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Host-microbiota relationship in the pathophysiology of aseptic abscess syndrome: protocol for a multicentre case-control study (ABSCESSBIOT)

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ABSTRACT

Introduction Aseptic abscess (AA) syndrome is a rare disease whose pathophysiology is unknown. It is often associated with inflammatory bowel disease and characterised by sterile inflammation with collections of neutrophils affecting several organs, especially the spleen. Microbiota are known to influence local and systemic immune responses, and both gut and oral microbiota perturbations have been reported in diseases associated with AA syndrome. However, interactions between these factors have never been studied in AA syndrome. The purpose of this translational case-control study (ABSCESSBIOT) is to investigate gut and/or oral microbiota in patients with AA syndrome compared with healthy controls. Moreover, microbiota associated metabolites quantification and Treg/Th17 balance characterisation will give a mechanistic insight on how microbiota may be involved in the pathophysiology of AA syndrome.

Methods and analysis This French multicentre case-control study including 30 French centres (University hospital or regional hospital) aims to prospectively enrol 30 patients with AA syndrome with 30 matched controls and to analyse microbiota profiling (in stools and saliva), microbial metabolites quantification in stools and circulating CD4+ T cell populations.

Ethics and dissemination This study protocol was reviewed and approved by an independent French regional review board (n° 2017-A03499-44, Comité de Protection des Personnes Ile de France 1) on 10 October 2022, and declared to the competent French authority (Agence Nationale de Sécurité du Médicament et des produits de santé, France). Oral and written informed consent will be obtained from each included patient and the control participant. Study results will be reported to the scientific community at conferences and in peer-reviewed scientific journals.

Trial registration number Clinical Trials web-based platform (NCT05537909).

INTRODUCTION

Aseptic abscess (AA) syndrome is a rare entity, well individualised among systemic inflammatory diseases and characterised by deep and circumscribed collections of neutrophils. The term abscess refers to macroscopic and histological appearance of the lesions and should not be confused with an infectious disease, which should be ruled out before making the diagnosis. The first case was published in 1995.1 In 1999, a French registration was created (Commission nationale de l’informatique et des libertés (CNIL) 1999, June 1999) and the last publication reported 71 French patients.2

Clinical overview

This inflammatory disease often presents with fever accompanied by variable painful symptoms depending on the location of the abscesses, which are usually intra-abdominal. AAs can affect all organs, but for some unknown reason, the spleen is the preferred site. In the main series, it was involved in 71.8% of cases, alone or in association with other organs. C reactive protein level is high and is associated with polymorphonuclear leucocytosis. The results of bacterial, viral and fungal investigations are negative as well as the autoimmune workup except sometimes anti-Saccharomyces cerevisiae antibodies. Useful imaging tests include ultrasound, CT scan and positron emission tomography.
scan. Colonoscopy is also part of the examinations to be performed, to look for underlying inflammatory colitis, especially in case of transit disorders. Pathological examination of abscesses can contribute to the diagnosis. When performed, it typically shows a central zone of more or less altered neutrophils surrounded by a pali-sading arrangement of histiocytes and sometimes giant cells. Antibiotic therapy is ineffective. Corticosteroid therapy generally allows a significant improvement on both clinical and biological levels. Colchicine can also be used as a first-line treatment. Immunosuppressive drugs may be necessary. The biotherapies are effective, notably anti-tumour necrosis factor (TNF)-α and interleukin (IL)-1 receptor antagonists. The evolution is usually marked by relapses.

Pathophysiology
AA syndrome is usually associated with other diseases especially inflammatory bowel disease (IBD) (42% in the last publication). Various alterations of the gut microbiota have been reported in patients with IBD (for review). The gut microbiome demonstrated reduced diversity, expansion of proinflammatory bacteria, and depletion of phyla with anti-inflammatory effects such as Firmicutes. A role of this dysbiosis has been reported in the pathogenesis in the disease activity and in the response to the treatment. The NOD2/CARD15 gene encodes a protein of the same name involved in innate immunity and has polymorphisms associated with Crohn's disease. A polymorphism associated with a more severe phenotype of AA syndrome was described. NOD2 was described as a 'bacterial sensor' controlling interaction between inflammation and microbiota. Patients with IBD with NOD2 mutations have characteristic microbiota with decreased abundance of Faecalibacterium species and increased abundance of Escherichia species.

Several studies demonstrated the importance of the intestinal microbiota in human physiology, especially for immune regulation. Antigens produced by the commensal microbiota provide immune stimulation without which the development of the immune system is affected, as observed in axenic mice. T lymphocytes play a critical role in cellular immune function. CD4+ T cells include several subgroups including Th1, Th2, Th17 and regulatory T cells (Treg). Many lymphocytes are present in the mucosa of the gastrointestinal tract and associated lymphoid tissues. Th17 and Treg are considered essential to the host-microbiota relationship and participate in inflammatory and anti-inflammatory reactions via IL-17 and IL-10 secretion, respectively. A perturbation of the balance Th17/Treg was described in IBD but also in other inflammatory diseases such as Behçet’s disease or spondylarthritids. Some commensal germs directly impact Th17/Treg balance: segmented filamentous bacteria are involved in the induction of Th17 cells, while the bacterium Faecalibacterium prausnitzii would instead promote Treg responses. This bacterium producing short chain fatty acids (SCFAs) such as butyrate is poorly represented in patients with inflammatory colitis.

The human body does not have the appropriate enzymes to hydrolyse complex carbohydrates such as fibres. These food components are thus fermented by gut microbiota resulting in the production of SCFA. The main ones are acetate, butyrate and propionate whose concentrations in the intestine follow the ratio 3:1:1. Acetate and propionate are primarily produced in the small intestine whereas butyrate is mainly produced in the caecum and colon. Acetate is derived from pyruvate from two different pathways: enteric bacteria and acetogenic bacteria (eg, Blautia hydrogenotrophica). Propionate is formed via succinate by Bacteroidetes and via the lactate signalling pathway by Firmicutes. Finally, butyrate is produced via acetyl-CoA by Firmicutes. Butyrate can modulate immune response mediated by neutrophils. Other microbiota-associated metabolites could influence inflammation loop. Primary bile acids (BAs) are metabolised in secondary BAs by colonic microbes. Dysbiosis can modify BA metabolism. Anti-inflammatory effects of some BAs was demonstrated in mice through their role in the development of colonic RORγ̂ Tregs. Tryptophan is also metabolised by gut microbiota. Some of the metabolites can act as aryl hydrocarbon receptor (AhR) ligands. AhR is a transcription factor involved in T cell differentiation and function, especially through IL-22, a cytokine implicated in neutrophil recruitment.

Patients with AA syndrome have oral manifestations such as aphthous lesions with or without definite Behçet’s disease. Oral microbiota have been studied in healthy subjects and is one of the most diverse of all human microbial communities. Strictly speaking, there is no ‘single oral microbiota’ because its composition is very heterogeneous in the different sites of the oral cavity, but the term is commonly used to encompass all such sites. However, the salivary microbiota exhibits long-term stability and can be considered an important reservoir containing microorganisms from all distinct niches of the oral cavity. Oral bacteria play a central role in the genesis of human oral diseases, mainly dental caries and periodontitis, but also in non-oral diseases such as IBD.

Immune responses in sites remote from the gut may also be regulated by the gut microbiota. Absence of microbiota or dysbiosis affect the expression of autoimmune diseases remote from the gut in animal models of rheumatoid arthritis, multiple sclerosis or autoimmune uveitis. Thus, some diseases require the presence of microbiota to develop. They are improved in patients treated with antibiotics or are less expressed in germ-free mice.

Thus, the intestinal microbiota has been implicated in many studies on inflammatory or autoimmune diseases. Its role and its interaction with the immune response in the AA syndrome remain to be explored.
Study aim
This study aims to assess its impact in the pathophysiology of AA syndrome. For this, gut and oral microbiota will be compared between patients with AA syndrome and healthy subjects living in the same environment. Microbiota-associated metabolites and circulating Th17 and Treg will also be studied, in order to better understand how potential microbiota disruption in patients could influence their inflammatory response.

METHODS AND ANALYSIS
Study design and setting
ABSCESSBIOT is a French multicentre case-control study, including 30 French centres (University hospital or regional hospital). All patients with AA syndrome (according to Andre et al criteria) will be systematically proposed participation to the study, aiming inclusion of 30 patients and 30 healthy matched controls from 2023 to 2024. They could be patients from the AA syndrome French register (CNIL 99.149, June 1999) or newly diagnosed patients. After oral and written informed consent has been obtained, patients will be enrolled and data will be collected using an electronic database (RedCap; Research Electronic Data Capture) hosted at the University Hospital of Clermont-Ferrand, France. All data regarding patients/family history, treatments and clinicobiological characteristics will be recorded at inclusion. Then data on gut microbiota composition, oral microbiota composition, microbial metabolites levels in stools and Th17/Treg balance will be studied (figure 1).

Objectives and endpoints
The main objective of this project is to study the gut microbiota profiling (alpha and beta diversity, composition and function) of patients with AA syndrome compared with healthy controls.

The secondary objectives are to study the oral microbiota profiling, the level of microbial metabolites (SCFAs, tryptophan metabolites, BAs) in stools and the circulating Th17/Treg balance of patients with AA syndrome compared with healthy controls.

Figure 1  Design of the ABSCESSBIOT study.
**Eligibility criteria**

**Inclusion criteria**

Eligible patients will be adults meeting the diagnostic criteria for AA syndrome described by André et al.36

1. Deep abscesses on radiological examination with neutrophilic features proven by pathological analysis of a surgical specimen or biopsy when performed.

2. Negative blood cultures; negative serological tests for bacteria including _Yersinia enterocolitica_; sterile pus during surgery or aspiration (according to standard cultures, acid-alcohol-resistant bacilli and fungal tests); failure of antibiotic therapy, when prescribed, after at least 2 weeks for conventional antibiotic therapy and at least 3 months for antituberculosis treatment.

3. Rapid clinical improvement the day after the prescription of corticosteroids (at least 1–2 mg/kg prednisone or equivalent) followed by radiological improvement after 1 month of corticosteroids, sometimes in association with immunosuppressive treatments.

Eligible controls will be adults living in the same environment as the matched cases. This can include both family members and non-family members living in the same household. They will be included during the same visit as the cases. A particular attention will be also paid on comparability of groups on age and sex in addition to environment including nutrition.

**Exclusion criteria**

Pregnant women, incapable patients and patients deprived of liberty will be excluded. Patients with antibiotic therapy administered within 6 weeks prior to inclusion will also be excluded.

**Enrolment and follow-up**

All patients fulfilling the inclusion and non-inclusion criteria will be proposed the study during an outpatient visit or a hospitalisation. Both oral and written information will be given. After oral and written informed consent has been obtained, patients will be enrolled. During an outpatient visit or a hospitalisation, samples (blood, stool and saliva) will be collected from the patient. There will be no follow-up. Healthy matched controls will be recruited during the same visit as the cases.

**Gut and oral microbiota**

Stool and saliva samples will be collected, aliquoted and immediately frozen (−80°C) in each centre. They will then be moved to the Biological Resource Centre (BRC) at the University Hospital of Clermont-Ferrand. After thawing, DNA will be extracted from the sample using Maxwell RSC PureFood GMO kit according to the manufacturer’s instructions. The full 16S gene will be amplified and Illumina high-throughput sequencing will be then performed on a MiSeq following the manufacturer’s guidelines.32

**Microbial metabolites quantification**

After thawing, stool samples will be prepared as previously described in the literature.33 Acetate, propionate and butyrate will be then analysed by gas chromatography-mass spectrometry. After sample preparation as previously described in the literature,29 fecal and sera BA will be analysed by liquid chromatography tandem mass spectrometry. Serum concentrations of tryptophan metabolites will be also be measured by liquid chromatography coupled with high resolution mass spectrometry.34

**Treg/Th17 balance**

Whole blood will be collected in each centre and then moved to the BRC at the University Hospital of Clermont-Ferrand. Peripheral blood mononuclear cells (PBMCs) will be isolated on ficoll, aliquoted and immediately frozen (−80°C). After thawing, CD4+ T cell populations will be assessed by flow cytometry. In particular, Tregs will be characterised as CD3+ CD4+ CD25+ CD127− cells and Th17 as CD3+ CD4+ CXCR3− CCR4+ CCR6+ CCR10− CD185−, as previously reported in the literature.35

Total RNA will be extracted from PBMCs using RNeasy Mini Kit (Qiagen, France) following the manufacturer’s instructions. RNA quality will be assessed by a NanoPhotometer (Implen, Germany). Reverse transcription will be performed using a PrimeScript RT Reagent Kit (TaKaRa, France) followed by qPCR quantification (iTaq Universal SYBR Green Supermix, Bio-Rad, France) and FOXP3 and RORCT expression will be compared with that of the housekeeping gene GAPDH.

**Statistical considerations**

The main objective of this study will be to compare the gut microbiota of patients with AA syndrome with that of controls matched according to the environment. As this is a rare disease, the number of subjects was set according to (1) The number of patients included in published studies dealing with the intestinal or salivary microbiota in Internal Medicine (n=22 patients in a study on Behçet and microbiota36 and n=21 patients in a study on Lupus and microbiota37) and (2) The estimation of effect size in terms of the primary endpoint (alpha and beta divergence) with respect to the recommendations proposed by Coher38 who defined the following effect size limits: small (ES: 0.2), moderate (ES: 0.5) and large (ES: 0.8, ‘grossly perceptible’). In view of these elements, at least 22 subjects per group will be needed to highlight an effect size around one for a two-sided type I error at 5% and a 90% statistical power. To improve the statistical power, we aim to recruit 30 patients and 30 matched controls.

**Statistical analysis**

Statistical analyses will be performed with Stata V.15 (StataCorp, College Station, Texas, USA) and R (https://
cran.r-project.org/). All statistical tests will be performed with a two-sided alpha level of 5%. Continuous variables will be presented as means and SDs or medians and IQRs. The assumption of normality will be analysed using the Shapiro-Wilk test. Patients and controls will be described and compared according to the following variables: epidemiological characteristics, clinical characteristics and treatments. A description of deviations from the protocol, the subjects distributed according to these deviations and the reasons for dropout will also be studied.

The analysis of the microbiota will be performed by sequencing of the 16S gene. Taxonomic affiliation will be performed using referenced databases such as GreenGenes and Ribosomal Database Project (RDP). The alpha and beta diversity of the samples will be determined after normalisation of the sample size. The analyses will also determine the baseline microbiota of each group. Thus, to compare the diversity indices between patients with AA syndrome and controls, the comparisons will be performed using Student’s t-test or Mann-Whitney test if the assumptions to apply t-test are not met. The homoscedasticity will be studied by the Fisher-Snedecor test. The results will be expressed using effect sizes and 95% CIs.

This analysis will be completed by a multivariate analysis (ie, multiple linear regression) with covariates determined according to the univariate results and according to the epidemiological relevance, specially treatments and IBD status. The normality of residuals from these linear models will be analysed as aforementioned. If necessary, a logarithmic transformation of the dependent variables (alpha and beta diversity) could be applied. Due to the sample size, the robustness of this multivariate analysis will be discussed.

In addition to these analyses, multidimensional analyses (principal coordinate analysis and linear discriminant analysis affect size) will be carried out in order to identify important potential bacterial groups. The functional analyses will also be addressed using the putative functional activities of each microbe and their representation using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (functions deduced from the putative gene content deduced from the bacterial taxonomy). This will be verified and compared with metagenomic data allowing the determination of a fraction representative of the entire microbiota content. The sequences will be analysed and affiliated to cellular functions using MG-RAST (metagenomics rapid annotation using subsystems technology associated with a dedicated database).

Furthermore, mixed factorial data will be also conducted to analyse the assets as elements of categorical and continuous variables related to clinical and gut microbiota characteristics.

No missing data will be expected. However, in case of missing data, a sensitivity analysis will be performed to study the statistical nature of missing data in order to propose the most appropriate data imputation method.

Patient and public involvement

None.

ETHICS AND DISSEMINATION

This study protocol was reviewed and approved by an independent French regional review board (n° 2017-A03499-44, Comité de Protection des Personnes Ile de France one on 10 October 2022) and declared to the competent French authority (‘Agence Nationale de Sécurité du Médicament et des produits de santé’, France). The study is registered at ClinicalTrials.gov (NCT05337909).

Patients and controls will be informed of the objectives and constraints of the study, of possible risks and of their rights to decline any participation in the study or the possibility of withdrawing at any time. Patient’s and control’s oral and written informed consent will be collected by the investigator.

Study results will be reported to the scientific community at conferences and in peer-reviewed scientific journals.

DISCUSSION

The main expected purpose of this study is the identification of specific microbial signatures associated with AA syndrome. We will match patients with healthy controls from the same environment to look for the particular effect of the disease. Due to the low number of patients, we will include patients with active and inactive disease, although this heterogeneity could impact our analysis. We will assess whether the level of colonisation by some specific bacterial species and/or the loss of protective bacteria and/or the level of microbiota-associated metabolites could be associated with the disease and implicated in the pathophysiology of AA syndrome, especially by modulating Treg/Th17 balance.

Another impact of this study could be to validate microbial signatures in stools and/or oral samples in order to obtain a non-invasive biomarker in this disease, hard to diagnose and usually needing invasive intervention. In case of relevant results, this combination of factors would therefore allow improved diagnostics and follow-up of patients.

To the best of our knowledge, no translational studies have investigated the interactions between gut/oral microbiota, microbiota-associated metabolites and immune response in AA syndrome. The ABSCESSBIOT project will be the first study assessing this global approach in this rare disease. It could improve the understanding of its pathophysiology but also lead to innovative and clinically relevant proposals. In addition to the description aspect of microbiota, immune response will be explored. If specific bacterial species, abnormal microbiota-associated metabolites and/or Treg/Th17 imbalance are associated to AA syndrome, other studies could be conducted to demonstrate their role in the pathophysiology. All these data could thus constitute a basis for the future development
of therapies targeting microbiota or the immune response in AA syndrome.

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Competing interests None declared.

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