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Immune Phenotype Correlates With Survival in Patients With GBM Treated With Standard Temozolomide-based Therapy and Immunotherapy

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Abstract. *Background/Aim: The need for more effective treatment modalities that can improve the clinical outcome of patients with glioblastoma multiforme remains imperative. Dendritic cell vaccination is a fast-developing treatment modality, currently under exploration. Functional immune cell subpopulations may play a role in the final outcome. Materials and Methods: Data from 101 patients drawn from the HGG-2010 trial, including baseline patient characteristics and fluorescence-activated cell sorting of immune cell subpopulations, were analyzed by statistical and machine-learning methods. Results: The analysis revealed strong correlations between immune profiles and overall survival, when the extent of resection and the vaccination schedule were used as stratification variables. Conclusion: A systematic, in silico workflow detecting strong and statistically significant correlations between overall survival and immune profile-derived quantities obtained at the start of dendritic cell vaccination was devised. The derived correlations could serve as a basis for the identification of prognostic markers discriminating between potential long- and short-term survivors of patients with glioblastoma multiforme.*

High grade gliomas (HGG) are the most frequent primary tumors of the central nervous system and consist of anaplastic/malignant gliomas (WHO grade III) and glioblastoma multiforme (GBM, WHO grade IV). The average incidence of GBM is about 3 to 4 per 100,000 adults (1). Standard treatment of these patients consists of resection, radiotherapy and chemotherapy. Even after maximal treatment, prognosis for patients with GBM remains poor, with a median progression-free survival of 6.9 months and a median overall survival (OS) of 14.6 months (2, 3). Relapse is universal and believed to be due to the extensive spread of tumor cells into the surrounding healthy brain tissue (4). Hence, there is a need for more effective treatment modalities that can improve clinical outcome.

Dendritic cell (DC) vaccination is an emerging treatment modality currently being explored in preclinical research and clinical trials (5-11). With DCs being the most potent antigen-presenting cells of the immune system, DC vaccination aims to activate the patient's immune system against the tumor. Additionally, induction of immunological memory might theoretically establish long-term anti-tumoral protection. Despite the promising effect of DC-based immune therapy for HGG, its clinical benefit may be restricted to only a subgroup of patients emerging as a "tail in the OS curve" repetitively found in survival analyses of vaccinated patients (12-14). Unfortunately, biomarkers at diagnosis predicting long-term outcome after active specific immunotherapy as part of standard treatment fail. Here we investigated whether profiles of immune cells at diagnosis and prior to DC vaccination are correlated with the final OS of patients undergoing radiochemotherapy, maintenance chemotherapy and DC vaccination.

Materials and Methods

The Computational Horizon in Cancer (CHIC) platform. As part of the European CHIC research project (www.chic-vph.eu), data from 101 patients with GBM and treated according to the HGG-2010 phase IIb randomized clinical trial (EudraCT 2009-018228-14) and subsequently sampled in the retrospective Glioma Translat study

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(15) became available for analysis. The treatment schedule has been published elsewhere (5). The Glioma Translat study was approved by the Ethical Committee, and all patients gave written informed consent. Data were uploaded by the responsible clinicians into the CHIC electronic platform after double pseudonymisation *via* a trusted third party, leading to effective anonymization for analysis. All patients underwent at least subtotal resection and were treated with radiochemotherapy with temozolomide as chemotherapeutic agent, and finally with maintenance temozolomide as described by Stupp *et al*. (2, 3).

Patient treatment protocol. Each patient underwent leukapheresis prior to radiochemotherapy, performed around 1 week after complete withdrawal from pre-operative corticosteroids. Circulating white blood cells were harvested, from which peripheral blood mononuclear cells were isolated and later differentiated into DCs. Isolation and differentiation processes were described in detail by Rutkowski *et al*. (16). DCs were loaded with tumor lysate-derived proteins and matured as described elsewhere (17-19). After leukapheresis, patients were randomized for immediate DC vaccination before and during maintenance temozolomide *versus* delayed DC vaccination after chemotherapy (5). Patient recursive partitioning analysis (RPA) risk profile classes, as defined by pretreatment and treatment-related prognostic factors, were used as a stratification variable (20, 21). The treatment schedule for the patients with immediate DC vaccination (four weekly DC vaccinations and further boost vaccines with only lysate) was identical to that of the published HGG-2006 trial (22). A similar time schedule for DC vaccination was used after finishing maintenance temozolomide for patients with delayed immunotherapy.

Immune profiles. Blood samples from all patients were taken before (V0) and after (V1) radiochemotherapy. Peripheral blood mononuclear cells were frozen for storage, and thawed immediately prior to analysis. Markers on circulating lymphocytes were determined by fluorescence-activated cell sorting (FACS). Specifically, these data concern the following immune cell populations: Natural killer cells (NK; $CD3^-CD56^+$ cells), total T-cells ($CD45^+CD3^+$), cytotoxic T-cells (CTL; $CD45^+CD3^+CD8^+CD4^-$), T-helper cells (Th; $CD45^+CD3^+CD4^+CD8^-$), and regulatory T-cells (Treg; $CD45^+CD3^+CD4^+CD8^-CD25^+CD127^-$). The fraction of $CD45^+CD3^+CD4^+CD8^-$ cells that were not Tregs was also calculated. Each of the aforementioned cell populations was measured as a percentage of a specific parent population as depicted in Figure 1. For each of the CTL, Th and Treg populations, the percentages of respective $CD69^+$ and $PD1^+$ subpopulations were also determined. The percentages of the respective $CD69^-$ and $PD1^-$ populations were calculated. The described gating strategy also enabled the calculation of all meaningful ratios (also referred to as 'features' hereafter) between immune cell subpopulations, by calculating the ratio of their percentages with respect to a common parent population.

Statistical methods. The Pearson correlation coefficients for each single variable mentioned above *versus* OS were calculated. Next, canonical correlation analysis (CCA) was performed using combinations of two or more variables *versus* OS (23). The result Q is the sum of quantities of the form [coefficient × (*feature* – mean of *feature*)] that correlates best with OS. Q was plotted against the survival period fixed at data sampling with or without event *minus* the average survival of the respective patient group.

Results

Clinical data. Data from 101 patients, 65 males and 36 females, all treated in the HGG-2010 study, and subsequently analyzed in the Glioma Translat study, were delivered into the CHIC platform. The age ranged between 35 and 70 years with a median of 58 years. RPA classification showed 17% class III, 73% class IV and 10% class V patients. Overall the distribution of sex, age and RPA was comparable between the CHIC study sample and the 132 patients studied in the Glioma Translat study. Median OS and 2-year OS were calculated for the entire group of 101 patients, and for each of four subgroups defined by extent of resection [zero *versus* nonzero residual tumor volume (RTV)] and the time schedule of vaccination (during *versus* after maintenance temozolomide, also referred to as early *versus* late vaccination). Data are shown in Table I.

Median OS for the entire population was 19 months with a 2-year OS of 33.66% (95% CI: 24.66-42.88). There was no difference in OS for patients treated with DC vaccination during *versus* after maintenance temozolomide. Patients with postoperative RTV had a significantly worse median OS as compared to patients who underwent complete resection with no RTV (17 *vs*. 22 months; Log-rank, p=0.036).

CCA between immune profiles and OS. It has been suggested that relative quantities (*i.e*. ratios) of immune system cells and their evolution throughout radiochemotherapy may play a role in treatment outcome (22). This assumption has also been explored for other types of cancer (24). Based on these observations, FACS data derived as described in Figure 1 were used to systematically quantify immune profiles. Specifically, at two time points, namely before and after radiochemotherapy (V0 or V1), all meaningful ratios between immune cell subpopulations were calculated, thereby quantifying the relative proportions of immune cells in the blood. To quantify immune profile changes during radiochemotherapy, for each such quantity, the ratio of its values after to that before radiochemotherapy (V1/V0) was also taken into consideration. This procedure resulted in essence in an enriched data set which contained 765 numerical features. The Pearson correlation coefficient for each one of these features *versus* OS was calculated, firstly for the entire patient population, and separately for each vaccination group. In all cases, none of these features showed strong correlation with OS.

We next hypothesized that there might be combinations of two or more of these features with a potential influence on the ultimate outcome. For this, CCA was performed (23). Specifically, given two or more selected features, CCA was used to calculate a corresponding number of coefficients for which the resulting weighted sum of the selected features correlated best with OS.

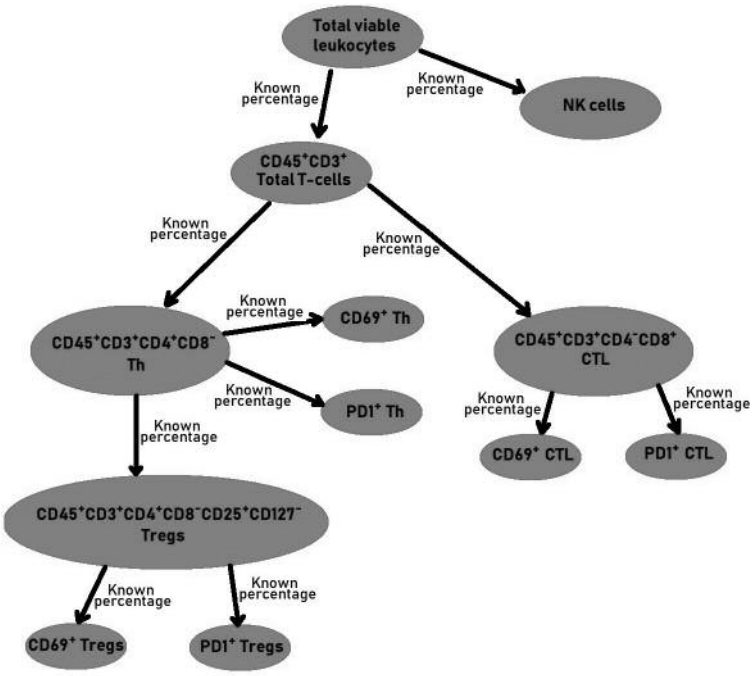

Figure 1. *Methodology to determine the immune profile. Fluorescent cytometric measurements provide relative quantities of immune system cells in terms of the depicted known percentages. CTL: Cytotoxic T-cells; NK: natural killer cells; PD1: programmed death receptor 1; Th: T-helper cells; Treg: regulatory T-cells.*

We first focused on features derived by FACS measurements obtained at time point V1, hence after radiochemotherapy and at time of the first DC vaccine in the early vaccination group. By first disregarding highly correlated features, CCA was performed for all possible combinations of features taken up to 7 at a time *versus* OS. For the entire patient population, no such feature combination correlating strongly with OS was detected. We noted that this exhaustive consideration of all possible combinations places severe restrictions on the maximum number of features in the combination. Consideration of combinations of eight features or more were computationally infeasible.

Patients were subsequently stratified into two subgroups according to vaccination schedule and the same analysis was performed for each of them. A small number of combinations, each consisting of seven features and correlating strongly with OS (>0.70) was found for each subgroup. The strongest of them are provided in Table II.

In all CCA results, a positive coefficient for a feature implies that if the specific feature has a value above its mean, it positively influences OS; if the specific feature has a value below its mean, it negatively influences OS. On the contrary, a negative coefficient implies that if the specific feature has a value above its mean, it negatively influences OS and conversely a value below the mean positively influences OS. It should be stressed that this association of a feature with OS should be considered only in the context of the feature combination it belongs to and the immune profile this combination implies, and not as a separate quantity associated with OS. It is the resulting weighted sum of the features (denoted hereafter by Q) of a specific combination that correlates with OS, and not each feature considered separately.

Table I. *Overall survival (OS) data of the total study population and subgroups residual tumor volume (RTV).*

Patient group	No. of patients	Median OS (months)	2-Year OS rate (%)	95%CI
Total group	101	19	33.66	24.66-42.88
Early vaccination, RTV=0	19	22	40.2	18.4-61.2
Late vaccination, RTV=0	29	23	44.8	26.5-61.5
Early vaccination, RTV>0	28	19	25	11-41.7
Late vaccination, RTV>0	25	16	28	12.4-46

CI: Confidence interval.

The extent of resection (*i.e.* RTV) is a known prognostic factor in GBM (25). Patients with RTV>0 had a significantly worse prognosis than patients without residual tumor

Table II. *Correlation between the immune profile (features obtained after radiochemotherapy) and overall survival (OS) for patients subgrouped according to vaccination schedule. Only V1 fluorescence-activated cell sorting measurements were considered.*

Vaccination subgroup[1]	Calculation[2]	Pearson correlation[3]	Immune profile associated with a high OS (*i.e.* above the mean)[4]
During TMZm: 47 patients	Q=−98.16× [(TregsCD69+ V1) − 0.13]	0.71 ($p=10^{-4}$)	TregsCD69+ V1<0.13 (−)
Mean OS=21.75	+44.39×[(TregsPD1+ V1) − 0.43]		TregsPD1+ V1>0.43 (+)
	−3.18×[(NK/Th V1) − 1.41]		NK/Th V1<1.41 (−)
	−92.17× [(TregsPD1+/CTL V1)− 0.08]		TregsPD1+/CTL V1<0.08 (−)
	+12.37×[(ThCD69+/CTL_CD69+ V1) − 0.88]		ThCD69+/CTL_CD69+ V1>0.88 (+)
	−0.25×[(ThPD1+/CTL_CD69+ V1) − 21.79]		ThPD1+/CTL_CD69+ V1<21.79 (−)
	+13.09×[(TregCD69+/ThCD69+ V1) − 1.02]		TregCD69+/ThCD69+ V1>1.02 (+)
After TMZm: 54 patients	Q=−651.66× [(ThCD69+ V1) − 0.02]	0.70 ($p=2.8\times10^{-5}$)	ThCD69+ V1<0.02 (−)
Mean OS=19.78	−45.27× [(CTL_PD1+ V1) − 0.54]		CTL_PD1+ V1<0.54 (−)
	−7.19× [(CTL/NK V1) − 1.44]		CTL/NK V1<1.44 (−)
	+580.72×[(TregCD69+/NK V1) − 0.02]		TregCD69+/NK V1>0.02 (+)
	−48.32× [(ThPD1+/CTL V1) − 0.40]		ThPD1+/CTL V1<0.40 (−)
	−481.89× [(TregCD69+/CTL_PD1+ V1) − 0.03]		TregCD69+/CTL_PD1+ V1<0.03 (−)
	+406.18×[(ThCD69+/CTL_PD1− V1) − 0.06]		ThCD69+/CTL_PD1- V1>0.06 (+)

TMZm: Maintenance temozolomide. [1]Mean OS calculated from all individual OS data obtained at time of data sampling. [2]Quantity Q was calculated for each patient. Q is a sum of quantities of the form [coefficient × (value of feature observed for the specific patient *minus* the mean of that feature in the specific patient subgroup)]. [3]Respective coefficient of correlation between the quantities Q and quantities (OS *minus* mean OS) for each patient, and *p*-value. [4]Description of the immune profile characteristics associated with a high value of OS, as they are implied by the calculation of quantity Q in the second column; (+) means the higher the better; (−) means the lower the better. Each feature should be considered only in the context of the feature combination it belongs to and the immune profile this combination implies, and not as a separate quantity associated with OS.

Table III. *Correlation between the immune profile (features obtained after radiochemotherapy) and overall survival (OS) for patients subgrouped according to vaccination schedule and extent of resection. Only V1 fluorescence-activated cell sorting measurements were considered.*

Patient subgroup[1]	Calculation[2]	Pearson correlation[3]	Immune profile associated with a high OS (*i.e.* above the mean)[4]
Vaccination: During TMZm,	Q=0.49 ×[(ThCD69−/CTL CD69+ V1) − 67.70]	0.85 ($p=6\times10^{-4}$)	ThCD69−/CTL CD69+ V1>67.70 (+)
RTV=0: 19 patients	−6.99×[(T(CD3+)/CTL PD1+ V1) − 5.28]		T(CD3+)/CTL PD1+ V1<5.28 (−)
Mean OS=23.26	−2.89×[(ThCD69−/CTL PD1− V1) − 5.73]		ThCD69−/CTL PD1− V1<5.73 (−)
	+5.36×[(ThPD1+/Treg V1) − 3.79]		ThPD1+/Treg V1>3.79 (+)
Vaccination: After TMZm,	Q=1.06×[(CTL CD69−/CTL CD69+ V1) − 36.38]	0.72 ($p=9\times10^{-4}$)	CTL CD69−/CTL CD69+ V1>36.38 (+)
RTV=0, 29 patients	−11.16×[(Treg/CTL CD69+ V1) − 3.67]		Treg/CTL CD69+ V1<3.67 (−)
Mean OS=21.76	+177,24×[(ThCD69−/T(CD3+) V1) − 0.38]		ThCD69−/T(CD3+) V1>0.38(+)
	+408.31×[(TregCD69−/ThCD69− V1) − 0.11]		TregCD69−/ThCD69− V1>0.11 (+)
Vaccination: During TMZm,	Q=−1.18 ×[(NK/ThPD1− V1) − 2.98]	0.75 ($p=6\times10^{-4}$)	NK/ThPD1− V1<2.98 (−)
RTV>0: 28 patients	+4.12×[(ThCD69+/TregCD69+ V1) − 3.80]		ThCD69+/TregCD69+ V1>3.80 (+)
Mean OS=20.21	+5.52×[(TregPD1+/ThCD69+ V1) −3.07]		TregPD1+/ThCD69+ V1>3.07 (+)
	−0.49×[(TregCD69−/TregCD69+ V1) − 24.14]		TregCD69−/TregCD69+ V1<24.14 (−)
Vaccination: After TMZm,	Q=−393.42×[(ThCD69− V1) − 0.98]	0.90 ($p=2\times10^{-7}$)	ThCD69− V1<0.98 (−)
RTV>0, 25 patients	−6.50×[(CTL PD1−/NK V1) − 0.71]		CTL PD1−/NK V1<0.71 (−)
Mean OS=17.48	−45.92×[(ThCD69−/T(CD3+) V1) − 0.39]		ThCD69−/T(CD3+) V1<0.39 (−)
	−102.53×[(TregPD1+/ThPD1+ V1) − 0.13]		TregPD1+/ThPD1+ V1<0.13 (−)

TMZm: Maintenance temozolomide; RTV=0: no residual tumor volume; RTV>0: observed residual tumor volume on postoperative magnetic resonance imaging. [1]Mean OS calculated from all individual OS data obtained at time of data sampling. [2]Quantity Q was calculated for each patient. Q is a sum of quantities of the form [coefficient × (value of feature observed for the specific patient *minus* the mean of that feature in the specific patient subgroup)]. [3]Respective coefficient of correlation between the quantities Q and quantities (OS *minus* mean OS) for each patient, and *p*-value. [4]Description of the immune profile characteristics associated with a high value of OS, as they are implied by the calculation of quantity Q in the second column; (+) means the higher the better; (−) means the lower the better. Each feature should be considered only in the context of the feature combination it belongs to and the immune profile this combination implies, and not as a separate quantity associated with OS.

Table IV. *Correlation between the immune profile (features obtained before and after radiochemotherapy) and overall survival (OS) for patients subgrouped according to vaccination schedule and extent of resection. Both V0 and V1 fluorescence-activated cell sorting measurements were considered.*

Patient subgroup[1]	Calculation[2]	Pearson Correlation[3]	Immune profile associated with a high OS (*i.e.* above the mean)[4]
Vaccination: During TMZm, RTV=0: 19 patients Mean OS=23.26	Q=0.54× [(CTL CD69−/CTL CD69+ V0) − 28.79] +6.99× [(CTL/ThPD1− V1) − 2.06] −20.79×[(CTL CD69+/T(CD3+) V1/V0) − 1.31] −4.08× [(TregPD1+/CTL CD69+ V1/V0) − 2.16]	0.93 (p=3×10^{-6})	CTL CD69−/CTL CD69+ V0>28.79 (+) CTL/ThPD1− V1>2.06 (+) CTL CD69+/T(CD3+) V1/V0<1.31 (−) TregPD1+/CTL CD69+ V1/V0<2.16 (−)
Vaccination: After TMZm, RTV=0: 29 patients Mean OS=21.76	Q=340.79×[(CTL CD69+ V0) − 0.04] +18.42×[(T(CD3+) V1/V0) − 1.05] −13.29×[(ThCD69−/ThCD69+ V1/V0) − 1.19] −18.62×[(ThCD69+/TregCD69− V1/V0) − 0.71]	0.76 (p=2×10^{-4})	CTL CD69+ V0>0.04 (+) T(CD3+) V1/V0>1.05 (+) ThCD69−/ThCD69+ V1/V0<1.19 (−) ThCD69+/TregCD69− V1/V0<0.71 (−)
Vaccination: During TMZm, RTV>0: 28 patients Mean OS=20.21	Q=13.19× [(CTL/ThPD1− V0) − 1.67] +493.31×[(TregPD1−/CTL V0) − 0.05] −5.11× [(TregPD1−/CTL CD69+ V1) − 2.89] +5.31× [(TregPD1−/ThCD69+ V1/V0) − 3.04]	0.84 (p=7×10^{-6})	CTL/ThPD1− V0>1.67 (+) TregPD1−/CTL V0>0.05 (+) TregPD1−/CTL CD69+ V1<2.89 (−) TregPD1−/ThCD69+ V1/V0>3.04 (+)
Vaccination: After TMZm, RTV>0: 25 patients Mean OS=17.48	Q=0.95× [(ThPD1+/TregPD1+ V0) − 12.12] −119.4×[(TregPD1+/NK V1) − 0.07] +1.21× [(Th/CTL CD69+ V1) − 24.09] −2.21× [(ThPD1−/CTL CD69+ V1) − 13.35]	0.92 (p=4×10^{-8})	ThPD1+/TregPD1+ V0>12.12 (+) TregPD1+/NK V1<0.07 (−) Th/CTL CD69+ V1>24.09 (+) ThPD1−/CTL CD69+ V1<13.35 (−)

TMZm: Maintenance temozolomide; RTV=0: no residual tumor volume; RTV>0: observed residual tumor volume on postoperative magnetic resonance imaging. [1]Mean OS calculated from all individual OS data obtained at time of data sampling. [2]Quantity Q was calculated for each patient. Q is a sum of quantities of the form [coefficient × (value of feature observed for the specific patient *minus* the mean of that feature in the specific patient subgroup)]. [3]Respective coefficient of correlation between the quantities Q and quantities (OS *minus* mean OS) for each patient, and p-value. [4]Description of the immune profile characteristics associated with a high value of OS, as they are implied by the calculation of quantity Q in the second column; (+) means the higher the better; (−) means the lower the better. Each feature should be considered only in the context of the feature combination it belongs to and the immune profile this combination implies, and not as a separate quantity associated with OS.

volume. Stratifying patients into four subgroups, defined by extent of resection (RTV=0/>0) and vaccination schedule (during/after maintenance temozolomide) and performing the aforementioned CCA analysis for each subgroup, revealed many combinations consisting of 4 features, each one correlating more strongly with OS. The strongest of these correlations are depicted in Table III.

Finally, additionally taking into consideration features derived from FACS measurements obtained at V0, after operation but prior to radiochemotherapy, thereby also including the change in immune profile due to radiochemotherapy, the analysis revealed more combinations of four features correlating more strongly with OS. The strongest of them are provided in Table IV and depicted in Figure 2.

In all subgroups, CTL, Th cells and Tregs were found to play a relative role in the modeling. However, the relative weight of the three cell populations, and their activated status demonstrated *via* CD69 or PD1 expression, were different. In general, it seems that elevated numbers of CTL CD69+ cells and decreasing numbers of Tregs favor a better OS outcome, however, they are never the only factors at play. Th and NK cells also appear to play a role, but their role is less clear. The data in Tables II-IV suggest quite complex interactions between CTLs, Tregs, Th, their activated and non-activated subpopulations, and NK cells, which however, appear to have a potential influence on the ultimate OS outcome.

Discussion

The assumption that immune cell subpopulations, their relative quantities and their timely evolution may play a role in the final outcome for patients with cancer has already been suggested and explored for various types of cancer (22, 24). To the best of our knowledge, in the context of GBM, no strong correlations between a single such quantity and OS outcome have been reported. It is therefore natural to ask if there are combinations of two or more such quantities whose combined effects may play a role in the OS outcome of patients with GBM. This work describes a systematic and straightforward analysis aiming to explore this assumption. The proposed workflow detects strong and statistically significant correlations between biologically meaningful, immune-profile-derived quantities obtained at the start of standard treatment plus DC vaccination on the one hand and OS many months later on the other hand. These correlations suggest a strong influence of a patient's immune status on the

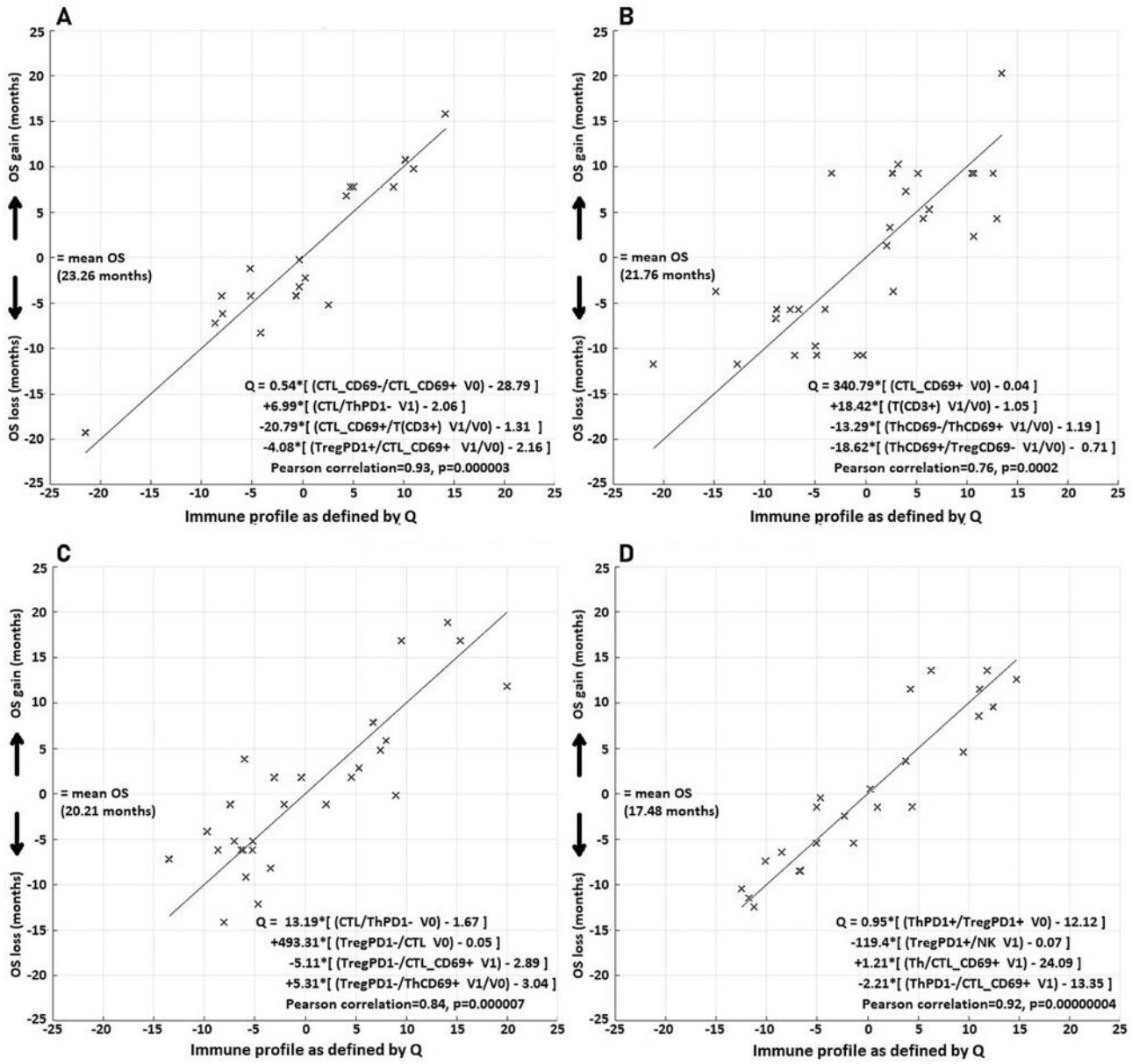

Figure 2. *Canonical correlation analysis of immune profiles versus overall survival (OS). The immune profile is composed of the different FACS-derived quantities (features). Diagrams for four subgroups are shown. A: Vaccination during temozolomide maintenance chemotherapy (TMZm), no residual tumor volume after resection; B: vaccination after TMZm, no residual tumor volume after resection; C: vaccination during TMZm, non-zero residual tumor volume after resection; D: vaccination after TMZm, non-zero residual tumor volume after resection. Each diagram depicts the strong correlation between Q (x-axis), where Q is the sum of quantities of the form: coefficient × [feature − mean of feature in the specific subgroup], and OS (y-axis) expressed as the quantity OS being the individual OS in months minus the mean OS, i.e. mean OS for the specific subgroup.*

OS outcome. Owing to the limited number of available patients, the question of prospective validity of the detected statistical patterns remains open. However, the proposed workflow could serve as a basis for more elaborate analyses aiming at the identification of prognostic markers able to discriminate between potential long- and short-term survivors.

Although the prognosis of GBM is universally dismal, different clinical and biological characteristics do have an influence on the ultimate outcome of the patients. The role of the grading of the tumor (grade III *versus* grade IV), the age of the patient, the Karnofsky Performance Index, the Minimental State, the extent of resection and the quality of radiotherapy are

reflected in the RPA and have a prognostic significance (21), even in the context of additional DC vaccination (26). In particular the extent of resection has been demonstrated to be crucial (25, 27). At the molecular level, the methylation status of O^6-methylguanine-DNA methyltransferase (*MGMT*) in GBM has been correlated to the response to temozolomide treatment and OS (28). The distinct epigenetic and biological subgroups of GBM defined by hotspot mutations in H3 histone family member 3A (*H3F3A*) and cytosolic isocitrate dehydrogenase [NADP(+)] 1 (*IDH1*) have different OS curves (29). The same observation was made in the original Glioma Translat study population from which data for this analysis were sampled [(15), data not published].

It is generally accepted, and demonstrated in at least three reviews (12-14), that DC vaccination contributes to improved OS in patients with GBM. Compared to the patient group described by Stupp *et al*. (3), RPA class IV patients were more represented in the current study cohort. The median OS of the total group in our study (19 months) exceeded the published median OS of 16 months for RPA class IV patients, in particular for the patients without RTV (early vaccination 22 months, late vaccination 23 months) and for patients with RTV and early vaccination (19 months). The 2-year OS of 33% of the total study group was higher than the published 2-year OS of 29%, due to the remarkable 2-year OS data in both RTV=0 subgroups (40% and 45%, respectively). Although not statistically significant at the level of median OS, there was a trend for an improved 2-year OS rate in the late vaccination subgroups as compared to their respective early vaccination subgroups.

The production of patient-specific DCs is labor intensive and expensive because of its categorization as an Advanced Therapy Medicinal Product. Therefore, a search for biomarkers that correlate with improved survival and that can be easily quantified might be of help in counseling patients for adding DC vaccination to the standard combination treatment. Most GBMs are considered as immune 'cold' tumors with a lower level of antitumoral immune cell infiltration due to immunosuppressive soluble and membrane-bound molecules and the presence of Tregs and myeloid-derived suppressor cells (30). The presence of $CD3^+CD8^+$ tumor-infiltrating immune cells has been correlated with prolonged survival in patients with GBM (31). In addition, systemic immune function is affected not only by the tumor but also by corticosteroids (31, 32). The systemic immune system recovers after tumor resection (32), but is further influenced by adjuvant radiochemotherapy (22). Therefore, one can argue that for patients with (sub)totally resected GBMs, as was the case in this series of patients, the (change of) immune populations in the peripheral blood compartment immediately prior to and after radiochemotherapy can be studied as eventual biomarkers for potential gain in OS upon addition of DC vaccination to the standard combination therapy. The data on peripheral blood immune profiles and their strong correlation to OS depicted here provide the first step for this. The relative role of the fraction of Th, CTL, Tregs and NK cells without or with CD69 or PD1 expression is different for the four clinical subgroups. These correlations are remarkable, taking into account the extremely complex biological processes between the blood sampling and the survival time of the patient.

In conclusion, immune profiles of T-cells detected in the blood and measured in the periods before and after radiochemotherapy were correlated with ultimate OS of the patients. Clinical conditions, such as RTV and time of DC vaccination influenced the correlations. Prospective validation studies should be performed to demonstrate the peripheral blood immune profiles as potential biomarkers in the context of DC vaccination for GBM.

Conflicts of Interest

There are no conflicts of interest in relation to this study.

Authors' Contributions

MA performed the mathematical/computational analyses and wrote the first draft of the manuscript. SVG provided the clinical data and wrote the clinical parts of the manuscript. DD, GS, NG reviewed the manuscript and suggested important further improvements. GS and NG supervised the CHIC project and consortium, to which this subproject belonged.

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