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Yamanishi, Shunsuke ; Nagashima, Hiroaki ; Tanaka, Kazuhiro ; Uno, Takiko ; Ikeuchi, Yusuke ; Iwahashi, Hirofumi ; Hashiguchi, Mitsuru ;…

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Title: Association of preoperative seizures with reduced expression of soluble CD163, an M2 macrophage marker, in the cerebrospinal fluid in isocitrate dehydrogenase wild-type glioblastoma

Authors: Shunsuke Yamanishi, M.D.[1], Hiroaki Nagashima, M.D., Ph.D.*[1], Kazuhiro Tanaka, M.D., Ph.D.[1], Takiko Uno[1], Yusuke Ikeuchi, M.D.[1], Hirofumi Iwahashi, M.D., Ph.D. [1], Mitsuru Hashiguchi, M.D., Ph.D. [1], Shintaro Horii[2], Tomoo Itoh, M.D., Ph.D.[3], Yoshihiro Muragaki, M.D., Ph.D.[4], and Takashi Sasayama, M.D., Ph.D.[1]

Affiliations:

1. Department of Neurosurgery, Kobe University Graduate School of Medicine
2. Division of Radiology, Department of Medical Technology, Kobe University Hospital
3. Department of Diagnostic Pathology, Kobe University Graduate School of Medicine
4. Center for Advanced Medical Engineering Research and Development, Kobe University

*Corresponding author: Hiroaki Nagashima
Department of Neurosurgery, Kobe University Graduate School of Medicine,
7-5-2, Kusunoki-cho, Chuo-ku, Kobe, 650-0017 Japan
E-mail: hn0628hn@med.kobe-u.ac.jp
Tel.: +81-78-382-5966, Fax: +81-78-382-5979

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Abstract

Purpose: To investigate the relationship between the tumor microenvironment (TME), tumor-related seizures (TRS), and cerebrospinal fluid (CSF) markers that predict preoperative seizures in patients with glioblastoma.

Methods: In total, 47 patients with isocitrate dehydrogenase (IDH) wild-type glioblastoma who underwent preoperative CSF examination, 3-T magnetic resonance spectroscopy (MRS), and neurological surgery between January 2017 and December 2023 were included. We measured the concentrations of soluble CD163 (sCD163), a soluble form of the M2 macrophage marker, in the CSF, the metabolite concentration on MRS, and the number of CD163-positive M2 macrophages in the tumor tissue. Factors associated with preoperative seizures were examined.

Results: Twelve patients (25.5%) had preoperative seizures. sCD163 levels in the CSF were positively correlated with the number of CD163-positive M2 macrophages in the tumor tissue, and both were significantly lower in the preoperative seizure group than in the non-preoperative seizure group ($p = 0.0124$ and $p < 0.0001$, respectively). MRS indicated that only glutathione (GSH) concentrations were higher in the preoperative seizure group than in the non-preoperative seizure group (2.55 mM and 1.87 mM, respectively; $p = 0.0171$). CD163-positive M2 macrophages were inversely correlated with GSH levels. sCD163 in the CSF had a high predictive accuracy (sensitivity, 91.7%, specificity 54.3%) for preoperative seizures.

Conclusions: The CSF level of sCD163 is useful for predicting the TME and preoperative seizures in IDH wild-type glioblastoma.

Keywords: CD163, isocitrate dehydrogenase wild-type glioblastoma, seizure, M2 macrophages, cerebrospinal fluid

Introduction:

Glioblastoma (GBM), the most malignant form of glioma, has a median survival of <2 years, even with combined modality treatment involving radiation therapy and chemotherapy[1–3]. In particular, patients with isocitrate dehydrogenase (IDH) wild-type GBM have a poor prognosis. Tumor-related seizures (TRS) are common manifestations of glioma. Seizures occur in 49% of patients with GBM, and at least one seizure occurs in up to 80% of patients with high-grade gliomas at some point during the course of the disease[4, 5]. Seizures in patients with GBM significantly reduce their quality of life; however, the etiology of TRS remains unknown.

The mechanism of TRS can be affected by a multifocal process and several factors, including tumor location, histopathology, neuroinflammation, chronic hypoxia, specific chemical factors, and excessive electrical excitation of cerebral neurons secreted by the tumor[6–11]. One possible hypothesis is that high levels of inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, released by activated inflammatory cells, including microglia, astroglia, and macrophages, lead to increases in the concentration of synaptic glutamate (Glu) and seizure onset[12].

Macrophages are myeloid immune cells, which infiltrate the glioma tissues and form the tumor microenvironment (TME)[13–15]. There are two types of macrophages, M1 and M2[16]. M1 macrophages release inflammatory cytokines, such as TNF-α and IL-1β, and exert pro-inflammatory effects[17, 18], while M2 macrophages, whose marker is CD163, have immunoregulatory functions and promote wound healing[15, 19]. Soluble CD163 (sCD163) is a cleaved and soluble form of CD163 that exists in the cerebrospinal fluid (CSF). Cytokine levels in the CSF are associated with macrophage infiltration into

the tumor tissues of glioma patients[20]; however, few studies have investigated the relationships among sCD163 levels in the CSF, TME, and TRS.

We previously reported that metabolite concentrations detected using magnetic resonance spectroscopy (MRS) could predict seizures[21]. Both MRS and CSF examinations are less invasive studies and no additional procedures beyond the usual preoperative examinations are required. Therefore, we believe that preoperative CSF examination and MRS may be useful for predicting the preoperative sizure and TME in patients with GBM. This study aimed to assess the sCD163 concentration in the CSF and metabolite concentrations detected by MRS and their predictive value for preoperative seizures by comparing preoperative and non-preoperative seizure groups of patients with IDH wild-type GBM.

Methods:

Patients and sample collection

This study was approved by the ethics review board of our institution (approval numbers: #B230181 for clinical research, #1497 for MRS, and #1579 for the use of glioma samples). Informed consent was obtained from all the patients.

Among the patients with GBM diagnosed and treated at the Department of Neurosurgery, Kobe University Hospital, between January 2017 and December 2023, 47 were enrolled in this retrospective study. All patients were newly diagnosed histologically as having GBM, IDH wild-type, and WHO grade 4. The following inclusion criteria were applied: 1) supratentorial GBM; 2) preoperative CSF collection; 3) preoperative magnetic resonance imaging (MRI) and MRS; and 4) previous surgical removal or biopsy. We used MRS as a non-invasive method for quantifying metabolite concentrations[21–23].

Data collection

We retrospectively reviewed the patient's medical records and radiological images. Patient characteristics included age at admission, sex, main location (frontal, temporal, parietal, occipital, and others), and metabolite concentrations detected by MRS. Patient charts were reviewed for the presence of any kind of seizure, such as focal awareness, focal impaired awareness, or focal to bilateral tonic-clonic seizures. Only clinical seizures with documented preoperative clinical presentations were included; electrographic seizures were excluded. Antiseizure medications (ASMs) were not routinely administered before surgery. Corticosteroids were administered after CSF sampling if necessary.

Measurement of sCD163

For the preoperative diagnosis, 2–6 ml of CSF was collected via lumbar puncture within 1 week before surgery. Lumbar puncture was not performed in patients with suspected elevated intracranial pressure. The CSF samples were immediately centrifuged and stored at −80°C until the time of analysis to prevent protein degradation. None of the samples was thawed prior to further analysis. A Human CD163 Quantikine ELISA Kit (R&D Systems, Inc. Minneapolis, MN, USA) was used to measure the concentration of sCD163. The assay was performed according to the manufacturer's instructions.

Immunohistochemistry

Anti-CD163 antibody (rabbit antibody, ab182422) was purchased from Abcam (Cambridge, UK). Anti-CD68 antibody (mouse antibody, M0814) was purchased from Dako (Glostrup, Denmark). Serial sections were deparaffinized, immersed in methanol

with 3% hydrogen peroxide, and heated in 1 mM Tris-EDTA (pH 8.0) or 0.01 M citrate (pH 6.0) buffer for 15 min by autoclaving at 121°C under 2 atm of pressure. The sections were subsequently incubated with primary antibodies at 4°C overnight. The sections were allowed to react with either a peroxidase-conjugated anti-rabbit or anti-mouse IgG polyclonal antibody (Histofine Simple Stain MAX-PO; Nichirei, Tokyo, Japan) for 60 min, after which the reaction products were visualized by immersing the sections in 0.03% diaminobenzidine solution containing 2 mM hydrogen peroxide for 5 min.

Assessment of staining

Immunohistochemical staining for CD163 and CD68 was evaluated and scored by two neuro-oncologists, K.T. and Y.I., both of whom were blinded to the clinical data and patient outcomes. The mean percentage of the immunoreactive area (% area) was calculated from three randomly selected fields per section under ×200 middle-power magnification. The final percentage area was calculated by averaging the mean values of each observer. Images were analyzed using ImageJ software ver. 1.48 (National Institutes of Health, Bethesda, MD, USA). Only areas containing the tumor were analyzed; areas without tumor cells, including those with necrosis, fibrosis, and blood vessels, were excluded from the analysis. The number of positive pixels was divided by the total number of pixels (negative + positive) in the analyzed area and multiplied by 100 to calculate the percentage of positive pixels.

MRI and MRS protocols

The MRS signal was acquired with a 3-T MRI/^{1}H-MRS scanner (Achieva; Philips Medical Systems, Best, Netherlands) An 8-channel head MRI coil was used for signal

reception and a quadrature body MRI coil was used for transmission of the radiofrequency pulses. Following routine preoperative MRI, FLAIR, and T2* images were obtained to localize the corresponding targets. Single-voxel localized magnetic resonance spectra were acquired using a double-echo point-resolved spectroscopic sequence (PRESS) with chemical-shift-selective water suppression. The MRS acquisition parameters were as follows: volume of interest (VOI), 1.5 × 1.5 × 1.5 cm3; repetition time/echo time = 2000/35 ms; number of acquisitions, 128 averages, and 1,024 complex points for the spectral data. The VOIs were localized in representative areas of solid tumors. Regions with necrosis, hemorrhage, and peripheral edema were excluded from the corresponding region. An unsuppressed water signal was obtained using these parameters. The full width at half maximum (FWHM) and signal-to-noise ratio of the magnetic resonance spectra were assessed. MRS data were quantified with LC-Model version 6.3-1R (Stephen Provencher, Oakville, Ontario, Canada) using a basis set developed with magnetic resonance experiment simulation software (GAMMA, Radiology, Duke University Medical Center, Durham, NC), and calibrated with an MRS phantom. Metabolites were quantified using Cramer–Rao lower bounds (CRLBs). The CRLB values were calculated from the residual error and Fisher matrix of the partial derivatives of the concentrations[24]. These CRLB values reflect the variance of the spectral fitting estimate but not its accuracy and indicate how close repeated measurements are to the current measurement under similar experimental conditions[22].

Statistical analysis

Between-group comparisons were performed using the Mann–Whitney *U* test and unpaired *t*-test, whereas the correlation between the groups was assessed using

Spearman's rank test. Kaplan–Meier survival analysis and log-rank test were used to consider the relationship between preoperative seizures and prognosis. The cutoff values for the % area of tCD163 and tCD68 were set as the mean values of the counts. To determine the optimal cutoff value of the CD163 concentration for providing the highest combination of sensitivity and specificity for differentiating between patients with and without seizures, we conducted receiver operating characteristic (ROC) curve analysis. Statistical significance was set at $p < 0.05$. Statistical analyses were performed using the GraphPad Prism 9 software (GraphPad Software, La Jolla, CA, USA).

Results:

Patient characteristics

The patient characteristics are shown in Table 1. Forty-seven patients met the eligibility criteria. The mean age was 64.9 (range, 38–86) years, and 25 patients (53.2%) were male. Of the 47 patients, 12 (25.5%) had a history of at least one epileptic seizure before surgery. The mean age was lower in patients with preoperative seizures than in those without seizures (58 and 67 years, respectively; $p = 0.0273$). There were no significant sex differences between patients with (M:F = 7:5) and without (M:F = 18:17) preoperative seizures. Furthermore, there were no significant differences in the tumor locations ($p = 0.997$). Preoperative seizures were not associated with overall survival ($p = 0.452$).

A reduced concentration of CD163 is associated with preoperative seizure

We examined the concentration of sCD163 in the CSF using a Human CD163 Quantikine ELISA Kit. The median sCD163 concentration was significantly lower in patients with preoperative seizures than in those without preoperative seizures (44.15

ng/ml and 78.4 ng/ml, respectively; p = 0.0124; Table 1, Fig. 1A). We then measured the amount of CD163 in the tissues of GBM patients (tCD163). The median % area of tCD163 was significantly lower in patients with preoperative seizures than in those without preoperative seizures (4.04% and 10.56%, respectively; p = 0.0171; Table 1, Fig. 1B). Moreover, there was a strong correlation between sCD163 and the % area of tCD163 (p <0.0001; Fig. 1C).

Next, we examined the levels of CD68, a pan-macrophage marker, in tissues of patients with GBM (tCD68). Spearman's rank test showed a significant correlation between sCD163 levels in the CSF and the percentage of CD163-positive M2 macrophages (p <0.0001; Fig. 2A). The median percentage of CD163-positive M2 macrophages was lower in patients with preoperative seizures than in those without preoperative seizures (39.8% and 92.7%, respectively; p <0.0001; Fig. 2B), whereas there was no significant difference in the percent of tCD68 (10.8% and 12.2%, respectively; p = 0.507; Fig. 2C). These results indicated that sCD163 levels in the CSF of patients with GBM were correlated with the number of M2 macrophages in the tissue and negatively correlated with preoperative seizures.

The GSH concentration was inversely correlated with M2 macrophage levels

To evaluate the relationship between the TME and intratumoral metabolites, we used MRS to measure the concentrations of Glu, glutathione (GSH), glutamine (Gln), and gamma-amino butyric acid (GABA), all of which had been reported to have a relationship with the occurrence of seizures (Table 1)[21, 25–27]. Only the concentration of GSH had a significant difference between the patients with preoperative seizures and those without preoperative seizures (2.55 mM and 1.87 mM, respectively; p = 0.0171; Table 1, Fig. 3A).

GSH induces macrophage activation and M1 polarization. Interestingly, the GSH concentration was inversely correlated with the sCD163 concentration and the percentage of CD163-positive M2 macrophages ($p = 0.00176$ and $p < 0.001$, respectively; Fig. 3B and C). These results suggest that not M1 but M2 polarization suppresses preoperative seizures.

Clinical application of predicting preoperative seizures using MRS assessment of GSH and ELISA levels of sCD163

Next, we investigated whether GSH levels in the tumor and sCD163 levels in the CSF could be used to predict preoperative seizures using the ROC curve method. ROC curve analysis demonstrated that GSH concentrations >2.0 mM were 83% sensitive and 65.7% specific for the prediction of postoperative seizures (area under the curve (AUC) = 0.731; 95% confidence interval (CI): 0.587–0.875; Fig. 4A). sCD163 concentrations <74.0 ng/ml was 91.7% sensitive and 54.3% specific for the prediction of postoperative seizures (AUC = 0.745; 95% CI: 0.601–0.89; Fig. 4B).

Discussion:

This study demonstrated that the concentration of sCD163, a soluble form of an M2 macrophage surface marker, correlated with the amount of tCD163, suggesting that sCD163 in the CSF reflects M2 macrophage infiltration in tumor tissues. Moreover, both the sCD163 and tCD163 levels were significantly lower in patients with preoperative seizures than in those without seizures. We calculated the percentage of CD163-positive M2 macrophages to predict the M1/M2 phenotype balance and TME. Interestingly, a lower percentage of CD163-positive M2 macrophages predicted preoperative seizures

and had an inverse correlation with the GSH concentration assessed by MRS, suggesting that polarization to M2 macrophages reduces M1 macrophages and preoperative seizures.

Multiple mechanisms contribute to seizure onset. A major pathological mechanisim involves Glu-related signaling. Downregulation of the Glu transporter leads to an increase in the extracellular Glu levels[28, 29], resulting in seizure activities in gliomas. Glu release also occurs via the xCT system, a cystine-glutamate exchanger that releases Glu in exchange for cystine and is imported for the synthesis of the cellular antioxidant GSH[30]. High xCT expression is an independent marker of glioma-associated seizures at diagnosis, perticularly in high-grade glioma[31]. In our study, the concentration of intratumoral Glu on MRS was not associated with preoperative seizures, whereas the GSH concentration was higher in patients with preoperative seizures. Gonen et al. reported that GSH concentration was significantly higher in patients with idiopathic generalized epilepsy by using 7-T MRS[26]. These results suggest that cystine import for GSH synthesis and Glu export to the extracellular space via the xCT may be associated with preoperative seizures.

GSH upregulates pro-inflammatory cytokines, stimulates the production of immune mediators, including nitric oxide and PGE_2, and promotes M1-like macrophage polarization[32]. M1 macrophages secrete inflammatory cytokines, such as TNF-α and IL-1β, and are associated with the pathogenesis of seizures[11, 17, 18]. On the other hand, M2 macrophages have immunoregulatory functions and mitigate inflammatory responses[15, 19]. The TME determines the mutual conversion between M1 and M2 macrophages, and in many types of tumors, the majority of tumor-associated macrophages are M2 macrophages[15]. M2 macrophages are known to express CD163 on their surface. In the present study, we found that a lower sCD163 concentration in the

CSF and a lower percentage of CD163-positive M2 macrophages were inversely correlated with GSH concentration detected by MRS, and were associated with higher seizure frequency, supporting previous results.

The ability to predict seizure development in a clinical setting using CSF and MRS has two main benefits. First, predicting seizure development can help to decide whether to initiate or discontinue preoperative ASM administration. Seizures significantly reduce the quality of life of patients and adjusting ASM administration may lead to control of seizure frequency and side effects of ASMs. Moreover, the relationship between sCD163 in the CSF and preoperative seizure revealed in this study may be useful in elucidating the mechanism of TRS, and in developing new ASMs. Second, conventional 3-T MRS is a noninvasive technique that evaluates metabolite concentrations in the brain and can be incorporated into routine pretreatment examinations. CSF can be obtained less invasively and is often used to differentiate primary central nervous system lymphoma from glioma, especially when the lesion is ring-enhanced[33–35]. In addition, routine CSF parameters are often examined in patients with first or newly diagnosed seizure[36]. No additional procedures beyond the usual preoperative examinations were required to predict seizure onset. Although lumber puncture for CSF collection is somewhat invasive, small amounts were collected only in cases without brain herniation or elevated intracranial pressure, and no adverse events due to lumber puncture were observed in our study.

To the best of our knowledge, this is the first study to show that low concentrations of sCD163 in the CSF are associated with the incidence of seizures in IDH wild-type GBM; however, some limitations should be mentioned. First, this was a retrospective study performed at a single institution, and the sample size was small. Second, we did not evaluate the actual metabolite concentrations in tumor tissue. Direct metabolite analysis

should be performed to confirm the actual concentrations of GSH, Glu, Gln, and GABA in tissues. Moreover, we did not test the number of M1 macrophages in the tissue or the concentration of pro-inflammatory mediators in the CSF. Third, metabolite heterogeneity in tumors was excluded. The VOI was manually set according to the MR image and thus may have been biased by the examiner. Fourth, we did not consider the effects of corticosteroids administration. Corticosteroids promote the repolarization of M1 to M2 macrophages[17, 37]. However, we administered corticosteroids to the patients after CSF collection if necessary. Corticosteroids might affect to the percentage of CD163-positive M2 macrophages, but not to the concentration of sCD163 in the CSF. Fifth, we did not examine the relationship between the concentration of sCD163 and postoperative seizures. We need further study to decide whether to initiate or discontinue postoperative ASM administration. Finaly, the data were merely snapshots of metabolites or the TME at a single time point; thus, basic experiments and a larger cohort are required to confirm our findings.

Conclusions:

This study demonstrated that a lower sCD163 concentration in the CSF is associated with preoperative seizures in patients with IDH wild-type GBM. Moreover, the sCD163 concentration was correlated with the percentage of CD163-positive M2 macrophages in the tumor tissue and inversely correlated with the GSH concentration detected using 3-T MRS. These results indicate that investigating sCD163 in the CSF is useful for predicting the TME, and polarization to M2 macrophages reduces the levels of M1 macrophages in tumor tissue and subsequent preoperative seizures.

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Author Contributions: All authors contributed to the study conception and design. Conceptualization: S.Y., H.N. and T.S.; Data curation: S.Y., H.N., K.T., H.I., M.H. and S.H.; Formal analysis: S.Y. and H.N.; Funding acquisition: H.N., K.T., Y.M. and T.S.; Investigation: S.Y., H.N., T.U., Y.I. and T.I.; Methodology: S.Y., H.N. and K.T.; Resources: H.N., K.T., T.I. and T.S.; Supervision: H.N., K.T. and T.S.; Visualization: S.Y. and H.N.; Writing-original draft: S.Y.; Writing-review and editing: S.Y., H.N. and T.S. All authors read and approved the final manuscript.

Data Availability: The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval: This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the ethics review board of Kobe University (B230181).

Consent to participate: Informed consent was obtained from all individual participants included in the study.

Figure Legends:

Figure 1. Relationship between the M2 macrophage marker CD163 and preoperative seizures.

(A) sCD163 concentrations in the CSF of patients with and without preoperative seizures.

(B) The % area of CD163 in the tumor tissue of patients with and without preoperative seizures.

(C) Correlation between sCD163 in the CSF and the % area of CD163 in the tumor tissue.

P-values were calculated using the Mann–Whitney nonparametric *U*-test and Spearman rank test. sCD163, soluble CD163; CSF, cerebrospinal fluid.

Figure 2. Relationship between CD163-positive M2 macrophages and preoperative seizures.

(A) Correlation between sCD163 in the CSF and the % of CD163-positive M2 macrophages.

(B) The % of CD163-positive M2 macrophages of patients with and without preoperative seizures.

(C) The % of CD68, a pan-macrophage marker, was determined in the tumor tissues of patients with and without preoperative seizures.

P-values were calculated using the Mann–Whitney nonparametric *U*-test and Spearman rank test. sCD163, soluble CD163; CSF, cerebrospinal fluid.

Figure 3. Relationship between GSH detected by MRS and preoperative seizures.

(A) GSH concentrations in patients with and without preoperative seizures.

(B) Correlation between GSH and sCD163 in the CSF.

(C) Correlation between GSH and the % of CD163-positive M2 macrophages.

P-values were calculated using the Mann–Whitney nonparametric *U*-test and Spearman rank test. GSH, glutathione; MRS, magnetic resonance spectroscopy; sCD163, soluble CD163; CSF, cerebrospinal fluid.

Figure 4. Predictive value of GSH and sCD163 for preoperative seizures.

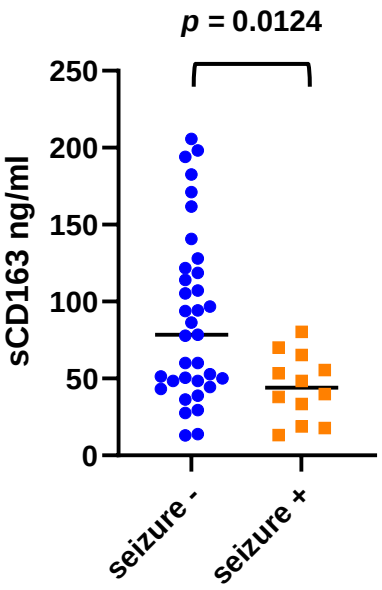
(A) ROC curve of GSH in differentiating preoperative from non-preoperative seizures in IDH wild-type glioblastomas.

(B) ROC curve of sCD163 for differentiating preoperative seizures from non-preoperative seizures in IDH wild-type glioblastomas.

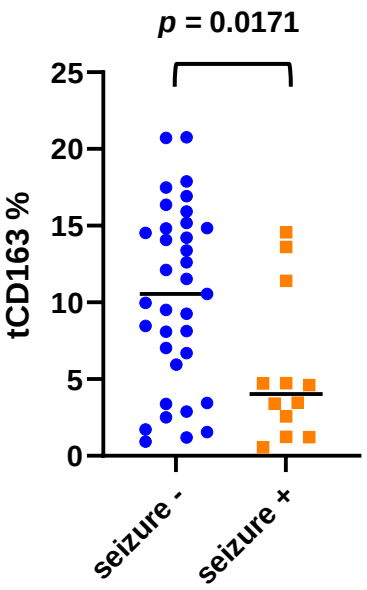
ROC, receiver operating characteristic; GSH, glutathione; sCD163, soluble CD163.

Figure 1

A


p = 0.0124
sCD163 ng/ml
250
200
150
100
50
0
seizure -
seizure +

B


p = 0.0171
tCD163 %
25
20
15
10
5
0
seizure -
seizure +

C

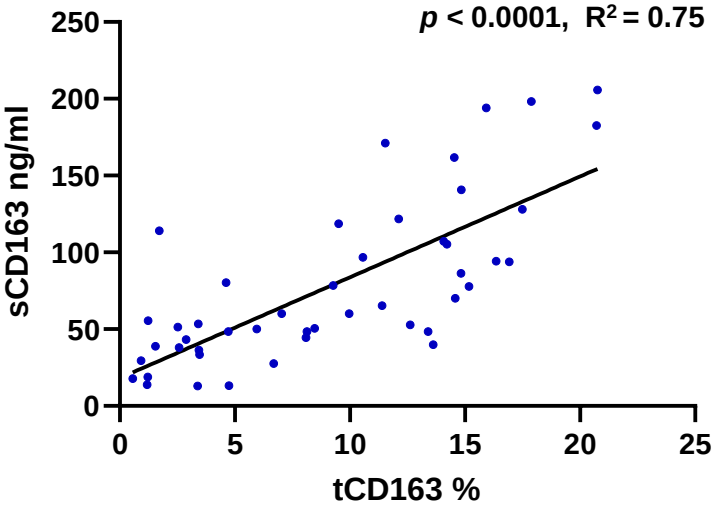
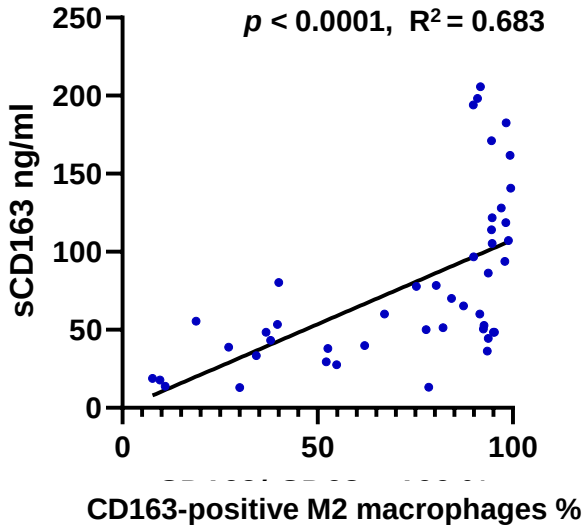
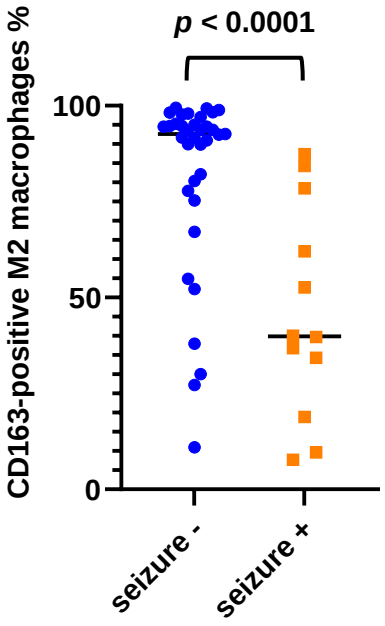

p < 0.0001, R2 = 0.75
sCD163 ng/ml
250
200
150
100
50
0
0
5
10
15
20
25
tCD163 %

Figure 2

A


p < 0.0001, R² = 0.683
sCD163 ng/ml
250
200
150
100
50
0
0
50
100
CD163-positive M2 macrophages %

B


p < 0.0001
CD163-positive M2 macrophages %
100
50
0
seizure -
seizure +

C

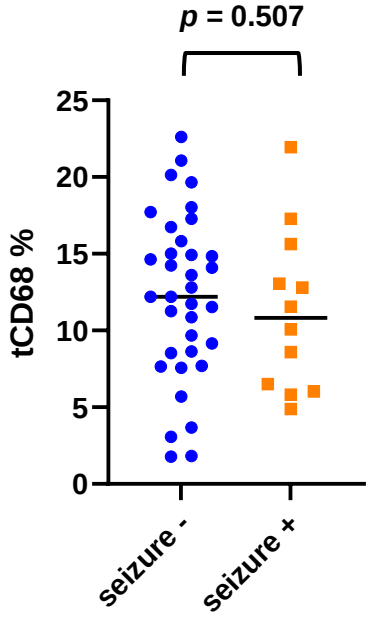
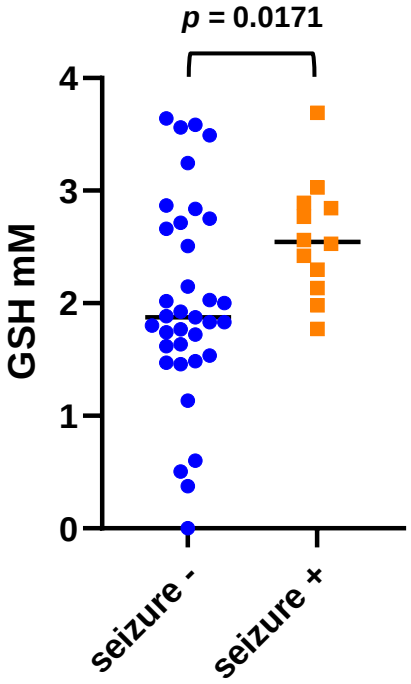
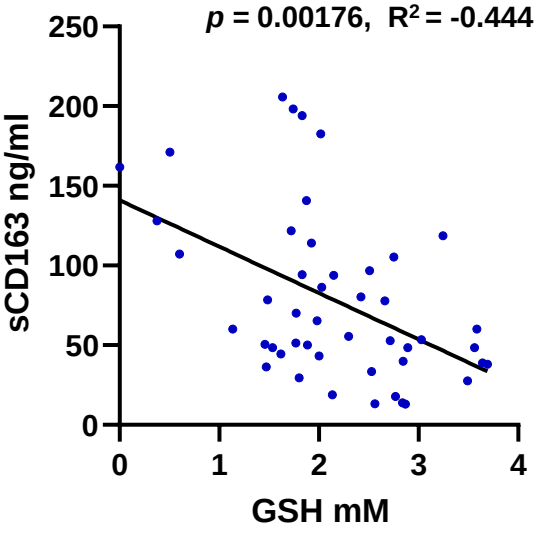

p = 0.507
tCD68 %
25
20
15
10
5
0
seizure -
seizure +

Figure 3

A


p = 0.0171
GSH mM
4
3
2
1
0
seizure -
seizure +

B


p = 0.00176, R2 = -0.444
sCD163 ng/ml
250
200
150
100
50
0
0
1
2
3
4
GSH mM

C

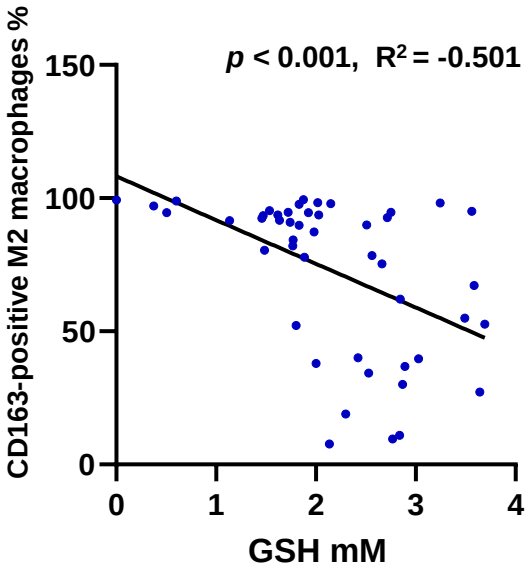
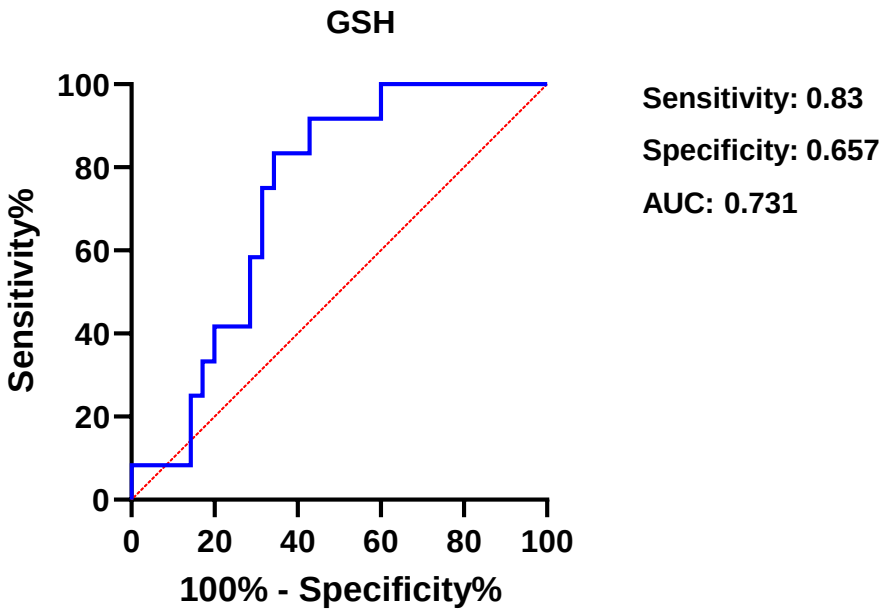

p < 0.001, R2 = -0.501
CD163-positive M2 macrophages %
150
100
50
0
0
1
2
3
4
GSH mM

Figure 4

A


GSH
Sensitivity%
100% - Specificity%
0
20
40
60
80
100
Sensitivity: 0.83
Specificity: 0.657
AUC: 0.731

B

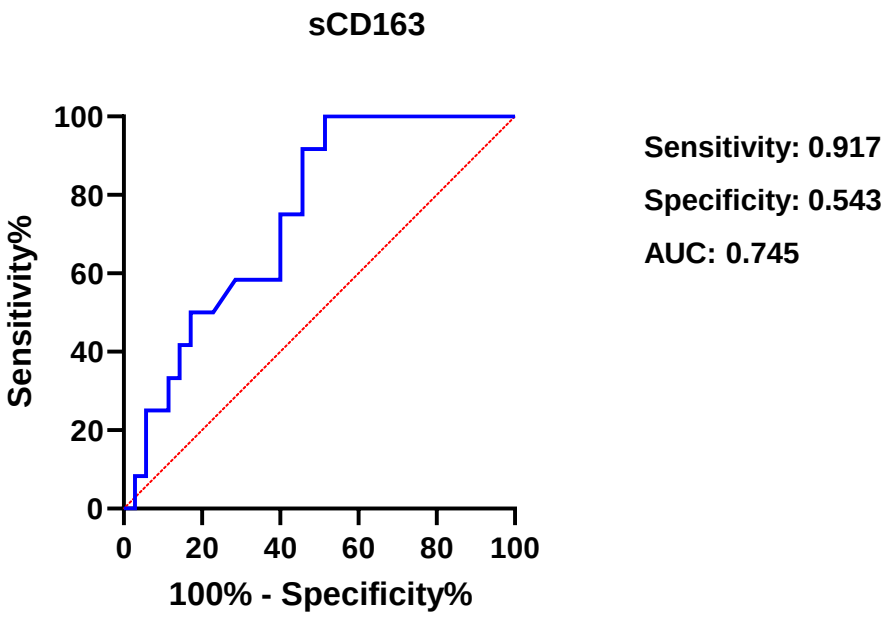

sCD163
Sensitivity%
100% - Specificity%
0
20
40
60
80
100
Sensitivity: 0.917
Specificity: 0.543
AUC: 0.745

Table 1. Patient characteristics with and without preoperative seizure.

Variate	preoperative seizure - (n= 35)	preoperative seizure + (n= 12)	p Value
Age, year	67 (47-86)	58 (38-77)	**0.0273**
Sex			
Male	18	7	0.687
Female	17	5	
Tumor location			
Frontal	11	3	0.997
Temporal	10	6	
Parietal	8	3	
Occipital	1	0	
Others	5	0	
Concentration of sCD163, ng/ml	78.4	44.15	**0.0124**
Immunohistochemistry, %			
tCD163	10.56	4.04	**0.0171**
tCD68	12.2	10.8	0.507
Metabolite concentration, mM			
Glu	3.32	4.95	0.079
GSH	1.87	2.55	**0.0171**
Gln	4.54	5.35	0.116
GABA	0.213	0.17	0.76

P-values were calculated using the Mann–Whitney nonparametric *U*-test. sCD163, soluble CD163; tCD163, the amount of CD163 in the tissues; tCD68, the amount of CD68 in the tissues; Glu, glutamate; glutathione; Gln, glutamine; GABA, gamma-amino butyric acid.