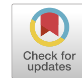
Inhibition of endogenous blood glutamate oxaloacetate transaminase enhances the ischemic damage



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Glutamate oxaloacetate transaminase 1 (GOT1) enzyme plays a critical role in the cell metabolism by participating in the carbohydrate and amino acid metabolism. In ischemic stroke, we have demonstrated that recombinant GOT1 acts as a novel neuroprotective treatment against the excess of extracellular glutamate that accumulates in the brain following ischemic stroke. In this study, we investigated the inhibitory effect of GOT1 on brain metabolism and on the ischemic damage in a rat model of ischemic stroke by means of a specific antibody developed against this enzyme. Inhibition of GOT1 caused higher brain glutamate and lactate levels and this response was associated with larger ischemic lesion. This study represents the first demonstration that the inhibition of the blood GOT1 activity leads to more severe ischemic damage and poorer outcome and supports the protective role of GOT1 against ischemic insults. (Translational Research 2021; 230:68–81)

Abbreviations: AbGOT1 = Antibody against GOT1; AST = aspartate aminotransferase; ADC = Apparent diffusion coefficient; CK = Creatine kinase; CM = Cistern magna; CSF = Cerebrospinal fluid; FOV = Field of view; GGT = Gamma-glutamyltransferase; GOT1 = Glutamate oxaloacetate transaminase 1; GPT = Glutamate pyruvate transaminase; HRP = Horse radish peroxidase; HCG = Human Chorionic Gonadotropin; MCAO = Middle Cerebral Artery occlusion; MRA = Magnetic resonance angiography; MRI = Magnetic resonance imaging; MRS = Magnetic resonance spectroscopy; NMDA = N-methyl-D-aspartate; STAIR = Stroke Therapy Academic Industry Roundtable

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AT A GLANS COMMENTARY

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Background

Glutamate oxaloacetate transaminase 1 (GOT1) enzyme plays a critical role in the cell metabolism. In this study we produced and purified a polyclonal antibody against GOT1 to block the metabolic activity of this enzyme and to evaluate its repercussions on the energetically compromised brain ischemic tissue in an animal model of ischemic stroke.

Translational Significance

This study supports the protective role of the recombinant form of GOT1 against ischemic insults.

INTRODUCTION

The cytoplasmatic form of the enzyme glutamate-oxaloacetate transaminase 1 (GOT1) is involved in the reversible transamination of oxaloacetate and glutamate to aspartate and α-ketoglutarate, which is an essential pathway in the carbohydrate and amino acid cell metabolism.[1] Indeed, under normal physiological circumstances, this pathway maintains the energy homeostasis and coordinates catabolic and anabolic metabolic programs.[1]

In the field of ischemic stroke, based on the capability of GOT1 to metabolize glutamate and use it as an energy substrate in anaerobic conditions as ischemia, the recombinant form of GOT1 (rGOT1) has been suggested as a novel protective drug for the acute phase of stroke.[2] In stroke it has been well established that there is a large increase of glutamate concentration in the extracellular space of the brain which leads to a cellular overload of calcium, mainly through its action on calcium-permeable N-methyl-D-aspartate receptors.[3] In addition, glutamate excess causes necrosis and breakdown of cellular structures, including membrane phospholipids, proteins and DNA, via an excitotoxic mechanism. Thus, we have previously demonstrated that the intravenous (i.v.) administration of the human rGOT1, alone or in combination with oxaloacetate, in a rat model of cerebral ischemia, decreases both the glutamate concentration in blood and brain and induces a reduction of the ischemic lesion after stroke.[4] These effects were also associated with a significant reduction in the sensorimotor deficits. To confirm that the protective effect was mediated by a decrease in the brain glutamate levels, we performed magnetic resonance spectroscopy (MRS) in the infarct region. Spectroscopic analysis revealed that the increase in brain glutamate seen in control animals after middle cerebral artery occlusion (MCAO) was significantly reduced in rGOT1-treated animals. These experimental findings were also in line with our previous retrospective study with more than 400 ischemic patients, where it was described that endogenous blood activity levels of GOT1, higher than 17 U/L at hospital admission, were associated with lower blood glutamate concentration and good outcome at 3 months after stroke.[5,6]

Other authors have also described that, besides the role as blood glutamate scavenger, GOT enables an abridged tricarboxylic acid cycle under conditions of stroke-induced hypoglycemia such that otherwise excitotoxic glutamate undergoes a role reversal to become a source of energy and survival factor.[7] However; the question how the inhibition of GOT1 affects the brain metabolism and the ischemic damage has not been tested far. To address this question, in the present study we have developed a specific antibody against GOT1 (AbGOT1) and its effects were tested on brain glutamate and lactate levels and on cerebral damage in ischemic rats induced by transient MCAO.

MATERIAL AND METHODS

Animals. Experimental protocols were approved by the University Clinical Hospital of Santiago de Compostela Animal Care Committee, according to the European Union (EU) rules (86/609/CEE, 2003/65/CE and 2010/63/EU) and with the ARRIVE guidelines. Male Sprague-Dawley rats (7−8 weeks age) with a weight of 250−300 g were used. Rats were watered and fed *ad libitum*. Surgical procedures and magnetic resonance analysis were performed under anesthetic conditions induced by inhalation of 5% sevoflurane in a nitrous oxide/oxygen mixture (70/30). Rectal temperature was maintained at 37°C + 0.5°C by using a feedback-controlled heating pad (Neos Biotec, Pamplona, Spain). Glucose levels were analyzed before surgery (ranging from 180 to 220 mg/dl). A total of 103 animals were used in the study. Total animals excluded and included are summarized in the Fig **S1**.

Synthesis and production of anti-rGOT1 antibody. Human GOT1 cDNA was cloned from the human hepatoma cell line hepG2, and the purified human identical recombinant enzyme (rGOT1) was expressed in

Escherichia coli cells as previously described[4,8] and was used as the antigen for immunization. Two albino rabbits were injected intramuscular (i.m.) with rGOT1 (50 μg in complete Freund's adjuvant) and boosted 3 times at an interval of 3 weeks. Test bleedings were performed 3 weeks after each inoculation and the titer of the blood antibodies against rGOT1 were tested by ELISA in plastic plates coated with rGOT1 (5 μg/mL) and determined with a secondary goat-anti Rabbit antibody, (Jackson Immunoresearch Labs, West Grove, PA) conjugated to horse radish peroxidase diluted 1:50000. Color development was with Tetra Methyl Benzidine substrate (Medicago, Quebec, CA) and determined in an ELISA reader. Immune serums with the highest anti-GOT1 titer were purified on a Hi-trap Protein A HP 5 ml column (GE Healthcare 17-0403-0, Chicago, IL) using the binding buffer and diluent, as recommended by the manufacturer. Yields of the Protein A purified anti-rGOT1 antibodies were approximately 10 mg/mL serum and the purified AbGOT1 at a dilution of 1:1.000 (17 μg/mL) neutralized >95% of the activity of human rGOT1 (1.3 ng/mL). The purified AbGOT1 also reacted on Western blots with rGOT1 at high dilution (1:5000).

In vitro dose-response analysis of anti-GOT1 antibody. The blocking efficacy of the rabbit anti human-GOT1 antibody (AbGOT1) on GOT1 enzyme activity was tested on blood samples obtained from the tail vein of healthy rats (500 μL). Different amounts of AbGOT1 (0, 0.5, 2, 5, and 10 μg) were added to serum samples to final volume of 50 μL (20 μL of a blood and 30 μL AbGOT1 diluted in PBS). The dilutions were incubated at 37°C during 30 minutes and the GOT1 enzymatic activity was determined by means of Reflotron GOT specific activity tests following the manufacturer's technical specifications (Roche, Basel, Switzerland). To validate the specificity of the AbGOT1 on GOT1 activity, an isotype form of the antibody (Rabbit IgG Isotype Control, Thermo Fisher Scientific; Catalog Number. 105009, Waltham, Massachusetts) were tested on the enzyme activity.

Inhibition of blood GOT1 activity by AbGOT1 in healthy animals. After the *in vitro* analysis, different doses of AbGOT1 were tested on the endogenous blood GOT1 activity in healthy rats (250−300 g). Control animals were administered intraperitoneal (i.p.) with 1 mL of PBS (drug vehicle), and AbGOT1 treated animals with 1 mL (i.p.) of different doses of antibody (1, 2.5, 4, and 5 mg/rat, $n = 3$/group). Blood samples (200 μL) were obtained from tail vein, under basal conditions (before the injection) and 1, 2, 3, 4, 5, 6, and 8 hours and 1, 2, 3, 4, 6, 8, 10, and 14 days after injection. Blood GOT activity was determined in all samples.

AbGOT1 effect in ischemic rat model animal. To evaluate the effect of blood GOT blocking on ischemia, the AbGOT1 dose previously selected from the dose-response study in healthy animals was tested in ischemic rats. In this study, 2 experimental groups were studied, one group treated (i.p.) with PBS (drug vehicle) and a second group treated (i.p.) with 1 mL of AbGOT1 (5 mg/rat; $n = 10$/each group). PBS or AbGOT1 were injected 1 hour before ischemic surgery. Blood samples (500 μL) for GOT, glutamate-pyruvate transaminase (GPT) and glutamate analysis were collected under basal conditions (before the administrations) and 1 hour after the administrations of PBS and AbGOT1 (but before ischemia). Blood samples were also collected after MCA reperfusion (75 minutes) and 2, 4, and 6 hours and 1, 2, 4, 7, and 14 days after ischemia onset.

Cerebral infarct lesion was determined by means of *in vivo* Magnetic Resonance Imaging (MRI) during arterial occlusion defined as (T_0) and 1, 7, and 14 days after ischemia. Functional assessments were performed under healthy conditions (1 day before surgery) and 7 and 14 days after ischemia.

In addition, in an independent group of experimental animals, brain glutamate and lactate levels were determined in control and AbGOT1-treated animals ($n = 3$ per group) before ischemia, during occlusion (75 minutes) and 120 minutes after reperfusion, in both contralateral and ipsilateral hemispheres, using the non-invasive MRS technique.

Surgical procedures. Transient focal ischemia was induced in rats by using the transient MCAO model following the surgical procedures previously described.[4,9,10] In brief, under an operating microscope, MCAO was performed by exposing the left common, external and internal carotid arteries through a midline neck incision. The left external carotid artery and pterygopalatine artery of the internal carotid artery were separated and ligated by 6-0 silk sutures. A silicon rubber-coated monofilament (403512PK5Re; Doccol Corporation, Sharon, Massachusetts) was inserted through the external carotid into the left common carotid artery and advanced into the internal carotid artery to 20 mm from the bifurcation to occlude the origin of the MCA. A laser-Doppler flow probe (tip diameter 1 mm) attached to a flowmeter (PeriFlux 5000, Perimed AB, Stockholm, Sweden) was located over the thinned skull in the MCA territory (4 mm lateral to the bregma) to obtain a continuous measure of relative cerebral flow during the arterial occlusion. Once the artery occlusion had been achieved, as indicated by Doppler signal reduction, each animal was carefully moved from the surgical bench to the MRI system for ischemic lesion assessment using apparent diffusion coefficient (ADC)

maps (defined as T_0 ischemic lesion). MR angiography (MRA) was also performed to ensure that the artery remained occluded throughout the MR procedure. After MR analysis, animals were returned to the surgical bench and the Doppler probe was repositioned. The suture was removed after 75 minutes of occlusion. After suture removal, the left pterygopalatine artery was reperfused while left external carotid (used to introduce the suture) remained tied to avoid bleeding. As we have described in elsewhere,[10] this surgical ischemic protocol, based on the combination of laser Doppler monitoring in combination with ADC and MRA represents a reliable inclusion protocol during ischemic surgery to reduce variability intergroups and to guarantee the reproducible of the infarct volumes.

The following exclusion criteria were used: (1) less than 70% reduction in relative cerebral blood flow; (2) vascular abnormalities, as determined by MRA; (3) baseline lesion volume of less than 25% or greater than 45% with respect to the ipsilateral hemisphere, as measured using ADC maps, and (4) absence of reperfusion or prolonged reperfusion (more than 10 minutes until achievement of at least 50% of the baseline cerebral blood flow) after filament removal. All excluded or deceased animals were replaced until the total number of animals indicated for each group was attained.

Experimental procedures were performed following 5 criteria derived from the Stroke Therapy Academic Industry Roundtable group guidelines for preclinical evaluation of stroke therapeutics,[11,12] which are: (1) cerebral blood flow was measured to confirm the vascular occlusion as an index of the reliability of the ischemic model; (2) animals were randomly assigned to treatment groups of the study; (3) researchers were blinded to treatment administration; (4) researchers were blinded to treatments during outcome assessment; and (5) temperature was controlled during the ischemic period.

***In vivo* MRI.** Infarct size was assessed by means of MRI. MRI studies were conducted on a 9.4-T horizontal bore magnet (Bruker BioSpin, Ettligen, Germany) with 12-cm wide actively shielded gradient coils (440 mT/m). Radiofrequency (RF) transmission was achieved with a birdcage volume resonator; signal was detected using a 4-element arrayed surface coil, positioned over the head of the animal, which was fixed with a teeth bar, earplugs and adhesive tape. Transmission and reception coils were actively decoupled from each other. Gradient-echo pilot scans were performed at the beginning of each imaging session for accurate positioning of the animal inside the magnet bore.

ADC maps were acquired during MCA occlusion (40 minutes after the onset of ischemia) using a spin-echo echo-planar imaging sequence with the following acquisition parameters: echo time (ET) = 26.91 ms, repetition time (RT) = 4 seconds, spectral bandwidth (SW) = 200 KHz, 7 b-values of 0, 300, 600, 900, 1200, 1600, and 2000 s/mm^2, flip angle (FA) = 90°, number of averages (NA) = 4, 14 consecutive slices of 1 mm, 24 × 16 mm^2 FOV (with saturation bands to suppress signal outside this FOV), a matrix size of 96 × 64 (isotropic in-plane resolution of 250 μm/pixel × 250 μm/pixel) and implemented with fat suppression option. Based on previous studies, the values of ADC in the healthy rat brain normally do not fall below 0.55 × 10^{-3} mm^2 s^{-1}; therefore, this threshold provides a convenient means of segmenting abnormal tissue.[13]

MCAO status was evaluated in a noninvasive manner with the time-of-flight magnetic resonance angiography (TOF-MRA). TOF-MRA scan was performed with a 3D-Flash sequence with an echo time (ET) = 2.5 ms, repetition time (RT) =15 ms, flip angle (FA)= 20°, number of averages (NA) = 2, spectral bandwidth (SW) = 98 KHz, 1 slice of 14 mm, 30.72 × 30.72 × 14 mm^3 FOV (with saturation bands to suppress signal outside this FOV), a matrix size of 256 × 256 × 58 (resolution of 120 μm/pixel × 120 μm/pixel × 241 μm/pixel) and implemented without fat suppression option.

Ischemic lesions were determined from T2-maps calculated from T2-weighted images acquired 24 hours, 7 and 14 days after the onset of ischemia using a MSME sequence: with an echo time (ET) = 9 ms, repetition time (RT) = 3 seconds, 16 echoes with 9 ms echo spacing, flip angle (FA) = 180°, number of averages (NA) = 2, spectral bandwidth (SW) = 75 KHz, 14 slices of 1 mm, 19.2 × 19.2 mm^2 FOV (with saturation bands to suppress signal outside this FOV), a matrix size of 192 × 192 (isotropic in-plane resolution of 100 μm/pixel × 100 μm/pixel) and implemented without fat suppression option.

***In vivo* MRS.** MRS was acquired as previously described.[14,15] Local shimming was performed by manual adjustment of first- and second-order shim coil currents using a proton-stimulated-echo acquisition mode (STEAM)-waterline sequence. The field homogeneity in a 3 × 3 × 3 mm^3 voxel typically resulted in signal line widths of 10−20 Hz for the water signal. *In vivo* 1H magnetic resonance spectra of both hemispheres of the rat brain were acquired by using a STEAM-1H sequence with an echo time (ET) = 3 ms, mixing time (TM) =10 ms, repetition time (RT) =1500 ms, flip angle (FA) = 90°, number of averages (NA) = 128, cubic voxel= 3 × 3 × 3 mm^3 and acquisition time = 3:15 minutes. Water signal was suppressed by variable power RF pulses with optimized relaxation delays.

Spectra were processed using MestReNova software (Mestrelab Research, Santiago de Compostela, Spain). For the quantitative analysis, glutamate and lactate signals were normalized to the creatine peak/

phosphocreatine areas for each single spectrum. MRS was acquired during the occlusion (75 minutes) and after reperfusion (40, 80, and 120 minutes after reperfusion).

Image analysis. Images were processed using ImageJ (Rasband WS, National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/) on an independent computer workstation. Ischemic damage was determined from ADC maps and T2-maps by manually selecting areas of reduced ADC values or hyperintense T2 signal by a researcher blinded to the animal protocols. Infarct size was indicated as the percentage of ischemic damage with respect to the ipsilateral hemisphere volume, corrected for brain edema.

Analysis of GOT1 levels in cerebrospinal fluid and brain tissue. The cerebrospinal fluid (CSF) was obtained from the cisterna magna (CM) and was carried out using the protocol described previously.[16] Briefly, by using the occipital crest as a reference point, a midline incision was made between the ears. The fascia was retracted and muscles dissected until the CM was exposed, which appears as a tiny inverted triangle, outlined by the cerebellum above and medulla below, behind the translucent dural membrane. Once identified the CM, a glass capillary was inserted and a volume of 5 μL of CSF was collected at every puncture. Clear CSF was collected in healthy and ischemic animals ($n = 5$/group) under basal conditions (before treatment administration) and 2, 4, and 24 hours after administrations (in case of healthy animals) or arterial reperfusion (in ischemic animals). CSF was transferred to a tube and kept frozen at −80°C. GOT activity in CSF was determined by Aspartate Aminotransferase Activity Assay Kit (Abcam, Ref: ab105135, Cambridge, UK) which requires a minimum volume of 5 μL to perform the analysis. GOT activity was analyzed in the brain tissue of ischemic animals 2 hours after ischemic reperfusion. Animals were sacrificed with overdose of anesthesia and immediately perfused with cold saline, the brain was removed and homogenized on ice to preserve protein levels. Samples were stored at −80°C until further analysis, which was carried out by means of Reflotron GOT specific activity tests following the manufacturer's technical specifications (Roche, Basel, Switzerland). Total protein content in samples was determined by the BCA protein assay (Thermo Scientific, Waltham, Massachusetts, USA). The results were expressed in U/L per μgr of protein.

Blood glutamate analysis. Blood samples were collected in test tubes (BD Microtainer SST Tubes. Ref: 365968, Franklin Lakes, New Jersey) and centrifuged at 3000 rpm (5804, Eppendorf, Hamburg, Germany) for 7 minutes. Serum was removed and immediately frozen and stored at −80°C. Serum glutamate concentration was determined by high performance liquid chromatography (1260 Infinity II, Agilent Technologies, Santa Clara, California) using the AccQ-Tag Pre-column derivatization method for amino acid analysis (Waters, Milford, MA), following a previously described method.[17]

Blood GOT and GPT activity analysis. Thirty-five μL of blood were collected in a microtainer BD (Microtainer K2E Tubes. Ref: 365975, Franklin Lakes, New Jersey, USA) and GOT/GPT activity were determined by means of Reflotron GOT/GPT specific activity tests strips following the manufacturer's technical specifications (Roche, Basel, Switzerland).

Sensorimotor tests. In order to assess the sensorimotor deficits after ischemic insult 3 different tests were done as modified versions of previously described methods.[18-20]

A) Cylinder test: this test consists of evaluation of asymmetry of limbs during the exploratory activity. For this test, the animal was put in a cylinder of transparent base of 20 cm diameter. A video camera is located under this transparent cylinder for recording the vertical exploratory movement of the animal with forelimbs during 5 minutes. For recording analysis, the Virtual Dub software was used. Analyzed behaviors were as follows: number of times that the animal touches the cylinder wall and independent use of each limb in contact with the cylinder wall in each upward movement. Laterality index was calculated (the number of times that the animal touches the cylinder with the impaired forelimb during the ascendant movement divided the number of impaired and nonimpaired forelimb contacts).This index is close to 0.5 for healthy animals, and tends to be 0 or 1 for animals that have a preferential use of the right or left paw, respectively.
B) Neuroscore: briefly, the animal was suspended by the tail, with its front paws touching the table or another stable surface. The rodent's walking pattern was observed during 1 minute. A sensorimotor score on a scale of 0 to 3 was assigned to rats on the basis of their gait behavior, being: 0: walking to both sides equally; 1: partial circling; 2: fully circling; 3: death of the animal.
C) Rotarod test: the test was performed using a rotarod apparatus (47650, UgoBasile, Comerio, Italy). The

animals were trained for 3 days before ischemia, 3 times per day. The speed was set at 20 rpm and the time that the animal could stay on the rotarod was measured, establishing 120 seconds as the cut-off limit.

All the sensorimotor tests were performed during the darkness cycle of animal housing, with environmental conditions consistently maintained across examinations and by a researcher blinded to the animal grouping. These tests were performed 1 day before surgery and 7 and 14 days after ischemia. Baseline functional evaluation is required to test for preoperative bias, because, on occasions, some animals display independent use of one limb.

Statistical analysis. All data are expressed as mean ± SEM. The data were analyzed using GraphPad Prism v.6.05 for Windows. The criterion for statistical significance was $P < 0.05$. Two-way ANOVA test was used to identify significant differences in multiple comparisons. The Student-test was used to identify significant differences between 2 groups. Data were first examined to assess distribution using the D'Agostino and Pearson omnibus normality test. To evaluate the effect of AbGOT1 in ischemia, 10 animals per group were required to detect the desired effect with a power (1-β) of 0.8 and an α of 0.05. N was calculated using EPIDAT software (http://www.sergas.es/Saude-publica/EPIDAT-4-2) and based on previous studies.[4]

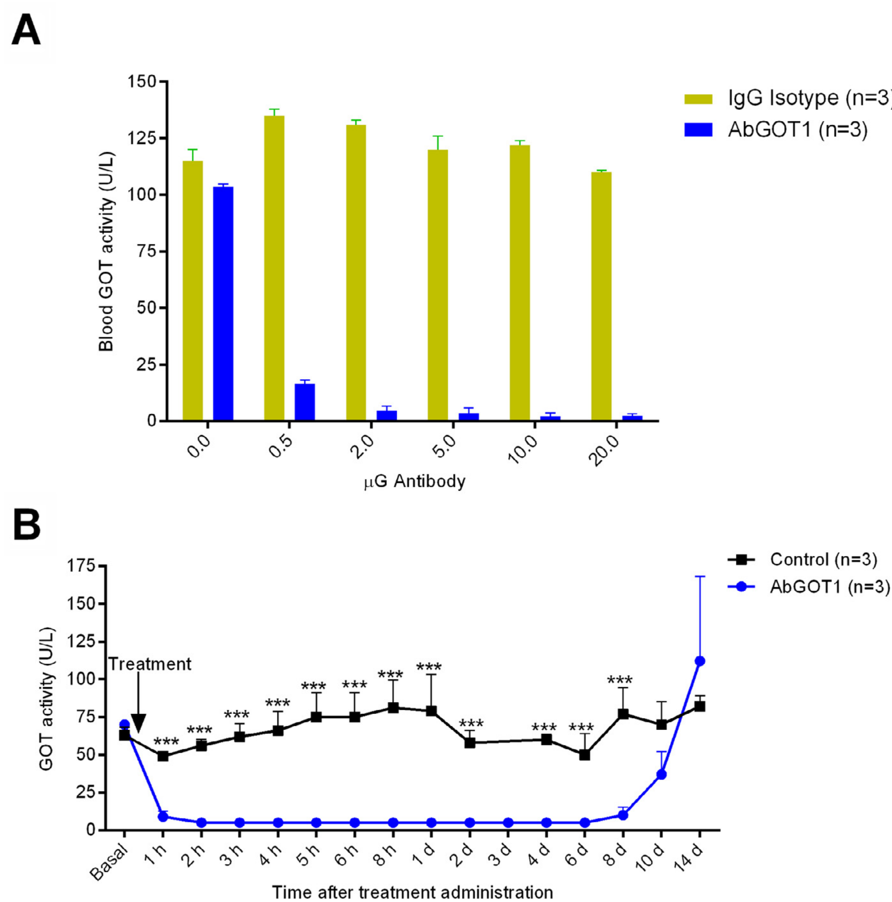

Figure 1. *In vitro* analysis of AbGOT1 and IgG isotype on blood GOT activity. The blocking efficacy of the AbGOT1 on GOT1 enzyme activity was tested on blood samples obtained from the tail vein of healthy rats (500 μL) (**A**). *In vivo* analysis of AbGOT1 on blood GOT activity in healthy animals. Control animals were treated (i.p.) with 1 mL of PBS (drug vehicle), and AbGOT1 treated animals with 1 mL (i.p.) 5 mg/rat. Basal samples were obtained before treatment (**B**). Data are shown as mean ± SEM. ***$P < 0.001$ compared with the control group ($n = 3$).

Table I. Dose-response analysis of AbGOT1 on blood GOT activity in healthy animals

AbGOT1 mg/rat	GOT (U/L) day 1	GOT (U/L) day 2	GOT (U/L) Day 4	GOT (U/L) day 8	GOT (U/L) day 14
Control	78	82	86	80	89
1 mg/rat	45	105	95	92	91
2.5 mg/rat	18	19	17	22	97
4.0 mg/rat	<5	<5	<5	15	92
5.0 mg/rat	<5	<5	<5	10	112

RESULTS

***In vitro* and *in vivo* analysis of AbGOT1 on GOT1 activity.** To validate the blocking efficacy of the developed antibody on the enzyme activity first, the AbGOT1 was tested in *in vitro* blood samples and latter in healthy experimental animals. Analysis of AbGOT1 on blood GOT activity showed that a minimal amount of 0.5 μg of antibody was enough to induce a reduction >80% of the enzyme activity (Fig 1, *A*). Doses of 2 μg of antibody showed an inhibition > 95% of GOT activity with

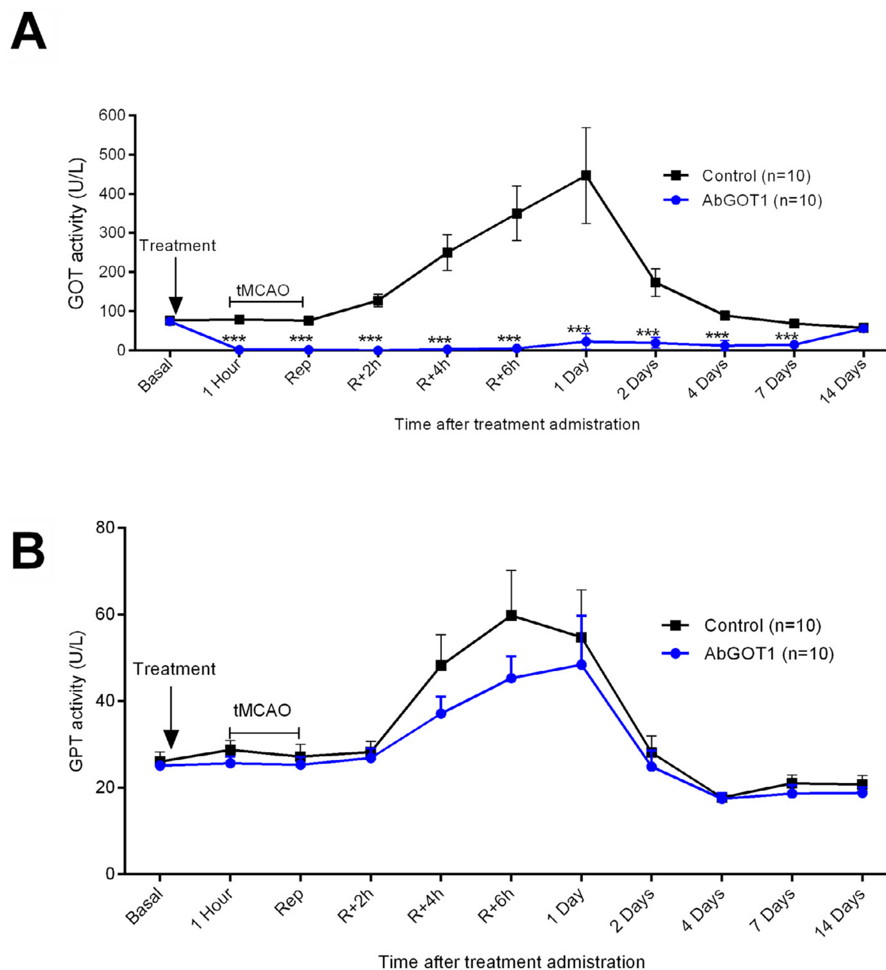

Figure 2. Temporal analysis of the effect of the AbGOT1 on blood GOT activity in ischemic animals (**A**). Temporal analysis of the effect of the AbGOT1 on blood GPT activity in ischemic animals (**B**). Control animals were treated (i.p.) with 1 mL of PBS (drug vehicle) and AbGOT1 treated animals with 1 mL (i.p.) 5 mg /rat. Treatments were injected 1 hour before ischemic surgery. Transient MCAO was induced during 75 minutes. Basal samples were obtained before treatment and surgery. Data are shown as mean ± SEM. $^{***}P < 0.001$ compared with the control group ($n = 10$).

respect to the basal levels. The IgG isotype control did not affect the GOT activity. *In vivo* dose-response analysis of AbGOT1 on blood GOT activity in healthy animals confirmed the same inhibitory effect observed in the previous *in vitro* study as summarized in Table I. Intraperitoneal administration of 1 ml of AbGOT1 (1 mg/rat) induced a reduction of 55% (with respect to the control group) of the GOT activity one day after administration. A higher dose (2.5 mg/rat) induced an inhibition of 80% (with respect to the control group), and this inhibition lasted for at least 4 days after administration. However, with a dose of 4 and 5 mg/rat, the endogenous blood GOT activity decrease was almost complete and below detectable values (<5 U/L) during at least 8 days after treatment administration. Temporal analysis during 1, 2, 3, 4, 5, 6, and 8 hours and 1, 2, 3, 4, 6, 8, 10, and 14 days after AbGOT1 confirmed that a single i.p. administration of 5 mg induced a significant reduction [F (1, 64) = 98.4, $P < 0.001$] of blood GOT activity which lasted for 8 days. Endogenous GOT1 activity recovered 14 days after antibody administration (Fig 1, *B*). Based on this inhibition, the dose of 5 mg/rat of AbGOT1 was used for subsequent studies in ischemic animals.

Effect of AbGOT1 in the ischemic animal model. Cerebral ischemic injury in control animals induced a significant increase of blood GOT1 levels during at least 2 days after ischemia with respect to basal levels (before ischemia). Maximum increase was observed one day after ischemic damage (446 ± 122 U/L) compared with basal levels (77 ± 11 U/L). Blood GOT1 levels returned to basal levels 4 days after ischemia (89 ± 7 U/L; Fig 2, *A*). In line with the findings observed in healthy animals, in ischemic animals, AbGOT1 treatment led to a complete inhibition of blood GOT1 below detectable levels (< 5U/L) one hour after administration and also abolished the natural increase of endogenous GOT1 enzyme activity caused by the ischemic damage which was observed in the control group [F (1, 200) = 101.9, $P < 0.001$]. GPT activity, the other transaminase blood-resident enzyme involved on the blood glutamate homeostasis,[21] was also increased after brain injury during at least one day after ischemia, and returned to basal levels one day later. The maximum increase was observed at 6 hours after ischemic damage (59 ± 10 U/L), as compared to the basal levels (26 ± 2 U/L). In contrast to what was seen with GOT1, this increase in blood GPT activity was not affected by the AbGOT1 treatment (Fig 2, *B*). No changes in blood glutamate levels were observed during the follow-up period in both control and AbGOT1 treated groups (Fig 3). Meanwhile, comparative analysis of ischemic lesions between controls and treated animals showed that blocking of endogenous GOT1 blood activity by AbGOT1 caused larger lesions following ischemia induction, 7 and 14 days compared with the control group [F (1, 64) = 9.1, $P = 0.003$)] (Fig 4). Doppler analysis of the cerebral blood flow determined before and after

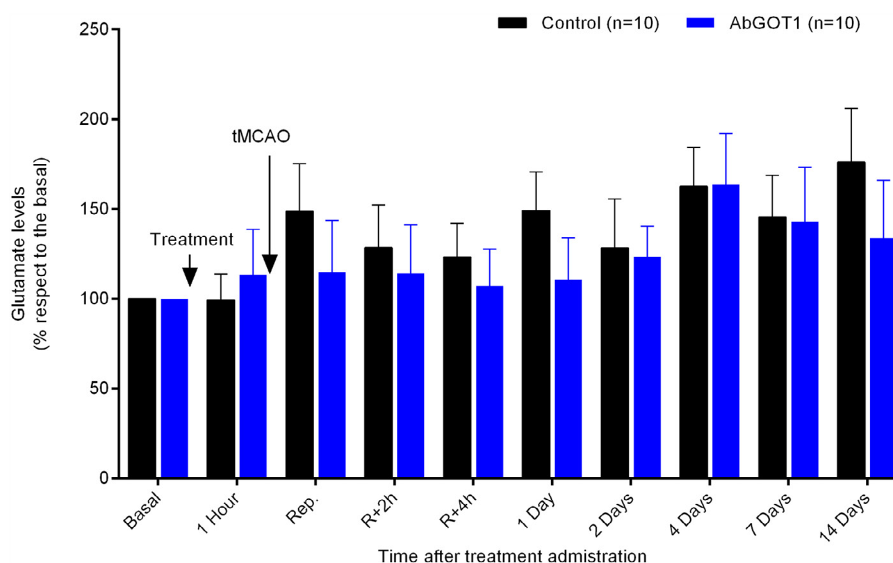

Figure 3. Temporal analysis of the effect of the AbGOT1 on blood glutamate levels in ischemic animals. Control animals were treated (i.p.) with 1 mL of PBS (drug vehicle) and AbGOT1 treated animals with 1 mL (i.p.) 5 mg/rat. Treatments were injected 1 hour before ischemic surgery. Transient MCAO was induces during 75 minutes. Basal samples were obtained before treatment and surgery. Data are shown as mean ± SEM (% respect the basal levels ($n = 10$).

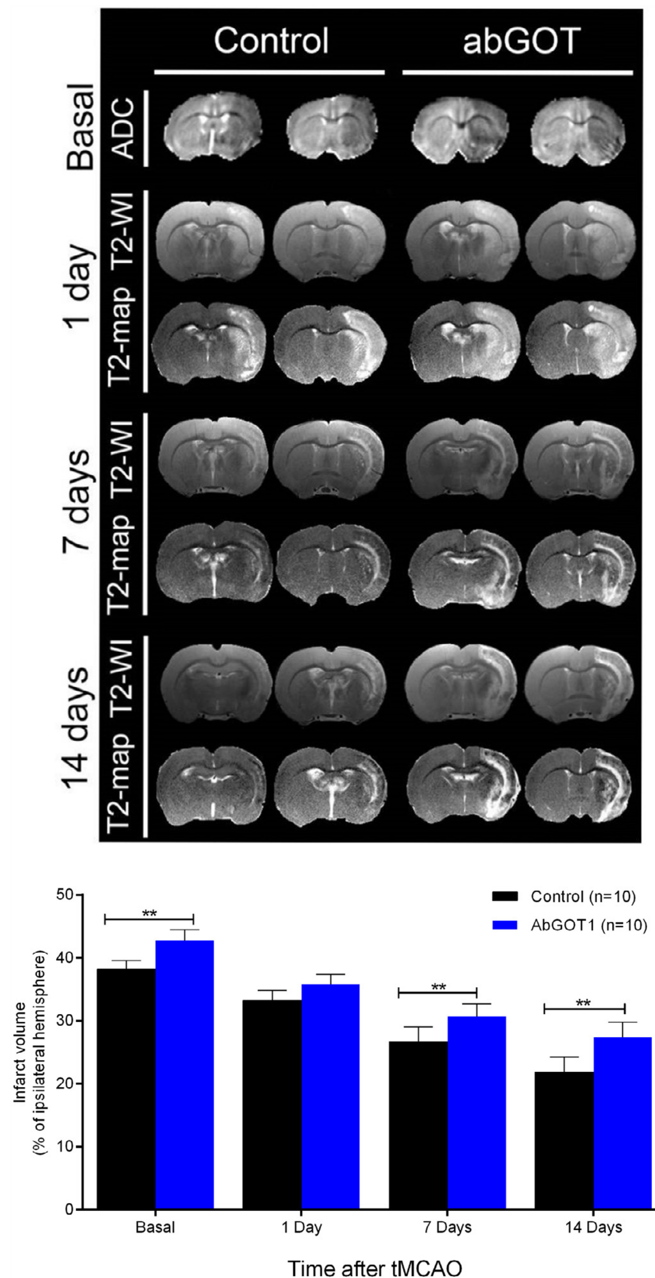

Figure 4. Magnetic Resonance Imaging assessments of ischemic injury evolution. Apparent diffusion coefficient (ADC) maps were recorded during cerebral artery occlusion (defined as basal). Lesion volume evolution was assessed using T2-weighted images recorded 24 hours, 7 days, and 14 days after ischemia induction. Control animals were treated (i.p.) with 1 mL of PBS (drug vehicle), and AbGOT1 treated animals with 1 mL (i.p.) 5 mg/rat. Treatments were injected 1 hour before ischemic surgery. Transient MCAO was induces during 75 minuted. T2 lesion are represented as % adjusted to the ipsilateral hemisphere. Data are shown as mean±SEM. $^{**}P < 0.01$ compared with the control group ($n = 10$).

AbGOT1 administration in healthy animals, showed that the blocking of the GOT1 activity had no impact on this physiological parameter (see **Fig S2**).

Analysis of sensorimotor deterioration in control and treated animals with AbGOT1. Ischemic injury was associated with neurological deficits, evaluated using the cylinder test, neuroscore and rotarod test. AbGOT1 treatment caused higher deficits as detected with the rotarod test [F (1, 47) = 5.3, P= 0.02] at 7 days compared with control groups; Fig 5, *A*). In the cylinder test and the neuroscore, although the differences observed were not statistically significant between the

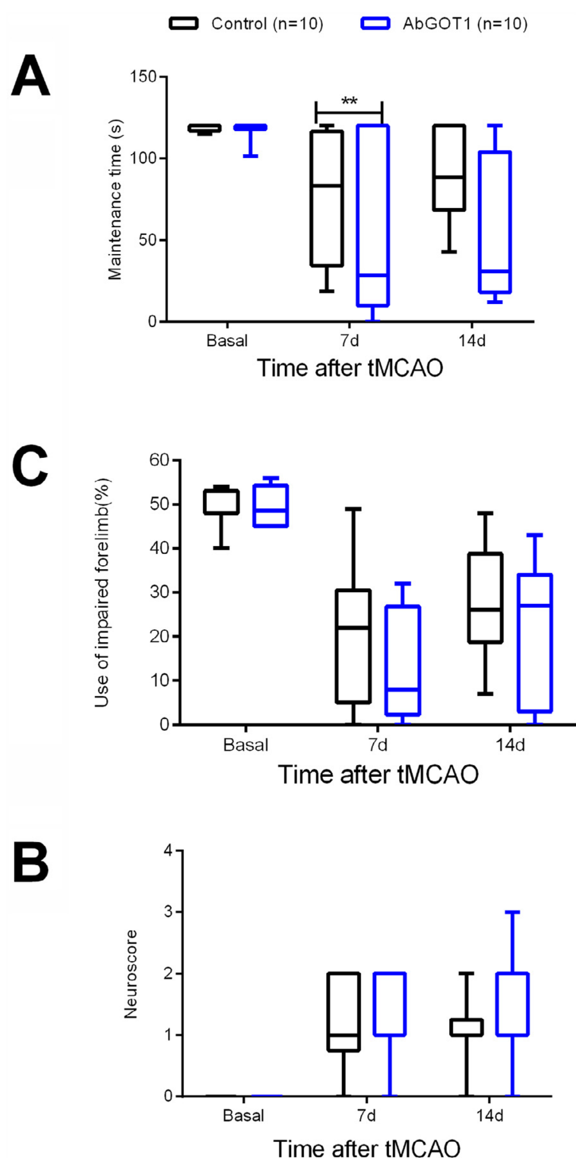

Figure 5. Box plots show the assessment of sensorimotor function using rotarod test (**A**) cylinder test (**B**) and neuroscore (**C**). Functional tests were performed before ischemic injury (baseline), 7 days and 14 days after injury in control (vehicle) and treated animals. Control animals were treated (i.p.) with 1 mL of PBS (drug vehicle) and AbGOT1 treated animals with 1 mL (i.p.) 5 mg/rat. Treatments were injected 1 hour before ischemic surgery. Transient MCAO was induced during 75 minutes. Boxes represent interquartile ranges. The line across each box indicates the median and the whiskers (T) are the highest and lowest values. Baseline reflects pretreatment and preoperative functional evaluation. Data are shown as mean ± SEM. $^{**}P < 0.01$ compared with the control group ($n = 10$)

groups, the animals treated with AbGOT1 presented a trend toward worse recovery (Fig 5, *B* and *C*). Functional evaluation performed 1 day before surgery (before AbGOT1 treatment and ischemia induction), defined as baseline, confirmed that none animals included in the study showed prestroke disability.

Effect of AbGOT1 on brain glutamate and lactate levels in the ischemic animal model. Analysis of cerebral brain glutamate levels determined by MRS technique revealed a persistent increase in brain glutamate after occlusion of MCA in the AbGOT1 treated group with respect to the basal levels and the contralateral brain region (healthy region) [F (3, 48) = 16.26, $P < 0.001$; Fig 6, A-C). In line with the brain glutamate response, analysis of lactate levels, used as a marker of ischemic brain lesion,[22,23] showed a significant increase in the ischemic region in animals that were submitted to the GOT1 blocking (Fig 6, *A,B* and *D*).

GOT activity in CSF and brain tissue. To investigate whether the administration of AbGOT1 had any impact on the activity of GOT1 in the brain, GOT activity was determined in healthy and ischemic animals both in CSF and brain tissue samples. As we have already observed, AbGOT1 treatment caused an inhibition of blood GOT (Fig 7, *A* and *C*) and a reduction in the GOT activity in CSF for at least 24 hours in both healthy (Fig 7, *B*) and ischemic animals (Fig 7, *B* and *D*); although, this inhibition was significantly lower (around 50% respect to CSF basal levels) compared with the effect observed in the blood (>95%). In order to confirm the specificity of AbGOT1 not only on the blood GOT1, the isotype form of the antibody was tested on brain GOT1 in a new group of animals. The findings confirmed again the inhibition of brain and blood GOT1 activity by AbGOT1 (Fig 8, *A* and *B*, respectively); however, no effect was observed under the isotype form.

DISCUSSION

Nowadays, the use of the recombinant GOT1 has been suggested as a novel neuroprotective drug based on its properties to act as a blood glutamate scavenger, and its use has been proposed for the acute phase of cerebral ischemia with the aim to reduce the neurological damage caused by glutamate excitotoxicity.[4]

However, due to the critical role of GOT1 on energetic cell metabolism, and to complement our earlier studies, in this study we decided to produce and purify a polyclonal antibody against GOT1 to block the metabolic activity of this enzyme, and evaluate its repercussions on the energetically compromised brain ischemic tissue, in the same animal model of ischemic stroke where we previously demonstrated the beneficial neuroprotective effect of the administration rGOT1.[4]

Our study showed that an inhibition of the blood GOT1 activity leads to higher ischemic damage and

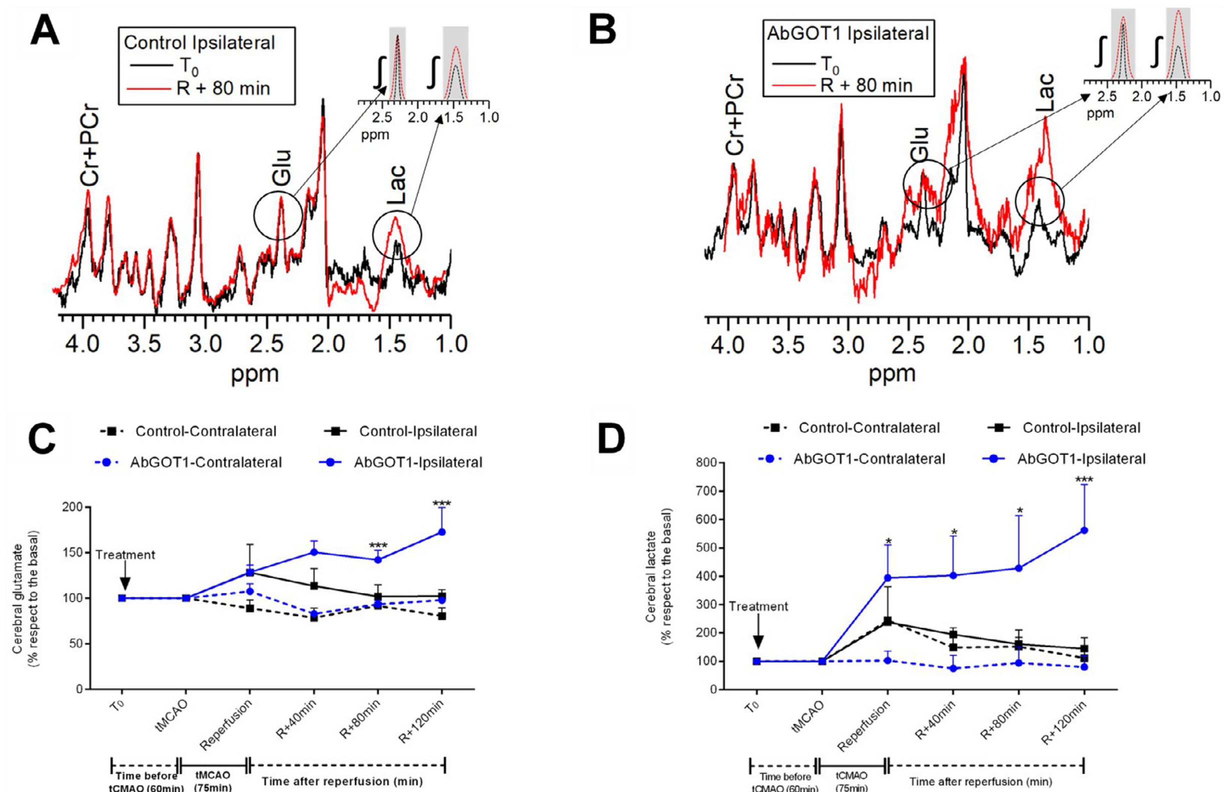

Figure 6. Brain glutamate and lactate levels were determined in control (**A**) and AbGOT1 (**B**) treated animals before ischemia (T_0), during occlusion (75 minutes) and 40, 80, and 120 minutes after reperfusion (R) using Magnetic resonance spectroscopy (MRS) technique. Glutamate (Glu), and lactate (Lac) peaks in the MR spectrum were normalized to the creatine (Cr) peak/phosphocreatine (PCr) areas for each single spectrum and then represented as % adjusted to basal levels. On the right and up panels are represented the automatic software integration of the area of glutamate and lactate spectral peaks. Figures **A** and **B** represent the differences in the glutamate and lactate peaks at T_0 and at 80 minutes after reperfusion in a control and treated animal respectively. Control animals were treated (i.p.) with 1 mL of PBS (drug vehicle), and AbGOT1 treated animals with 1 mL (i.p.) 5 mg/rat. Treatments were injected 1 hour before ischemic surgery. Transient MCAO was induced during 75 minutes. Graph **C** shows the cerebral glutamate levels in a control animal and treated animals, and lactatate levels in the graph **D**. Data are shown as mean ± SEM. * $P < 0.05$, ***$P < 0.001$ compared with the control group ($n = 3$).

poorer outcome in comparison to ischemic control animals which were not treated with AbGOT1. Our results seem to confirm that cell GOT1 contributes to maintain the cell viability in ischemic conditions and subsequently reducing the neuronal damage. Previously a somewhat similar observation was reported in stroke patients that had high natural levels of blood GOT (> 18 U/L). Such patients were found to have significantly less neurological damage at 3 months after the stroke than patients with low endogenous levels of GOT.[5,6]

MRS analysis of the brain parenchyma of animals treated with AbGOT1 demonstrated a significant increase in glutamate levels. In this regard, recent reports[24,25] have proposed that, besides the blood glutamate scavenger property, GOT1 can, alternatively, metabolize glutamate in the cerebral ischemic site as an alternative source of energy to maintain the cellular viability and to protect the brain against the excitotoxic damage. Thus, overexpression of GOT1 in the brain parenchyma caused a reduction of the ischemic stroke lesion volume and improved the poststroke sensorimotoric functions. Based on these previous findings, we could observe that AbGOT1 induced a partial blocking of the GOT1 activity in the brain, and therefore this might contribute to the increase of glutamate in the extracellular space of the brain of ischemic animals treated with AbGOT1, that could explain why they had a larger ischemic lesion and poorer outcome.

The differences observed on infarct volume between AbGOT1-treated and untreated animals during arterial occlusion (defined as basal volume) were ruled out to be caused by some alteration of cerebral blood supply as no change on this parameter was observed for 150 minutes after AbGOT1 treatment. In this regard, current studies in tumoral cell lines have reported that inhibition of GOT1 increases the lactate levels and generates a metabolic

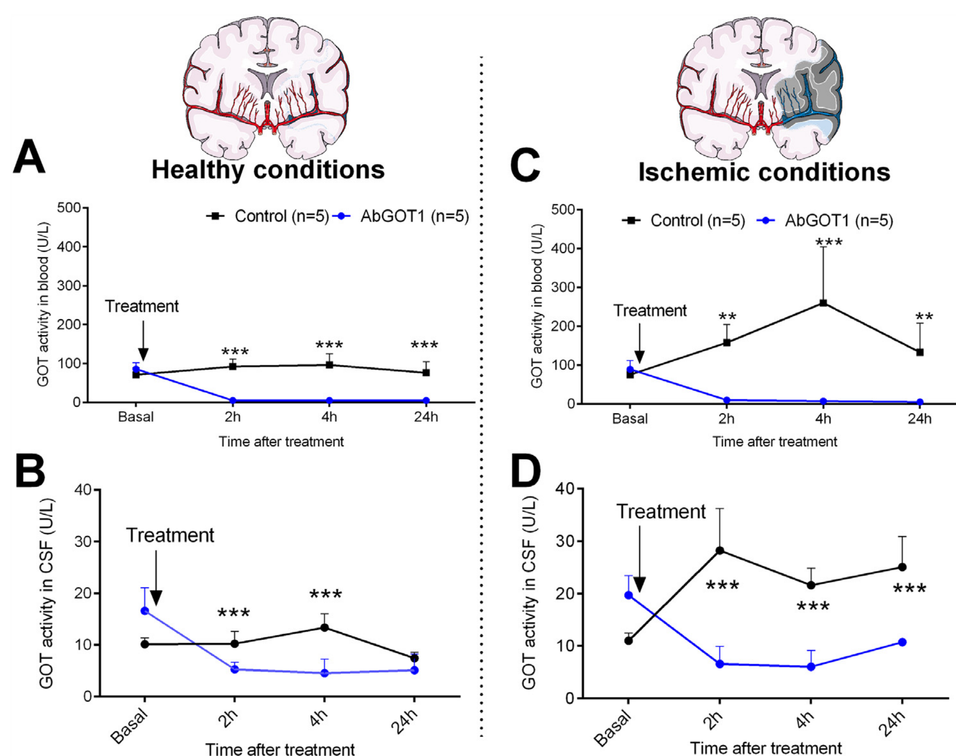

Figure 7. GOT activity was determined in blood and CSF from healthy and ischemic animals with AbGOT1 and saline. In healthy animals, the analysis of GOT activity in blood (**A**) and CSF (**B**) showed that the administration of AbGOT1 causes a decrease in GOT activity in comparison to control group, in which GOT activity remains unchanged. GOT activity in blood (**C**) and CSF (**D**) of ischemic animals revealed that, as in healthy animals, AbGOT1 reduces GOT activity in comparison to controls. In contrast to healthy control animals, in ischemic control animals, an increase in GOT activity was observed in both CSF and serum with respect to basal levels after ischemia. Data are shown as mean ± SEM. $^{**}P < 0.01$, $^{***}P < 0.001$compared with the control group ($n = 5$).

situation glucose-dependent, making the cells more vulnerable to damage.[26,27] In line with these cancer cell studies, brain MRS analysis confirmed this increase of lactate caused by GOT1 inhibition, probably explained through the conversion of pyruvate to lactate via the lactate dehydrogenase activity, and this lactate raise might explain the differences observed on the basal infarct volume determined immediately after ischemic damage induction.

To confirm that the results observed were caused by the specific inhibition of GOT1 and not due to other blocking of other blood transaminase enzyme, the effect of AbGOT1 treatment was also evaluated on the activity of GPT (as well-known as ALT). GPT, like GOT, is a glutamate transaminase enzyme that catalyzes the reversible transamination between alanine and α-ketoglutarate to generate pyruvate and glutamate and therefore has a role similar to GOT1 in the homeostasis of glutamate.[21] Basal GPT activity and its increase were observed after ischemia induction but were not altered by the AbGOT1 treatment, which demonstrates that the results observed were mostly mediated by the inhibition of GOT1.

Based on the key role of GOT in the glutamate metabolism, contrary to what was expected, the administration of AbGOT1 did not cause a significant change in blood glutamate levels in ischemic animals compared with the untreated group. A plausible explanation for this response is that other enzymes involved in the blood glutamate homeostasis, such as the GPT, could compensate the inhibition of the GOT1 activity; however, we are aware that the mechanism(s) for unchanged glutamate levels are presently unclear.

In conclusion, due to the critical role of GOT1 on energetic cell metabolism, inhibition of GOT1 activity during the ischemic period causes an increase in the damage and leads to a poorer outcome, that supports the use of rGOT1 as a promising drug for the acute phase of cerebral ischemia.

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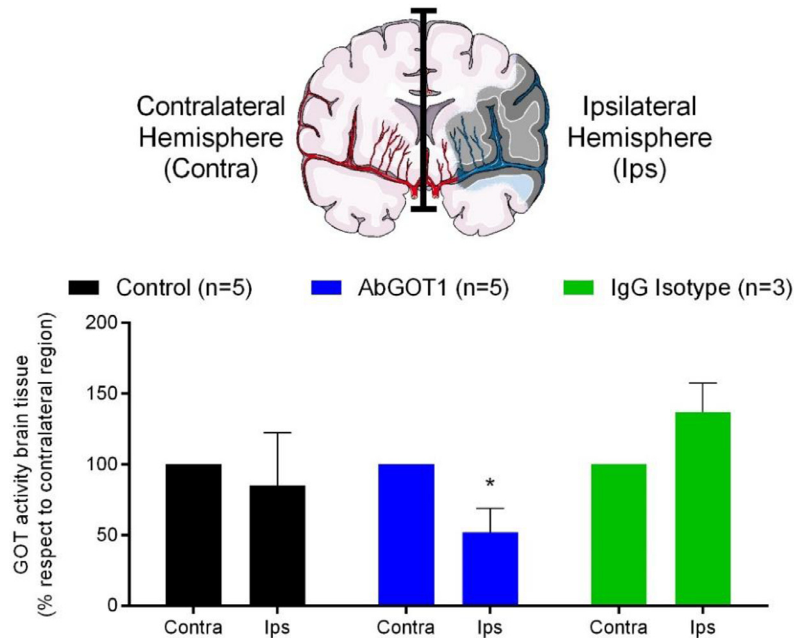

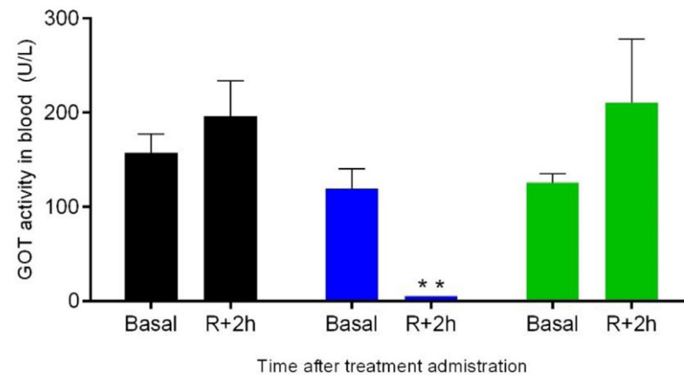

Figure 8. The specificity of AbGOT1 on GOT activity was determined in the brain tissue and blood of ischemic animals using an isotype form. In **A,** a 50% reduction of the GOT activity in the brain tissue of the animals treated with AbGOT1 was observed, while the isotype form had no effect on the GOT activity. Also, in the same group of ischemic animals GOT activity was measured in the blood (**B**), and the same results were observed as previously described: AbGOT significantly reduced GOT activity and the isotype form did not induce changes in GOT activity. Data are shown as mean ± SEM. $*P < 0.05$, $**P < 0.01$ Compared with the control group ($n = 5$).

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Author contributions: DM and FC designed and conceived the study. AD-L, MP-M, A d S-C A and A B-C performed all experiments. AR synthesized the antibody. RI-R performed the MR scan and analysis. AD-L, MP-M, DM, JC and FC wrote the manuscript. AD-L, MP-M, TS, JC, DM and FC discussed the results and their implications and commented on the manuscript at all stages. All authors have read the journal's policy on conflicts of interest. The

authors have no conflicting interests to declare. All authors have read the journal's authorship agreement.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2020.10.004.

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