







# Association of programmed cell death ligand 1 and circulating lymphocytes with risk of venous thromboembolism in patients with glioma

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## ABSTRACT

**Introduction** The role of the adaptive immune system in the pathophysiology of cancer-associated venous thromboembolism (VTE) has not been investigated in detail. Programmed cell death ligand 1 (PD-L1) is an immune checkpoint molecule responsible for immune evasion in several cancer entities, as expression on tumour cells silences the T cell-mediated immune response. Given the interrelation between inflammation, haemostasis and cancer, we aimed to investigate the association of players of the adaptive immunity (eg, lymphocytes, tumour PD-L1) with risk of VTE in patients with glioma, one of the most prothrombotic cancer types.

**Methods** In this prospective observational single-centre cohort study, patients with newly diagnosed glioma or regrowth after resection were included. Primary endpoint was objectively confirmed VTE. At study inclusion, a blood draw was performed. Tumour PD-L1 expression was assessed via immunohistochemistry.

**Results** In total, 193 patients were included. PD-L1 expression in  $\geq 1\%$  of tumour cells was observed in 20/193 (10.4%) glioma. In multivariable cox-regression analysis, on adjustment for age, sex and WHO grade IV, systemic lymphocyte counts were significantly associated with risk of VTE (HR per 1 G/L increase (95% CI): 1.15 (1.03 to 1.29),  $p=0.013$ ). In contrast, no significant difference in risk of VTE was found regarding the PD-L1 status: the cumulative 24 months probability of VTE was 17.0% in patients with no PD-L1 and 11.8% in those with PD-L1 expressing tumours ( $p=0.663$ ).

**Conclusion** In summary, PD-L1 expression was not associated with risk of VTE. Interestingly, peripheral lymphocytes, which are key players in adaptive immunity, were linked to an increased risk of glioma-associated VTE.

## INTRODUCTION

Cancer patients have an increased risk of venous thromboembolism (VTE) and malignant progression is one of the major related risk factors.<sup>1–3</sup> Particularly, high-grade gliomas belong to one of the cancer entities with the highest VTE risk.<sup>4</sup> Various risk factors have been previously published for glioma including several systemic haematological

## Key questions

### What is already known about this subject?

- ▶ Venous thromboembolism (VTE) is a frequent and life-threatening complication in patients with malignant primary brain tumours.
- ▶ Hypercoagulability and inflammation are important hallmarks of both cancer and thrombosis.
- ▶ The association of players of the adaptive immunity (eg, lymphocytes, tumour programmed cell death ligand 1 (PD-L1)) with risk of VTE in patients with glioma has not been investigated so far.

### What does this study add?

- ▶ Systemic lymphocyte counts are associated with an increased risk of VTE in patients with glioma.
- ▶ The immunomodulatory functions of PD-L1 on tumour cells seem not to impact the risk of VTE in patients with glioma.
- ▶ So far, it was assumed that the innate immune system plays a main role in VTE development. This exploratory study supports a potential role of the adaptive immune system in cancer-associated VTE.

### How might this impact on clinical practice?

- ▶ Peripheral lymphocytes might serve as novel easy-to-obtain biomarker for risk stratification of VTE in patients with glioma.

parameters (eg, leucocytes, platelets, soluble (s)P-selectin, D-dimer) as well as local tumour characteristics such as an isocitrate dehydrogenase 1 (IDH1) wildtype status and the upregulation of the potent platelet activator podoplanin on brain tumour cells.<sup>5–7</sup>

Next to hypercoagulability, inflammation is another hallmark of cancer.<sup>8–10</sup> Mechanistically, an interrelation between inflammation, innate immunity and thrombosis is well-recognised.<sup>11</sup> A role of the adaptive immune system in the development of VTE has not been investigated in detail. Interestingly, cancer cells are able to modulate the adaptive

immune response in order to escape the host immune surveillance. One mechanism how tumour cells evade the immune system is the upregulation of so-called immune checkpoint molecules including programmed cell death ligand 1 (PD-L1). PD-L1 is an immunosuppressive protein that represents an important regulator for the adaptive immune response. By interacting with PD-1 on lymphocytes, PD-L1 inhibits the T cell function and thereby leads to a limited immune response.<sup>12</sup> Recently, immune checkpoint inhibitors targeting the PD-1/PD-L1-axis have revolutionised the medical field of oncology and improved the overall survival in several extracranial malignancies.<sup>13–15</sup> Of note, PD-L1 expression was also observed on glioma cells, pointing out a potential target for immune checkpoint inhibitors in this patient group.<sup>16,17</sup> Nonetheless, no significant survival benefit in glioblastoma was yielded on treatment with immune checkpoint inhibitors so far.<sup>18</sup>

Given the tight interrelation between cancer and inflammation as well as the high risk of glioma-associated thrombosis,<sup>4,9</sup> we aimed at exploring the association of mediators of the adaptive immune response (eg, circulating lymphocytes, tumour PD-L1 expression) with the risk of future VTE in patients with glioma.

## METHODS

### Study design and study population

This study was conducted within the framework of the Vienna Cancer and Thrombosis Study (CATS). CATS is a prospective observational single-centre cohort study, which has been initiated at the Medical University of Vienna with the aim to identify risk factors for VTE in patients with cancer. The study design has been described previously in detail.<sup>5,7,19</sup> Briefly, in this analysis, patients are recruited at time of brain cancer diagnosis or at time of regrowth after tumour resection and are then observed for a maximum time period of 2 years. Primary endpoint of the study is objectively confirmed VTE. Patients with glioma recruited from October 2003 to March 2014 with available tumour samples were included in the current study. Ethical approval was obtained from the institutional ethics committee according to the declaration of Helsinki (EC number: 126/2003, ethik-kom@meduni-wien.ac.at). Before study inclusion, all patients gave their written informed consent.

### Blood parameter analysis

At study inclusion and prior to chemotherapy, a single blood draw was collected via sterile venipuncture in Vacutainer K3-EDTA tubes (Vacuette, Greiner BioOne, Kremsmuenster, Austria). Blood cell counts were analysed within 2 hours from sampling with a Sysmex XE-5000 haematology analyzer (Kobe, Japan). Data on sP-selectin and D-dimer were available from our previous reports.<sup>5,19,20</sup>

### Immunohistochemistry

Immunohistochemical staining was performed using a Ventana Benchmark Ultra immunostaining system.

Formalin-fixed and paraffin-embedded (FFPE) tissue blocks of glioma were cut into serial 3–5 µm slices. PD-L1 expression was assessed via the monoclonal mouse anti-human PD-L1 antibody (1:50, Clone 22C3, Dako North America). The PD-L1 signal was semiquantitatively scored based on the percentage of tumour cells presented with a specific membranous signal as previously described.<sup>16</sup> Samples with ≥1% distinct membranous anti-PD-L1 labelling of all tumour cells were classified as PD-L1 positive. FFPE tissue blocks of human placenta were used as PD-L1 positive control. Slides were digitalised using a NanoZoomer slide scanner (Hamamatsu Photonics, Hamamatsu, Japan). Data on IDH1 R132H mutation and intratumoural podoplanin expression were available from our previous studies.<sup>5,6</sup>

### Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics (V.2014, IBM, Armonk, New York, USA). Continuous variables were summarised as medians (IQR) and categorical variables as absolute frequencies (%). Differences between continuous variables were assessed with Mann-Whitney U-tests and categorical variables with  $\chi^2$ -tests and Fisher's exact tests. The observation time was defined from study inclusion until VTE, death or censoring alive at 2 years after baseline. Median follow-up was estimated with the reverse Kaplan-Meier method according to Schemper and Smith.<sup>21</sup> The probability of VTE was calculated with 1-Kaplan-Meier estimators. VTE incidences between groups were compared with log-rank tests. Hazards of VTE were modelled with univariable and multivariable cox-regression models.  $P \leq 0.05$  was defined as cut-off for statistical significance.

## RESULTS

### Study population

In the framework of the CATS study, 285 patients with primary brain tumours were included from October 2003 until March 2014. After reevaluation, 13 patients had to be excluded because they did not meet the exact inclusion and exclusion criteria. In 66 cases, tumour samples were not available, and 13 patients did have other central nervous system tumours than glioma. Thus, a total of 193 patients with glioma were eligible for the present study. Absolute lymphocyte counts (G/L) were available in 171 of those patients.

Baseline characteristics of the study population are listed in [table 1](#). Patients were recruited for a median of 17 days after tumour surgery or tumour biopsy. According to the WHO classification, 146 (75.6%) patients had a glioblastoma (WHO grade IV), 38 (19.7%) patients had an anaplastic glioma (WHO grade III) and 9 (4.7%) patients had a diffuse glioma (WHO grade II). IDH1 R132H mutation was present in 40 (20.7%) patients with glioma while 153 (79.3%) patients had an IDH1 wildtype status.

**Table 1** Baseline characteristics

	Total cohort, n=193	PD-L1 (-), n=173	PD-L1 (+), n=20	P value
<b>Demographics</b>				
Age at study entry	55 (44–66)	55 (44–65)	56 (40–67)	0.879
Female sex	72 (37.3%)	65 (37.6%)	7 (35%)	1.000
Body mass index (kg/m <sup>2</sup> )	25.7 (23.0–27.8)	25.7 (23.2–27.8)	26.0 (23.3–30.5)	0.504
Newly-diagnosed malignancy	171 (88.6%)	151 (87.3%)	20 (100%)	0.136
<b>Tumour grade</b>				
WHO grade IV	146 (75.6%)	129 (74.6%)	17 (85.0%)	
WHO grade III	38 (19.7%)	35 (20.2%)	3 (15.0%)	
WHO grade II	9 (4.7%)	9 (5.2%)	0 (0%)	
<b>Histology</b>				
Glioblastoma	144 (74.6%)	128 (74.0%)	16 (80.0%)	
Gliosarcoma	2 (1.1%)	1 (0.6%)	1 (5.0%)	
Anaplastic astrocytoma	30 (15.5%)	27 (15.6%)	3 (15.0%)	
Anaplastic oligodendroglioma	7 (3.6%)	7 (4.0%)	0 (0%)	
Anaplastic ependymoma	1 (0.5%)	1 (0.6%)	0 (0%)	
Diffuse astrocytoma	7 (3.6%)	7 (4.0%)	0 (0%)	
Diffuse oligodendroglioma	2 (1.1%)	2 (1.2%)	0 (0%)	
<b>Immunohistochemistry</b>				
IDH1 R132H mutation	40 (20.7%)	39 (22.5%)	1 (5%)	0.082

IDH1, isocitrate dehydrogenase 1; PD-L1, programmed cell death ligand 1.

### Systemic lymphocyte levels

In the current glioma patient cohort, the median lymphocyte count was 1.384 G/L (IQR: 0.917–1.771) (n=171). There was no significant difference in patients with IDH1 mutation compared with those with IDH1 wildtype status (median (IQR): 1.519 (1.047–1.872) vs 1.366 (0.871–1.765), p=0.218).

### PD-L1 expression in glioma

Membranous PD-L1 expression of ≥1% of tumour cells was detected in 20 (10.4%) glioma specimens. Median percentage of tumour cells presenting with strong, complete, membranous PD-L1 expression was 0% (range 0%–30%). Online supplementary figure 1 shows representative immunohistochemical staining of PD-L1 in glioma. In subgroup-analysis, PD-L1 expression in ≥1% of the tumour cells was observed in 11.6% (17/146) of glioblastoma (WHO grade IV) and in 10.0% (3/30) of anaplastic astrocytoma (WHO grade III). In total, only 1 out of 20 (5%) patients with PD-L1 expression had an IDH1 R132H mutation, while 19/20 (95%) PD-L1 positive tumours were IDH1 wildtype.

### Probability of VTE during follow-up

Median follow-up for VTE of the total study cohort was 402 days. During the observation time, 26 (13.5%) patients with glioma developed objectively confirmed VTE. In 1-Kaplan-Meier analysis, the cumulative 6-month, 12-month, 24-month probabilities of VTE were 10.5%, 14.1% and 16.5%, respectively.

### Circulating lymphocytes and risk of VTE in patients with glioma

In univariable cox-regression analysis, lymphocyte counts were significantly associated with occurrence of VTE (HR per 1 G/L increase (95% CI): 1.162 (1.048 to 1.289), p=0.004, [table 2](#)). This result prevailed on adjustment for potential confounders such as sex, age and WHO grade IV (adjusted HR: 1.152 (1.030 to 1.289), p=0.013, [table 2](#)) and also after adjustment of biomarkers previously

**Table 2** Univariable and multivariable cox-regression analyses of circulating lymphocyte levels with the risk of VTE in patients with glioma

	HR*	95% CI	P value
<b>Univariable analysis</b>			
Lymphocytes (G/L)	1.162	1.048 to 1.289	0.004
<b>Multivariable analysis</b>			
Lymphocytes (G/L)†	1.152	1.030 to 1.289	0.013
Lymphocytes (G/L)‡	1.166	1.054 to 1.290	0.003
Lymphocytes (G/L)§	1.184	1.067 to 1.314	0.002
Lymphocytes (G/L)¶	1.149	1.037 to 1.273	0.008

\*Per 1 G/L increase.

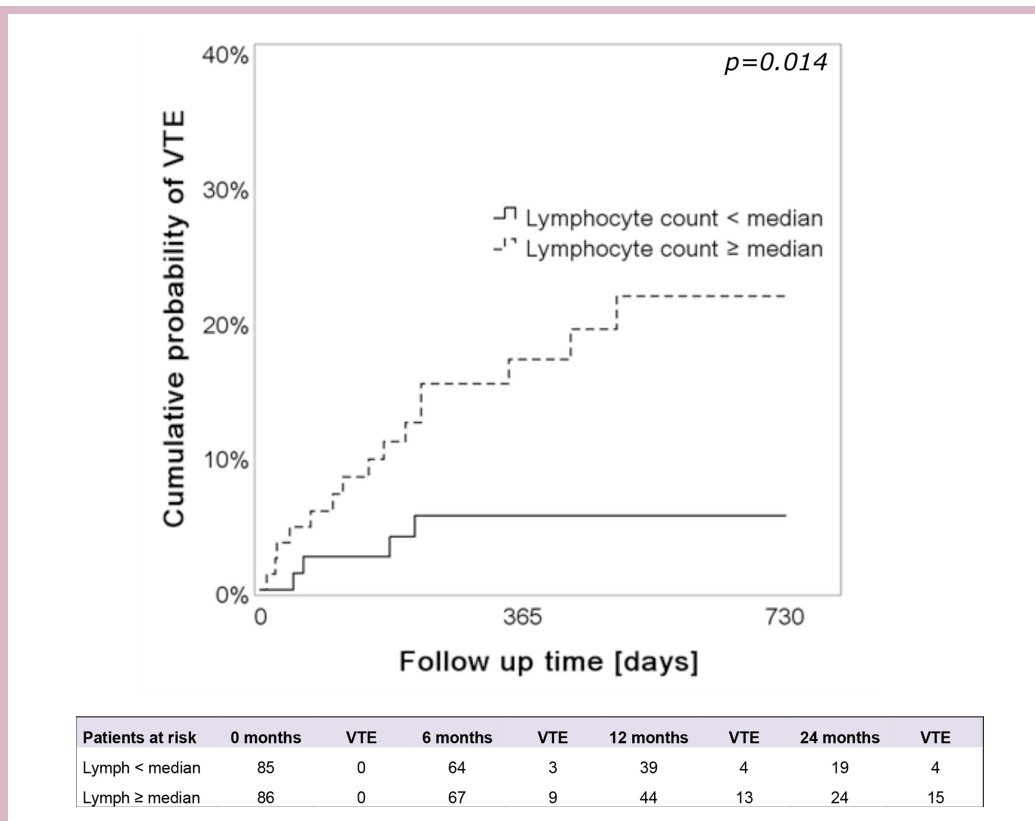
†Adjusted for sex, age and glioblastoma.

‡Adjusted for platelet count, D-dimer and sP-selectin.

§Adjusted for podoplanin expression.

¶Adjusted for IDH1 mutation status.

IDH1, isocitrate dehydrogenase 1; sP-selectin, soluble P-selectin; VTE, venous thromboembolism.



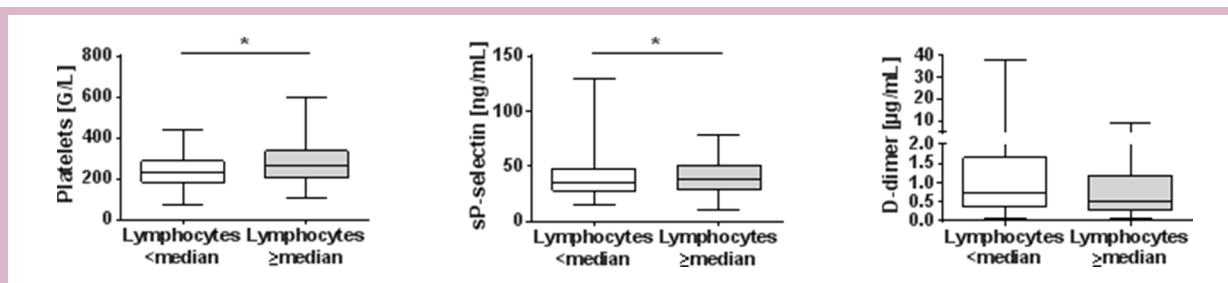
**Figure 1** Cumulative probability of VTE according to circulating lymphocytes in patients with glioma (cut-off: median=1.38 G/L). During the observation time of 2 years, patients with higher lymphocyte counts had an increased risk to develop VTE compared with those with lower lymphocyte counts. VTE, venous thromboembolism.

identified to predict risk of cancer-associated VTE such as intratumoural podoplanin (adjusted HR: 1.184 (1.067 to 1.314),  $p=0.002$ ), IDH1 mutation status (adjusted HR: 1.149 (1.037 to 1.273),  $p=0.008$ ) and haemostatic parameters (eg, platelet count, sP-selectin and D-dimer; adjusted HR: 1.166 (1.054 to 1.290),  $p=0.003$ ). In 1-Kaplan Meier analysis, the cumulative 6-month, 12-month and 24-month probability of VTE was 11%, 17.2% and 21.8% in patients with high lymphocyte counts ( $\geq$ median) compared with 3.9%, 5.5% and 5.5% in patients with low lymphocyte counts ( $<$ median) (log-rank,  $p=0.014$ , [figure 1](#)). Additional subgroup-analyses regarding the IDH1 wildtype status and WHO grade IV, respectively, are shown in online

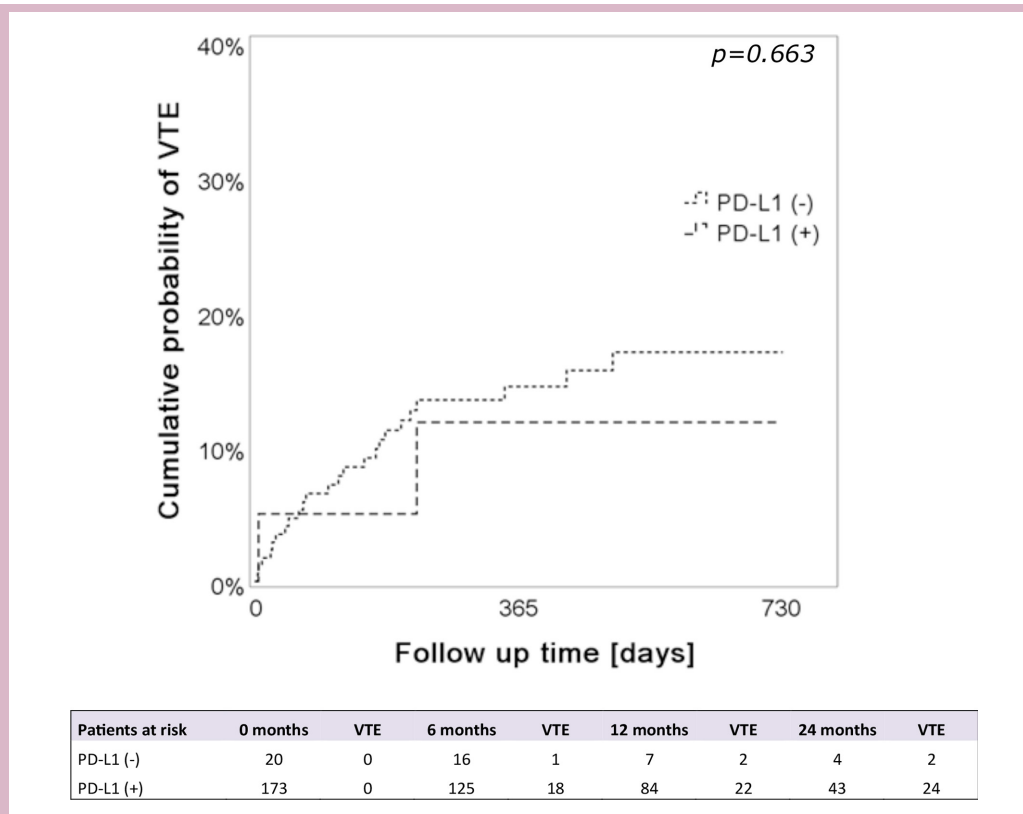
supplementary figures 2 and 3. Furthermore, patients with high lymphocyte counts had significantly higher platelet counts (median: 266 vs 229 G/L,  $p<0.0001$ ) and sP-selectin levels (38.3 vs 32.6 ng/mL,  $p=0.05$ ) compared with patients with low lymphocyte counts ([figure 2](#)). In contrast, no significant association of systemic lymphocyte levels with D-dimer levels was found (0.51 vs 0.71  $\mu$ g/mL,  $p=0.186$ ).

#### PD-L1 expression and risk of VTE in patients with glioma

In univariable cox-regression analysis, the HR of VTE in patients with PD-L1 expression as compared with no PD-L1 expression was 0.726 (95% CI): 0.172 to 3.076



**Figure 2** Association of systemic lymphocyte levels with previously described VTE-related laboratory parameters such as platelet count, sP-selectin and D-dimer. Patients with higher lymphocyte counts had significantly higher platelet counts as well as sP-selectin levels compared with patients with lower lymphocyte counts. In contrast, no significant difference in D-dimer levels was found. sP-selectin, soluble P-selectin; VTE, venous thromboembolism.



**Figure 3** Cumulative probability of VTE according to PD-L1 status in patients with glioma. No significant association between the PD-L1 status and the risk to develop VTE was found. PD-L1, programmed cell death ligand 1; VTE, venous thromboembolism.

( $p=0.664$ ) and did not change after adjustment for potential confounders such as sex, age and WHO grade IV (adjusted HR: 0.651 (0.153 to 2.759),  $p=0.560$ ) or for previously identified VTE predictors such intratumoural podoplanin expression (adjusted HR: 0.589 (0.139 to 2.503),  $p=0.474$ ) and IDH1 mutation status (adjusted HR: 0.570 (0.134 to 2.415),  $p=0.445$ ). The cumulative 6-month, 12-month and 24-month probabilities of VTE were 5%, 11.8% and 11.8% in patients with PD-L1 positive tumours and 11.2%, 14.4% and 17.0% in glioma patients with no PD-L1 expression (log-rank,  $p=0.663$ , figure 3). Also, PD-L1 did not correlate with biomarkers previously identified to predict risk of VTE in cancer patients such as D-dimer (PD-L1 positive vs PD-L1 negative, median: 0.640 vs 0.645  $\mu\text{g}/\text{mL}$ ,  $p=0.628$ ), sP-selectin (38.0 vs 33.3  $\text{ng}/\text{mL}$ ,  $p=0.254$ ) and platelets (251 vs 240  $\text{G}/\text{L}$ ,  $p=0.583$ ), respectively.

## DISCUSSION

In the present study, we investigated the association of circulating lymphocytes and tumour PD-L1 expression, both players of the adaptive immune system, with risk of VTE in patients with glioma. PD-L1 expression on brain tumour cells did not increase the risk of venous thrombotic events, while higher baseline lymphocyte counts were associated with a predisposition for VTE development in the future. This is a novel insight, since it is widely

accepted that the innate immunity is predominantly involved in the pathophysiology of cancer-related VTE.<sup>11</sup>

So far, the role of lymphocytes, as mediators of the adaptive immune response,<sup>22</sup> for risk of cancer-related VTE has not been investigated in detail. In the present study, increased levels of circulating lymphocytes were linked to a higher risk of VTE in patients with glioma. This association was independent from previous established VTE related risk factors in glioma including local tumour characteristics (eg, WHO grade IV, IDH1 wild-type, podoplanin) as well as several laboratory parameters (eg, platelet count, sP-selectin, D-dimer).<sup>5-7, 23</sup> Also, there was no statistically significant difference in systemic lymphocyte counts between patients with IDH1 wildtype compared with those with IDH1 mutant glioma. Surprisingly, patients with high lymphocyte counts also had higher platelet counts. Of particular note, in brain tumours, a low platelet count is established as a risk factor for VTE whereas in other solid tumours, a high platelet count is linked to an elevated VTE risk, altogether leading to the assumption that different mechanisms might be involved in the pathophysiology of cancer-related VTE.<sup>5, 7, 24</sup>

In line with our observation, a lower platelet-to-lymphocyte ratio, reflecting an inflammatory state, has been previously linked to a higher risk of VTE in patients with primary brain tumours while no association with the neutrophil-to-lymphocyte ratio, which is also considered

as a surrogate of inflammation, was found.<sup>8</sup> Moreover, another study observed an increased CD4/CD8 T cell ratio and decreased peripheral CD8 T cells in patients with a history of VTE compared with healthy controls.<sup>25</sup>

So far, little is known about the interaction of adaptive immune cells with haemostatic and coagulatory processes. Interestingly, subpopulations of T cells and B cells are able to express tissue factor, which is the main initiator of the coagulation cascade.<sup>26–27</sup> Moreover, lymphocytes are able to release numerous proinflammatory cytokines, which potentially might contribute to an increased hypercoagulable state.<sup>28,29</sup> Interestingly, a potential mechanistic link between platelets and the adaptive immunity has been reported in several previous studies. In particular, activated platelets were linked to the modulation of T cell and B cell immune responses via the platelet CD40 ligand (CD40L), which is known to initiate and propagate the adaptive immune response.<sup>30–32</sup>

In our patient cohort, 10.4% glioma stained positive for membranous PD-L1 expression and in subgroup-analysis of glioblastoma, 11.6% patients were positive for PD-L1. This is within the range of previous studies reporting a rate of 9%–38% PD-L1 positive cases in glioblastoma.<sup>16–17</sup> PD-L1 plays a significant role in modulating the T cell-mediated immune response and the expression of PD-L1 on tumour cells was found to be linked to increased PD-1 positive tumour-infiltrating lymphocytes as well.<sup>12–33</sup> It is currently unknown whether the immunomodulatory functions of PD-L1 might be involved in thromboinflammatory processes, which could translate into risk of VTE. However, in the current study, PD-L1 upregulation on brain tumour cells seems not to impact the development of VTE.

Traditionally, it is assumed that the innate immune response plays the crucial role in the formation of a venous thrombus.<sup>11</sup> In particular, neutrophil extracellular traps, which primarily consist of DNA that is released by neutrophils on different stimuli, are able to activate haemostasis and platelets and trigger thrombosis.<sup>34–35</sup> However, only a few studies focused on a potential involvement of the adaptive immunity in coagulation and venous thrombosis. In this study, we were able to demonstrate a potential involvement of adaptive immune cells with the development of glioma-associated VTE, as supported by the association of higher lymphocyte counts with risk of VTE. In order to develop a new algorithm for VTE risk prediction in patients with glioma, we propose independent cohort studies with the focus to validate our results. Future studies investigating *in vivo* mouse models of venous thrombosis with deficient lymphocyte subsets could provide mechanistic insights into the role of peripheral lymphocytes in the pathophysiology of VTE.

Some limitations of the current study need to be discussed. Although we found a significant association between circulating lymphocytes with the risk of VTE in glioma, we did not investigate specific lymphocyte subsets (eg, CD4 T cells, CD8 T cells, B cells), which have distinct functions during the adaptive immune response. Future

studies should address the role of lymphocyte subsets in haemostasis and their ability to stratify risk of VTE. To perform such studies, freshly taken whole blood would be needed. Also, it remains an open question, which antigens might cause the increase in systemic lymphocyte levels. Interestingly, previous studies reported that infection and preactivation with the cytomegalovirus (CMV) can lead to a T cell boost and that CMV-specific DNA and protein products were found in the majority of glioma tissue.<sup>36–38</sup> Of particular note, we did not systematically record the application of corticosteroids in our study population, which could have affected systemic lymphocyte and neutrophil levels as well.<sup>39</sup> However, Berghoff *et al* reported that the application of corticosteroids did not correlate with the density of tumour-infiltrating lymphocytes in glioma neither with PD-L1 expression in the tumour microenvironment.<sup>16</sup> Moreover, we did not record clinical symptoms such as hemiparesis or the Karnofsky performance status (KPS) in all patients, which might be potential confounders for developing VTE.<sup>40–42</sup> Another limitation of the current study is that no standardised protocol for immunohistochemical PD-L1 staining in glioma is available so far. Previous studies used various antibodies to detect PD-L1 expression in glioma and two different staining patterns were reported. In the current study, we used the Food and Drug Administration-approved anti-PD-L1 antibody from Dako, which is also used in diagnostic testing of PD-L1 in lung cancer.<sup>43</sup> Furthermore, we did not evaluate the expression of PD-L1 in tumour-associated macrophages as we primarily focused on adaptive immunity and tumour-specific expression of PD-L1. Nevertheless, we acknowledge that PD-L1 expression on macrophages has immunological importance in the inflammatory microenvironment as well.<sup>44–45</sup>

To conclude, tumour PD-L1 expression did not correlate with VTE occurrence. In contrast, circulating lymphocytes were associated with a higher risk of VTE in patients with glioma, indicating that the adaptive immune system might be involved in thrombogenesis in these patients. Future studies are needed to provide further insight into the mechanism underlying our observation.

**Contributors** PMSN, IP and CA designed the study. PMSN, ASB, MP, GR, JR, LH and JAH designed and performed the experiments. JR, MP, CM and CA recruited patients. PMSN, CA, FM and FP performed statistical analyses. PMSN and CA wrote the article, which was reviewed, edited and approved by all other authors.

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