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Serum HDL subfractions are impaired by COVID-19 vaccine in patients with thymic epithelial tumors

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Patients with thymic epithelial tumors (TET), who have a high risk of developing immunological disorders such as immunodeficiency and autoimmunity, were included among frail patients eligible for the COVID-19 vaccinal program. We previously found an increase of serum biomarkers of inflammation in 25 of 44 (56,8%) TET patients after the second dose (T2) of mRNA vaccine (BNT162b2 from Pfizer-BioNTech), although none developed immune-related complications at this time point. In this study we have investigated the metabolic process of high-density lipoproteins (HDL) that are among the main players in inflammatory and immune modulation. To verify the impact of the vaccine on HDL metabolism in TET patients, we prospectively evaluated serum HDL and HDL subfractions at baseline and T2. Among the 45 TET patients, we observed two different trends: 24 ones (53.3%, subgroup 1) showed a significant decrease of serum small HDL ($p < 0.0001$) class and/or HDL 10 subfraction (HDL 10) ($p < 0.0001$) at T2 as compared to baseline values (T0), the other 21 patients (subgroup 2) did not show significant variations. Serum values of small HDL class in subgroup 1 at T2 were inversely correlated with serum interleukin (IL)-6, activated T lymphocytes and regulatory T cells. These findings suggested that the COVID-19 vaccine-related inflammation induces the HDL remodeling with a reduction of anti-inflammatory small HDL class. Further studies need to clarify why the vaccine causes inflammation in about 50% of TET patients and whether such response is peculiar to COVID-19 vaccine or if it would be induced also by other mRNA vaccines.

Keywords TET, HDL, HDL subfractions, Biomarkers, Inflammation

Abbreviations

TET	Thymic epithelial tumors
HDL	High density lipoproteins
IL	Interleukin
SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
IQR	Interquartile range
LDL	Low-density lipoproteins
VLDL	Very-low-density lipoproteins
r_s	Spearman correlation coefficient
AD	Autoimmune diseases

In March 2020, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) became a pandemic that has caused more than 7 million deaths so far. An extraordinary research effort allowed to obtain antiviral vaccines[1] since the end of 2020 and their impact in preventing severe infections has been rapidly highlighted[2]. In Italy, a three-dose vaccination schedule was recommended for high-risk groups among which physicians and frail patients, with a first administration followed by a second dose after 21 days and a third booster dose after 6

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months. The goal of vaccine administration is to induce an immune response, and some degree of inflammation can be expected after vaccination[3]. Patients with thymic epithelial tumors (TET) are classified by histological analysis as type A, type AB, type B (separated into B1, B2, and B3) thymoma and thymic carcinoma[4], and they were included among frail patients eligible for the vaccine, although reduced immunological self-tolerance makes such patients more prone to autoimmunity[5,6]. Our group[7,8] and others[9] have described the safety and efficacy of the COVID-19 vaccine in patients with different types of TET as well as the absence of immune-related complications[8,10]. Furthermore, lymphocyte subpopulations were evaluated as potential predictive markers of immunological response to the COVID-19 vaccine in TET patients[9,10]. However, inflammation with cases of acute respiratory distress syndrome was sporadically reported as complications of the COVID-19 vaccine[3] and we recently demonstrated that about 50% of patients with TET showed an increase of serum biochemical markers of inflammation after the second dose of vaccine[11]. High-density lipoproteins (HDL) are among the main players in inflammatory and immune modulation[12]. During infections, the reduction of cholesterol and HDL and the remodeling of HDL in blood compromise the anti-inflammatory activity of these molecules[13]. In fact, we found relevant changes in lipid metabolism during acute COVID-19-related inflammation[14]. HDL can be divided into various subclasses, according to its density, size, electrophoretic mobility and apolipoprotein cargo[15]. In our study, HDL were separated according to their size by polyacrylamide gel electrophoresis (Quantimetrix), a method that allows the subdivision of HDL into 10 subfractions, which are subsequently grouped into three main classes: Large (from HDL-1 to HDL-3), Intermediate (from HDL-4 to HDL-7) and Small (from HDL-8 to HDL-10). An increased level of large HDL and a reduced level of small HDL have been observed in a chronic inflammatory disease, i.e., rheumatoid arthritis, together with an inverse correlation between C-reactive protein and small HDL[16]. A reduction of small HDL has been also observed in multisystem inflammatory syndrome in children, a severe post-COVID condition due to a delayed hyperimmune response to SARS-CoV-2[17]. Therefore, the aim of the present study was to explore serum HDL and HDL subfractions in patients with TET at baseline and after the second dose of COVID-19 vaccine.

Results

At baseline (T0) and one month after the second dose (T2), Cholesterol and HDL values showed a normal distribution within normal ranges, although for HDL we found a minimum value of 19 mg/dL in two patients. At T2, HDL levels were not significantly different, compared to T0 (Table 1). Also, large and intermediate HDL classes did not show significant differences between the two times, whereas small HDL class resulted significantly lower at T2 than T0. Supplementary Table 1 showed comparisons of values of the ten HDL subfractions at T0 and T2. Only HDL 10 subfraction resulted significantly decreased at T2 as compared to T0.

We observed that, based on the serum levels of the small HDL class and/or the HDL-10 subfraction, two distinct trends emerged in the 45 patients. A group of 24 patients (53.3%) showed a significant decrease of serum small HDL class and/or HDL 10 at T2 as compared to baseline values (subgroup 1); and another group of 21 patients (46.7%) did not show significant variations of these parameters (subgroup 2), as reported in Supplementary Table 2. Furthermore, we found no statistical differences between the two subgroups for the main characteristics, for the presence of comorbidities and treatment (Table 2). Moreover, during the observation period, no specific dietary or physical activity regimen was recommended to the patients. Figure 1A shows the bar chart of serum HDL, small HDL class and HDL 10 subfraction in subgroup 1 at T0 and T2. The values of both small HDL and HDL 10 were significantly different ($p < 0.0001$) between T2 and T0, while HDL levels were not significantly different. Figure 1B shows the trend of HDL, small HDL class and HDL 10 subfraction in subgroup 1 at T0 and T2.

As shown in Table 3, we evaluated the correlations between HDL, small HDL class and HDL 10 subfraction *versus* previously studied IL-6 values[11] and previously studied lymphocyte subpopulations[10] in subgroup 1 at T0 and T2. The values of these parameters are reported in Supplementary Table 3. Serum HDL, small HDL class and HDL 10 subfraction values at T2 were significantly and inversely correlated with serum IL-6 values at T2, whereas no correlation was found for values at T0. Furthermore, serum HDL values at T2 were significantly and inversely correlated with percentage of activated Th17 lymphocytes at T2, while at T0 no correlation was found. In addition, small HDL class values at T2 were significantly and inversely correlated with percentage of activated T lymphocytes at T2, whereas no correlation was found at T0. Finally, both small HDL class and the HDL 10 subfraction at T2 exhibited significant negative correlations with the percentage of regulatory T cells, with no corresponding association at T0.

	T0	T2	*p* value
Cholesterol	178 (32)	182 (36)	0.248
HDL	41.4 (13.7)	40.5 (13.6)	0.481
Large HDL	19 (14.0.-26.5)	20 (13.5–27.5)	0.620
Intermediate HDL	14.7 (3.5)	14.3 (3.4)	0.351
Small HDL	5.3 (2.1)	4.6 (1.9)	**0.038**

Table 1. Comparison of values (mg/dL) of Cholesterol, HDL and HDL classes at T0 and T2 in 45 TET patients. T0: baseline; T2: one month after the second dose. Significant *p* values are reported in bold. Continuous variables were expressed as mean (± standard deviation), while non-parametric variables were presented as median and interquartile range (IQR).

	TET patients (n = 45)	Subgroup 1 (n = 24)	Subgroup 2 (n = 21)
Age (years)	55.0 (47.5–64.0)	54.5 (47.0–64.0)	55.0 (46.5–64.0)
Male, n (%)	17 (38)	9 (38)	8 (38)
BMI	25.8 (23–32.5)	26.4 (23.3–33.6)	25 (20.5–31.1)
Diabetes, n (%)	11 (24)	6 (25)	5 (24)
Smoker, n (%)	15 (33)	8 (33)	7 (33)
Dyslipidemia, n (%)	11 (24)	5 (21)	6 (29)
Statins, n (%)	7 (16)	3 (13)	4 (19)
Beta-blockers, n (%)	18 (40)	12 (50)	6 (29)
Treatment, n (%) *	21 (47)	12 (50)	9 (43)
Autoimmune diseases, n (%)	17 (38)	9 (38)	8 (38)
Miastenia gravis (MG), n (%)	8 (18)	4 (17)	4 (19)
Severe immunodeficiency, n (%)	24 (53)	13 (54)	11 (52)

Table 2. Characteristics of study population. TET: thymic epithelial tumors, *etoposide and/or somatostatin analog.
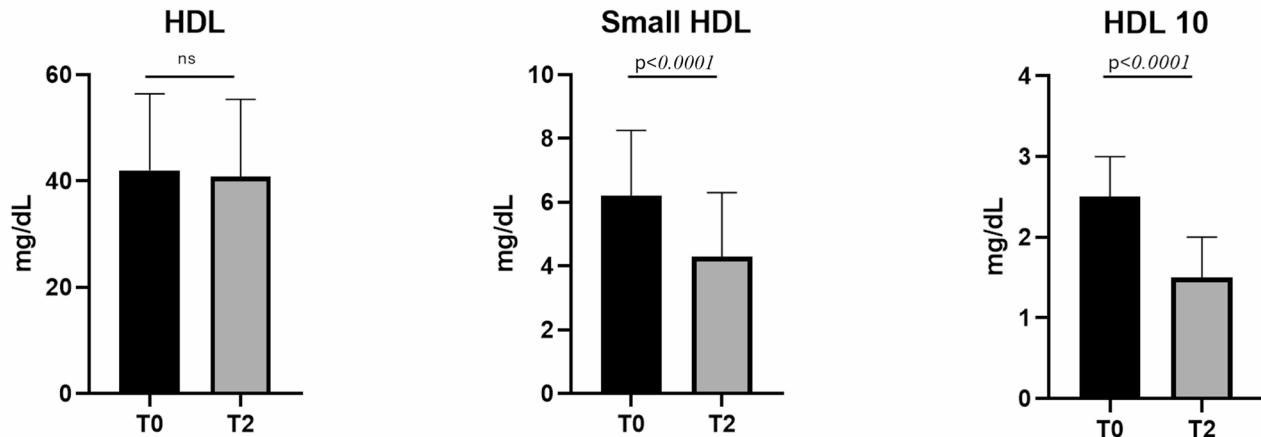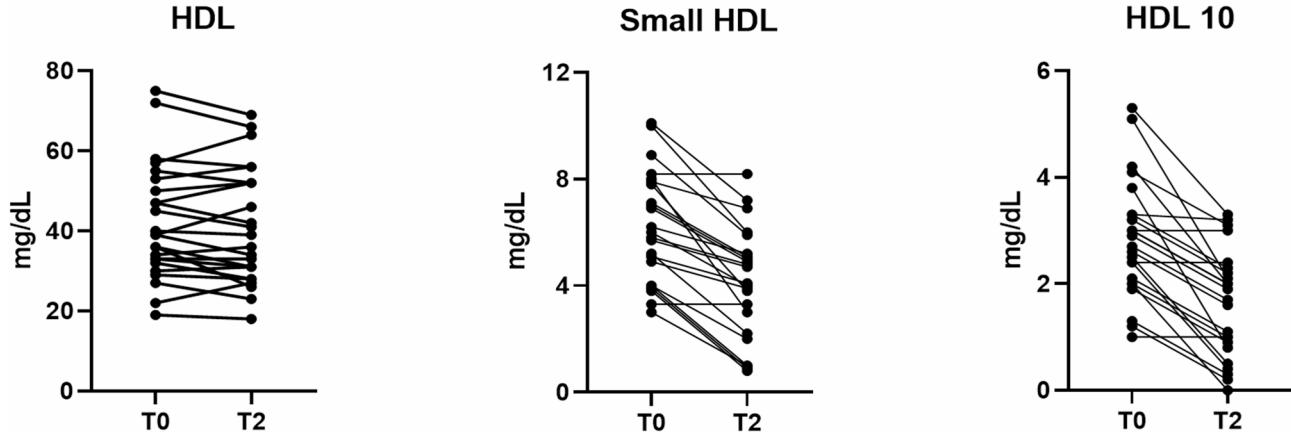
**Fig. 1**. (**Panel A**) Bar chart of serum HDL, small HDL class and HDL 10 subfraction in patients with TET (subgroup 1) at baseline (T0) and one month after the second dose of vaccine (T2). (**Panel B**) Trend of serum HDL, small HDL class and HDL 10 subfraction in patients with TET (subgroup 1) at baseline (T0) and one month after the second dose of vaccine (T2).

	HDL		Small HDL		HDL 10	
	p value	r_s	*p* value	r_s	*p* value	r_s
IL-6 (pg/ml) T0	0.080	−0.364	0.420	−0.172	0.696	−0.084
IL-6 (pg/mL) T2	**0.008**	−0.525	**0.019**	−0.475	**0.030**	−0.444
Lymphocytes (%) T0	0.972	0.008	0.119	0.327	0.125	0.322
Lymphocytes (%) T2	0.834	0.045	0.377	0.189	0.357	0.196
T lymphocytes (%) T0	0.820	−0.049	0.583	0.117	0.406	0.177
T lymphocytes (%) T2	0.522	0.137	0.181	0.282	0.211	0.264
Activated T (%) T0	0.745	0.070	0.434	−0.167	0.593	−0.115
Activated T (%) T2	0.901	−0.026	**0.023**	−0.460	0.064	−0.383
Activated Th 1 (%) T0	0.542	−0.130	0.788	0.057	0.518	0.138
Activated Th 1 (%) T2	0.138	−0.312	0.299	−0.220	0.573	−0.120
Activated Th 17 (%) T0	0.855	−0.039	0.479	−0.151	0.449	−0.162
Activated Th 17 (%) T2	**0.033**	−0.435	0.276	−0.231	0.191	−0.276
Regulatory T cells (%) T0	0.792	0.056	0.511	−0.141	0.466	−0.156
Regulatory T cells (%) T2	0.969	−0.008	**0.002**	−0.589	**0.003**	−0.573

Table 3. Spearman correlation analysis of serum levels of HDL, small HDL class, and HDL 10 subfraction at T2 versus IL-6 and T lymphocyte populations in subgroup 1. IL: interleukin; r_s: Spearman's correlation coefficient. Significant values are in bold.

Discussion

It is well known that vaccines work by introducing antigens that stimulate the immune system to develop targeted protection. These antigens activate innate immune receptors, triggering cytokine release and inflammatory responses similar to those caused by natural infections. Such reactions are essential for building adaptive immunity but can also lead to mild local or systemic side effects like pain, swelling, fever, or fatigue. Maintaining a balance between immune activation and minimal reactogenicity is key to vaccine safety[18].

We found that the COVID-19 vaccine causes a remodeling of serum HDL in about 50% of patients with TET after the second dose. Specifically, we found a significant reduction of small HDL class and HDL 10 subfraction with known anti-inflammatory activity[17,19,20]. These results agree with our recent observation of an increase of serum inflammatory biomarkers in about half of TET patients after the second dose of vaccine (T2)[11]. In fact, the levels of small HDL class and HDL 10 subfraction were inversely correlated with serum IL-6 levels after the second dose of vaccine, whereas no correlations were found at baseline. Such data reinforce the pivotal role of HDL in the pathogenesis of inflammation[12]. A previous study reported a proinflammatory response 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[21] but, differently from our results, in that study the proinflammatory response correlated to the magnitude of the humoral response. Our data show that only the subgroup 1 of TET patients showed a significant increase of serum biomarkers and a reduction of anti-inflammatory HDL class, whereas the subgroup 2 did not develop significant change. We confirm that these two subgroups are similar both in terms of major complications and adverse events to the vaccine that were not observed in any patient. Also, no differences were observed in terms of humoral and cell immunity development toward the vaccine. The S protein of the virus may trigger the inflammatory response to the vaccine[22]. The cytokine release and the remodeling of HDL would be related to activated T-lymphocytes, particularly activated Th17 (whose values were inversely correlated to small HDL class and to HDL levels, respectively), as we observed in both acute COVID-19[23] and multisystem inflammatory syndrome in children[24,25]. Moreover, the role of HDL in maintaining immune homeostasis is underscored by evidence showing that levels of the small HDL class and the HDL 10 subfraction are inversely correlated with regulatory T cells, which are crucial for preserving peripheral tolerance, preventing autoimmunity, and controlling chronic inflammatory responses[26].

Indeed, the reduction and the remodeling of HDL with a depletion of small HDL class were observed in both healthy subjects after the infusion of the lipopolysaccharide and in patients with infectious diseases[20], and in severe COVID-19[27]. The present study firstly describes HDL and their subfractions in TET patients after the vaccine and demonstrates that also in such conditions in about half of the subjects there is a remodeling that reduces subfractions with a higher content of apoA-1, antioxidant enzymes, and L-cholesterol acyltransferase[28]. These alterations share the pathogenic mechanism previously observed in bacterial sepsis[29] and in patients with common variable immunodeficiency with infectious and autoimmune complications[12]. In fact, the cytokine storm causes the alteration of cholesterol release and the impairment of cholesterol reverse transport[30,31]. This is confirmed by the significant inverse correlation of small HDL class and HDL 10 *versus* serum IL-6. As consequence of the impaired cholesterol release and reverse transport, there is a reduction of circulating HDL, that causes the hyperactivation of TLR2 and TLR4 receptors with the subsequent inhibition of ATF3 expression and further release of cytokines by macrophages[12,13] creating a vicious circle. Although our study included a low number of patients with TET, they were clinically homogeneous and carefully monitored during the vaccine cycle. After the second dose of vaccine, none of the patients with TET had further immune-related complications, although we cannot exclude that in the long term an immune-related disease may develop in TET patients with elevated levels of inflammatory markers. In fact, it has been shown that excessive secretion of

IL-6 can lead to the onset of autoimmune diseases (AD)[32]. Moreover, we previously found significantly higher levels of serum IL-6 in TET patients with AD compared to TET patients without AD suggesting a role of this cytokine in sustaining these diseases[5]. Therefore, the inverse association between small HDL class and HDL 10 subfraction versus IL-6, observed in this study, suggests these parameters as useful potential markers for the identification of patients at increased risk of inflammatory complications and/or AD. A study limitation is represented by the lack of comparisons between the different types of TET due to the limited number of patients in each group. Furthermore, it was not possible to study the trend of HDL subfractions in healthy subjects since most of the population had already started the COVID-19 vaccination cycle and the baseline sample was not available. Finaly, there is a lack of data regarding dietary or physical activity, since we did not recommend any specific regimen during the study period.

Methods

Study design and participants

The study was approved by the Ethics Committee of our institution (#76.21). Forty-five patients affected by TET (median age: 55 years, interquartile range (IQR): 4–65, 17 males), referred to the Regional Coordination Center for Rare Tumors of the Campania Region (Federico II University Hospital of Naples) from April to November 2021, were prospectively enrolled. Study inclusion criteria (reported in a previous study[10] that evaluated the humoral and cellular response to the vaccine[11,33]), were: age greater or equal to 18 years, histological diagnosis of TET or thymic carcinoma (biopsy or surgical specimen); and known status (presence vs absence) of autoimmune disorders. Immune-related disorders were diagnosed according to national guidelines[34], using the following criteria. Severe immunodeficiency: hypogammaglobulinemia, low or absent B cells, abnormal CD4/CD8 T-cell ratio, CD4 T-cell lymphopenia, and impaired T-cell mitogenic responses, associated with increased susceptibility to infections due to encapsulated bacteria, fungi, or viruses. Myasthenia gravis (MG): antibodies directed against postsynaptic antigens (acetylcholine receptor, muscle-specific kinase, or LRP4), with or without clinical signs such as ptosis, diplopia, or limb/bulbar muscle weakness. All patients were enrolled at baseline, before the first dose of COVID-19 mRNA vaccine (BNT162b2 from Pfizer-BioNTech). SARS-CoV-2 infection was not found in any of the patients until T2. Serum biomarkers were assessed at two time points, i.e., baseline (T0) and 1 month after the second dose (T2). We next divided the patients into two subgroups: subgroup 1, patients that showed a significant decrease of serum small HDL class and/or HDL 10 subfraction at T2 as compared to baseline values; subgroup 2, patients that did not show significant variations of these parameters.

Biochemical parameters

Serum HDL levels were measured by an automated biochemistry analyzer (Architect ci 16200 Integrated System, Abbott Diagnostics, Rome, Italy) using specific commercial kits (Abbott Diagnostics, Rome, Italy). For all patients, samples collected at T0 and T2 were analyzed in the same laboratory. Interleukin (IL)-6 levels were analyzed by automated microfluidic immunoassay cartridges on the ProteinSimple Ella (Bio-Techne), and the results were previously described[11]. The analysis of HDL subfractions was performed by polyacrylamide gel electrophoresis using the Quantimetrix kit (Quantimetrix Corp., CA, USA). In brief, serum (25 μL) was added to the precast high-resolution polyacrylamide gel tubes along with Lipoprint HDL Loading Gel solution (300 μL). After mixing, the tubes were photopolymerized at room temperature for 30 min. Electrophoresis with tubes containing sera samples was performed at a constant of 3 mA/tube for 50 min. Then, the tubes were scanned and analyzed using the Lipoprint LDL/HDL subfractions system. Subfraction lanes were identified by their mobility using low-density lipoproteins (LDL) + very-low-density lipoproteins (VLDL) as the starting reference point (LDL/VLDL = 0) and albumin as the ending reference point (Albumin = 1). The HDL subfractions were differentiated between LDL/VLDL and albumin peaks and the ten HDL subfractions were grouped into Large (from HDL-1 to HDL-3), Intermediate (from HDL-4 to HDL-7) and Small (from HDL-8 to HDL-10) classes. Although this kit is currently validated for research analysis only, it allows to resolve HDL in ten subfraction relative to particle size[35]. Lymphocyte populations were analyzed by multicolor flow cytometry (Facs Canto II; Becton Dickinson); the gating strategy was as previously described[23,36,37]. In brief, lymphocytes were gated based on CD45 vs. SSC-A, and this population was used to identify T lymphocytes (CD45+, CD3+). Within this subset, T helper (TH) and cytotoxic T cells were defined as CD3+, CD4+ and CD3+, CD8+, respectively. Naïve and memory T cells were characterized by CD45RA+ and CD45RO+ expression, respectively. Regulatory T cells were identified as CD3+, CD4+, CD25+, CD127−. Activated T lymphocytes were defined within the lymphocyte gate (CD45 vs. SSC-A) as CD3+, HLA-DR+. Among TH cells (CD3+, CD4+), TH17 and TH1 subsets were identified as CCR6+, CXCR3− and CCR6−, CXCR3+, respectively, while their activated forms were determined by CD38+ and HLA-DR+ expression.

Statistical analyses

Continuous parametric and non-parametric data were reported as average (standard deviation) and median (IQR), respectively. The Shapiro–Wilk test was applied to evaluate the normality of the distributions. Paired comparisons were evaluated using the paired t test and Wilcoxon test, as appropriate. 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 Chi-square test. The correlations between variables were assessed using Spearman correlation analysis. Statistical analyses were performed by SPSS (version 28, IBM SPSS Statistics). Graphing was performed using Graph Pad Prism 8 Software (GraphPad Software, San Diego, CA, USA). p values < 0.05 were considered significant.

Data availability

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions
Design of the work: G.Ce, G.Ca. and M.Ge.; Methodology, investigation and data analysis: G.Ce., P.d.P, E.P, M.T.; Manuscript writing and validation: G.Ce., G.P., M.Gi., G.Ca. and M.Ge. All authors read and approved the final manuscript.

Declarations

Competing interests
The authors declare no competing interests.

Ethics approval and consent to participate
The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the University Federico II of Naples (#76.21). Informed consent was obtained from all participants.

Additional information
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