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

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

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44 **Abbreviations**

45	ASA	acetylsalicylic acid
46	BJV	bovine jugular vein
47	BP	bovine pericardium patch
48	CH	cryopreserved pulmonary homograft
49	ClfA	clumping factor A
50	CFU	colony-forming unit
51	EC	endothelial cell
52	Fnbp	fibronectin binding protein
53	Fg	fibrinogen
54	Fn	fibronectin
55	IE	infective endocarditis
56	O/N	overnight
57	PBS	phosphate-buffered saline
58	PPVI	percutaneous pulmonary valve implantation
59	<i>S. aureus</i>	<i>Staphylococcus aureus</i>
60	SEM	Scanning electronic microscopy
61	RVOT	right ventricular outflow tract
62	VWF	von Willebrand factor
63	vWbp	VWF-binding protein

Abstract

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 grafts used for valve replacement. Thus, adherence of *Staphylococcus aureus* (*S. aureus*) and platelets to 5 graft tissues were studied quantitatively in perfusion chambers, assisted by microscopic analysis. We also evaluated standard anti-platelet therapy to prevent onset of *S. aureus* endocarditis.

Results: Of all tissues, bovine pericardium (BP) showed the highest fibrinogen binding. Perfusion of all plasma-precoated tissues identified BP and BJV_{wall} with the highest affinity for *S. aureus*. Perfusions of anticoagulated human blood over all tissues also triggered more platelet adhesion to BP and BJV_{wall}, 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 $\alpha_{IIb}\beta_3$ and coated or soluble fibrinogen, respectively, interactions abrogated by the $\alpha_{IIb}\beta_3$ -antagonist eptifibatide. Also, standard anti-platelet 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 $\alpha_{IIb}\beta_3$. *S. aureus* then attaches from blood, to (activated) bound platelet $\alpha_{IIb}\beta_3$ via plasma fibrinogen. Dual anti-platelet therapy appears a realistic approach to prevent endocarditis and its associated mortality.

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 $\alpha_{IIb}\beta_3$ and plasma fibrinogen.

Perspective Statement:

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

1. Introduction

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) conduit³. Recent studies focus on the increased risk of IE after PPVI using a BJV conduit, a risk as high as 2.6 % per patient year¹, suggesting it to be higher 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 *S. 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 bacterium from host defense pathways⁸.

Staphylococcal clumping factor A (ClfA) triggers bacterial adhesion to endothelial cells (ECs) *in vitro* and inflammation in animal models⁹⁻¹¹. Activated ECs release von Willebrand factor (VWF), to which *S. aureus* adheres via the VWF-binding protein (vWbp) in shear stress conditions^{10, 12}. Histological observations have revealed that fibrin deposition occurs on these leaflets even in the absence of infection¹³. As *S. aureus* has a strong affinity for fibrin and fibrinogen (Fg) via ClfA¹⁴, 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* binding¹⁵.

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 (Fnbp) A^{10, 12}, interactions inhibited by $\alpha_{IIb}\beta_3$ antagonists¹⁸. 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 haemostatic and infectious complications¹⁹.

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 PPVI¹. So far, its potential in IE prevention remains unclear.

In the present study, we have, therefore, investigated *in vitro* bacterial adhesiveness, 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 pharmacological anti-platelet intervention strategies were investigated for their efficacy to reduce the incidence of *S. aureus* endocarditis.

2. Methods

2.1 Bacterial strains

S. aureus strains 8325-4 and Newman (wild type and *clfA*-deletion mutant clinical strains)²⁰ were cultured at 37 °C on Mueller-Hinton blood agar and in tryptic soy broth (TSB)

liquid medium. For the mutant strain, 5 µg/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.

2.2 Graft tissues

Bovine pericardium patch (BP; Supple Peri-Guard®; Synovis Surgical Innovations, St. Paul, Minnesota, USA), cryopreserved pulmonary homograft (CH; human origin, European Homograft Bank, Brussels, Belgium) and bovine jugular vein conduit (BJV; Contegra®, Medtronic, Minneapolis, USA) were used to investigate bacterial and platelet adhesion under perfusion. Detailed graft processing is specified in the supplement. To avoid artefactual binding of bacteria and blood constituents to cut graft borders, only the central (native) graft surfaces were exposed to perfusate in appropriately manufactured perfusion chambers²¹. Before studying bacterial adhesion, graft tissues were investigated for Fg binding, which is described in detail in the supplement.

2.3 Bacterial adhesion to graft tissues

After various overnight (O/N) coatings in different conditions, tissues were perfused at a shear rate of 1,000 s⁻¹ for 10 min at RT with bacterial suspensions of 10⁶-10⁷ 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 detail²¹ and in the Supplement. Briefly, after perfusion, tissues were washed in PBS under flow, transferred to a tube containing 1 mL of 0.9 % NaCl and sonicated for 10 min using a sonication bath (VWR Ultrasonic Cleaner; VWR, Radnor, Pennsylvania). Next, bacterial suspensions were serially diluted

and spread on Mueller-Hinton blood agar plates and incubated O/N at 37 ° C to count colony forming units (CFUs).

For imaging purposes, bacteria (10^6 CFU/mL) were labeled with 5(6)-carboxy-fluorescein N-hydroxy-succinimidyl ester (30 µg/mL, Sigma-Aldrich) ²¹, mixed with whole blood (containing Rhodamine 6G stained platelets) and perfused for 15 min 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 h 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 Scientific) for 10 min and the co-presence of Fg and bacteria was visualized as mentioned above.

Where indicated, anti-platelet drugs such as eptifibatide, acetylsalicylic acid (ASA) and ticagrelor were added to the mixture of whole blood and bacteria (10^6 CFU/mL). Perfusions were performed for 15 min 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 the Data Supplement.

In stepwise experiments, graft tissues (precoated with plasma) were preperfused with whole blood (stained for platelets or not) for 20 min, followed by washing with plasma or PBS (15 min) and were finally perfused with suspensions of 10^6 CFU/mL in PBS, plasma or serum for 10 min (detailed protocol of serum preparation in the Supplement). When indicated, the $\alpha_{IIb}\beta_3$ 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 above, 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.

2.4 Statistical analysis

Statistical analysis revealed that not all data followed a Gaussian distribution and therefore non-parametric analyses were performed. Statistical significance between groups was analyzed by using the T-test with the non-parametric Mann-Whitney U-test. Data analyses were performed with GraphPad Prism 8.0d (GraphPad Software, San Diego California USA). Values are reported as median and distribution plots, via 25-75% percentile boxes with respective min and max binding whiskers. P -values < 0.05 were considered significant.

3. Results

3.1 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 CH_{wall} (median near 1.0, Fig. 1), whereas BP showed the highest apparent affinity (median around 1.9; $p < 0.001$ compared to CH wall). BJV_{leaflet}, BJV_{wall} and CH_{leaflet} displayed intermediate values, the latter two characterized by a wide spread (Fig. 1).

3.2 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 CH_{wall} recruited 7 to 10-fold less bacteria than the bovine grafts, BJV_{wall} and BP, respectively (Fig. 2A). 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, higher than that achieved after albumin or serum coating and in general comparable to that shown in Fig. 2A for BP (Fig. 2B). Similar results were found for bacterial adhesion experiments on BJV_{wall} (Fig. 2C), i.e. 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 BJV_{wall} (Fig. 2C) and BP (Fig. 2B). 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 (Fig. S1). In further support, microscopic analysis of labeled *S. aureus* (red) and Fg (green) showed partial co-localization of both labels (Fig. 2D). Or, higher Fg-binding, especially to BP and BJV_{wall} predisposes to *S. aureus* recruitment on coated Fg.

As *S. aureus* ClfA is a bacterial adhesin with affinity for Fg²², we investigated the adherence of a deletion mutant of Newman *S. aureus*, $\Delta clfA$, to the BJV conduit. The $\Delta clfA$ bound substantially less to BJV_{wall} by about 42 % (Supplementary Fig. S2). Hence, this finding confirmed that ClfA participates in *S. aureus* adhesion to plasma-coated grafts.

3.3 Different reactivity of platelets with various graft tissues via coated Fg-integrin $\alpha_{IIb}\beta_3$

Prior to 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 higher to BJV_{wall} and BP than to CH_{wall}, CH_{leaflet} and BJV_{leaflet}, reaching approximately 0.5 % bound platelets (Fig. 3A). Upon whole blood perfusion, scanning electron microscopy (SEM) of BJV_{wall} conduit showed single adhered platelets, spread and with an activated morphology (marked pseudopods), rather than platelet aggregates (Fig. 3B). In addition, platelet adhesion was noted to both the surface of ECs (Fig. 3B, left panel) and the sub-endothelial matrix (Fig. 3B, right panel). Interestingly, addition of the integrin $\alpha_{IIb}\beta_3$ antagonist eptifibatide abrogated platelet adherence to BJV_{wall} by > 80 % (Fig. 3C). Despite low thrombogenicity (perfusion over 20 min), we conclude, that BJV_{wall} 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 BJV tissue to result from platelet $\alpha_{IIb}\beta_3$ 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 BJV_{wall} and BP constitute a group of 2 reactive tissues, compared to the group of CH_{wall}, CH_{leaflet} and BJV_{leaflet}, with low reactivity. High *S. aureus* binding parallels high platelet binding, presumably to the same ligand, i.e. coated Fg (Supplementary Fig. S3). Therefore, the remainder of the work was done with BJV_{wall} and BP exclusively.

3.4 Impact of platelet-graft tissue interactions on *S. aureus* adhesion

In comparison to perfusions over BJV_{wall} and BP with *S. aureus* suspended in PBS (Fig. 2A-C) or in plasma (Fig. 4A, first bar) with comparable levels of bacterial adhesion, when *S. aureus* was suspended in blood directly, bacterial adhesion to BJV_{wall} appeared to be significantly smaller (Fig. 4A, second bar), with a degree of *S. aureus* binding around 0.1 % of the total amount of perfused bacteria. Interestingly, inclusion of eptifibatide (10 μ g/mL) to the perfused blood eradicated both platelet adhesion (Fig. 3C) and attachment of *S. aureus* by about 52 % (Fig. 4A, striped bar), down to residual levels of 26 % adhesion

compared to plasma and 48 % adhesion compared to 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 ClfA and coated Fg, we “separated” the critical participants in blood and perfused them sequentially. Thus, Fig. 4B shows, that when blood is first perfused over plasma-precoated BJV_{wall}, *S. aureus* will bind to the same degree to deposited platelets as in Fig. 4A (second red bar), but only when suspended in plasma and not in PBS (Fig. 4B, 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 BJV_{wall} conduit, after having perfused blood over plasma-precoated tissue already. Fig. 4C shows that in this case no *S. aureus* bound (Fig. 4C, 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 two-step perfusions) binds to activated adhered platelet $\alpha_{IIb}\beta_3$ via Fg (Fig. 4A-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 (Supplementary Fig. S4).

Separate perfusions over plasma-coated BJV_{wall} in which bacteria were suspended in plasma vs. PBS (Fig. 4A green 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 findings²³.

Together, these results show that, in case *S. aureus* is perfused in blood over BJV_{wall} 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 process²⁴.

Fg is the common ligand, mediating both platelet deposition on BJV_{wall} or BP and enabling *S. aureus* binding to platelets in the next step.

3.5 Aspirin and ticagrelor reduce bacterial adhesion to graft tissues

Fig. 5A shows how platelets spread on graft tissues and how they recruit bacteria in a platelet-controlled manner.

Because eptifibatide reduced bacterial attachment (Fig. 4), we have also tested *in vitro* whether current anti-aggregant agents, i.e. as used in dual anti-platelet 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 Fig. 5B. ASA (100 µg/mL) alone significantly reduced bacterial attachment by nearly 55 % versus the control blood condition - similar to the effect of eptifibatide (Fig. 4A). 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 (Supplementary Fig. S5).

Bacterial adhesion to BJV_{wall} in plasma in the presence and absence of the ASA / ticagrelor combination used in Fig. 5 showed similar *S. aureus* binding to coated Fg (Supplementary Fig. S5), confirming the effect of pharmacological platelet inhibition on *S. aureus* recruitment.

4. Discussion

IE after PPVI remains a severe complication and the risk to acquire IE seems to be higher after application of heterologous BJV conduits compared to 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 adhesion to cardiac graft tissues. Therefore, the importance of interactions between bacteria and platelets for adhesion were investigated in blood perfusion experiments under shear stress. Bacterial adhesion to BJV in blood could be significantly reduced by anti-platelet agents (Fig. 6, graphical abstract), 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 $\alpha_{IIb}\beta_3$. Single adhered and activated platelets were visualized by SEM. 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 $\alpha_{IIb}\beta_3$ and *S. aureus* suspended in blood. Standard dual anti-platelet treatment potently blocks bacterial adhesion, providing evidence that this strategy may be beneficial in IE prevention.

Despite tissue processing prior to 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_{wall} was more prone for platelet binding than BJV_{leaflet}, in agreement with the knowledge that valvular (leaflet part) ECs are less reactive in inflammatory adhesion, in part because of lower expression of von Willebrand factor and

a higher expression of thrombomodulin, in turn promoting the endothelial anticoagulant system, contributing to an anti-inflammatory state via protein C activation²⁹. Some of these properties can be expected to be preserved to some degree, even in fixed tissues.

“Simple” perfusions, i.e. those where *S. aureus*, suspended in plasma, serum or PBS, were performed over BJV_{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_{wall} or BP were precoated with serum and was restored by the supplementation of serum with Fg. Also, platelet binding occurred to coated Fg and the $\alpha_{IIb}\beta_3$ antagonist eptifibatide 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 $\alpha_{IIb}\beta_3$ antagonist, single platelet deposition is observed to ligands as VWF primarily, but also to fibronectin (Fn) and laminin³⁰. 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 $\alpha_V\beta_3$ and $\alpha_5\beta_1$ 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 tissues³¹. Platelet glycoprotein VI (GPVI) has been reported to bind to fibrin and Fg^{32, 33}, but in view of the strong inhibition of platelet binding by eptifibatide, a role for GPVI appears highly unlikely, including binding to subendothelial matrix collagen (Fig. 3). Platelet adhesion to surface-bound Fg is followed by platelet activation and spreading via $\alpha_{IIb}\beta_3$ ³⁴, presently also found for BJV and BP-bound platelets, a process interrupted by the $\alpha_{IIb}\beta_3$ antagonist eptifibatide.

When *S. aureus* is directly suspended in whole blood, subsequent perfusions over BP or BJV_{wall} 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 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 $\alpha_{IIb}\beta_3$ antagonists elicits passivation of the graft tissue, as has been described for platelet integrin $\alpha_{IIb}\beta_3$ antagonists, triggering passivation of prothrombotic surfaces in flowing human blood²⁴. 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 surfaces³⁵, findings which may explain why $\alpha_{IIb}\beta_3$ -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 (two-step approach).

Two-step perfusions enabled the separation of 1. the platelet binding step and 2. the *S. aureus* recruitment step. Addition of $\alpha_{IIb}\beta_3$ 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* 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 $\alpha_{IIb}\beta_3$ to *S. aureus* (ClfA) facilitates IE initiation (Fig. 7, central picture). We presently found that ClfA substantially mediated adhesion to BJV tissue. FnbpA also binds Fg²⁰ and recently,

we have shown that both interact comparably with ECs via $\alpha_5\beta_1$ -bound Fn and $\alpha_v\beta_3$ -bound Fg²³. 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 30-35 % during plasma clotting³⁶.

Anti-platelet or anticoagulant medications are under discussion for their potential protective effect against IE, some centers giving preventive treatment with ASA after PPVI, yet there is no proven evidence¹. In our model, we noted that the approximate number of bacterial entities recovered from BJV grafts after perfusion oscillated around 20 – 30 CFUs per mm², an amount which may suffice to predispose to IE onset. Since this binding could be reduced via anti-platelet agents like eptifibatide and classical dual anti-platelet therapy, this study shows that pharmacological targeting of platelets has the potential to significantly reduce bacterial attachment to BJV tissue (Fig. 6, graphical abstract).

It should be noted that the `dual prophylaxis` strategy reduced bacterial attachment towards 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 process²⁵. Investigations in a low-bacteremia IE rat model also showed significant protection against IE by dual anti-platelet therapy, the result of disrupted platelet-platelet interactions in those studies¹⁸. 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 anti-aggregant prevention in IE^{2, 18, 37}. As a clinical study in *S. aureus* sepsis patients found a reduced mortality in patients under aspirin treatment, anti-platelet strategy could be of value especially for *S. aureus* endocarditis, which accounts for up to 50 % of mortality in patients with prosthetic valve IE^{18, 8, 37}. Together with previous studies, our data provide a mechanistic basis for patient studies

investigating the benefit of preventive anti-platelet 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 anti-platelet drugs showed that plasma Fg potentiates *S. aureus* adhesion particularly to BJV_{wall} tissue-bound platelets. Pharmacological animal studies and clinical studies on existing “at-risk” cohorts will provide the required evidence for the application of anti-platelet agents in *S. aureus* endocarditis prevention.

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Authors' Contributions

MH, RH, BD, TRV, MBD, FR, and PM designed the research. BD, TRV and MBD performed the experiments and analyzed the data. BD, MBD, PM, PB, MH and RH interpreted the data. RJ provided cryopreserved homografts and PB, PM and FR provided other materials and resources. PB performed SEM imaging and evaluation. MBD, BD, MH and RH wrote the manuscript. All authors revised and approved the final manuscript.

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Legends to the figures

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: $n \geq 6$), bovine pericardium patch, BP ($n > 6$) graft tissues with fluorescently labeled Fg ($30 \mu\text{g/mL}$). 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 non-outliers. T-test with non-parametric Mann-Whitney U-test was applied: $** = p < 0.01$, $*** = p < 0.001$.

Figure 2. Adhesion of *S. aureus* to cardiovascular grafts under shear stress.

Micro-parallel plate flow chamber perfusion for 10 min over (A) all graft tissues, O/N precoated with hPlasma ($n \geq 6$); (B) bovine pericardium (BP) and (C) bovine jugular vein (BJV_{wall}), O/N precoated as indicated with fibrinogen (Fg) ($30 \mu\text{g/mL}$, $n > 6$), hAlbumin (200 mg/mL , $n > 6$), hPlasma ($n > 6$), hSerum ($n = 5$; $n > 6$), hSerum plus Fg ($300 \mu\text{g/mL}$), also containing dabigatran ($1 \mu\text{M}$), $n > 6$); (D) or fluorescently labeled Fg with suspensions of 10^7 CFU/mL of (A-C) unlabeled or (D) labeled *S. aureus* (Texas Red) in PBS at a shear rate of $1,000 \text{ s}^{-1}$. (A-C) Adhesion of *S. aureus* was evaluated as the percentage of adherent CFUs to the total dose of CFUs over the perfusion interval. (D) Representative images show co-staining of *S. aureus* (Texas Red) and Fg (AF-488) on the surface of BP, illustrating partial colocalization of labels. 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 non-outliers. T-test with non-parametric Mann-Whitney U-test was applied: * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Figure 3. Platelet attachment to graft tissues in perfused blood. Citrate anticoagulated blood perfusion over (A, C) all plasma O/N precoated graft tissues in a micro-parallel plate flow chamber for 20 min, at a shear rate of $1,000 \text{ s}^{-1}$ ($n \geq 6$). SEM images of (B) bovine jugular vein (BJV) showing single platelets on the surface of endothelial cells (left panel) and on subendothelial areas (right panel); white arrow indicates a spread platelet with marked protrusions. Effect of (C) the anti-platelet drug eptifibatide ($10 \mu\text{g}/\text{mL}$) on platelet adhesion to BJV. Adhesion of platelets was calculated as the percentage of adherent platelets (determined by an acid phosphatase assay) to the total dose of platelets 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 non-outliers. T-test with nonparametric Mann-Whitney U-test was applied: ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$.

Figure 4. Interaction analysis between platelets and bacteria on graft tissue surfaces.

Micro-parallel plate flow chamber perfusions over plasma O/N precoated bovine jugular vein (BJV_{wall}) with $10^6 \text{ CFU}/\text{mL}$ of *S. aureus*, suspended (A) in hPlasma, or in whole blood without or with eptifibatide ($10 \mu\text{g}/\text{mL}$) added, as indicated by the legends inside the figure, for 15 min. Two-step perfusions (B, C), where *step 1* consisted of a pre-perfusion

with whole blood or PBS for 20 min, as indicated below the x-axis, followed by a perfusion step 2 for 10 min of 10^6 CFU/mL of *S. aureus* in PBS, hPlasma or hSerum at a flow rate of $1,000\text{ s}^{-1}$ ($n > 6$), as indicated by the legends inside the figure. Eptifibatide ($10\text{ }\mu\text{g/mL}$) 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 non-outliers. T-test with non-parametric Mann-Whitney U-test was applied: $*$ = $p < 0.05$, $**$ = $p < 0.01$, $***$ = $p < 0.001$, $****$ = $p < 0.0001$.

Figure 5. Inhibition of bacterial adherence to BJV by anti-platelet treatment.

(A) Microscopic images of platelet and *S. aureus* adhesion to plasma O/N precoated bovine jugular vein tissue (BJV_{wall}), after perfusion of 10^6 CFU/mL *S. aureus*, suspended in whole citrate-anticoagulated blood, in a micro-parallel plate flow chamber for 15 min at a shear rate of $1,000\text{ s}^{-1}$. (A) upper: representative SEM micrography of BJV reveals a single activated platelet (asterisk) with attached *S. aureus* (arrows) on its surface; lower: fluorescent micrography showing a single activated platelet (red, Texas Red) surrounded by 5 individual bacteria (green, carboxyfluorescein labeling). (B) Bacterial adhesion after perfusion of 10^6 CFU/mL *S. aureus*, suspended in whole citrate-anticoagulated blood over plasma O/N precoated BJV at a shear rate of $1,000\text{ s}^{-1}$ ($n \geq 6$). Where indicated, ASA ($100\text{ }\mu\text{g/mL}$) and ticagrelor (TICA, $2\text{ }\mu\text{g/mL}$) were added to the perfusates, alone or in combination, as indicated. Adhesion of *S. aureus* was evaluated as the percentage of adherent CFUs, relative 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 non-outliers. T-test with non-parametric Mann-Whitney U-test was applied: $* = p < 0.05$, $** = p < 0.01$, $*** = p < 0.001$.

Figure 6 (Graphical Abstract). General experimental study design and impact of dual anti-platelet treatment on infective endocarditis initiation.

Discs of various graft tissues were placed in a micro-parallel 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 colony forming units (CFU counting). 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 $\alpha_{IIb}\beta_3$ receptor and bacterial ligands via fibrinogen. This *in vitro* flow chamber system, therefore, made it possible to investigate if inhibiting platelet binding with anti-aggregant agents would eradicate the platelet-assisted *S. aureus* recruitment to the graft tissue. We found that bacterial adhesion to bovine jugular vein (BJV) in citrate anticoagulated blood was eliminated by anti-platelet agents such as acetylsalicylic acid (ASA) and ticagrelor (TICA), in combination. These results provide a basis for clinical studies on the use of anti-platelet drugs in prevention of *S. aureus* infections, such as infective endocarditis.

Figure 7 (Central Picture). 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 surface integrins as well as to subendothelial ligands. The middle and right part of the scheme (A, A') illustrate, in the (non-physiological) 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 $\alpha_{IIb}\beta_3$, illustrated in the second-last right part of the scheme (B). Adhering and activated platelets expose activated integrin $\alpha_{IIb}\beta_3$ and spread on the tissue surface. The interaction between bound platelet integrin $\alpha_{IIb}\beta_3$ 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 the 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 $\alpha_{IIb}\beta_3$ and *S. aureus* ClfA and other adhesins, presumably FnbpA and B. To develop clinical lesions, progressive platelet clustering via $\alpha_{IIb}\beta_3$, soluble Fg/(fibrin) and inclusion of *S. aureus* will generate vegetations, as further illustrated in the middle part of the scheme (D), in agreement with established mechanisms.

Video. Overview of the importance and relevance of the study together with an associated operative procedure.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5**A** **B**

Infective endocarditis

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SUPPLEMENTARY DATA

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

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METHODS

Preparation of graft tissues for experimental work

BP and BJV conduit were purchased and stored according to the manufacturers' instructions. CH tissue was processed by the EHB and stored in the vapor phase of liquid nitrogen, prior to use. Handling of CH prior to use followed the EHB instructions¹. Graft tissues were prepared as described before². Briefly, 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, USA). 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 prior to use.

Depending on the experimental setting, tissues were incubated overnight at 4 °C in Phosphate-buffered saline (PBS, Gibco), human albumin (200 g/L, C.A.F.-D.C.F., Brussels), either alone or supplemented with fibrinogen from human plasma (Fg, 30 µg/mL, Sigma-Aldrich), in human platelet-depleted pooled plasma (from 6 healthy donors) or in human serum (protocol of serum preparation below).

Fibrinogen binding assay

Prepared graft tissues were placed in a 96-well microtiter plate and incubated in 200 µL of human albumin for 2 h at 37 °C, briefly rinsed with PBS and followed by incubation with 100 µL of fluorescent human Fg (Alexa Fluor 488 conjugate, Invitrogen) diluted in human albumin (30 µg/mL) for another 2 h at 37 °C. Then, specimens were washed 3 times with PBS for 5 min 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 (Thermo Scientific Nunc). The mounted samples were imaged using a high-throughput

fluorescence scanner (InCell Analyzer 2000, GE Healthcare Life Sciences, Pittsburgh) using FITC excitation and emission parameters. As internal controls of background fluorescence, individual tissues were also incubated with human albumin only. After image acquisition, ImageJ 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 micro-parallel plate flow chamber (in-house design) based on a previously developed system ². 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 (RT) with whole blood, bacterial suspensions or combinations of both, as specified, using a high accuracy multi syringe pump (PHD 2000 Programmable, Harvard Apparatus, Cambridge, Massachusetts, United States) at a flow rate of $1,000 \text{ s}^{-1}$. 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 min, corresponding to a ratio of adherent bacterial CFUs (colony forming units) or platelets 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 (ACN).

Platelet adhesion to graft tissues

After overnight incubation with human plasma at 4°C , graft tissues were mounted in

the micro-parallel plate flow chamber and perfused for 20 min at RT 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 min, tissues were removed from the chamber 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 ⁴). The method allowed us to count reliably in the range between 0.3×10^6 and 2.5×10^6 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. Briefly, the acid phosphatases were released from platelets during 60-min 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 Scientific) and 0.1 % Triton X-100 (Thermo Scientific) followed by the addition of 100 µL of 2 M NaOH to develop the colorimetric reaction. To test the specificity of the assay towards 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 below.

As anti-platelet treatments, drugs such as eptifibatide (Integrilin, 10 µg/mL, GSK), acetylsalicylic acid (ASA, Aspegic Injectable, Sanofi Belgium) and ticagrelor (TICA, Cayman Chemical) 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 ⁵.

Additionally, 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 anti-platelet or anti-coagulant agents in the prior 10 days.

Serum generation from human pooled plasma

Pooled human plasma was treated with human alpha-thrombin native protein (Thermo Scientific) at a final concentration of 10 U/mL for 3 h 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 O/N slow residual depletion of Fg. Next day serum was centrifuged for 15 min at 14,500 rpm using a bench-top centrifuge (Centrifuge 5424, Eppendorf), carefully transferred to a new tube and passed through a 0.45 µm filter. Prepared serum was verified for residual Fg content, using a sandwich ELISA, then aliquoted and stored at -20 °C.

Microscopic investigation (electron and fluorescence microscopy)

Samples were prepared for scanning electron microscopy (SEM) by repeated osmication to increase their conductance. Briefly, 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 (or until further processing). After 3x washing in 0.1 M Na-cacodylate buffer, followed by H₂O, the samples were incubated in a mixture of 1 % osmium tetroxide and 1.5 % ferrocyanide in H₂O for 60 min on ice in the dark. Then, after incubation for 30 min in 0.2 % tannic acid, the samples were again exposed to 1

% osmium tetroxide for 30 min in the dark at RT. This step was repeated after an intermittent incubation in 1 % tetracarbohydrazide for 20 min. 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 Leica CPD300 critical point dryer. Finally, they were mounted on pin stubs, coated with 8 nm 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 min at RT after perfusions. After 3x washing in PBS the samples were mounted between two microscope coverslips (24 x 50 mm, VWR International) 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 S1 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.

Figure S1. Adhesion of fluorescently labeled *S. aureus* (Texas Red) to BP surface. Micro-parallel plate flow chamber perfusion of bacteria for 10 min over bovine pericardium patch (BP). (A) Microscopic illustration of *S. aureus* (red) on the surface of BP pre-coated 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, $n \geq 6$). 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 non-outliers. T-test with non-parametric Mann-Whitney U-test was done, * = $p < 0.05$. BP = bovine pericardial tissue, O/N = overnight.

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 ⁶. We therefore studied whether the *S. aureus* Newman strain equally adheres to the BJV conduit. Since the main manuscript shows that Fg is the only ligand involved, the Newman strain, which lacks the receptor for fibronectin (FnBPA) ⁷ could be adequately selected to study the role of ClfA, in view of the availability of a ClfA negative mutant, $\Delta clfA$. Figure S2 shows the binding of Newman *S. aureus*, in comparison to that of $\Delta clfA$, which represents the *clfA* deletion mutant.

Figure S2. Adherence of Newman *S. aureus* versus *clfA* mutant strain to BJV.

Plasma precoated BJV_{wall} was perfused for 10 min with suspensions of 10^7 CFU/mL of bacteria in medium (PBS) at a flow rate of $1,000 \text{ s}^{-1}$ ($n > 6$). Adhesion of Newman *S. aureus* and $\Delta clfA$ 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 non-outliers. T-test for the difference in binding was applied with non-parametric Mann-Whitney U-test: * = $p < 0.05$. BJV = bovine jugular vein tissue.

Plots of *S. aureus* adhesion vs. platelet adhesion show that the experimental tissues examined in the present study can be divided in two groups. The first group (BP and

BJV_{wall}) shows a high degree of interaction both with platelets and *S. aureus*. The second group (CH_{wall}, CH_{leaflet} and BJV_{leaflet}) shows hardly any affinity for *S. aureus* or for platelets (Figure S3).

Figure S3. Relation 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 pre-coating with plasma, at a flow rate of 1,000 s⁻¹ for 10 or 20 min 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.

The main manuscript (Fig. 4) shows perfusions over plasma precoated BJV_{wall} where *S. aureus* binds 2-fold more weakly to the graft tissue, when suspended in whole blood (presence of platelets), compared to suspension in plasma (absence of platelets). To better understand the platelet-bacterial interactions in blood, we have additionally performed two-step perfusions. The figure below (Fig. S4) presents bacterial adhesions to BJV in plasma. Step 1 consisted of platelet preloading on the tissue

surface by perfusing whole blood in the presence or absence of the $\alpha_{IIb}\beta_3$ antagonist eptifibatide (10 $\mu\text{g/mL}$) over plasma precoated BJV; step 2 constitutes a perfusion step with *S. aureus* (10^6 CFUs/mL) suspended in plasma. This “inhibited-platelet” preperfusion step resulted in even lower *S. aureus* adhesion (step 2) as in Fig. 4A 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.

Figure S4. Effect of eptifibatide on bacterial adhesion to BJV in two-step perfusion. Plasma precoated BJV was perfused for 20 min with whole blood (step 1), followed by the washing with PBS and 10 min perfusions with suspensions of 10^6 CFU/mL of bacteria in plasma (step 2) at a flow rate of $1,000 \text{ s}^{-1}$ ($n \geq 6$). Where indicated, eptifibatide (EPTI) was added to step 1 at a concentration of $10 \mu\text{g/mL}$. Adhesion of *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. T-test for the difference in binding was used with non-parametric Mann-Whitney U-test: **** = $p < 0.0001$. BJV = bovine jugular vein tissue.

In view of the strong inhibition of bacterial binding in Fig. 5 of the main manuscript, we have double-checked that the combination aspirin/ticagrelor 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 Fig. 5 of the main manuscript. These perfusions did not reveal any inhibitory effect of aspirin and ticagrelor on *S. aureus* viability itself.

Figure S5. Adhesion of *S. aureus* to BJV tissue in the presence of anti-platelet agents. Plasma pre-coated BJV tissue was perfused with *S. aureus* ($10^7/\text{mL}$) for 10 min at a flow rate of $1,000 \text{ s}^{-1}$ ($n = 5$), suspended in plasma in the presence and absence of ticagrelor 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. T-test with non-parametric Mann-Whitney U-test was applied: ns = non-significant. TICA = ticagrelor; ASA = acetylsalicylic acid; BJV = bovine jugular vein tissue.

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