Antiplatelet therapy abrogates platelet-assisted Staphylococcus aureus infectivity of biological heart valve conduits

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Objective: Although recent advances in pulmonary valve replacement have enabled excellent hemodynamics, infective endocarditis remains a serious complication, particularly for implanted bovine jugular vein (BJV) conduits.

Methods: We investigated contributions by platelets and plasma fibrinogen to endocarditis initiation on various graft tissues used for valve replacement. Thus, adherence of Staphylococcus aureus and platelets to 5 graft tissues was studied quantitatively in perfusion chambers, assisted by microscopic analysis. We also evaluated standard antiplatelet therapy to prevent onset of S aureus endocarditis.

Results: Of all tissues, bovine pericardium (BP) showed the greatest fibrinogen binding. Perfusion of all plasma-precoated tissues identified BP and BJV with the greatest affinity for S aureus. Perfections of anticoagulated human blood over all tissues also triggered more platelet adhesion to BP and BJV as single platelets. Several controls confirmed that both S aureus and platelets were recruited on immobilized fibrinogen. In addition, perfusions (and controls) over plasma-coated tissues with whole blood, spiked with S aureus, revealed that bacteria exclusively bound to adhered platelets. Both the platelet adhesion and platelet-mediated S aureus recruitment required platelet αIIbβ3 and coated or soluble fibrinogen, respectively, interactions abrogated by the αIIbβ3 antagonist eptifibatide. Also, standard antiplatelet therapy (aspirin/ticagrelor) reduced the adherence of S aureus in blood to BJV 3-fold.

Conclusions: Binding of plasma fibrinogen to especially BJV grafts enables adhesion of single platelets via αIIbβ3. S aureus then attaches from blood to (activated) bound platelet αIIbβ3 via plasma fibrinogen. Dual antiplatelet therapy appears a realistic approach to prevent endocarditis and its associated mortality. (J Thorac Cardiovasc Surg 2019; ■-■:1-11)

CENTRAL MESSAGE

In the onset of infective endocarditis on heart valve conduits, single platelets adhere to biological heart valve conduit tissues upon fibrinogen coating and recruit bacteria via platelet integrin αIIbβ3 and plasma fibrinogen.

PERSPECTIVE

Infective endocarditis is an underestimated, highly lethal disease, the prevalence of which is rapidly increasing as a consequence of medicsurgical interventions. Results provide a strong rationale that antiplatelet agents may be beneficial in the prevention of S aureus endocarditis, by reducing platelet adhesion and subsequently diminishing bacterial attachment.

See Commentary on page XXX.
Infective endocarditis (IE) remains a diagnostic and therapeutic challenge, associated with high morbidity and mortality in patients requiring heart valve replacement.\(^1,2\)

In current clinical practise, replacement of a pulmonary valve can be achieved by percutaneous implantation of a pulmonary (stent-mounted) valve (PPVI) and by surgical placement of a cryopreserved pulmonary homograft (CH) or a bovine jugular vein (BJV) conduit.\(^3\) Recent studies have focused on the increased risk of IE after PPVI using a BJV conduit, a risk as high as 2.6% per patient year,\(^1\) suggesting it to be greater than for other tissues.\(^4,5\) In this patient cohort, IE is associated with high morbidity and a frequent need for re-intervention, especially when caused by *Staphylococcus aureus*.\(^6\) So far, the mechanisms underlying the different susceptibilities for IE across different grafts remain unexplained.

*S. aureus* is among the most important bacterial pathogens, responsible for severe endovascular infections, causing an inflammatory response, platelet recruitment, and a prothrombotic state, in which the host coagulation cascade is activated.\(^7,8\) *S. aureus* staphylocoagulase mediates encapsulation during the development of cardiac vegetations, rich in fibrin, platelets, bacteria, and inflammatory cells, thus hiding the bacteria from host defense pathways.\(^8\)

Staphylocoecal clumping factor A (ClfA) triggers bacterial adhesion to endothelial cells (ECs) in vitro and inflammation in animal models.\(^9,11\) Activated ECs release von Willebrand factor (VWF), to which *S. aureus* adheres via the VWF-binding protein in shear stress conditions.\(^10,12\)

Histologic observations have revealed that fibrin deposition occurs on these leaflets even in the absence of infection.\(^13\) As *S. aureus* has a strong affinity for fibrin and fibrinogen (Fg) via ClfA,\(^13\) fibrin might act as a critical precursor for bacteria and platelet attachment, at the expense of VWF. Recently, our group demonstrated that heart leaflet damage indeed promotes fibrin deposition, serving to recruit *S. aureus*, whereas heart valve inflammation triggered massive VWF-mediated platelet deposition, in turn promoting *S. aureus* binding.\(^15\)

Several molecular pathways have been proposed to explain platelet activation by *S. aureus*, leading to activation of platelet \(\alpha_{IIb}\beta_3\),\(^16,17\) allowing it to bind Fg (or fibrin), not only causing platelet aggregation, but also binding of *S. aureus* ClfA and fibronectin binding protein A,\(^10,12\) interactions inhibited by \(\alpha_{IIb}\beta_3\) antagonists.\(^18\) It remains poorly understood whether these mechanisms contribute to the onset of conduit IE, especially since the BJV tissue is chemically pretreated to maximally avoid hemostatic and infectious complications.\(^19\)

Since current guidelines on IE prevention limit the use of prophylactic antibiotics, a need for evidence-based alternative measures emerges. Aspirin is being discussed as a preventive measure in patients after PPVI.\(^1\) So far, its potential in IE prevention remains unclear.

In the present study, we have, therefore, investigated in vitro bacterial adheriveness, the role of human plasma, Fg/fibrin, and blood platelets on various valve conduit tissues under flow, to understand differences in the interaction between these tissues and *S. aureus*. Furthermore, available pharmacologic antiplatelet intervention strategies were investigated for their efficacy to reduce the incidence of *S. aureus* endocarditis.

### METHODS

#### Bacterial Strains

*S. aureus* strains 8325-4 and Newman (wild type and cflA-deletion mutant clinical strains)\(^20\) were cultured at 37°C on Mueller–Hinton blood agar and in tryptic soy broth liquid medium. For the mutant strain, 5 \(\mu\text{g/mL}\) erythromycin was added to the media. Bacteria were stored in liquid medium supplemented with 15% (vol/vol) glycerol at –80°C. Where indicated, Newman strain was used; otherwise, “*S. aureus*” refers to the 8325-4 strain.

#### Graft Tissues

Bovine pericardium patch (BP; Supple Peri-Guard; Synovis Surgical Innovations, St Paul, Minn), CH (human origin, European Homograft Bank, Brussels, Belgium), and bovine jugular vein (BJV) conduit (Contegra; Medtronic, Minneapolis, Minn) were used to investigate bacterial and platelet adhesion under perfusion. Detailed graft processing is specified in Appendix E1. To avoid artifactual binding of bacteria and blood constituents to cut graft borders, only the central (native) graft surfaces were exposed to perfusate in appropriately manufactured perfusion chambers.\(^21\) Before studying bacterial adhesion, we investigated graft tissues for Fg binding, which is described in detail in Appendix E1.

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**Abbreviations and Acronyms**

ASA = acetylsalicylic acid  
BJV = bovine jugular vein  
BP = bovine pericardium patch  
CFU = colony-forming unit  
CH = cryopreserved pulmonary homograft  
ClfA = clumping factor A  
EC = endothelial cell  
Fg = fibrinogen  
Fn = fibronectin  
IE = infective endocarditis  
O/N = overnight  
PBS = phosphate-buffered saline  
PPVI = percutaneous pulmonary valve implantation  
VWF = von Willebrand factor
Bacterial Adhesion to Graft Tissues

After various overnight (O/N) coatings in different conditions, tissues were perfused at a shear rate of 1000 s⁻¹ for 10 minutes at room temperature with bacterial suspensions of 10⁶ to 10⁷ colony-forming units (CFU)/mL, verified by CFU counting, in phosphate-buffered saline (PBS), human plasma (plasma), human serum (serum), serum reconstituted with Fg (300 μg/mL), or human anticoagulated blood according to a protocol, recently reported in detail in Appendix E1. In brief, after perfusion, tissues were washed in PBS under flow, transferred to a tube containing 1 mL of 0.9% NaCl, and sonicated for 10 minutes using a sonication bath (VWR Ultrasonic Cleaner; VWR, Radnor, Pa.). Next, bacterial suspensions were serially diluted and spread on Mueller–Hinton blood agar plates and incubated O/N at 37°C to count CFUs.

For imaging purposes, bacteria (10⁶ CFU/mL) were labeled with 5(6)-carboxy-fluorescein N-hydroxy-succinimidyl ester (30 μg/mL; Sigma-Aldrich, St Louis, Mo.,) mixed with whole blood (containing Rhodamine 6G stained platelets) and perfused for 15 minutes over previously prepared tissues. After washing in PBS and fixation in 4% paraformaldehyde, specimens were imaged under the fluorescence microscope. Where indicated, BP patch after O/N incubation with human albumin (200 g/L) was incubated with labeled Fg-AF488 (50 μg/mL in albumin 200 g/L) for 1 hour at 37°C, washed with PBS, and imaged in fluorescence microscopy. In some cases, coating of labeled Fg-AF488 was followed by perfusions with labeled bacteria (Texas Red, 20 μg/mL; Thermo Fisher Scientific, Waltham, Mass.) for 10 minutes, and the copresence of Fg and bacteria was visualized as mentioned previously.

Where indicated, antiplatelet drugs such as eptifibatide, acetylsalicylic acid (ASA), and ticagrelor were added to the mixture of whole blood and bacteria (10⁶ CFU/mL). Perfusions were performed for 15 minutes over the tissues and bacteria were quantified after being plated on blood agar. Platelet preparation and assessment of the amount of adhered platelets by an optimized acid phosphatase assay (colorimetric assay) are specified in detail in Appendix E1.

In stepwise experiments, graft tissues (precoated with plasma) were preperfused with whole blood (stained for platelets or not) for 20 minutes, followed by washing with plasma or PBS (15 minutes), and were finally perfused with suspensions of 10⁶ CFU/mL in PBS, plasma, or serum for 10 minutes (detailed protocol of serum preparation in Appendix E1). When indicated, the αIIbβ₃ antagonist eptifibatide was added to the first or the second perfusion step. After a washing step with PBS, the serial dilution method was performed as described previously, or microscopic analysis was applied.

The plasma and serum, as well as the respective bacterial suspensions, were verified to be endotoxin free (endotoxin levels <0.1 EU/mL; Thermo Scientific Pierce LAL Chromogenic Endotoxin Quantitation Kit). All buffers were freshly used.

Statistical Analysis

Statistical analysis revealed that not all data followed a Gaussian distribution and therefore nonparametric analyses were performed. Statistical significance between groups was analyzed by using the t test with the nonparametric Mann–Whitney U test. Data analyses were performed with GraphPad Prism 8.0d (GraphPad Software, San Diego Calif.). Values are reported as median and distribution plots, via 25%-75% percentile boxes with respective min and max binding whiskers. P values <.05 were considered significant.

RESULTS

Fibrinogen Binds Differently Across Various Graft Tissues

We first investigated the binding of fluorescently labeled Fg to all 5 graft tissues compared in this study. Fg hardly bound to CHwall (median near 1.0, Figure 1), whereas BP showed the greatest apparent affinity (median around 1.9; P < .001 compared with CH wall). BJVleaflet, BJVwall, and CHleaflet displayed intermediate values, the latter 2 characterized by a wide spread (Figure 1).

Plasma-Derived Fg Enhances S aureus Adhesion to Graft Tissues Under Shear Stress

After O/N coating of the tissue discs with plasma, bacterial adherence analysis revealed that the plasma-precoated CHwall recruited 7- to 10-fold less bacteria than the bovine grafts, BJVwall, and BP, respectively (Figure 2, A). To confirm that the impact of plasma coating is due to Fg, BP was coated either with Fg (30 μg/mL), albumin (200 mg/mL), plasma, or serum. Coating with Fg and plasma triggered comparable bacterial adhesion, greater than that achieved after albumin or serum coating and in general comparable with that shown in Figure 2, A, for BP (Figure 2, B). Similar results were found for bacterial adhesion experiments on BJVwall (Figure 2, C), ie, coating with serum and albumin showed similarly low S aureus adherence. Yet, reconstituting serum with low Fg concentration (300 μg/mL) increased bacterial binding to the levels reached in plasma, indicating that Fg is solely capable of supporting S aureus adhesion to plasma-coated BJVwall (Figure 2, C) and BP (Figure 2, B). Direct fluorescence visualization and image analysis, likewise, detected significantly more bacterial foci on plasma-treated BP than on albumin-treated BP, providing also direct support for the role of plasma Fg (Figure E1). In further support, microscopic analysis of labeled S aureus (red) and Fg (green) showed partial colocalization of both labels.

FIGURE 1. Relative binding of fibrinogen (Fg) to graft tissues in static conditions. Incubation of cryopreserved pulmonary homograft, CH (walls: n > 6 and leaflets: n = 4) and bovine jugular vein, BJV (walls and leaflets: n ≥ 6), bovine pericardium patch, BP (n > 6) graft tissues with fluorescently labeled Fg (30 μg/mL). Fg binding is calculated as a fluorescence signal relative to the autofluorescence of each graft tissue and is shown as a fold change. Data are presented as box and whisker dot plots with the upper and lower borders of the box representing the 25% and 75% percentile (upper and lower quartiles). The middle horizontal line represents the median and the upper and lower whiskers the maximum and minimum values of nonoutliers. T test with nonparametric Mann–Whitney U test was applied: **P < .01, ***P < .001.
or greater Fg-binding, especially to BP and BJWwall predisposes to S aureus recruitment on coated Fg. As S aureus ClfA is a bacterial adhesin with affinity for Fg,22 we investigated the adherence of a deletion mutant of Newman S aureus, D clfA, to the BJW conduit. The D clfA bound substantially less to BJW wall by about 42% (Figure E2). Hence, this finding confirmed that ClfA participates in S aureus adhesion to plasma-coated grafts.

Different Reactivity of Platelets With Various Graft Tissues via Coated Fg-Integrin αⅡbβ3

Before investigating the reactivity of S aureus in blood, we first analyzed platelet adhesion to graft tissues after perfusion with whole citrate-anticoagulated blood. Platelet adhesion was 2.5-fold greater to BJWwall and BP than to CHwall, CH leaflet, and BJW leaflet, reaching approximately 0.5% bound platelets (Figure 3, A). Upon whole blood perfusion, scanning electron microscopy of BJWwall conduit showed single adhered platelets, spread and with an activated morphology (marked pseudopods), rather than platelet aggregates (Figure 3, B). In addition, platelet adhesion was noted to both the surface of ECs (Figure 3, B, left panel) and the subendothelial matrix (Figure 3, B, right panel). Interestingly, addition of the integrin αⅡbβ3 antagonist eptifibatide abrogated platelet adherence to BJWwall by >80% (Figure 3, C). Despite low thrombogenicity (perfusion over 20 minutes), we conclude that BJWwall and BP are prone to adhere and activate (single) platelets. The potent inhibition of platelet binding by eptifibatide strongly suggests the interaction between platelets and BJW tissue to result from platelet αⅡbβ3 interactions with coated Fg. Indeed, when the degree of S aureus binding is matched with that of platelets, for all 5 tissues investigated, it is clear that BJWwall and BP
Constitute a group of 2 reactive tissues compared with the group of CHwall, CHLeaflet, and BJVLeaflet, with low reactivity. High *S. aureus* binding parallels high platelet binding, presumably to the same ligand, ie, coated Fg (Figure E3). Therefore, the remainder of the work was done with BJVwall and BP exclusively.

**Impact of Platelet–Graft Tissue Interactions on *S. aureus* Adhesion**

In comparison with perfusions over BJVwall and BP with *S. aureus* suspended in PBS (Figure 2, A–C) or in plasma (Figure 4, A, first bar) with comparable levels of bacterial adhesion, when *S. aureus* was suspended in blood directly, bacterial adhesion to BJVwall appeared to be significantly smaller (Figure 4, A, second bar), with a degree of *S. aureus* binding around 0.1% of the total amount of perfused bacteria. Interestingly, inclusion of eptifibatide (10 μg/mL) to the perfused blood eradicated both platelet adhesion (Figure 3, C) and attachment of *S. aureus* by about 52% (Figure 4, A, third bar), down to residual levels of 26% adhesion compared with plasma and 48% adhesion compared with blood. Because these findings suggested that in blood, *S. aureus* adhesion occurred almost exclusively to adhering platelets and no longer as a result of direct interactions between CIIA and coated Fg, we “separated” the critical participants in blood and perfused them sequentially. Thus, Figure 4, B, shows that when blood is first perfused over plasma-precoated BJVwall, *S. aureus* will bind to the same degree to deposited platelets as in Figure 4, A (second bar), but only when suspended in plasma and not in PBS (Figure 4, B, first vs second bar). Because these findings suggested a role for plasma Fg in *S. aureus* binding, we also suspended *S. aureus* in serum and perfused it over BJVwall conduit, after having perfused blood over plasma-precoated tissue already. Figure 4, C shows that in this case no *S. aureus* bound (Figure 4, C, first bar). In addition, when the perfusion step was performed with *S. aureus* suspended in plasma containing eptifibatide, we also observed that *S. aureus* adhesion was abrogated. Eptifibatide added in this step removed <30% of the already-adhered platelets (not shown), further excluding that the lack of *S. aureus* binding in the presence of eptifibatide resulted from platelet removal. Finally, in agreement with the model in which *S. aureus*, suspended in blood or plasma (in 2-step perfusions) binds to activated adhered platelet αIIbβ3 via Fg (Figure 4, A–C), we added eptifibatide to the blood perfusion step to eradicate platelet binding. Subsequent perfusion with *S. aureus* suspended in plasma in this case did not lead to bacterial binding (Figure E4).
**FIGURE 4.** Interaction analysis between platelets and bacteria on graft tissue surfaces. Microparallel plate flow chamber perfusions over plasma O/N precoated bovine jugular vein (BJV<sub>wall</sub>) with 10<sup>6</sup> CFUs/mL of S aureus, suspended (A) in hPlasma, or in whole blood without or with eptifibatide (10 µg/mL) added, as indicated by the legends inside the figure, for 15 minutes. Two-step perfusions (B, C), where step 1 consisted of a pre-perfusion with whole blood or PBS for 20 minutes, as indicated below the x-axis, followed by a perfusion step 2 for 10 minutes of 10<sup>6</sup> CFUs/mL of S aureus in PBS, hPlasma, or hSerum at a flow rate of 1000 s<sup>-1</sup> (n > 6), as indicated by the legends inside the figure. Eptifibatide (10 µg/mL) was also included (C) in the step 2 perfusate, as indicated. Adhesion of S aureus was evaluated as the percentage of adherent CFUs to the total dose of CFUs over the perfusion interval. Results are presented as box and whisker dot plots with the upper and lower borders of the box representing the 25% and 75% percentile (upper and lower quartiles). The middle horizontal line represents the median, the upper and lower whiskers the maximum and minimum values of nonoutliers. T test with non-parametric Mann–Whitney U test was applied: *P < .05, **P < .01, ***P < .001, ****P < .0001. PBS, Phosphate-buffered saline.

Separate perfusions over plasma-coated BJV<sub>wall</sub> in which bacteria were suspended in plasma vs PBS (Figure 4, A, first bar and B, third bar) confirmed that S aureus in PBS or plasma binds in the same manner to coated Fg, in agreement with previous findings.23

Together, these results show that is case S aureus is perfused in blood over BJV<sub>wall</sub> or BP, it binds less to adhered platelets than to coated Fg in the absence of platelets, with a 2-fold reduced median value. Blocking platelet adhesion and/or their activation shows a reduction in S aureus recruitment. Platelet inactivation does not reactivate direct Fg-mediated S aureus recruitment, suggestive of a platelet-induced passivation process.24

Fg is the common ligand, mediating both platelet deposition on BJV<sub>wall</sub> or BP and enabling S aureus binding to platelets in the next step.

**Aspirin and Ticagrelor Reduce Bacterial Adhesion to Graft Tissues**

**Figure 5, A,** shows how platelets spread on graft tissues and how they recruit bacteria in a platelet-controlled manner. Because eptifibatide reduced bacterial attachment (Figure 4), we have also tested in vitro whether current antiaggregant agents, ie, as used in dual antiplatelet therapy (combination of ASA and the platelet ADP receptor antagonist ticagrelor) might mitigate bacterial adherence of graft tissues.

Their effects (separate and in combination) on S aureus adherence to BJV graft under shear stress are presented in Figure 5, B. ASA (100 µg/mL) alone significantly reduced bacterial attachment by nearly 55% versus the control blood condition—similar to the effect of eptifibatide (Figure 4, A). Ticagrelor (2 µg/mL) alone similarly reduced bacterial attachment by 54%, whereas the combination of ASA and ticagrelor showed a stronger, additive reduction reaching 71% inhibition. A potential bactericidal effect of ASA and ticagrelor25 was inexistent at the concentrations used, such as validated via separate incubation and perfusion tests (Figure E5).

Bacterial adhesion to BJV<sub>wall</sub> in plasma in the presence and absence of the ASA/ticagrelor combination used in Figure 5 showed similar S aureus binding to coated Fg (Figure E5), confirming the effect of pharmacologic platelet inhibition on S aureus recruitment.

**DISCUSSION**

IE after PPVI remains a severe complication, and the risk to acquire IE seems to be greater after application of heterologous BJV conduits compared with CH.5,26 Differences in bacterial adherence to BJV and CH tissues cannot be attributed to intrinsic tissue specificities,27,28 a finding triggering the present study on the role of platelets and plasma proteins in bacterial adherence to cardiac graft tissues. Therefore, the importance of interactions between
bacteria and platelets for adhesion were investigated in blood perfusion experiments under shear stress (Video 1). Bacterial adhesion to BJV in blood could be significantly reduced by antiplatelet agents (Figure 6), a major finding in the present study.

Our results reveal that plasma Fg binds especially to bovine grafts and mediates bacterial adhesion in the absence of blood cells. In circulating blood, platelets adhere more to BJV and BP than to CH tissue and single adhering platelets in turn drive bacterial recruitment mediated via activated platelet $\alpha_{\text{IIb}}\beta_3$.

Single adhered and activated platelets were visualized by scanning electron microscopy. Fg is crucial, not only in the platelet adhesion step, but also in the $S. aureus$ recruitment step, because plasma Fg mediates the interaction between activated platelet $\alpha_{\text{IIb}}\beta_3$ and $S. aureus$ suspended in blood. Standard dual antiplatelet treatment potently blocks bacterial adhesion, providing evidence that this strategy may be beneficial in IE prevention.

Despite tissue processing before implantation, aiming to reduce surface thrombogenicity in vivo, the perfusion of blood provokes adhesion of a measurable number of platelets, specifically single, activated platelets, to especially bovine graft surfaces at endothelialized and denuded areas. BJV$_{\text{wall}}$ was more prone for platelet binding than BJV$_{\text{leaflet}}$, in agreement with the knowledge that valvular (leaflet part) ECs are less reactive in inflammatory adhesion, in part because of lower expression of VWF and a greater expression of thrombomodulin, in turn promoting the endothelial anticoagulant system, contributing to an anti-inflammatory state via protein C activation. Some of these properties can be expected to be preserved to some degree, even in fixed tissues.

“Simple” perfusions, ie, those in which $S. aureus$, suspended in plasma, serum, or PBS, were performed over BJV$_{\text{wall}}$ and BP, uncovered that coated Fg was the only required ligand to induce $S. aureus$ binding. Thus, $S. aureus$ adhesion was abrogated when BJV$_{\text{wall}}$ or BP was precoated with serum and was restored by the supplementation of serum with Fg. Also, platelet binding occurred to coated Fg and the $\alpha_{\text{IIb}}\beta_3$ antagonist epitifibatide strongly reduced platelet deposition. Initially, we anticipated platelet adhesion to occur to traditional ligands on damaged and intact EC surfaces. Indeed, when blood perfusions are performed in the presence of an $\alpha_{\text{IIb}}\beta_3$ antagonist, single platelet deposition is observed.
to ligands as VWF primarily, but also to fibronectin (Fn) and laminin. Since adhering platelets use Fg as a surface-bound ligand, it is clear that Fg needs to first coat onto the graft surface by plasma incubations. Fg can bind to integrins $\alpha_\text{V}\beta_3$ and $\alpha_\text{IIb}\beta_3$ on ECs, but also to collagen, exposed as a result of chemical processing of the valvular tissue, factors we do not fully control in the prepared tissues. Platelet glycoprotein VI has been reported to bind to fibrin and Fg, but in view of the strong inhibition of platelet binding by eptifibatide, a role for platelet glycoprotein VI appears highly unlikely, including binding to subendothelial matrix collagen (Figure 3). Platelet adhesion to surface-bound Fg is followed by platelet activation and spreading via $\alpha_\text{IIb}\beta_3$, presently also found for BJV and BP-bound platelets, a process interrupted by the $\alpha_\text{IIb}\beta_3$ antagonist eptifibatide.

When $S$ aureus is directly suspended in whole blood, subsequent perfusions over BP or BJV revealed that bacterial binding occurred to adhered platelets exclusively and with a 2-fold lower recruitment rate as from perfusions without blood cells (platelet-poor plasma). Correspondingly, preventing platelet adhesion with eptifibatide abolished $S$ aureus binding to these grafts. This observation raises an interesting paradigm: inhibition of the platelet-assisted recruitment of $S$ aureus does not

**FIGURE 6.** General experimental study design and impact of dual antiplatelet treatment on infective endocarditis initiation on bovine jugular vein (BJV) tissue. Discs of various graft tissues were placed in a microparallel flow chamber, allowing perfusion of bacteria, suspended in whole blood over graft tissue surfaces in laminar flow conditions. The optimized setup enables assessment of bacterial adhesion by fluorescence microscopy and a serial dilution method to determine CFUs. Also, platelet adhesion in the flow chamber was quantitatively measured by a colorimetric assay, based on an acid phosphatase test. In the course of these studies, we uncovered that bacteria are exclusively recruited to graft cardiac tissues by adhered active platelets, bridging their activated $\alpha_\text{IIb}\beta_3$ receptor and bacterial ligands via fibrinogen. This in vitro flow chamber system, therefore, made it possible to investigate whether inhibiting platelet binding with antiaggregant agents would eradicate the platelet-assisted $S$ aureus recruitment to the graft tissue. We found that bacterial adhesion to BJV in citrate anticoagulated blood was eliminated by antiplatelet agents such as ASA and TICA, in combination. These results provide a basis for clinical studies on the use of antiplatelet drugs in prevention of $S$ aureus infections, such as infective endocarditis. ASA, Acetylsalicylic acid; TICA, ticagrelor; CFU, colony-forming unit.
promote *S. aureus* binding to coated Fg, i.e., revert the system to the “simple” binding of *S. aureus* to coated Fg. This can only be explained when perfused $\alpha_{\text{IIb}}\beta_{3}$ antagonists elicit passivation of the graft tissue, as has been described for platelet integrin $\alpha_{\text{IIb}}\beta_{3}$ antagonists, triggering passivation of prothrombotic surfaces in flowing human blood.\textsuperscript{24} We did not investigate such passivation mechanism in larger detail in the present study, but collectively, our findings substantiate that all bacterial binding in blood perfused over BJV conduit tissue and BP results from *S. aureus* recruitment onto bound and activated platelets. Platelet activation also includes microvesicle release by those platelets and their binding to prothrombotic surfaces,\textsuperscript{35} findings, which may explain why $\alpha_{\text{IIb}}\beta_{3}$-antagonized platelets and platelet-derived microvesicles reduce the Fg availability for direct *S. aureus* binding, as seen in plasma, in the absence of blood cells (2-step approach).

Two-step perfusions enabled the separation of (1) the platelet binding step and (2) the *S. aureus* recruitment step. Addition of $\alpha_{\text{IIb}}\beta_{3}$ antagonists in each step eradicated bacterial adhesion effectively. Eptifibatide in step 1 inhibits platelet binding, in turn eliminating *S. aureus* recruitment in step 2 (passivation). Eptifibatide in step 2 blocks *S. aureus* recruitment to already-bound platelets, a conclusion supported by the finding that eptifibatide in step 2 only provoked minor detachment of platelets, adhered in step 1. This strategy also confirmed that *S. aureus*

![FIGURE 7. Interactions between *S. aureus*, platelets, and bovine jugular vein grafts. This scheme summarizes the different modes of interaction uncovered in this study. Especially, cardiac bovine graft tissues comprise zones of intact endothelium and subendothelium, exposing extracellular matrix (ECM). Fibrinogen in blood can bind both to endothelial cell (EC) surface integrins as well as to subendothelial ligands. The middle and right part of scheme (A, A’) illustrate, in the (nonphysiological) absence of blood cells, how bound fibrinogen (Fg) can directly recruit *S. aureus*, partly via *S. aureus* ClfA–Fg interactions. However, in blood, circulating resting platelets adhere to Fg-coated (sub)endothelial surfaces as single platelets via integrin $\alpha_{\text{IIb}}\beta_{3}$, illustrated in the second-last right part of scheme (B). Adhering and activated platelets expose activated integrin $\alpha_{\text{IIb}}\beta_{3}$ and spread on the tissue surface. The interaction between bound platelet integrin $\alpha_{\text{IIb}}\beta_{3}$ and soluble plasma Fg then enables recruitment of bacteria to the cardiac tissue, primarily via Fg–*S. aureus* interactions, as illustrated in the lower segment of the middle part of scheme (C). Thus, presently we found that *S. aureus* initially interacts exclusively with single adherent activated platelets and that soluble plasma Fg is the bridging ligand between integrin $\alpha_{\text{IIb}}\beta_{3}$ and *S. aureus* clumping factor A (ClfA) and other adhesins, presumably fibronectin binding protein and B. To develop clinical lesions, progressive platelet clustering via $\alpha_{\text{IIb}}\beta_{3}$, soluble Fg/ (fibrin) and inclusion of *S. aureus* will generate vegetations, as further illustrated in the middle part of scheme (D), in agreement with established mechanisms.

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suspended in serum (step 2) was ineffective for bacterial binding.

Collectively, our findings indicate that Fg is important as a first ligand recruiting platelets and as a second soluble ligand, which by the binding of activated platelet α5β3 to S aureus (ClfA) facilitates IE initiation (Figure 7). We presently found that ClfA substantially mediated adhesion to BJV tissue. Fibronectin binding protein A also binds Fg20 and recently, we have shown that both interact comparably with ECs via α5β1-bound Fn and αvβ3-bound Fg.23 As serum itself could only substitute for plasma after reconstitution with Fg, Fg appears to be the major ligand involved, reducing the impact of the partial loss of Fn concentrations by 30% to 35% during plasma clotting.36

Antiplatelet or anticoagulant medications are under discussion for their potential protective effect against IE, with some centers administering preventive treatment with ASA after PPVI, yet there is no proven evidence.1 In our model, we noted that the approximate number of bacterial entities recovered from BJV grafts after perfusion oscillated around 20 to 30 CFUs per mm2, an amount that may suffice to predispose to IE onset. Since this binding could be reduced via antiplatelet agents like epifibatide and classical dual antiplatelet therapy, this study shows that pharmacologic targeting of platelets has the potential to significantly reduce bacterial attachment to BJV tissue (Figure 6).

It should be noted that the “dual- prophylaxis” strategy reduced bacterial attachment toward baseline in all our tests. Accordingly, recent results in a mouse model showed reduced biofilm growth upon ticagrelor treatment, mechanistically explained as a platelet-driven process.25 Investigations in a low-bacteremia IE rat model also showed significant protection against IE by dual antiplatelet therapy, the result of disrupted platelet–platelet interactions in those studies.18 Vegetation weight and valve infection rate were significantly diminished. Beneficial use of aspirin in humans is under debate, and several authors suggested a re-evaluation of antiaggregant prevention in IE.2,5,16,18 As a clinical study in patients with S aureus sepsis found a reduced mortality in patients under aspirin treatment, antiplatelet strategy could be of value especially for S aureus endocarditis, which accounts for up to 50% of mortality in patients with prosthetic valve IE.8,18,37 Together with previous studies, our data provide a mechanistic basis for patient studies investigating the benefit of preventive antiplatelet treatment in IE onset, especially in patients with bioprosthetic valve conduits.

In conclusion, our investigation of the “S aureus–platelet–tissue” interplay and effects of antiplatelet drugs showed that plasma Fg potentiates S aureus adhesion particularly to BJV wall tissue-bound platelets. Pharmacologic animal studies and clinical studies on existing “at-risk” cohorts will provide the required evidence for the application of antiplatelet agents in S aureus endocarditis prevention.

Conflict of Interest Statement
Dr Gewillig is a proctor for Medtronic outside the area of this work. All other authors have nothing to disclose with regard to commercial support.

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References

Key Words: infective endocarditis, platelets, fibrinogen, *S aureus*, cardiac graft tissues
APPENDIX E1. METHODS

Preparation of Graft Tissues for Experimental Work

Bovine pericardium patch (BP) and bovine jugular vein (BJV) conduit were purchased and stored according to the manufacturers’ instructions. Cryopreserved pulmonary homograft (CH) tissue was processed by the European Homograft Bank and stored in the vapor phase of liquid nitrogen before use. Handling of CH before use followed the European Homograft Bank instructions.†11 Graft tissues were prepared as described previously.†2 To summarize, both wall and valvular leaflet tissues were used from the BJV conduit and CH. Circular pieces of tissues were cut with a 6-mm disposable skin biopsy punch (Acu-Punch; Acuderm Inc, Fort Lauderdale, Fla). If necessary, disposable sterile scalpels were used to cut all tissue patches to the same height. All tissue discs were rinsed with 0.9% NaCl before use.

Depending on the experimental setting, tissues were incubated overnight at 4°C in phosphate-buffered saline (PBS; Gibco), human albumin (200 g/L; CAF-DCF, Brussels, Belgium), either alone or supplemented with fibrinogen from human plasma (Fg, 30 μg/mL; Sigma-Aldrich, St Louis, Mo), in human platelet-depleted pooled plasma (from 6 healthy donors) or in human serum (National Institutes of Health, Bethesda, Md) was used to perform an acid phosphatase assay based on a colorimetric method for the measurement of platelet adhesion at 405 nm in microtiter plates (adapted from Bellavite and colleagues†14). The method allowed us to count reliably in the range between 0.3 × 10⁶ and 2.5 × 10⁶ of adherent platelets on the surface of tissue discs. To provide a linear relationship between optical density at 405 nm and platelet number (standard curve), platelet-rich pooled plasma was serially diluted with platelet-poor plasma. To summarize, the acid phosphatases were released from platelets during a 60-minute incubation at 37°C with 150 μL of a lysis solution consisting of 0.1 M citrate buffer, pH 5.4, 5 mM p-nitrophenyl phosphate (Acros Organics; Thermo Fisher Scientific) and 0.1% Triton X-100 (Thermo Fisher Scientific) followed by the addition of 100 μL of 2 M NaOH to develop the colorimetric reaction. To test the specificity of the assay toward platelets, experiments with reconstituted blood from the concentrate of red blood cells were performed, thereby validating that enzyme activity after perfusion with reconstituted blood was negligible compared to that with whole blood.

In parallel, grafts were fixed in 4% paraformaldehyde (Sigma-Aldrich) and investigated under confocal microscopy as described to follow.

As antiplatelet treatments, drugs such as aspirin (Integrilin, 10 μg/mL; GlaxoSmithKline, Dublin, Ireland), acetylsalicylic acid (ASA, Aspecg Injectable; Sanofi

Platelet Adhesion to Graft Tissues

After overnight incubation with human plasma at 4°C, graft tissues were mounted in the microparallel plate flow chamber and perfused for 20 minutes at room temperature with citrate-anticoagulated whole blood†3 stained with 10 μg/mL Rhodamine 6G (Sigma-Aldrich), when indicated (red staining of platelets). After washing with 5 mM HEPES buffer (Sigma-Aldrich), 150 mM NaCl, pH 7.5, for 15 minutes, tissues were removed from the chamber to an exposure number of CFUs or platelets in perfusates. Additionally, for labeled bacteria, fluorescent foci were counted per image and a degree of adhesion is derived as an average count number.

Fibrinogen Binding Assay

Prepared graft tissues were placed in a 96-well microtiter plate and incubated in 200 μL of human albumin for 2 hours at 37°C, briefly rinsed with PBS, and followed by incubation with 100 μL of fluorescent human Fg (Alexa Fluor 488 conjugate; Invitrogen, Carlsbad, Calif) diluted in human albumin (30 μg/mL) for another 2 hours at 37°C. Then, specimens were washed 3 times with PBS for 5 minutes and finally each circular tissue segment was placed with its treated surface downward on a single drop of antifade mounting medium (ProLong Diamond; Invitrogen) in a 6-well plate (Nunc; Thermo Fisher Scientific, Waltham, Mass). The mounted samples were imaged using a high-throughput fluorescence scanner (InCell Analyzer 2000; GE Healthcare Life Sciences, Pittsburgh, Pa) using fluorescent isothiocyanate excitation and emission parameters. As internal controls of background fluorescence, individual tissues were also incubated with human albumin only. After image acquisition, ImageJ (National Institutes of Health, Bethesda, Md) was used to quantify absolute (auto)fluorescence units, to express the degree of Fg binding as a relative signal to the internal control, as a fold change.

In Vitro Perfusion Experiments

Identically prepared tissue discs were mounted in a microparallel plate flow chamber (in-house design) based on a previously developed system.†5 Taking into consideration the channel height across all tissue patches, the distance between the mounted graft and the medium inlet (the entrance length) and the Reynolds number, our setup guaranteed fully developed laminar flow. Graft tissues were perfused at room temperature with whole blood, bacterial suspensions, or combinations of both, as specified, using a high-accuracy multi-syringe pump (PHD 2000 Programmable; Harvard Apparatus, Cambridge, Mass) at a flow rate of 1000 s⁻¹. Obtained results are expressed as a percentage of adhesion of the total amount of S aureus or platelets perfused over a standard interval of respectively 10 and 15 or 20 minutes, corresponding to a ratio of adherent bacterial CFUs (colony-forming units) or platelets to an exposure number of CFUs or platelets in perfusates.

Preparation of Fibrinogen from Human Plasma (Fg, 30 g/L; Sigma-Aldrich) and investigated under confocal microscopy as described to follow.
Belgium, Machelen, Belgium) and ticagrelor (TICA, Cayman Chemical Co, Ann Arbor, Mich) were added to whole blood before perfusion over the tissues. Concentrations of ASA (100 µg/mL) and TICA (2 µg/mL) were selected, based on clinically documented serum salicylate peak concentrations in previous studies.\textsuperscript{E5} In addition, these concentrations were double checked for their inhibitory effects on platelet aggregation in a Chronolog aggregometer using arachidonic acid and ADP as platelet agonists.

Blood was donated by healthy subjects, upon informed consent, who were not receiving any antiplatelet or anticoagulant agents in the previous 10 days.

**Serum Generation From Human Pooled Plasma**

Pooled human plasma was treated with human alpha-thrombin native protein (Thermo Fisher Scientific) at a final concentration of 10 U/mL for 3 hours at 37°C. From time to time during the incubation, the clot was agitated. Subsequently, the clot was discarded whereas the serum was placed at 4°C for overnight slow residual depletion of Fg. The next day, serum was centrifuged for 15 minutes at 14,200 x g to remove platelets, and passed through a 0.45-µm filter. Prepared serum was verified for residual Fg content, using a sandwich enzyme-linked immunosorbent assay, then aliquoted and stored at –20°C.

**Microscopic Investigation (Electron and Fluorescence Microscopy)**

Samples were prepared for scanning electron microscopy by repeated osmication to increase their conductance. To summarize, tissue pieces were fixed by immersion in fixation buffer containing 4% paraformaldehyde, 2.5% glutaraldehyde (Sigma-Aldrich), and 0.2% picric acid in 0.1 M Na-cacodylate buffer pH 7.2 overnight at 4°C. Then, after incubation for 30 minutes in 0.1% tannic acid (Centrifuge 5424 R; Eppendorf, Hauppauge, NY), carefully transferred to a new tube, and passed through a 0.45-µm filter. Samples were prepared for scanning electron microscopy by repeated osmication to increase their conductance. To summarize, tissue pieces were fixed by immersion in fixation buffer containing 4% paraformaldehyde, 2.5% glutaraldehyde (Sigma-Aldrich), and 0.2% picric acid in 0.1 M Na-cacodylate buffer pH 7.2 overnight at 4°C. Then, after incubation for 30 minutes in 0.1% tannic acid, the samples were again exposed to 1% osmium tetroxide for 30 minutes in the dark at room temperature. This step was repeated after an intermittent incubation in 1% tetracarboxyhydradiazide for 20 minutes. All of the osmication steps were in H2O with intermittent washing in H2O. Then, samples were dehydrated in a graded ethanol series and finally critical point dried in 100% dried ethanol in a CPD300 critical point dryer (Leica Microsystems, Wetzlar, Germany). Finally, they were mounted on pin stubs, coated with 8 nm of chromium in a Leica ACE600 coating unit, and imaged in a Zeiss Sigma scanning electron microscope operated at 5 kV.

For fluorescence microscopy of labeled bacteria and platelets, tissue pieces were washed in PBS and fixed in 4% paraformaldehyde for 10 minutes at room temperature after perfusions. After 3× washing in PBS, the samples were mounted between 2 microscope coverslips (24 × 50 mm; VWR International, Radnor, Pa) using droplets of antifade mountant. Finally, the specimens were imaged using a confocal microscope (Zeiss) after necessary parameters of excitation and emission wavelengths have been preset.

**RESULTS**

Based on the fluorescent detection of bound *S aureus*, image analysis in Figure E1 confirms data in the main manuscript that show that Fg from plasma coated (immobilized) onto the BP surface triggers bacterial adhesion to a larger extent than albumin coating.

Clumping factor A (ClfA) is a major bacterial ligand mediating binding of *S aureus* to Fg, which we studied before as a determinant of bacterial interactions with (sub)endothelium.\textsuperscript{E6} We therefore studied whether the *S aureus* Newman strain equally adheres to the BP conduit. Since the main manuscript shows that Fg is the only ligand involved, the Newman strain, which lacks the receptor for fibronectin (FnBPA)\textsuperscript{E7} could be adequately selected to study the role of ClfA, in view of the availability of a ClfA negative mutant, ΔclfA. Figure E2 shows the binding of *Newman S aureus*, in comparison with that of ΔclfA, which represents the clfA deletion mutant.

Plots of *S aureus* adhesion versus platelet adhesion show that the experimental tissues examined in the present study can be divided in 2 groups. The first group (BP and BJV\textsubscript{wall}) shows a high degree of interaction both with platelets and *S aureus*. The second group (CH\textsubscript{wall}, CH\textsubscript{leaflet} and BJV\textsubscript{leaflet}) hardly any affinity for *S aureus* or for platelets (Figure E3).

The main manuscript (Figure 4) shows perfusions over plasma precoated BJV\textsubscript{wall} where *S. aureus* binds 2-fold more weakly to the graft tissue when suspended in whole blood (presence of platelets), compared with suspension in plasma (absence of platelets). To better understand the platelet–bacterial interactions in blood, we have additionally performed 2-step perfusions. Figure E4 presents bacterial adhesions to BIV in plasma. Step 1 consisted of platelet preloading on the tissue surface by perfusing whole blood in the presence or absence of the e\textsubscript{H3}δ\textsubscript{3} antagonist eptifibatide (10 µg/mL) over plasma precoated BJV; step 2 constitutes a perfusion step with *S aureus* (10⁶ CFUs/mL) suspended in plasma. This “inhibited-platelet” preperfusion step resulted in even lower *S aureus* adhesion (step 2) as in Figure 4, A, of the main manuscript, indicative of occurrence of a “passivation process” induced by antagonized platelets (as discussed in the main manuscript), repelling bacteria from the platelet-free tissue surface.
In view of the strong inhibition of bacterial binding in Figure 5 of the main manuscript, we have double-checked that the combination ASA/TICA did execute its effects only because of interference with platelet function. Therefore, we studied adhesion of *S. aureus* to plasma-coated BJV tissue in the absence and presence of ASA/TICA at the same concentrations tested in Figure 5 of the main manuscript. These perfusions did not reveal any inhibitory effect of acetylsalicylic acid and ticagrelor on *S. aureus* viability itself.

E-References


**FIGURE E1.** Adhesion of fluorescently labeled *S. aureus* (Texas Red) to BP surface. Microparallel plate flow chamber perfusion of bacteria for 10 minutes over bovine pericardium patch (BP). (A) Microscopic illustration of *S. aureus* (red) on the surface of BP precoated with plasma O/N. (B) Degree of bacterial adhesion to BP treated O/N with albumin or plasma, based on red fluorescence intensity measurements. Background of the tissue is visible in green. Adhesion levels are presented as an Average Count Number of red foci per fluorescent image (ACN, n ≥ 6). Data are presented as box and whiskers dot plots with the upper and lower borders of the box representing the 25% and 75% percentile (upper and lower quartiles). The middle horizontal line represents the median, the upper and lower whiskers the maximum and minimum values of nonoutliers. T test with nonparametric Mann–Whitney *U* test was done, *P < .05.*

**FIGURE E2.** Adherence of Newman *S. aureus* versus clfA mutant strain to BJV. Plasma precoated BJV wall was perfused for 10 minutes with suspensions of 10⁷ CFU/mL of bacteria in medium (PBS) at a flow rate of 1000 s⁻¹ (n > 6). Adhesion of Newman *S. aureus* and ΔclfA were evaluated as the percentage of adherent CFUs to the total dose of CFUs over the perfusion interval. Data are presented as box and whisker dot plots with the upper and lower borders of the box representing the 25% and 75% percentile (upper and lower quartiles). The middle horizontal line represents the median, the upper and lower whiskers the maximum and minimum values of nonoutliers. T test for the difference in binding was applied with non-parametric Mann–Whitney *U* test: *P < .05.* BJV, Bovine jugular vein tissue; clfA, clumping factor A.

**FIGURE E3.** Relationship between tissue origin and affinity distribution for *S. aureus* and platelets. Distribution in a high-affinity (BP and BJV wall) and a low-affinity tissues group (CH wall, CH leaflet, and BJV leaflet) delineated by dotted line boxes, based on their degree of interaction with *S. aureus* and platelets, perfused over their surface, after O/N precoating with plasma, at a flow rate of 1000 s⁻¹ for 10 or 20 minutes, respectively. Data are presented as dot plots, representing the median values and 25-75 percentiles for both types of binding, respectively. BP, Bovine pericardial tissue; BJV, bovine jugular vein tissue; CH, cryopreserved homograft tissue; wall and leaflet, respective parts of the valve.
FIGURE E4. Effect of epifibatide (EPTI) on bacterial adhesion to BJV in 2-step perfusion. Plasma precoated BJV was perfused for 20 minutes with whole blood (step 1), followed by the washing with PBS and 10-minute perfusions with suspensions of $10^6$ CFUs/mL of bacteria in plasma (step 2) at a flow rate of 1000 $\text{s}^{-1}$ ($n \geq 6$). Where indicated, EPTI was added to step 1 at a concentration of 10 $\mu$g/mL. Adhesion of \textit{S. aureus} was evaluated as the percentage adherent CFUs of the total dose of CFUs over the perfusion interval. Data are presented as box and whisker dot plots with the upper and lower borders of the box representing the 25% and 75% percentile (upper and lower quartiles). The middle horizontal line represents the median, the upper and lower whiskers the maximum and minimum values of non-outliers. \textit{T} test for the difference in binding was used with nonparametric Mann–Whitney \textit{U} test: **** $p < .0001$. \textit{BJV}, Bovine jugular vein tissue; \textit{EPTI}, epifibatide.

FIGURE E5. Adhesion of \textit{S aureus} to BJV tissue in the presence of antiplatelet agents. Plasma-precoated BJV tissue was perfused with \textit{S aureus} ($10^7$CFUs/mL) for 10 minutes at a flow rate of 1000 $\text{s}^{-1}$ ($n = 5$), suspended in plasma in the presence and absence of TICA and ASA, at the indicated concentrations. Data are presented as box and whisker dot plots with the upper and lower borders of the box representing the 25% and 75% percentile (upper and lower quartiles). The middle horizontal line represents the median, the upper and lower whiskers the maximum and minimum values of non-outliers. \textit{T} test with nonparametric Mann–Whitney \textit{U} test was applied. \textit{BJV}, Bovine jugular vein tissue; \textit{ASA}, acetylsalicylic acid; \textit{TICA}, ticagrelor; \textit{ns}, nonsignificant.
Antiplatelet therapy abrogates platelet-assisted *Staphylococcus aureus* infectivity of biological heart valve conduits

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In the onset of infective endocarditis on heart valve conduits, single platelets adhere to biological heart valve conduit tissues upon fibrinogen coating and recruit bacteria via platelet integrin $\alpha_{\text{IIb}}\beta_3$ and plasma fibrinogen.