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# CD8+CD103+PD1+TIM3+ T cells in glioblastoma microenvironment correlate with prognosis

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## Abstract

Glioblastoma, isocitrate dehydrogenase-wildtype (GB), is the most common and aggressive primary brain malignancy with poor outcome. Immune checkpoint inhibitors (ICIs) have been tested in GB and, despite disappointing results, the identification of a small subgroup of responders underlies the need to improve our understanding of the tumour microenvironment (TME) immunity. This study aimed to determine whether the expression of selected immune checkpoints on tissue-resident memory T cells (Trm) may predict patient outcome. We conducted a single cohort observational study. Tumour samples were collected from 45 patients with histologically confirmed GB (WHO grade 4) and processed to obtain single-cell suspensions. Patients were assessed for the correlation of Trm phenotype with overall survival (OS) or progression-free survival (PFS) using multiparametric flow cytometry and uni/multivariate analyses. Levels of Trm expressing programmed cell death protein 1 (PD1) and T cell immunoglobulin and mucin domain-containing protein 3 (TIM3) were found to be linked to clinical outcome. Low frequency of Trm expressing PD1 or TIM3 or both markers defined subgroups as independent positive prognostic factors for patient survival. On multivariate analysis, low CD8+CD103+PD1+TIM3+ Trm and Karnofsky performance status (KPS) ≥70 were confirmed to be the most

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predictive independent factors associated with longer OS (hazard ratios—HR [95%CI]: 0.14 [0.04–0.52] $p < 0.001$, 0.39 [0.16–0.96] $p = 0.04$, respectively). The CD8+CD103+ Trm subgroups were also age-related predictors for survival in GB.

**KEYWORDS**
age, disease outcome, glioblastoma, PD1, predictors, TIM3, tissue-resident memory T cells (Trm)

## INTRODUCTION

Glioblastoma (GB) is the most common and malignant primary tumour of the central nervous system (CNS) in adults [1]. The standard-of-care treatment consists of surgery followed by radiotherapy with concomitant and adjuvant temozolomide (TMZ) [2]. The prognosis is poor, with a median overall survival (OS) of approximately 18 months and a 2-year survival rate lower than 20% [3].

Several factors hinder the efficacy of GB therapies. Among them, the immunosuppressive tumour microenvironment (TME) [4], is thought to be the major cause of failure of many clinical trials with immunotherapies. Despite this, in the first clinical trial using nivolumab, 8% of patients showed responses longer than bevacizumab (11.1 months vs. 5.3 months) [5].

Several studies indicated that multiple therapies for GB may lead to substantial changes in the TME [6], whose immune contexture is one of the most important players for tumour progression and response to therapies in many cancer types [7]. TME is a highly complex and dynamic entity that is responsible for defining GB as a cold tumour [8], dominated by a highly immunosuppressive milieu and dysfunction of T cells [9]. A diverse rate of tumour-infiltrating lymphocytes (TILs), including tissue-resident memory T cells (Trm), and the expression of specific stimulatory or inhibitory molecules are determinant factors in defining TME immune reactivity [10]. Accordingly, T cells infiltrating GB express multiple immune checkpoints, such as programmed cell death protein 1 (PD1) and T cell immunoglobulin and mucin domain-containing protein 3 (TIM3), and exhibit impaired function [11].

Solid tumours show enrichment of CD4+ and CD8+ Trm that, upon recruitment into the tissue and in the presence of local inflammatory signals, undergo maturation with CD103 up-regulation [12]. CD8+CD103+ T cells, also defined as CD8+CD103+ Trm, are the major anti-tumour effector cells, and their high rates correlate with longer OS in many tumour types [13]. CD8+CD103+ Trm populate the human brain, playing a key role in immune surveillance [14], and have also been implicated in the response to neoadjuvant vaccination of GB patients [15]. However, the role of CD8+CD103+ Trm in GB needs to be further elucidated.

Age-dependent factors have been reported to be critical for GB prognosis [16]. For example, elderly GB patients have a better prognosis based on O6-methylguanine (O6-MeG)-DNA methyltransferase (*MGMT*) status [17]. Although immune dysfunction increases with age [17], in GB, patient age has not been linked to specific immune parameters yet.

Here, we analysed intratumoural T cells in GB and we postulate a major role of CD8+CD103+ Trm in shaping TME immune contexture. Importantly, these cells hold prognostic significance since the low frequency of CD8+-CD103+ Trm expressing PD1 and TIM3 predicts longer survival.

## MATERIALS AND METHODS

### Study design

Enrolled patients underwent GB resection and standard therapeutic and follow-up approaches in Neuro-Oncology. Main collected data: baseline demographics, Karnofsky Performance Status (KPS), gross total resection (GTR) versus non-GTR, Stupp therapy, isocitrate dehydrogenase status, MGMT methylation status, progression free survival (PFS) and overall survival (OS).

### Biological sample processing

Freshly resected tumour samples were processed to obtain single-cell suspensions to be stored in liquid nitrogen; PBMCs were collected and stored in liquid nitrogen. Both types of samples were thawed to perform experiments.

### Multiparametric flow cytometry and in vitro TIL expansion and characterisation

Thawed tumour samples were depleted from myelin debris and 10 μL of each cell suspension was stained with two specific antibody mixtures to evaluate T cell subsets. For TILs, whole tumour cell suspensions were cultured in the presence of IL-2 and anti-CD3/anti-CD28 stimuli with or without anti-TIM3 Sabatolimab and anti-PD1 Pembrolizumab. After 15 days, cells were stained with a mixture of

**TABLE 1** Clinical features of patients enrolled in the study.

| Variable | All | Age | | | Sex | | | MGMT | | | KPS | | | GTR | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | ≤63 | ≥63 | *p* | Male | Female | *p* | Met | Unmet | *p* | ≥ 70 | ≤ 70 | *p* | Yes | No | *p* |
| Number (%) | 45 (100) | 22 (48.9) | 23 (51.1) | | 24 (53.3) | 21 (46.7) | | 27 (60) | 18 (40) | | 34 (74.5) | 11 (25.5) | | 32 (71.1) | 13 (28.9) | |
| Age at diagnosis median (range) | 64 (45–78) | 57 (45–63) | 71 (64–8) | **<0.0001** | 61 (45–78) | 65 (49–76) | 0.62 | 65 (45–78) | 63.5 (52–76) | 0.75 | 60.5 (45–78) | 72 (55–76) | **0.02** | 61 (49–78) | 67 (45–77) | 0.58 |
| Sex *n* (%) | | | | 0.55 | | | | | | 0.76 | | | 1 | | | 0.2 |
| Male | 24 (53) | 13 (59) | 11 (47.8) | | | | | 15 (55.5) | 9 (50) | | 18 (0.53) | 6 (54.5) | | | | |
| Female | 21 (47) | 9 (41) | 12 (52.1) | | | | | 12 (44.5) | 9 (50) | | 16 (0.47) | 5 (45.5) | | 17 (53) | 4 (31) | |
| Tumour site *n* (%) | | | | | | | | | | | | | | | | |
| Temporal | 19 (42.2) | 7 (31.8) | 12 (52.1) | 0.23 | 11 (4.8) | 8 (38.1) | 0.76 | 11 (40.7) | 8 (44.4) | 1 | 14 (41.2) | 5 (45.4) | 1 | 15 (46.9) | 4 (30.8) | 0.5 |
| Frontal | 15 (33.3) | 8 (36.3) | 7 (30.4) | 0.75 | 4 (1.6) | 11 (5.4) | 0.02 | 10 (37) | 5 (27.7) | 0.74 | 12 (35.3) | 3 (27.3) | 0.72 | 12 (37.5) | 3 (23) | 0.49 |
| Parietal | 8 (17.8) | 5 (22.7) | 3 (13) | 0.45 | 7 (29.1) | 1 (4.7) | **0.05** | 4 (14.8) | 4 (22.2) | 0.69 | 5 (14.7) | 3 (27.3) | 0.38 | 4 (12.5) | 4 (30.8) | 0.2 |
| Occipital | 1 (2.2) | 1 (4.5) | 0 (0) | 0.48 | 1 (4.2) | 0 (0) | 1 | 1 (3.7) | 0 (0) | 1 | 1 (2.9) | 0 | 1 | 1 (3.1) | 0 | 1 |
| Multicentric | 2 (4.5) | 1 (4.5) | 1 (4.3) | 1 | 1 (4.2) | 1 (4.7) | 1 | 1 (3.7) | 1 (5.5) | 1 | 2 (5.9) | 0 | 1 | 0 (0) | 2 (15.4) | 0.07 |
| KPS median (range) | 70 (40–90) | 80 (50–909 | 70 (40–90) | 0.05 | 70 (40–90) | 80 (40–90) | 0.75 | 70 (60–90) | 75 (40–90) | 0.64 | | | | 80 (50–90) | 70 (40–80) | 0.003 |
| ≥70 (%) | 34 (75.5) | 20 (90.9) | 14 (60.9) | 0.73 | 18 (75) | 16 (76.2) | 0.9 | 24 (88.9) | 10 (55.6) | 0.007 | | | | 27 (84.4) | 7 (53.8) | 0.1 |
| <70 (%) | 11 (24.5) | 2 (9.1) | 9 (39.1) | 0.45 | 6 (25) | 5 (23.8) | 0.34 | 3 (11.1) | 8 (44.4) | 0.17 | | | | 5 (15.6) | 6 (46.2) | 0.17 |
| GTR *n* (%) | | | | 0.51 | | | 0.2 | | | 0.73 | | | **0.05** | | | |
| Yes | 32 (71.1) | 17 (77.3) | 15 (65.2) | | 15 (62.5) | 17 (81) | | 20 (74.1) | 12 (67.7) | | 27 (79.4) | 5 (45.4) | | | | |
| No | 13 (28.9) | 5 (22.7) | 8 (34.8) | | 9 (37.5) | 4 (19) | | 7 (25.9) | 6 (33.3) | | 7 (20.6) | 6 (54.6)) | | | | |
| Stupp therapy *n* (%) | | | | 1 | | | 0.7 | | | 0.23 | | | 0.08 | | | 0.2 |
| ≥6 cycles | 37 (82) | 18 (81) | 19 (82) | | 19 (79) | 18 (86) | | 24 (89) | 13 (72) | | 30 (88) | 7 (64) | | 28 (87) | 9 (69) | |
| <6 cycles | 8 (18) | 4 (19) | 4 (18) | | 5 (21) | 3 (14) | | 3 (11) | 5 (28) | | 4 (12) | 4 (36) | | 4 (13) | 4 (31) | |
| IDH mutation *n* (%) | 0 (0) | 0 (0) | 0 (0) | | 0 (0) | 0 (0) | | 0 (0) | 0 (0) | | 0 (0) | 0 (0) | | 0 (0) | 0 (0) | |
| MGMT *n* (%) | | | | 1 | | | 0.76 | | | | | | **0.01** | | | 0.73 |
| Met | 27 (60) | 13 (59.1) | 19 (82) | | 15 (62.5) | 12 (57.1) | | | | | 24 (70.6) | 3 (27.3) | | 20 (62.5) | 7 (53.8) | |
| Unmet | 18 (40) | 9 (40.9) | 4 (18) | | 9 (37.5) | 9 (42.9) | | | | | 10 (29.4) | 8 (72.7) | | 12 (375) | 6 (46.2) | |
| OS (months) median (range) | 12 (2–31) | 14.7 (5–31) | 10 (2–24) | **0.01** | 12.7 (5–25) | 12 (2–30) | 0.8 | 14.5 (6.5–30) | 10.5 (2–31) | 0.1 | 14.7 (7–31) | 7.5 (7–21) | **0.0007** | 15 (2–31) | 9 (2–25) | **0.004** |
| PFS (months) median (range) | 6 (1–22) | 8 (3–22) | 5 (1–16) | **0.02** | 6 (2–15) | 6 (1–22) | 0.9 | 6 (2–18.5) | 6 (1–22) | 0.7 | 8 (2–22) | 6 (1–13) | **0.002** | 8 (2–22) | 5 (1–10) | **0.002** |

*Note*: Statistical tests: Mann Whitney *U* test for continuous variables (Age, KPS), Fischer's exact test for categorical variables (Sex, GTR, Chemotherapy, MGMT status, Tumour site). $p < 0.05$ was considered to indicate the statistical significance and is highlighted in bold.
Abbreviations: GTR, gross total resection; IDH, isocitrate dehydrogenase; KPS, Karnofsky performance status; MGMT, O6-methylguanine-DNA-methyltransferase; OS, overall survival; PFS, progression free survival.

antibodies to evaluate the phenotypic and functional parameters of CD8+ T cell subsets. All stained cells were evaluated by the cytometry Beckman Coulter system.

## Statistical analysis

Kruskal–Wallis rank test for unpaired distributions and Wilcoxon matched-pairs signed-rank test were used to identify differences between immune cell subsets. Categorical variables were analysed by Fisher's exact test. Spearman's rank analysis was used to correlate continuous variables. The Contal-O'Quigley cut-off method was applied to dichotomise continuous variables and to identify the optimal cut-off value for grouping patients into high and low groups. Survival was assessed by Kaplan–Meier analysis (statistical significance: $p < 0.05$) and their differences were assessed by log-rank test. Cox proportional hazard model was used for univariate and multivariate models.

Detailed information on methods and Materials are described in the Supporting Information. Clinical Trial Registration: Prot. 5755 Comitato Etico FPG.

# RESULTS

## Study design

This study included 45 newly diagnosed GB patients (Table 1). Twenty seven patients (60.0%) had *MGMT* promoter methylation, and 24 (53.3%) were males (Figure 1a). Median age at diagnosis was 64 years; median age of young patients (≤63 years) versus elderly patients (>63 years) was 57 versus 71, respectively. Males and females exhibited equally distributed baseline features, except for no significant trend towards younger age in males (median age in males vs. females 61 vs. 65 years; $p = 0.62$). The median follow-up was 12 months. Predictors of survival were KPS ≥70 and GTR (Table 1). Kaplan–Meier analysis showed that OS differed significantly according to age (young vs. elderly, 14.7 vs. 10 months, $p = 0.01$), KPS (≥70 vs. <70, 14.7 vs. 7.5 months, $p = 0.0007$), and GTR (GTR vs. non-GTR, 15 vs. 9 months, $p = 0.004$). Patients with methylated *MGMT* promoter had a longer median OS compared to those with unmethylated promoter (14.5 vs. 10.5 months; Table 1), but the difference was not significant. Conversely, PFS was significantly different only in patients grouped by KPS (median ≥70 vs. <70 group, 8 vs. 6 months, $p = 0.002$) and GTR (median GTR vs. no-GTR, 8 vs. 5 months, $p = 0.002$; Figure 1b; Table 1). These data were confirmed by the univariate analysis, which showed a significant correlation with better OS of patients aged ≤63 years, KPS ≥70, and GTR (hazard ratios—HR [95%CI]: 0.42 [0.20–0.89] $p<0.001$, 0.26 [0.12–0.58] $p<0.001$, 0.31 [0.15–0.65] $p<0.001$, respectively). Better PFS was associated only with KPS ≥70 and GTR (HR [95%CI]: 0.36 [0.18–0.74] $p = 0.01$, 0.35 [0.17–0.74] $p = 0.01$, respectively). HR for OS and PFS were not significantly different between sexes (Figure 1c; Table S1). Overall, the clinical features of enrolled patients are in line with current literature.

## Significance of PD1 and TIM3 expression on CD8+CD103+ Trm

Although the immune infiltrate in GB has been largely characterised [18], the association of immune checkpoint expression on intratumoural T cell subsets with disease outcome needs to be further be exploited. Therefore, we performed a deep characterisation of T cells in tumour samples collected at surgery for lineage, differentiation, memory, activation and inhibition markers. The frequency of total immune infiltrates, identified as cells expressing high CD45 levels as opposed to microglial cells characterised by low CD45 expression (Figure S1), was extremely variable among GB tumours, ranging from 0.01% to 60.3% of viable cells (median 1.7%; Figure 2a). As well, high variability among patients was found in the frequency of CD45+CD3+ T lymphocytes (Figure S2) or of CD4+ and CD8+ T cells (CD8+ T cells: 32.4% [7.4%–74.9%]; CD4+ T cells: 34.6% [0%–59.1%]; Figure 2b). A significantly higher frequency of CD4+ and CD8+ T lymphocytes expressing PD1 compared to those expressing TIM3 or both immune checkpoints were also found (Figure 2c). Similarly to other solid tumours, GB also recruits CD103+ Trm, which are characterised by the concomitant expression of Trm-linked markers CD103 and CD69 [14] (Figure S1), herein referred as CD103+ Trm. CD4+CD103+ Trm rate resulted significantly lower than that of CD8+CD103+ Trm (median: 1.8% vs. 7.0%, $p = 0.0002$). In addition, the frequency of CD8+CD103+PD1+ Trm was significantly higher than CD4+CD103+PD1+ Trm (median: 6.3% vs. 1.4%, $p = 0.0002$) whereas TIM3 was present at comparable levels on the two T subpopulations (Figure 2d). The further analysis of the diverse subgroups within CD8+CD103+-Trm showed the significant prevalence of cells expressing PD1 compared to those expressing TIM3 and both immune checkpoints (Figure 2e). A deeper assessment of the correlation between the diverse intratumoural T subsets was performed by Spearman's analysis. The immune infiltrate was found to positively correlate with CD3+ T cells ($R = 0.58$, $p<0.0001$). In turn, CD3+ T cells displayed a strong correlation with CD8+ T cells ($R = 0.53$, $p = 0.0001$), a weak association with CD4+ T cells ($R = 0.34$, $p = 0.02$) and a strong inverse correlation with all CD4+CD103+ Trm

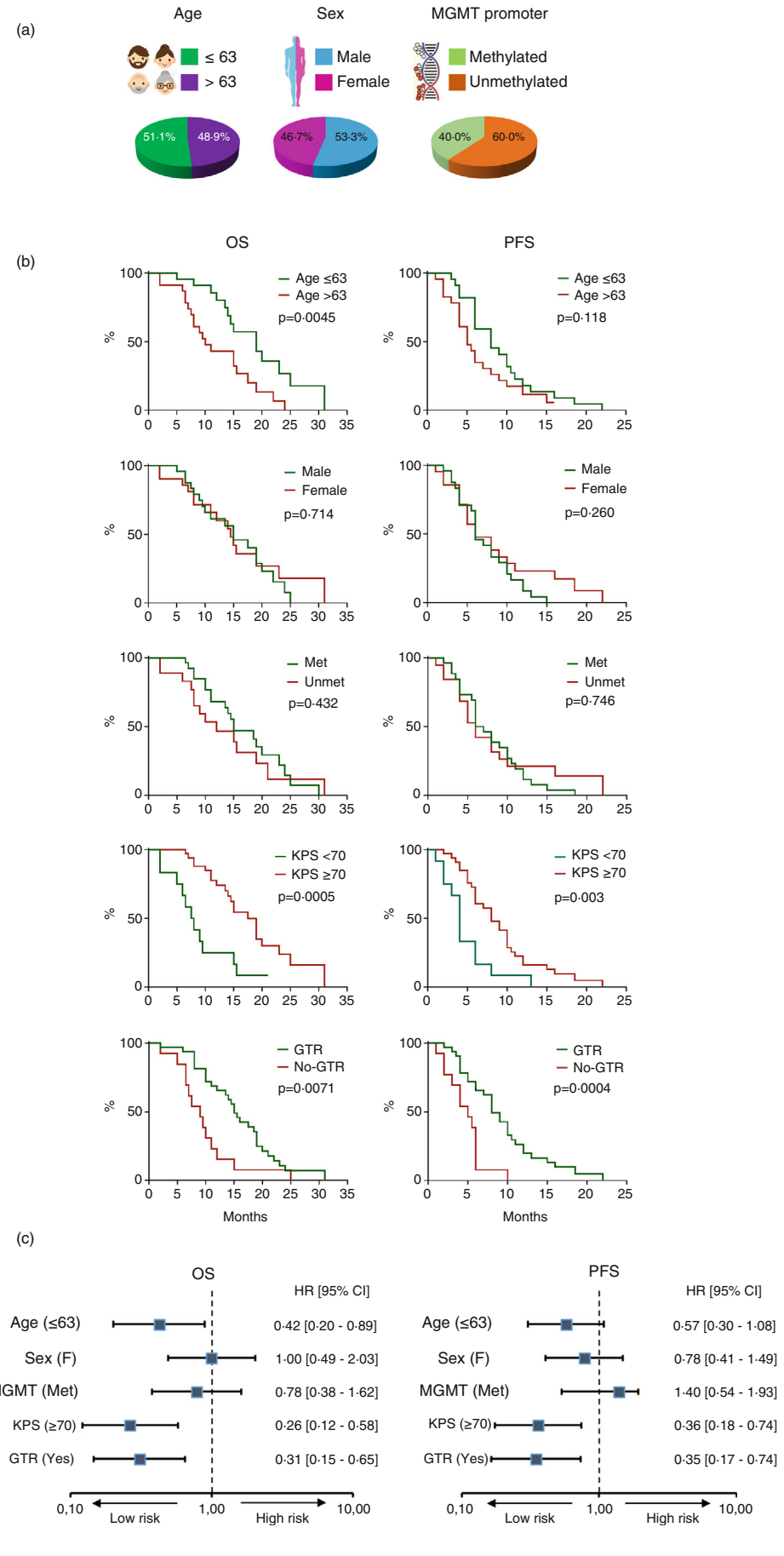


**FIGURE 1** (a) Schematic representation of the study parameters. GB cohort consists of 45 patients stratified by sex (female, males), age (≤63 years, >63 years) and *MGMT* promoter methylation status (methylated promoter = Met; unmethylated MGMT promoter = Unmet); (b) Kaplan–Meier analysis for OS and PFS in GB patients stratified by age, sex, *MGMT* promoter methylation, KPS and GTR. *p* values were calculated by the Log-rank (Mantel-Cox) test; (c) Forest plot showing hazard ratios with 95% confidence intervals of sample classifiers age, sex and *MGMT* promoter methylation status for OS and PFS. GB, glioblastoma; GTR, gross total resection; KPS, Karnofsky performance status; OS, overall survival; PFS, progression-free survival.

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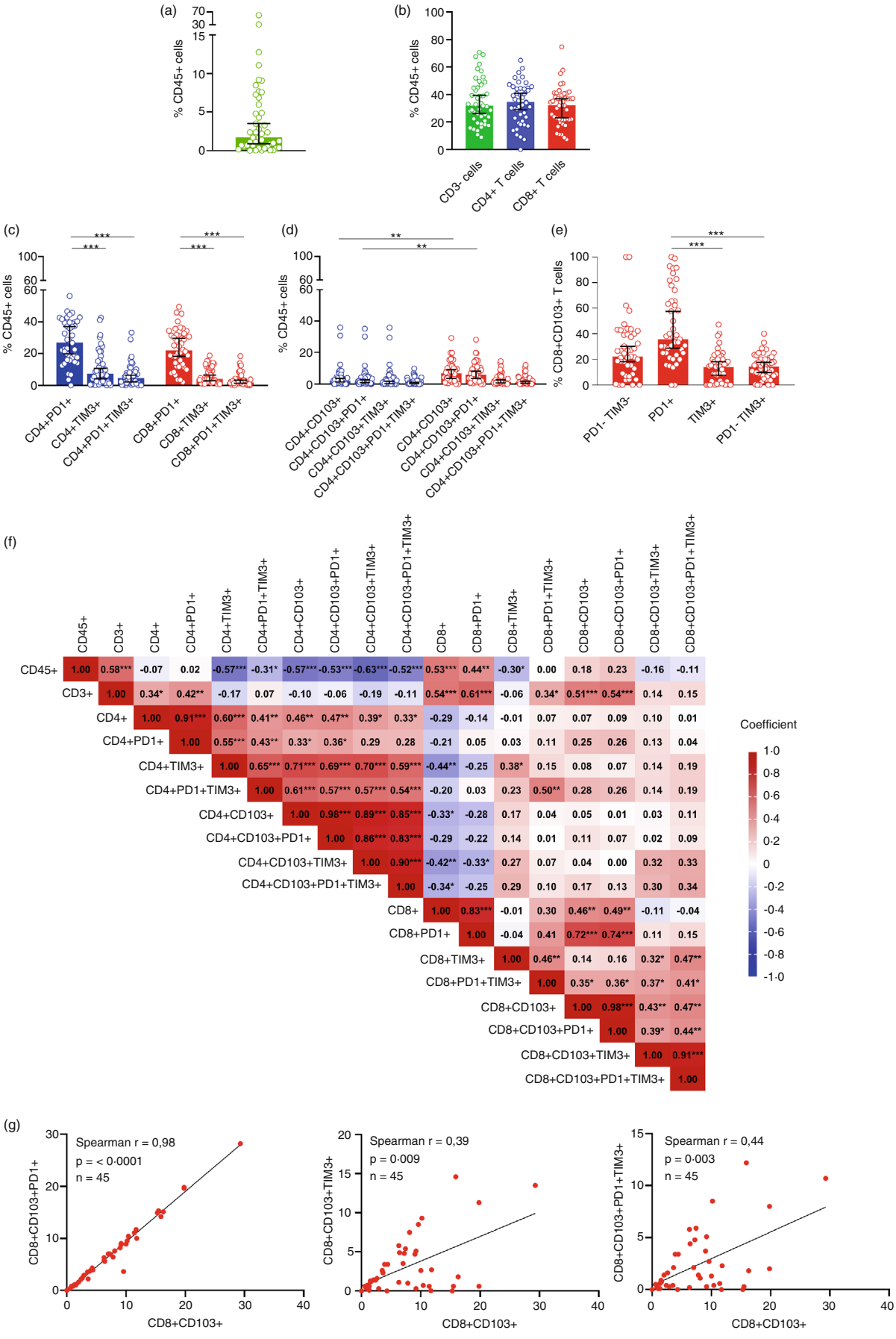


**FIGURE 2** Legend on next page.

subsets. Conversely, strongly positive associations were found between CD8+ and CD8+CD103+ Trm ($R = 0.46$, $p = 0.001$), CD8+CD103+ Trm and CD8+CD103+PD1+ Trm ($R = 0.98$, $p < 0.0001$), CD8+CD103+TIM3+ Trm and CD8+CD103+PD1+TIM3+ Trm ($R = 0.91$, $p < 0.0001$) (Figure 2f). Essentially, CD8+CD103+ Trm were strongly correlated with the presence of CD8+CD103+-PD1+, CD8+CD103+TIM3+ and CD8+CD103+PD1+-TIM3+ Trm subsets (Figure 2g). These data suggest that the intratumoural immune infiltrate in GB is positively associated with CD8+CD103+ Trm, and in particular with CD8+CD103+ Trm expressing PD1 and TIM3 rather than CD4+ Trm.

Next, we analysed the correlation of intratumoural T cells expressing PD1 or TIM3 or both inhibitory markers with prognosis, by categorising each T cell subset as 'high or low' frequency on the cut-off value from the asymptotic distribution of re-scaled rank statistic using the Contal-O'Quigley method. Four out of six CD8+ T subsets were found to be predictors of patient outcome with all CD8+CD103+ Trm subsets resulting the most statistically significant (Figure 3a). Specifically, low CD8+-CD103+PD1+ Trm were associated with significantly better OS and PFS ($p = 0.02$ and 0.0031, respectively) and even higher significant was the correlation with better OS and PFS of low CD8+CD103+TIM3+ Trm ($p = 0.0002$ and 0.0033, respectively) and CD8+CD103+-PD1+TIM3+ Trm ($p < 0.0001$, both) (Figure 3b). On univariate analysis, low CD8+CD103+PD1+, CD8+-CD103+TIM3+ and CD8+CD103+PD1+TIM3+ Trm subsets resulted independent predictors of improved OS (HR [95%CI]: 0.49 [0.35–0.92] $p < 0.001$, 0.23 [0.09–0.54] $p < 0.001$, 0.08 [0.02–0.25] $p = 0.04$, respectively) and PFS (HR [95%CI]: 0.37 [0.18–0.77] $p < 0.001$, 0.32 [0.14–0.74] $p < 0.001$, 0.13 [0.05–0.39] $p < 0.001$, respectively; Figure 3c; Table S2). Next, we used a multivariate model to explore the relationship between each CD8+CD103+ Trm subset and clinical parameters, namely age, sex, MGMT methylation, KPS and GTR, in predicting survival. Better OS was observed with low CD8+CD103+-PD1+ Trm (HR [95%CI]: 0.29 [0.12–0.74] $p = 0.01$) associated with GTR, KPS ≥70 and age ≤ 63, with low CD8+CD103+TIM3+ Trm (HR [95%CI]: 0.21 [0.08–0.53] $p < 0.001$) associated with KPS ≥70 and age ≤ 63, and lastly with low CD8+CD103+PD1+TIM3+ Trm (HR [95%CI]: 0.14 [0.04–0.52] $p < 0.001$) in association with KPS ≥70 only (Figure 4a; Table S3). Better PFS resulted associated with low CD8+CD103+PD1+ Trm and GTR and KPS, and low CD8+CD103+TIM3+ and CD8+CD103+PD1+TIM3+ Trm with KPS (Figure S3). Then, high or low CD8+CD103+ Trm subsets were evaluated for predicting OS in GB patients grouped by KPS and age. Kaplan–Meier plots showed that all three high CD8+CD103+ Trm subsets associated with KPS≥70 in predicting better OS (Figure 4b). Of note, elderly patients with low CD8+CD103+ Trm subsets had significantly better OS when compared with same age patients distinguished by high immune subsets; in particular low CD8+CD103+TIM3+ and CD8+CD103+PD1+TIM3+ Trm resulted the most significant predictors ($p = 0.0002$) (Figure 4c). In addition, CD8+CD103+ Trm subsets were statistically significant predictors of longer OS for young patients as compared to elderly ones. Confirming the multivariable analysis, young patients with high CD8+-CD103+PD1+TIM3+ Trm were absent in the Kaplan–Meier plot (Figure 4c). Low CD8+CD103+ Trm subsets were also found to be predictors of improved PFS in patients with KPS ≥70 and aged ≥63 (Figure S4a,b). Finally, a multivariate analysis including all CD8+ Trm subsets and clinical variables confirmed that low frequency of CD8+CD103+PD1+TIM3+ Trm was associated with KPS≥70 in predicting better OS (HR [95%CI]: 0.14 [0.04–0.52] $p < 0.001$) and PFS HR [95%CI]: 0.25 [0.08–0.80] $p = 0.02$, respectively (Figure 4d; Table S4).

**FIGURE 2** Lymphocyte composition and immunophenotypic characterisation of intratumoural T subsets in GB patients. Intratumoural T cell subsets were analysed by flow cytometry on viable CD45+ cells; dots represent single patients. (a) Distribution of intratumoural CD45+ cell frequencies in the GB cohort. (b) Median frequencies of CD4+ and CD8+ T cells with respect to total tumour CD45+ T cells. (c) PD1 and TIM3 expression on CD4+ and CD8+ T cells gather the subpopulations CD4+PD1+ T cells, CD4+TIM3+ T cells, CD4+PD1+TIM3+ T cells, CD8+PD1+ T cells, CD8+TIM3+ T cells, CD8+PD1+TIM3+ T cells. (d) Frequencies of CD103+ Trm subsets assessed within total CD45+ cells; PD1 and TIM3 expression on CD103+ Trm gathers the following subsets: CD4+CD103+PD1+, CD4+CD103+TIM3+, CD4+CD103+PD1+TIM3+, CD8+CD103+PD1+, CD8+CD103+TIM3+, CD8+CD103+PD1+TIM3+. (e) Frequencies of CD8+CD103+PD1-TIM3−, CD8+CD103+PD1+, CD8+CD103+TIM3+, CD8+CD103+PD1+TIM3+ subsets assessed within total CD8+CD103+ Trm. T cell subsets in (a, c–e) were compared using Kruskal–Wallis test and statistically significant differences are indicated with asterisks (**$p < 0.005$; ***$p < 0.0005$). (f) Heatmap of Spearman's correlations among subpopulations of intratumoural T cells. Red indicates a positive correlation and blue represents a negative correlation; the absence of correlation is indicated by white. The values of Spearman's coefficient are reported, while asterisks mark the significance level (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$). (g) Correlation plot of CD8+CD103+ Trm with the subsets expressing PD1, TIM3 or both molecules. The line shows the LOESS fit to the data.

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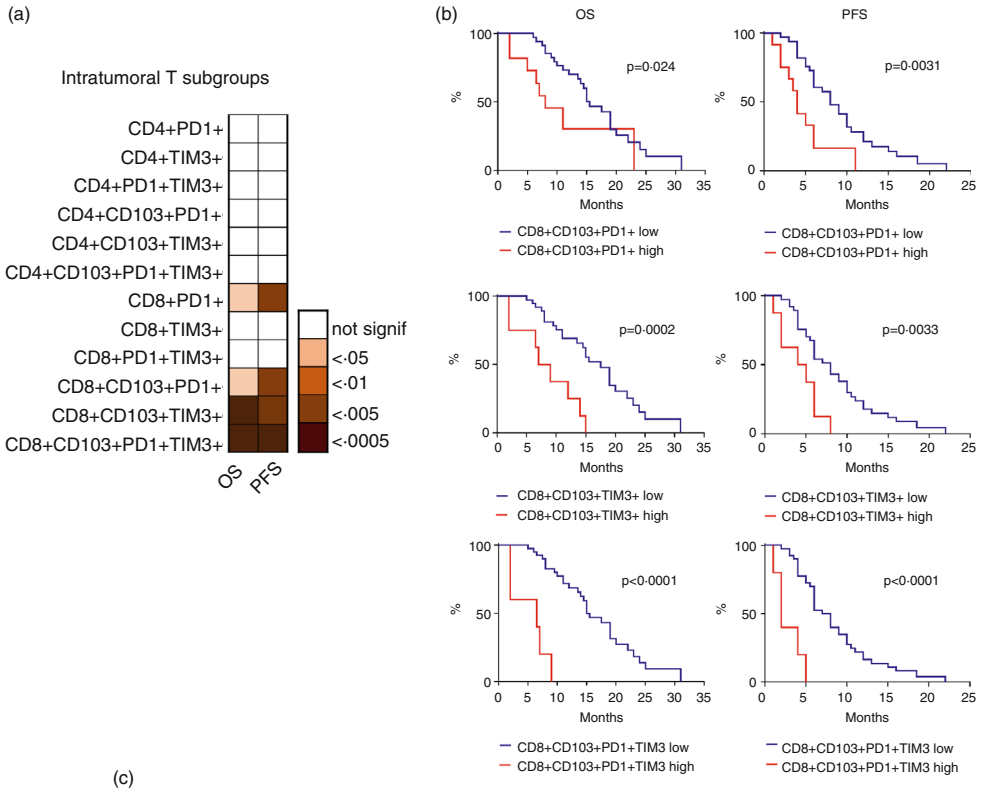
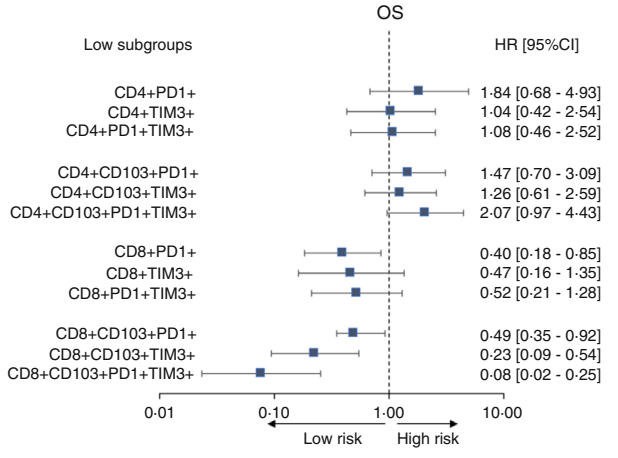



(c)



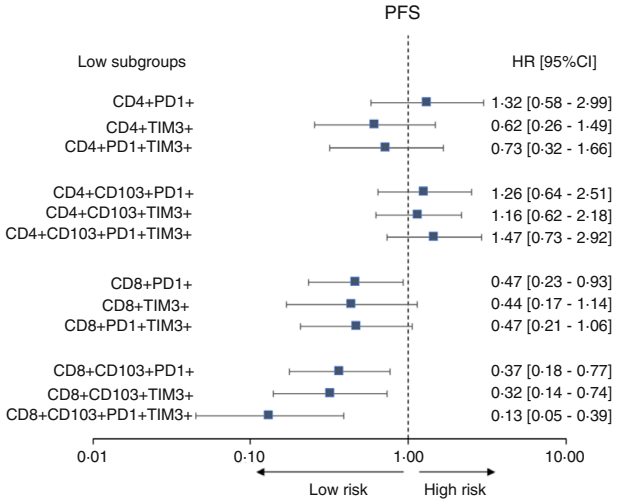




**FIGURE 3** Correlation of intratumoural T cell subsets with OS and PFS patients. (a) Plot of Kaplan–Meier analysis summarising the correlation of PD1- and TIM3-expressing T cell subsets with OS and PFS patients. The analysis was performed to associate low or high percentages of PD1- and TIM3-expressing T lymphocytes, established based on the cut-off of each subset, with OS and PFS; *p* values were calculated by log-rank (Mantel–Cox) test; brown colours indicate negative correlation of high frequencies of PD1- and TIM3-expressing T cells with OS and PFS. (b) Kaplan–Meier curves showing the significant correlations with OS and PFS of low and high frequencies of CD8+CD103+PD1+, CD8+CD103+TIM3+ and CD8+CD103+PD1+TIM3+ Trm subsets. (c) Univariate regression analysis for effects of T cell subsets over prognosis of patients in terms of OS and PFS; comparisons were performed using log-rank (Mantel–Cox) test and corresponding error bars show 95% CI. OS, overall survival; PFS, progression-free survival.

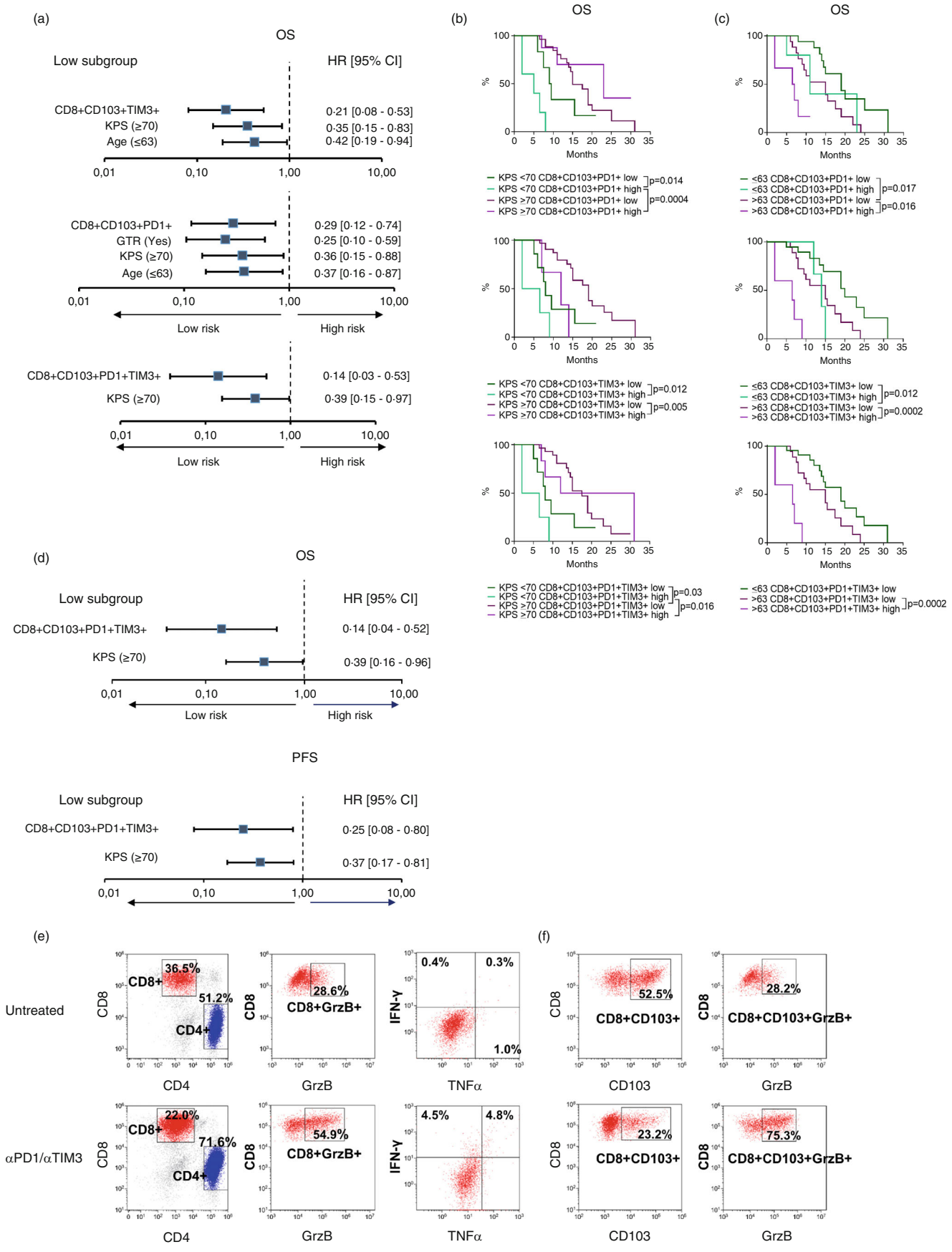



**FIGURE 4** Legend on next page.

## Inhibition of PD1-TIM3 on CD8+CD103+ T cells enhances their anti-tumour activity

To functionally characterise CD8+CD103+ Trm, we cultured cells from whole tumour samples for 14 days in the presence of IL-2 with or without anti-PD1 (pembrolizumab) and anti-TIM3 (Sabatolimab) monoclonal antibodies, in co-culture with tumour and antigen-presenting cells. Treatment with these antibodies led to an increased capability of CD8+ T cells to produce Granzyme B (GrzB), IFN-γ and TNF-α, despite their number reduction (Figure 4e). Likewise, a reduced percentage of anti-PD1/TIM3-treated CD8+CD103+ T cells was found to enhance GrzB production (Figure 4f), suggesting a key role of CD8+CD103+ Trm within GB intratumoural T cells.

## Lack of prognostic significance of PD1 and TIM3 expression on T cells of peripheral blood

The frequency of lymphocytes as well as CD3+, CD4+ and CD8+ T cells in the peripheral blood (PB) showed a high variability among patients (Figure S5a–d). Similarly to TME, the frequencies of both CD4+PD1+ and CD8+-PD1+ T cells were higher than the relative subsets expressing only TIM3 or both TIM3 and PD1 (Figure 5a). In addition, Spearman's analysis revealed a stronger positive correlation within CD4+ rather than CD8+ T cell subsets. In particular, CD4+PD1+TIM3+ T cells robustly linked with CD4+PD1+ T cells ($R = 0.59$, $p < 0.005$), CD4+TIM3+ T cells ($R = 0.74$, $p < 0.0005$) and CD4+ T cells ($R = 0.58$, $p < 0.005$). On the other hand, a strongly positive association was observed between CD8 + PD1+ T cells and CD8+ T cells ($R = 0.71$, $p < 0.0005$) as well as CD8+PD1+TIM3+ T cells and CD8+TIM3+ T cells ($R = 0.54$, $p < 0.005$; Figure 5b). However, Kaplan–Meier analysis demonstrated the absence of prognostic value of high and low frequencies of T cell subsets for both OS and PFS (Figure S6). Of note, the frequency of both CD4+ and CD8+ T cells was higher in the immune infiltrates of tumours than in the PB whereas the CD4+/CD8+ ratio was similar (Figure 5c,d). In addition, although with no prognostic significance, CD8+ T subpopulations expressing PD1 or TIM3 or both markers were found significantly more frequent in tumours than in blood samples ($p < 0.001$; Figure 5e).

## DISCUSSION

In this study, we found that low frequency of intratumoural CD8+CD103+ Trm expressing PD1 and TIM3 correlate with significantly reduced risk of death in GB, suggesting a key role of these immune checkpoints in affecting the anti-tumour immune response.

Intratumoural CD8+CD103+ Trm are positively associated with good prognosis in several high-grade tumours and with response to immunotherapy [19]. Nevertheless, in some cases, high frequency of CD8+-CD103+ Trm associated with poor outcome [20], generating contradictory results that need to be further exploited. Here, we show that CD8+CD103+ Trm abundantly infiltrate GB, and low frequency of these cells expressing PD1, or TIM3 or both molecules strongly correlates with better patient survival whereas the high frequencies mark poor disease outcomes.

The phenotype of intratumoural Trm differs among tumour types and can be organ and tissue specific [21]. In the brain, Trm are under strict control of immune checkpoint molecules limiting their immune reactivity but with preserved functionality upon activation [14]. Trm are deemed to exert anti-tumour immunity eliminating transformed cells through the release of GrzB and cytokines to recruit and activate other immune cells [22]. Although CD8 + CD103+ Trm are able to recognise their cognate antigen within the TME, they fail to control tumour growth in the long term. Likely, the chronic stimulation within the tumour results in increased expression of exhaustion markers, such as PD1 and TIM3, driving immune function downregulation [23]. Of interest, a high density of intratumoural CD8+CD103+ Trm have been reported to correlate with poor prognosis in some types of cancer [24]. Here, we show that CD8+CD103+

**FIGURE 4** Identification of the interdependency of low and high CD8+CD103+ Trm subsets with clinical variables in predicting GB outcome. (a) HR and *p*-value of Cox stepwise multivariate regression including CD8+CD103+ Trm subsets and all clinical variables. (b) Kaplan–Meier plots showing CD8+CD103+ Trm subset prediction of OS in GB patients stratified by KPS. (c) Kaplan–Meier analysis of OS displaying CD8+CD103+ Trm subset prediction in GB patients stratified by age. (d) Multivariate analysis including all CD8+CD103+ Trm subsets and all clinical variables showing the stronger predictor value of CD8+CD103+PD1+TIM3+ and KPS ≥70 for OS and PFS. (e) Functional effects of anti-PD1 and anti-TIM3 antibody treatment on expanded T cells from culture of whole tumour cells. Untreated and antiPD1/anti-TIM3-treated CD8+ T cells were evaluated by flow cytometry for GrzB (middle panels), IFN-γ and TNF-α production (right panels). (f) Untreated and anti-PD1/anti-TIM3-treated CD8+CD103+ Trm were gathered in intratumoural CD8+ T cells (left panels) and assayed for GrzB production (right panels). GB, glioblastoma; HR, hazard ratios; KPS, Karnofsky performance status.

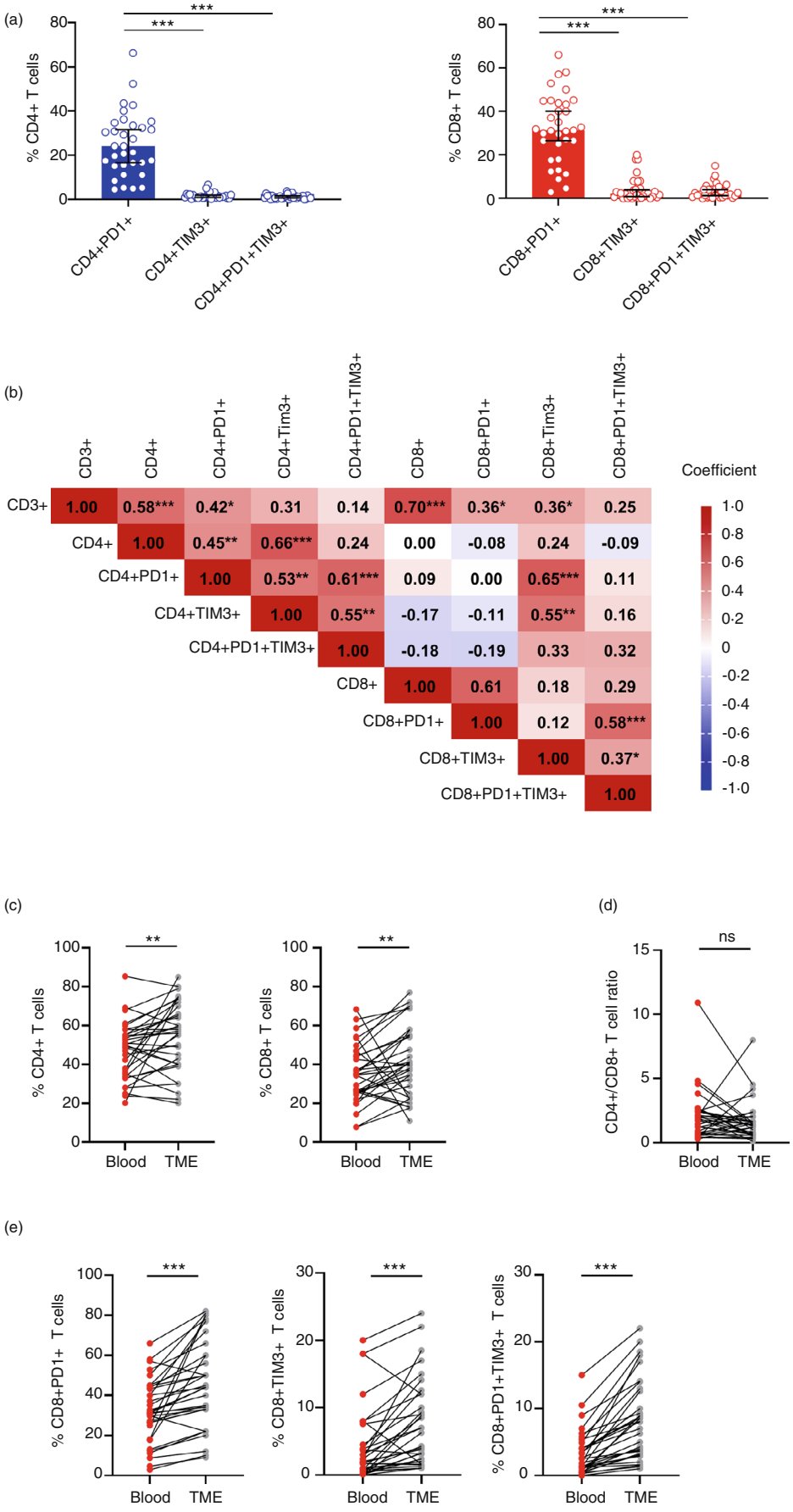



**FIGURE 5** Composition and phenotypic characterisation of T subsets in the blood of GB patients. Blood lymphocyte populations were analysed by flow cytometry on viable CD45+ cells. (a) Scatter plots of median values of T cell subsets in blood were compared using Kruskal–Wallis test. (b) Heatmap of Spearman's correlations showing binary associations within subpopulations of T cells in blood. Red, positive correlation; blue, negative correlation; white, no correlation (*$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$). (c) Pairwise comparisons between CD4+ and CD8+ T cells between blood and TME using Wilcoxon matched-pairs signed-rank test. (d) Pairwise comparisons of TME blood CD4+/CD8+ T cell ratio. (e) Pairwise comparisons of CD4+PD1+, CD4+TIM3+, CD4+PD1+TIM3+, CD8+PD1+, CD8+TIM3+ and CD8+PD1+TIM3+ T cell subsets between blood and TME using Wilcoxon matched-pairs signed-rank test. Dots represent single patients. Statistically significant differences are indicated with asterisks (**$p < 0.005$; ***$p < 0.0005$). GB, glioblastoma; TME, tumour microenvironment.

Trm are present at variable rates within GB tumours, and the rate of Trm subsets expressing PD1 or TIM3 or both molecules predicts prognosis since high frequencies of CD8+CD103+PD1+, CD8+CD103+TIM3+ and CD8+-CD103+PD1+TIM3+ Trm strongly correlate with a bad disease outcome. Specifically, we first found that intratumoural immune infiltrate was positively associated to total CD8+ T cells rather than CD4+ T cells, highlighting that even in a cold tumour like GB, the prevalence of intratumoural CD8+ T cells could be therapeutically exploited [25]. However, while total intratumoural CD8+ T cells lacked prognostic significance, low frequencies of CD8+CD103+ Trm expressing PD1 or TIM3 or both molecules were to various extents associated with better prognosis, pinpointing these immune checkpoints as determinants for their exhausted function [26]. Low CD8+CD103+PD1+, CD8+CD103+TIM3+ and CD8+-CD103+PD1+TIM3+ Trm subsets significantly associated with both better PFS and OS, suggesting that the release from checkpoint controls could have a role in the therapy of GB. In particular, the low frequency of CD8+CD103+PD-1+TIM-3+ Trm was found to be the most predictive immune marker of better prognosis, suggesting that the low frequency of such terminally exhausted T cells is the most important determinant for the antitumor immune response [27].

We also showed that the frequencies of PD1- and TIM3-expressing CD8+CD103+ Trm associated with the clinical variable KPS and age in predicting GB outcome [28]. To our knowledge, this study is the first to correlate intratumoural low frequencies of CD8+-CD103+ Trm subsets with high KPS and younger age, identifying GB patients with better prognosis. Low CD8+CD103+PD1+ and CD8+CD103+TIM3+ Trm subsets were significant independent predictors of better survival since it was true for patients with both KPS $<70$ and $> 70$ as well as those aged $\leq 63$ and $>63$. It is worth noting, that a low frequency of CD8+CD103+-PD1+TIM3+ Trm was found to predict better outcome in patients with KPS $<70$ and $> 70$ but only aged $>63$ since no young patients in our cohort had a high frequency of the most exhausted CD8+CD103+PD1+-TIM3+ Trm. Accordingly, young GB patients with KPS $\geq 70$ and low frequencies of CD8+CD103+ Trm subsets were the group with the best clinical outcome. However, while the association between Trm subsets and age is relevant weakening the immune function in the elderly [29], there is not an obvious link between the above cited immune populations and KPS; in this regard, we can speculate on the significance of tumour location in terms of immune infiltrates [30]. Importantly, we found little to no expression of CD103 on PB CD8+ T cells differently from what was recently reported [31], and the expression of PD1 and TIM3 in PB T cells was not predictive of disease outcome. Altogether, these data confirm that CD8+CD103+ Trm are present in the human brain and may play a master role in immune surveillance under a tight control of inhibitory checkpoints. Consistent with this finding, our study also disclosed that in vitro blocking of PD1 and TIM3 released the capability of intratumoural T cells to produce the effector cytokines IFN-γ and TNF-α, and the cytotoxic factor GrzB. Altogether, our results support the concept that a timely and targeted use of ICIs could reactivate antitumor Trm function.

PD1-based immunotherapies have been tested in a multitude of phase I/II and phase III clinical trials [32]. In line with the finding that high intratumoural TIM3 expression is linked to glioma severity and progression [33], anti-TIM3 therapy is being explored in GB [34]. However, although to date no obvious clinical benefits of immune checkpoint blockade have been reported in GB, few patients have shown long-term responses suggesting a potential benefit for selected patient populations. The concept that boosting the immune system properly leads to an effective antitumor response in GB has been recently demonstrated by the phase III trial with DCVax-L vaccine which has met both primary and secondary endpoints with extended patient survival for several months [35]. This evidence is in line with our finding that the frequency of CD8+-CD103+ Trm expressing PD1 and TIM3, evaluated at the surgery, may shape anti-GB immunity affecting prognosis. Accordingly, these immune biomarkers were found tightly associated with the clinical variables KPS and age in stratifying patients with diverse disease outcome. In addition, the negative impact of PD1 and TIM3 expression on CD8+CD103+ Trm cells may be reversed by specific immune checkpoint blockade as it occurred in our in vitro experiments leading to production of T effector and cytotoxic mediators. Our study has the limitation that it was conducted in a relatively small cohort, however it allowed a deeper understanding of the biology of CD8+CD103+ Trm, identifying them as intratumoural potentially reactive T cells whose function could be restored by PD1-TIM3 blockade. For this reason, the adjuvant administration of immune checkpoint inhibitors may represent a therapeutic option to improve clinical course of GB patients.

## AUTHOR CONTRIBUTIONS

Lucia Gabriele and Roberto Pallini conceived the study and wrote the manuscript. Giulia Romagnoli performed the experiments, processed the data and contributed writing the manuscript. Quintino Giorgio D'Alessandris was responsible for sample collection and patient data

management and assisted in analysing data. Imerio Capone contributed to the concept development and study design, data analysis, data interpretation and manuscript writing. Andrea Tavilla performed all statistical data analyses. Caterina Lapenta, Irene Canini, Mariachiara Buccarelli, Valentina Tirelli, Massimo Sanchez and Alessandra Fragale contributed performing the experiments. Lucia Ricci-Vitiani and Mauro Biffoni assisted in sample and experiment management. Martina Giordano, Stefano Giannetti, Rina Di Bonaventura and Liverana Lauretti assisted in data and patient management. LG was responsible for the overall content as guarantor. All authors read and approved the manuscript.

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## CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

## ETHICS STATEMENT

The study followed the principles set forth in the World Medical Association Declaration of Helsinki and was approved by the Institutional Ethics Committee of Fondazione Policlinico Gemelli IRCCS (Prot. ID 2253).

## PATIENT CONSENT STATEMENT

Informed consent was obtained from all participants included in the study.

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