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## CD32A-expressing platelets determine the severity of experimental anaphylaxis.

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**Title:****CD32A-expressing platelets determine the severity of experimental anaphylaxis****Authors:**

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**One Sentence Summary:** Platelet numbers and expression of the activating IgG receptor CD32A determine the severity of acute hypersensitivity reactions (anaphylaxis).

**Abstract:**

Platelets are key regulators of vascular integrity; however, their role in anaphylaxis, a life-threatening systemic allergic reaction characterized by the loss of vascular integrity and vascular leakage, remains unknown. Anaphylaxis is a consequence of inappropriate cellular responses triggered by antibodies to generally inoffensive antigens, resulting in a massive mediator release and rapidly occurring organ dysfunction. Human platelets express receptors for IgG antibodies and can release potent mediators, yet their contribution to anaphylaxis has not been previously addressed in mouse models, probably because mice do not express IgG receptors on platelets. Here, we investigated whether platelets contribute to IgG anaphylaxis in human IgG receptor-expressing mouse models and a cohort of patients suffering from drug-induced anaphylaxis. We found that platelet counts dropped immediately and dramatically at anaphylaxis induction, only when they expressed the human IgG receptor Fc $\gamma$ RIIA/CD32A. Platelet depletion attenuated anaphylaxis whereas thrombocytopenia drastically worsened its severity. Fc $\gamma$ RIIA-expressing platelets were directly activated by IgG immune complexes *in vivo* and were sufficient to restore susceptibility to anaphylaxis in resistant mice. Serotonin released by activated platelets contributed to anaphylaxis severity. Data from a cohort of patients suffering from drug-induced anaphylaxis indicated that platelet activation was associated with anaphylaxis severity and that anaphylaxis occurrence was accompanied by a reduction in circulating platelet numbers. Our findings identify platelets as critical novel players in IgG anaphylaxis and provide a rationale for the design of platelet-targeting strategies to attenuate the severity of anaphylactic reactions.



**[Main Text: ]**

## **Introduction**

Anaphylaxis is an acute and life-threatening multisystem allergic reaction. It results from inappropriate cellular responses triggered by antibodies to generally inoffensive antigens, and is characterized by massive mediator release (1), and rapidly occurring vascular and organ dysfunction. Experimental models have elucidated two different antibody-dependent anaphylaxis pathways. The 'classical' pathway relies on antibodies of the IgE class, which, when bound to the high affinity FcεRI receptor on basophils and mast cells, trigger cell activation upon exposure to the causative allergen (2, 3). The 'alternative' anaphylaxis pathway is engaged by IgG antibodies, which, when aggregated by a specific antigen, activate myeloid cells *via* IgG receptors (FcγRs) (4-6). Even though human anaphylaxis has been classically viewed as an IgE antibody-dependent reaction, accumulating evidence indicates that IgG antibody-dependent reactions may be responsible for human anaphylaxis cases, particularly in the context of drug-induced anaphylaxis (7).

Both clinical and experimental anaphylaxis are associated with immediate release of histamine and lipid mediators. Notably, the lipid mediator platelet-activating factor (PAF) has been proposed to contribute to the severity of anaphylaxis in humans (8) and in several experimental models (9-11). The cellular source and target(s) of PAF in such reactions remain unknown, but it is reasonably established that IgG anaphylaxis induction entails the FcγR-dependent activation of myeloid cells that can release PAF. In particular, both resident cells - mast cells and macrophages- and circulating cells -neutrophils, monocytes and basophils- have

been found to contribute to anaphylaxis, but to largely varying degrees depending on the animal model and the inciting allergic agent used (4, 6, 12).

Among the five classical human Fc $\gamma$ Rs (Fc $\gamma$ RI, Fc $\gamma$ RIIA, Fc $\gamma$ RIIB, Fc $\gamma$ RIIIA, Fc $\gamma$ RIIIB) (13), hFc $\gamma$ RIIA/CD32A is the most widely expressed Fc $\gamma$ R in humans; an activating IgG receptor present on all cells of myeloid origin, including platelets. When expressed in transgenic mice, hFc $\gamma$ RIIA can induce severe IgG-dependent anaphylaxis that relies on the contribution of neutrophils and monocytes/macrophages (14). Mice expressing four human Fc $\gamma$ Rs (Fc $\gamma$ RIIA, Fc $\gamma$ RIIB, Fc $\gamma$ RIIIA and Fc $\gamma$ RIIIB) in place of endogenous mouse Fc $\gamma$ RIIB, Fc $\gamma$ RIII and Fc $\gamma$ RIV, also demonstrated severe systemic IgG-induced anaphylaxis that was abolished following hFc $\gamma$ RIIA blockade (11), suggesting a major role for hFc $\gamma$ RIIA in IgG-dependent anaphylaxis induction, even when several other human Fc $\gamma$ Rs are expressed.

On human platelets, hFc $\gamma$ RIIA is expressed as the sole Fc $\gamma$ R (15). Considering that wild-type mice do not express any IgG receptor on platelets, we hypothesized that a putative platelet contribution to human IgG-induced anaphylaxis might have been overlooked due to the inappropriateness of classical mouse models. Indeed, platelets are equipped with a plethora of bioactive molecules, which they release upon activation. Among them, serotonin is a prime candidate to contribute to clinical signs of anaphylaxis, since serotonin can increase vascular permeability (16, 17), trigger vasoconstriction or vasodilation (18), and induce bronchoconstriction in the context of allergic asthma (19).

To test whether hFc $\gamma$ RIIA-expressing platelets actively contribute to IgG anaphylaxis, we developed new mouse models expressing either only hFc $\gamma$ RIIA and no other Fc $\gamma$ R, or mice recapitulating the human Fc $\gamma$ R complexity in place of mouse Fc $\gamma$ Rs. We then corroborated our findings with patient samples from clinical cases of drug-induced anaphylaxis.

Our results demonstrate that platelets are critical players in hFcγRIIA-induced anaphylaxis. Human FcγRIIA-expressing platelets were directly activated by aggregated hIgG *in vitro* and *in vivo* and were sufficient to restore susceptibility to anaphylaxis in resistant mice. Activated platelets released serotonin, which contributed to the severity of anaphylaxis; accordingly, thrombocytopenia aggravated IgG-dependent anaphylaxis, whereas thrombocytopenia was protective. Data from a clinical study on perioperative drug-induced anaphylaxis further support the notion that platelets contribute to human anaphylaxis.

## Results

### *Platelets are required for hFcγRIIA-induced anaphylaxis*

In mice expressing human FcγRs in place of endogenous mouse FcγRs, whether as transgenes or knock-in inserts, anaphylaxis can be induced by injection of heat-aggregated human IgG (HA-hIgG), as a surrogate for human IgG-immune complexes (11, 14, 20). As expected, hFcγRIIA<sup>tg</sup> mice (on a FcγR<sup>null</sup> background, thus expressing only hFcγRIIA and no other mouse or human FcγRs) developed a rapid hypothermia upon HA-hIgG injection, a primary readout for anaphylaxis, associated with hemoconcentration and augmented vascular permeability (Fig.S1A-B). Surprisingly, the reaction was accompanied by a profound thrombocytopenia (Fig.1A), detectable as early as 5 minutes following anaphylaxis induction, and that persisted for 24 hours (Fig.1B). As expected, FcγR<sup>null</sup> mice (expressing no FcγRs) suffered no hypothermia, vascular leakage or hemoconcentration and only a mild and transient decrease in platelet counts (Fig.1A-B/S1A-B). To test whether thrombocytopenia was merely a marker of systemic cell activation or if platelets were critical for anaphylaxis induction, we depleted platelets 72 h before anaphylaxis induction using anti-GPIbα mAbs. Antibody-

dependent platelet clearance was efficient even in FcγR<sup>null</sup> mice (Fig.S1C), demonstrating that this depletion method is FcγR-independent (21), and therefore should not interfere with FcγR-dependent cell activation thereafter. Platelet depletion abolished hypothermia in hFcγRIIA<sup>tg</sup> mice (Fig.1C/S1C-D), suggesting that platelets are required for IgG-dependent anaphylaxis. We then tested whether an increase in platelet numbers would lead to an escalation in anaphylaxis severity. hFcγRIIA<sup>tg</sup> mice treated with a thrombopoietin receptor agonist, romiplostim, exhibited a 3-fold increase in circulating platelet numbers as compared to untreated mice (Fig.1D) (22). This increase in platelet counts rendered hFcγRIIA<sup>tg</sup> mice extremely sensitive to anaphylaxis induction, with a high accompanying mortality (Fig.1E). Importantly, thrombocytopenia alone was not sufficient to augment sensitivity to anaphylaxis, as thrombocytopenic FcγR<sup>null</sup> mice remained resistant (Fig.1D-E).

#### *hFcγRIIA<sup>tg</sup>-expressing platelets are sufficient to induce anaphylaxis in resistant mice*

Platelet activation during anaphylaxis may result from direct engagement with circulating HA-hIgG and could therefore contribute to - or even be responsible for - anaphylaxis induction. Washed platelets from hFcγRIIA<sup>tg</sup> mice aggregated *in vitro* upon incubation with HA-hIgG in a dose-dependent manner, and to the extent reached by classical inducers of platelet aggregation, *i.e.* thrombin or adenosine 5'-diphosphate (ADP) (Fig. 2A). Markers of platelet activation accompanied *in vitro* aggregation: expression of both CD62P and activated αIIbβ3 integrin were significantly increased on the platelet surface (Fig.2B). Importantly, washed platelets from FcγR<sup>null</sup> mice aggregated and exhibited markers of activation only upon stimulation with thrombin or ADP, but not with HA-hIgG (Fig.S2A/B), indicating that the effect of the latter requires IgG receptor expression. Supporting this observation, blocking hFcγRIIA with an anti-

hFcγRIIA Fab-fragment prevented activation and aggregation of washed platelets from hFcγRIIA<sup>tg</sup> mice by HA-hIgG (Fig.2A/B). These data demonstrate that IgG immune complexes can directly activate hFcγRIIA<sup>tg</sup>-expressing mouse platelets *in vitro*, and suggest that this may also occur *in vivo*.

Accordingly, 30 minutes after injection of HA-hIgG, circulating platelets in hFcγRIIA<sup>tg</sup> mice, but not in FcγR<sup>null</sup> mice, exhibited increased CD62P expression whether present as single platelets or as platelet-neutrophil aggregates (Fig.2C/D). About 80% of circulating neutrophils (Fig.2E) and >90% of Ly6C<sup>hi</sup> monocytes (Fig.S2C/E) in hFcγRIIA<sup>tg</sup> but not FcγR<sup>null</sup> mice were covered with platelets during anaphylaxis (t=30 minutes) but not after the reaction (t=5 hours). In addition, single neutrophils in circulation of hFcγRIIA<sup>tg</sup> mice, but not of FcγR<sup>null</sup> mice, displayed an activated phenotype as reflected by their reduced CD62L expression (Fig.2F). This suggests that both hFcγRIIA<sup>tg</sup>-expressing platelets and neutrophils become activated during HA-hIgG anaphylaxis independently of their association. Indeed, injection of an anti-CD62P antibody abolished the formation of platelet-myeloid cell aggregates following injection of HA-hIgG (Fig.2G/Fig.S2E), without affecting anaphylaxis severity (Fig.2H), or neutrophil (Fig.S2F) and platelet activation (Fig.S2G/H).

We next evaluated whether expression of hFcγRIIA exclusively on platelets may be sufficient to induce anaphylaxis, without the requirement of other FcγR-mediated myeloid cell activation. We therefore devised an *in vivo* model in which the anaphylactic inducer, *i.e.* HA-hIgG, could engage only platelets: we transferred washed platelets from hFcγRIIA<sup>tg</sup> mice, or from FcγR<sup>null</sup> mice as a negative control, into FcγR<sup>null</sup> mice prior to injection with HA-hIgG. Platelets from hFcγRIIA<sup>tg</sup> mice, but not from FcγR<sup>null</sup> mice, conferred FcγR<sup>null</sup> mice with a susceptibility to hypothermia in response to HA-hIgG injection (Fig.2I). Therefore, hFcγRIIA-

mediated activation of platelets is sufficient for anaphylaxis induction in resistant mice, in the absence of other Fc $\gamma$ R-mediated myeloid cell activation.

*Platelet-released serotonin contributes to hypothermia in mice undergoing HA-hIgG-anaphylaxis*

We next investigated whether platelets activated *via* hFc $\gamma$ RIIA release mediators that are able to directly induce a hypothermic response. Isolated platelets from hFc $\gamma$ RIIA<sup>tg</sup> mice were stimulated *in vitro* with HA-hIgG to induce platelet aggregation and secretion, and the supernatant containing the entire platelet releasate was recovered by centrifugation. The releasate from HA-hIgG-activated platelets, but not the buffer alone, induced significant hypothermia when injected i.v. into recipient Fc $\gamma$ R<sup>null</sup> mice (Fig.3A). This releasate contained significant levels of serotonin, but only minute quantities of histamine (337 $\pm$ 52ng serotonin *versus* 1.7 $\pm$ 0.2 ng histamine /10<sup>9</sup> platelets) (Fig.S3A). Furthermore, a 15-fold increase in serotonin concentration was observed in the circulation of hFc $\gamma$ RIIA<sup>tg</sup> mice 5 minutes after anaphylaxis onset, whereas histamine levels were barely increased (Fig.S3B). To test whether serotonin contributes to HA-hIgG-induced anaphylaxis, we inhibited serotonin biosynthesis in hFc $\gamma$ RIIA<sup>tg</sup> mice prior to anaphylaxis induction by repeated injections of 4-chloro-phenylalanine (PCPA), an inhibitor of the essential enzyme tryptophan hydroxylase (23). Pre-treatment with PCPA reduced anaphylactic hypothermia in hFc $\gamma$ RIIA<sup>tg</sup> mice more than 2-fold (Fig.3B) without altering platelet consumption (Fig.3C), indicating that serotonin indeed contributes to HA-hIgG anaphylaxis. Accordingly, intravenous injection of serotonin led to a dose-dependent hypothermia in WT mice (Fig.3D). Finally, the transfer of the releasate from serotonin-deprived HA-hIgG-activated washed platelets (Fig.S3C) induced a milder temperature drop in recipient Fc $\gamma$ R<sup>null</sup> mice than that

from serotonin-sufficient platelets (Fig.3A). Together these results indicate that hFcγRIIA-expressing platelets release serotonin upon activation with HA-hIgG, which contributes to hypothermic symptoms of HA-hIgG-induced anaphylaxis.

*Platelets contribute to IgG-anaphylaxis in a mouse model reproducing human FcγR complexity*

hFcγRIIA is one of four human activating IgG receptors, along with hFcγRI, hFcγRIIA and hFcγRIIIB, in addition to the inhibitory IgG receptor hFcγRIIB. We therefore investigated if platelet activation contributes also to anaphylaxis induction in the context of full human FcγR complexity by using a novel mouse model knocked-in for all human FcγRs (hFcγR<sup>KI</sup>), which recapitulates hFcγR expression (Fig.S4/S5). These mice are susceptible to hIgG-induced anaphylaxis, demonstrating rapid hypothermia following HA-hIgG injection, which was likewise accompanied by a profound thrombocytopenia (Fig.4A). Blocking hFcγRIIA *in vivo* was sufficient to abolish hypothermia and thrombocytopenia in HA-hIgG-injected hFcγR<sup>KI</sup> mice (Fig.4B), illustrating that hFcγRIIA is the FcγR dominantly contributing to anaphylaxis induction, as was suggested by our previous studies using another mouse model (24). Platelet depletion in hFcγR<sup>KI</sup> mice reduced the nadir of hypothermia 2-fold (Fig.4C/Fig.S1C), confirming the contribution of platelets to anaphylaxis, albeit that this effect was less pronounced than in hFcγRIIA<sup>tg</sup> mice, in which platelet depletion abolished hypothermia (Fig.1C). As observed in hFcγRIIA<sup>tg</sup> mice (Fig.2), platelets rapidly associated with neutrophils during HA-hIgG-induced anaphylaxis in hFcγR<sup>KI</sup> mice (Fig.S6A), and pretreatment of hFcγR<sup>KI</sup> mice with an anti-CD62P antibody also abolished the formation of these aggregates (Fig.S6A) without affecting the anaphylaxis outcome (Fig.S6B) or platelet consumption (Fig.S6C). These data confirm the contribution of platelets to anaphylaxis pathophysiology, in a more complex model of cognate

hFcγR expression, while simultaneously arguing for the additional activation of other cellular pathways (9, 14, 24).

*Releasate from HA-hIgG-activated human platelets induces hypothermia in mice*

Having demonstrated the crucial role of hFcγRIIA-expressing mouse platelets in HA-IgG anaphylaxis, we next investigated whether this was also the case for human platelets. As observed with hFcγRIIA-expressing mouse platelets (Fig.2A), washed human platelets aggregated *in vitro* upon incubation with HA-hIgG in a dose-dependent manner, and to the extent reached by classical inducers of platelet aggregation, *i.e.* thrombin or ADP (Fig.5A). *In vitro* aggregation was accompanied by upregulation of platelet activation markers: expression of P-selectin (Fig.5B) and of activated αIIbβ3 integrin as revealed by PAC-1 antibody binding (Fig.5C). hFcγRIIA was required for the activation of human platelets by HA-hIgG; as blocking hFcγRIIA prevented aggregation and expression of P-selectin and activated αIIbβ3 integrin on the platelet surface (Fig.5A-C). These data demonstrate that IgG immune complexes can directly activate human platelets *via* hFcγRIIA *in vitro*, and suggest that this may also occur during anaphylactic reactions in humans without the requirement of accessory cells.

We next investigated if human platelets activated with HA-hIgG release mediators that can induce a temperature drop *in vivo*. Transfer of the releasate from washed human platelets activated with HA-hIgG induced significant hypothermia in recipient mice, which was not observed upon injection of HA-hIgG-containing buffer alone (Fig.5D). As observed in the releasate of hFcγRIIA-expressing mouse platelets (Fig.3), human platelet releasate contained significant serotonin concentrations ( $177\pm 45$  ng/ $10^9$  platelets), but only a minute amount of

histamine ( $7 \pm 1$  ng/ $10^9$  platelets) (Fig.5E). Thus, serotonin released from HA-hIgG-activated human platelets may contribute to hypothermia in recipient mice.

#### *Evidence for platelet activation during human drug-induced anaphylaxis*

Having demonstrated that hFcγRIIA-expressing platelets contribute to IgG anaphylaxis severity in mouse models, we sought to evaluate the translation of these findings to the clinic. To this end, we investigated circulating platelet numbers and activation status in blood samples from 67 patients suffering from mild or severe anaphylaxis to perioperative neuromuscular blocking agent (NMBA) administration and their matched controls, in the case-control “NASA” study (<https://clinicaltrials.gov/ct2/show/NCT01637220>). Significantly fewer single platelets, identified as CD61<sup>+</sup>/FSC<sup>lo</sup> cells by flow cytometry (Fig.S7), were detected 30 minutes after anaphylaxis induction (*i.e.* NMBA injection) in the blood of patients suffering from severe *versus* mild anaphylaxis ( $1.30 \pm 0.14 \times 10^4 / \mu\text{L}$  *versus*  $1.76 \pm 0.18 \times 10^4 / \mu\text{L}$ ), or compared to matched control patients undergoing anesthesia without anaphylaxis ( $1.80 \pm 0.09 \times 10^4 / \mu\text{L}$ ). Six-to-eight weeks post-anaphylaxis, platelet counts from patients that had suffered a severe anaphylactic reaction were comparable ( $2.06 \pm 0.15 \times 10^4 / \mu\text{L}$ ) to those of controls (Fig.6A), indicating that this phenotype was not inherent to this patient group. Of note, the platelet counts represented herein are unusual as only single CD61<sup>+</sup>/FSC<sup>lo</sup> cells were considered (thus excluding platelets in aggregates) and because blood was drawn on heparin in this clinical protocol, which is known to affect platelet responsiveness (25). The sampling method should not, however, be responsible for the variations we observe between patient groups, since all blood was sampled identically. Nonetheless, to complement this analysis we took advantage of four case/control pairs among all patients in this study, who underwent cardiac surgery and therefore assessment of their

differential blood counts before, during and after surgery. In this patient sub-group, we could confirm thrombocytopenia in anaphylactic patients compared to controls, particularly in the +3/4h interval following anesthesia (Fig.6B). This effect could not be accounted for merely by hemodilution, since simultaneously monitored leukocyte counts did not change significantly (Fig.6C). Finally, and similar to results from our pre-clinical models, platelet CD62P expression and the percentage of CD62P-positive platelets were significantly higher in patients suffering from severe *versus* mild NMBA-induced anaphylaxis, or *versus* control groups (Fig.6D), indicating that human platelets are activated during anaphylaxis.

## Discussion

This work identifies platelets as critical novel players in the effector phase of IgG anaphylaxis using two different pre-clinical mouse models sharing the particularity of expressing the activating human IgG receptor hFcγRIIA (CD32A) on platelets. Severe thrombocytopenia and platelet activation required hFcγRIIA expression on platelets, whereas anaphylaxis severity increased with augmented platelet numbers and was reduced in their absence. hFcγRIIA-expressing platelets were directly activated by aggregated hIgG *in vitro* and *in vivo* and were sufficient to restore susceptibility to anaphylaxis in resistant mice. Activated platelets released serotonin, which contributed to anaphylaxis severity. These observations were partly corroborated by clinical data from a cohort of patients suffering from drug-induced anaphylaxis, showing that platelet activation was associated with anaphylaxis severity and that anaphylaxis occurrence was accompanied by a reduction in circulating platelet numbers.

Our analysis identifies platelet-derived serotonin as a mediator in hFcγRIIA-induced anaphylaxis. Serotonin is released by both human and mouse platelets following HA-hIgG activation: the releasate from HA-hIgG-activated human or mouse platelets contained high concentrations of serotonin, and was able to induce significant hypothermia when transferred into recipient mice, whereas the releasate from serotonin-depleted platelets induced only a mild hypothermia. Mice exhibited elevated circulating serotonin concentrations rapidly after anaphylaxis induction, and developed only a mild hypothermia when serotonin biosynthesis was inhibited *in vivo* prior to anaphylaxis induction. Furthermore, injection of serotonin alone into mice induced a hypothermic response. Supporting our findings, release of platelet serotonin was observed following incubation of neuromuscular blockers with blood samples of patients who

had previously an anaphylactic reaction to a neuromuscular blocker (26). Serotonin has also been described to trigger bronchoconstriction when released in the lungs of asthmatic patients (19), to induce either vasoconstriction or vasodilation depending on the type of serotonin receptor and vascular bed (18) and to increase vascular permeability through the formation of gaps in the endothelium (16, 17), suggestive of pleiotropic effects when released during anaphylaxis.

Depletion of platelet serotonin stores by inhibition of serotonin biosynthesis attenuated but did not totally protect mice from anaphylaxis, indicating that serotonin is not the sole mediator of anaphylaxis in the pre-clinical models used herein. Circulating histamine concentrations did not change significantly during anaphylaxis in our models, and antihistamine pretreatment had no effect on anaphylaxis severity (Fig. S6D). PAF receptor antagonists, however, attenuated HA-hIgG-induced anaphylaxis in hFcγR<sup>KI</sup> mice, as reported for other models of IgG anaphylaxis (9-11), suggesting that PAF, but not histamine, may contribute along with serotonin to IgG anaphylaxis. Whether PAF originates from platelets and/or from independently activated myeloid cells remains an open question.

Experimental anaphylaxis in hFcγRIIA-expressing mice was associated with transient hypothermia, augmented vascular permeability and hemoconcentration, and was accompanied by a rapid and severe thrombocytopenia. Importantly, anaphylaxis-induced thrombocytopenia occurred within minutes and preceded significant hypothermia in our models, suggesting that thrombocytopenia is an early sign of anaphylaxis triggered by direct platelet activation. In support of the notion that platelets may trigger anaphylaxis induction, IgG receptor expression exclusively on platelets was sufficient to induce hypothermia. Furthermore, these results may explain the dominance of hFcγRIIA over other human FcγRs in anaphylaxis induction in hFcγR<sup>KI</sup> mice (expressing hFcγRI, hFcγRIIA, hFcγRIIB, hFcγRIIIA and hFcγRIIIB), or in mice

expressing a more restricted set of hFcγRs, as reported previously (11), as hFcγRIIA is the only activating IgG receptor expressed by platelets (15).

The role of hFcγRIIA-induced platelet activation is best established in the pathophysiology of immune-mediated thrombocytopenia and thrombosis, a group of pathologies that encompass thrombosis triggered by certain therapeutic monoclonal antibodies and sepsis-associated or heparin-induced thrombocytopenia (27-30). In most of these conditions, disease-causing IgG is binding *via* its antigen-recognition site to the surface of platelets, thereby facilitating interactions of its Fc portion with platelet hFcγRIIA. Whether this is a general requirement for hFcγRIIA activation is not known. It may, however, imply that in the context of IgG-dependent anaphylaxis, IgG complexes must first be retained by one hFcγRIIA molecule on the platelet surface to then be able to trigger platelet activation *via* another hFcγRIIA molecule on the same or adjacent platelet (*i.e.* in *cis* or *trans*). Indeed, hIgG readily bound to the platelet surface in hFcγRIIA<sup>tg</sup> mice injected with HA-hIgG, in a dose dependent manner, which led to a corresponding reduction in detectable hFcγRIIA expression on platelets (Fig.S8).

Other studies investigating IgG-induced inflammatory reactions reported that platelets might rather play a regulatory role, preventing exaggerated neutrophil-mediated inflammation or inducing anti-inflammatory cytokine (*e.g.* IL-10) production by monocytes (31, 32). Importantly, however, these studies have been performed in mice expressing mouse FcγRs, and therefore in the absence of the sole activating IgG receptor on platelets, hFcγRIIA. In light of the data presented herein and previous reports on hFcγRIIA function *in vivo*, it may be pertinent to reevaluate the role of platelets during inflammation using hFcγRIIA-expressing mice, to ascertain whether protective or regulatory functions are preserved when platelets express hFcγRIIA.

We found that the large majority of circulating neutrophils and Ly6C<sup>hi</sup>-monocytes were covered with activated platelets; which, however, neither accounted for the reduction in circulating platelet numbers nor for the activation of either platelets or myeloid cells, because anti-CD62P antibodies blocked these interactions yet had no significant effect on anaphylaxis severity or thrombocytopenia. Other phenomena *in vivo* such as platelet-platelet aggregation, phagocytosis or adhesion to the vascular endothelium (33) may contribute to the thrombocytopenia observed during anaphylaxis. Also, our data imply that activation of platelets (increased CD62P expression) and neutrophils (CD62L shedding) occurs independently of their association. Prior studies on platelet-neutrophil interactions have suggested that platelet binding to neutrophils may facilitate neutrophil effector functions, such as extravasation into inflamed tissues and the formation of neutrophil extracellular traps (34-36). Our data indicate that these physical interactions are not required for anaphylaxis induction, but rather that platelets and myeloid cells are each activated through direct FcγR engagement. Indeed we have shown previously that antibody-mediated neutrophil depletion had a beneficial effect on IgG anaphylaxis in hFcγRIIA<sup>tg</sup> mice (14) and in mice expressing a more restricted set of human FcγRs (11) than that of hFcγR<sup>KI</sup> mice.

Inherently, data obtained using engineered mouse models may over- or underestimate pathways leading to disease. Platelet contribution to anaphylaxis was observed to be greater in the hFcγRIIA<sup>tg</sup> mice than in hFcγR<sup>KI</sup> mice. This may be attributed to differences in hFcγRIIA expression levels on platelets, which is greater in hFcγRIIA<sup>tg</sup> mice than in hFcγR<sup>KI</sup> mice. Another possibility is that expression of multiple human IgG receptors compared to expression of a single IgG receptor (hFcγRIIA) might lead to the concurrent activation of additional cellular pathways in hFcγR<sup>KI</sup> mice compared to hFcγRIIA<sup>tg</sup> mice. Future clinical studies evaluating

hFcγRIIA expression levels on platelets with respect to anaphylaxis susceptibility may resolve this distinction. Our data indicate that, regardless of whether high or moderate, hFcγRIIA expression on platelets promotes platelet contribution to anaphylaxis severity. Importantly, our *in vivo* data were supported by our *in vitro* studies on human washed platelets, which like their mouse counterparts, aggregated and expressed activation markers in response to hFcγRIIA engagement by HA-hIgG. Furthermore, we observed reduced numbers of circulating platelets in severe anaphylactic cases, along with an activated platelet phenotype. Of note, these observations are consistent with the sole other report in the literature that studied platelet numbers during human anaphylaxis: in which two anaphylactic cases were accompanied by >60% reduction in circulating platelet counts (37). Thus, clinical data are in line with our data obtained in preclinical mouse models, and substantiate the premise that platelets contribute to human anaphylaxis. Further clinical studies will be necessary to confirm these observations and evaluate whether platelets or platelet-derived mediators such as serotonin may represent new targets to intervene and ameliorate anaphylactic reactions in patients.

This report offers new insights into the mechanisms underlying anaphylaxis as it may occur in humans and identifies platelets and their serotonin release as critical players. Our work provides a rationale for future studies to assess the role of platelets in more complex clinical and pre-clinical settings, in which both IgE and IgG anti-allergen antibodies may contribute to anaphylaxis.

## Materials and Methods

### *Clinical study design*

Detailed description of the NASA clinical study design and cohort can be found in (38 and Jönsson, de Chaisemartin *et al*, submitted). In brief, the multi-center NASA (“*Neutrophil Activation in Systemic Anaphylaxis*”) study involved 11 hospital anesthesia departments in the Ile-de-France region in France (<https://clinicaltrials.gov/ct2/show/NCT01637220>). The purpose of the NASA study was to evaluate the role of neutrophils during anaphylactic reactions to NMBA in humans through a case/control study design, with a 6-8 weeks’ follow-up in cases, with clinical signs consistent with intra-anesthetic anaphylaxis to NMBA whatever the grade of the reaction. Patients were retrospectively classified in severity grade according to the Ring and Messmer (39) classification by two independent evaluations of clinician experts. A severe acute hypersensitivity reaction (AHR) was defined as a grade 3 or 4 (i.e. a severe organ failure or a cardiac/respiratory arrest). As case patients were not in a medical and psychological state to provide study consent during the AHR, their consent was obtained as soon as they were judged able. If the patients did not recover the day after the AHR, consent was obtained from a next of kin. Non-inclusion criteria were the absence of consent. The study protocol was approved by a local ethics committee (committee for the protection of Individuals “Ile-de-France X”) and prospectively registered at ClinicalTrials.gov (Identifier: NCT0163722). The biological collection was approved and labeled by the National Health Authority, and registered within the French Ministry of Research and ARH (“Agences Régionales de l’Hospitalisation”). As recommended by International/National Guidelines (40-42) on management of perioperative AHR, a venous blood sample was collected 30 minutes following the onset of the AHR when the patient’s condition had been stabilized. Platelet enumerations were performed by flow cytometry

on diluted total human blood using anti-CD61 and anti-CD62P antibodies. All patients that suffered an AHR were treated following the French and international practice guidelines (40, 41, 43, 44).

### ***Mice***

hFcγR<sup>KI</sup> mice were generated by intercrossing of VG1543 mice (24) with VG6074 mice (Fig.S4A) designed and generated by Regeneron Pharmaceuticals, Inc. on a mixed 62.5% C57BL/6N, 37.5% 129S6/SvEv genetic background, and backcrossed one generation to C57BL/6N. FcγR<sup>null</sup> mice were described previously (45). Mice expressing the fcgr2a transgene (46) were bred to FcγR<sup>null</sup> mice to generate FcγRIIA<sup>tg</sup> FcγR<sup>null</sup> mice (referred to as hFcγRIIA<sup>tg</sup> herein). All mice were bred at Institut Pasteur and used for experiments at 7-11 weeks of age. hFcγR<sup>KI</sup>, FcγR<sup>null</sup> or hFcγRIIA<sup>tg</sup> mice demonstrate normal development and breeding patterns. Experiments using mice were validated by the CETEA ethics committee number 89 (Institut Pasteur, Paris, France) under #2013-0103, and by the French Ministry of Research under agreement #00513.02.

### ***Antibodies and reagents***

ADP (Adenosine 5'-diphosphate sodium), prostacyclin PGI<sub>2</sub>, apyrase, PAF, ACD (acid-citrate-dextrose) anticoagulant and p-chlorophenylalanine (PCPA) were obtained from Sigma Aldrich, TNP<sub>(21-31)</sub>-BSA from Santa Cruz, thrombin from Roche, anti-GPIIbα (polyclonal rat IgG) and isotype control from Emfret Analytics and heparin from Tocris. Human albumin and fibrinogen were provided by LFB Biomédicaments (2014-01029). Anti-FcγRIIA (mouse IgG2b,

clone IV.3) and isotype control mIgG2b (clone Gork; hybridoma from B. Heyman (Uppsala Universitet, Uppsala, Sweden)) antibodies were purified as described (47). The antibodies used for flow cytometry staining of human CD61 (clone VI-PL2), CD62P (clone AK-4 or CLBThromb/6), CD64 (clone 10.1), CD16 (clone 3G8) and activated human  $\alpha$ IIB $\beta$ 3 integrin (clone PAC1), mouse CD62P (clone KO2.3), CD41 (clone MWReg30), Ly6G (clone 1A8), Ly6C (clone AL-21), CD11b (clone M1/70) and CD45 (clone 30-F11), were purchased from BD Pharmingen, CD62L (clone MEL14-H2.100) was purchased from Miltenyi Biotech;  $\alpha$ IIB $\beta$ 3 (clone JON/A) antibody from Emfret Analytics; anti-human CD32A (clone IV.3) from Stemcell Technologies and isotype controls mIgG1 (clone P3.6.2.8.1) from eBioscience and mIgG2b (clone MPC-11) from BioLegend. Serotonin Fast Track and histamine ELISA kits were obtained from Labordiagnostika Nord and Abcam, respectively.

### *Passive Systemic Anaphylaxis*

Human Intravenous Immunoglobulins (IVIG; Gamunex<sup>®</sup>, Grifols) were heat-aggregated (HA-hIgG) by incubation at 20 or 25 mg/mL in BBS (0.17 M H<sub>3</sub>BO<sub>3</sub>, 0.12 M NaCl, pH8) for 1 hour at 63°C, then diluted to 10 mg/mL in 0.9% NaCl for i.v. injection at 100  $\mu$ L per mouse, resulting in a dose of 0.05 mg/g of body weight. Central temperature was monitored using a digital thermometer (YSI) with rectal probe. Control non-aggregated hIgG (NA-hIgG) was similarly diluted without heating. For the illustration of vascular leakage, 100  $\mu$ L of 0.5% Evans Blue was injected i.v. 5 minutes prior to anaphylaxis induction. 15 minutes later, mice were sacrificed and front paws prepared for extraction in formamide (48h, RT). Optical density of extracted dye was measured at 620 nm for quantification.

### ***Platelet enumerations and hematocrit determination***

In selected patients (4 cardiac surgery anaphylactic cases and 4 paired controls) platelets from EDTA-treated whole blood were enumerated using the Sysmex TS 500 Hemocytometer (Kobe, Japan). For analysis of mouse blood, 10  $\mu$ L of blood was collected at the tail vein directly in EDTA (20 mM final), and platelet numbers and hematocrit assessed using a hematometer (SCIL Vet abc Plus, Horiba Medical).

### ***In vivo blocking, depletion, romiplostim treatment***

For platelet depletion, mice were injected i.v. with 50  $\mu$ g/mouse of anti-GPIIb $\alpha$  (anti-CD42b) or isotype control (rat IgG) 72 h before challenge. Blocking of Fc $\gamma$ RIIA was achieved by i.v. injection of 40  $\mu$ g/mouse blocking anti-Fc $\gamma$ RIIA mAb (clone IV.3) or isotype control (mIgG2b) 24 h and 12 h before challenge. For CD62P blocking experiments, 50  $\mu$ g/mouse of anti-CD62P REA antibody (clone REA344; Miltenyi) or an isotype control were injected 30 minutes before challenge. To induce neo-production of platelets, mice received s.c. injections of romiplostim (50  $\mu$ g/kg of body weight; NPLATE<sup>®</sup> Amgen) on day 0 and 3 and were used for experiments on day 6, adapted protocol from (22). For *in vivo* serotonin depletion, mice received i.p. injections of p-chlorophenylalanine (PCPA, 300 mg/kg) or vehicle (PBS-Tween 20 5% (v/v)) for 7 consecutive days. On the third day of treatment, a mild thrombocytopenia was induced with 5  $\mu$ g/mouse of anti-GPIIb $\alpha$  mAb to remove serotonin-loaded platelets from the circulation. Mice were used for experiments on day 7 when platelet counts had recovered.

### ***Preparation of washed platelet suspensions, in vitro stimulation and transfer***

*Preparation of washed human or mouse platelets.* Washed platelets from mouse blood were prepared as previously described (48). Platelets were suspended in Tyrode's buffer (137 mM NaCl, 2 mM KCl, 12 mM NaHCO<sub>3</sub>, 0.3 mM NaH<sub>2</sub>PO<sub>4</sub>, 1 mM MgCl<sub>2</sub>, 2 mM CaCl<sub>2</sub>, 5.5 mM glucose, 5 mM HEPES, pH7.3) containing 0.35% human serum albumin and apyrase (0.02 U/ml). Suspension of washed platelets was adjusted at the indicated concentration and maintained at 37°C until use. For generation of serotonin-depleted washed platelets, mice received i.p. injections of p-chlorophenylalanine (PCPA, 300 mg/kg) or vehicle (PBS-Tween 20 5% (v/v)) for 5 consecutive days and were used for experiments on day 6.

*In vitro stimulation.* Platelet aggregation was measured at 37°C in an aggregometer APACT 4004 (ELITechGroup). A 270 µL aliquot of washed human ( $2 \times 10^6$  platelets/µL) or mouse ( $2 \times 10^5$  platelets/µL) platelets was stirred at 1,100 rpm and activated by addition of the appropriate agonist and human fibrinogen (0.8 mg/mL), in a final volume of 300 µL. The extent of aggregation was quantified by measuring the maximum curve height above baseline. For flow cytometric analysis washed human or mouse platelets were stimulated for 10 minutes at 37°C.

*Transfer of washed mouse platelets.*  $1.7 \times 10^9$  washed mouse platelets in 300 µL Tyrode's albumin buffer (or buffer alone) were injected i.v. into recipient mice 20 minutes before anaphylaxis induction.

*Transfer of platelet releasates.* Washed human or mouse platelets ( $6 \times 10^6$  platelets/µL) were activated by HA-hIgG (1 mg/mL) for 30 minutes, followed by centrifugation at 15 000 g for 10 minutes and releasates were collected for i.v. injection into recipient mice (200 µL/mouse). Tyrode's albumin buffer incubated with HA-hIgG (1 mg/mL) served as negative control.

### ***Flow cytometric analysis of platelets, leukocytes and their aggregates***

Flow cytometric analysis of NASA study patients: As recommended by International/National Guidelines (40-42) on management of perioperative AHR, a venous blood sample was collected 30 minutes following the onset of the AHR. It should be noted that platelets analysis was not an original objective of the NASA study and that blood samples were taken using heparin as anti-coagulant. Platelet enumerations and assessment of CD62P expression were performed by flow cytometry. Diluted total human blood was incubated for 15 min at room temperature in the dark in the presence of anti-CD61 antibodies, together with anti-CD62P antibodies or its isotype control (mIgG1). Mouse blood cells or washed human or mouse platelets were stained with indicated fluorescently-labeled mAbs for 15 minutes at room temperature. In mouse whole blood, single platelets were defined as  $FSC^{lo}/CD41^+$  cells, neutrophils as  $CD45^+/CD11b^+/Ly6G^{hi}$  cells and  $Ly6C^{hi}$ -monocytes as  $CD45^+/CD11b^+/Ly6G^{lo}/Ly6C^{hi}$  cells. Within these populations, aggregates were identified using  $CD41^+$  as a marker. Platelet activation of single platelets and on aggregates was assessed by CD62P expression and neutrophil activation by CD62L shedding.

### ***Statistical analysis***

Mouse data and experiments with human washed platelets were analyzed using one-way or two-way ANOVA with Tukey's post-test or unpaired t-test for comparison of two groups of measurements. A  $p$ -value less than .05 was considered significant: ( $*p < .05$ ;  $**p < .01$ ;  $***p < .001$ ;  $****p < .0001$ ). If not stated otherwise, data are represented as mean  $\pm$  SEM. Statistical

differences between different patient groups were assessed using a Kruskal-Wallis test with Dunn's correction for multiple testing (\$), differences between measures acquired at two different time points in the same patients were assessed using a Wilcoxon test (#). If not stated otherwise, data are represented as mean  $\pm$  SEM.

## Supplementary Materials

Supplementary Materials:

NASA study group

Fig. S1. Clinical signs of HA-hIgG anaphylaxis and platelet depletion using anti-GPIb $\alpha$  antibodies.

Fig. S2. Platelets associate with Ly6C<sup>hi</sup>-monocytes during IgG anaphylaxis and require Fc $\gamma$ RIIA expression for activation.

Fig. S3. Serotonin but not histamine concentration is augmented during HA-hIgG anaphylaxis.

Fig. S4. Generation of hFc $\gamma$ R<sup>KI</sup> mice.

Fig. S5. Expression of hFc $\gamma$ Rs in blood of humans or transgenic mice.

Fig. S6. Formation of platelet/neutrophil aggregates is dispensable for HA-hIgG anaphylaxis in hFc $\gamma$ R<sup>KI</sup> mice.

Fig. S7. Platelet gating in NASA study.

Fig. S8. Platelet-bound hIgG and hFc $\gamma$ RIIA expression on platelets *ex vivo* following HA-hIgG injection into hFc $\gamma$ RIIA<sup>tg</sup> mice.

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## Figure Legends:

**Fig. 1. Platelets are required for hFcγRIIA-induced anaphylaxis.** (A/B) Change in body temperature (left) and platelet count (right) during HA-hIgG-anaphylaxis in hFcγRIIA<sup>tg</sup> (circles, n=4) and FcγR<sup>null</sup> (squares, n=4) mice. (C) HA-hIgG-anaphylaxis-induced changes in body temperature in hFcγRIIA<sup>tg</sup> (circles) and FcγR<sup>null</sup> (squares) mice (n=4), pretreated with anti-GPIIbα antibody (red symbols) or isotype control (black symbols), (n=4). (D) Platelet counts in hFcγRIIA<sup>tg</sup> (circles) and FcγR<sup>null</sup> (squares) mice, pretreated (green symbols, n=5) or not (open symbols, n=4 or 5) with romiplostim. (E) Change in body temperature (left) and survival (right) during HA-hIgG-anaphylaxis in hFcγRIIA<sup>tg</sup> (circles) and FcγR<sup>null</sup> (squares) mice pretreated (green symbols) or not (open symbols) with romiplostim, n=3. ↓: Death of all romiplostim-treated hFcγRIIA<sup>tg</sup> mice before the first temperature read-out. (A-E) Temperature data are represented as mean ± SEM, platelet counts from individual mice are indicated together with the mean ± SEM. Data in (A-E) are representative of at least 2 independent experiments. \*P<0.05, \*\*P<0.01, \*\*\*P<0.001, \*\*\*\*P<0.0001.

**Fig. 2. hFcγRIIA-expressing platelets are sufficient to induce anaphylaxis in resistant mice.** (A) Maximal aggregation (%) of washed platelets from hFcγRIIA<sup>tg</sup> mice compared to baseline and (B) expression of platelet activation markers following incubation with indicated agonist. Data are pooled from three independent experiments. (C-F) hFcγRIIA<sup>tg</sup> (circles) and FcγR<sup>null</sup> (squares) mice were challenged or not with HA-hIgG; n=4. (C/D) ΔGeoMean CD62P expression (C) on single platelets and (D) within platelet-neutrophil aggregates, (E) percentage of neutrophils associated to CD41<sup>+</sup>-platelets and (F) ΔGeoMean CD62L expression on single neutrophils. (G/H) Indicated groups of mice were pretreated with anti-CD62P mAb or isotype control (black circles, n=4) and challenged or not with HA-hIgG; all groups n=4. (G) Percentage of neutrophils associated to CD41<sup>+</sup>-platelets 30 minutes post challenge and (H) changes in body temperature over time. (I) Washed platelets from hFcγRIIA<sup>tg</sup> mice (red squares, left panel) or from FcγR<sup>null</sup>

mice (grey squares, right panel), or Tyrode's albumin buffer (TyAlb) alone (open squares), were transferred intravenously into  $Fc\gamma R^{null}$  mice prior to challenge with HA-hIgG (all groups, n=3).  $hFc\gamma RIIA^{tg}$  mice receiving i.v. injection of TyAlb followed by challenge with HA-hIgG served as a positive control (open circles, n=5). Changes in body temperature were recorded. (C-I) Data are representative of at least 2 independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

**Fig. 3. Platelet-released serotonin contributes to hypothermia in mice undergoing HA-hIgG-anaphylaxis.** (A) Releasates (Rel.) from HA-hIgG-activated washed platelets from  $hFc\gamma RIIA^{tg}$  mice left untreated (black squares, n=6), treated with PCPA (red squares, n=8) or HA-hIgG-containing Tyrode's albumin buffer alone (open squares, n=9) were transferred intravenously into  $Fc\gamma R^{null}$  mice and changes in body temperature were recorded. (B) Changes in body temperature during HA-hIgG-anaphylaxis in  $hFc\gamma RIIA^{tg}$  (circles) and  $Fc\gamma R^{null}$  (squares) mice, pretreated with PCPA (black symbols, n $\geq$ 8) or vehicle control (open symbols n $\geq$ 8). (C) Platelet counts from the experiment represented in panel B 70 minutes post anaphylaxis induction. (D) Changes in body temperature in WT mice injected intravenously with 0  $\mu$ g (n=4), 20  $\mu$ g (n=3) or 100  $\mu$ g (n=3) of serotonin. Data are representative of 3 independent experiments. (A-C) Data are pooled from 3 (A) or 2 (B/C) independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ .

**Fig. 4. Platelets contribute to IgG-anaphylaxis in mouse models reproducing human  $Fc\gamma R$  complexity.** (A-C) Change in body temperature (left panels) and platelet count (right panels) during HA-hIgG- anaphylaxis in (A)  $hFc\gamma R^{KI}$  (triangles, n=4) and  $Fc\gamma R^{null}$  (squares, n=4) mice. (B)  $hFc\gamma R^{KI}$  mice pretreated with anti- $hFc\gamma RIIA$  mAb (red triangles, n=4) or isotype control (black triangles, n=4). (C)  $hFc\gamma R^{KI}$  pretreated with anti-GPIIb $\alpha$  antibody (red triangles, n=4) or isotype control (black triangles, n=4) and  $Fc\gamma R^{null}$  mice pretreated with anti-GPIIb $\alpha$  antibody (red squares, n=4). (A-C) Temperature data are represented as mean  $\pm$  SEM, platelet counts from

individual mice are indicated together with the mean  $\pm$  SEM. Data are representative of at least 2 independent experiments. \*P<0.05, \*\*\*P<0.001, \*\*\*\*P<0.0001.

**Fig. 5. Releasate from HA-hIgG-activated human platelets induces hypothermia in WT mice. (A)**

Direct activation of washed human platelets by HA-hIgG *in vitro*. Maximal aggregation (%) of stimulated washed human platelets compared to baseline and (B/C) % of platelets expressing the activation markers CD62P (B) and activated  $\alpha$ IIb $\beta$ 3 integrin (C) following *in vitro* incubation with indicated stimuli. Data are pooled from 5 independent experiments. (D) Transfer of releasate from activated washed human platelets induces hypothermia. Releasate from washed human platelets activated with HA-hIgG (black diamonds, n=12), or HA-hIgG-containing Tyrode's albumin buffer (TyAlb) alone (open diamonds, n=8) was transferred intravenously into WT mice and changes in body temperature were immediately recorded. Data are pooled from 3 independent experiments. (E) Serotonin (n=4) and histamine (n=5) content in the releasate of HA-hIgG-stimulated washed human platelets. (A-E) Data are represented as mean  $\pm$  SEM and are representative of at least 3 independent experiments. \*P<0.05, \*\*\*P<0.001, \*\*\*\*P<0.0001.

**Fig. 6. Evidence for platelet activation during human drug-induced anaphylaxis. (A)**

Platelet counts in blood of individuals from the NASA study; presenting no (n=67), mild (n=37) or severe (n=30) anesthesia-induced anaphylaxis and the same severe patients during follow-up visit (n=30). (B) Platelet and (C) leukocyte counts of 4 cardiac surgery cases (black symbols) and their matched control (open symbols) pairs (identical symbols within a pair) from the NASA cohort indicated as percentage of baseline (left panels) and absolute counts (right panels). A smoothed trend line has been added to indicate average behavior of each group (2<sup>nd</sup> order polynomic, 3 neighbors: red, patients; green, controls). \*p<0.05, unpaired t-test of cases *versus* controls, considering all values in the 3-4 hours' interval (grey area). (D) CD62P-expressing platelets (left, percentage; right, geometric mean CD62P minus isotype staining) from the NASA study patients presenting no (n=73), mild (n=35) or severe (n=29) anesthesia-induced anaphylaxis or the same mild (n=35) or

severe (n=29) patients during follow-up visit. (**A/D**) Statistically significant differences between groups of anesthetized subjects: \$ P<0.05, \$\$\$\$ P<0.0001, and within a group of subjects comparing two different time points: ## P<0.01, ### P<0.001; ##### P<0.0001.