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Short title: Antibacterial effect of platelets.

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Abstract:

Background:

The involvement of platelets in anti-infectious immunity has been widely demonstrated. Molecules secreted mainly by alpha granules are involved in reducing the growth of certain bacterial species. However, the effect of antiplatelet treatments on the platelet antibacterial ability remains poorly understood. The aim of this study was to evaluate the platelet antibacterial effect against *Staphylococcus aureus*, and to evaluate the influence of antiplatelet drugs on this effect.

Materiel and methods:

Blood samples were collected from healthy donors or patient treated with antiplatelet therapy. Six bacterial strains of *S. aureus* were included. Bacteria were incubated with platelets for four hours. Colonies were counted on blood agar. The supernatant's effect was evaluated. The effect of *in vitro* antiplatelet agents and salicylic acid was also tested. **CD62P** expression rate was evaluated under different conditions of infection and platelet treatment.

Results:

Platelets slowed the growth of the six strains of *S. aureus* (p= 0.006 for P6134, p= 0.001 for P6170 and P6138 and p=0.003 for the other strains *vs* bacteria alone). The supernatant of platelets pre-infected with bacteria and that of platelets pre-treated with TRAP, retained this antibacterial effect (Platelet-bacteria supernatant: p= 0.018; TRAP: p= 0.011 vs bacteria alone). The treatment of platelets by antiplatelet drugs significantly decreased this antibacterial effect (Aspirin: p= 0.027; Ticagrelor: p=0.0263; combination: p=0.0092 *vs* untreated platelets). Salicylic acid also induced inhibition of this antibacterial effect (p=0.042 vs untreated platelets).

Conclusion:

Our study showed that antiplatelet agents decreased antibacterial effect of platelets on *S. aureus*.

Key words:

Platelets, Bacteria, aspirin, P2Y12 inhibitors, *Staphylococcus aureus*.

Introduction:

Platelets have long been linked to hemostatic processes only [1]. To date, many studies have demonstrated that platelets are involved in immunity as sentinels that are an integral part of our immune system [2].

Platelets α granules can store hundreds of proteins. In addition to their involvement in the hemostasis process, some of these molecules also play a role in anti-infectious immunity [3]. This concerns thrombin Platelet Microbicidal Proteins (tPMP), such as Platelet Factor 4 (PF4), or thrombocidins (TC1 and 2), a carboxy-terminal diamino acid truncated versions of neutrophil-activating peptide-2 (NAP-2) and connective tissue-activating peptide-III (CTAP-3) respectively [4]. Staphylococcal α toxin has been shown to induce platelet degranulation, releasing anti-bacterial proteins [5]. In addition, after stimulation by the same toxin, platelets secrete the beta defensin 1 (HBD 1), a molecule stored in the extra granular platelet, with bactericidal activity [6]. Previous studies have suggested that these proteins act by increasing membrane permeability of the bacterium, leading to its death [6].

Antiplatelet therapy has widely shown a reduction in mortality from coronary heart disease [7]. However, their influence on antibacterial effect of platelets have not been thoroughly studied. The few studies conducted on this subject have shown that the secretion of PMP requires the ADP pathway [8]. It has also been demonstrated that salicylic acid (SAL), the main metabolite of aspirin, reduces the secretion of staphylococcal α toxin [9]. However, the indirect link between SAL and the secretion of platelet antibacterial proteins through staphylococcal α toxin has not yet been experimentally established.

Although the antibacterial effect of platelets was widely described, to date, no studies have evaluated the influence of antiplatelet drugs on this effect. The aim of this study was therefore to analyze the platelet antibacterial effect against six strains of *Staphylococcus*

74 *aureus*, and to evaluate the influence of antiplatelet drugs such as aspirin and $P2Y_{12}$
75 antagonists on this effect.

Material and methods :

1. Platelet preparation:

Blood was drawn by venipuncture into sodium citrate from healthy subjects or from patients with daily anti-platelet medication (Aspirin, $P2Y_{12}$ receptor antagonist or a combination of both). The protocol was approved by the ethic committee of the IHU méditérranée-infection (Reference 2016-002). Platelet-Rich Plasma (PRP) was prepared according to the ISTH recommendations [10]. Then, platelet count determination was performed using a hematology analyzer. PRP was again centrifuged at 1100g for 10 minutes to obtain a platelet pellet that was suspended in Tyrode's buffer to obtain 4.10^8 e/mL. Platelets were then kept at 37°C in order to prevent activation.

2. Bacterial Preparation :

Six different strains of *S. aureus* from the CSUR (Collection des souches de l'unité des Rickettsies, IHU Mediterranée infection, Marseille France) were used. Four of them Methicillin sensitive (MSSA: P6299, P6175, P2188 and P6134) and two resistants (MRSA: P6170 and P2138). These strains were previously isolated from positive blood cultures. The strains were identified by Maldi Toff mass spectrometry using the Bio Typer database (Bruker, Dresden, Germany). Methicillin susceptibility profiles were determined by the disc diffusion method. Strains were cultured on 5% sheep blood-enriched Columbia agar (COS, BioMérieux, Marcy l'Etoile, France). After 18 hours of incubation at 37°C, colonies were removed and suspended in 0.9‰ NaCl medium to obtain the required concentrations: 1.5, 3 or 10 x 10^8 CFU/ mL.

3. Bacteria- platelets mixture and culturing:

Bacteria and platelets were mixed at a Multiplicity of infection of 1 (except for the part where a range of bacterial concentrations was tested) during 4 hours at 37 ° C under rotation [6]. After four hours of incubation, the aliquots were diluted in series in Tyrode's buffer to

provide dilutions from 10^{-1} to 10^{-7} times the initial concentrations. Dilutions were spread on COS agar (BioMérieux, Marcy l'Etoile, France). After 18 hours of incubation at 37 ° C, colony forming units (CFU) were counted and a comparison was made between the bacterial-platelet mixture and the control consisting of the bacteria incubated with the Tyrode's buffer. The percentage of growth was determined as follows: Number of CFU obtained for each condition X 100 / number of CFU obtained for bacteria incubated with the Tyrode's buffer.

4. Preparation of the supernatant resulting from the incubation of the bacteria with the platelets:

Platelets (4.10^8 e/mL) were incubated with *S. aureus* (P2188, strain chosen randomly, 3.10^8 CFU/ mL) at 37°C for one hour, allowing activation and platelet degranulation. Then, the mix was centrifuged twice, the first at 1100 g for 10 minutes to remove platelets and the second at 5000 g for 10 minutes to eliminate bacteria. The supernatant containing the secreted platelet compounds was thus filtered using Minisart High Flow Stérile luer mâle - 0.2 µm (Sartorius Stedim) to eliminate all cells. This supernatant was again incubated with the same strain for a period of 4 hours at 37°C under rotation. In addition, the supernatant of resting platelet and of platelets pre-activated by 10 µM of Thrombin Receptor-Activating Peptide (TRAP) (STAGO, France) were also tested.

5. Effect of antiplatelet drugs *in vitro*:

PRP of healthy subjects was treated with aspirin (Sanofi, Toulouse, France) at a final concentration of 2 mM [11, 12], or by ticagrelor (AstraZeneca AB S-151 85 Södertälje Suède) at a final concentration of 10 µM [13], or by the combination of both drugs for 1 hour at 37°C. A part of PRP remained untreated. After washing, platelets of different conditions were incubated with 3x 10^8 CFU/ mL of *S. aureus* (strain: P2188, strain chosen randomly). Bacteria incubated in the Tyrode's buffer and bacteria incubated with untreated platelets were used as

controls. In addition, bacteria incubated with single drugs alone without platelets were performed as a control on the interference of these drugs on bacterial growth.

6. Analysis of platelet activation by flow cytometry:

180 µL of treated or untreated platelets (250 G/L) were incubated with 20µL of *S. aureus* P2188 (10^9 CFU) for 1 hour at 37 C°. Resting and TRAP (10 µM) activated platelets were used as controls. After incubation, 4 µL of Anti CD62P- PC 5 (IgG,κ monoclonal, BD Biosciences) were added to 50 µL of samples and vortexed. Samples were incubated at room temperature and under static conditions in the dark for 30 min, 200 µL of PBS were added before analysis by flow cytometer (Beckman Coulter FC500).

7. Effect of salicylic acid:

Platelets of healthy subjects were incubated with 3x 10^8 CFU/ mL of *S. aureus* (strain P6299, strain chosen randomly) with or without SAL (Cooper, France) to a final concentration of 2 mM [11, 12] according to the same protocol described above. Bacteria incubated in Tyrode's buffer and bacteria incubated with untreated platelets were used as controls. Also, bacteria incubated with SAL alone without platelets was performed as a control on the interference of this compound on bacterial growth.

8. Statistical analysis :

Statistical analyzes were performed using IBM-SPSS-Statistics software version 23. Comparison was made between absolute numbers of CFUs that appeared for each condition. Statistical differences were evaluated using the Student Paired t test when samples were normally distributed or Kruskal Wallis, Mann-Whitney U test, Wilcoxon signed ranks test for samples non normally distributed with a p-value ≤ 0.05 considered to be significant.

Results:

1. Platelets inhibited *S. aureus* growth:

After 4 hours of incubation, the platelets significantly decreased bacterial growth of all the six tested strains of *S. aureus* compared to controls. (n=11: p= 0.006 for P6134, p= 0.001 for P6170 and P6138 and p=0.003 for the other strains, Wilcoxon signed ranks test) (Fig 1 A). The inhibition of bacterial growth by platelets remained significant whatever the concentration tested compared to controls, which passed to 7.53% ± 11.7% with 1.5 x 10^8 CFU/ mL (p=0.028), 6.83% ± 5.7% with 3 x 10^8 CFU/ mL (0.027) and 13.69% ± 9.09% with 10^9 CFU/ mL (p=0.028) (n= 6; Wilcoxon signed ranks test).

2. Supernatant of the mix Platelet-*S. aureus* had a similar effect to that of platelets:

The supernatant of resting platelets showed no effect on bacterial growth (p = 0.682; Paired t test). Interestingly, the supernatant of platelets pre-infected with bacteria and that of platelets pre-activated with TRAP showed significant inhibition of S. *aureus* growth compared to bacteria incubated with the Tyrode's buffer and to the supernatant of resting platelets (n= 7, Platelet-bacteria supernatant: p= 0.018 and 0.0013 respectively. TRAP: p= 0.011 and 0.0014 respectively. Student t test) **(Fig 1B)**.

3. *In vitro* treatment by anti-platelet drugs decreased the anti-staphylococcal effect of platelets:

The treatment of platelets by antiplatelet drugs significantly decreased their antibacterial effect **(Fig 2A)**. Although the decrease in growth was significant compared to the bacteria incubated with Tyrode's buffer (n=5: Aspirin: p= 0.002; Ticagrelor: p<0.001; combination: p=0.0043), this decrease was significantly lower compared to that obtained with the untreated platelets (Aspirin: p= 0.027; Ticagrelor: p=0.0263; combination: p=0.0092). Drugs used alone, at the same concentrations as above, showed no effect on *S. aureus* growth

(Aspirin: p=0.256; Ticagrelor: p=0.144; combination: p=0.32; Paired t test) **(Fig 2C; Supp Fig 1)**.

4. Antiplatelet drugs decreased the phenotype of platelet activation induced by *S. aureus*:

As expected, platelets infected with *S. aureus* P2188 showed significant increase in mean intensity of CD62P expression compared to untreated platelets (n=5, p=0.0009). In addition, pretreatment of platelets with anti-platelet drugs before incubation with *S. aureus* decreased significantly the mean intensity of CD62P expression compared to untreated platelets infected with *S. aureus* (n= 5. Aspirin: p= 0.046; Ticagrelor: p= 0.0001, Association: p=0.0015). Interestingly, Ticagrelor and the association of both drugs showed higher inhibition rates compared to Aspirin (n=5; p= 0.003 and p=0.0378 respectively; Paired t test) **(Fig 3)**.

5. *In vitro* treatment of platelets by Salicylic acid inhibited the platelet anti-staphylococcal effect:

To determine whether SAL could influence bacterial growth, the antibacterial effect of platelets was tested with and without SAL. The presence of SAL significantly decreased the platelet antibacterial effect compared to untreated platelets (n=5; untreated platelets:4.31% ± 2.6%; SAL condition:52.86% ± 29.79%; p=0.042; Wilcoxon signed ranks test). SAL, without platelets, used at the same concentration showed no effect on bacterial growth (p=0.160; Paired t test) **(Fig 2C; Supp Fig 1)**.

6. Platelets of patients with daily anti-platelet treatment decreased their antibacterial effect on *S. aureus*:

As shown in **Fig 1A**, platelets from healthy subjects significantly reduced *S. aureus* P2188 growth compared to control (n=14; p= 0.001). Interestingly, platelets from patients treated daily with antiplatelets exhibited a significant decrease in *S. aureus* bacterial growth

197 inhibition whatever the treatment (n= 8 for aspirin, p< 0.001; n=5 for $P2Y_{12}$ receptor
198 antagonists, p=0.001; n=10 for combination treatment, p< 0.001; Mann Whitney test) **(Fig**
199 **2B)**.

Discussion:

We showed that platelets have an antibacterial effect on *S. aureus*. This effect was bound to compounds secreted during platelet activation and was maintained even in the presence of high bacterial concentrations. For the first time, our results showed that anti-platelet treatment acting either on the ADP or the arachidonic acid (AA) pathways reduces this antibacterial effect.

With regard to the overall platelet effect, we obtained a significant inhibition on all six tested strains. This effect could be related to multiple types of interactions already described, such as engulfment and degranulation, including both killed bacteria and those with slow growth by platelet sequestration. Using supernatant fraction, the anti-bacterial effect was observed only with that of activated platelets by TRAP or *S. aureus* with growth inhibition comparable to that using whole platelets, suggesting that this effect was mostly the consequence of platelet secretion induced by bacteria. A recent study has also shown that most of the antibacterial effect of platelets was associated to their secretory capacity reporting a rare observation of engulfing bacteria by platelets [14]. The exact composition of the *S. aureus*-platelet supernatant related to antibacterial activity is not yet completely clear, although we note the main molecules already reported, consisting in N-serine and N-aspartate versions of PMP-1, basic peptides and derivatives, CTAP-3 [8] as well as HBD 1. The latter is an extra-granular platelet peptide, also found in epithelial cells, forming part of a large group of small cationic peptides (defensins), having activity against a board range of pathogens, including Gram-positive and Gram-negative bacteria, by broadly targeting bacterial membranes. [5, 6, 15].

Platelets treated *in vitro* or derived from patients under long-term antiplatelet therapy, aspirin and $P2Y_{12}$ inhibitors, exhibited a reduced effect on bacterial growth. This suggest the involvement of the two pathways in this secretory process. This was in accordance with flow

cytometry data in which treated platelets were associated with low level in CD62P expression induced by bacteria. This therefore reflects a decrease in the spill of the α granules induced by the bacteria [16]. It was shown that platelet signaling pathways leading to PMP release primarily depend on P2 receptors of ADP [8]. In our study, we observed a decrease in the effect with both $P2Y_{12}$ inhibitors and aspirin, suggesting the involvement of AA pathway also in the secretion mechanism of antibacterial platelet molecules.

Regarding the use of aspirin and SAL *in vitro*, we confirmed the effect observed with *in vivo* treated platelets and dissociated the effect of the active molecule on platelets from its main metabolite. Indeed, while aspirin probably reduced this effect by inhibiting the AA pathway, SAL has note yet any documented effect on platelet function. However, previous studies have reported that SAL can modulate the expression of several genes regulation systems in *S. aureus*, resulting, inter alia, in the inhibition of the synthesis of α toxin [9, 17]. The latter being involved in the secretion of platelet antibacterial peptides [5, 6]. The decrease in anti-bacterial platelet effect observed with both molecules suggests that in patients treated with aspirin, the attenuation of the antibacterial effect depends not only on the direct pathway of AA but might also be indirectly related to the decrease in staphylococcal α toxin secretion.

Several studies have reported a beneficial effect of antiplatelet agents in infectious models such as infectious endocarditis and sepsis [18, 19]. However, other studies have reported contradictory results [20]. The relationship between the infectious agent and the platelets seems to be bi-directional. On one hand, the infectious agent leads to platelet aggregation with further negative consequences. On the other hand, platelets act as an immune actor involved in the elimination of the pathogen. In our study, we reported an inhibitory effect of antiplatelet agents on the immune capacity of platelets, but we did not

assess their effect on hemostatic consequences in this context where antiplatelet agents may retain their well-known beneficial effect.

Conclusion:

We have confirmed that platelets have an anti-staphylococcal effect and we have shown for the first time that aspirin and $P2Y_{12}$ antagonists inhibit this effect. We also demonstrated that this effect was mediated by secreted-compounds, and that the influence of aspirin long-term treatment in patients on the anti-bacterial effect of derived platelets was due, not only to the action of the active molecule on the platelets, but also to its main metabolite on the bacteria.

Future studies should focus on the composition of effective supernatants generated from activated platelets in order to characterize deeply each peptide or component and their specific antibacterial activity.

Declarations

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Competing Interests: No conflict of interest

Ethical Approval: Not required

Reference:

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Figures:

Figure 1: Secreted platelet compounds impede *S. aureus* growth:

(A). Effect of whole platelets on *S. aureus* growth. Values are expressed as Medians and ranges. Black stars show significant differences between bacteria incubated with platelets compared to bacteria incubated with Tyrode's buffer considered as 100%. n=11; **: $0.001 \leqslant p < 0.01$; Wilcoxon signed ranks test.

(B). Effect of platelet supernatant on *S. aureus* growth. Values are expressed as mean ± SD. Black stars show significant differences between bacteria incubated with platelet supernatants under different conditions and bacteria incubated with Tyrode's buffer. Gray stars show significant differences between bacteria incubated with supernatant of pre-infected or pre-activated platelets and bacteria incubated with supernatant of resting platelets. n=7; *: $0.01 \leqslant p < 0.05$; **: $0.001 \leqslant p < 0.01$.; student t test.

Figure 2: Platelets treated *in vitro* or obtained from subjects with daily antiplatelet drugs have a reduced effect on bacterial growth.

(A) Influence of *in vitro* treatment of platelets on their antibacterial effect. Values are expressed as mean ± SD. n=5; Paired t test.

(B). Effect of daily antiplatelet treatment. Values are expressed as Medians and ranges. n= 8 for aspirin; n=5 for P2Y12 antagonists; n=10 for combination; Mann-Whitney Test.

(C). Effect of drugs used alone on bacterial growth. Values are expressed as mean ± SD. n=5; Paired t test.

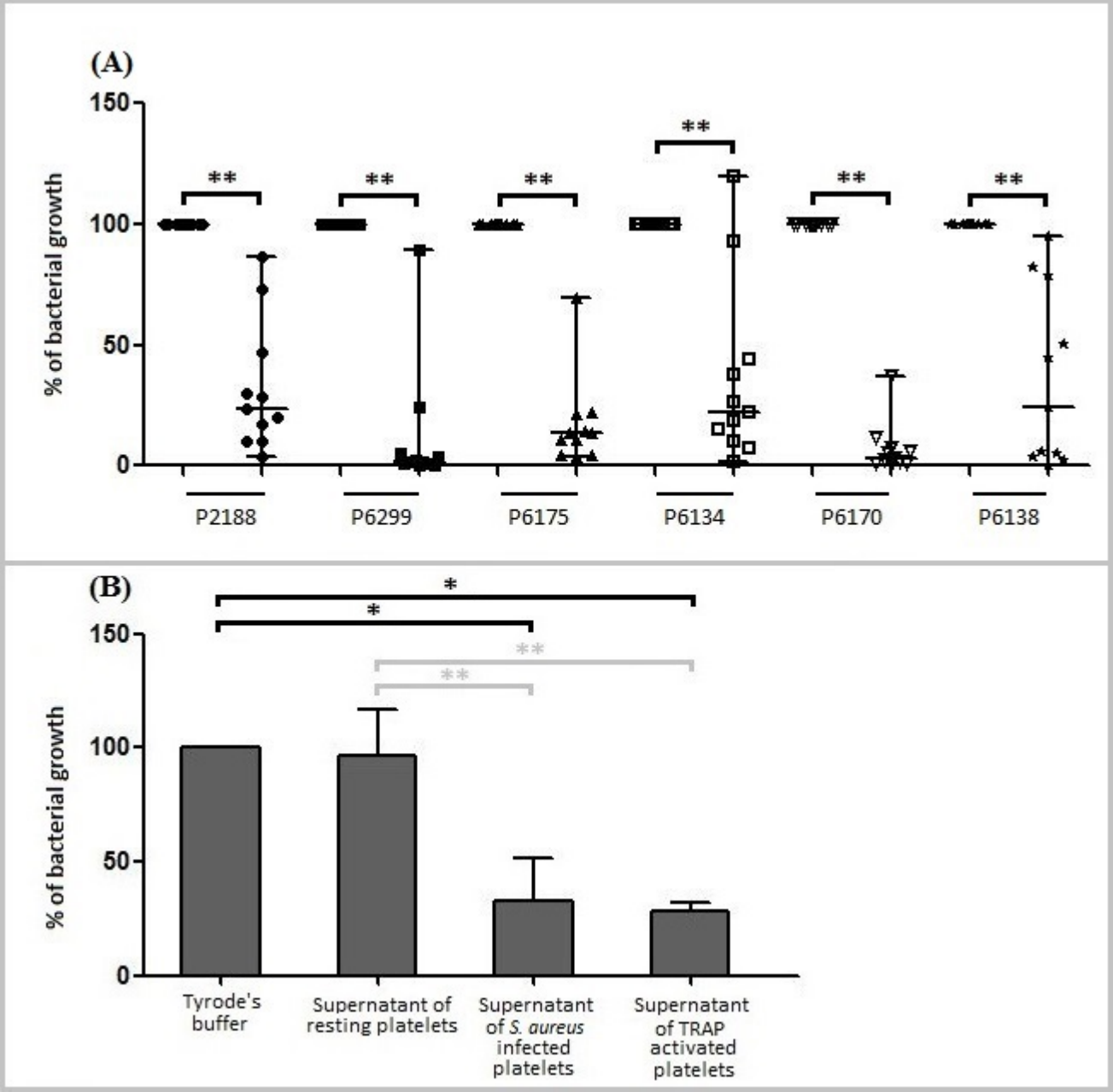
Grey stars depict the statistical differences between antiplatelet conditions compared to untreated platelet condition. Black stars depict statistical differences between treated or untreated platelet conditions compared to the bacteria incubated with Tyrode's buffer. NS: Not significant difference; *: $0.01 \leqslant p < 0.05$; **: $0.001 \leqslant p < 0.01$ and ***: $p < 0.001$.

Figure 3: Antiplatelet agents decreased the platelet CD62P expression induced by bacteria.

(1) untreated, (2) Aspirin, (3) Ticagrelor and (4) combination (Ticagrelor + Aspirin). Median fluorescence intensity (MFI) of expressed CD62P are shown. Values are expressed as mean ± SD. (A) shows significant difference on CD62P mean intensity compared to untreated platelets. (B) shows significant difference on CD62P mean intensity compared to bacteria-untreated platelets. Black stars show significant difference between the different treatment conditions. n=5. $p<0.05$. Paired t test.

Supplementary figure 1:

(A) Growth curve of *S. aureus* P2188 growth in trypticase soy agar (TSB), in the absence of SAL and in the presence of SAL at doses of: 1mM, 2mM, 5mM, 10mM, and 20mM. No apparent difference was perceived until the concentration of 20 mM. (B) Growth curve of *S. aureus* P2188 growth in TSB, in the absence of aspirin and in the presence of aspirin at doses of: 1mM, 2mM, 5mM, 10mM, and 20mM. No apparent difference was perceived until the concentration of 20 mM. (C) Growth curve of *S. aureus* P2188 growth in TSB, in the absence of ticagrelor and in the presence of ticagrelor at doses of: 2.5 µM, 5 µM, 10 µM, 20 µM and 30µM. No apparent differences were perceived with all the concentrations tested.


(A)
% of bacterial growth
150
100
50
0
**
**
**
**
**
**
P2188
P6299
P6175
P6134
P6170
P6138
(B)
% of bacterial growth
150
100
50
0
*
*
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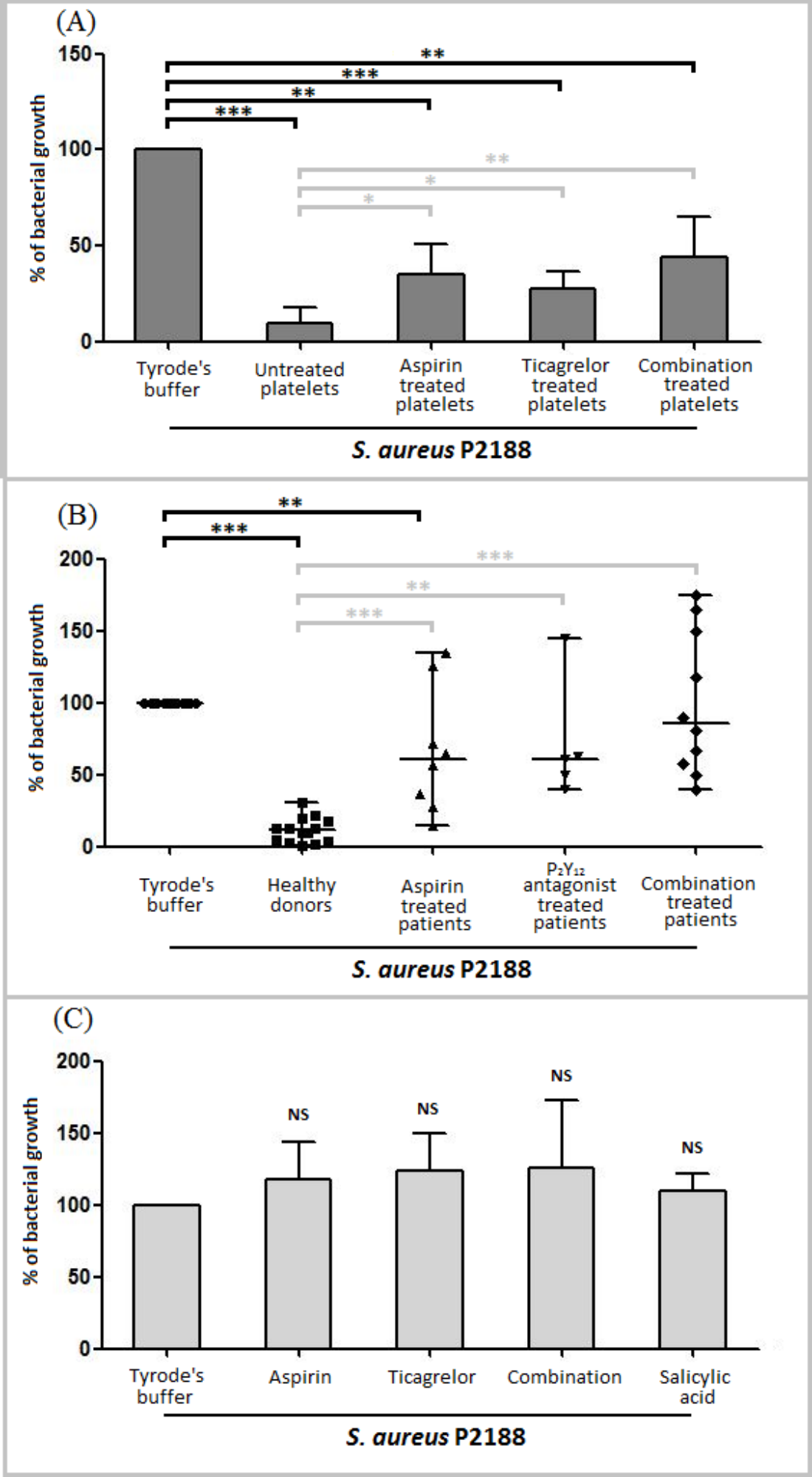
Tyrode's buffer
Supernatant of resting platelets
Supernatant of S. aureus infected platelets
Supernatant of TRAP activated platelets


(A)
% of bacterial growth
150
100
50
0
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**
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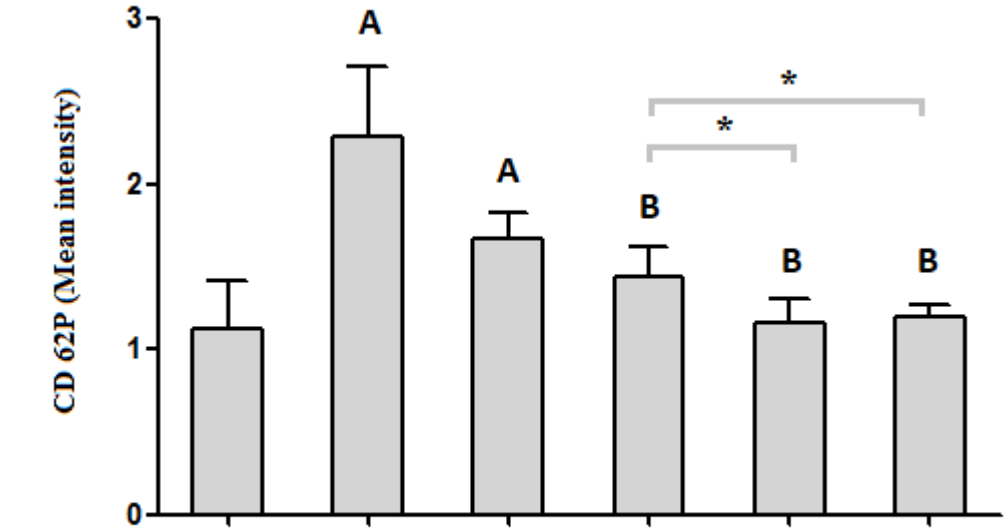
*
*
Tyrode's buffer
Untreated platelets
Aspirin treated platelets
Ticagrelor treated platelets
Combination treated platelets
S. aureus P2188
(B)
% of bacterial growth
200
150
100
50
0
**
***
***
**
***
Tyrode's buffer
Healthy donors
Aspirin treated patients
P2Y12 antagonist treated patients
Combination treated patients
S. aureus P2188
(C)
% of bacterial growth
200
150
100
50
0
NS
NS
NS
NS
Tyrode's buffer
Aspirin
Ticagrelor
Combination
Salicylic acid
S. aureus P2188



S. aureus	-	-	+	+	+	+
Drugs		TRAP	-	ASA	TCG	ASA + TCG