Ultra-Large von Willebrand Factor Fibers Mediate Luminal *Staphylococcus Aureus* Adhesion to an Intact Endothelial Cell Layer under Shear Stress

**Running title:** Pappelbaum et al.; *S. aureus* adhesion to undamaged endothelium

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Abstract:

Background—During pathogenesis of infective endocarditis *S. aureus* adherence often occurs without identifiable pre-existing heart disease. However, molecular mechanisms mediating initial bacterial adhesion to morphologically intact endocardium are largely unknown.

Methods and Results—Perfusion of activated human endothelial cells with fluorescent bacteria under high shear rate conditions revealed 95% attachment of the *S. aureus* by ultra-large von Willebrand factor (ULVWF). Flow experiments with VWF deletion mutants and heparin indicate a contribution of the A-type domains of VWF to bacterial binding. In this context analyses of different bacterial deletion mutants suggest the involvement of wall teichoic acid but not of staphylococcal protein A. Presence of inactivated platelets and serum increased significantly ULVWF-mediated bacterial adherence. ADAMTS13 caused a dose-dependent reduction of bacterial binding and a reduced length of ULVWF, but single cocci were still tethered by ULVWF at physiological levels of ADAMTS13. To further prove the role of VWF in vivo, we compared wild type mice with VWF knock-out mice. Binding of fluorescent bacteria was followed in TNFα stimulated tissue by intravital microscopy applying the dorsal skinfold chamber model. In comparison to wild type mice (n=6) we found less bacteria in postcapillary (60±6 vs. 32±5 bacteria) and collecting venules (48±5 vs. 18±4 bacteria, *P*<0.05) of VWF knock-out mice (n=5).

Conclusions—Our data provide first evidence that ULVWF contribute to the initial pathogenic step of *S. aureus*-induced endocarditis in patients with an apparently intact endothelium. An intervention reducing the ULVWF formation with heparin or ADAMTS13 suggests novel therapeutic options to prevent infective endocarditis.

Key words: von Willebrand factor, infection, endothelium, cardiovascular disease, blood flow
Introduction

*Staphylococcus aureus* is a major pathogen responsible for various infections in humans and meanwhile the most frequent cause of infective endocarditis in industrialized countries.\(^1\) Prerequisite for the pathogenesis of infective endocarditis is the infection of the endocardium.\(^2\) Therefore, one of the first and essential steps is bacterial adhesion to the endothelium. In contrast to other pathogens causing infective endocarditis, *S. aureus* appears to be able to bind not only to damaged but also to intact endothelium.\(^3\)-\(^5\) Fibrinogen, fibronectin and platelets in combination with *S. aureus* clumping factor and fibronectin-binding proteins are involved in the pathogenesis of infective endocarditis.\(^6\)-\(^8\) In addition wall teichoic acids (WTA) of *S. aureus* have been implicated in adhesion to the endothelial cell (EC) layer.\(^9\) However, molecular mechanisms of the very first adhesion steps of *S. aureus* to the undamaged endothelium are still largely unknown.

Recruitment of host cells to the endothelium is a crucial step during inflammation and coagulation. It has been shown previously that under shear von Willebrand factor is a potent binding partner for platelets and a key molecule for the extravasation of leukocytes during inflammation on intact endothelium.\(^10\) VWF is a large multimeric glycoprotein that is mainly produced by ECs and stored in Weibel-Palade-bodies. Acute activation of ECs is followed by a rapid release of VWF to the luminal side of ECs.\(^11\) After secretion, VWF is elongated due to blood flow and forms ultra-large VWF fibers on an intact endothelial layer.\(^12\) In contrast to globular VWF, ULVWF under shear exposes the cryptic GPIb\(\alpha\) platelet binding site to which platelets bind with high affinity.\(^12\) To maintain homeostasis ADAMTS13 (a disintegrin and metalloproteinase with thrombospondin motifs 13) rapidly degrade ULVWF fibers stretched upon high shear stress as present by passage through the microcirculation.\(^12\) Lack or
consumption of ADAMTS13 activity under pathophysiological conditions such as thrombotic thrombocytopenic purpura (TTP), sepsis or other inflammatory diseases prohibit VWF cleavage and can lead to vessel occlusion due to thrombus formation.13, 14

Since the release of VWF to the luminal side of ECs occurs without cell damage, we have tested whether VWF might function as potential bridging molecule for the initial adherence of staphylococci to the endothelium. Indeed, previous studies have shown an interaction of VWF with S. aureus using purified VWF, or surface immobilized recombinant VWF under low shear conditions and indicate that staphylococcal protein A might serve as specific binding partner.15, 16

This study has been designed to investigate the role of ULVWF fibers on S. aureus adherence to an intact human endothelial layer under high shear stress conditions to which the heart valve endothelium is subjected.17 Because current animal models for infective endocarditis depend on damaging the heart valves18 we established an artificial blood vessel. This microfluidic system mimics the pathophysiological conditions of endocarditis reflecting flow rates of large arteries.19 To verify the role of VWF for bacterial adhesion in vivo we used a non-traumatic animal model.

Methods

Bacteria

The staphylococcal strains and derivatives used in this study are listed in Table 1. Staphylococcal strains were cultivated as described in the online-only Data Supplement. After cultivation bacteria were stained either with fluorescein-isothiocyanate FITC (Sigma-Aldrich, Steinheim, Germany) or with tetramethylrhodamine-5- (and 6)-isothiocyanate TRITC (Sigma-Aldrich, Steinheim, Germany) as previously described.20 Optical densities of bacterial
suspensions were adjusted to an OD<sub>540</sub> of 1 corresponding to 3 x 10<sup>9</sup> CFU/ml.

**In vitro perfusion assay of HUVEC**

*In vitro* experiments with primary human umbilical vein endothelial cells (HUVECs) were performed under flow conditions, applying an air pressure pump system. This device allows a unidirectional, continuous perfusion of an intact endothelium. The general pump technique of the IBIDI air pressure pump (IBIDI GmbH, Munich, Germany) system has been described previously. In brief, HUVECs (1 x 10<sup>7</sup> cells/cm<sup>2</sup>) were seeded on gelatine-coated μ-Slide I<sup>0.2</sup> Luer (IBIDI GmbH, Munich, Germany) and cultivated under slight flow (1 dyne/cm<sup>2</sup>) to confluence in EGM2 medium (Lonza, Basel, Switzerland). Fluorescent-labeled bacteria (3.6 x 10<sup>7</sup> CFU/ml) were resuspended in HEPES-buffered ringer solution (10 mM HEPES, 5 mM glucose, 1 mM calcium chloride, 1 mM magnesium chloride, 5 mM potassium chloride, 140 mM sodium chloride) which was supplemented with 25% washed red blood cells in order to increase buffer viscosity and to approximate cellular constituents of physiological blood conditions. Where indicated, platelets and serum were additionally added. Prior to the experiments, platelets were inhibited with prostaglandin and apyrase. Serum was either heat-treated to inactivate ADAMTS13 or remained non-treated. Measurements of the ADAMTS13 activity (ACTIFLUOR™ ADAMTS-13 Activity kit, American diagnostica, Pfungstadt, Germany) revealed a lack of any activity in the heat-treated sample, while we found an activity of 744 ± 168 ng/ml in non-treated serum.

Histamine-activated (100 μM) HUVECs were perfused with fluorescent-labeled live bacteria at corresponding flow rates for 20 min. Where indicated purified human fibrinogen (750 μg/ml) (Sigma-Aldrich, Steinheim, Germany) or recombinant human ADAMTS13 (600 ng/ml) was added. Immunofluorescence staining of luminally secreted VWF was performed to
analyze bacterial adherence to VWF on intact endothelium. Detailed protocols are available in the online-only Data Supplement.

**In vitro perfusion assay of functionalized surfaces**

Functionalized surfaces coated either with human full length recombinant wild type VWF (rwtVWF) or VWF deletion mutants lacking the A1 (VWFdelA1), the A2 (VWFdelA2) or the A3 domain (VWFdelA3) were flushed with fluorescent-labeled *S. aureus* SA113 (3.6 x 10^7 CFU/ml) for 4 min at 10 dyne/cm². To analyze the platelet binding properties of these deletion mutants we perfused washed, inactivated and Celltrace™ Calcein Red-Orange, AM (Invitrogen, Darmstadt, Germany) stained platelets over the functionalized surfaces. Bacterial and platelet adherence was analyzed by fluorescence microscopy using a microscope with 40 X objective (Zeiss, Jena, Germany). Adhesion was quantified at 12 fields of view for each microfluidic channel. More details of the VWF mutants and the coating are provided in the online-only Data Supplement.

**Intravital fluorescence microscopy of the mouse dorsal skinfold chamber**

After TNFα stimulation of the tissue of the dorsal skinfold chamber either VWF knock-out mice or wild type (wt) mice were challenged with fluorescent-labeled live *S. aureus*. Thirty minutes after injection, intravital fluorescence microscopy was performed as described previously.²³ Quantitative off-line analysis of bacterial adherence (given as clusters per mm² of endothelial surface) in postcapillary and collecting venules was performed as described previously.²³ Bacterial adherence was analyzed in postcapillary and collecting venules. Detailed protocols are available in the online-only Data Supplement.

**Statistical analysis**

*In vitro* perfusion assays with HUVECs based on several independent batches of ECs, thus VWF
release and bacterial adhesion varied considerably between the experiments. Therefore, we normalized the result of the overall bacterial adhesion of each single experiment to 100%. Adhesion was classified into VWF-mediated and VWF-independent binding and percentage of binding of each class was calculated. To compare the results with another dataset (e.g. bacterial binding after ADAMTS13 addition) binding of the second group is represented relative to bacterial adhesion of the first group.

Values of the *in vitro* experiments performed with endothelial cells are expressed as means + SD. Values of the *in vitro* experiments performed on functionalized surfaces are expressed as means + SEM. Statistical significance of the *in vitro* experiments was tested with the unpaired Student’s *t*-test. All values of the *in vivo* experiments are expressed as means + SEM. Differences between groups were tested with the unpaired Student’s *t*-test and Mann-Whitney *U*-test. Statistical tests were performed with the SAS software (SAS Institute Inc., Cary, USA). *P* values below 0.05 were considered as statistically significant and are based on the unpaired Student’s *t*-test.

**Results**

**Luminally released VWF mediates *S. aureus* adhesion to intact endothelium *in vitro***

To analyze the impact of luminally released VWF on *S. aureus* adhesion to confluent endothelium we flushed histamine-activated HUVECs with suspensions of two different *S. aureus* strains (Cowan I and SA133) at a shear stress of 10 dyne/cm². In agreement with previous publications, formation of ULVWF fibers on the intact endothelium was shear flow dependent (*Figure 1A*). Adherence of individual *S. aureus* cells or small bacterial clusters to an intact layer of ECs was almost exclusively mediated by ULVWF fibers (*Figure 1A, B*.
**Figure IA, B** in the online-only Data Supplement). The highly dynamic process of the interaction between *S. aureus* with ULVWF fibers under high shear conditions is illustrated in a live image (**Figure II** in the online-only Data Supplement) and by real time movies showing the collapse and re-elongation of the ULVWF under stop-flow conditions and the waving of the fiber under continuous flow (Movie 1 and 2 in the online-only Data Supplement). Entrapment of *S. aureus* by ULVWF fibers was also confirmed by high resolution atomic force microscopy (**Figure III** in the online-only Data Supplement). Quantification of the fluorescence images proved that totally 73 ± 29 cocci/mm² of *S. aureus* SA113 (n = 11) and 49 ± 28 cocci/mm² of *S. aureus* Cowan I (n = 7) adhered to the intact endothelium. In both cases approximately 95% of *S. aureus* co-localized with ULVWF while VWF-independent adhesion was rare (96 ± 2% of *S. aureus* SA113 and 95 ± 6% of *S. aureus* Cowan I VWF-mediated versus 4 ± 2% of *S. aureus* SA113 and 5 ± 6% of *S. aureus* Cowan I VWF-independent, respectively, mean ± SD) (**Figure 1C**).

Our data indicate that endothelial-derived ULVWF functions as a dominant binding partner for *S. aureus*. To further verify our results, we cut established ULVWF by addition of recombinant ADAMTS13 (**Figure 2A**). Perfusion with ADAMTS13 caused a dramatic reduction of ULVWF fibers (**Figure 2C**) and ULVWF-mediated *S. aureus* adhesion (**Figure 2B**) (95 ± 6% adhesion without (n = 7), 50 ± 14% adhesion with ADAMTS13 (n = 5), mean ± SD).

**Fibrinogen has no effect on initial VWF-mediated *S. aureus* binding to endothelium**

Since recent studies showed that fibrinogen acts as bridging molecule for staphylococcal adhesion to the endothelium,7,25 we analyzed the impact of fibrinogen on *S. aureus* adhesion under high shear flow. Although fibrinogen induced bacterial cluster formation as expected25, neither VWF-mediated nor VWF-independent *S. aureus* adherence was affected (VWF-
mediated: 95 ± 6% without fibrinogen (n = 7) versus 87 ± 11% with fibrinogen (n = 3), VWF-independent: 5 ± 6% without fibrinogen versus 13 ± 11% with fibrinogen, mean ± SD) (Figure 3A). Figure 3B shows large adherent *S. aureus* clusters formed in presence of fibrinogen which were entrapped by ULVWF.

**VWF-mediated *S. aureus* binding via protein A is shear flow dependent**

To analyze the impact of staphylococcal protein A (SpA), a surface associated molecule, for the bacterial interaction with VWF we compared an isogenic SpA deficient mutant with its parental wt strain (*S. aureus* Cowan I). Within a paired experiment both bacterial strains were perfused over activated HUVECs with a shear stress of 2.5 dyne/cm² (n = 3) or 10 dyne/cm² (n = 5). Adhesion of the SpA mutant to VWF was significantly decreased at low shear flow (2.5 dyne/cm²) compared to the wt strain (54 ± 3% *S. aureus* SpA adhesion versus 93 ± 3% *S. aureus* Cowan I adhesion, mean ± SD) (Figure 4A). However, at high shear flow conditions (10 dyne/cm²) *S. aureus* SpA binding to VWF (116 ± 9%, mean ± SD) was even significantly increased compared with the wt adhesion (95 ± 6%, mean ± SD) (Figure 4B). Figure 4C shows a representative immunofluorescence image of *S. aureus* SpA binding to ULVWF fibers under high shear flow.

**Bacterial binding to VWF under high shear stress is multi-factorial involving diverse**

**S. aureus** **virulence factors**

Since protein A does not account for *S. aureus* binding to luminally released VWF at high shear flow, we investigated the influence of other bacterial adhesion molecules. Therefore, suspensions with different bacterial mutants were perfused over activated HUVEC layers with a shear stress of 10 dyne/cm². The apathogenic *S. carnosus* which does not possess major *S. aureus* surface proteins8, 26 showed a low VWF binding capacity (17 ± 3%, mean ± SD (n = 4)) compared to
S. aureus SA113 (96 ± 2%, mean ± SD (n = 11)) (Figure 5). The S. aureus mutants srtA and tagO are deficient in LPXTG-anchored surface proteins and WTA, respectively. Binding of both mutants to VWF was significantly reduced in comparison to S. aureus SA113 wt (S. aureus srtA 32 ± 1% (n = 4); S. aureus tagO 44 ± 2% (n = 5), mean ± SD) (Figure 5).

The A1 and A3 domains of VWF mediate bacterial adherence

VWF has binding sites for various molecules such as platelet glycoprotein Ib, collagens or glycosaminoglycans like heparin. These functional binding sites are located mainly within the A-type domains consisting of A1, A2 and A3. Presence of unfractionated heparin reduced the binding of S. aureus to VWF suggesting the involvement of the VWF A1 domain (VWF-mediated: 95 ± 6% adhesion without (n = 7) versus 16 ± 2% adhesion with heparin (n = 3), mean ± SD) (Figure 6A). However, heparin is not a specific blocker of the A1 domain but able to influence other binding pathways of S. aureus. Analysis of the VWF-independent bacterial adherence revealed a slightly reduced binding upon heparin treatment (VWF-independent: 5 ± 6% adhesion without (n = 7) versus 3 ± 2% adhesion with heparin (n = 3), mean ± SD) (Figure 6A) pointing towards an interference of heparin with bacteria trapped by the endothelial glycocalyx. To further specify the contribution of the A-type domains for S. aureus binding, we synthesized genetically modified recombinant VWF molecules lacking the A1 (VWFdelA1), A2 (VWFdelA2) or A3 (VWFdelA3) domain and perfused S. aureus over surfaces functionalized with this recombinant VWF. S. aureus SA113 (n = 10) adhesion to VWFdelA1 and VWFdelA3 was significantly decreased compared to the adhesion to recombinant wild type VWF (rwtVWF) (Figure 6B). Adhesion to VWFdelA2 was only slightly reduced (Figure 6B). Next we perfused VWF-functionalized surfaces with S. aureus tagO (n = 5) and S. aureus srtA (n = 5). However, this gave no further insight into the molecular mechanisms responsible for the interaction
between VWF and *S. aureus*, since the adherence patterns were very similar in all mutants (Figure 6B).

To prove the functionality of our VWF-mutants we measured their capacity to bind platelets. As expected we found a reduced platelet binding only towards VWFdelA1 while binding to VWFdelA2 and VWFdelA3 was not affected (n = 5) (Figure 6C).^{31}

**Bacterial binding is affected by platelets and ADAMTS13**

Serum contains several proteins like immunoglobulins or plasmatic VWF that could interact with *S. aureus*, while platelets bind with high affinity via GPIbα to the A1 domain exposed by stretched VWF under shear. An increased competition of the acting binding partners for specific VWF binding might be the consequence. Therefore, we further upgraded our in vitro flow device with inactivated platelets and serum, lacking ADAMTS13 activity (Figure 7). However, we found an approximately two fold increased bacterial adherence to the endothelium and an even more dominant role of ULVWF (n = 5) (Figure 7A). Immunofluorescence stainings revealed that ULVWF fibers appear to be thicker and more robust in the presence of serum and platelets, implicating potentially more binding sites for *S. aureus* (Figure 7B), as already hypothesized previously.^{33}

Upon systemic inflammation, ADAMTS13 is consumed by overwhelming amounts of VWF released into the blood^{13}, suggesting also a reduced concentration of ADAMTS13 in the course of endocarditis. In addition, it has been proposed that interleukin 6, which was found to be elevated in the blood of endocarditis patients,^{34} could block ADAMTS13 activity. To understand the role of ADAMTS13 for the ULVWF-mediated adhesion of *S. aureus* we analyzed bacterial attachment in the presence of platelets and inactivated serum supplemented with different amounts of recombinant ADAMTS13 (n = 4) or serum with a physiological
ADAMTS13 activity (n = 4). Figure 7C shows an inverted dose-dependency between ADAMTS13 activity and VWF-mediated *S. aureus* adherence. Although 4-fold less bacteria attached to the endothelium in the presence of 100 ng/ml ADAMTS13 and 8-fold less bacteria in the presence of 200 ng/ml ADAMTS13 we could still detect VWF-mediated adhesion even at physiological levels of ADAMTS13 (744±168 ng/ml). Quantification of the ULVWF fiber length proves that with increasing concentrations of ADAMTS13 the ratio of large fibers to small fibers decreases. However, functional ULVWF fibers were still formed in the presence of high levels of ADAMTS13 (Figure 7D).

**VWF is required for bacterial adherence in vivo.**

To obtain further evidence for the biological relevance of the *S. aureus*-VWF interaction on undamaged endothelium we performed *in vivo* experiments applying the dorsal skinfold chamber model. Bacterial binding in the microcirculation was analyzed by intravital fluorescence microscopy. Release of VWF from the endothelium was induced by TNF-α topically applied to the investigated skin area 30 minutes prior to the experiment. Fiber formation within the lumen of vessels was controlled by immunofluorescence stainings prepared on tissue sections (Figure IV in the online-only Data Supplement). In total we analyzed 160 vessels of five VWF knock-out mice (n = 5) and six wt mice (n = 6). After challenging the mice with bacteria, we found a markedly reduced bacterial adhesion in postcapillary venules (10 – 30 μm diameter) and collecting venules (31 – 50 μm diameter) of the VWF knock-out mice compared to wt mice (60 ± 6 adherent bacteria/mm² in wt mice versus 32 ± 5 adherent bacteria/mm² in VWF knock-out mice in postcapillary venules, and 48 ± 5 adherent bacteria/mm² in wt mice versus 18 ± 4 adherent bacteria /mm² in VWF knock-out mice in collecting venules, mean ± SEM) (Figure 8). We found a significant difference of bacterial adhesion in the collecting venules according to the
unpaired Student’s t-test. Additionally, significance was confirmed with the nonparametric Mann-Whitney U-test.

**Discussion**

*S. aureus* is one of the main pathogens causing endocarditis. Initial adhesion to the endothelium is a crucial step for pathogenesis of this intravascular disease. Once bound to the vessel wall *S. aureus* is able to overcome the EC barrier and to spread to the surrounding tissue leading to the establishment of infection.

Most of the previously performed studies focused on bacterial adhesion at sites of vascular injury. However, *S. aureus* is also able to infect physically undamaged endothelium. The molecular mechanisms of initial *S. aureus* adherence to intact endothelium are mostly unknown, although fibronectin and fibrinogen are required as bridging molecules. In contrast to those studies we aim to investigate bacterial adherence to the intact endothelium during acute inflammatory conditions under distinct shear flow conditions. Thus, we have established and adapted a microfluidic system to study activated ECs under arterial shear flow conditions.

In the current study we demonstrate that luminally released ULVWF acted as a predominant adhesive factor for *S. aureus* mediating the binding of the bacterium to an intact EC layer under high shear flow conditions (Figure 1, 2). Only less than 5% of the attached bacteria interact in a VWF-independent manner that might be due to EC surface associated fibronectin or surface exposed glycosaminoglycans (Figure 1, 6A). Previous studies already demonstrated plasmatic fibrinogen as a potent binding partner of *S. aureus* that triggers the formation of bacterial clusters and the bacterial adhesion to the endothelium. Therefore, we also investigated whether the presence of fibrinogen could affect the VWF-mediated interaction
between the intact endothelium and *S. aureus*. Although fibrinogen led to the formation of bacterial clusters, we found that neither the VWF-mediated nor the VWF-independent amount of colony-forming units of endothelium-bound bacteria was increased in the presence of fibrinogen. Accordingly, our data suggest that binding of *S. aureus* to the endothelium is initiated by VWF and that VWF is not only able to sequester single bacteria but also to entrap large bacterial clusters (**Figure 3**).

One potential binding partner of VWF expressed by *S. aureus* is protein A. In this context Hartleib et al showed a specific interaction of SpA with VWF under low shear flow.\(^{15}\) Therefore, we compared the VWF binding capacities of an isogenic *S. aureus* wt/SpA strain pair within our experimental setting. In line with the previous results we observed a SpA-dependency at low shear flow conditions. However, at high shear flow conditions we measured a SpA-independent *S. aureus* adhesion (**Figure 4**). Notably, these findings of a shear flow dependency are supported by results obtained by O´Seaghdha and coworkers showing that *L. lactis* derivatives expressing the SpA bind to recombinant human VWF under low shear rates but not under high shear rates.\(^ {35}\)

To reveal the potential bacterial binding partner for VWF under high shear flow conditions we first determined the interaction between VWF and *S. aureus* srtA which lacks all cell wall-anchored surface proteins.\(^ {27}\) Adhesion of *S. aureus* srtA to ULVWF was higher than the adhesion of the non-pathogenic *S. carnosus* which lacks all relevant surface associated adhesins\(^ {26}\), suggesting the involvement of additional bacterial surface molecules for VWF adhesion (**Figure 5**). Therefore, we analyzed the role of wall teichoic acids (WTAs) which are highly abundant, surface exposed glyco-polymers incorporated into bacterial cell walls. Our data suggest indeed the attendance of WTAs, since we found a significantly reduced adhesion of the *S. aureus* tagO mutant compared to *S. aureus* wild type adhesion (**Figure 5**). Interaction of host lectins such as
galectins and WTA has already been debated and is subject of ongoing investigations in our laboratories.\textsuperscript{27} Interestingly, galectin 1 and 3 are both expressed by human ECs and released in complex with VWF.\textsuperscript{36}

The A1 domain of luminally exposed, stretched VWF provides not only binding sites for the platelet receptor GPIb but also for collagen and heparin.\textsuperscript{31} The A3 domain contains binding sites for collagen type I and III\textsuperscript{29}, whereas the A2 domain has no adhesive properties but contains the cleavage site for ADAMTS13.\textsuperscript{37} Our experiments indicate that \textit{S. aureus} is mainly immobilized on stretched VWF fibers exposing the A-type domains. Blocking of the A1 domain by using heparin revealed a 6-fold reduction of the VWF-mediated adhesion of \textit{S. aureus} to the endothelium (Figure 6A). In agreement with our data also Li et al previously showed a heparin-dependent reduction of bacterial binding to VWF.\textsuperscript{38} We confirmed the impact of the A1 domain by a reduced adherence of \textit{S. aureus} to functionalized surfaces coated with recombinant VWF lacking the A1 domain. Interestingly, bacterial adhesion to surfaces coated with recombinant VWF lacking the A3 domain was also strongly diminished, indicating a significant impact of the A3 or collagen binding domain of VWF in bacterial binding (Figure 6B). To the best of our knowledge there are to date no confirmed \textit{S. aureus} surface molecules with specific sequence-based homologies with collagen type I and III. However, it might be possible that quaternary structures create de-novo binding sites which are based on the structural configuration rather than on direct sequence similarity.

The shear activated VWF A2 domain provides a cleavage site for the plasma protease ADAMTS13 which efficiently cleaves ULVWF fibers under physiological conditions.\textsuperscript{12,37} Therefore, VWF fibers are postulated to exist only upon reduced ADAMTS13 activity as known in patients suffering from TTP.\textsuperscript{14} However the amount and the activity of ADAMTS13 were
found to be also reduced during systemic inflammation such as sepsis leading to the persistent formation of ULVWF fibers. These studies showed that ADAMTS13 activity inversely correlates with patients outcome. Secondary deficiency of ADAMTS13 is either related to the consumption of ADAMTS13 due to an increased amount of VWF in the circulation or due to its inhibition by inflammatory cytokines. To our knowledge, existence or absence of ADAMTS13 in patients suffering from endocarditis are not yet investigated. Therefore we performed experiments with different concentrations of ADAMTS13 to predict its role in the onset of ULVWF-mediated binding of S. aureus. Although the amount and the length of ULVWF fibers exposed on the endothelial surface were drastically diminished in the presence of physiological amounts of ADAMTS13 (744 ± 168 ng/ml), we still found bacteria tethered by VWF. Assuming a consumption or inhibition of ADAMTS13 in affected patients, our experiments predict an even more significant contribution of ULVWF for bacterial binding. In agreement with our in vitro data, we were also able to detect luminal released VWF fibers in the vessels of mice treated with TNF-α suggesting the formation of ULVWF in vivo.

To further prove the pathological relevance of our results we studied the interaction between human endothelium and S. aureus not only in the presence of red blood cells but together with platelets and serum. Although we expect a decoration of S. aureus with IgG or soluble VWF on the one hand and the occupation of the A1 domain by platelets on the other hand, we found an even increased contribution of ULVWF to bacterial binding. Interestingly, we also found that ULVWF strings decorated with platelets are thicker and more robust than ULVWF without platelets suggesting an improved docking of bacteria. To investigate the pure effect of ULVWF for S. aureus adherence we performed our flow experiments with inactivated platelets preventing the exposure of active GPIIb/IIIa, a well-known binding partner for
S. aureus.

The major drawback of current animal models for infective endocarditis is the artificial destruction of the endothelium prior to bacterial inoculation.\textsuperscript{18} We aimed to circumvent this strategy applying our recently described \textit{in vivo} model of the dorsal skinfold chamber.\textsuperscript{23} To measure the role of VWF for bacterial trapping we compared wild type mice with mice deficient in VWF. It is worth to note that the lack of VWF leads also to a lack of Weibel-Palade-bodies accompanied by a disturbed processing of various endothelial-derived factors such as P-selectin.\textsuperscript{42} Although P-selectin is a potent binding partner for leukocytes and potentially responsible for the anchorage of the ULVWF to the EC surface\textsuperscript{43} it has been excluded as adhesion molecule for \textit{S. aureus} recently.\textsuperscript{44}

Applying our experimental \textit{in vivo} setting together with intravital fluorescence microscopy we showed a significant reduction of bacterial adhesion in VWF knock-out mice (Figure 8). This further underlines the physiological relevance of VWF-mediated attachment of \textit{S. aureus} to intact microvasculature.

In conclusion, we could show that binding of \textit{S. aureus} to the intact endothelial cell layer is predominantly mediated by luminally secreted ULVWF fibers involving A1 and A3 domains. Bacterial adhesion to VWF is resistant to even high shear flow conditions since shear flow activated ULVWF fibers support \textit{S. aureus} binding. Our data shed light on the initial early pathogenetic steps of \textit{S. aureus} endocarditis in patients with an apparently intact endothelium. Based on our findings one can speculate that therapeutic intervention with recombinant ADAMTS13, as it is discussed for patients suffering from TTP\textsuperscript{45}, or heparin could be a novel approach as adjunctive therapy besides the current antibiotic based therapy for infective endocarditis.\textsuperscript{46}
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**Conflict of Interest Disclosures:** None.

**References:**


Table 1. List of analyzed staphylococcal strains regarding deficiency of adhesins, applied experimental flow conditions and origin.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Properties</th>
<th>Shear rate conditions [dyne/cm²]</th>
<th>Reference or source</th>
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<td>Wild type</td>
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<td>WTA-deficient</td>
<td>10</td>
<td>(28)</td>
</tr>
<tr>
<td>S. aureus SA113 srtA</td>
<td>Sortase A-deficient</td>
<td>10</td>
<td>(27)</td>
</tr>
<tr>
<td>S. carnosus TM300</td>
<td>Wild type</td>
<td>10</td>
<td>(26)</td>
</tr>
</tbody>
</table>

Figure Legends:

Figure 1. *S. aureus* adherence to intact endothelium is mediated by luminally released VWF.

Stimulated, confluent HUVECs were flushed with bacteria at a shear stress of 10 dyne/cm². (A)

Fluorescence image of *S. aureus* SA113 (green) bound to ULVWF fibers (red) released from an
intact endothelial cell layer (bright field). Scale bars correspond to 50 μm. (B) Magnification of
*S. aureus* SA113 (green) co-localized with ULVWF fibers (red). Scale bar corresponds to 20 μm.
(C) Quantification of *S. aureus* strains SA113 (n = 11) and Cowan I (n = 7) attached to an intact
endothelium. Adhesion was classified into VWF-mediated and VWF-independent. Total
bacterial adhesion was normalized to 100%. Data are expressed as means + SD (*P < 0.001).

**Figure 2.** *S. aureus* binding is specific to ULVWF fibers. (A) Representative
immunofluorescence image of ULVWF fibers on stimulated endothelium without and with
ADAMTS13 after perfusion of stimulated, confluent HUVECs at a shear stress of 10 dyne/cm².
Scale bars correspond to 50 μm. (B) Percentage of relative *S. aureus* Cowan I adherence to an
intact endothelium with (n = 5) and without (n = 7) ADAMTS13. Bacterial adhesion in the
presence of ADAMTS13 is represented relative to bacterial adhesion in the absence of
ADAMTS13 which was normalized to 100%. Data are expressed as means + SD (*P < 0.05).
(C) Quantification of the luminally released ULVWF fiber amount. Each data point reflects one
independent experiment. Black bars correspond to the median.

**Figure 3.** Fibrinogen addition did not affect *S. aureus* adherence to VWF. (A) Quantification of
*S. aureus* Cowan I binding to endothelium in the presence (n = 3) or absence of fibrinogen (n =
7), after perfusion of stimulated, confluent HUVECs at a shear stress of 10 dyne/cm². Total
bacterial adhesion was normalized to 100%. Data are expressed as means + SD. (B)
Representative fluorescence image of *S. aureus* Cowan I (green) cluster bound via ULVWF
fibers (red, indicated by arrows) to an intact HUVEC layer (bright field) after fibrinogen
addition. Scale bars correspond to 20 μm.
**Figure 4.** *S. aureus* binding to VWF via protein A was shear flow-dependent. (A) Under low shear flow conditions adhesion of *S. aureus* SpA was decreased compared to *S. aureus* Cowan I (n = 3). (B) Under high shear flow conditions adhesion of *S. aureus* SpA was increased (n = 5). *S. aureus* Cowan I adhesion was normalized to 100% and adhesion of *S. aureus* SpA is represented relative to *S. aureus* Cowan I adhesion. Data are expressed as means +SD (*P<0.05, ** P < 0.001). (C) Representative fluorescence image of *S. aureus* Cowan I (green) and *S. aureus* SpA (red) bound to ULVWF fiber (white) under high shear flow conditions. Scale bar corresponds to 20 μm.

**Figure 5.** *S. aureus* interaction with VWF comprises various adhesion factors. Percentage of adherent bacteria after perfusion over stimulated HUVECs. *S. aureus* SA113 adhesion was normalized to 100% and bacterial adhesion of *S. aureus* tagO (n = 5), of *S. aureus* srtA (n = 4) and *S. carnosus* (n = 4) is represented relative to *S. aureus* SA113 (n = 11) adhesion. Data are expressed as means + SD (*P<0.001).

**Figure 6.** *S. aureus* bound to VWF A1 and A3 domains. (A) Quantification of adherent bacteria to HUVECs with (n = 3) and without (n = 7) heparin at a shear stress of 10 dyne/cm². *S. aureus* Cowan I wt adhesion in the presence of heparin is represented relative to bacterial adhesion in absence of heparin which was normalized to 100%. Data are expressed as means + SD (*P<0.05). (B) Quantification of adherent bacteria after perfusion of *S. aureus* SA113 (n = 10), of *S. aureus* tagO (n = 5) and of *S. aureus* srtA (n = 5) over functionalized surfaces coated with rwtVWF, VWFdIA1, VWFdIA2 or VWFdIA3 at a shear stress of 10 dyne/cm². Data are expressed as means + SEM (*P<0.05) (C) Quantification of adherent platelets after perfusion...
over functionalized surfaces coated with rwtVWF or VWF deletion mutants (n = 5). Data are expressed as means + SEM (*P<0.05).

**Figure 7.** Bacterial binding is affected by platelets and ADAMTS13. (A) Quantification of *S. aureus* SA113 binding to endothelium in the presence of red blood cells (RBC) (n = 11) and in the presence of RBC, platelets and inactivated serum (n = 5), after perfusion of stimulated, confluent HUVECs at a shear stress of 10 dyne/cm². Adhesion was classified into VWF-mediated and VWF-independent. *S. aureus* adhesion was normalized to 100% and adhesion in the presence of RBC, platelets and inactivated serum is represented relative to bacterial adhesion in the presence of RBC. Data are expressed as means ± SD. (B) Representative immunofluorescence images of ULVWF fibers on stimulated endothelium after perfusion either with RBC or with RBC, platelets and inactivated serum at 10 dyne/cm². Scale bars correspond to 50 μm. (C) Quantification of *S. aureus* SA113 binding to endothelium in the presence of RBC, platelets and either ADAMTS13 active serum (n = 4) or inactivated serum supplemented with 100 ng/ml (n = 4) or 200 ng/ml (n = 4) of recombinant ADAMTS13, after perfusion of stimulated HUVECs at a shear stress of 10 dyne/cm². Bacterial adhesion in the presence of ADAMTS13 is represented relative to bacterial adhesion in the absence of ADAMTS13 which was normalized to 100%. Data are expressed as means ± SD. (D) Quantification of the length of luminally released ULVWF fibers after perfusion with RBC, platelets and either ADAMTS13 active serum or inactivated serum supplemented with different concentrations of recombinant ADAMTS13. Total fiber amount was normalized to 100%. Data are expressed as means ± SD.

**Figure 8.** Reduced bacterial binding in VWF knock-out mice. (A) Intravital fluorescence
microscopy of attached CFSE-labeled *S. aureus* Cowan I in postcapillary and collecting venules of the dorsal skinfold chamber after local TNF-α-exposure. Scale bars correspond to 100 μm. (B) Magnification of *S. aureus* bound to collecting venules. Scale bars correspond to 50 μm. (C) Quantitative evaluation of adherent bacteria (per mm² endothelial surface) in venules of TNF-α-exposed dorsal skinfold chambers of wt mice (n = 6) or VWF knock-out mice (VWF -/-, n = 5). Data are assessed by intravital fluorescence microscopy and expressed as means ± SEM (*P<0.05).
Figure 2


Panel C: Scatter plot showing ULVWF fiber amount [fibers/mm²] with and without ADAMTS13.
Figure 3

(A) Relative bacterial adherence [%]

- VWF-dependent
- VWF-dependent

(B) ULVWF fibers

S. aureus Cowan I

Bright field / merge

Legend:
- n.s.: not significant
Figure 4
Figure 5

Relative bacterial adherence [%]

- SA113
- tagO
- srtA
- S. carnosus

VWF-mediated | VWF-independent
Figure 6
Figure 7
Figure 8
Ultra-Large von Willebrand Factor Fibers Mediate Luminal Staphylococcus Aureus Adhesion to an Intact Endothelial Cell Layer under Shear Stress
Karin I. Pappelbaum, Christian Gorzelanny, Sandra Grässle, Jan Suckau, Matthias W. Laschke, Markus Bischoff, Corinne Bauer, Marina Schorpp-Kistner, Christopher Weidenmaier, Reinhard Schneppenheim, Tobias Obser, Bhanu Sinha and Stefan W. Schneider

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SUPPLEMENTAL MATERIAL

for

Ultra-large von Willebrand factor fibers mediate luminal Staphylococcus aureus adhesion to an intact endothelial cell layer under shear stress

Karin I. Pappelbaum, MSc; Christian Gorzelanny, PhD; Sandra Grässle, MSc; Jan Suckau, cand. med.; Matthias W. Laschke, MD, PhD; Markus Bischoff, PhD; Corinne Bauer, cand. med.; Marina Schorpp-Kistner, PhD; Christopher Weidenmaier, PhD; Reinhard Schneppenheim, PhD; Tobias Obser, BTA; Bhanu Sinha, MD, PhD*; Stefan W. Schneider, MD*

* Corresponding authors and equal contribution
Supplemental Methods

Cultivation of bacteria for in vitro assays

Staphylococcal strains were grown stationary in Mueller-Hinton broth (Roth, Karlsruhe, Germany) for 18h at 37°C. The culture media for the mutants contained appropriate antibiotics for marker selection (5 µg/ml tetracycline (Amdipharm, St. Helier, Jersey), 2.5 µg/ml erythromycin (Roth, Karlsruhe, Germany)).

Cultivation of bacteria for in vivo application

Bacteria cells of exponential growth phase cultures (A_{600nm} of OD1) of S. aureus strain Cowan I were washed twice with phosphate-buffered saline, fluorescent-labeled with 50 µM of CFSE (5-[6]-carboxyfluorescein diacetate succinimidyl ester (Life Technologies, Darmstadt, Germany) for 15 min at 37°C and 500 rpm, washed for an additional three times with PBS to remove unbound fluorescent dye and 100 µl of the fluorescent-labeled cell suspension (containing 6.4-8.9x10^7 cfu) were applied to the animals.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were cultivated to confluence in M199 medium (Invitrogen, Darmstadt, Germany) supplemented with 10% heat-inactivated fetal calf serum (PAA Laboratories GmbH, Pasching, Austria), antibiotics (penicillin and streptomycin; PAA Laboratories GmbH, Pasching, Austria), 5 U/ml heparin (Biochrom, Berlin, Germany) and 1% growth factor supplement derived from bovine retina as described previously.¹
**Immunofluorescence staining of VWF**

HUVECs and bacteria were fixed with 4% paraformaldehyde in PBS for 10 min at 37°C under flow. For VWF staining endothelial cells were blocked for 1 h with 2% BSA. To avoid a co-staining of *S. aureus* by antibody binding to protein A bacteria were blocked for 30 min with normal goat IgG (2.5 µg/ml) (R&D, Minneapolis, USA). Immunofluorescence staining was performed with anti-VWF rabbit antibody (15 µg/ml) (DAKO, Hamburg, Germany) and with anti-rabbit Alexa 350 (5 µg/ml) (Invitrogen, Darmstadt, Germany). Adherence of fluorescent-labeled bacteria to endothelial cells was analyzed by fluorescence microscopy using a microscope with 40 X objective and appropriate fluorescence filters (Zeiss, Jena, Germany). To quantify bacterial attachment to the intact endothelium 20 – 30 fields of view per experiment were analyzed. Analysis of images was performed with the software AxioVS40 V 4.8.0.0. (Zeiss, Jena, Germany).

**In vitro perfusion of functionalized surfaces**

Human full length recombinant wild type VWF (rwtVWF) and VWF deletion mutants lacking the A1 (VWFdelA1: deletion of amino acid residues E1260-G1479), A2 (VWFdelA2: deletion of amino acid residues N1493-E1673) or the A3 domain (VWFdelA3: deletion of amino acid residues G1672-G1874) were expressed in 293-EBNA cells (Invitrogen, Darmstadt, Germany) as previously described.² Perfusion experiments with functionalized surfaces were performed with the BioFlux 200 flow system (Fluxion, San Francisco, USA) as previously described.³ In brief, channels of 24 Well BioFlux plates were coated either with rwtVWF, VWFdelA1, VWFdelA2 or VWFdelA3 applying a solution of 100 µg/ml for 2h at 37°C. To remove excess VWF, channels were flushed with HEPES-buffered ringer solution.
(10 mM HEPES, 5 mM glucose, 1 mM calcium chloride, 1 mM magnesium chloride, 5 mM potassium chloride, 140 mM sodium chloride) which was supplemented with 25% washed red blood cells. To evaluate bacterial attachment, channels were flushed with HEPES-buffered ringer solution supplemented with 25% washed red blood cells and fluorescent-labeled S. aureus SA113.

Preparation of the mouse dorsal skinfold chamber and intravital fluorescence microscopy

All experiments were conducted in accordance with the German legislation on protection of animals and the NIH Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, Washington, USA) and were approved by the local governmental animal care committee. Fifteen to 20-week-old VWF knock-out mice (B.6.129S2-VWF<sup>tm1Wgr</sup>/J)<sup>4</sup> and wild type (wt) mice (C57BL/6) (The Jackson Laboratory, Maine, USA) with a body weight of 22–25 g were used for the study. Dorsal skinfold chambers were prepared in mice as described previously in detail.<sup>5</sup> After the surgery, the animals were allowed to recover from anesthesia and surgery for at least 48 h before the microcirculatory analyses. For intravital fluorescence microscopy, the animals were anesthetized (75mg/kg body weight ketamine intraperitoneal injection (i.p.) (Pharmacia GmbH, Erlangen, Germany) and 15mg/kg body weight xylazin i.p. (Bayer, Leverkusen, Germany)) and a fine polyethylene catheter (PE10, 0.28 mm internal diameter) was inserted into the A. carotis for application of bacteria and fluorescent dyes. For endothelial cell stimulation, the tissue of the dorsal skinfold chamber was exposed to TNF-α (topical application of 2000 U dissolved in 100 µL PBS) (Roche, Mannheim, Germany) for 30 min. Subsequently, the mice were attached to the microscopic stage. After analysis of the
intravascular bacterial adherence, 0.1 ml of 5% FITC-labeled dextran 150,000 was additionally injected for contrast enhancement by staining of blood plasma to determine microhemodynamic parameters, including vessel diameter (µm) and centerline red blood cell velocity (µm/s). Intravital fluorescence microscopy was performed by means of a Zeiss Axiotech microscope (Zeiss; Oberkochen, Germany) with a 100W mercury lamp attached to an epi-illumination filter block for blue (450-490nm excitation; >520nm emission wavelength), green (530- 560nm; >580nm) and ultraviolet (330- 390nm; >430nm) light and 10x and 20x long distance objectives. The microscopic images were recorded by a charge-coupled device video camera (FK6990; Pieper, Schwerte, Germany) and transferred to a DVD system for off-line evaluation.

**Atomic force microscopy (AFM)**

Plasmatic VWF (1 µg) (Merck, Darmstadt, Germany) was adsorbed to freshly cleaved mica (Science Service GmbH, Munich, Germany). Subsequently *S. aureus* Cowan I (1 µl of a suspension of an OD$_{540}$ of 1) was flushed. Intermittent contact mode AFM (the NanoWizard I, JPK Instruments, Berlin, Germany) was performed in air, applying a cantilever with a spring constant of 1 N/m and a resonant frequency of 100 kHz (NSC36, MicroMash, Tallinn, Estonia).
Supplemental Figures and Figure Legends

Supplemental Figure 1. VWF-dependent *S. aureus* adherence to intact endothelium. Stimulated, confluent HUVECs were flushed with bacteria at a shear stress of 10 dyne/cm². (A) Fluorescence image of FITC-labeled *S. aureus* Cowan I (green) bound to ULVWF fibers (red) released from an intact endothelial cell layer (bright-field). Scale bars correspond to 50 µm. (B) Magnification of *S. aureus* Cowan I (green) co-localized with ULVWF fibers (red). Regions of co-localization are represented in yellow. Scale bar corresponds to 20 µm.

Supplemental Figure 2. *S. aureus* adhesion to ULVWF fibers. Live image of fluorescent-labeled *S. aureus* SA133 (green) bound to ULVWF fibers (red) under high shear flow conditions (10 dyne/cm²). ULVWF fibers were stained with fluorescent-labeled antibody under flow conditions. Scale bars correspond to 50 µm.
Supplemental Figure 3. *S. aureus* is covered by ULVWF fibers. (A) High resolution surface topography of *S. aureus* Cowan I (arrowhead) that were entrapped by ULVWF fibers (arrow) obtained by atomic force microscopy. Height is false-color coded as indicated by the color bar on the right. Scale bar corresponds to 1 µm (B) Corresponding 3D image of *S. aureus* enveloped by ULVWF fibers. Scale bar corresponds to 1 µm.
Supplemental Figure 4. ULVWF fiber formation in vivo after stimulation. (A) Cryosections of TNFα-stimulated and unstimulated mouse tissue. ULVWF fibers (green) and endothelial CD31 (red) were stained with fluorescent-labeled antibody. Nuclei (blue) were stained with DAPI. Scale bars correspond to 20 µm. (B) Magnification of ULVWF fibers (arrows) stretched within the vessel lumen after TNFα stimulation (right). Without stimulation VWF are located within the vessel wall (left).

Supplemental Movie Legend

Supplemental Movie 1. S. aureus adhesion to ULVWF fibers after collapse and reelongation under stop-flow. Perfusion of fluorescent-labeled S. aureus SA133 over histamine-stimulated HUVECs resulted in the formation of string-like structures that tethers bacteria. Interruption of flow (time 00:03:17.) lead to the contraction of ULVWF fibers until
flow starts again (time 00:09:85.). Timestamp in hh:mm:ss. as indicate. Arrow corresponds to 20 μm and arrowhead indicates the flow direction.

Supplemental Movie 2. S. aureus adhesion to ULVWF fibers that are waving in the direction of flow. Perfusion of fluorescent-labeled S. aureus SA133 over histamine-stimulated HUVECs resulted in the formation of string-like structures that tethers bacteria and waving under continuous flow. Timestamp in hh:mm:ss. as indicate. Arrow corresponds to 20 μm and arrowhead indicates the flow direction.

Supplemental References

5. Laschke MW, Vollmar B, Menger MD. The dorsal skinfold chamber: window into the dynamic interaction of biomaterials with their surrounding host tissue. Eur Cell Mater. 2011;22:147-164; discussion 164-147