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ORIGINAL ARTICLE

Platelet and immune signature associated with a rapid response to the BNT162b2 mRNA COVID-19 vaccine

Davide Flego[1] | Simone Cesaroni[1] | Giulio F. Romiti[1] | Bernadette Corica[1] | Ramona Marrapodi[1] | Noemi Scafa[1] | Francesca Maiorca[1] | Ludovica Lombardi[1] | Davide Pallucci[1] | Fabio Pulcinelli[2] | Valeria Raparelli[3,4] | Marcella Visentini[1] | Roberto Cangemi[1] | Silvia Piconese[5] | Domenico Alvaro[1] | Antonella Polimeni[6] | Stefania Basili[1] | Lucia Stefanini[1] | Vax-SPEED-IT Study Group

[1]Department of Translational and Precision Medicine, Sapienza University of Rome, Rome, Italy

[2]Department of Experimental Medicine, Sapienza University of Rome, Rome, Italy

[3]Department of Translational Medicine, University of Ferrara, Ferrara, Italy

[4]Faculty of Nursing, University of Alberta, Edmonton, Alberta, Canada

[5]Department of Internal Clinical Sciences, Anaesthesiology and Cardiovascular Sciences, Sapienza University of Rome, Rome, Italy

[6]Department of Oral and Maxillo-Facial Sciences, Sapienza University of Rome, Rome, Italy

Correspondence
Lucia Stefanini, Department of Translational and Precision Medicine, Sapienza University of Rome, Viale dell'Università, 37 - 00185 Rome, Italy.
Email: lucia.stefanini@uniroma1.it

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Abstract

Background: A rapid immune response is critical to ensure effective protection against COVID-19. Platelets are first-line sentinels of the vascular system able to rapidly alert and stimulate the immune system. However, their role in the immune response to vaccines is not known.

Objective: To identify features of the platelet-immune crosstalk that would provide an early readout of vaccine efficacy in adults who received the mRNA-based COVID-19 vaccine (BNT162b2).

Methods: We prospectively enrolled 11 young healthy volunteers (54% females, median age: 28 years) who received two doses of BNT162b2, 21 days apart, and we studied their platelet and immune response before and after each dose of the vaccine (3 and 10 ± 2 days post-injection), in relation to the kinetics of the humoral response.

Results: Participants achieving an effective level of neutralizing antibodies before the second dose of the vaccine (fast responders) had a higher leukocyte count, mounted a rapid cytokine response that incremented further after the second dose, and an elevated platelet turnover that ensured platelet count stability. Their circulating platelets were not more reactive but expressed lower surface levels of the immunoreceptor tyrosine-based inhibitory motif (ITIM)-coupled receptor CD31 (PECAM-1) compared to slow responders, and formed specific platelet-leukocyte aggregates, with B cells, just 3 days after the first dose, and with non-classical monocytes and eosinophils.

Conclusion: We identified features of the platelet-immune crosstalk that are associated with the development of a rapid humoral response to an mRNA-based vaccine (BNT162b2) and that could be exploited as early biomarkers of vaccine efficacy.

Flego and Cesaroni equally contributed to the study.

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KEYWORDS
COVID-19, immunity, platelet activation, platelet count, vaccines

1 | INTRODUCTION

Coronavirus disease 2019 (COVID-19) is a systemic, potentially life-threatening disease, triggered by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). To date, 340 million cases and more than 5 million deaths have been attributed to SARS-CoV-2 worldwide (https://covid19.who.int/). Challenged by this public health emergency, the scientific community developed several vaccines within less than 12 months, an unprecedented achievement for modern medical science. All licensed vaccines employ novel antigen-delivery platforms. Among these, BNT162b2 (Pfizer-BioNTech) and mRNA-1273 (Moderna) represent the first broad-scale application of the mRNA-based vaccine technology.[1] When administered with a two-dose regimen, they have been shown to provide a 95% protection against COVID-19 in persons 16 years of age or older.[2,3] Studies in humans and mice have shown that a rapid humoral response is critical to ensure protection against COVID-19.[4,5] However, the determinants influencing the kinetics and efficacy of the vaccine-induced immune response in a real-world setting are still under active investigation.

After erythrocytes, platelets are the most prevalent blood component and the first to encounter immune response sites.[6] Best known for their role in thrombosis and hemostasis, platelets actively participate in the immune response through the regulated expression of adhesive and immune receptors on their surface and the release of several mediators.[7] During bacterial and viral infections, platelets can rapidly alert the immune system, stimulate innate effector cells, accelerate leukocyte migration to immune response sites, modulate antigen presentation, and enhance adaptive immune responses.[8-10] However, it is not known whether platelets contribute to the immune response after vaccinations.

In this study, we aimed to characterize the early platelet and immune response after the administration of the BNT162b2 mRNA COVID-19 vaccine, with the hypothesis that biomarkers of the platelet-leukocyte crosstalk could be an early readout of vaccine efficacy. Therefore, we longitudinally characterized the platelet and immune phenotype of young healthy volunteers before and after the vaccination and stratified them based on their antibody response kinetics. We found that study participants achieving an effective level of neutralizing antibodies before the second injection (fast responders), compared to slow responders, mounted a faster inflammatory response that sustained the platelet turnover, expressed less CD31 (PECAM-1) on the platelet surface, and formed specific platelet-leukocyte aggregates shortly after the injection. Thus, our data identify platelet indices that discriminate individuals able to establish a strong immune response after only one dose and that could be exploited to optimize future vaccine design and administration.

Essentials

- The determinants of the antibody response rate to mRNA vaccines in healthy adults are not known.
- The study identifies early indices associated with the response rate to the BNT162b2 vaccine.
- Fast responders have a rapid cytokine response, fast platelet turnover, and lower platelet CD31.
- Fast responders display platelet-B cell aggregates as early as 3 days after the first dose.

2 | METHODS

2.1 | Study population

We prospectively enrolled 11 healthy adults over 18 years old, without signs of infection or significant comorbidities, scheduled for vaccination with the COVID-19 mRNA-based BNT162b2 vaccine (Pfizer-BioNTech) at our institution (Sapienza University Hospital Policlinico Umberto I, Rome, Italy). We excluded individuals with severe immunosuppression, who were pregnant or breastfeeding, and subjects who underwent recent transfusions of platelets or plasma and that used antiplatelet or anticoagulant medications in the 10 days before enrolment. As controls we included 4 subjects that had been vaccinated at least 3 months prior to the study. All participants gave written informed consent. At baseline, each participant completed a short questionnaire on previous relevant medical history, smoking, and previous known history of exposure to SARS-CoV-2. The study was approved by the Ethics Committee of the Policlinico Umberto I of Rome (ClinicalTrials.gov Identifier: NCT04844632).

2.2 | Blood sampling and storage

Venous blood samples were taken at the following timepoints: 0–1 day before the first dose of the vaccine; 3 days after the first dose of vaccine administration; 10 ± 2 days after the first dose of vaccine administration; 0–1 day before the second dose of vaccine (i.e., 20–21 days after first dose); 3 days after the second dose of vaccine administration (i.e., 24 days after first dose); 10 ± 2 days after the second dose of vaccine administration (i.e., 28–31 days after first dose). Sodium-citrate anti-coagulated whole blood was used for platelet functional and phenotypical analysis. EDTA-anticoagulated blood was used to determine cell counts and the platelet morphological parameters with a Sysmex KX-21N

(Sysmex Corp.). Serum and plasma samples were stored at −80°C until batch analysis.

2.3 | Detection of circulating SARS-CoV-2 neutralizing antibodies

Serum SARS-CoV-2 neutralizing antibodies were detected using COVID-19 Immunorank™ MICRO-ELISA (Leinco Technologies) and the neutralization index, expressed as percent, was calculated following the manufacturer's specifications. The experiment was done in duplicate for each subject. We defined fast responders and slow responders the subjects that reached a neutralization index above or below the median of the overall cohort (43%), respectively, 20 days after the first vaccine (0–1 days before the second dose of vaccine).

2.4 | Platelet--leukocyte aggregate quantification

Within 15 min from blood withdrawal, 50 µl of whole blood was incubated with fluorochrome-conjugated monoclonal antibodies α-CD66b-PE, α-CD56-PEDazzle594, α-CD19-PerCPCy5.5, α-CD16-PECy7, α-CD14-APC, α-CD4-Alexa700, α-CD25-Bv421, α-CD3 Bv510, α-CD8 Bv605 (all from Sony Biotechnology Inc.) and α-CD41-BB515 (BD Biosciences). After 15 min of incubation, red cell lysis was performed with BD FACS Lysing Solution (BD Biosciences). At least 20,000 events in the singlet gate were acquired. Fluorescence-activated cell sorting (FACS) analysis was conducted with BD LSR Fortessa and the data were analyzed with FlowJo, version 10.7.1 (FlowJo LLC). Fluorescence-minus-one controls were performed to ensure proper gating. The complete gating strategy to identify the leukocyte subsets is specified in Figure S1. The absolute count of each leukocyte subtype was determined by multiplying the total white blood cell count by the differential percentage for that cell type based on the flow cytometry analysis. Platelet--leukocyte aggregates (PLA) were identified based on the expression of CD41a in the individual leukocyte subpopulations and expressed as fold increase over baseline before vaccine administration.

2.5 | Platelet phenotypic and functional analysis

Platelet analyses were performed within 30 min from blood withdrawal in whole blood anticoagulated with sodium-citrate and diluted 1:10 with Tyrode's buffer, containing mmol/L (137 NaCl, 0.34 Na_2HPO_4, 2.9 KCl, 12 $NaHCO_3$, 10 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 5 D-glucose, 0.35% bovine serum albumin) pH 7.3. To assess changes in the expression of surface receptors, platelets were labelled with saturating concentrations of α-CD41-APC in combination with α-CD31-FITC or α-GPVI-PE or α-CD42-PE (all from BD Biosciences) and the expression of each receptor was quantified based on the median fluorescence intensity among $CD41^+$ events. To monitor the activation status of circulating platelets, diluted blood was labelled for 10 min with α-CD62P-PE (BD Biosciences) in the presence or absence of 10ng/ml convulxin. All samples were fixed with phosphate-buffered saline 1% paraformaldehyde. FACS analysis was conducted on a BD Accuri C6 Plus and the data were analyzed with FlowJo, version 10.7.1 (for complete gating strategy see Figure S2).

2.6 | Cytokine array

Plasmatic cytokine concentrations were assessed using the LEGENDplex™ bead-based immunoassay (Inflammatory cytokines panel I; BioLegend) following manufacturer's specifications. Plasma samples were analyzed in batch, in double. Briefly, 12.5 µl of plasma was diluted 1:1 in the assay buffer and incubated with fluorescence-encoded beads and cytokine-specific antibodies, which allow simultaneous quantification of 13 human inflammatory cytokines and chemokines, including interleukin (IL)-1β, interferon (IFN)-α2, IFN-γ, tumor necrosis factor (TNF)-α, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23, IL-33, and monocyte chemoattractant protein (MCP)-1. Six thousand events in the FSC/SSC gate that identified the beads were acquired with a BD Accuri C6 Plus and the data were analyzed with the LEGENDplex software, version 2020.12.15. The concentration of each cytokine expressed in picogram/milliliter (pg/ml) was calculated based on the fluorescence intensity of each bead by sigmoidal interpolation of a standard curve.

2.7 | Statistical analysis

Categorical variables were expressed as counts and percentages. Continuous variables were reported as median and interquartile range. Appropriate two-tailed non-parametric tests were used to evaluate differences between individual timepoints of the study (Mann-Whitney test for fast vs. slow responders, Friedman test followed by Dunn's multiple comparison test or Wilcoxon-signed rank test for paired comparisons). To assess which parameters correlated the most with the antibody response we conducted Spearman rank Correlations. A two-sided *P*-value < .05 was considered statistically significant. All analyses were performed using GraphPad Prism 9, version 9.1.0.

3 | RESULTS

3.1 | Different antibody response rates in BNT162b2 mRNA vaccine recipients

We enrolled 11 young, healthy volunteers (mean age ± standard deviation [SD]: 28.7 ± 1.4 years, 54% females) who received two doses of BNT162b2, 21 days apart, and we studied their platelet and

immune response before and after each dose of the vaccine (3 and 10 ± 2 days post-injection). Within 1 month from the first dose, all subjects presented high levels of neutralizing antibodies against the SARS-CoV-2 spike protein. We observed differences in the kinetics of the antibody response, with six participants showing high levels of neutralizing antibodies before administration of the second dose (fast responders, 54%) and five participants developing comparable antibody levels only 10 days after the second dose (slow responders, 46%; Figure 1). We excluded that different response rates were due to previous SARS-CoV2 infections because neutralizing antibodies were undetectable at baseline and none of the study participants reported to have contracted COVID-19 prior to the study.

3.2 | Short-term immune response after the BNT162b2 mRNA vaccine

The most notable short-term response after the injections of BNT162b2 mRNA vaccine was the increase of the absolute monocyte count, which reached statistical significance after the second dose (Figure 2A). Detailed peripheral blood immunophenotyping by multiparameter flow cytometry revealed the expansion of all three monocyte subpopulations within 3 days from each injection, with an incremental effect between the first and the second dose (Figure 2B and Figure S3). The fold increase was greater for non-classical ($CD14^{dim}$ $CD16^{+}$) and intermediate ($CD14^{+}$ $CD16^{+}$) monocytes compared to classical ($CD14^{+}$ $CD16^{-}$) monocytes. Concomitantly we detected a progressive reduction in the absolute count of B cells ($CD19^{+}$ $CD3^{-}$) and natural killer T (NKT)-like cells ($CD56^{+}$ $CD3^{+}$) that reached statistical significance 3 days after the second dose, a transient drop in the regulatory T (Treg, $CD3^{+}CD4^{+}CD25^{high}$) cell count 10 days after the first dose, and a modest increase of the $CD8^{+}$ T cell ($CD3^{+}CD8^{+}$) count 10 days after the second dose (Figure S3).

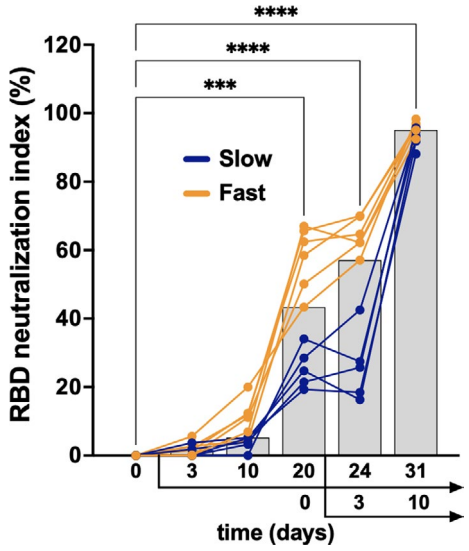

FIGURE 1 Antibody response following the BNT162b2 mRNA COVID-19 vaccine. Serum levels of neutralizing antibodies against the receptor binding domain (RBD) domain of the Sars-CoV2 spike protein, before (0) and after vaccine administration, in fast responders (orange dots and lines; $n = 6$) and slow responders (blue dots and lines; $n = 5$), expressed as percentage of neutralization index. Arrows indicate the measurements performed after the first and the second vaccine injection. Bars show the median at each time point for all study participants ($n = 11$). Two-tailed Friedman test and Dunn's multiple comparisons test. ***$P < 0.001$ ****$P < 0.0001$

When we stratified the subjects based on their antibody response, we observed that slow responders had lower absolute white blood cell counts, compared to the fast responders (Figure 2C). The most significant differences were detected among lymphocytes. Even before the vaccination, fast responders had a higher $CD4^{+}$ T cell ($CD4^{+}$ $CD3^{+}$) count and less circulating NKT cells and this difference persisted after the vaccination. After vaccination, NK cells ($CD56^{+}$ $CD3^{-}$) increased in fast responders but decreased among slow responders. Moreover, in the fast responders the B cell count expanded 3 days after the first injection and then declined gradually, while in the slow responders the B cell count was steady until day 20 and then dropped to levels comparable to the fast responders only after the second injection.

We observed a similar lag time in the cytokine response of the two groups of subjects (Figure 3A and Figure S4). Fast responders experienced increased plasmatic cytokine levels 3/10 days after the first dose and 10 days after the second dose of the vaccine. Slow responders reached their maximum cytokine concentrations 20/24 days after the first dose of the vaccine. To assess which cytokines correlated the most with the antibody response we conducted a Spearman rank correlation at each timepoint (Figure S5). We observed a significant positive correlation between the antibody response at day 20 post-vaccination, timepoint at which we measured the greatest difference between slow and fast responders, and the fold change of IL-1β. The positive correlation was detectable very early (3 days) and was highly significant 10 days after the first injection (antibody response and IL-1β on day 10: $r = 0.76$, $P = 0.009$; Figure 3B). Ten days after the second injection we found that the antibody response rate correlated with a different cytokine signature featuring IL-10, IL-17a, and IL-23 (antibody response and IL-10, day 31: $r = 0.82$, $P = 0.003$; antibody response and IL-17a, day 31: $r = 0.84$, $P = 0.002$; antibody response and IL-23, day 31: $r = 0.69$, $P = 0.023$; Figure S5D).

3.3 | Platelet turnover following the BNT162b2 mRNA vaccine

The median platelet count did not change significantly during the observation period, except for a minimal downward fluctuation 3 days after the first dose (Figure 4A). Despite being within the normal range for healthy subjects, the platelet morphological parameters increased significantly by day 10 post-vaccination, with a maximum platelet size (mean platelet volume [MPV] and platelet large cell ratio [P-LCR]) after 20 days (Figure 4B, C), and a peak in

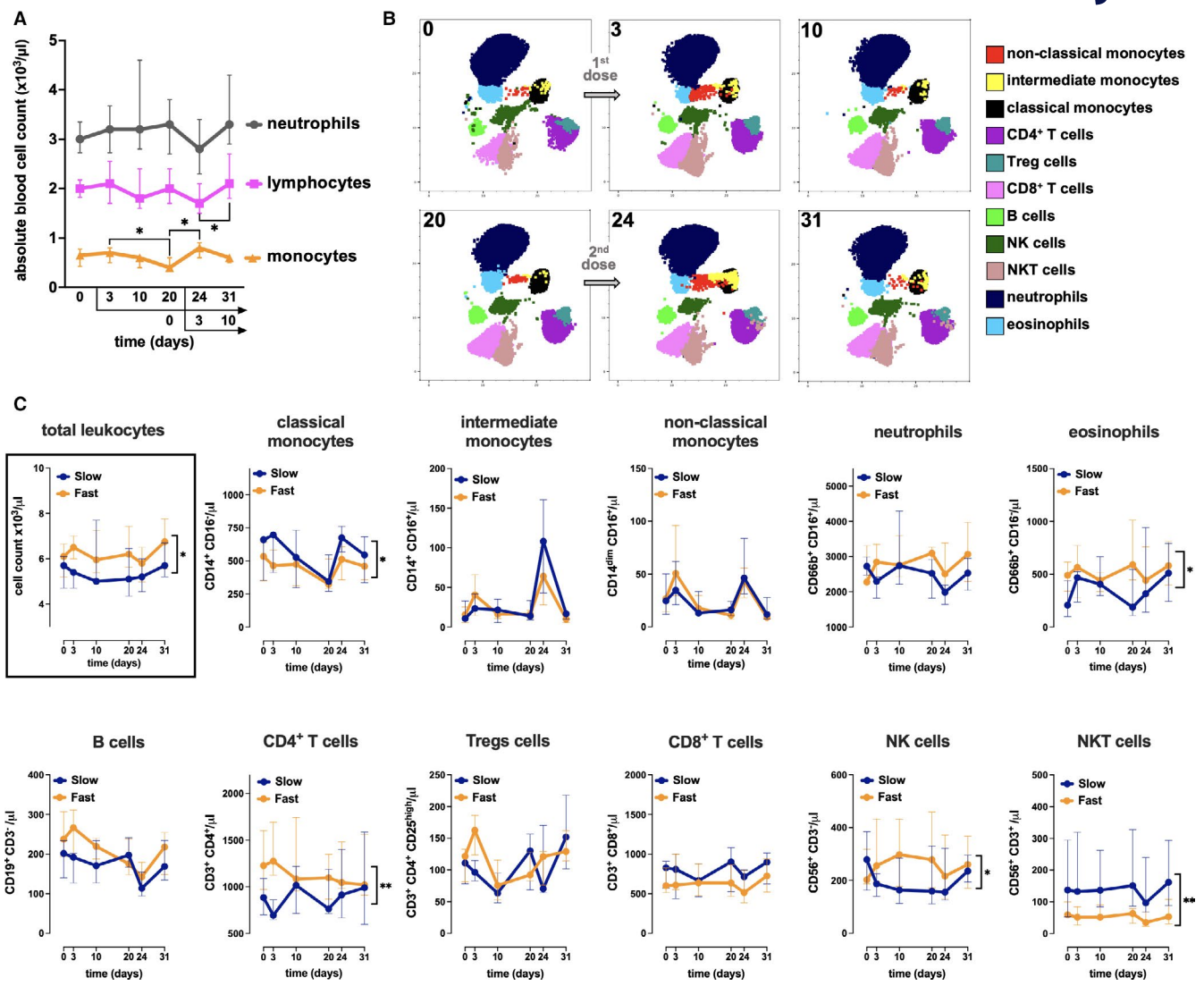

FIGURE 2 Short-term immune response following the BNT162b2 mRNA COVID-19 vaccine. A, Absolute monocyte (orange), lymphocyte (pink), and neutrophil (gray) count, before and after each dose of the vaccine (arrows indicate when injections were performed) measured with a Sysmex KX-21N Hematology Analyser. B, Representative Uniform Manifold Approximation and Projection (UMAP) dot plot of leukocyte subpopulations before (0) and after vaccine injections (days after immunization specified in the top left corner). C, Absolute count of total leukocytes (rectangular insert) and of the leukocyte subpopulations in slow responders (blue) versus fast responders (orange) as detected by multiparameter flow cytometry on a BD LSR Fortessa and analyzed with FlowJo software, version 10.7.1. Shown is median ± interquartile range. Two-tailed Friedman test and Dunn's multiple comparisons test for intragroup analysis, Mann-Whitney test for intergroup analysis. $^{*}P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$ $^{****}P < 0.0001$

platelet size variability (platelet distribution width [PDW]) after 24 days (Figure 4D). Because repetitive blood draws, as performed in this study, could in theory produce similar results, we monitored the blood count of four control subjects who had been vaccinated at least 3 months before the blood draws. Only the participants vaccinated immediately before the study showed an increased platelet size (Figure S6), which could be indicative of increased platelet turnover.

MPV and P-LCR showed a significant positive correlation with the antibody response 10 days after the first dose (antibody response and MPV, day 10: $r = 0.66$, $P = 0.032$; antibody response and P-LCR, day 10: $r = 0.73$, $P = 0.014$; Figure 4I and Figure S7). Among subjects with a fast humoral response, platelet size increased as early as 10 days post-vaccination, and this change was not accompanied by a change in platelet count (Figure 4E–H), indicating that platelet consumption and platelet production were balanced. In the slow responders, the increase in platelet size was delayed and reached the maximum 20 days post-vaccination. In this group of subjects, we observed a small but significant drop in the platelet count shortly after the vaccination, which may suggest that an increase in platelet consumption was not counterbalanced by a timely increase in platelet production. Interestingly, there was correspondence between the timing of the platelet size changes and that of the cytokine variations and we detected a strong positive association (Figure S8) between the platelet size parameters and IL-1β (IL-1β and MPV, day 10: $r = 0.78$, $P = 0.007$; IL-1β and P-LCR, day 10: $r = 0.84$, $P = 0.002$).

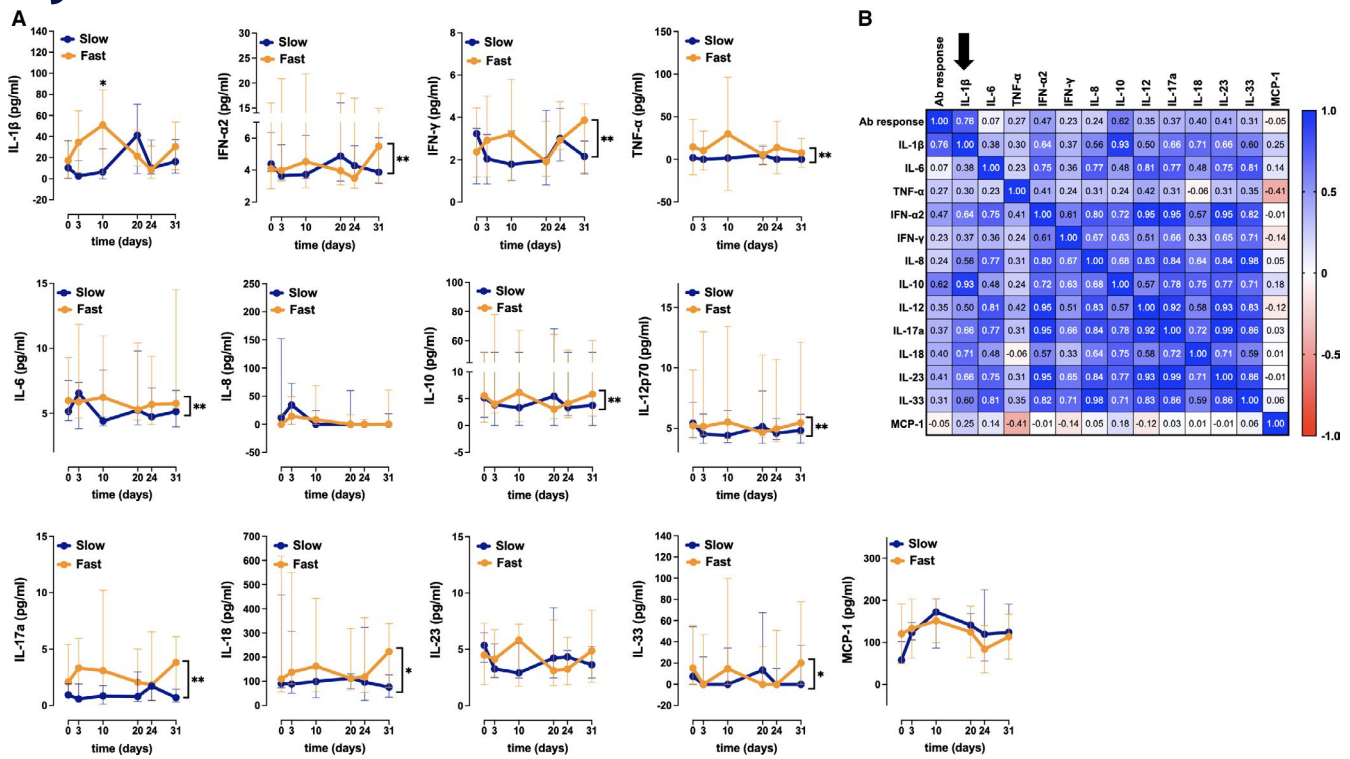

FIGURE 3 Cytokine response following the BNT162b2 mRNA COVID-19 vaccine. A, Plasmatic concentrations (pg/ml) of 13 inflammatory cytokines/chemokines measured by multiple bead assay of fast (orange) versus slow (blue) responders before (0) and (3, 10, 20, 24, 31 days) after vaccine administration. Shown is median ± interquartile range. Mann-Whitney test for intergroup analysis. $^{*}P < 0.05$, $^{**}P < 0.01$. B, Spearman rank correlation matrix between the antibody response (Ab response) and the fold increase of each cytokine 10 days after the first dose compared to pre-vaccine levels. In each box is indicated the correlation coefficient r, which ranges from +1 (positive correlation, blue) to −1 (negative correlation, red). r is indicated in white fonts when the correlation is statistically significant ($P < 0.05$). The arrow highlights that the fold increase of the plasmatic concentration of interleukin (IL)-1β significantly correlates with the antibody response

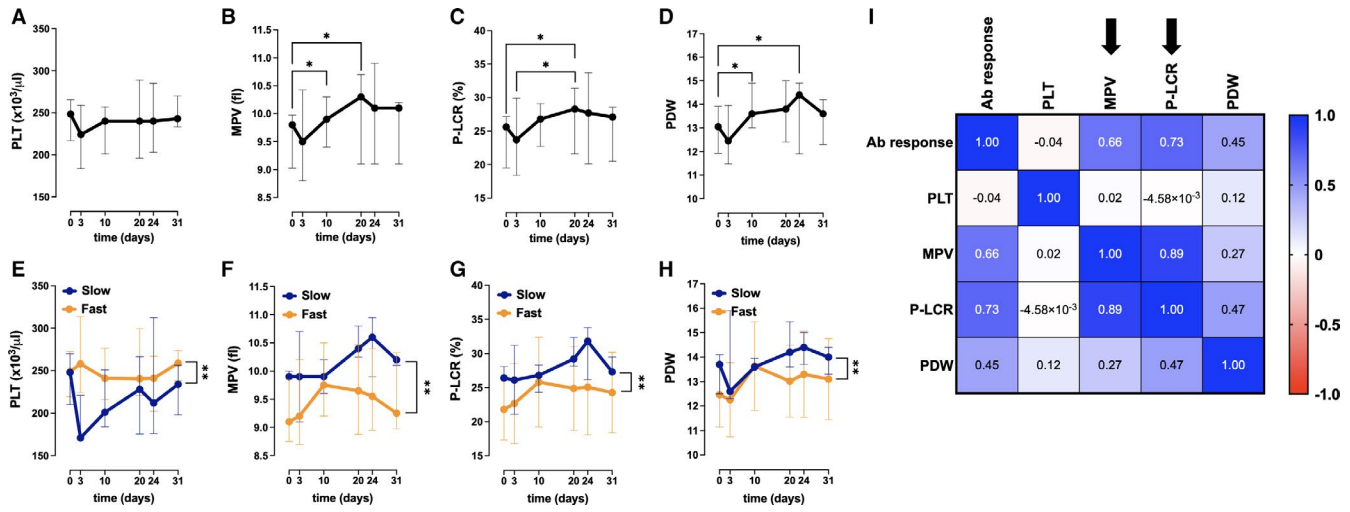

FIGURE 4 Platelet turnover following the BNT162b2 mRNA COVID-19 vaccine. A,E, Platelet count (PLT), (B,F) mean platelet volume (MPV), (C,G) platelet large cell ratio (P-LCR, expressed as percentage), and (D,H) platelet distribution (PDW) measured with a Sysmex KX-21N Hematology Analyser before (0) and (3, 10, 20, 24, 31 days) after vaccine administration in all participants (black) and in slow responders (blue) versus fast responders (orange). Shown is median ± interquartile range. Two-tailed Friedman test, Dunn's multiple comparisons test for intragroup analysis, Mann-Whitney test for intergroup analysis. $^{*}P < 0.05$ $^{**}P < 0.01$. I, Spearman rank correlation matrix between the antibody response (Ab response) and the fold change of platelet-related hematological parameters 10 days after the first dose compared to pre-vaccine levels. In each box is indicated the correlation coefficient r, that ranges from +1 (positive correlation, blue) to −1 (negative correlation, red). r is indicated in white fonts when the correlation is statistically significant ($P < 0.05$). The arrows highlight that the fold increase of MPV and P-LCR significantly correlates with the antibody response

3.4 | Platelet phenotype following the BNT162b2 mRNA vaccine

Following vaccination, we observed that on circulating platelets the surface expression level of integrin αIIb (CD41a; Figure 5A) and of the immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptor glycoprotein (GP)VI (Figure 5B) decreased by day 20 and remained downregulated throughout the study, in a reciprocal manner to MPV. Conversely, the expression of the immunoreceptor tyrosine-based inhibitory motif (ITIM)-coupled receptor CD31 (PECAM-1) transiently increased on day 10 (Figure 5C). The surface abundance of glycoprotein Ibα (GPIbα, CD42b), fluctuated around the median of the control group (Figure S9) except for some reduction around day 10 (Figure 5D), possibly due to proteolytic cleavage or internalization.[11]

Although fast and slow responders experienced the same vaccine-induced phenotypical changes (Figure 5E–H), slow responders expressed significantly higher levels of the inhibitory receptor CD31 on their platelet surface compared to fast responders and CD31 surface expression negatively correlated (Figure S10) with the antibody response rate (antibody response and CD31, day 10, $r = -0.58$, $P = 0.06$). Moreover, we observed that the surface

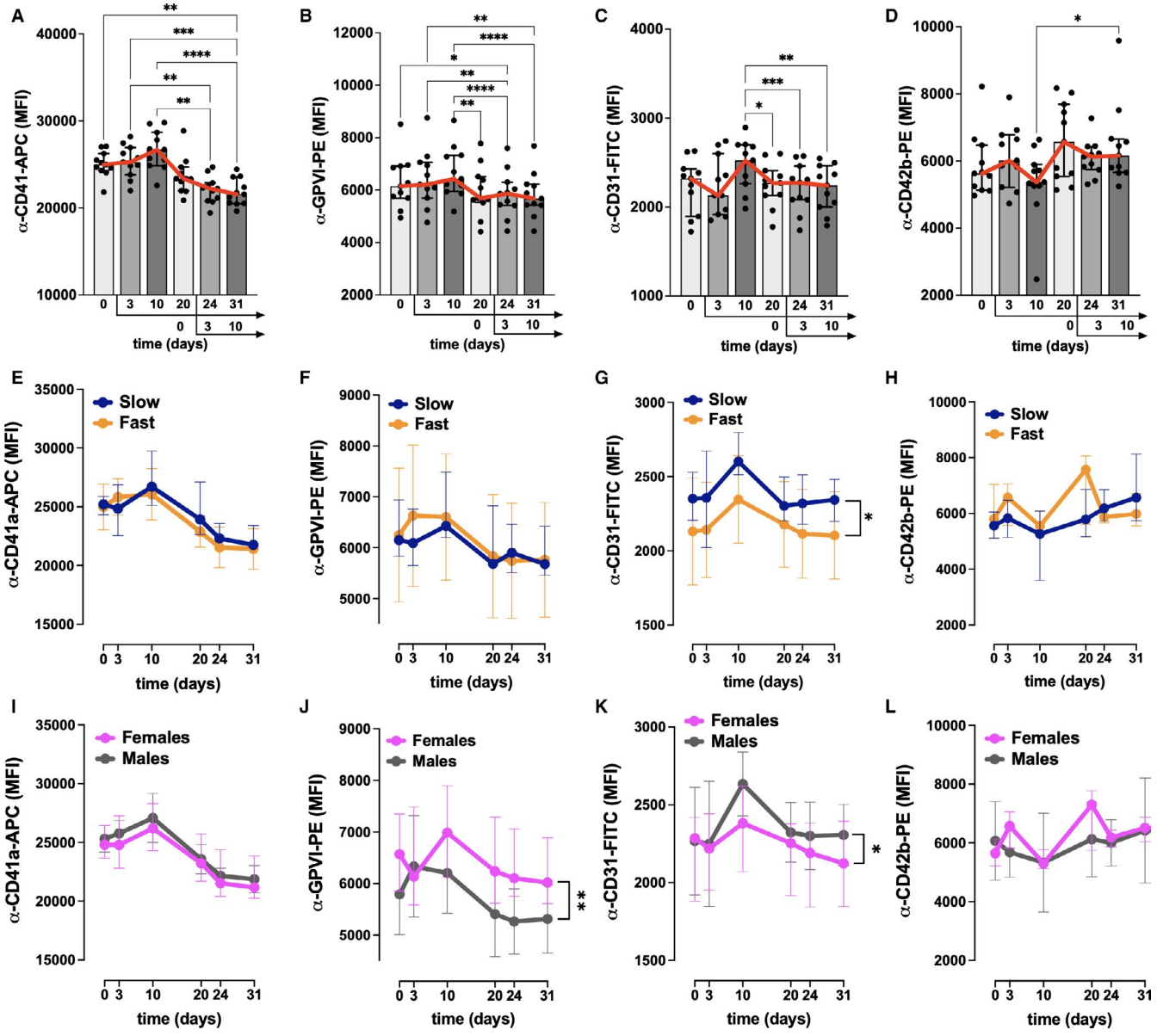

FIGURE 5 Platelet surface receptor expression following the BNT162b2 mRNA COVID-19 vaccine. Time course of the platelet surface expression of (A,E,I) CD41a (integrin αIIb), (B,F,J) GPVI (glycoprotein VI), (C,G,K) CD31 (PECAM-1) and CD42b (glycoprotein Ibα), (D,H,L) measured by flow cytometry with a BD Accuri C6 Plus, in all participants (bar graph with red trend lines connecting the median), in females (pink) versus males (gray) or in fast (orange) versus slow (blue) responders. Shown are median fluorescence intensity (MFI) ± interquartile range. Two-tailed Friedman test and Dunn's multiple comparisons test for intragroup analysis, Mann-Whitney test for intergroup analysis. *$P < 0.05$ **$P < 0.01$ ***$P < 0.001$ ****$P < 0.0001$

expression of GPVI and CD31, which mediate opposing signaling pathways,[12] were inversely related between the sexes, with males expressing more CD31 and females more GPVI, before and after vaccine injection (Figure 5I–L).

3.5 | Platelet reactivity following the BNT162b2 mRNA vaccine

To assess whether platelets from slow and fast responders had different reactivity, we measured the surface expression of platelet P-selectin (CD62P),[13] which is translocated from α-granules to the plasma membrane upon platelet activation and mediates platelet--leukocyte and platelet--endothelial interactions. Surface P-selectin was very low on circulating non-stimulated platelets and stayed low throughout the study except for a small but significant increase 10 days after the second dose (Figure 6A). P-selectin exposure in response to convulxin,[14] a specific agonist for the immune-like receptor GPVI, induced two waves of time-dependent increase in P-selectin exposure in the 10 days following each dose, with an incremental effect between the first and the second dose when it reached statistical significance compared to the control group (Figure 6B).

The activation state of circulating platelets and their responsiveness to agonist stimulation was the same in all study participants independently of their antibody response rate (Figure 6C, D).

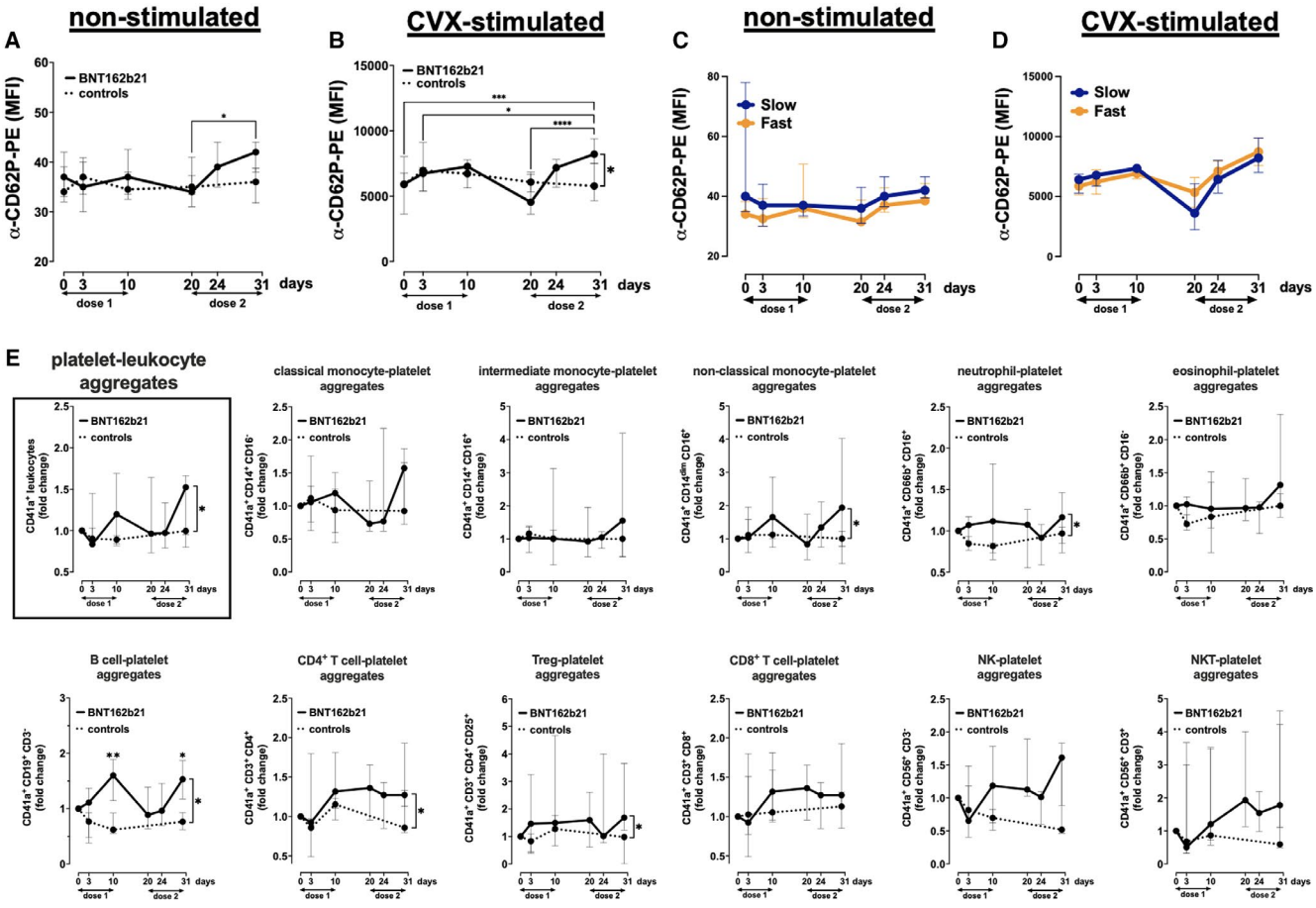

FIGURE 6 P-selectin expression and platelet--leukocyte aggregate formation following the BNT162b2 mRNA vaccine. A,B, Surface expression of P-selectin (CD62P) on circulating platelets non-stimulated or stimulated with the glycoprotein (GP)VI-specific agonist, convulxin (CVX), before (0) and (3, 10, 20, 24, 31 days) after vaccine administration. Shown is the median fluorescence intensity (MFI) ± interquartile range of vaccinated (straight line) and control (dotted line) subjects. C,D, Comparison of the surface expression of P-selectin (CD62P) among slow responders (blue) and fast responders (orange). Shown is median ± interquartile range. E, Relative frequencies of the circulating platelet aggregates with total leukocytes (squared insert), classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$) and non-classical ($CD14^{dim}CD16^+$) monocytes, neutrophils ($CD66^+CD16^+$), eosinophils ($CD66^+CD16^-$), B-cells ($CD3^-CD19^+$), $CD4^+$ ($CD3^+CD4^+$), regulatory ($CD3^+CD4^+CD25^{high}$) and $CD8^+$ ($CD3^+CD8^+$) T cells, natural killer (NK, $CD3^-CD56^+$) and natural killer T-cells (NKT, $CD56^+CD3^+$). Shown is the median ± interquartile range of the fold change relative to baseline (before vaccine administration) of the $CD41a^+$ events in each population for vaccinated (straight line) and control (dotted line) subjects. Flow cytometry acquisition was performed on a BD LSRFortessa and analyzed with FlowJo software, version 10.7.1. Two-tailed Friedman test and Dunn's multiple comparisons test for intragroup analysis, Mann-Whitney test for intergroup analysis. $^*P < 0.05$ $^{**}P < 0.01$ $^{***}P < 0.001$ $^{****}P<0.0001$

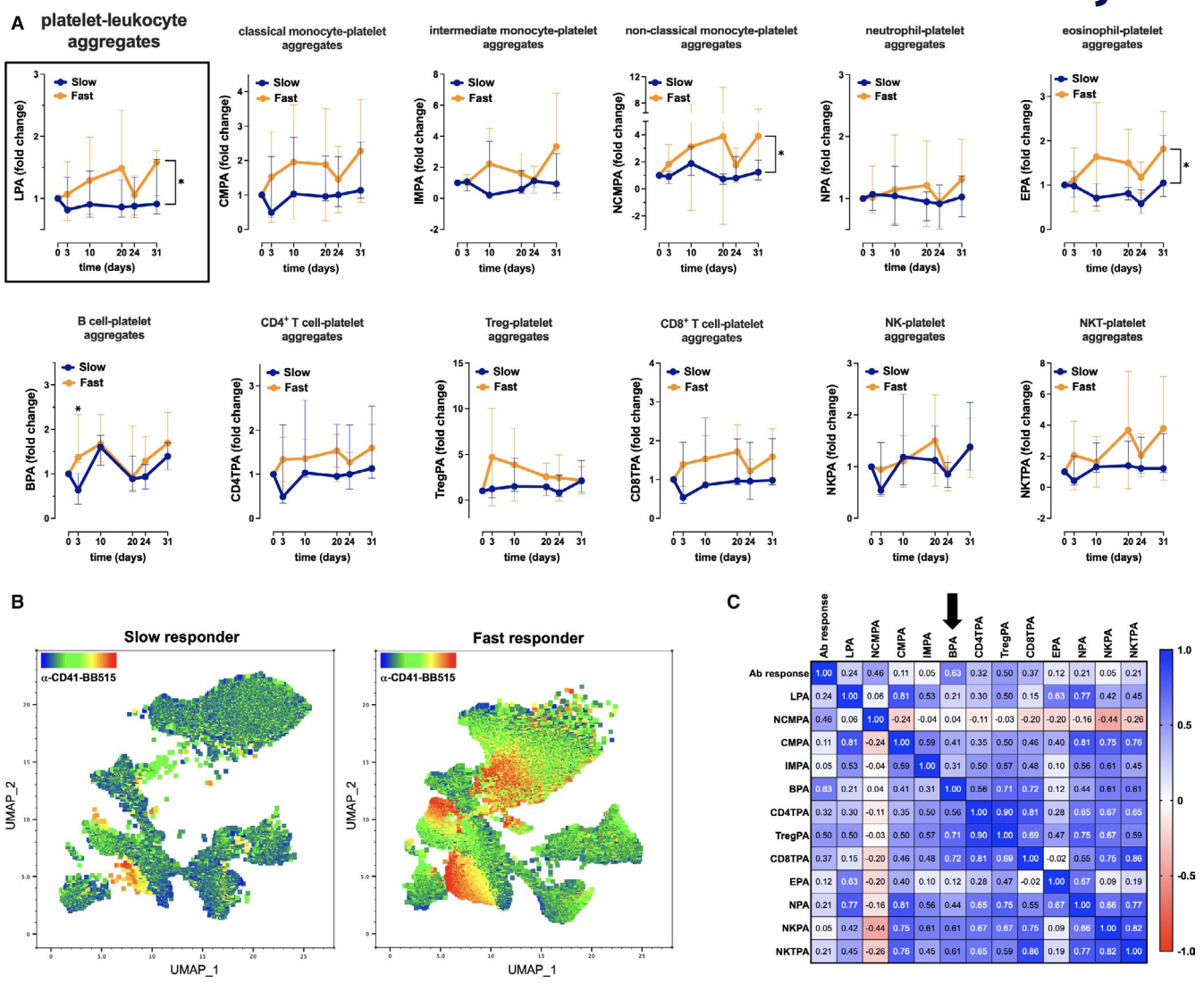

FIGURE 7 Distinct platelet--leukocyte aggregate formation in fast and slow responders shortly after the BNT162b2 mRNA vaccine. A, Relative frequencies among fast (orange) versus slow (blue) responders of circulating platelet aggregates with total leukocytes (squared insert); classical (CMPA), intermediate (IMPA) and non-classical (NCMPA) monocytes; neutrophils (NPA); eosinophils (EPA); B-cells (BPA); $CD4^+$ (CD4TPA); regulatory (TregPA) and $CD8^+$ (CD8TPA) T cells; natural killer (NKPA) and natural killer T-cells (NKTPA). Flow cytometry acquisition was performed on a BD LSRFortessa and analyzed with FlowJo software, version 10.7.1. Shown is the median ± interquartile range of the fold change relative to baseline (before vaccine administration) of the $CD41a^+$ events in each population. Mann-Whitney test for intergroup analysis. $^*P < 0.05$. B, Representative Uniform Manifold Approximation and Projection (UMAP) dot plot of $CD41a^+$ leukocytes among leukocyte subpopulations of a slow responder (left panel) and a fast responder (right panel) 20 days after administration of the first dose of the vaccine (1 day before second dose). Red dots indicate higher CD41a expression. C, Spearman rank correlation matrix between the fold change of platelet--leukocyte aggregates 3 days after the first dose and the antibody response (Ab response). In each box is indicated the correlation coefficient r, which ranges from +1 (positive correlation, blue) to −1 (negative correlation, red). r is indicated in white when the correlation is statistically significant ($P < 0.05$). The arrow highlights that the fold increase of the circulating B cell-platelet aggregates 3 days after the first dose significantly correlates with the antibody response

3.6 | Platelet--leukocyte interactions following the BNT162b2 mRNA vaccine

To characterize in depth the crosstalk between platelets and the immune system we measured the interaction of platelets with 11 leukocyte subtypes by multicolor flow cytometry (Figure S1).[15] We observed that circulating levels of PLA increased incrementally after each dose of the vaccine. Compared to the control group, the leukocyte populations binding the most to platelets were non-classical monocytes, B cells, $CD4^+$ T cells, and Treg cells and, to a lesser extent, neutrophils (Figure 6E). The highest level of PLA was detected 10 days after the second dose consistently with the peak of P-selectin exposure. We also measured a significant 1.5-fold increase in the proportion of B cells bound to platelets 10 days after the first dose and an almost 2-fold increase in NKT-platelet interactions 20 days after the first dose, even though the abundance of these populations in peripheral blood decreased over time (Figure S3). In a subgroup of patients (n=3) we compared the B cell expression of CD69, an early lymphocyte activation marker, and we found that B cells bound to platelets were more active than unbound B cells (Figure S11). Interestingly, the slow responders displayed impaired

ability to form PLA (Figure 7A,B). We detected significant differences between fast and slow responders in the binding of platelets with non-classical monocytes, eosinophils, and B cells. Formation of platelet-B cell aggregates 3 days after the first dose positively correlated (Figure 7C and Figure S14) with the antibody response rate (antibody response and platelet-B cell aggregates on day 3: $r = 0.63$, $P = 0.044$).

4 | DISCUSSION

Understanding what modifies the kinetics of the adaptive immune response is critical to optimize vaccine design and administration. Our study identifies four early features that positively correlate with a rapid antibody response following the BNT162b2 mRNA COVID-19 vaccine: (1) a rapid cytokine response, (2) an increased platelet turnover, (3) lower platelet expression of the inhibitory receptor CD31 (PECAM-1), and (4) formation of specific platelet-leukocyte aggregates, specifically with active B cells.

Our data show that the efficacy of the BNT162b2 vaccine (i.e., the ability to evoke SARS-CoV-2 neutralizing antibodies) is approximately 50% after only one injection, in agreement with previous larger studies.[16,17] In vaccine recipients achieving an effective level of neutralizing antibodies before the second injection (fast responders), administration of the mRNA-based vaccine BNT162b2 evoked a rapid rise in the systemic concentration of cytokines 3–10 days after the first injection followed by a second wave of cytokine response 10 days after the second injection (Figure 3A). Slow responders had a higher IL-6 response shortly after the vaccine but had an otherwise lower cytokine response that peaked only 20 days after the first injection. The cytokines showing differential changes in response to the injections included molecules with both pro-inflammatory (IL-1β, IFN-α2, IFN-γ, TNF-α, IL-12p70, IL-17A, IL-18, IL-23, and IL-33) and anti-inflammatory functions (IL-10). The cytokine that associated the most to the antibody response rate was IL-1β, which is a determining factor in the transition from local inflammation to the induction of an adaptive response because it stimulates antigen presenting cells and promotes the differentiation of T cells.[18] In agreement with Bergmaschi et al.[19] we also found that the vaccine evoked an interferon response. The fast responders showed increased IFN-γ levels after both the first and the second dose, while the slow responders showed a more transient IFN-γ response only after the second dose. A likely source of IFN-γ were the NK cells that expanded after the first injection only in the fast responders (Figure 2C). Numerous studies demonstrate that NK cells, through the release of IFN-γ, potentiate vaccine efficacy by promoting the differentiation of $CD4^+$ T cells, isotype switching and differentiation into antibody-secreting cells of B cells, and the antigen-presenting functions of myeloid cells.[20] In agreement with recent reports[21] we also detected a significant increase of the plasmatic levels of MCP-1/CCL2, one of the key chemokines that regulate migration and infiltration of monocytes/macrophages. However, the MCP-1 concentration and the monocyte blood count were comparable in the fast and the slow responders.

Several studies have demonstrated that the adaptive response to vaccines is modulated by early innate immunity. Platelets are the first cells to detect immune response sites and have been shown to modulate the adaptive immune response like innate immune cells. Thus, we tested the hypothesis that morphological, phenotypical, or functional features of platelets could be used as an early biomarker of effective development of vaccine-induced humoral responses. One of the features that distinguished fast and slow responders were the platelet size parameters (MPV and P-LCR), that can be easily monitored via low-cost blood tests. An increase in platelet size is generally the consequence of an accelerated platelet turnover and increased production of immature reticulated platelets, which are larger in size.[22-24] Previous studies show that various inflammatory mediators, such as IL-1β, IL-6, and C-C motif chemokine ligand 5 (CCL5), can trigger megakaryopoiesis and modify the process of proplatelet production and release.[25-28] Accordingly, we calculated a positive correlation between IL-1β and MPV/P-PLCR and we observed a correspondence between the timing of the cytokine variations (Figure 3A and Figure S4) and of the platelet size changes (Figure 4). While the fast responders experienced a cytokine response shortly after each dose of the vaccine (which was paralleled by an increase of the platelet turnover that maintained the platelet count stable), slow responders displayed a delay between the vaccine injections and the cytokine response. This uncoupling was accompanied by a delayed platelet turnover and a reduction of the platelet count shortly after the first dose of the vaccine. These data support the idea that the low-grade inflammation may be driving the increased rate of platelet turnover and that a timely innate immune response to the vaccine is necessary to ensure not only the rapid development of the adaptive response, but also the balance between platelet consumption and platelet production.

Another platelet feature that associated with the antibody response rate was the expression of the ITIM-coupled receptor CD31. ITAMs and ITIMs are opposing signalling modules that control the cellular activation within the immune system. In leukocytes, proteolytic cleavage of CD31 is associated to cellular hyper-reactivity[29-32] and, in platelets, lack of CD31 leads to hyper-responsiveness of the ITAM-coupled receptor GPVI.[12,33] Consistently, we found that the slow responders expressed significantly higher levels of CD31, suggesting that these subjects may have platelets less responsive to immune stimuli. Moreover, females, who generally exhibit stronger immune responses to vaccinations,[34] expressed more GPVI and less CD31, thus were potentially more responsive to platelet immune stimulation. We are currently investigating if the modulation of the balance between ITAM/ITIM signalling pathways occurs after other types of vaccines and whether it applies also to leukocytes. Further high-throughput studies are needed to appreciate the full spectrum of phenotypical changes occurring in platelets following COVID-19 vaccination.

We did not detect any difference in platelet reactivity among fast and slow responders. However, we found that vaccinated subjects were more responsive to GPVI stimulation compared to controls, as if the vaccine had a priming effect on ITAM signalling. Klug and colleagues recently reported that platelet reactivity was unchanged after BNT162b2 vaccine administration, when platelets were stimulated with TRAP, a G-protein coupled receptor (GPCR) agonist.[35] A possible reason ITAM, but not GPCR, signalling needs to be primed is that the platelet ITAM receptors, GPVI and CLEC-2, are necessary to secure vascular integrity at sites of inflammation[36] while the GPCRs for thrombin are dispensable for inflammation-mediated hemostasis.[37]

Another reason priming of ITAM-dependent P-selectin exposure might be beneficial in healthy individuals is that P-selectin facilitates platelet-leukocyte interactions. In our cohort we found that individuals who developed a slower antibody response failed to form vaccine-specific PLA and expressed higher levels of platelet CD31, which antagonizes ITAM signalling. The leukocytes that bound the most to the platelets of the fast responders, but not to the platelets of the slow responders, were non-classical monocytes. This monocyte subpopulation is specialized in producing inflammatory cytokines in response to viruses and nucleic acid via Toll like receptor (TLR) 7 and 8[38] and, in preclinical studies, it was shown to be among the cells recruited at the injection site and in the draining lymph nodes after lipid nanoparticle (LNP)-mRNA administration and able to internalize LNP, translate the mRNA, and present antigen.[39] Because platelets are more adhesive than leukocytes, the formation of heterotypic aggregates may be physiologically important to increase the adhesive capacity of leukocytes, selectively enhance their trafficking and infiltration at immune response sites, and ultimately accelerate the development of an effective adaptive response. CD31, which is more expressed in slow responders, may be reducing platelet-leukocyte binding by promoting active detachment, as it was shown previously between leukocytes and macrophages.[40]

Moreover, 10 days after the first dose of the vaccine we detected a significant increase of platelet-B cell aggregates. Previous studies have demonstrated that, in coculture, platelets induce B cell activation and immunoglobulin production[41] and, in a mouse model of viral infection, platelets promote B cell isotype switching and enhanced protection against viral rechallenge.[42] We are currently investigating in more detail which B cell subpopulations are more prone to bind platelets and if there is a causal relationship between PLA formation, B cell activation, and stimulation of a rapid immune response post-vaccine. At this stage, however, we cannot tell if the platelet interaction contributes to B cell activation or whether the increased platelet binding simply reflects the increased adhesiveness of activated lymphocytes. Nevertheless, we can use the detection of platelet-leukocyte aggregates as a very sensitive tool to discriminate which leukocyte subpopulations are active after the vaccine. Indeed, larger activated T cells and memory cells have greater platelet-binding ability than naïve cells[43] and we observed that platelet-bound B cells are significantly more active than unbound B cells in three vaccinated subjects (Figure S11). Moreover, platelet-B cell aggregates form as early as 3 days after the first injection, long before antibodies are detected in circulation, and are the earliest detectable feature that discriminates slow and fast responders; thus, it

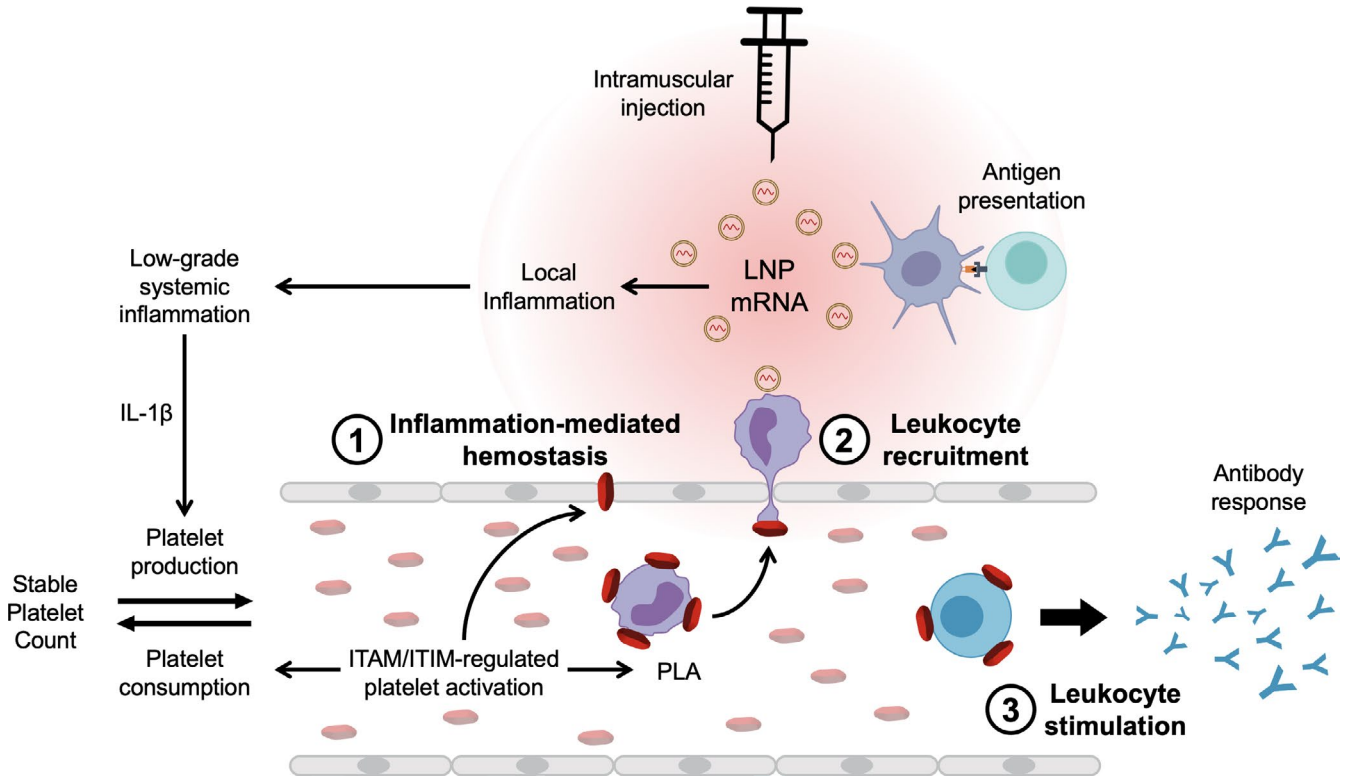

FIGURE 8 Conceptual framework of the study. Based on our evidence we hypothesize that shortly after intramuscular injection of the lipid nanoparticles containing the BNT162b2 mRNA COVID-19 vaccine (LNP-mRNA), the local activation of the immune response results in the systemic increase of interleukin (IL)-1β concentration that promotes platelet production and maintains the platelet count steady. Circulating platelets are primed respond to immunoreceptor tyrosine-based activation motif (ITAM)-coupled receptor stimulation and to form specific platelet-leukocyte aggregates (PLA). Elevated expression of the immunoreceptor tyrosine-based inhibitory motif (ITIM)-coupled receptor CD31 (PECAM-1), a delayed cytokine response and platelet turnover and impaired PLA formation associate to a slower antibody response in immunized subjects. Thereby our study suggests that regulated platelet activation following immunization may be beneficial to (1) ensure hemostasis at sites of inflammation, (2) facilitate the recruitment of leukocytes to the site of immune response, and (3) stimulate leukocyte activation and ultimately accelerate the onset of the adaptive immune response

could be used as a very early biomarker of effective development of vaccine-induced humoral responses.

Early stratification of vaccine responders is critical to mitigate the pandemic on a global scale because it could aid the optimal distribution of the second doses in countries with a limited vaccine supply and enable the proper allocation of the third dose (booster) in countries in which many citizens have already received the second dose but that are experiencing new waves of infection.

In the last few months, the adenoviral vector-based COVID-19 vaccines, ChAdOx1nCoV-19 (AstraZeneca/COVISHIELD) and Ad26.COV2.S (Johnson & Johnson/Janssen) have been linked to rare cases of vaccine-induced immune thrombotic thrombocytopenia (VITT).[44-50] Rare cases of immune thrombocytopenia (ITP) have also been reported following the mRNA COVID-19 vaccines and other anti-viral vaccines, but proof linking vaccines to *de novo* autoimmune disorders are lacking.[51-53] The frequency of these side effects is extremely low and the benefits of the vaccines in saving lives far outweighs the risk of thrombocytopenia or thrombosis in the general population. Moreover, our findings suggest that, in healthy adults, controlled ITAM-mediated platelet activation could be beneficial to support inflammation-induced hemostasis and to form platelet-leukocyte aggregates, which could accelerate the development of an effective immune response. Because of the small sample size of our cohort, we cannot tell if formation of PLA, in particular with B cells, could evoke the emergence of pre-existing autoreactive conditions. Further effort should be made to understand platelet regulation in response to vaccines to identify the groups of subjects at risk of these rare side effects.

These findings should be interpreted in light of potential limitations. We could analyze only 11 young adults receiving BNT162b2, potentially limiting the statistical power of our findings, as well as the transferability to other COVID-19 vaccines. Due to the filter setup of the flow cytometer, we could not include in our multicolor panel markers to univocally distinguish T regulatory cells from activated $CD4^+$ T cells. For future studies we have now designed specific panels to resolve the functional state of the interacting platelets and leukocytes. Moreover, the study design did not contemplate a 24-hour post-injection timepoint that would have captured the acute phase of the inflammatory response to the vaccine and possibly even earlier features distinguishing fast and slow responders.

In summary, our study provides evidence that platelets may be assisting leukocytes for the development of the immune response to an mRNA-based vaccine (Figure 8). Moreover, our data suggest that platelet indices, such as platelet size, the surface expression of platelet CD31, or circulating levels of platelet-leukocyte aggregates, could be exploited to discriminate individuals able to establish a more rapid and effective immune response, in order to optimize future vaccine design and administration.

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CONFLICTS OF INTEREST

D. Alvaro received a research grant from InterceptPharma, outside the scope of this manuscript, and is a consultant for Shionogi and Aboca. S. Basili received a research grant from Merck Sharp & Dohme, outside the scope of this manuscript. All other authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

D. Flego performed the experiments, analyzed the data, and contributed to the final manuscript; S. Cesaroni performed the experiments, analyzed the data, and contributed to the final manuscript; G. F. Romiti enrolled the study subjects, analyzed the data, and contributed to the final manuscript; B. Corica enrolled the study subjects and contributed to the final manuscript; R. Marrapodi performed experiments; N. Scafa performed experiments; F. Maiorca analyzed the data; L. Lombardi performed experiments; D. Pallucci performed experiments; F. Pulcinelli performed experiments and revised the manuscript; V. Raparelli analyzed the data and contributed to the final manuscript; M. Visentini analyzed the data and contributed to the final manuscript; S. Piconese performed experiments, analyzed the data, and contributed to the final manuscript; R. Cangemi analyzed the data and contributed to the final manuscript; D. Alvaro critically revised the manuscript; A. Polimeni critically revised the manuscript; S. Basili designed the study and critically revised the manuscript; L. Stefanini designed the study, analyzed and interpreted the data, and wrote the first draft of the manuscript.

ORCID

Simone Cesaroni https://orcid.org/0000-0002-0932-6707
Giulio F. Romiti https://orcid.org/0000-0002-3788-8942
Bernadette Corica https://orcid.org/0000-0001-9460-4435
Francesca Maiorca https://orcid.org/0000-0002-5412-5760
Davide Pallucci https://orcid.org/0000-0001-6971-8684
Valeria Raparelli https://orcid.org/0000-0002-2100-5682
Lucia Stefanini https://orcid.org/0000-0001-7420-301X

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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APPENDIX 1

Vax-SPEED-IT Study Group participants:

Anisa Degjoni (Sapienza University of Rome, Rome, Italy).
Fabrizio Recchia (Sapienza University of Rome, Rome, Italy).
Giorgia Polti (Sapienza University of Rome, Rome, Italy).
Nicolò Sperduti (Sapienza University of Rome, Rome, Italy).
Paolo Ciacci (Sapienza University of Rome, Rome, Italy).
Patrizia Pacini (Sapienza University of Rome, Rome, Italy).
Stefano Perri (Sapienza University of Rome, Rome, Italy).
Alba Rosa Alfano (Sapienza University of Rome, Rome, Italy).
Federica Taccari (Sapienza University of Rome, Rome, Italy).
Elena Mereu (Sapienza University of Rome, Rome, Italy).