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TUMOR MARKERS AND SIGNATURES

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A novel serum extracellular vesicle protein signature to monitor glioblastoma tumor progression

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Abstract

Detection of tumor progression in patients with glioblastoma remains a major challenge. Extracellular vesicles (EVs) are potential biomarkers and can be detected in the blood of patients with glioblastoma. In our study, we evaluated the potential of serum-derived EVs from glioblastoma patients to serve as biomarker for tumor progression. EVs from serum of glioblastoma patients and healthy volunteers were

Abbreviations: AUC, area under the receiver operating characteristic curves; EV, extracellular vesicle; H3, histone H3; HBSS, Hank's balanced salt solution; HV, healthy volunteers; IDH, isocitrate dehydrogenase; MRI, magnetic resonance imaging; OS, overall survival; PD, progressive disease; RANO, response assessment in neuro-oncology; RT, room temperature; SD, stable disease; t1, baseline value before initiation of radio-chemotherapy; t-PD, time-point before progressive disease.

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separated by size exclusion chromatography and ultracentrifugation. EV markers were defined by using a proximity-extension assay and bead-based flow cytometry. Tumor progression was defined according to modified RANO criteria. EVs from the serum of glioblastoma patients (n = 67) showed an upregulation of CD29, CD44, CD81, CD146, C1QA and histone H3 as compared to serum EVs from healthy volunteers (*P* value range: <.0001 to .08). For two independent cohorts of glioblastoma patients, we noted upregulation of C1QA, CD44 and histone H3 upon tumor progression, but not in patients with stable disease. In a multivariable logistic regression analysis, a combination of CD29, CD44, CD81, C1QA and histone H3 correlated with RANO-defined tumor progression with an AUC of 0.76. Measurement of CD29, CD44, CD81, C1QA and histone H3 in serum-derived EVs of glioblastoma patients, along with standard MRI assessment, has the potential to improve detection of true tumor progression and thus could be a useful biomarker for clinical decision making.

KEYWORDS
biomarkers, extracellular vesicles, glioblastoma, protein-signature, tumor-progression

What's new?

It is challenging to detect tumor progression in glioblastoma. Here, the authors evaluated whether extracellular vesicles (EVs) circulating in the blood could serve as a reliable biomarker for tumor progression. They screened two independent cohorts including a total of 67 glioblastoma patients. Serum EVs collected from glioblastoma patients showed upregulation of CD29, CD44, CD81, CD146, C1QA and histone H3 relative to EVs from healthy volunteers. Among glioblastoma patients, tumor progression was accompanied by upregulation of C1QA, CD44 and histone H3, which was not observed in patients with stable disease.

1 | INTRODUCTION

Glioblastomas lacking mutations in isocitrate dehydrogenase (IDH; IDH-wildtype) are devastating brain tumors, with a median overall survival (OS) of 15 to 18 months.[1] First-line treatment consists of surgical resection, radiochemotherapy with concomitant temozolomide and maintenance chemotherapy with temozolomide.[1] Silencing of the *O-6-methylguanine-DNA methyltransferase* (*MGMT*) gene via promoter methylation enhances response to alkylating chemotherapy.[2] The randomized multicenter phase III CeTeG/NOA-09 trial recently showed that intensified alkylating chemotherapy with lomustine (CCNU) and temozolomide leads to a survival benefit in *MGMT*-promoter-methylated patients.[3] Treatment options for recurrent disease are limited.[4]

The standard monitoring for treatment efficacy is the sequential performance of contrast-enhanced magnetic resonance imaging (MRI), which are evaluated using the modified response assessment in neuro-oncology (RANO) criteria.[5] In some cases, a reliable MRI-based detection of tumor progression is challenging, since increasing contrast enhancement could be caused by pseudoprogression.[6] Thus, it would be beneficial to have additional parameters that would allow detection of true progression at—or prior to—the first sign of increasing contrast enhancement when, at least in some cases, progression remains uncertain. Under these circumstances, a minimally invasive liquid biopsy test would be highly desirable.

Extracellular vesicles (EVs) are small membrane-bound particles secreted from cells that contain functional molecules and play a major role in intercellular communication.[7] Especially in oncology, EVs mediate interaction between the tumor and the microenvironment and are involved in regulation of cell proliferation, migration, immunosuppression and angiogenesis.[8,9] While preliminary data show that EVs secreted by glioblastoma cells can be detected in human blood,[10-13] no markers have thus far been proven capable of detecting true tumor progression in longitudinal sequential analyses.

In our study, we screened for suitable serum EV markers and evaluated their potential for detecting tumor progression in two independent patient cohorts, one from the CeTeG/NOA-09 trial[3] and the other from a cohort of patients treated at the University Hospital of Bonn. Based on these findings, we propose a novel panel of protein EV markers that could be routinely used to detect tumor progression in the clinic.

2 | MATERIALS AND METHODS

2.1 | Characteristics of patients and healthy volunteers

Our study included two independent patient cohorts, characteristics of which are listed in Table S1. Characteristics of matched healthy volunteers are shown in Table S2. For the first patient cohort, all

36 patients from the phase III CeTeG/NOA-09 trial (CeTeG/NOA-09 cohort) had either at least two serum samples, one at a time-point prior to tumor progression and one at progression (progression subgroup) or had no progression, but at least three samples at time-points without progression (stable disease group). Serum was drawn at two to four consecutive MRI visits, scheduled every 3 months. Twenty-seven (75%) patients had an event of tumor progression, as defined per modified RANO criteria (n = 21)[3] or if the study center initiated recurrent treatment (n = 6) during the study follow-up (progressive disease; PD), as opposed to 9 (25%) patients that had stable disease (SD) throughout the study. Patients were recruited from June 2011 until April 2014 and follow-up time after enrollment of the last patient was 36 months.[3] Baseline values after surgery, but before the initiation of radio-chemotherapy (t1) were available for 25 (69.4%) patients. Values at the time-point before progressive disease (t-PD), that is, the last visit documented as having stable disease by MRI (t-PD) and at the time-point of documented PD were available for all patients with PD. Ten out of these patients—all with tumor progression (four with time-points t1, t-PD and PD and six with time-points t1 and PD)—were used for screening of putative EV markers using the Proximity Extension Assay (see below). For patients without tumor progression, at least three time-points were available.

The second patient cohort included 31 glioblastoma patients treated at the University Hospital of Bonn (Bonn cohort). Mainly *IDH*-wildtype patients were included (Table S1) and 23 (74.2%) had a tumor progression during the follow-up period of the study based on modified RANO criteria (3). Patients were recruited from June 2016 until April 2017 and follow-up after last patient enrollment was 35 months. Blood samples were collected for two to four consecutive MRI visits performed every 2 to 4 months. We only had 3 (9.7%) patients with baseline values before the initiation of radio-chemotherapy, however all patients with tumor progression had an MRI with pre-PD stable disease (t-PD). There were at least three time-points available for patients without tumor progression.

2.2 | Sample collection

Serum was collected from glioblastoma patients in 9 mL S-monovettes (Sarstedt, Nuembrecht, Germany) at different time-points and also from healthy volunteers at two different time-points, as previously described.[14] Briefly, samples were rested in an upright position for 30 minutes at room temperature (RT) and then centrifuged for 15 minutes at 2000*g* at RT. Then, samples were centrifuged again for 20 minutes at 3200*g* at 6°C to remove platelets. Subsequently, the supernatants were filtered with a 0.45 μm filter and stored at −80°C.

2.3 | Cell culture

Primary GB cells 46z and 106z were cultured as previously described.[15] Short-term expanded patient-derived glioblastoma cells were cultured adherently on laminin/poly-L-ornithine-coated plastic applying defined media that consisted of N2/B27-supplemented Neurobasal with addition of growth factors every other day (EGF and bFGF; 10 ng/mL each). HOG (RRID: CVCL_D354) were a kind gift from the laboratory of Prof. Xandra Breakfield, Harvard Medical School. The authenticity of all cell lines was confirmed within the last 3 years using STR profiling.

All experiments were performed with mycoplasma-free cells, which was confirmed with PlasmoTest-Mycoplasma detection kit (Invivogen, Toulouse, France). HOG were cultivated for up to 15 passages after thawing in DMEM (LifeTechnologies, Paisley, UK) supplemented with 10% FCS, 1% Penicillin-Streptavidin and sodium-pyruvate.

2.4 | EV separation

Small EVs from conditioned cell-culture media (30 mL per EV sample) were separated using the Exo-spin exosome purification kit (Cell Guidance Systems, Cambridge, UK), a combination of EV precipitation followed by size exclusion chromatography, according to the manufacturer's instructions. Small EVs from 0.5 mL serum samples were isolated as previously described.[14] In short, we used size exclusion chromatography using the sepharose-based qEV columns (iZON Science, Christchurch, New Zealand). The small EVs were eluted with Hank's balanced salt solution (HBSS). Fractions 8 to 10 of 500 μL each were collected and pooled and a protease inhibitor (Roche, Basel, Switzerland) was added. Subsequently, 1.5 mL of this mixture were concentrated to a final volume of 120 μL by ultracentrifugation (1 hour and 45 minutes, 110 000 *g*, 4°C) using the TLA-55 rotor and the Optima MAX-XP ultracentrifuge (both Beckman Coulter, Brea, California).

2.5 | Nanoparticle tracking analysis

ZetaView Nanoparticle Tracking Analyzer (Particle Metrix, Meerbusch, Germany) was used for nanoparticle tracking analysis, according to the manufacturer's protocol, as previously described.[14,16] The manufacturer's default software EV-settings were used. The videos were analyzed by the ZetaView Software 8.05.11 SP1. For details see Appendix S1.

2.6 | Flow cytometry assays

Surface Protein expression on EVs was analyzed by bead-assisted flow cytometry using 4 μm sized carboxylated polystyrene beads (Polysciences, Warrington, Pennsylvania). To allow binding, EV-suspensions (110 μL each) were incubated with 10 μL carboxylated polystyrene beads (5×10^7 beads/mL; Polysciences, Warrington, Pennsylvania) overnight at 4°C. Next, the beads were blocked with 1% BSA. The solution was then split into 10 wells of a 96 U-bottom plate and primary antibodies (Table S3) were added. After 25 minutes incubation at RT and two washing steps with 200 μL PBS (containing 1% BSA, 0.1% sodium azide) a phycoerythrin-labeled (PE) secondary antibody (Table S3) was applied for 20 minutes at RT. After two further washing steps, the beads were analyzed immediately with FACSCanto II (BD Biosciences, San Jose, California). The flow rate was set

on "medium" resulting in an event rate of ~200 events s^{-1}. Flow cytometer acquisition settings were maintained for all samples, including flow rate, threshold and voltages. Gating was performed based on FSC/SSC parameters excluding bead-doublets. The MIFlowCyt checklist[17] containing more details was completed and is attached in the Appendix S1. For analysis, CD63, suggested as the most reliable marker for EVs derived from the endosomal pathway,[18] was chosen for normalization since this marker showed the lowest variability within patients and HV (data not shown).

MACSPlex analysis: FACS analysis using MACSPlex exosome capture beads was performed according to the manufacturer's guidelines.[19] Briefly, 120 µL EV suspension was incubated with 15 µL bead-suspension overnight at RT. After a washing step, 15 µL MACSPlex Exosome detection-reagent cocktail (CD9-, CD63-, CD81-APC) were added and incubated for 1 hour at RT. After two further washing steps, the bead-coupled EVs were analyzed by FACS. After gating the bead population based on FSC/SSC parameters, different gates in the PE vs FITC channel for 37 different antigens were applied.

All data were acquired with LSRII or FACSCanto II and analyzed with FlowJo software, version 10 (all BD Biosciences, San Jose, California). The geometric mean fluorescence intensities (MFI) were background-corrected and negative values were excluded from the plot.

2.7 | Transmission electron microscopy

An established protocol was applied.[16] Briefly, 5 µL EV sample was loaded onto formvar-coated copper grids (Science Services, Munich, Germany) and incubated for 20 minutes. Then, the EVs were fixed with 2% paraformaldehyde for 5 minutes, washed with PBS, fixed again for 5 minutes with 1% glutaraldehyde, washed with ddH_2O and incubated with contrast dye (1.5% uranyl acetate) for 4 minutes. Images were taken with a Gatan OneView 4 K camera (Gatan, Pleasanton, California) mounted on a Jem-2100Plus microscope (JEOL) operating at 200 kV.

2.8 | Immunodetection of EV proteins

Simple Western technology (ProteinSimple, San Jose, California) was used for the detection of GAPDH, Apo-A1 and calnexin and EV markers (flotillin-1, TSG101 or CD9) (details in Table S4). The 12 to 230 kDa Wes Separation Module as well as the secondary anti-rabbit, anti-mouse and anti-goat antibody detection modules (all ProteinSimple) were used according to the manufacturer's instructions. For details see Appendix S1. Data analysis was performed with Compass software (ProteinSimple).

2.9 | Proximity extension assay

For screening of EV markers from patient material, serum small EVs were lysed with radioimmunoprecipitation assay (RIPA) buffer, proteins were analyzed using two ProSeek Multiplex Oncology panels with a total of 184 markers (Oncology II and Oncology III; Olink Bioscience, Uppsala, Sweden), as previously described.[12] Proximity extension assay consists of three steps: for each panel with 92 markers, 92 probe-pairs bind to their respective proteins, followed by a pre-amplification step of unique DNA-reporter sequences based on proximity extension. Finally, the reporter DNA sequences were detected using quantitative real-time PCR. Lysis buffer was used as a negative control and three spike-in controls served as positive controls. The assay-dependent limit of detection value (LOD) was estimated from negative controls plus three standard deviations. The obtained Cq values were corrected for background and extension control, thereby generating normalized protein expression values.

2.10 | Statistical analyses

For univariate analyses, GraphPad Prism (Version 8.2.1) software (La Jolla, California) was used. The Mann-Whitney *U* test was applied to detect EV-level-differences between glioblastoma patients and healthy volunteers. A Wilcoxon signed-rank test for each marker was applied separately to each cohort, in order to evaluate differences between the time-points.

The joint association of EV markers with disease status (progression vs stability) was explored in the pooled dataset from both cohorts using multivariable logistic regression models. Differences between first and second marker measurements were used as the outcome variable. For preselection of markers, simple logistic regression models, each with one marker as predictor, were set up and the areas under the receiver operating characteristic curves (AUCs) were calculated. Markers with an AUC of at least 0.55 were selected for further analysis. Afterwards, a multivariable logistic regression model with the selected markers but without further clinical markers (such as Karnofsky performance status or age) was set up. The performance of the model was assessed using the AUC values obtained from 100 times 3-fold cross-validation, as previously described.[20] Missing values were imputed, as previously performed.[21] Calculations were carried out using the R system for statistical computing (version 3.6.1; R Development Core Team, Vienna, Austria, 2019).

To detect differences in the expression of markers in primary glioblastoma tissue as well as their prognostic significance, The Cancer Genome Atlas (TCGA) database was interrogated using the OmicSoft Array-Studio V10.1 (Qiagen, Venlo, Netherlands). For expression differences an F-ANOVA test was performed and survival curves were generated using Kaplan-Meier plots, with survival differences detected using a log-rank test. Samples were classified based on the ranked gene expression of genes, selecting the top 50% as the high expression group and bottom 50% as the low expression group.

The analysis of data from the proximity extension assay was performed on normalized and log_2-transformed protein expression values. In line with the manufacturer's instructions, a marker was excluded from the analysis if 75% of the values were below the LOD,

thus 94 out of 184 proteins were included in the analysis. To account for the repeated measures design, patients were considered as blocking factors in the statistical-model fitting performed by the R package LIMMA (R version 3.6.2, LIMMA version 3.42.2[22]). The resulting *P*-values were adjusted for multiple testing using the Benjamini-Hochberg method. Similar to a *P* value of <.05, the false discovery rate was used to set a significance cut-off level for the proximity extension assay (in this case .05) and thereby correct for multiple testing. Differentially regulated proteins with a false discovery rate of <0.05 were considered as statistically significant.

3 | RESULTS

3.1 | Selection of putative EV markers suitable for liquid biopsy

In order to discover putative EV-based markers for glioblastoma, we used two screening approaches. The first was a bead-based FACS analysis of purified small EVs from conditioned media of two primary human glioblastoma cell lines, 46z and 106z (both without *MGMT* promoter methylation[23]) and the established human high grade glioma cell line, HOG, with *MGMT* promoter methylation.[24] The primary glioblastoma cells exhibit stem-cell-like properties that mimic the biological aggressiveness of a tumor in vitro.[23] All of these cell lines gave a high yield of small EVs (Figure S1). Using the MACSPlex assay analyzing the presence of 37 different antigens,[19] we observed high levels of the standard EV markers CD9, CD63 and CD81, but also of CD29, CD44 and CD146 (Figure 1) thereby identifying possible glioblastoma-associated EV-markers. While CD44 exhibited similar fluorescence intensity levels to those of standard EV markers, CD133 was barely detectable in small EVs from all three cell lines. Nanoparticle tracking analysis and transmission electron microscopy

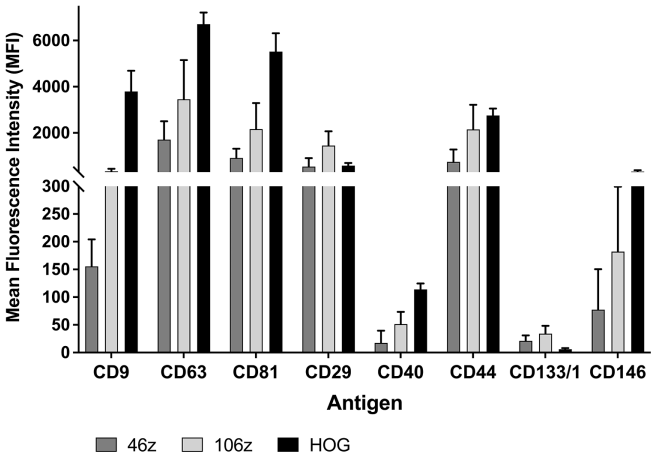

FIGURE 1 MACSPlex flow cytometry of EVs from primary glioblastoma cells (46z and 106z) as well as high grade glioma cell line HOG. Depicted is the geometric mean fluorescence intensity (MFI) of eight protein markers (n = 3)

show the expected size (~130 nm) and shape of small EVs from cell culture supernatant (Figure S1a,b). EV-purity was confirmed by immunodetection (Figure S1c).

Since it is known that the small EVs secreted by glioblastoma cells only represent a small fraction of all small EVs in patient serum,[11] we used a second screening approach for reactive EV markers (the proximity extension assay with two oncology panels[12]). For this screen, we analyzed small EVs from the serum of 10 glioblastoma patients from different time-points (see Section 2) and five healthy volunteers at two different time-points as controls (n = 5). While we did not observe any significant differentially regulated proteins between baseline glioblastoma-small EVs and small EVs at the time of tumor progression (PD; Figure 2A), we noted an upregulation of the complement C1qA chain (C1QA) at PD compared to t-PD (last pre-PD MRI visit showing stable disease; Figure 2B). No differences were observed between small EVs of healthy volunteers at different time points (Figure 2C) or between small EVs of healthy volunteers and baseline glioblastoma-small EVs (Figure 2D). Beyond the two experimental screening approaches and because histones are known to be upregulated in tumor EVs,[25] we also included histone H3 (H3) in our panel. The combination of these two screening approaches yielded markers that are either directly secreted by tumor cells or secreted by non-tumorigenic cells as a reaction to the tumor.

3.2 | Characteristics of EVs extracted from serum

Characterization of small EVs by nanoparticle tracking analysis, transmission electron microscopy and immunodetection for quality control showed the expected size (117.6 ± 14 nm) (Figure S2a) and shape (Figure S2d) for the isolated small EVs as well as the presence of the EV markers CD9 and flotillin-1. A lack of calnexin, a protein of the endoplasmic reticulum, indicates the purity of the small EVs following SEC and ultracentrifugation (Figure S2b). The lipoprotein Apo-A1 was still present, but strongly depleted up to 29 500-fold (Figure S2c). Furthermore, while no significant longitudinal changes were observed for glioblastoma patients, they had overall higher EV levels compared to healthy volunteers (Figure S3). Notably, the extent of resection did not have an impact on the serum particle concentration or levels of the identified markers in serum-derived small EVs at the baseline time-point (ie, before initiation of radiochemotherapy, Figure S4).

3.3 | TCGA data for the identified glioblastoma markers

In order to further support the relevance of the markers CD29, CD44, CD146 and C1Qa, we investigated the expression of each of these markers in glioblastoma patients using the TCGA database. We found an upregulation of all these markers in the RNAseq data of primary and recurrent glioblastoma tumors compared to normal tissue

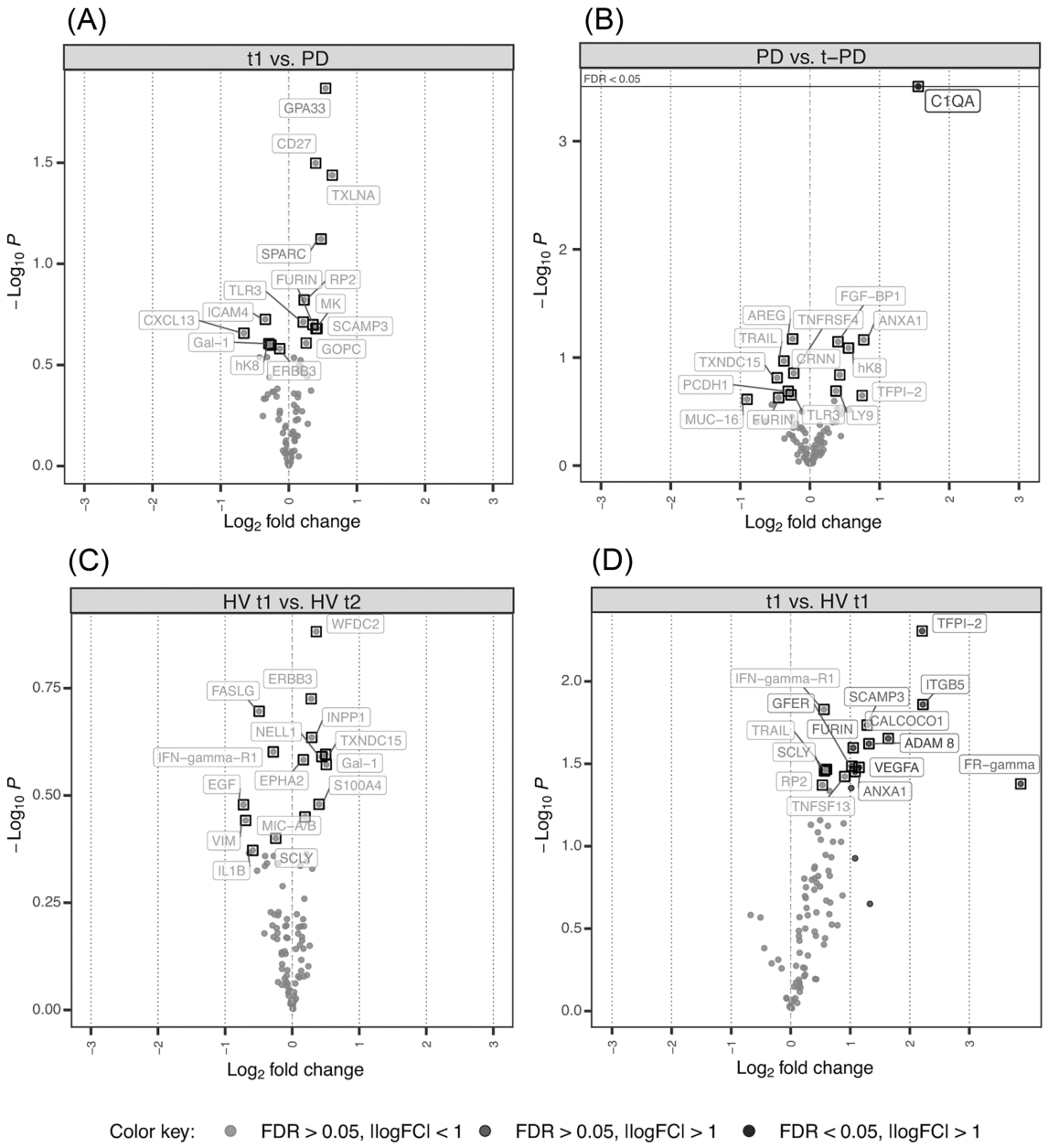

FIGURE 2 Volcano plots of protein levels identified from EVs as analyzed by the proximity extension assay (Olink Bioscience, Uppsala, Sweden) from glioblastoma patients (n = 10) at two or three time-points (t1, t-PD and PD) and healthy volunteers (HV, n = 5) at two time-points. Depicted is the $\log_2$ fold-change in the *x*-axis and the $-\log_{10}$ *P*-value in the *y*-axis. Note the increase of C1QA in EVs at PD compared to t-PD (false discovery rate [FDR] < 0.05) (B), while no change is detected in baseline glioblastoma-small EVs compared to small EVs at the time of tumor progression (A) or in EVs from healthy volunteers (C, D)

(Figure S5a-d; range of F-ANOVA *P*-values: 9.3×10^{-10} to .029). No data on histone H3 expression were available.

3.4 | Evidence for elevated levels of the identified markers in small EVs from glioblastoma patients compared to healthy donors

CD9, CD63, CD81 (all established EV markers) and CD29, CD44, CD146, C1Qa and H3 were analyzed on serum-derived small EVs of glioblastoma patients and compared to serum-derived small EVs from healthy volunteers using bead-based flow-cytometry. Serum-derived small EVs from 67 glioblastoma patients with two to three time-points each were compared to those of healthy volunteers (n = 22 with one to two time-points each). The CD9/CD63 ratio was slightly increased in healthy volunteers and we observed a nonsignificant trend towards a higher ratio of CD29/CD63 in glioblastoma patients. All of the other markers (CD44, CD146, C1QA and H3) and the standard EV marker CD81 showed significantly higher levels in glioblastoma patients compared to healthy volunteers (Figure 3).

3.5 | Evaluation of the identified markers in two independent glioblastoma cohorts

We next tested the potential of the protein markers on EV for detecting tumor progression in two independent patient cohorts. Glioblastoma markers CD29 and CD146 did not show a significant upregulation in small EVs upon tumor progression in either of the two cohorts (CD29: Figures 4D and 5D respectively; CeTeG: $P = .25$, Bonn: $P = .08$/CD146: Figure S6d, $P = .58$). CD81 was found to be increased upon tumor progression in the CeTeG/NOA-09 cohort

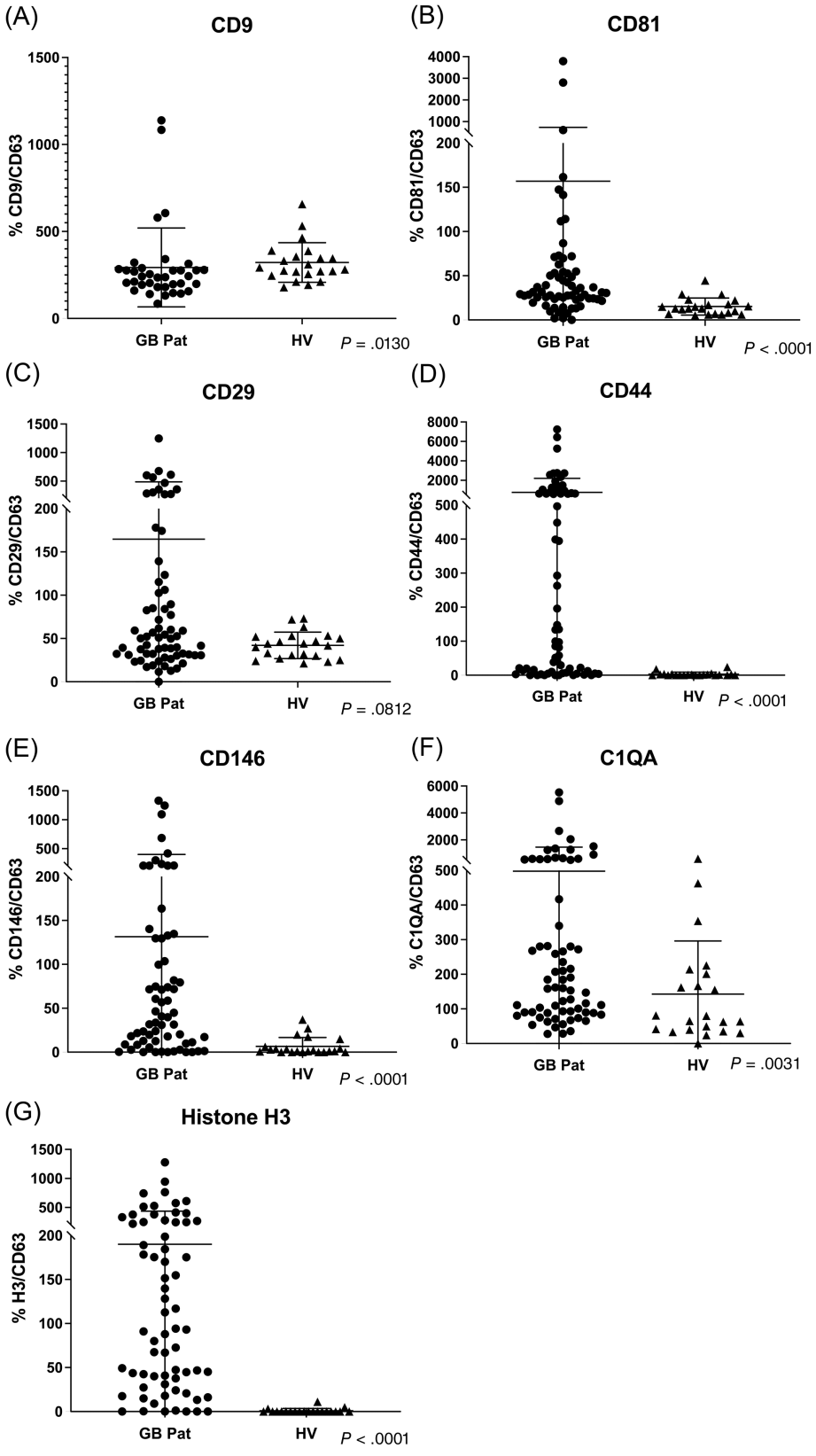

FIGURE 3 Bead-assisted flow cytometry data of seven marker (A-G) from serum-derived EVs from glioblastoma patients (n = 67) compared to healthy volunteers (n = 22) for each marker (MFI of each marker was normalized to CD63). Depicted are pooled data of glioblastoma patients from both glioblastoma cohorts using the average for all time-points collected per patient compared to healthy volunteers using the average ratio of 1 to 2 time-points. GB, glioblastoma. Bars represent median ± interquartile ranges

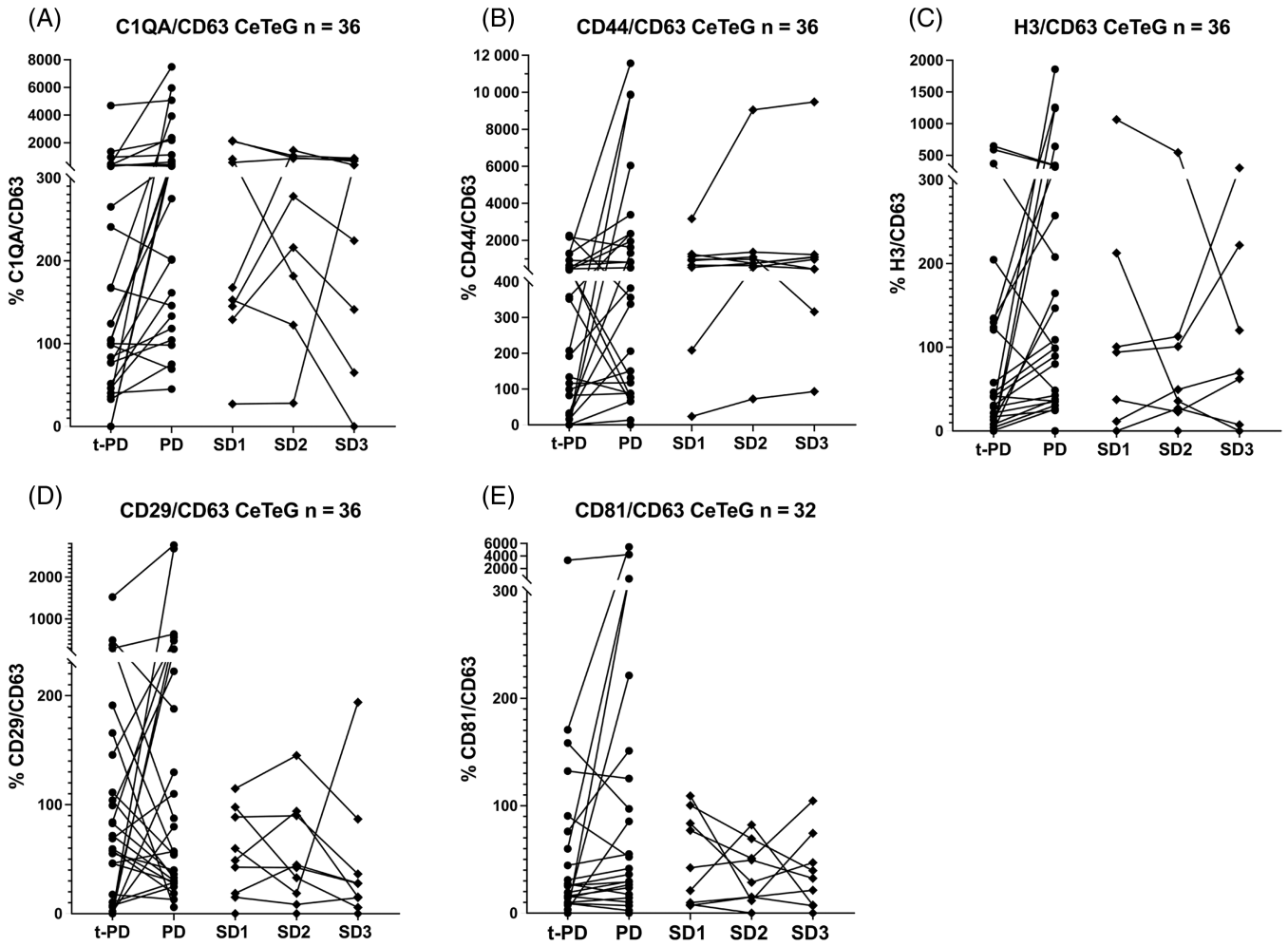

FIGURE 4 Longitudinal bead-assisted flow cytometry data of EVs from serum of glioblastoma patients from the CeTeG/NOA-09 trial (n = 36). Depicted are the time-points t-PD and PD for 27 patients with tumor progression (PD) and three time-points (SD1, SD2, SD3) for 9 patients with stable disease (SD) during the study follow-up. For (A) C1QA, (B) CD44, (C) H3 and (E) CD81, levels of these markers increased upon tumor progression ($P < .0001$, $P = .027$, $P = .049$ and $P = .015$, respectively), while no significant change was found in patients with SD (SD1 vs SD2: (A) $P = 1.0$, (B) $P = .25$, (C) $P = .81$ and (E) $P = .36$, SD2 vs SD3: (A) $P = .1$, (B) $P = 1.0$, (C) $P = .69$ and (E) $P = 1.0$, respectively). For (D) CD29, no clear upregulation was detected at the time of tumor progression ($P = .3$)

(increased in 15 out of 23 patients; 65.2%; Figure 4E; $P = .06$), but not during earlier disease stages in these patients (Figure S6e; $P = .73$) or in patients without progression during our study (Figure 4E; $P = .31$ and $P = 1.0$ for SD1-SD2 and SD2-SD3 respectively), yet this finding was not confirmed in the Bonn cohort (Figure 5E; 7/23; 30.4%; $P = .2$). C1QA, CD44 and H3 showed a significant increase upon tumor progression in both patient cohorts (Figures 4A-C and 5A-C; C1QA: increase in 25 out of 27 patients; 92.6%; $P < .0001$ and 16/23; 69.6%; $P = .03$/CD44: increase in 19 out of 27 patients; 70.4%; $P = .02$ and 15/23; 65.2%; $P = .03$/H3: increase in 17 out of 27 patients; 62.9%; $P = .03$ and 15/23; 65.2%; $P = .02$). The exclusion of the two patients with IDH-mutant tumors and one patient with unknown IDH status did not change the finding of a significant increase of C1QA, CD44 and H3 upon progression (data not shown). For all three of these markers, no significant increase was noted in patients with stable disease (Figures 4A-C and 5A-C) or at earlier time-points of patients with tumor progression from the CeTeG/NOA-09 study (Figure S6a-c). Consistent with our data from the proximity extension assay, patients of the CeTeG/NOA-09 trial showed a decrease in C1QA levels in 14/21 patients (66.6%; $P = .18$; Figure S6a) after baseline, which then increased upon tumor progression. Contrary to the EV-based analyses, study of histone H3 and C1QA protein levels in total serum did not allow for the detection of tumor progression (Figure S7).

Notably, 6 out of 27 patients with PD of the CeTeG/NOA-09 cohort did not fulfill the RANO criteria at the time-point of suspected progression (example shown in Figure S8) and while further confirmatory MRIs are lacking, they all had a lower OS compared to the rest of the cohort (0.35-2.33 years). Interestingly, they all showed elevation of at least two out of three markers (CD44, C1QA and H3). MRI examples and the corresponding levels of serum EV markers for three patients (two with concordance between MRI and markers and one

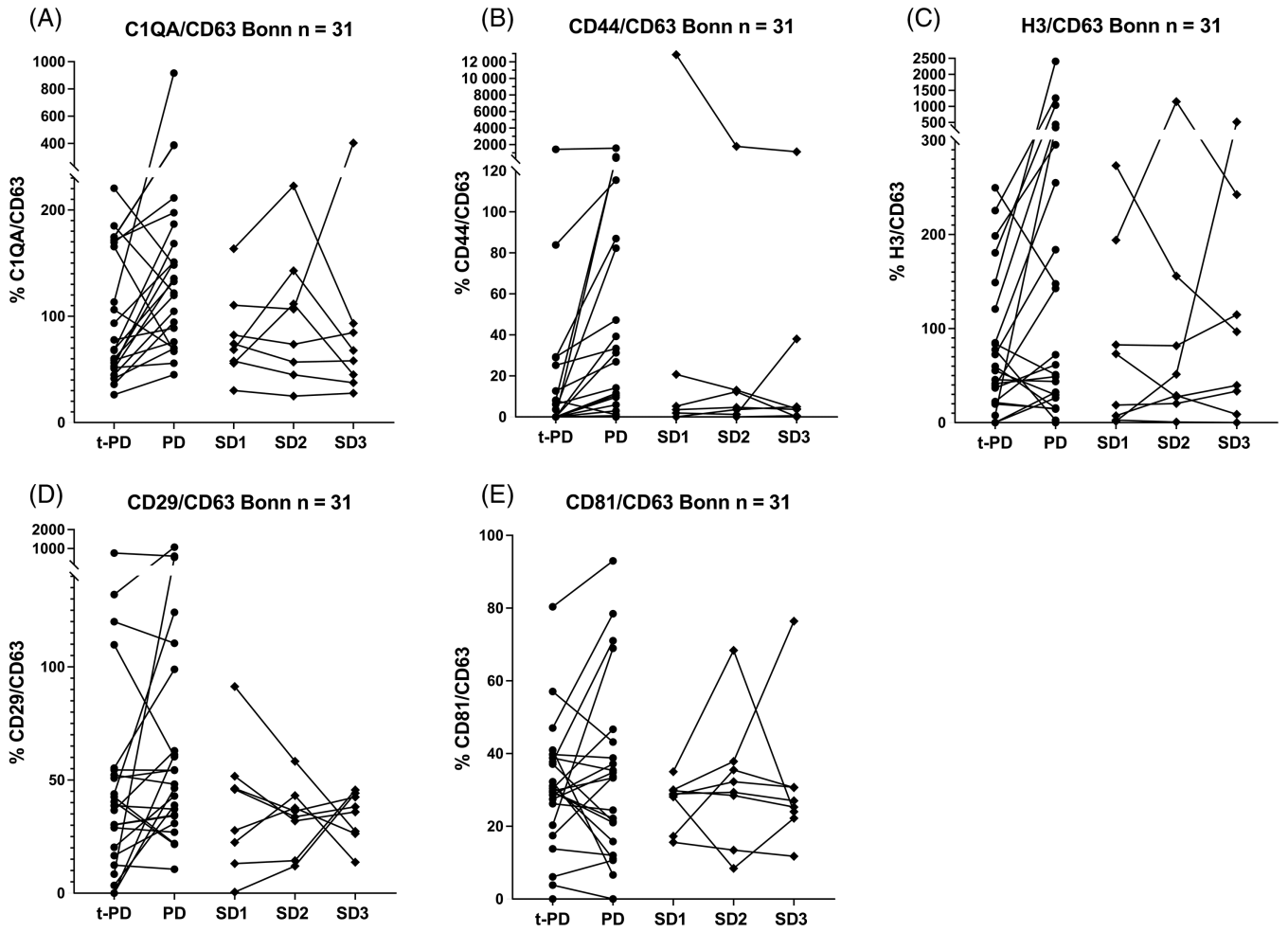

FIGURE 5 Longitudinal bead-assisted flow cytometry data of EVs from serum of glioblastoma patients from the Bonn cohort (n = 31). Depicted are t-PD and PD for 23 patients with tumor progression (PD) and three time-points (SD1, SD2, SD3) for eight patients with stable disease (SD) during the study follow-up. For (A) C1QA, (B) CD44 and (C) H3, levels of these markers increased upon PD ($P = .0083$, $P < .0001$ and $P = .023$, respectively), while no significant change was found in patients with SD (SD1 vs SD2: (A) $P = .74$, (B) $P = .84$ and (C) $P = .84$, SD2 vs SD3: (A) $P = .74$, (B) $P = .58$ and (C) $P = .95$, respectively). For (D) CD29 and (E) CD81, no clear upregulation was detected at PD ($P = .13$ and $P = .73$, respectively)

with uncertain MRI results, but increase in markers and shorter OS) are shown in Figure S8.

3.6 | Multivariable analysis for detection of tumor progression

To assess whether a combination of markers can predict tumor progression using a multivariate logistic regression analysis, we performed a pooled cohort analysis with summary statistics shown in Table S5. Complete information was available for 60 out of 67 (90%) patients. Four patients with missing CD81 information and three patients with missing CD146 information were among patients with PD. The EV markers CD81 (AUC, 0.60), C1QA (0.68), H3 (0.66), CD29 (0.58), CD44 (0.59) were selected, whereas CD146 (0.52) did not fulfill the selection criterion. The multivariable logistic regression model with the selected five markers had an apparent AUC of 0.76. Cross-validation yielded an AUC of 0.66.

4 | DISCUSSION

Reliable diagnosis of progressive disease in glioblastoma is a major challenge. In our study, we found evidence that a panel of proteins on the surface of serum-derived small EVs from glioblastoma patients is useful to detect tumor progression. Based on the findings of two different screening methods (one in vitro and one using patient samples), we analyzed six protein markers (CD81, CD29, CD44, CD146, C1QA and H3) in two independent glioblastoma cohorts. In our data, three out of the six markers (C1QA, CD44 and H3) were significantly upregulated in serum-derived small EVs upon tumor progression, and a combination of CD81, CD29, CD44, C1QA and H3 was suitable to indicate progression in glioblastoma patients.

These markers have previously been suggested to play a role in immune response and oncology. In a recent study, C1QA was shown to be highly concentrated in the perivascular niche of glioblastoma, where it increases invasiveness and induces angiogenesis.[26] While never before described in glioblastoma-EVs, C1QA has been implicated as an immune-stimulatory molecule contained in EVs that are derived from tumor-associated macrophages in a colon carcinoma model.[27] Since the complement system is known to be upregulated whenever a danger signal is present,[28] our data could imply an upregulation of C1QA in small EVs at baseline as a part of the postoperative immune response, which then declines and emerges again at the time of tumor progression.

CD44 has been demonstrated to be associated with invasiveness and proliferation.[29,30] It has also been described as a marker for glioma-initiating cells, interestingly, not always overlapping with CD133.[30,31] In EVs from primary glioblastoma cells bearing stem-cell properties,[23] we observed low levels of CD133, but high levels of CD44, which might indicate a role for CD44 in glioma-propagating cells. In the evaluation of publicly available TCGA data, CD44 was the marker with the highest expression level in primary tissue and even higher levels upon recurrence of the disease, compared to normal tissue (Figure S5b). While the RNA profile of glioblastoma tissue is not necessarily reflective of the EV profile of these cells, small EVs have been shown to carry CD44.[25] Notably, *EGFRvIII*-positive glioma cells secrete EVs with higher CD44 levels and a more invasive phenotype compared to *EGFRvIII*-negative cells.[32] Our CD44 data could thus point to a phenotypic shift in the tumors towards a more invasive subpopulation at the time of progression, which can be detected in bloodstream. While we cannot exclude the possibility that CD44-containing EVs are also released by nontumor cells as a response to tumor growth (eg, T lymphocytes),[33] we did not find any significant changes in the numbers of leukocytes between different time-points in either of our cohorts (data not shown).

Similarly, CD29 has previously been described as a marker for biological aggressiveness in glioblastoma and has also been reported to increase EV-uptake in glioma cells after irradiation.[34] Histones and their associated modifications, which lead to epigenetic remodeling, are hallmarks of cancer, especially in neuro-oncology. In various diseases, extracellular histones have been shown to function as danger signals[35] and to be present in both small and large EVs.[25] To our knowledge, EV-associated H3 and CD29 have not yet been described as biomarkers for high-grade gliomas, but one possible mechanism could include their release upon hypoxic stress and tumor-associated necrosis at disease progression. While CD81 is considered to be a standard EV marker,[36] Ricklefs et al demonstrated that it is upregulated in EVs secreted by glioblastoma cells and that plasma-derived EVs of glioma patients show elevated levels of CD81 compared to healthy donors,[11] which is in line with our findings.

In our study, we used bead-assisted flow cytometry to detect EV markers, which only allows for a bulk analysis of EVs. Ricklefs et al recently showed that $CD63^+/CD81^+$ EVs are much more frequently secreted by glioma cells and can be detected in plasma EVs from glioma patients.[11] Whether our markers define a specific EV population in patient serum or are diffusely distributed across different EV subgroups, could be highly relevant to their use as biomarkers and remains to be assessed in single EV analyses in future studies. However, if our results are confirmed in validation studies, bead-assisted EV analysis will be a feasible method to implement in clinical routine, as it does not require special flow cytometers or expertise.

The potential of EVs for use as liquid biopsy is supported by their known functional role in tumor biology for glioblastoma and other tumors.[37] Interestingly, it has been suggested that the study of serum EVs could be superior to total serum analyses.[14,38] Our findings from total serum sample support this notion, yet the small number of patients studied do not allow us to draw definitive conclusions. More importantly, the EV-separation technique and flow cytometry assay used here are feasible (time-/cost-efficient) for a typical diagnostic laboratory.

The main limitations of our study are the relatively low patient number and the heterogeneity of our patient cohorts, one from a clinical trial with only *MGMT*-methylated patients and the other from a single center, yet also including non-*MGMT*-methylated patients. Nevertheless, with an AUC of 0.76, we were able to establish a good correlation between the EV biomarker panel and the true progression results obtained according to modified RANO criteria. Thus, this is to our knowledge the first report introducing a feasible biomarker panel with novel (C1QA and H3) and known (CD81, CD29 and CD44) EV markers for serum small EVs that correlate with true progression in glioblastoma patients. If confirmed in prospective trials with larger patient numbers, this panel could provide a useful tool for clinicians making treatment decisions, who often face the problem of equivocal MRI findings. In this context, pseudoprogression can possibly lead to unjustified withdrawal of an effective treatment and conversely, if true progression is not reliably detected, the initiation of a recurrent treatment may be unnecessarily delayed. In the CeTeG/NOA-09 cohort, 6/27 patients did not fulfill RANO criteria at the time of suspected progression, but showed an elevation of at least one out of three markers (C1QA, CD44 and histone H3) and had a much lower OS compared to the median OS of the cohort. Thus, if MRI shows equivocal results in the context of glioblastoma progression monitoring, a test for EV markers could be used as an adjunct to distinguish pseudoprogression from true progression. Future studies are warranted to define the cut-off values for a significant increase of these marker levels to confirm progressive disease. At this stage however, this is to our knowledge the first study identifying a panel of serum EV markers able to detect tumor progression in glioblastoma patients even in cases with equivocal MRI results.

Ideally, our liquid biopsy strategy will be part of future improvements to the response criteria for glioblastoma treatment, using an integrated approach of clinical evaluation, MRI assessment and EV-biomarker diagnostics, thereby helping to solve one of the most pertinent problems of glioblastoma therapy.

AUTHOR CONTRIBUTIONS

Theophilos Tzaridis: Study concept; Experiments; Statistical/bioinformatical analyses; Drafted article; Investigation; Writing,

review and editing; Resources. **Johannes Weller:** Statistical/bioinformatical analyses; Writing, review and editing. **Daniel Bachurski:** Experiments; Writing, review and editing; Resources. **Farhad Shakeri:** Statistical/bioinformatical analyses; Writing, review and editing. **Christina Schaub:** Writing, review and editing; Resources. **Peter Hau:** Writing, review and editing; Resources. **Andreas Buness:** Statistical/bioinformatical analyses; Writing, review and editing. **Uwe Schlege:** Writing, review and editing; Resources. **Joachim-Peter Steinbach:** Writing, review and editing; Resources. **Clemens Seide:** Writing, review and editing; Resources. **Roland Goldbrunner:** Writing, review and editing; Resources. **Niklas Schäfer:** Writing, review and editing; Resources. **Robert J. Wechsler-Reya:** Writing, review and editing; Resources. **Michael Hallek:** Writing, review and editing; Resources; Resources. **Björn Scheffler:** Study concept; Writing, review and editing. **Martin Glas:** Study concept; Writing, review and editing. **Lothar Haeberle:** Statistical/bioinformatical analyses; Writing, review and editing. **Ulrich Herrlinger:** Study concept; Drafted article; Investigation; Writing, review and editing. **Christoph Coch:** Study concept; Drafted article; Investigation; Writing, review and editing. **Katrin S. Reiners:** Study concept; Experiments; Statistical/bioinformatical analyses; Drafted article; Investigation; Writing, review and editing. **Gunther Hartmann:** Study concept; Writing, review and editing. All authors approved the final version of this article. The work reported in the article has been performed by the authors, unless clearly specified in the text.

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CONFLICT OF INTEREST

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DATA AVAILABILITY STATEMENT

Handling of the publicly available dataset used in our study is described in the Materials and Methods. Further information and other data that support the findings of our study are available from the corresponding author upon request.

ETHICS STATEMENT

The studies were approved by the local ethics committee of the University of Bonn (Protocol number for Bonn cohort: 182/08; CeTeG/NOA-09 cohort: 093/10; healthy volunteers: 007/17) and were conducted in accordance to the tenets of the Declaration of Helsinki. The registration number of the CeTeG/NOA-09 trial is NCT01149109. Written informed consent was obtained by all donors.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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