Porphyromonas gingivalis Participates in Pathogenesis of Human Abdominal Aortic Aneurysm by Neutrophil Activation. Proof of Concept in Rats

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Abstract

Background: Abdominal Aortic Aneurysms (AAAs) represent a particular form of atherothrombosis where neutrophil proteolytic activity plays a major role. We postulated that neutrophil recruitment and activation participating in AAA growth may originate in part from repeated episodes of periodontal bacteremia.

Methods and Findings: Our results show that neutrophil activation in human AAA was associated with Neutrophil Extracellular Trap (NET) formation in the IntraLuminal Thrombus, leading to the release of cell-free DNA. Human AAA samples were shown to contain bacterial DNA with high frequency (11/16), and in particular that of Porphyromonas gingivalis (Pg), the most prevalent pathogen involved in chronic periodontitis, a common form of periodontal disease. Both DNA reflecting the presence of NETs and antibodies to Pg were found to be increased in plasma of patients with AAA. Using a rat model of AAA, we demonstrated that repeated injection of Pg fostered aneurysm development, associated with pathological characteristics similar to those observed in humans, such as the persistence of a neutrophil-rich luminal thrombus, not observed in saline-injected rats in which a healing process was observed.

Conclusions: Thus, the control of periodontal disease may represent a therapeutic target to limit human AAA progression.

Introduction

Abdominal Aortic Aneurysms (AAAs) may be considered as a particular form of atherothrombosis characterized by high levels of proteolytic activity [1,2,3] leading to dilation and eventually to rupture of the aortic wall. AAA progression towards rupture is not linear, but usually presents periods of stability alternating with periods of growth (“staccato” growth) [4,5]. A multilayered IntraLuminal Thrombus (ILT) usually lines the aneurysm and presents an interface with the circulating blood components [6]. This blood-ILT interface generates biological activity linked to activation of platelets and the coagulation cascade [7], red blood cell retention and hemoglobin release [8], and neutrophil accumulation leading to retention and/or release of proteases and oxidative activities [6,9,10]. Neutrophil activation leads to the release of markers measurable in plasma of patients with AAA, such as MMP-9, elastase-z1 antitrypsin complexes and myeloperoxidase [11]. Elastase that remains associated with the most luminal layer of the ILT inhibits the colonization of the fibrin network by mesenchymal cells, thus impeding the subsequent healing process [12]. We and others have pointed at potential mediators of neutrophil recruitment such as L and P-selectin [7,13] and chemoattractants such as RANTES, IL-8 and Leukotriene B4 [10]. Nevertheless, these mediators alone do not appear sufficient to explain the abundance of activated neutrophils within the ILT and the staccato progression observed in human AAA.

Recent epidemiological data indicate that chronic periodontitis, the most common form of periodontal disease, is associated with
occlusive atherothrombotic plaque progression [14,15,16,17]. However, epidemiological studies linking AAA progression to periodontal disease, or to other sources of weak pathogens, are lacking. Nevertheless, bacterial DNA corresponding to periodontal pathogens has been detected in cardiovascular tissues, including AAA tissue samples [18,19,20]. Recently, Aoyama et al. [21] reported that challenge with P. gingivalis, but not with A. actinomycescomitans (an aggressive periodontal pathogen), could promote progression of aortic diameter in an experimental model AAA. There are rational arguments suggesting that weak pathogens, such as periodontal pathogens, could participate in AAA progression:

- As well established in arthropods, coagulation and innate immunity are also interdependent in mammals. Neutrophils and the coagulation system were recently reported to interact and promote bacterial adhesion. This biological process prevents bacterial dissemination and facilitates neutrophil-induced bacterial destruction via the formation of Neutrophil Extracellular Traps (NETs).

- In humans, fibrin and hemoglobin are the most abundant proteins in the ILT. The fibrin network may represent a platform for bacterial adhesion [22,23] and is a substrate for gingipains [24]. Hemoglobin may also promote the binding of P. gingivalis [25] to the ILT and serve as a necessary source of nutrition for various periodontal pathogens, including P. gingivalis [26].

- AAA growth is discontinuous [4], suggesting weak transitory but repeated episodes of acute enrichment of the luminal layer of the ILT in biological activity associated with neutrophil accumulation, as suggested by its multilayered aspect. Repeated episodes of bacteremia could be one explanation for this observation.

In the present study, we hypothesized that repeated retention of P. gingivalis by the ILT of AAA could enhance neutrophil recruitment and subsequent activation, and thus participate in aneurysmal progression. We first assessed neutrophil activation and NET formation associated with the presence of P. gingivalis in human AAA samples. In a second part, we provide an experimental proof of concept, showing that repeated intravenous injection of P. gingivalis in a rat model of AAA led to enhanced aortic dilation associated with neutrophil retention and persistence of a non-healing luminal thrombus, mimicking human physiopathology.

**Materials and Methods**

**Human tissue and plasma samples**

AAA tissues (n = 16) were obtained from patients undergoing surgery and enrolled in the RESAA protocol (ReLeft Sanguin de l’évolutivité des Anevrismes de l’Aorte abdominale). All patients gave informed written consent, and the protocol was approved by a French ethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, CCPRB Paris-Cochin, approval no 2005). Control aortas (n = 10) were sampled from dead organ donors with the authorization of the French Biomedicine Agency (PFS 09-007). These control aortic samples were macroscopically normal, devoid of early atheromatous lesions.

Plasma samples of AAA patients were obtained from AMETHYST (Aneurysm Metalloproteinases and Hypertension) study (Table 1). Amethyst is an ongoing study promoted by INSERM (Institut National de la Santé et de la Recherche Médicale) that involves a cohort of patients with asymptomatic AAAs (group 1 (n = 11): aortic diameter 3-5 cm; group 2 (n = 21): aortic diameter >5 cm). These patients were age- and sex-matched with healthy volunteers (n = 15). All study participants gave informed consent. The study was approved by an ethic committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale, CCPRB Paris-Cochin approval nos. 1930 and 1931). Exclusion criteria for patients were cancer, infection and any immune-mediated disease. Peripheral blood was drawn in standardized conditions (fasting subjects at rest for 10 minutes, between 8 and 10 AM), with minimal stasis, into prechilled EDTA tubes. No later than 30 minutes after collection, two centrifugations were performed to separate the plasma from the blood cells (2500 rpm, 15 min, 12°C; 2500 rpm, 15 min, 4°C). Plasma samples were stored at −80°C until use.

**Determination of human AAA thrombus characteristics and carotid intima-media thickness**

AAA diameter and thrombus volume were determined by computed tomographic (CT) angiography using a dedicated software [27]. Briefly, the main steps consisted of: 1) user identification of AAA lumen entry and exit points located near the celiac trunk and iliac bifurcation, respectively; 2) automatic segmentation of 3D lumen; 3) automatic curved multiplanar reformation computation of lumen path; 4) semi-automated aneurysm wall segmentation on curved multiplanar reformations based on active contour processing; 5) interactive contour validation and editing if needed. Finally, 3D mathematical models of the AAA components were reconstructed and automatic calculation of maximal AAA diameter, and thrombus volume were performed. All CT examinations were anonymized and processed by an experimented CT technologist blinded to the radiology report. Repeated measurements allowed calculation of coefficients of variation (CV) for AAA size (16%, intra-class coefficient of correlation: 0.80) and thrombus size (10%, intra-class coefficient of correlation: 0.85).

Carotid intima media thickness (IMT): ultrasonography of both left and right common carotid arteries was performed using a high-resolution B-mode system with a 7.5-MHz linear array transducer (ATL Apogee 800+). The arterial wall segments were assessed in a longitudinal view, strictly perpendicular to the ultrasound beam, with both walls clearly visible to achieve diameter measurements. The actual IMT measurements were performed on the far wall along a minimum 10-mm length of an arterial segment with a high-quality image automatic acquisition using IOTEC software (IODP). Adventitia-to-adventitia diameter and intraluminal diameter of the common carotid artery were also measured. The intra-observer reproducibilities were 3.6±4%.

**Human aneurysmal conditioned medium**

Briefly, the ILT was dissected into three parts [28]: luminal (at the interface with the circulating blood), intermediate, and abluminal layers. The media of AAA and control aortas was separated from the adventitia and each layer was cut into small pieces (5 mm³), separately incubated (24 hours at 37°C) in a standardized volume (6 mL/g of wet tissue) of RPMI 1640 medium supplemented with antibiotics and an antimycotic. The conditioned medium was centrifuged, and the supernatant was aliquoted and frozen at −80°C until use.

**Porphyromonas gingivalis culture**

P. gingivalis strain (T103683) was purchased from the Collection de l’Institut Pasteur (Paris, France) and was grown on M20...
medium, consisting of 3% (w/v) tryptone, 1.5% (w/v) agar, 2% (w/v) yeast extract, 0.05% (w/v) cysteine hydrochloride, 0.5% (w/v) glucose and 2.5% (v/v) hemin solution (0.1% (w/v) hemin chloride, 4% (v/v) triethanolamine), in an anaerobic chamber at 37°C (Bio-Merieux, Lyon, France). Bacteria were subcultured once a week, 1 mL of the cellular suspension was centrifuged (5,000 g, 5 minutes) and resuspended in 12 mL of M20 medium.

**Experimental model of AAA**

Experimental AAA was induced by implanting a segment of sodium dodecyl sulfate (SDS)-decellularized guinea pig aorta in rat aorta as previously described [29]. Briefly, guinea pig infrarenal aortas (1.5 cm) were dissected out under deep pentobarbital anesthesia and decellularized by SDS treatment (0.1%, overnight 4°C). The next day after washing in saline, decellularized guinea pig aortas were orthotopically transplanted into the Lewis rat. One week after the surgery, *P. gingivalis* (10^7 CFU in 500 μL of saline) and saline suspension or saline alone was injected once a week via the jugular vein for 4 weeks. Two days after the fourth injection, rats were anesthetized by sodium pentobarbital (50 mg/kg, IP) and sacrificed. Blood was collected in citrated tubes and the diameters of the AAA and the thoracic aorta were measured before removal. The aneurysmal wall was fixed in paraformaldehyde (3.7%), embedded in paraffin and sectioned at 6 μm (Bio-Merieux, Lyon, France). The study was designed with 80% power to detect a relative difference in aneurysmal size between 5% (w/v) tryptone, 1.5% (w/v) agar, 2% (w/v) yeast extract, 0.05% (w/v) cysteine hydrochloride, 0.5% (w/v) glucose and 2.5% (v/v) hemin solution (0.1% (w/v) hemin chloride, 4% (v/v) triethanolamine), in an anaerobic chamber at 37°C (Bio-Merieux, Lyon, France). Bacteria were subcultured once a week, 1 mL of the cellular suspension was centrifuged (5,000 g, 5 minutes) and resuspended in 12 mL of M20 medium.

**Values are means ± standard deviation or n (%). AAA: abdominal aortic aneurysm, ACEI: angiotensin converting enzyme inhibitors, ARB: angiotensin II receptor antagonists, wall + thrombus volume: [total - lumen] volumes, p: p-value from the Fisher’s (discrete variables) or Mann-Whitney (continuous variables) tests.**

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| AAA>5 cm n = 21 | AAA≤5 cm n = 11 | All patients n = 32 | p* |
|----------------|----------------|---------------------|----|
| Age (years)    | 76±7           | 70±9                | 74±8 | 0.13 |
| Gender male    | 19 (90)        | 11 (100)            | 30 (94) | 0.53 |
| BMI (kg/m²)    | 27.0±3.6       | 26.7±2.2            | 26.9±3.2 | 0.91 |
| BMI (%)        | 80%            | 77%                 | 76%  | 0.805 |
| BMI (kg/m²)    | 26.6±2.7       | 26.7±2.2            | 26.9±3.2 | 0.91 |
| Prevalent history of: | | | | |
| Diabetes       | 2 (10)         | 3 (27)              | 5 (16) | 0.31 |
| Hypertension   | 15 (71)        | 8 (73)              | 23 (72) | 1.00 |
| Past or current smoking | 18 (86) | 10 (91) | 28 (88) | 1.00 |
| Dysplasia      | 19 (90)        | 9 (82)              | 28 (88) | 0.59 |
| Concomitant drugs: | | | | |
| Statins        | 16 (76)        | 8 (73)              | 24 (75) | 1.00 |
| Anticoagulants or antithrombectomy | 5 (24) | 0 (-) | 5 (16) | 0.14 |
| ACEI or ARB    | 11 (52)        | 6 (55)              | 17 (53) | 1.00 |
| AAA characteristics: | | | | |
| Greater diameter (cm) | 5.4±0.7 | 4.3±1.0 | 5.0±1.0 | 0.008 |
| Total volume (ml) | 141±42 | 93±37 | 124±46 | 0.008 |
| Wall + thrombus volume (ml) | 92±41 | 46±27 | 76±42 | 0.003 |
| Intima-media thickness (mm) | 0.81±0.20 | 0.83±0.13 | 0.82±0.17 | 0.36 |

**Animal sample size calculation**

The study was designed with 80% power to detect a relative 50% difference in aneurysmal size between *P. gingivalis* and saline groups. Statistical testing was performed at the two-tailed (alpha) level of 0.05 using a t-test. Based on preliminary data indicating that the average aneurysmal size at 5 weeks after xenograft surgery was 5.41 mm, standard deviation: 1.83, we used 11 rats for each group (*P. gingivalis* or saline). A computer-based randomization was used to allocate *P. gingivalis* or saline injection to each rat.

**Immunofluorescence**

Human AAA, normal aorta and rat aorta were fixed in paraformaldehyde (PFA) 3.7%, embedded in paraffin and sectioned at 6 μm. The sections were deparaffinized in toluene and hydrated in graded series of ethanol. After blocking in 5% goat serum, mouse anti-human elastase clone 265-3K1 (1 μg/mL, Hycult Biotechnology), rabbit anti-MPO (1:100, Dako), mouse anti-Histone H1 (4 μg/mL, Santa-Cruz Biotechnology), rabbit anti-Histone H4 Cit5 (1:200, Millipore) or rabbit anti-*P. gingivalis* (1:100, a generous gift from Dr Bonnaure-Mallet) were applied to sections and incubated for 1 hour at room temperature. After washing with PBS, appropriate secondary antibodies (goat antimouse or anti-rabbit conjugated with either Alexa 555 or 488, 2 μg/mL) and DAPI (100 ng/mL) were added for 15 minutes and slides were mounted using Fluoprep mounting medium (Dako). All steps are separated by 3 washes by PBS. The method of terminal dUTP nick-end labeling (TUNEL) was used to visualize DNA fragmentation (Roche Diagnostic, Meylan, France).

**Western-Blot**

Twenty μg of proteins of human tissue-conditioned medium (thrombi and arterial wall of AAA and normal aortas) were loaded on a 10% polyacrylamide gel for their separation under denaturing (SDS) and reducing conditions, before being transferred to a nitrocellulose membrane (Hybond, Amersham Biosciences, England). After blocking in 5% nonfat dried milk, the membrane was incubated with rabbit polyclonal anti-citrullinated histone
were rinsed with H2O and then incubated for 20 hours in a buffer eliminated by a 2.5% triton X-100 solution (2x 30 min). The gels containing 1% of type 1 gelatin. After electrophoresis, SDS was samples were loaded onto an SDS-10% polyacrylamide gel

Determination of cell-free DNA concentrations

Cell-free DNA (cf-DNA) concentration was determined in the conditioned medium of aortic samples and in plasma, in both humans and in the experimental AAA model in rats, using Quant-it™ Picogreen® ds DNA Reagent (Invitrogen). Briefly, 10 μL of samples and Lambda DNA standard (1 ng/μL - 1 μg/μL) were diluted in TE buffer (200 mM Tris-HCl, 20 mM EDTA, pH 7.5, 100 μL final) before addition of 100 μL Picogreen® dsDNA reagent. After mixing, and incubation for 5 minutes at room temperature in the dark, the fluorescence was measured using a microplate reader (excitation 480 nm, emission 520 nm). Intra-assay coefficient of variation (CV) was estimated at 3.8%.

Determination of Myeloperoxidase (MPO), MMP-9 and MPO-DNA complexes

The concentration of MPO in conditioned medium and in plasma of rats was determined using the rat MPO ELISA kit from Hycult Biotechnology (Uden, The Netherlands, intra-assay CV: 3.66%). MMP-9 activity in conditioned medium and plasma of rats was determined by gelatin zymography [30]. Briefly, 20 μL of samples were loaded onto an SDS-10% polyacrylamide gel containing 1% of type 1 gelatin. After electrophoresis, SDS was eliminated by a 2.5% triton X-100 solution (2x 30 min). The gels were rinsed with H2O and then incubated for 20 hours in a buffer containing 50 mM Tris and 2.5 mM CaCl2 before staining by Coomassic blue.

MPO-DNA complexes were quantified in human and rat samples (conditioned medium and plasma) by combining two different ELISA tests as previously described [31]. First, MPO was captured onto a 96-well plate coated with a monoclonal antibody against MPO (rat or human MPO ELISA kit, Hycult Biotechnologie). Diluted samples (1:10) were added to the plate and incubated for 1 hour at room temperature, and non captured material was eliminated by thorough washing steps. Second, a peroxidase-labeled anti-DNA monoclonal antibody (component number 2 of the Cell death detection ELISA kit, Roche) was added and incubated for 2 hours with gentle shaking. After washing, the peroxidase substrate ABTS (Sigma-Aldrich) was added, and the absorbance at 405 nm was measured after 30 minutes of incubation in the dark. Intra-assay CV was estimated at 4.22%.

In order to determine the percentage of cf-DNA deriving from NETs, cf-DNA concentration was evaluated in the samples before and after MPO immunocapture.

Determination of bacterial endotoxin

Endotoxins released by the ILT and the residual arterial wall into the conditioned medium (n = 16) were quantified using the Limulus Amebocyte Lysate (LAL) chromogenic endpoint assay (Hycult Biotechnology) according to the supplier’s instructions. Briefly, samples diluted at 1:5 (50 μL final) were incubated with LAL reagent (50 μL) for 30 minutes at room temperature and a stop solution was added before reading on a spectrophotometer at 405 nm.

DNA extraction and bacterial DNA amplification by PCR

Samples of AAA ILT, media and adventitia and control aortas were pulverized using a freezer mill (Spex Certiprep Ltd) and DNA was extracted from the powder by the QIamp DNA blood Midi kit (Qiagen). Briefly, 100 mg of tissue were incubated with lysozyme (20 mg/mL) diluted in 20 mM Tris-HCL pH 8.0, 2 mM EDTA, 1.2% Triton for 30 min at 37°C. Samples were then incubated with 20 μL of proteinase K (Qiagen) at 56°C until the tissue was completely digested and the protocol for DNA extraction was then followed according to the manufacturer’s instructions. Ten ng of DNA were loaded on a 1% agarose gel and stained by ethidium bromide for quality control before amplification. The same protocol was used to isolate DNA of rat aortas.

The extracted DNA was amplified using either an ubiquitous primer set that matches almost all bacterial 16S ribosomal RNA (Forward: 5’-AGC GAT GGT AGC AAT ACC TGT C-3’; Reverse: 5’-TTC GCC GGG TTA TCC CTC-3’, Tm 55°C) or by a pair of specific primers corresponding to a sequence encoding 16S rRNA of P. gingivalis (Forward: 5’-AGC GAG CTT GCC ATA CTG CG-3’; Reverse: 5’-ACT GTT AGC AAC TAC CGA TGT-3’, Tm 60°C) [32]. Briefly, PCR was carried out in a mixture containing 7 μL of DNA (50 ng), 7 μL of H2O, 4 μL of Master Mix and 2 μL of 0.2 mM PCR primer set and Mix (50 cycles) was carried out by real time PCR using a LightCycler® system with SYBR green detection (Roche Applied Biosystems).

Products of amplification were then analyzed by electrophoresis on a 1% agarose gel stained by ethidium bromide. Genomic DNA extracted from P gingivalis strain 381 was used as a positive control.

Determination of anti- P. gingivalis antibodies by ELISA

The presence of antibodies against P. gingivalis in conditioned medium and in serum was investigated as previously described [33]. Briefly, a suspension of P. gingivalis bacteria was centrifuged (10,000 g for 30 min at 4°C). The pellet was washed by 0.05 M sodium carbonate buffer and then resuspended in the same buffer to an optical density of 1.0 at 640 nm. The bacteria were heated at 60°C for 45 min, diluted 1:10 in sodium carbonate buffer and dispensed in 96-well plates. The plates were then incubated at 37°C for 4 hours and then overnight at 4°C. The excess bacteria were removed by washing in 0.005% Tween 20 in PBS and plates were allowed to air-dry before storage at 20°C until use. Each serum sample (dilution 1:100, in 1% BSA-PBS) was added to the P. gingivalis-coated plate and incubated at 37°C for 2 hours. After washing, 100 μL of peroxidase-labelled rabbit anti-human IgG,A,M (dilution 1:500 in 1% BSA-PBS) were added to each well and incubated for 2 hours at 37°C. After washing, TMB was used as substrate for peroxidase and the reaction was stopped with 0.5N H2SO4 before reading at 450 nm. Intra-assay CV was estimated at 9.32%.

Neutrophil isolation and in vitro stimulation of NETs formation

Human neutrophils were isolated from healthy donors using a dextran/Ficol method. Briefly, leukocytes were separated from red blood cells by sedimentation after hemaglutination in 1% dextran (20 minutes, room temperature) followed by a Ficoll-Paque centrifugation (616 g, 25 minutes, 20°C) and hypo-osmotic lysis of erythrocytes [34]. After washing in PBS, cells were counted on a Hemalog H1 device (Technicon Instruments Corp., Tarritown, NJ, USA) and adjusted to 1.106 cells/mL in HBSS without Ca2+. Cells were seeded in 8-well Lab-Tek® chamber slides (Permanox, Thermo scientific) at 250,000 cells/well for immuno-fluorescence staining and in a 96-well plate for cell-free DNA determination. After adhesion (1 hour, 37°C), neutrophils were
activated by adding formyl-Methionyl-Leucyl-Phenylalanine (fMLP, 100 nM) or *P. gingivalis* (1.10⁷ to 1.10⁸ CFU/mL) for 2 hours at 37°C. The conditioned media were centrifuged (2,500 g, 10 minutes, room temperature) and the supernatants were stored at −20°C. Neutrophils were fixed in PFA 3.7% for immunofluorescence.

**Statistical analysis**

Results are expressed as box plots in which the boxes represent the 25th and 75th percentiles, the line within the box represents the median value and the lines outside the boxes represent the 5th and the 95th percentiles. Differences between control and AAA subjects or saline- and *P. gingivalis*- injected rats were assessed by the Mann-Whitney non-parametric test (Prism 5, GraphPad software). The correlations were determined by the Least Squares method. Statistical significance was accepted when *p* < 0.05.

**Results**

AAA intraluminal thrombus is enriched with neutrophil extracellular traps

Pathogen-induced neutrophil activation was recently reported to induce the formation of NETs [35], consisting of extracellular, highly decondensed chromatin (histones and DNA) associated with neutrophil granule proteins (elastase, myeloperoxidase, etc.) [36], and thought to play a pivotal role in antibacterial defense. Immunodetection of histone H1 revealed strong staining in the luminal part of the ILT, associated with disorganized nuclei, stained by DAPI (Figure 1A). No staining was observed in the abluminal part of the ILT, almost devoid of cells. In contrast, immunostaining revealed intense histone H1 positivity in the adventitia of AAA relative to that of control aortas. Interestingly, H1 staining was not observed in intact nuclei due to the low accessibility of the histones within condensed chromatin to the antibody, under the experimental conditions used for immunofluorescence staining (performed without permeabilization).

Histone citrullination (H3 and H4) was previously reported to be a hallmark of NET formation, accompanying chromatin decondensation in neutrophils [37]. Citrullinated histone H4 (Cit-H4) immunostaining was observed in areas of neutrophil accumulation, as shown by elastase co-staining, mainly in the luminal part of the ILT (Figure 1B) and in the adventitia of AAA (data not shown). The release of citrullinated histones was then assessed by western-blot in the conditioned medium of each layer of the ILT and of the arterial wall (Figure 1C). Cit-H3 was shown to be mainly released by the luminal part of the ILT and by the adventitia of AAA samples, whereas conditioned medium from control aortic wall did not contain detectable levels of Cit-histones. Finally, detection of fragmented DNA by TUNEL showed a positive staining in the luminal part of the ILT that did not exactly co-localize with NETs (Figure 1D).

![Figure 1. Characterization of neutrophil extracellular traps (NETs) in human AAA samples.](#)

The presence of NETs in AAA intraluminal thrombus (ILT) and in aneurysmal wall was demonstrated by co-immunostaining of histone H1, citrullinated histone H4 (cit-H4) and elastase. Nuclei were stained with DAPI (100 ng/mL, 15 minutes). (A) Immunostaining for histone H1 (green) showing destructured nuclei in the luminal (lum) part of ILT as well as in the adventitia (Adv). (B) Immunostaining for cit-H4 and elastase demonstrates specific neutrophil activation prior to DNA expulsion. (C) The release of cit-H4 was analyzed by western blot in the conditioned medium from the different layers of ILT (luminal: Lum, intermediate: Int and abluminal: Abl) and of the remaining aortic wall (media: Med and adventitia: Adv). (D) Terminal-transferase dUTP Nick End Labelling (TUNEL) staining in the luminal part of ILT (green). TUNEL-positive cells are indicated by grey arrowheads. Extracellular nucleosomes (NETs) are indicated by white arrowheads. Merged images were obtained using Archimed software.

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Increased cell free-DNA (cf-DNA) concentration in conditioned medium and in plasma of AAA patients vs healthy subjects

As shown for citrullinated histone-H3, we postulated that NET formation in the tissue might be reflected by the release of cf-DNA into the conditioned medium [38]. The release of cf-DNA by AAA arterial wall (media and adventitia) was significantly higher relative to that of normal aortas (Figure 2A). The luminal part of the ILT was the main source of cf-DNA as compared to intermediate and abluminal layers (p<0.001). These results are consistent with the enrichment of the luminal layer in neutrophils [12].

In plasma, the concentration of cf-DNA was significantly increased in patients with AAA compared to that of control subjects (p<0.0001), and positively correlated with AAA diameter (*n* = 32, *r* = 0.579, *p* < 0.01), and thrombus volume (*n* = 32, *r* = 0.604, *p* < 0.01, Figure 2B). A significant difference was observed between large (>5 cm) and small (3–5 cm) AAA (p<0.01).

Increased circulating MPO-DNA complexes in AAA

Although often used to quantify circulating NETs [38], cf-DNA assay measures all types of DNA able to interact with Picogreen, whatever its origin (coming from NETosis, necrosis or apoptosis of

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![Figure 2](image-url). Increased cell-free DNA (cf-DNA) in the conditioned medium and in plasma of human AAA. (A) The concentration of cf-DNA was determined in the conditioned medium obtained from the arterial wall of control and aneurysmal aortas, from AAA thrombus and (B) in plasma of healthy subjects and patients with a small (<5 cm) or a large (>5 cm) AAA. Results are presented as box plots in which the median is shown. **p<0.01; ***p<0.001 (Mann-Whitney analysis). The correlation (n = 32) between AAA diameter, thrombus volume and cf-DNA concentration were obtained by the Least Squares method.

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cell types other than neutrophils). In order to show that cf-DNA did indeed reflect circulating NET content, we measured the MPO-DNA complexes, by immunoprecipitation of MPO followed by detection with an anti-DNA conjugated with horseradish peroxidase [31] in conditioned medium (AAA and normal aorta samples) and in plasma. The concentration of the MPO-DNA complexes was significantly higher in conditioned medium of aneurysmal wall as compared to that of normal aorta (Figure 3A), and also in that from the luminal thrombus layer compared to that from intermediate and abluminal layers.

In plasma, the concentration of MPO-DNA complexes was significantly higher in patients with AAA than in controls (Figure 3B): p<0.05 between controls and small AAA patients, p<0.0001 between controls and large AAA patients. No significant difference between small and large aneurysms was observed. Moreover, the concentration of MPO-DNA complexes was positively correlated with levels of cf-DNA (r = 0.545, p = 0.0015). The concentration of cf-DNA was also determined in plasma samples before and after MPO immunocapture, in order to evaluate the proportion of cf-DNA originating from NETs. Our results show that approximately 20% of plasma cf-DNA was MPO-associated DNA (data not shown), suggesting that a part of cf-DNA release is dependent on NET formation.

### Presence of bacteria in AAA samples

Bacteria represent one of the major triggers of NET formation. To test our hypothesis that AAA ILT could be a substrate for bacterial retention and therefore participate in the aneurysmal progression, we first quantified endotoxin (lipopolysaccharide or LPS) in the conditioned medium of the different layers of the ILT (Figure 4A) by the Limulus Amebocyte Lysate assay. Out of 16 AAA ILT samples, only 4 of them had undetectable levels of bacterial LPS (Figure 4A). The abluminal thrombus layer was shown to release more LPS than its luminal layer (p = 0.005). The presence of bacteria was then investigated by PCR on the total DNA extracted from the ILT and arterial wall samples (in both control and aneurysmal aortas). Amplification of DNA encoding for bacterial 16S ribosomal RNA was performed using specific primers. 10/16 thrombi and 11/16 aneurysmal walls were positive for bacterial DNA (Figure 4B). *P. gingivalis* is the major anaerobic pathogen responsible for periodontal disease and may produce chronic bacteremia subsequent to chewing or toothbrushing. *P. gingivalis* was detected by PCR in 6/16 thrombi and 7/16 aneurysmal wall tested (Figure 4C). In contrast, all control aortic walls were negative for 16S ribosomal RNA for all bacteria and *P. gingivalis* in particular (data not shown). In addition, *P. gingivalis* was indirectly assessed by quantification of antibodies immunoreactive against *P. gingivalis* either released from AAA adventitia

![Figure 3. MPO-DNA complexes in conditioned medium and in plasma of human AAA.](image-url)

MPO-DNA complexes released by the intra-luminal thrombus (ILT) of AAA and by the arterial wall of control aorta and AAA were quantified in the conditioned media (A) as well as in plasma (B). A sandwich ELISA was used, consisting of an anti-human MPO for immunocapture and a peroxidase-conjugated anti-DNA antibody for detection. Results are presented as box plots in which the median is shown. *p<0.05, **p<0.01; ***p<0.0001 (Mann-Whitney analysis).

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(containing tertiary lymphoid organs or ATLOs) or contained in the serum of patients with AAA versus controls. Figure 5A indicates that adventitia from aneurysmal aortas released higher amounts of anti-\textit{P. gingivalis} immunoglobulins relative to adventitia from normal aorta. Furthermore, serum of AAA patients contained more immunoreactive antibodies against \textit{P. gingivalis} compared to control subjects (Figure 5B). Although analysis of the correlation between adventitial and serum antibodies against \textit{P. gingivalis} could only be performed on a limited number of samples \((n = 15)\), a positive association was found \((r = 0.54, p = 0.039)\). Strong positive correlations were observed between the titer of anti-\textit{P. gingivalis} immunoglobulins in serum and cf-DNA, AAA diameter and thrombus volume (Figure 5C, \(n = 32\)). It is noteworthy that no correlation (Figure 5C) between the thrombus volume and the intima-media thickness was observed \((r = 0.027, p = 0.905)\).

\textit{P. gingivalis} promotes NET formation \textit{in vitro}

The presence of \textit{P. gingivalis} in AAA samples shown by PCR and the correlations between anti- \textit{P. gingivalis}/cf-DNA and anti- \textit{P. gingivalis}/AAA diameter led us to hypothesize that these bacteria could participate in the chronic renewal of the ILT via recruitment and activation of neutrophils. We found that \textit{P. gingivalis} were able to trigger the formation of NETs, as shown by immunofluorescent
staining of histone H1 and citrullinated histone H4 (Figure 6A). *P. gingivalis* promoted neutrophil DNA expulsion in a dose-dependent manner as shown by quantification of cf-DNA in the supernatant of neutrophils incubated with different concentrations of *P. gingivalis* (Figure 6B). Induction by *P. gingivalis* of NET formation and subsequent trapping was further demonstrated by epifluorescence (Figure 6C) and confocal microscopy (Figure 6D).

Chronic *P. gingivalis*-bacteremia induces neutrophil recruitment in experimental AAA

In order to provide an experimental proof of concept that *P. gingivalis* may impact on aneurysm progression, we used the decellularized xenograft model of aneurysm in rats [29]. To mimic chronic bacteremia associated with periodontal disease, *P. gingivalis* was injected by the intravenous route once a week for 4 weeks (10^7 CFU/rat) without producing significant modification of their general health status (no difference in body weight between control (346.3 ± 6.8 g) and *P. gingivalis*-injected (338.7 ± 6.1 g) rats, no signs of prostration and no macroscopically visible alterations of visceral organs: lung, liver and kidney at necropsy). Repeated *P. gingivalis* bacteremia induced a significant increase in the aneurysm size as compared to saline-injected rats (Figure 7A, B, median±IQR, *P. gingivalis* (n = 9): 8.12±2.68 mm vs saline (n = 11): 5.25±2.75 mm, p<0.03). Histological analysis showed that ILTs of rats infected by *P. gingivalis* were larger than those of non-infected rats and...
exhibited a significant enrichment in neutrophils (Figure 8A). As expected in this experimental model, mesenchymatous cell colonization associated with important fibrosis was observed in the ILT of non-infected rats, suggesting the beginning of the healing process (Figure 8B). In contrast, *P. gingivalis*-injected rats exhibited a large ILT containing neutrophils in the luminal part, mimicking what is observed in human pathology, without any sign of healing. We have also observed strong histone H1 immunostaining in the neutrophil-rich area associated with disorganized nuclear structure, suggesting the presence of NETs, similar to those observed in human ILT (Figure 8C). Double immunostaining for *P. gingivalis* and histone H1 showed the presence of *P. gingivalis* trapped by extracellular nucleosomes at the luminal pole of the ILT (Figure 9A). *P. gingivalis* colonization was shown to be specific of the ILT since neither the AAA of saline-injected rats nor the thoracic aorta of *P. gingivalis*-infected rats were positive for Pg 16S rRNA (PCR) (Figure 9B).

To further demonstrate the impact of neutrophil enrichment in the *P. gingivalis*-infected aneurysmal samples (wall+thrombus), MMP-2/MMP-9 activities (assessed by gelatin zymography), MPO and cf-DNA concentrations were measured in conditioned medium. MMP-9 activity was significantly increased in medium conditioned by AAA samples from *P. gingivalis*-infected rats compared to AAA samples obtained from non-infected rats (Figure 10A). In contrast, greater amounts of pro-MMP-2 were released by aneurysmal segments of non-infected rats relative to *P. gingivalis*-infected rats (p = 0.05), reflecting an ongoing healing process in non-infected rats, since pro-MMP-2 is preferentially secreted by mesenchymatous cells. The ratio MMP-9/MMP-2 was therefore significantly higher in conditioned medium from *P. gingivalis*-infected rats.
gingivalis-infected rats (0.163 vs 0.061 in saline-injected rats, p = 0.008). MPO concentration was strongly increased in AAA conditioned medium from P. gingivalis-infected rats as compared to non-infected rats (Figure 10B, p = 0.0003). In plasma, a trend towards increased MPO levels was observed in P. gingivalis-infected rats without reaching statistical significance (p = 0.065). The medium conditioned by the aneurysmal segment of the abdominal aorta contained more cf-DNA than the medium obtained by incubation of the adjacent aortic segment from the same rat (thoracic aorta, Figure 11A). Moreover, cf-DNA concentration was increased in both conditioned medium and plasma of rats injected by P. gingivalis compared to saline-injected rats (Figure 11A). This suggests that cf-DNA is released by the AAA segment and not by the rest of the aorta potentially infected by P. gingivalis. More importantly, a strong positive correlation was observed between the concentrations of cf-DNA in both conditioned medium and plasma, and the AAA diameter (r = 0.635, p<0.03 and r = 0.83, p<0.001, respectively). Finally, NETs contributed, at least in part, to cf-DNA measured in plasma and conditioned medium since MPO/DNA complexes were detected in higher amounts in P. gingivalis-infected rat samples (Figure 11B).

Discussion

Several lines of evidence led us to hypothesize that bacteria, and in particular periodontal pathogens, may participate in the development of AAA. Epidemiological data suggest an association between periodontal and cardiovascular diseases [39]. The nature of this association is however still a matter of debate; in particular, whether periodontal disease impacts directly on the pathogenesis of cardiovascular diseases or indirectly by increasing background inflammation is still not settled. Interestingly, atherothrombosis and periodontal disease share risk factors such as age, smoking and male gender [16,40]. However, to date, no epidemiological study has been reported linking AAA development and periodontal disease. In the present study, we sought to establish a potential causal link between P. gingivalis, a highly prevalent periodontopathogen, and AAA progression.

Neutrophil recruitment and activation in human AAA thrombus – Potential role of P. gingivalis

The persistence of neutrophils in the most luminal part of the ILT, interfacing with the bloodstream cannot be explained solely by their passive trapping during the process of fibrin formation. We have recently reported that conditioned medium from the luminal layer of ILT was chemoattractant for neutrophils in human AAA. However, blocking strategies interfering with the interleukin 8 pathway and RANTES only produced a 50% inhibition of neutrophil chemoattraction [10]. Since neutrophils constitute the first line of defense against bacteria and are strongly attracted by lipopolysaccharide (LPS) and bacterial peptides, we tested for the presence of endotoxin in AAA samples. We show that both the mural ILT and the residual AAA wall contained and released LPS that may account for neutrophil chemoattraction and activation. LPS from P. gingivalis was shown to stimulate neutrophils via LPS-binding protein in the serum of patients with periodontal disease [41]. In vitro studies have shown that neutrophil exposure to a variety of different microbial pathogens

![Figure 7. Chronic P gingivalis (Pg) infection fostered AAA development in rats. (A) Experimental AAA was induced by implanting a segment of a SDS-decellularized guinea pig aorta in the rat aorta. Rats were or not infected weekly with Pg (1.10⁷ CFU/500 μL/rat, for 4 weeks). (B) At the end of treatment, rats were anaesthetized for blood sampling and sacrificed after measuring AAA diameter. Results (n = 10) are presented as box plots in which median is shown, **p<0.01 (Mann-Whitney Analysis). doi:10.1371/journal.pone.0018679.g007](#)
P. gingivalis in Abdominal Aortic Aneurysm

Figure 8. P. gingivalis (Pg) infection promoted neutrophil recruitment, NET formation and inhibited healing. (A) Hematoxylin/Eosin staining showing the presence of a thrombus and neutrophil accumulation at its luminal pole (right, inset) in Pg-infected rats. The presence of mesenchymatous cells is observed in saline-injected rats (left, inset). (B) Masson’s trichrome staining. Fibrosis associated with healing is observed in green in saline-injected rats whereas red staining highlights the presence of a thrombus in Pg-infected rats. (C) Immunostaining for histone H1 (red), nuclei appear in blue (DAPI). Merged images show the presence of extracellular H1 associated with disorganized DNA (inset), but also intact neutrophils characterized by their multilobed nuclei (bottom, right).

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**P. gingivalis** in human AAA:

The presence of bacterial DNA was investigated by PCR. DNA encoding for bacterial 16S ribosomal RNA was detected in 11/16 of the AAA samples tested whereas 7/16 were positive for detection of *P. gingivalis* DNA. These results are in line with a previous study [46], which tested the presence of 7 periodontopathic bacteria by PCR in AAA mural ILT and arterial wall and showed that more than 80% of AAA samples tested contained *P. gingivalis* DNA. The lower incidence in our study may be explained not only by different procedures for DNA extraction and primers used for detection of *P. gingivalis* DNA, but also by the higher prevalence of chronic periodontitis in the Japanese population compared with other developed countries[46]. In another recently published study by Nakano et al.[21], detection of *P. gingivalis* by PCR was reported in 8/76 aneurysm samples.
However, no distinction was made between thoracic (TAA) and abdominal aortic aneurysms, which correspond to totally different pathologies. Interestingly, these authors distinguished between these two locations for assessment of the presence of *P. gingivalis* detection in dental plaque: 12/19 AAA were positive for oral *P. gingivalis* versus only 5/16 for TAA. More importantly, in our study, we have detected increased levels of anti- *P. gingivalis* antibodies released by AAA adventitia relative to those from normal aortic adventitia, probably associated with the presence of adventitial tertiary lymphoid organs in AAA [2]. In addition, serum titers of anti- *P. gingivalis* immunoglobulins were increased in AAA patients and strongly positively correlated with AAA thrombus volume \( r = 0.731, p = 0.0001 \). This correlation is much stronger than those observed for other biological markers of AAA (\( r = 0.293, p = 0.28 \)), MMP-9/AAA expansion: \( r = 0.53, p = 0.48 \)), and thioredoxin/AAA diameter: \( r = 0.5, p = 0.48 \)), and thus strengthens the potential link between *P. gingivalis* infection and AAA development. Interestingly, no correlation was observed between carotid intima-media thickness (IMT) and thrombus volume in our cohort of AAA patients (\( r = 0.25, p = 0.28 \)). This suggests that *P. gingivalis* is specifically linked to AAA pathogenesis rather than being an additional risk factor increasing systemic inflammation associated with subclinical diffuse atherosclerosis (i.e increased IMT).

Other infectious pathogens such as *Chlamydia pneumoniae*, *Helicobacter pylori*, *Cytomegalovirus* or *Herpes simplex* virus have been suggested to be involved in the pathogenesis of AAA [49]. Whereas anti-*Chlamydia pneumoniae* antibodies were shown to be increased in plasma of AAA patients [50], most studies failed to demonstrate a link between infectious burden and AAA disease [49]. In addition, DNA corresponding to *C. pneumoniae* could not be detected in AAA samples [51,52,53]. Antibiotic strategies have been evaluated to thwart infectious agents and in particular *C. pneumoniae*. A study by Mosorin et al. [54] suggested that doxycycline may favorably alter the outcome of patients with AAA. Tetracyclines and particularly doxycycline, directly inhibit the activities of human matrix metalloproteinases (MMPs) in human AAA walls [55]. More recently, doxycycline was shown to reduce AAA neutrophil and cytotoxic T-cell content [56]. This antibiotic is known to reduce the severity and progression of periodontal disease in animal models and humans [57]. Doxycycline and derivatives have also the potential to inhibit the *P. gingivalis* proteinases such as gingipains [58,59]. The beneficial effects of doxycycline on AAA progression could therefore also be attributed, at least in part, to its action against periodontal pathogens.

In contrast to lung or gastric bacteria, subgingival plaque pathogens can easily reach the bloodstream several times a day *via* chewing and toothbrushing, especially in patients with periodontal disease [60,61]. Fimbriae and in particular fimbrillin A (FimA, the major fimbrin subunit) are involved in most adherence properties exhibited by *P. gingivalis*. Fim A was shown to promote adhesion to fibrin and fibrin/platelet matrices, which occur in ILT [62,63]. Hemoglobin may also mediate *P. gingivalis* adhesion to the ILT and...
be used as a source of nutrient [25,26]. However, since *P. gingivalis* are strict anaerobic bacteria, they are not likely to proliferate in an aerobic environment such as that observed in the ILT (PO$_2$ = 100 mm Hg). Chronic bacteremia could therefore allow subclinical infection of different cardiovascular tissues including the ILT of AAA, principally composed of fibrin, platelets and hemoglobin.

**P. gingivalis** in an experimental model of AAA in rats

In order to provide a proof of concept that *P. gingivalis* may be an actor of AAA progression, we have used an experimental model of AAA in rats [29]. This animal model is characterized by the formation of a thrombus about one week after grafting a decellularized guinea pig aorta in the abdominal position, associated with aortic dilation. In this model, like in all currently used AAA models, the mural ILT is rapidly colonized by mesenchymatous cells that initiate a fibrotic healing process. In contrast, after 4 weekly intravenous injections of *P. gingivalis*, the aortic diameter was not only significantly increased relative to saline-injected rats (p = 0.01), but the composition of the AAA was strikingly different. In *P. gingivalis*-injected rats, the mural ILT was persistent and exhibited a multilayered aspect, similar to what is observed in human AAA samples. The ILT was considerably enriched in neutrophils and all markers of their activation were increased in conditioned medium and in plasma of *P. gingivalis*- vs saline-injected rats. In the present study, we demonstrate that a
periodontal pathogen enhances the development of AAA by maintaining the presence of a neutrophil-rich ILT, leading to a pathophysiological pattern similar to that observed in humans. It cannot be excluded that other bacteria or their products may have similar effects, and may participate in AAA pathogenesis, but our data highlight the major role of \( P. \) gingivalis in AAA development.

Using a different model of experimental AAA in mice, Aoyama et al. [21] have reported that, in contrast to \( P. \) gingivalis, \( A. \) actinomycetemcomitans did not promote aortic dilation. However, these authors used a calcium-chloride model in mice, that consists in an external aggression of the aorta that does not lead to the formation of a thrombus, as often observed in humans.

**Strengths and limitations of the study**

Our study provides a combination of clinical and experimental data that could link periodontal disease to AAA formation. However, albeit reaching statistical significance, the number of human samples analyzed is quite small. Additional epidemiological studies linking AAA and periodontal diseases would be necessary to support our findings. The model that we used is characterized by the formation of a thrombus about one week after xenografting. The healing process then usually takes place in the absence of additional aggression that would maintain the recruitment of neutrophils. In the present study, we report for the first time that chronic injection of \( P. \) gingivalis leads to the formation of a thrombus, as often observed in humans.

![Cell-free DNA (CF-DNA) and MPO-DNA complexes are increased in rats infected with \( P. \) gingivalis (Pg).](image-url)
persistence of the ILT, similar to human pathophysiology. However, we did not test other periodontal pathogens that would be of interest, such as performed by Aoyama et al. [21]. Finally, we provide evidence that *P. gingivalis* DNA is present in AAA samples and that *P. gingivalis* material is sufficient to produce an advertitial immune response. However, the presence of living pathogens was not shown suggesting that *P. gingivalis* material, such as dead bacteria or LPS, may be sufficient to bind to the thrombus and promote its chronic renewal.

**Conclusion**

In conclusion, the results of the present study indicate that *P. gingivalis* accelerates AAA progression via recruitment and activation of neutrophils, leading to production of NETs which are detectable in the plasma of AAA subjects. Because repeated subclinical episodes of bacteremia are systematically associated with periodontal diseases, *P. gingivalis* could be therefore a key actor in human AAA progression.

Taken together, our results demonstrate that a common pathogen may have a causal role in the pathogenesis of AAA. These findings bring significant new information to the field of AAA pathogenesis but should be strengthened by both epidemiological and observational studies in humans before one can envisage potential therapeutic strategies based on the treatment of periodontal disease to prevent AAA evolution towards rupture.

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**Author Contributions**

Conceived and designed the experiments: OM SD J-BM. Performed the experiments: SD J-MA CJ LL OM. Analyzed the data: SD CJ OM PR. Contributed reagents/materials/analysis tools: MB-M PR LL YC RR. Wrote the paper: OM SD J-BM PB.

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