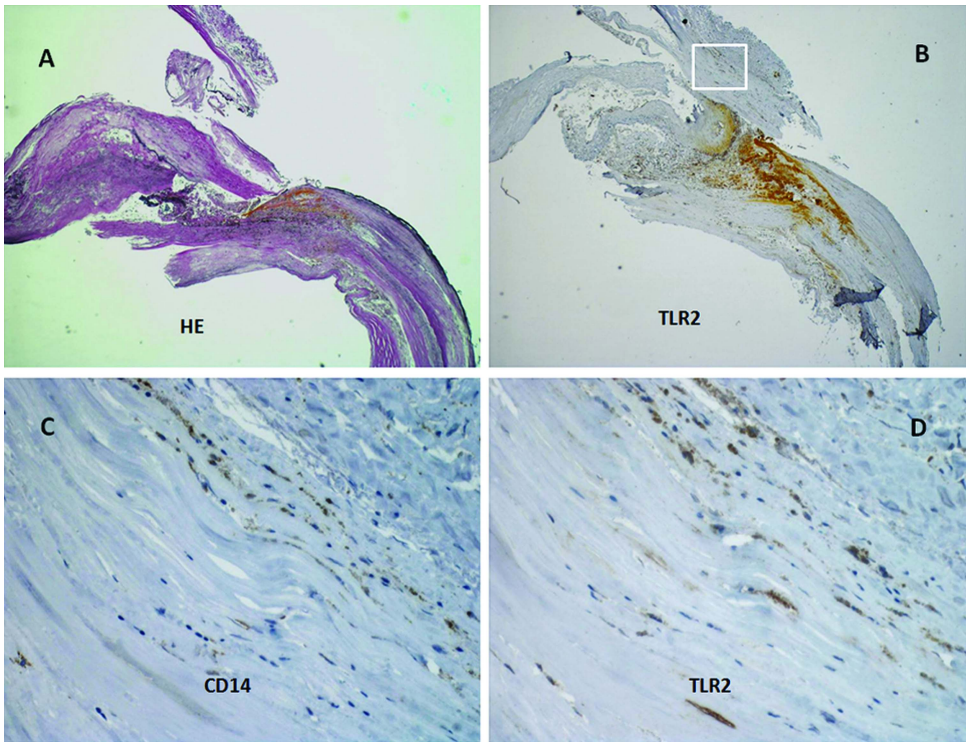

Figure 2 H&E staining of the ruptured aneurysm wall (A). The highly intensive immunohistochemical staining of toll-like receptor-2 (B, D) and CD14 (C) in the same aneurysm.

for each subject. Those samples were used to exclude the possible bacteria background due to the sampling method and possible circulating bacterial DNA of peripheral blood. It is not ethically acceptable to take the healthy part of cranial artery for control. Therefore, we think that the clinically healthy piece of cranial artery (autopsy) and the peripheral blood sample of the same subject (patients) are the best control samples. By using the inner control and δ Ct methods[22] we confirmed that amount of bacterial DNA in aneurysms differed significantly from that in control samples within the same individual. As a limit of true finding for samples, twofold difference was used.[28 29] Therefore, all the presented positive results are true findings corrected with inner control. Moreover, we have used inner control approach also in our earlier study.[30] The study is therefore far from providing a definite solution to the formation of intracranial aneurysms. However, this is the first time that oral bacteria were found in the aneurysm wall.

CONCLUSIONS

The aim of this preliminary study was to assess the presence of oral and pharyngeal bacterial genome in ruptured intracranial aneurysms. Bacterial DNA was detected in 58% of specimens and highly intensive staining of CD14 and TLR-2 in ruptured aneurysms was observed. This is the first report showing evidence that dental infection could be a part of pathophysiology in intracranial aneurysm disease.

Acknowledgements We thank MSc Teppo Haapaniemi for his assistance with immunohistochemical stainings and all participating patients who gave their consent to be included in the study.

Contributors All the authors have taken part in the conception and design of this study. MJP, LMP and TP have done the analysis and interpretation of data. All the authors have done the drafting and revision of this manuscript and approved of the version to be published.

Funding Hilda Kauhanen Foundation.

Ethics approval Pirkanmaa University Hospital Ethics Committee and the National Supervisory Authority for Welfare and Health.

Provenance and peer review Not commissioned; externally peer reviewed.

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J Neurol Neurosurg Psychiatry 2013 84: 1214-1218 originally published online June 12, 2013
doi: 10.1136/jnnp-2012-304635