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Serum biomarkers of inflammation and vascular damage upon SARS-CoV-2 mRNA vaccine in patients with thymic epithelial tumors

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Abstract

Objectives: Thymic epithelial tumors (TET) patients are at high risk of autoimmune and hypimmune complications. Limited evidence is available on the potential risk of immune-related and inflammatory reactions induced by SARS-CoV-2 vaccine in this patient population.

Methods: In order to identify subjects at higher risk for vaccine complications, we prospectively evaluated a panel of serum biomarkers related to inflammation (TNF- α , IL-1 β , -6, -10, -12, and -17A, IFN- α , β and γ , MPO, MMP-9), and vascular damage (E- and P-selectin, VEGF-A, P-ANCA and MCP-1) in 44 TET patients and in 30 healthy controls along the whole SARS-CoV-2 vaccine cycle.

Results: About 50 % of subjects (either TET and controls) showed an increase of serum biochemical markers of inflammation and endothelial damage with a large heterogeneity of

values. Such increase appeared early, after the first dose in control subjects and later, after the second dose in TET patients (in which we observed mainly an increase of inflammatory biomarkers). The values normalized after about 3 months and did not increase after the third, booster dose. No autoimmune or vascular complications were observed in the study subjects and no difference was observed in terms of vaccine response among subjects showing serum biomarkers increase and those who experienced no changes.

Conclusions: Our data highlight the relevance of Sars-Cov-2 vaccine in TET patients, as it resulted safe and prevented severe COVID-19. However, further studies are awaited to explore the mechanisms and the potential consequences of the observed increase of serum inflammatory and vascular damage biomarkers.

Keywords: SARS-CoV-2; thymic epithelial tumors; inflammation; vascular damage; mRNA vaccine

Introduction

In March 2020 severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) became a pandemic causing so far millions of deaths. An unprecedented research effort has made it possible to obtain antiviral vaccines [1] since December 2020 and their impact in the prevention of serious infections was quickly clear [2]. In Italy, high-risk groups including physicians and frail patients underwent a three-dose vaccine schedule, with a first dose followed by a second administration after 21 days, and a booster dose performed after 6 months. A particularly frail category comprises patients with thymic epithelial tumors (TET), which, although rare, represents the most frequent neoplasia of the anterior mediastinum. TET include thymoma, thymic carcinoma, and thymic neuroendocrine tumors [3]. The presence of autoreactive T lymphocytes and paraneoplastic autoimmunity due to reduced immunological self-tolerance render such patients more prone to autoimmune complications [4] cautioning on the use of SARS-CoV-2 vaccine, also related to known cross-reactions of antibodies against vaccine spike protein with self-proteins [5]. On the other hand, a variety of autoimmune

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complications observed upon vaccine administration are emerging [6]. Moreover, most patients with TET display severe immunodeficiency mainly involving B-lymphocytes and Ig production with peculiar immunological signatures [7]. Our [8, 9] and other [10] groups have shown high efficacy of the SARS-CoV-2 vaccine in TET patients, who in most cases developed humoral and cellular immunity, as well as the absence of autoimmune complications [9, 11]. Moreover, we demonstrated the possibility of predicting response to vaccine in TET patients [11].

In addition, other vaccine side effects and complications have been reported, including excessive inflammation with cases of acute respiratory distress syndrome, vascular injury with episodes of severe p-ANCA-positive vasculitis, platelet activation with thrombotic events; the pathogenesis of such complications is still under study [12, 13] even if a relationship with immunity is emerging [14]. Serum biomarkers could help to cast light on the mechanisms of such effects and to identify subjects at risk [15].

In order to better understand the pathogenesis of vaccine complications and to identify subjects at higher risk, we prospectively evaluated a panel of serum biomarkers related to inflammation and vascular damage during the entire cycle of SARS-CoV-2 vaccination.

Materials and methods

Study design and participants

The study was approved by the Ethics Committee of the University of Naples Federico II (approval n. 76.21). Forty-four consecutive TET patients (median age: 55 years, interquartile range, IQR: 48–65, 18 males), referred to the Rare Tumors Regional Coordination Center of the Campania Region (Federico II University Hospital of Naples) from April 2021 to November 2021, were prospectively enrolled in the study. Detailed description of inclusion criteria, diagnostic criteria, and presence of autoimmune diseases (i.e., good syndrome [GS] and myasthenia gravis [MG]) has been reported in a previous study [11] that evaluated the humoral and cellular response [16] to the vaccine. All patients were enrolled before receiving the first dose of SARS-CoV-2 mRNA vaccine (BNT162b2 from Pfizer-BioNTech). Serum biomarkers were evaluated at different time points, including T0 (before first vaccine dose), T1 (2 weeks after first dose), T2 (1 month after second dose), T3 (3 months $\pm$ 2 weeks after second dose), T4 (before vaccine dose booster) and T5 (after the booster vaccine dose). A clinical and anamnestic examination, as per standard clinical practice, was performed every four weeks during and after the administration of the entire vaccination course up to 12 months, to identify humoral and/or clinical signs/symptoms suggestive of autoimmune diseases or other complications. In addition, we studied 30 healthy subjects (median age: 46 years; IQR: 43–60, 10 males). All enrolled subjects signed an informed consent at study entrance.

Sample collection and storage

Venous blood was collected in tubes without anticoagulant, and then centrifuged for serum separation for the analysis of biomarkers of inflammation and vascular damage. The serum samples were stored at -80°C until analysis. The storage times of samples were comparable among the six time points. EDTA blood samples were used for cytometric analyses and for lymphocytes isolation within 3 h of blood collection.

Analysis of serum biomarkers

The levels of serum tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, IL-10, IL-12, and IL-17A, interferon (IFN)- α , β and γ , myeloperoxidase (MPO), matrix metalloproteinase (MMP)-9, E-selectin, vascular endothelial growth factor (VEGF)-A, P-ANCA and monocyte chemo-attractant protein (MCP)-1 were analyzed by automated microfluidic immunoassay cartridges on ProteinSimple Ella (Bio-Techne), in accordance with the manufacturer's instructions. Serum P-selectin levels were measured by Human P-Selectin/CD62P Quantikine ELISA kit (R&D Systems, Minneapolis, Minnesota, USA), in accordance with the manufacturer's instructions. No reference intervals were provided from manufacturer for serum biomarkers. The intervals obtained in the 30 healthy subjects before vaccine were reported in Supplementary Material, Table S1.

The percentages and numbers of blood cells were measured by multicolor flow cytometry (Facs Canto II; Becton Dickinson Italia, Milan, Italy) as previously described [17]. The IFN γ Stimulation Index (SI) and anti-SARS-CoV-2 IgG antibodies were obtained as previously described [11].

Statistical analyses

Continuous data have been reported as median and IQR. The Shapiro–Wilk test was applied to evaluate the normality of distributions. Comparisons between two groups of independent samples were evaluated by the Mann–Whitney U test. Categorical data have been reported as number and percentage, and the comparisons were evaluated by the Fisher's test. Friedman's test was used for paired comparisons among the three or four time points. Correlations between variables were evaluated using Spearman correlation analysis. Statistical analyses were performed by SPSS (version 28, IBM SPSS Statistics). Graphics were done using KaleidaGraph software (version 4.5.4, Synergy, Reading, PA, USA) and Excel (Microsoft 365). p-Values <0.05 were considered significant.

Results

TET patients and controls show different trends of serum TNF- α levels during the vaccine cycle

We considered the increase of at least two folds the upper reference value of serum TNF- α , that was previously described as frequently enhanced in serum from subjects after the vaccine [13]. The analysis of serum TNF- α

Table 1: Distribution of patients with thymic epithelial tumor (TET) and controls within 3 subgroups according to the trend of serum tumor necrosis factor (TNF)- α during the vaccine cycle.

	TET	Controls	p-Value
n	44	30	
Subgroup 1 ^a	18 (40.9 %)	12 (40.0 %)	ns
Subgroup 2 ^b	1 (2.3 %)	15 (50.0 %)	<0.0001
Subgroup 3 ^c	25 (56.8 %)	3 (10.0 %)	<0.0001

Trend of serum TNF- α : ^ano significant change between T0, T1, T2 and T3;

^bsignificant increase in T1 vs. T0 and decline in T2 and T3; ^cno significant changes between T0 and T1, significant increase in T2 and decline in T3. ns, not significant.

demonstrated that both in patients with TET and in controls there were three different trends. A first group of subjects (including both TET patients and controls) did not show a change of serum TNF- α following the first and the second dose of vaccine (subgroup 1); a second group of subjects (again including both TET patients and controls) showed an early increase of serum TNF- α after the first dose of vaccine (T1) followed by a decline of the values after the second dose at T2 (subgroup 2); a third group of subjects did not show changes of serum TNF- α after the first dose of vaccine (T1), but they showed an increase after the second dose (T2) followed by a decline at three months (T3) from the second dose (subgroup 3). According to such classification, Table 1 compares the trends observed in patients with TET and in controls. In particular, 18/44 (40.9 %) patients with TET and 12/30 (40.0 %) controls were classified in the subgroup 1 (p not significant); 1/44 (2.3 %) patients with TET and 15/30 (50.0 %) controls were classified in the subgroup 2 (p<0.0001); 25/44

(56.8 %) patients with TET and 3 (10.0 %) controls were classified in the subgroup 3 (p<0.0001).

We then compared the 18 TET patients classified in the subgroup 1 with the 25 TET patients classified in the subgroup 3. As shown in Table 2, no significant differences were observed between these two subgroups of TET patients considering the number of subjects receiving anti-cancer treatments, as well as evidence of disease (i.e., locally-advanced or metastatic TET), GS, or autoimmune diseases. Moreover, no significant differences were observed between the two subgroups as regard to the values of IgG anti Sars-CoV-2, to the cellular response expressed by the IFN- γ stimulation index and to the B lymphocyte count at T0. Only the age and the T lymphocyte count at T0 was significantly different between the two subgroups. In particular, subgroup 3 showed higher age and T lymphocyte count than the subgroup 1 (Table 2).

Serum levels of inflammatory biomarkers in TET patients and in controls

As reported above, 25/44 (56.8 %) TET patients were classified in the subgroup 3, while 15/30 (50.0 %) controls were classified in the subgroup 2 according to the trend of serum TNF- α . We compared the data obtained in such two subgroups. Figure 1A shows the variation of serum TNF- α in such subgroups. In TET patients (continuous line) the values of TNF- α were not significantly different between T0 and T1, significantly enhanced at T2 as compared to T0 and were significantly

Table 2: Comparison between subgroups 1 and 3 of patients with thymic epithelial tumors (TET) classified on the basis of the trend of tumor necrosis factor (TNF)- α and clinical and laboratory parameters in 44 patients with TET.

	Subgroup 1 ^a	Subgroup 3 ^b	p-Value
n	18	25	ns
Gender, n (%) (female)	14 (78 %)	13 (52 %)	ns
Age, years	50 (44–55)	59 (50–66)	0.004
Body mass index	26.82 (23.14–33.85)	25.71 (22.28–31.44)	ns
in therapy, n of cases (%)	8 (44 %)	12 (48 %)	ns
Evidence of disease, n of cases (%)	8 (44 %)	12 (48 %)	ns
Good syndrome, n of cases (%)	10 (55 %)	9 (36 %)	ns
Autoimmune disease, n of cases (%)	11 (61 %)	18 (72 %)	ns
IgG anti-Sars-CoV-2, AU/mL	12,000 (1,750–22,500)	10,000 (0–21,000)	ns
IFN- γ stimulation index (fold change)	6.1 (3.0–44.0)	5.3 (2.9–16.0)	ns
B lymphocytes at T0, n/mm ³	35 (15–119)	38 (20–151)	ns
T lymphocytes at T0, n/mm ³	548 (428–1,078)	1,104 (609–1,567)	0.031
Cardiovascular disease	7 (39 %)	16 (64 %)	ns
Dyslipidemia	6 (33 %)	6 (24 %)	ns
Diabetes	4 (22 %)	6 (24 %)	ns

Trend of serum TNF- α : ^ano significant change between T0, T1, T2 and T3; ^bno significant changes between T0 and T1, significant increase in T2 and decline in T3. ns, not significant.

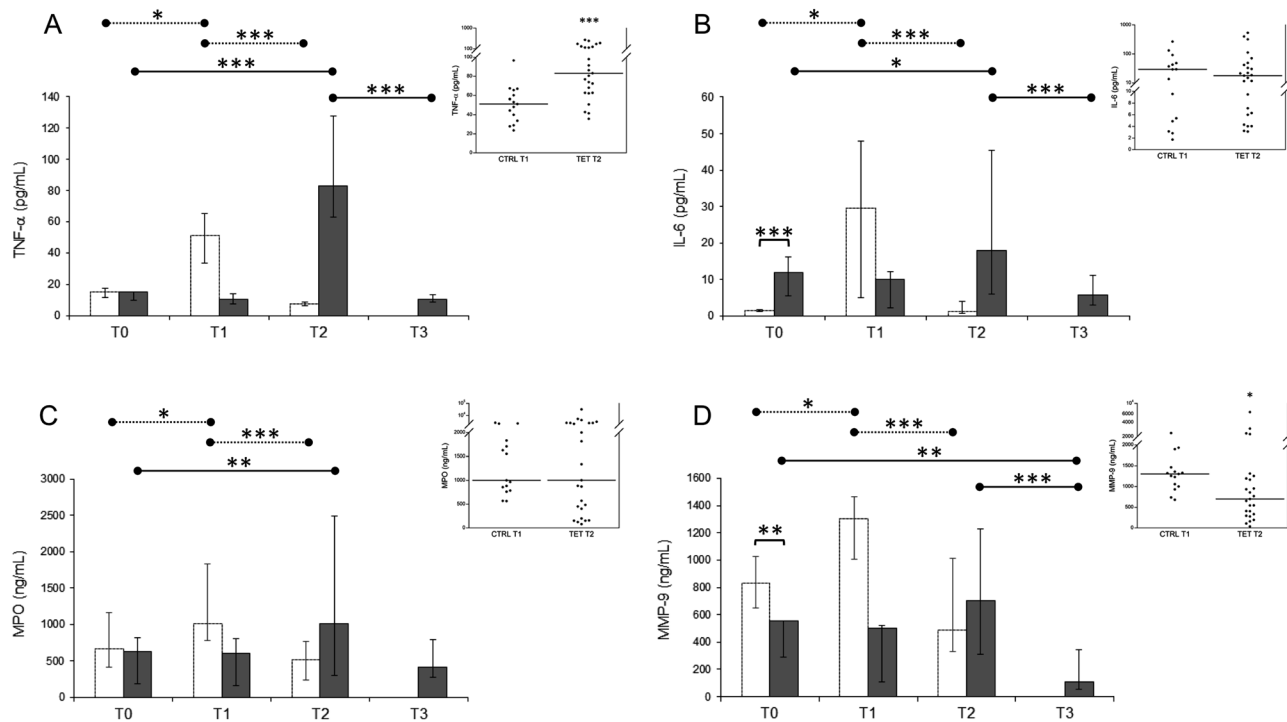


Figure 1: Serum levels of TNF- α (A), IL-6 (B), MPO (C) and MMP-9 (D) in CTRL-subgroup 2 and in TET-subgroup 3 at T0 in comparison to T1, T2 and T3. White and grey columns represent the trend in CTRL and TET, respectively. Dotted lines correspond to the comparisons among the different time points in CTRL group; continuous lines correspond to the comparisons among the different time points in TET group. Comparisons of data distributions in CTRL at T1 and TET at T2 are shown in the upper right of each panel. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. CTRL, healthy subjects; TET, thymic epithelial tumors.

lower at T3 as compared to T2, with no significant difference between T3 and T0. In controls (dotted line) the values of the biomarker were significantly higher at T1 as compared to T0, significantly reduced at T2 as compared to T1, with no significant difference between T2 and T0. No significant differences were observed for serum TNF- α levels at T0 between TET patients and controls, while the values of TNF- α at peak (i.e., T2 for TET and T1 for controls) were significantly higher in TET patients as compared to controls, although with relevant inter-individual variability both within TET patients with TET and controls (Figure 1A, scattergram).

The trend of serum IL-6 (Figure 1B), mirrored that observed for TNF- α , even if the values at T0 were significantly higher in TET patients as compared to controls and the values at peak were not significantly different between TET patients and controls (Figure 1B, scattergram). Again, the scattergram showed a large inter-individual variability of the serum IL-6 values both within patients with TET and controls.

Figure 1C shows the trend of serum MPO. In TET patients, the values of serum MPO were not significantly different between T0 and T1, they were significantly enhanced at T2 as compared to T0 and significantly lower at T3 as compared to T2, with no significant difference between T3 and T0. In controls, the values of the biomarker were significantly higher at T1 as compared to T0, they were significantly

reduced at T2 as compared to T1, with no significant difference between T2 and T0. No significant differences were observed for serum MPO at T0 and at peak between TET patients and controls, although we observed a relevant inter-individual variability of serum MPO both within TET patients and controls at peak (Figure 1C, scattergram).

Finally, the values of serum MMP-9 in TET patients were not significantly different between T0 and T1, they were enhanced (although not significantly) at T2 as compared to T0 and significantly lower at T3 as compared to T2 and to T0. In controls, the values of the biomarker were significantly higher at T1 as compared to T0, they were significantly reduced at T2 as compared to T1, with no significant difference between T2 and T0. Serum MMP-9 at T0 was significantly lower in patients with TET as compared to controls both at T0 and at peak again with relevant interindividual variability of serum MMP-9 both within TET patients and within controls (Figure 1D, scattergram).

For all the biomarkers of inflammation, i.e., TNF- α , IL-6, MPO and MMP-9, serum values at T4 and T5 (i.e., before and after the booster dose) were not significantly different as compared to T0 (data not shown). Moreover, serum levels of other inflammatory biomarkers, including IL-1 β , IL-10, IL-12, and IL-17A, and IFN- α , β and γ , were not significantly different among T0 and T1, T2, T3, T4 and T5 both in patients with TET and in controls (data not shown).

Serum levels of biomarkers of vascular damage in TET patients and in controls

Likely to the above-described analyses, we assessed the levels and changes of biomarkers of vascular damage, of the 25/44 (56.8 %) TET patients classified in the subgroup 3, and those of the 15/30 (50.0 %) controls classified in the subgroup 2.

Figure 2A shows the trend of serum E-selectin in such subgroups. In TET patients (continuous line), the values of serum E-selectin were not significantly different among T0, T1, and T2, whereas they were significantly enhanced at T3 as compared to T0; in controls (dotted line), the values of serum E-selectin were not significantly different among T0, T1 and T2. No significant differences were observed for serum E-selectin between TET patients with TET and controls, i.e., T1 for controls and T2 for TET patients (Figure 2A, scattergram). The values of serum P-selectin (Figure 2B) were not significantly different among T0, T1, T2 and T3 in patients with TET, while in controls they resulted significantly higher at T1 as compared to T2, and significantly declined at T2 as compared to T1, with no significant difference between T2 and T0. Furthermore, the values of P-selectin either at T0 or at peak (Figure 2B, scattergram) were significantly lower in TET patients as compared to controls.

In TET patients, serum VEGF (Figure 2C) was not significantly different among T0, T1 and T2 (although a non-significant trend of increase was observed at T2), and significantly lower in T3 as compared to T2. In controls, serum VEGF was significantly enhanced in T1 as compared to T0 and significantly declined in T2 as compared to T1, with no significant differences between T2 and T0. Moreover, both basal (T0) and peak values of serum VEGF resulted significantly higher in TET patients as compared to controls.

Finally, serum MCP-1 (Figure 2D) was not significantly different in TET patients among T0, T1, T2 and T3, while in controls it was significantly enhanced in T1 as compared to T0 and declined (although not significantly) in T2 as compared to T1. Moreover, both basal (T0) and peak values of serum MCP-1 resulted significantly higher in TET patients as compared to controls.

For all the biomarkers of endothelial damage, including E- and P-selectin, VEGF, MCP-1 serum values at T4 and T5 (i.e., before and after the booster dose) were not significantly different as compared to T0. Moreover, serum levels of P-ANCA were not significantly different at T0 as compared to T1, T2, T3, T4 and T5, both in TET patients and in controls (data not shown).

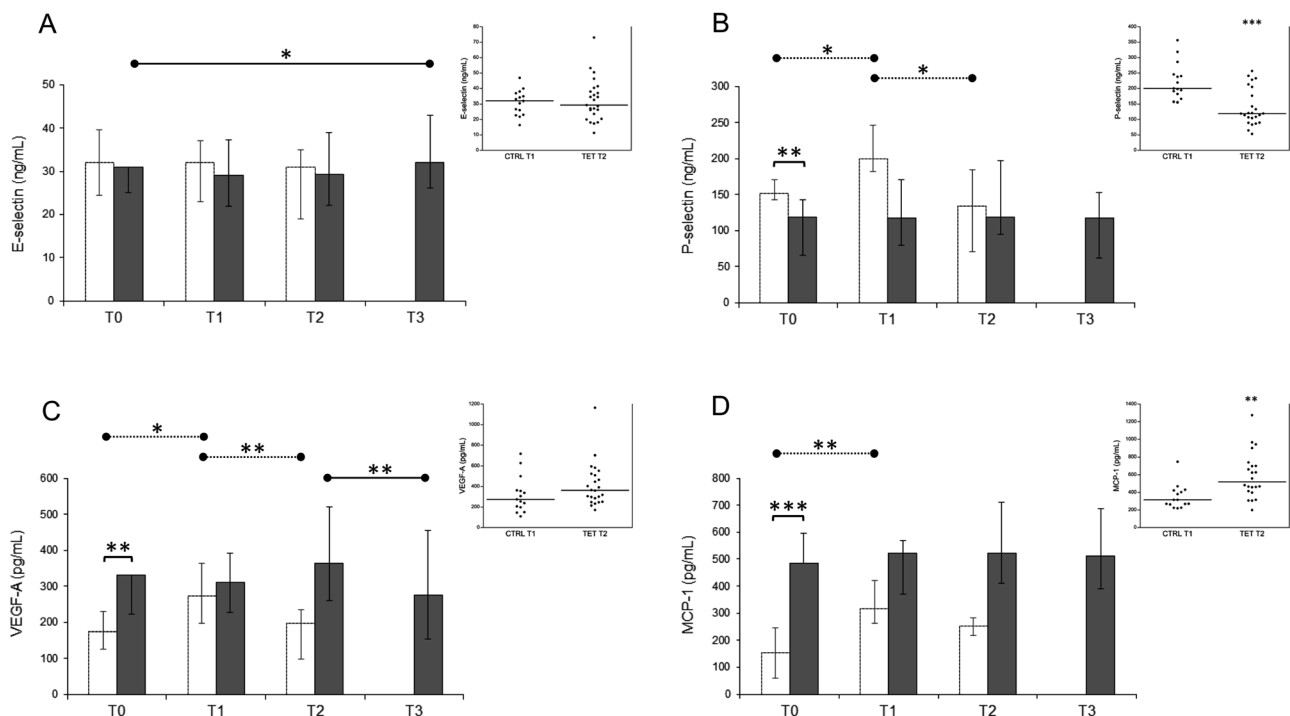


Figure 2: Serum levels of E-selectin (A), P-selectin (B), VEGF-A (C) and MCP-1 (D) in CTRL-subgroup 2 and in TET-subgroup 3 at T0 in comparison to T1, T2 and T3. White and grey columns represent the trend in CTRL and TET, respectively. Dotted lines correspond to the comparisons among the different time points in CTRL group; continuous lines correspond to the comparisons among the different time points in TET group. Comparisons of data distributions in CTRL at T1 and TET at T2 are shown in the upper right of each panel. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$. CTRL, healthy subjects; TET, thymic epithelial tumors.

Correlations between serum biomarkers

We evaluated the correlations between serum biomarkers, age and circulating cell populations in 25 TET patients classified in the subgroup 3 at T2. Statistically significant correlations were observed: (i) between biomarkers of inflammation (i.e., IL-6 with TNF- α , $p=0.004$ and MMP-9 with MPO, $p=0.001$); (ii) between biomarkers of inflammation and biomarkers of endothelial activation/damage (i.e., IL-6 with P-selectin, $p=0.001$ and MPO with E-selectin, $p=0.009$); and (iii) between serum biomarkers and cell sub-populations (i.e., IL-6 with Th1, $p=0.03$; E-selectin with Th17, $p=0.025$; VEGF and activated Th1, $p=0.027$) (Supplementary Material, Table S2).

Discussion

The Sars-Cov-2 vaccine causes an increase of serum biomarkers of inflammation and vascular damage although with relevant interindividual variability and different kinetics between controls and TET patients. Several studies demonstrated that Sars-Cov-2 vaccines induce inflammation [17] and endothelial/platelet activation [13] with an increase of serum biomarkers. Our data firstly show, both in TET patients and among healthy subjects, the existence of two different sub-populations, each including about a half of study subjects; one subgroup showed an increase of serum biomarkers (often relevant), and the other was not involved by such alterations. These two sub-populations seem to be similar both in terms of major complications and adverse events, which were not observed in any of study subjects, despite in several cases the levels of pro-inflammatory biomarkers were comparable to those observed in patients with severe acute COVID-19 [18]. Moreover, no differences were observed in terms of humoral and cell immunity development toward the vaccine. Whereas we observed a lower age in TET patients who did not develop the increase of serum biomarkers, however no significant correlations were found between the age and the levels of serum biomarkers. Interestingly, we found a lower number of T lymphocytes at T0 in TET patients who did not develop the increase of serum biomarkers. This evidence supports the hypothesis that COVID-19 inflammation and cytokine release are related to activated T-lymphocytes, particularly Th1 and Th17, as we observed either in acute COVID-19 [18] and in multisystem inflammatory syndrome in children [19, 20], and later in patients with TET following the vaccine [11].

Among biomarkers of inflammation, we observed a relevant TNF- α and IL-6 response, that we previously found in severe acute COVID-19 [18, 21] and in MIS-C [19]. Such response may be induced by the proinflammatory effect of

the S protein [12]. We observed different kinetics between controls and TET patients i.e., serum biomarkers of inflammation enhanced after the first dose of the vaccine in controls, while the response was induced only by the second dose in patients with TET. Differently, a previous study reported a proinflammatory response only after the second dose both in 20 healthy controls and in 22 patients with multiple sclerosis treated with the same vaccine of the present study [17]. Moreover, in that study the proinflammatory response correlated to the magnitude of the humoral response, while we excluded any correlation between the levels of pro-inflammatory markers and the levels of humoral or cellular response and no differences were observed in the immunity toward the vaccine in TET patients who had an increase of serum biomarkers comparing with those who did not experience such increase. Furthermore, besides the TNF- α , IL-6 and MCP-1 increase observed in half of our cohort, the aforementioned study also reported a relevant IFN response upon COVID-19 vaccine administration, which we lacked to observe. On the other hand, we excluded a relevant IFN response both in acute COVID-19 patients [18] and in MIS-C [19]. Finally, although the booster dose significantly improved both the humoral and the cell response in TET patients TET [11] we did not observe an increase of serum biomarkers after the third dose of vaccine both in controls and in TET patients, differently from the previous study [17].

Another study compared 55 healthy subjects who received the adenovirus-vector based vaccine with 55 who received mRNA vaccines analyzing several proinflammatory, endothelial- and platelet-activation biomarkers [13]. Although in both groups the vaccine induced an increase of serum biomarkers of inflammation, endothelial and platelet activation (among which, in agreement with us, serum IL-6, TNF- α , P- and E-selectin) they observed a stronger response in subjects treated with the adenovirus-vector based vaccine. In agreement with our data, the response occurred after the first dose of vaccine. Also, in that study, although the endothelial and platelet activation, no subject underwent thrombotic complications. However, in patients with TET we observed mainly an increase of proinflammatory biomarkers, while the enhancement of biomarkers of endothelial (E-selectin) and platelet (P-selectin) activation was less relevant as compared to controls. This may depend on the fact that endothelial/platelet activation is sustained by the immunity [14] that is less effective in TET patients, as compared to healthy subjects. Furthermore, in none of our study subjects the vaccine induced an increase of p-ANCA, previously described as associated to severe vasculitis after COVID-19 infection or vaccine [22, 23]. On the other hand, both patients with TET and healthy controls showed an increase of serum VEGF-A

following the Sars-Cov-2 vaccine administration, and the levels of such serum biomarker were higher in TET patients during the whole vaccine cycle. Such increase is likely due to the vaccine S-protein that competitively antagonizes the binding of VEGF to its NRP-1 co-receptor [24] altering the pathways of angiogenesis and nociception. The increase of unbound VEGF-A causes the interaction of the protein with other receptors inducing the long COVID-19 syndrome and rare complications of COVID-19 vaccine like myocarditis and RS3PE syndrome [25] and atrial arrhythmias through the intercalated disk remodeling [26]. We did not observe cases of such complications in TET patients and controls following the vaccine. However, considering that approximately 10 % of our patients and controls had an extraordinary increase in serum VEGF levels, and that patients with TET had higher values of serum VEGF-A also at T0, we suggest to further investigate the role of such biomarker.

A study limitation is represented by the relatively low number of subjects, however they were clinically homogeneous, and were carefully followed during the whole vaccine cycle with a recording of all clinical and laboratory data. Our data highlight the relevance of Sars-Cov-2 vaccine in TET patients, as it resulted safe and prevented severe COVID-19. However, we suggest monitoring both inflammatory and endothelial biomarkers (through the analysis of serum biomarkers), to select patients at higher risk for complications. Furthermore, it will be interesting to evaluate whether the proinflammatory and vascular damage response would be peculiar to COVID-19 vaccine or if it will be triggered also by other mRNA vaccines.

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Research ethics: The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Committee of the University Federico II, Naples (approval n. 76.21).

Informed consent: The participants provided their written informed consent to participate in this study.

Author contributions: GCr and MGz carried out the experiments, analyzed the data and drafted the manuscript.

GS carried out the experiments and analyzed the data. EP, MT, and PDP contributed to patient enrollment, clinical data collection and data analysis. MR carried out the experiments. GP contributed to patient enrollment, data analysis and interpretations of results. PF contributed to data analysis and interpretations of results. GC and MG planned and supervised the study, performed data analysis and results interpretation, and drafted the manuscript. All authors contributed to manuscript drafting and revision, and approved the submitted version.

Competing interests: The authors state no conflicts of interest.

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Data availability: The original contributions presented in the study are included in the article/Supplementary Material; further inquiries can be directed to the corresponding author.

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