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"Unconventional CD147-dependent platelet activation elicited by SARS-CoV-2 in COVID-19": Reply

Dear Editor,

We thank Dr Wada and collaborators for their interest in our recent study[1] and we fully agree on the importance of the early detection of platelet activation in patients with COVID-19. So far, several biomarkers that reflect disease severity have been published and compared (see for example Lore and collegues[2]), but the upstream role of platelets in the natural history of the response to SARS-CoV-2[3] makes them a particularly relevant object of study. Platelet activation can be monitored with several approaches. For example, platelets can be studied directly, evaluating their granules content, the expression of P-selectin, the state of the integrins and the exposure of anionic phospholipids on the membrane[1,3–7] or evaluating them functionally via the study of their ability to form heterotypic aggregates with leukocytes[5–7] or by monitoring the kinetics of homotypic aggregation and release reaction.[4] All of these approaches are time consuming and labor intensive, and are difficult to apply in centers that are not equipped with state-of-the-art technology, equipment or facilities.

A second approach, used by Wada and collaborators,[8] is based on surrogate markers, that is, on the detection in plasma of molecules that could be released from activated platelets, with the idea that the higher the concentration of molecules released from platelets, the greater the extent of platelet activation. In their study, Wada and collaborators make a case for the role of C-type lectin-like receptor 2 (CLEC-2).

CLEC-2 is a type II transmembrane receptor expressed by platelets, which is involved in the activation of glycoproteins Ib alpha and VI during primary hemostasis favoring the interaction with collagen and laminin.[9,10] Wada and collaborators show that CLEC-2 concentration is increased in plasma of patients with COVID-19, even though they fail to detect significant differences in the levels of soluble CLEC-2 between patients with mild or severe COVID-19. This is surprising given the apparent upstream role that platelets play in COVID-19 (see above).

An important caveat associated with studying surrogate markers of platelet activation in the blood is the fact that we cannot be sure that platelets are their only source. CLEC-2, for example, is expressed as a dimer not only on platelets, but also on neutrophils, dendritic cells and Kupffer cells.[9] Cells belonging to all of these lineages are heavily involved in the pathophysiology of COVID-19. Therefore, the simple assessment of the total blood concentration of soluble CLEC-2 may only partially reflect platelet activation, thus confusing the interpretation of the results. A similar limitation applies to the evaluation of soluble P-selectin. CLEC-2 as well as P-selectin are both type C lectin receptors, expressed by activated platelets and which can be shed from the platelet surface due to action of ADAMt and metalloproteases.[9,10] The expression of P-selectin is less wide, since the only other source besides platelets are activated endothelial cells. However, soluble P-selectin by itself has been found to be a suboptimal marker in patients with COVID-19.[1]

A third approach on which we have relied is based on the study of extracellular vesicles. They can be easily retrieved from plasma and can be analyzed in any institution equipped with a flow cytometer. There are two main advantages to this approach, namely the fact that the bioactive molecules associated with extracellular vesicles have a prolonged lifespan and bioactivity, due to their relative protection from proteases and other inactivating factors in the environment.[5] Second, they can be studied for the expression of lineage-specific markers, thus revealing what their source is.

Platelet-derived extracellular vesicles can be for example traced by the expression of the *bona-fide* marker CD61 (GPIIIa). Figure 1 show that the concentration of total extracellular vesicles in the plasma of patients with COVID-19 and controls. Panel A clearly shows that extracellular vesicles dramatically accumulate in COVID-19. However, virtually all cells release extracellular vesicles, particularly if they are stressed or activated, as normally occurs in an acute inflammatory state. Indeed, the concentration of total extracellular vesicles does not provide clues to the clinical severity of the disease (Panel B). Furthermore, it is not significantly associated with factors known to be crucial for COVID-19, such as the concentration of C-reactive protein (CRP, Panel D), of D-dimer (Panel E), of lactic dehydrogenase (LDH, Panel F), nor is it different in those patients who will eventually suffer acute respiratory distress syndrome. In contrast, extracellular vesicles expressing the prototypical platelet-derived DAMP, HMGB1 represent a much better indicator of severity and predictor of clinical outcome (Panel G and Maugeri and colleagues[1]). We selected HMGB1 and not P-selectin since the latter is less represented in extracellular vesicles,[5–7] while the fundamental role of platelet HMGB1 in guiding intravascular immunity makes the signal particularly relevant.[1,5–7]

The concentration in the plasma of soluble HMGB1, although higher in patients with COVID-19 than in controls, failed to correlate

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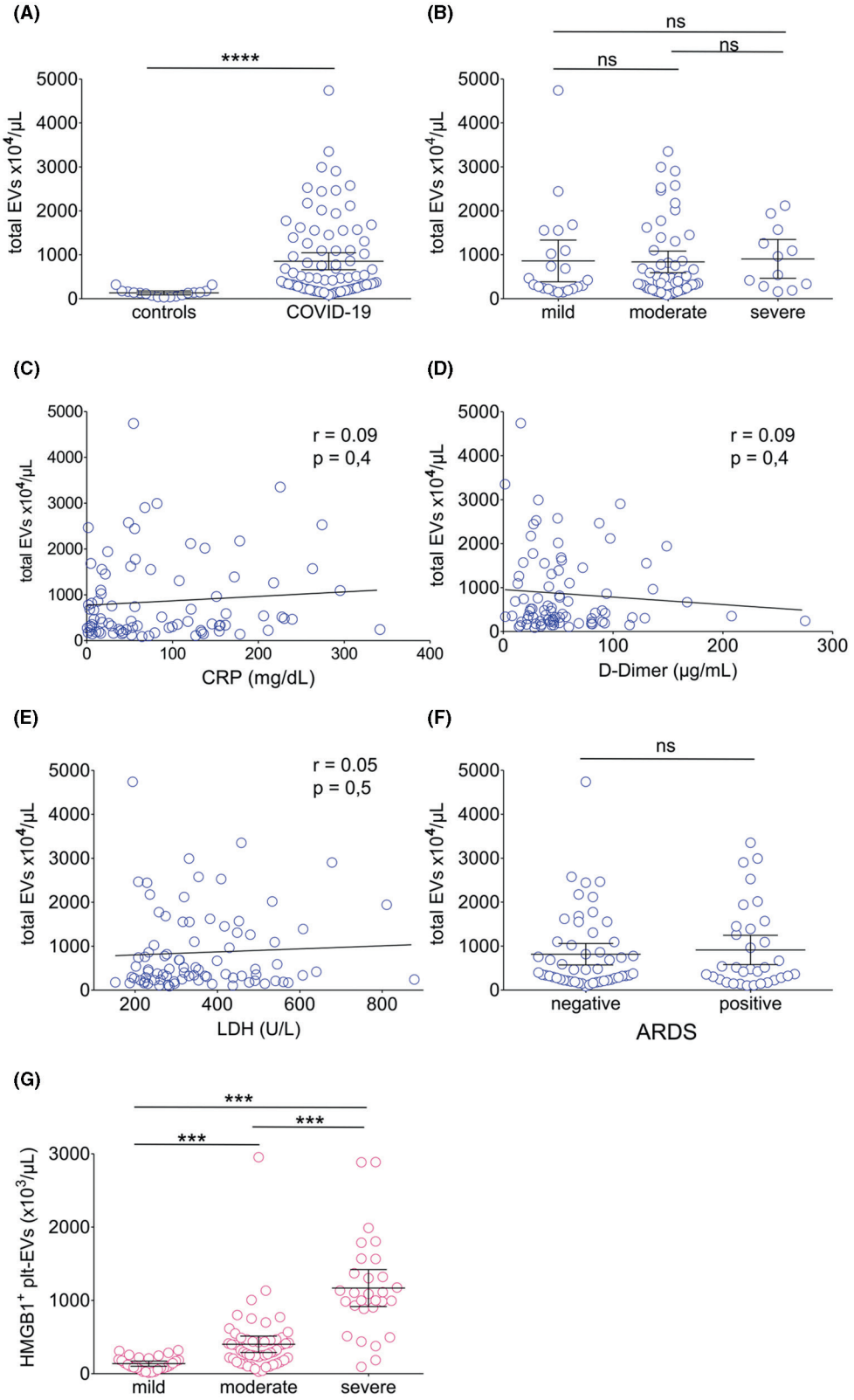

FIGURE 1 HMGB1+ plt-EVs concentration is associated with COVID-19 severity and predicts clinical outcomes. The concentration of total EV retrieved in the plasma of patients with COVID 19 are higher respect to healthy controls (A) and is not associated with the severity of disease (B). The concentration of total EV do not correlates with concentration of C-reactive protein (CRP; C), of D-dimer (D), of lactate dehydrogenase (LDH, E) neither is higher in patients who will develop acute respiratory distress syndrome (ARDS, F). On the contrary, the concentration of HMGB1+ plt-EV is significantly associated to disease severity and predict clinical outcomes (G). Symbols depict individual observations in each subject. ****$p < .0001$; ***$p < .001$.

with the severity of the disease.[2] Platelet-derived extracellular vesicles expressing HMGB1 thus better reflect the relevant events in COVID-19 than the total pool of soluble HMGB1 in the plasma. As suggested above, discussing the better performance of platelet-derived compared with unselected total extracellular vesicles, this possibly depends on the source of the molecule. Total plasma HMGB1 in fact does not derive from activated platelets only, but also from dying cells and activated inflammatory leukocytes.

A similar issue might apply to CLEC-2. Indeed, the moiety has been found to be associated with platelet-derived extracellular vesicles released from megakaryocytes and activated platelets.[10] Therefore, the platelet-derived CLEC-2 fraction expressed on extracellular vesicles may represent a better biomarker of COVID-19 severity than the concentration of the soluble molecule in plasma, since quantification of proteins using ELISA does not identify the cellular origin of the molecule assessed.

Altogether, the search for biomarkers of platelet activation must continue. A consensus on the requirements that a molecule would have to meet to be defined as a platelet activation biomarker would go a long way in highlighting the most valuable signals for clinical and pathogenetic studies, both in patients with COVID-19 and in vaccinated patients who develop thromboembolic events. These requirements should, in our opinion, include the clear demonstration of the platelet selectivity of these molecules and of their biological relevance, as revealed by their association with disease outcome.

AUTHOR CONTRIBUTIONS

Both authors contribute equally.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

ETHICS STATEMENT

All patients belonged to the COVID-19 San Raffaele clinical-biological cohort (Covid-BioB, ClinicalTrials.gov NCT04318366). The study conforms to the declaration of Helsinki and obtained ethical approval from the Institutional Review Board (protocol number 34/int/2020). Written informed consent was obtained by all patients.

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