Bacterial signatures in thrombus aspirates of patients with lower limb arterial and venous thrombosis

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ABSTRACT

Objective: Increasing data supports the role of bacterial inflammation in adverse events of cardiovascular and cerebrovascular diseases. In our previous research, DNA of bacterial species found in coronary artery thrombus aspirates and ruptured cerebral aneurysms were mostly of endodontic and periodontal origin, where *Streptococcus mitis* group DNA was the most common. We hypothesized that the genomes of *S mitis* group could be identified in thrombus aspirates of patients with lower limb arterial and deep venous thrombosis.

Methods: Thrombus aspirates and control blood samples taken from 42 patients with acute or acute-on-chronic lower limb ischemia (Rutherford I-IIb) owing to arterial or graft thrombosis (n = 31) or lower limb deep venous thrombosis (n = 11) were examined using a quantitative real-time polymerase chain reaction to detect all possible bacterial DNA and DNA of *S mitis* group in particular. The samples were considered positive, if the amount of bacterial DNA in the thrombus aspirates was 2-fold or greater in comparison with control blood samples.

Results: In the positive samples the mean difference for the total bacterial DNA was 12.1-fold (median, 7.1), whereas the differences for *S mitis* group DNA were a mean of 29.1 and a median of 5.2-fold. Of the arterial thrombus aspirates, 57.9% were positive for bacterial DNA, whereas bacterial genomes were found in 75% of bypass graft thrombosis with 77.8% of the prosthetic grafts being positive. Of the deep vein thrombus aspirates, 45.5% contained bacterial genomes. Most (80%) of bacterial DNA-positive cases contained DNA from the *S mitis* group. Previous arterial interventions were significantly associated with the occurrence of *S mitis* group DNA ($P = .049$, Fisher's exact test).

Conclusions: This is the first study to report the presence of bacterial DNA, predominantly of *S mitis* group origin, in the thrombus aspirates of surgical patients with lower limb arterial and deep venous thrombosis, suggesting their possible role in the pathogenesis of thrombotic events. Additional studies will, however, be needed to reach a final conclusion. (J Vasc Surg 2018;67:1902-7.)

Clinical Relevance: This is the first study to report the presence of bacterial DNA, predominantly of *Streptococcus mitis* group origin, in the thrombus aspirates of patients with lower limb arterial and deep venous thrombosis. Interestingly, the occurrence of *S mitis* group DNA seems to be significantly associated with previous vascular manipulations. It remains to be established whether these findings play a role in the actual thrombosis.

Bacterial inflammation has long been suggested to contribute to inflammation of atherosclerotic plaques, either directly or by indirect mechanisms, where inflammation at nonvascular sites can contribute to the progression of the lesions. Proposed bacteria include respiratory pathogens and periodontal bacteria as well as bacteria from the gastrointestinal tract.[1] One group of common oral bacterial species, namely, *Streptococcus mitis* group, has been shown to have an exceptional ability to adhere to an endothelial surface and bind platelets as well.[2,3] Previous studies[4-6] showed that *S mitis* group DNA was predominant in thrombus aspirates from coronary arteries and cerebral arterial wall samples in patients with acute coronary events and cerebral aneurysms respectively. The *S mitis* group comprises 13 species.[7] Most of these bacteria can be found in the oral cavity of healthy individuals.

Based on these previous studies, it is likely that oral pathogens may play a certain role in the pathogenesis of cardiovascular diseases.[4-6] To the best of our

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The study was financially supported by the Academy of Finland (grant no 286284 for T.L); Competitive State Research Financing of the Expert Responsibility area of Tampere University Hospital (grants X51001 and X51401); Finnish Foundation for Cardiovascular Research (T.L and PJK); Finnish Cultural Foundation; Tampere Tuberculosis Foundation (T.L and PJK); Viipuri Tuberculosis Foundation; Emil Aaltonen Foundation; Yrjö Jahnsson Foundation; Signe and Ane Gyllenberg Foundation; and Diabetes Research Foundation of Finnish Diabetes Association.

Author conflict of interest: none.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a conflict of interest.

0741-5214

http://dx.doi.org/10.1016/j.jvs.2017.05.090

knowledge, there are no studies depicting the role of dental or periodontal bacteria in the thromboembolic events of lower limbs and whether they are present in venous thrombosis. Thus, the target bacteria for our study were *S mitis* group because their DNA could be found in almost 80%[4] of the thrombus aspirates and in the majority of the arterial wall samples[5,6] of the patients with cardiovascular and cerebrovascular diseases, respectively. We hypothesize that the signs of these oral pathogens can equally be found in the thrombus aspirates of peripheral vessels of the lower limbs.

ARTICLE HIGHLIGHTS

- **Type of Research:** Prospective cohort study
- **Take Home Message:** Aspirates from acute arterial, bypass graft or deep vein thrombus contained bacterial DNA in 25 of 39 patients (64.1%), most frequently (77.8%) from acute thrombus formed in prosthetic bypass grafts. The majority 80% of bacterial DNA-positive cases contained DNA from the *Streptococcus mitis* group
- **Recommendation:** These data support the possible role of bacterial inflammation, caused predominantly by *S mitis*, in thrombotic events.

METHODS

Patients

This prospective study analyzed data from consecutive patients (N = 62) referred to Tampere University Hospital, Tampere, Finland, for acute or acute-on-chronic lower limb arterial, arterial bypass graft, or deep venous thrombosis from September 2014 to October 2016.

Inclusion criteria. Patients with symptoms or signs of acute or acute-on-chronic lower limb ischemia (Rutherford class I-IIb)[8] presenting with angiographic evidence of native artery or bypass graft thrombosis (n = 31) and patients presenting with deep venous thrombosis of the iliofemoral segment with symptoms' duration no longer than 2 weeks (n = 11).

Exclusion criteria. Patients with progressive sensorimotor changes or contraindications for thrombolysis (n = 20). Those patients were treated surgically and excluded from further analysis. Based on our previous experience, thrombus aspirates from those patients were not obtained owing to increased sample contamination risk.[9,10] The patients included in the study were treated with thrombolysis, and the actual study group thus comprised 42 cases (Table). The study was approved by the ethics committee of the hospital, and all the patients included gave informed consent.

Specimen processing

Aspiration of thrombus samples was performed aseptically in an angiography laboratory by an interventional radiologist. The antiseptic routinely used for skin disinfection was a denatured ethanol solution (A12T Dilutus 80%; Berner, Helsinki, Finland) being effective against both aerobic and anaerobic pathogens.[11] Patients received no antibiotic therapy at admission to the hospital or later during the procedure. The procedure was carried out within 3 hours after admission to the hospital in cases of acute arterial ischemia and within 12 hours in venous cases, if no significant venous ischemia or life-threatening conditions were present. An introducer sheath was placed, and the thrombus aspirates were obtained using 6-F angio catheters from the proximal parts of the arteries or grafts and usually distally in the cases of deep venous thrombosis before initiation of thrombolysis and placed into Eppendorf tubes. Control blood samples were taken through the introducer sheaths before the thrombus aspiration took place and stored in similar tubes. The specimens were frozen at −80°C after collection. DNA from the samples was extracted using a commercial QIAmp DNA Mini Kit (Qiagen Ltd, Calif) according to the instructions provided. Blood and whole collected thrombus aspirates were then analyzed using the real-time quantitative polymerase chain reaction (qPCR). The risk for contamination was reduced to minimal as the specimen handling was performed aseptically throughout the whole process.

Real-time qPCR

The presence of bacterial DNA was identified using qPCR with ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems, Foster City, Calif) as previously described[12] with Maxima Probe/ROX qPCR Master-Mix (Thermo Fischer Scientific, Waltham, Mass). Arterial thrombus aspirates were compared with arterial control blood samples, as opposed to venous to venous to reduce any potential bias caused by sampling from different sites and, subsequently, bias resulting from different conditions like flow dynamics and pressure. The presence of bacterial DNA in thrombus and in control blood samples were determined by using published primers and a probe for *Streptococcus* spp., mainly *S mitis* group,[12] and universal bacterial primers and a probe[13] using human housekeeping gene, RNAseP (Applied Biosystems), as a reference gene. Each measurement was performed as duplicates or quadruples in uncertain cases. The relative amounts of bacterial DNA in samples were calculated by the comparative threshold cycle (*C*t) method ($\Delta\Delta C$t, $\Delta C\text{t}_{\text{sample}} - \Delta C\text{t}_{\text{control}}$),[14] where the sample was a thrombus aspirate and control was a blood sample from the same patient. First, the differences of the *C*t values (ΔCt) between candidate bacteria and reference gene measurement (*C*t from candidate bacteria − *C*t from RNAseP) for each sample were calculated; then the comparative *C*t ($\Delta\Delta C$t) (ΔCt from thrombus − ΔCt from one patient's own arterial blood)