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(54) **Title:** IMAGING METHOD FOR PREDICTING THE ONSET OF MULTIPLE SCLEROSIS

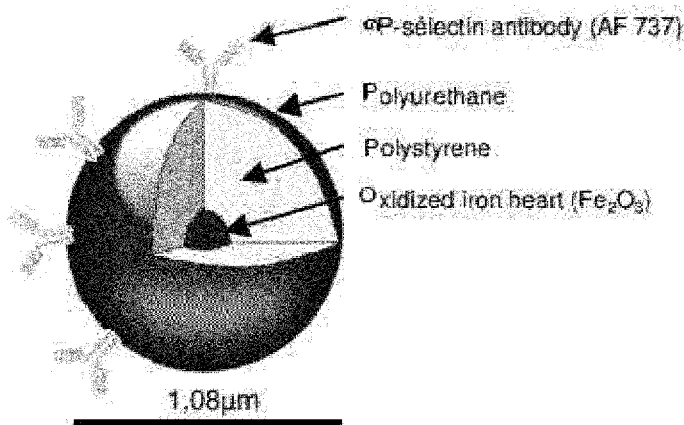


Figure 1

(57) **Abstract:** The present invention relates to an imaging method for predicting the onset of neurological symptoms or the remission from neurological symptoms in a patient suffering from remitting-relapsing multiple sclerosis (RRMS). The ability to predict the occurrence of a subsequent relapse or remission and to estimate the time when that process will occur has important clinical implications. However, to date, there exists no reliable method for predicting the risk of relapse in an asymptomatic RRMS patient or the chances of remission in a symptomatic RRMS patient. The inventors found that imaging targeting-p-selectin allows accurate prediction of the onset of neurological symptoms or the appearance of remission. Thus, the invention relates to an imaging agent for use in a method for predicting the onset of neurological symptoms or the remission from neurological symptoms in a patient suffering from RRMS, wherein said imaging agent, such as a MPIO particle, comprises a selectin ligand.



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Imaging method for predicting the onset of multiple sclerosis

BACKGROUND OF THE INVENTION

5 Multiple sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system, also known as disseminated sclerosis or encephalomyelitis disseminata. This damage disrupts the ability of parts of the nervous system to communicate, resulting in a wide range of signs and symptoms, including physical, mental, and sometimes psychiatric problems. MS takes several forms, with new symptoms either occurring in isolated attacks (relapsing forms) or
10 building up over time (progressive forms). Between attacks, symptoms may disappear completely; however, permanent neurological problems often occur, especially as the disease advances.

Relapsing-remitting multiple sclerosis (RRMS) is characterized by an unpredictable relapsing-remitting course, wherein clearly defined attacks of worsening neurologic function. -
15 often called relapses, flare-ups or exacerbations - are followed by partial or complete recovery periods (remissions). During recovery periods symptoms improve partially or completely, and there is no apparent progression of disease. RRMS is the most common disease course at the time of diagnosis. Approximately 85 percent of people are initially diagnosed with RRMS, compared to 10-15 percent with progressive forms of the disease.

20 Diagnosis of MS involves blood tests, lumbar punctures, evoked potentials and magnetic resonance imaging.

Today MRI diagnosis and therapeutic assessment of multiple sclerosis is mostly based on quantification of contrast-enhancing lesions and change of lesion load (Polman et al., 2011, Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. Ann Neurol.
25 2011 Feb;69(2):292-302).

New tools are required to improve the sensitivity of imaging in MS and to provide valuable information for disease physiopathological and therapeutic assessment. In particular, there is an unmet need for methods for predicting the risk of relapse in patients suffering from RRMS.

30 Indeed, the ability to predict the occurrence of a subsequent relapse (yes/no) and to estimate the time when that process will occur has important clinical and practical implications.

This knowledge can help in decisions related to treatment – e.g. either treat patients with more aggressive disease or avoid over-treatment of patients with a more favorable disease course. Prediction of the time to next relapse can also be useful in the design of clinical trials as an additional criterion for selecting active patients. For patients with clinically isolated syndrome (CIS), who have just experienced the first relapse, this knowledge may allow to estimate the probability to convert to definite MS by predicting the time until the second relapse.

Leukocyte recruitment is mediated by adhesion molecule expressed on endothelial cells (Rossi et al., 2011, Vascular inflammation in central nervous system diseases: adhesion receptors controlling leukocyte-endothelial interactions *J. Leukoc. Biol.*, 89 (2011), pp. 539–556).

Selectins are important cell adhesion molecules, with high affinities for carbohydrate moieties. They play a prominent and critical role in the initial stages of circulating cellular components and vascular wall interactions by mediating leucocytes/platelet and leucocytes/endothelium interactions. Three types of selectins have been discovered so far: P-selectin, E-selectin and L-selectin. L-selectin is constitutively expressed on almost all circulating leukocytes. The expression of E-selectin is inducible on vascular endothelium upon activation by various mediators including cytokines and endotoxin. P-selectin is contained in intracytoplasmic granules and is rapidly translocated to platelet or endothelial surfaces after cell exposure to thrombin or histamine.

The P-, L- and E-selectins are structurally similar transmembrane proteins. They all possess large, highly glycosylated, extracellular domains, a single spanning transmembrane domain, and a small cytoplasmic tail. At their extracellular amino termini, they have a single calcium-dependent (or C-type) lectin domain (L) followed by an epidermal growth factor (EGF)-like domain (E) and several complement regulatory domains (C). Selectin-mediated cell adhesion results from calcium-dependent interactions of the amino-terminal lectin domain with a large variety of carbohydrate-presenting molecules on the surface of target cells. While the affinity of each of the selectins varies depending on the ligand, they all bind a specific tetrasaccharide carbohydrate structure known as sialyl Lewis X (SLe^x), which contains sialic acid and fucose residues.

Selectins are considered as potentially useful markers for the diagnosis of some of these pathologies. Numerous efforts are in progress to image selectins predominantly through Magnetic

Resonance Imaging (MRI) (S. Bouty *et al.*, Contrast Media Mol. Imaging, 2006, 1: 15-22), scintigraphy (G. Hairi *et al.*, Ann. Biomed. Eng., 2008, 36: 821-830), and more recently using ultrasons (F.S. Villanueva *et al.*, Nat. Clin. Pract. Cardiovasc. Med., 2008, 5: S26-S32). Most selectin imaging agents developed so far are anti-selectin antibodies (B.A. Kaufman *et al.*, Eur. Heart J., 2007, 28: 2011-2017; G. Hairi *et al.*, Ann. Biomed. Eng., 2008, 36: 821-830; K. Licha *et al.*, J. Biomed. Opt., 2005, 10: 41205; and P. Hauff *et al.*, Radiology, 2004, 231: 667-673) and sialyl Lewis X analogs or derivatives (S. Bouty *et al.*, Contrast Media Mol. Imaging, 2006, 1: 15-22; F.S. Villanueva *et al.*, Circulation, 2007, 115: 345-352). These imaging agents have been suggested to allow the *in vivo* non-invasive detection of selectins in inflammation, neurodegenerative disorders, cancer and thrombosis.

However, to date, there exists no reliable method for predicting the risk of relapse in an asymptomatic patient suffering from RRMS, or for predicting the chances of remission in a symptomatic patient suffering from RRMS.

SUMMARY OF THE INVENTION

The inventors have found that imaging targeting-p-selectin allows non-invasive measurement of vascular inflammation and can thus be used as additional information inaccessible to conventionally used imaging techniques for the diagnosis, follow-up and prognosis of MS patients.

The inventors have developed a molecular imaging technique which can reveal the blood-spinal cord barrier (BSCB) dysfunctions together with the immune cell infiltration. This imaging technique can accurately predict the onset of neurological symptoms, notably in asymptomatic patients, but also the appearance of remission.

Thus, the present invention relates to an imaging agent for use in a method for predicting the onset of neurological symptoms or the remission from neurological symptoms in a patient suffering from remitting-relapsing multiple sclerosis, wherein said imaging agent comprises a selectin ligand.

The invention also relates to the use of spinal cord P-selectin as a biomarker for multiple sclerosis.

5 The invention also provides a method for predicting the onset of neurological symptoms or the remission from neurological symptoms in a patient suffering from remitting-relapsing multiple sclerosis, said method comprising the steps of:

- administering to said patient a detectable amount of an imaging agent comprising a selectin ligand,

10 - subjecting said patient to MRI,

- collecting the MRI signal,

- quantifying the percentage of signal void,

- comparing said percentage of signal void to a predetermined value,

wherein an increase in the percentage of signal void compared to the predetermined value is

15 indicative of a risk of relapse in a patient who does not present any neurological symptoms,

and/or wherein a decrease in the percentage of signal void compared to the predetermined value is indicative of a risk of remission in a patient who presents neurological symptoms.

20 **DETAILED DESCRIPTION OF THE INVENTION**

As described above, the present invention relates to an imaging agent for use in a method for predicting the onset of neurological symptoms or the remission from neurological symptoms in a patient suffering from remitting-relapsing multiple sclerosis (RRMS), wherein said imaging agent comprises a selectin ligand.

25 As used herein, the term “patient” refers to a human or another mammal (*e.g.*, mouse, rat, rabbit, hamster, dog, cat, cattle, swine, sheep, horse or primate). In many embodiments, the subject is a human being.

30 As used herein, the term “imaging agent” refers to a compound that can be used to detect specific biological elements (*e.g.*, biomolecules) using imaging techniques. Imaging agents of the

invention are molecules comprising a selectin ligand associated with at least one detectable moiety. Imaging agents of the present invention can be used to detect selectins in *in vitro* and *ex vivo* biological systems as well as in subjects.

The detectable moiety according to the invention is typically detectable through any one of the following imaging techniques Planar Scintigraphy (PS) or Single Photon Emission Computed Tomography (SPECT), Positron Emission Tomography (PET), ultrasound imaging such as contrast-enhanced ultrasonography (CEUS), Magnetic Resonance Imaging (MRI) or fluorescence spectroscopy.

Typically, said method for predicting the onset/relapse of neurological symptoms is carried out by Magnetic Resonance Imaging (MRI), or by ultrasound imaging such as CEUS.

Typically, said method for predicting the onset/relapse of neurological symptoms is carried out by imaging the central nervous system of said patient.

In one embodiment, said imaging technique, notably the MRI or ultrasound imaging, is carried out on the brain of said patient.

In another embodiment, said imaging technique is carried out on the spinal cord of said patient.

In one embodiment, said imaging technique, notably MRI imaging or ultrasound imaging, is carried out on the upper spinal cord, preferably the cervical spinal cord.

In one embodiment, said imaging technique, notably MRI imaging or ultrasound imaging is carried out on the lower spinal cord, preferably the lumbar spinal cord.

Without wishing to be bound by theory, it is believed that, in certain cases of MS, lesions in the spinal cord appear prior to brain lesions. Indeed, it has been reported that presence of asymptomatic spinal lesions may help confirm a diagnosis of MS when few or no brain lesions are present (Lycklama et al. Spinal-cord MRI in multiple sclerosis. Lancet Neurol. 2003 Sep;2(9):555-62).

As used herein, the term “neurological symptoms” refers to the clinical manifestations of MS. They include, but are not limited to:

- numbness or weakness in one or more limbs,
- partial or complete loss of vision, usually in one eye at a time, often with pain during eye movement,
- Prolonged double vision,
- 5 - Tingling or pain in parts of the patient's body
- Electric-shock sensations that occur with certain neck movements, especially bending the neck forward (Lhermitte sign)
- Tremor, lack of coordination or unsteady gait
- Slurred speech
- 10 - Fatigue
- Dizziness
- Problems with bowel and bladder function.

The patient according to the invention can be asymptomatic. Typically such a patient does not exhibit some of the more specific symptoms as mentioned above. For example, such a patient does not exhibit:

- numbness or weakness in one or more limbs,
- partial or complete loss of vision, usually in one eye at a time, often with pain during eye movement,
- 20 - Prolonged double vision,
- Tingling or pain in parts of the patient's body
- Electric-shock sensations that occur with certain neck movements, especially bending the neck forward (Lhermitte sign)
- Tremor, lack of coordination or unsteady gait
- 25 - Slurred speech

Preferentially, an asymptomatic patient does not exhibit any neurological symptoms as previously described.

The method of the invention can predict the risk of relapse in a patient suffering from RRMS.

As used herein, the term "relapse" has its general meaning in the art. It refers to episodes of inflammatory activity. A relapse is defined by the occurrence of new or worsening neurological

symptoms, during at least 24 hours, separated from the previous relapse by at least one month. Relapses are also called attacks, flare-ups, or exacerbations.

Advantageously, the method for predicting a relapse according to the invention can be useful for selecting and/or adapting the appropriate treatment regimen. Typically, if a risk of relapse is identified, the physician will prescribe a treatment in order to attenuate or prevent the neurological symptoms in said patient. Prior to the development of the prediction tool according to the present invention, treatment could only be initiated once the relapse had actually occurred, i.e. after the onset of the neurological symptoms.

Without wishing to be bound by theory, it is believed that the method of the invention enables a better management of the neurological symptoms.

Conversely, the method of the invention can predict the chances of remission from neurological symptoms in a patient suffering from RRMS who is experiencing symptoms at the time the imaging is carried out. This knowledge can also be very helpful in decisions related to treatment – e.g. it may avoid over-treatment of patients with a favorable disease course.

As used herein, “remission” has its general meaning in the art. It refers to a period between two relapse episodes, during which the number and/or intensity of the neurological symptoms is reduced.

The invention also provides a method for predicting the onset of neurological symptoms or the remission from neurological symptoms in a patient suffering from relapsing-remitting multiple sclerosis, said method comprising the steps of:

- administering to said patient a detectable amount of an imaging agent comprising a selectin ligand,
- subjecting said patient to an imaging technique,
- collecting the imaging signal from the imaging agent,
- quantifying the signal,
- comparing the level of said signal to a predetermined value,

wherein an increase in the level of the signal compared to the predetermined value is indicative of a risk of relapse in an asymptomatic patient, typically who does not present any neurological symptoms,

and/or wherein a decrease in the level of the signal compared to the predetermined value is indicative of a chance of remission in a patient who presents some neurological symptoms.

Typically, the invention provides a method for predicting the onset of neurological symptoms or the remission from neurological symptoms in a patient suffering from remitting-relapsing multiple sclerosis, said method comprising the steps of:

- administering to said patient a detectable amount of an imaging agent comprising a selectin ligand,
- subjecting said patient to MRI,
- collecting the MRI signal,
- quantifying the percentage of signal void,
- comparing said percentage of signal void to a predetermined value,

wherein an increase in the percentage of signal void compared to the predetermined value is indicative of a risk of relapse in a patient who does not present any neurological symptoms, and/or wherein a decrease in the percentage of signal void compared to the predetermined value is indicative of a chance of remission in a patient who presents neurological symptoms.

Prediction is based on the comparison of the signal collected by MRI in a given patient to a reference signal.

Typically, the MRI signal for a patient can be quantified by quantifying the percentage of signal void in the images collected from said patient, wherein the percentage of signal void is defined as the percent ratio of the selectin positive signal over the total area of the imaged zone (e.g. brain and/or spinal cord). The percentage of signal void is representative of the number of lesions present in the brain and/or spinal cord.

Typically said percentage of signal void is compared to a predetermined value v1 or v2. If the percentage of signal void measured for a patient is higher than the predetermined value v1, the patient is deemed to be at risk of presenting a relapse. Conversely, if the percentage of signal void measured for said patient is lower than the predetermined value v2, the patient is deemed to have an increased chance of entering into remission.

Typically, the predetermined values can standard values observed in the general population of healthy control individuals. Alternatively, the predetermined values can be values corresponding to patients suffering from MS at predetermined stages of the disease.

5 In one embodiment, the predetermined values are values obtained during previous MRI imaging carried out on the same patient. In this case, the method of the invention serves to monitor the evolution of the RRMS in a given patient. According to this embodiment, an increase in the number of lesions is indicative of a risk of relapse. A decrease in the number of lesions is indicative of a chance of remission.

10 In another embodiment, the method for predicting the onset of neurological symptoms or the remission from neurological symptoms in a patient suffering from remitting-relapsing multiple sclerosis comprises the steps of:

- administering to said patient a detectable amount of an imaging agent comprising a
15 selectin ligand,
- subjecting said patient to ultrasound imaging such as CEUS,
- collecting the ultrasound signal,
- quantifying the mean acoustic signal from retained imaging agents
- comparing said mean acoustic signal to a predetermined value,

20 wherein an increase of the mean acoustic signal compared to the predetermined value is indicative of a risk of relapse in a patient who does not present any neurological symptoms, and/or wherein a decrease in the percentage of signal void compared to the predetermined value is indicative of a chance of remission in a patient who presents neurological symptoms.

Prediction is based on the comparison of the signal collected by ultrasound imaging in a
25 given patient to a reference signal.

According to the present invention, the imaging agent comprises a selectin ligand.

As used herein, the term “selectin” has its art understood meaning and refers to any member of the family of carbohydrate-binding, calcium-dependent cell adhesion molecules that
30 are constitutively or inductively present on the surface of leukocytes, endothelial cells or

platelets. The term “E-selectin”, as used herein, has its art understood meaning and refers to the cell adhesion molecule also known as SELE; CD62E; ELAM; ELAM1; ESEL; or LECAM2 (Genbank Accession Numbers for human E-selectin: NM_000450 (mRNA) and NP_000441 (protein)). As used herein, the term “L-selectin” has its art understood meaning and refers to the
5 cell adhesion molecule also known as SELL; CD62L; LAM-1; LAM1; LECAM1; LNHR; LSEL; LYAM1; Leu-8; Lyam-1; PLNHR; TQ1; or hLHRc (Genbank Accession Numbers for human L-selectin: NM_000655 (mRNA) and NP_000646 (protein)). The term “P-selectin”, as used herein, has its art understood meaning and refers to the cell adhesion molecule also known as a SELP; CD62; CD62P; FLJ45155; GMP140; GRMP; PADGEM; or PSEL (Genbank Accession
10 Numbers for human P-selectin: NM_003005 (mRNA) and NP_002996 (protein)).

As used herein, the term “selectin ligand” refers to compound that specifically binds to selectin. Selectin ligands include, but are not limited to, anti-selectin antibodies, sialyl Lewis X analogs and fucoidans.

15 In one embodiment, the selectin ligand is an antibody that specifically binds to selectin, preferably P-selectin.

Anti P-selectin antibodies have been extensively described in the art. Typically, the anti P-selectin antibody can be the polyclonal goat anti-mouse antibody commercialized by R&D Systems under reference AF737.

20 In one embodiment, said anti P-selectin antibody is a humanized antibody recognizing human P-selectin. Methods for obtaining such a antibodies are known in the art.

Any MRI imaging agent that can be coupled to a selectin ligand can be used in the method according to the invention.

25 In one embodiment said imaging agent is selected in the group consisting of microparticles of iron oxide, iron-containing colloidal particles and gadolinium chelates.

Suitable imaging agents that specifically bind to selectin have been disclosed in the following international patent applications: WO2007/020450 (Isis Innovation Limited) and
30 WO2010116209 (INSERM), the content of which is hereby incorporated by reference.

Any ultrasound imaging agent that can be coupled to a selectin ligand as described above can also be used in the method according to the invention.

In one embodiment said imaging agent is selected from an acoustically active (gas-filled) microbubble or an acoustically active lipid particle (*i.e.*: a gas-filled liposome).

Example of suitable ultrasound imaging agents that may be used in the practice of the invention have been described in Lindner JR et al., Circulation 2001, 104:2107-2112 and in Villanueva FS & Wagner WR, Nat Clin Pract Cardiovasc Med 2008, 5:S26-S32).

The invention also relates to the use of P-selectin as a biomarker for multiple sclerosis. Preferably, the invention relates to the use of spinal cord P-selectin as a biomarker for multiple sclerosis.

The invention also provides a method for treating a patient suffering from RRMS, said method comprising the steps of:

- administering to said patient a detectable amount of an imaging agent comprising a selectin ligand,
- subjecting said patient to an imaging technique such as MRI or ultrasound imaging,
- collecting the imaging signal (*i.e.*: MRI signal or ultrasound signal),
- quantifying the level of the signal (typically the percentage of signal void for MRI or the mean acoustic signal for ultrasound imaging),
- comparing said level of the imaging to a predetermined value,
- if an increase in the level of the imaging signal compared to the predetermined value is observed, administering a treatment in order to attenuate or prevent the neurological symptoms in said patient, or
- if a decrease in the level of the imaging signal compared to the predetermined value is observed, reducing the treatment for attenuating or preventing the neurological symptoms .

FIGURES LEGENDS

Figure 1: Schematic representation of P-Sel/MPIOs (microparticles of iron oxide).

Figure 2: P-Sel/MPIOs allow diagnosis and spatio-temporal monitoring of inflammation in spinal cord of mice with chronic EAE. (A) Experimental design of the experiments. (B) Typical course of chronic EAE. Evolution of clinical score is given in function of time. (C, E) Representative images of molecular imaging for P-Sel showing P-Sel positive vessels in lower (C) and upper (E) spinal cord in sham mice and EAE-MOG mice at CS1, CS2, CS3 and CS4. (D, F) Corresponding quantification of signal void (D: lower spinal cord, E: upper spinal cord) due to P-Sel/MPIOs (n=5 per group, *p< 0.05, NS: non significant).

Figure 3: Spatial and temporal dissemination of blood-spinal cord opening in mice with chronic EAE. (A) Images of Evan's blue extravasation in the spinal cord of sham and EAE animals at indicated clinical scores. (B) Ex vivo detection of Evan's Blue fluorescence in the same animals and (C) corresponding quantifications (n=5 per group, *p< 0.05).

Figure 4: P-Sel/MPIOs allow diagnosis and spatio-temporal monitoring of inflammation in spinal cord of mice with relapsing-remitting EAE. (A) Experimental design of the experiments. (B) Typical course of relapsing-remitting EAE. Evolution of clinical score is given in function of time. (C, E) Representative images of molecular imaging for P-Sel showing P-Sel positive vessels in lower (C) and upper (E) spinal cord in sham mice and EAE-MOG mice during presymptomatic, surge, recovery and relapse phases. (D, F) Corresponding quantification of signal void (D: lower spinal cord, E: upper spinal cord) due to P-Sel/MPIOs (n=5 per group, *p< 0.05, NS: non significant).

Figure 5: Spatial and temporal dissemination of blood-spinal cord opening in mice with relapsing-remitting EAE. (A) Images of Evan's blue extravasation in the spinal cord of sham and EAE animals at indicated clinical phases. (B) Ex vivo detection of Evan's Blue fluorescence in the same animals and (C) corresponding quantifications (n=5 per group, *p< 0.05).

Figure 6: Prediction of first relapse and remission by P-Sel/MPIOs in relapsing-remitting EAE. (A) Representative images of molecular imaging for P-Sel showing P-Sel positive vessels in upper and lower spinal cord in mice with a clinical score of 0 (presymptomatic phase). Left:

animals with no or negligible P-Sel staining, Right: animals showing substantial P-Sel staining. Clinical score at the day of P-Sel imaging (d0) and the day after (d1) are indicated. (B) Representative images of molecular imaging for P-Sel showing P-Sel positive vessels in upper and lower spinal cord in mice with a clinical score of 3 (first relapse phase). Right: animals showing substantial P-Sel staining, Left: animals with no or negligible P-Sel staining. Clinical score at the day of P-Sel imaging (d0) and the day after (d1) are indicated.

Figure 7: Prediction of first relapse in asymptomatic relapsing-remitting EAE animal by P-Sel/MPIOs. (A) Time course of the experimental session. (B) Representative images of molecular imaging for P-Sel obtained from asymptomatic animals (with a clinical score of 0) 10 days after PLP-EAE induction and showing P-Sel positive vessels in upper and lower spinal cord. Left: animals with no or negligible P-Sel staining, Right: animals showing substantial P-Sel staining. (C) Mean percentage of signal void measured in both groups. (D) Mean clinical scores at the day of P-Sel imaging (Day 10) and the day after (Day 11). (E) Four asymptomatic mice exhibited low P-Sel staining at the day of MRI (D10) all of them showed no relapse the day after. Conversely, four other asymptomatic mice exhibited high P-Sel staining at the day of MRI, all of them had relapse the day after.

Figure 8: Prediction of remission in symptomatic relapsing-remitting EAE animals by P-Sel/MPIOs. (A) Time course of the experimental session. (B) Representative images of molecular imaging for P-Sel obtained from animals at EAE onset (with a mean clinical score around 2) showing P-Sel positive vessels in upper and lower spinal cord. Left: animals showing substantial P-Sel staining. Right: animals with no or negligible P-Sel staining. (C) Mean percentage of signal void measured in both groups. (D) Mean clinical scores at the day of P-Sel imaging (Day 15) and the day after (Day 16). (E) Four symptomatic mice exhibited high P-Sel staining at the day of MRI, none of them had remission the day after. Conversely, four other symptomatic mice at the day of MRI (D15) exhibited low P-Sel staining all of them had a remission the day after.

Figure 9: Clearance of the P-Sel-MRI signal from the tissues (A) Time course of the experimental session. (B) Representative images of molecular imaging for P-Sel obtained from

EAE animals and showing P-Sel positive vessels in upper and lower spinal cord. MRI sessions were performed 10 min (upper panel), 5 hours (middle panel) and 24 hours (lower panel) after MPIO-P-Sel injection. (C): Mean percentage of signal void obtained at each MRI session in caudal (C) and rostral (D) spinal cord.

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Figure 10: Statistical Correlation between P-Sel MRI signal and lymphocyte infiltration in relation to clinical scores (CS). CD4+ infiltration increases with the EAE clinical score (from Sham to CS4) and is also positively correlated with the percentage of signal void, both in rostral (left panel) and caudal (right panel) spinal cord.

10

EXAMPLES

Material and methods

Experimental Autoimmune Encephalomyelitis (EAE):

15 *Chronic EAE* was induced in 9-week-old male C57BL6/J mice. Mice were immunized subcutaneously by two investigators blinded to treatment with 200 μ g of MOG₃₅₋₅₅ peptide (EAE animals) or phosphate buffered saline (Sham-operated animals) in Complete Freund Adjuvant (CFA) (Sigma) containing 800 μ g of *M. tuberculosis* (H37Ra, Difco). All animals receive 200 ng of pertussis toxin (Sigma) intraperitoneally (i.p.) at the time of immunization and
20 48 hours later.

Relapsing remitting EAE was induced in 7-8 week-old SJL/J female mice (Janvier, France). Mice were immunized subcutaneously by two investigators blinded to treatment with 200 μ g of PLP₁₃₉₋₁₅₁ peptide (EAE animals) or phosphate buffered saline (Sham-operated animals) in Complete Freund Adjuvant (CFA) (Sigma) containing 800 μ g of *M. tuberculosis* (H37Ra,
25 Difco). The emulsion was administered at four injection sites above the shoulder and the flanks. All animals were additionally intraperitoneally (i.p.) injected with 200 ng pertussis toxin derived from *Bordetella pertussis* (Sigma) in 200 μ L saline at the time of, and after 48 hours following immunization.

Clinical score (CS) of EAE: Mice were examined daily for clinical signs of EAE and were
30 scored as followed: CS 0, no disease; CS1, limp tail; CS2, hindlimb weakness; CS3, complete

hindlimb paralysis; CS4, hindlimb paralysis plus forelimb paralysis; and CS5, moribund or dead. Animal were euthanized if they showed a loss of weight superior to 10%. Clinical score was assessed daily by one person blinded to the treatment.

Targeting-moiety conjugation to MPIOs and molecular imaging : Polyclonal goat anti-mouse

5 antibodies for P-selectin (R&D Systems, AF737), were covalently conjugated to MPIOs (40µg of antibody per mg of MPIOs). Mice received intravenous injection of 2.0mg Fe/kg of conjugated MPIOs (200µL) and were immediately subjected to contrast-enhanced MRI. 3D T2*-GEFC (weighted gradient echo imaging with flow compensation) images presented are minimum intensity projections of 5 consecutive slices (Z resolution: 350µm). Signal voids quantification on
10 3D T2*-weighted images using automatic triangle threshold in Image J software (v1.45r). The quality of conjugated MPIOs was systematically checked in a naïve mouse, by stereotaxic injection of lipopolysaccharide (LPS; 1µg in 1µL) in the striatum (0.5mm anterior, 2.0mm lateral, -3mm ventral to the bregma).

Ex vivo quantification of Evan's blue extravasation: Evan's Blue (200 µl, 2%) was
15 intravenously injected in EAE mice 4h before termination and quantified in perfused spinal cords using the BioSpace™ Photoimager.

Tissue sampling, histology and immunohistochemistry: Mice were perfused with heparinised saline. Brains and spinal cords were harvested. Cryomicrotome-cut sections were incubated in the presence of the corresponding antibodies. All sections were examined with a Leica DM6000
20 microscope. Images were digitally captured using a coolsnap camera and visualized with Metavue software.

Statistical analyses: Results are the mean ± SEM. Statistical analyses were performed using Kruskal-Wallis followed by Mann-Whitney's U-test. Statistical significance was concluded for p<0.05. All allocation of animals to experimental and control groups were randomized, and
25 masking of observers to group assignment were incorporated into the design of the study.

Results:

**P-Sel/MPIOs allow monitoring of the spatial and temporal dissemination of inflammation
30 in spinal cord of EAE mice.**

Our first step was to develop a tool for molecular imaging based on the immunodetection of P-Sel adhesion molecule (Figure 1). We selected an antibody against P-Sel for its specificity and its capacity to stain the neuroendothelium in EAE, but not sham, animals (data not shown). These antibodies were then coupled to MPIOs (Figure 1), which we showed were able to bind the endothelium in inflammatory animals after i.v. injection (data not shown).

Using this tool, we were able to show a spatial and temporal dissemination of endothelial activation in chronic EAE (Figure 2). Animals were injected i.v. with P-Sel/MPIOs (Figure 2a) at different stages of chronic EAE corresponding to clinical scores 1 to 4 (Figure 2b). Molecular MRI in the lower and upper part of the spinal cord showed a gradual increase in signal void in a caudo-rostral direction along with the increase in clinical score. (Figure 2c-f) This spatio-temporal dissemination matched the caudo-rostral gradient over time of blood-spinal cord opening shown by the optical and fluorescent assessment of Evan's Blue extravasation (Figure 3a-c). The downstream infiltration of T4 lymphocytes and neutrophils also occurred following this caudo-rostral progression over time (data not shown).

In a second part, we applied the same methods to assess the relevance of this imaging tool in relapsing-remitting EAE (Figure 4-5). P-Sel/MPIOs revealed endothelial activation in early disease, during surge and during relapse in lower and upper parts of the spinal cord (Figures 4c-f), while little if any hyposignal was observed in remitting animals (recovery, Figures 4c-f). Confirming what shown above in chronic EAE, signal void in relapsing remitting EAE matched blood-spinal cord opening (Figure 5) and leukocyte infiltration (data not shown).

Overall, these data indicate that P-Sel/MPIOs enable a follow-up by molecular imaging of disease progression in the spinal cord of animals subjected to two EAE models mimicking the progressive and relapsing-remitting forms of MS. Moreover, the hyposignal due to P-Sel/MPIOs is representative of endothelial activation, blood-spinal cord barrier opening and leukocyte infiltration.

P-Sel/MPIOs allow prediction of first relapse in asymptomatic animals and remission in symptomatic animals.

We further assessed whether molecular imaging using P-Sel/MPIOs could predict changes in symptomatology in relapsing-remitting EAE.

First, we assessed the ability of this tool to predict the appearance of a first relapse in asymptomatic animals (Figure 6a and Figure 7). Asymptomatic animals (clinical score 0 ± 0)

were injected i.v. with P-Sel/MPIOs at day 10 before EAE onset and subjected to molecular imaging (Figure 7A). Animals with little or no hyposignal (figure 7C) showed no relapse the day after MRI (Clinical score 0 ± 0 ; Figures 6a and 7D-E). Conversely, animals showing substantial hyposignal (Figure 7C) declared a first relapse the day after (Clinical score 2.5 ± 0.5 ; Figures 6a and 7D-E).

Furthermore, histological analysis demonstrated that the presence of P-Sel MRI signal in asymptomatic animals (at Day 10), which predicts the appearance of a first relapse is correlated with early demyelination in both rostral and caudal spinal cord (data not shown)

Second, we assessed the ability of this tool to predict a phase of remission in symptomatic animals (Figures 6b and 8). Symptomatic animals (clinical score 1.875 ± 1.057) were injected i.v. with P-Sel/MPIOs at disease peak (day 15) and subjected to molecular imaging (figure 8A). Animals with substantial hyposignal (Figure 8C) showed no remission the day after MRI (Clinical score 2.5 ± 0.58 ; Figures 6b and 8D-E). Conversely, animals showing little or no hyposignal (Figure 8C) experienced a remission the day after (Clinical score 0.5 ± 0.58 ; Figure 6b and 8D-E).

P-Sel MRO signal is cleared from the tissues within the first 24 hours after injection.

EAE Animals (Clinical score 2) were injected i.v. with P-Sel/MPIOs and subjected to successive molecular imaging sessions of the lower and upper parts of the spinal cord, performed 10 min, 8h and 24h after the injection (Figure 9A). The results showed a progressive decrease of the signal during the first 24 hours after the injection illustrating its clearance from the tissues.

P-Sel MRI signal appears specifically within the white matter of the spinal cord.

Our results showed (data not shown) that the signal obtained from EAE animals is indeed absent in grey matter, which matches the physiopathology of the disease.

Lastly we have shown that P-SEL is preferentially expressed in small vessels ($> 10 \mu\text{m}$ of diameter) of mice subjected to MS model (EAE mice) (data not shown) and a correlation can be established between P-Sel MRI signal and lymphocyte infiltration, in relation to clinical score (see Figure 9).

Conclusion:

The molecular imaging tool developed in this work, targeting the endothelial adhesion molecule P-Sel, allows diagnostic follow-up of animals subjected to chronic or relapsing-remitting EAE models of MS. The use of this tool revealed the spatio-temporal pattern of dissemination of

neuroendothelial activation along the spinal cord in these two models. While the chronic model showed a slow progression of neuroendothelial activation in a caudo-rostral direction, the relapsing remitting model showed a much more rapid progression, with neuroendothelial activation appearing in the whole spinal cord from the early stages. We also showed that, in both

5 models, P-Sel imaging, primarily targeting neuroendothelial activation, was representative of associated pathological processes, BSCB alteration and leukocyte infiltration. Again, differential patterns of BSCB alteration and leukocyte infiltration, matching those of endothelial activation, were noticed in the two models. Finally, we validated the use of P-Sel/MPIO as a predictive tool for relapse and remission in MS models. Indeed, the presence of P-Sel/MPIO-derived hyposignal

10 in asymptomatic animals specifically and accurately predicted the appearance of relapse. In addition, the disappearance of P-Sel/MPIO-derived hyposignal in symptomatic animals predicted their remission. Furthermore, we have established that the presence of the P-Sel MRI signal which predicts the appearance of a first relapse corresponds at the histological level to early demyelination.

15 This study is the first to show the validity of molecular imaging targeting P-Sel for exploration of MS-like disease in the spinal cord of mice subjected to chronic or relapsing-remitting EAE. It is also the first study to report the predictive value of this tool in the prognosis of relapse and remission in these models of multiple sclerosis.

CLAIMS

1. An imaging agent for use in a method for predicting the onset of neurological symptoms or the remission from neurological symptoms in a patient suffering from remitting-relapsing multiple sclerosis, wherein said imaging agent comprises a selectin ligand.
5
2. An imaging agent for use in a method according to claim 1, wherein said method comprises the step of imaging the central nervous system of said patient.
- 10 3. An imaging agent for use in a method according to claim 1 or 2, wherein said method comprises the step of imaging the spinal cord of said patient.
4. An imaging agent for use in a method according to claim 1 or 2, wherein said method comprises Magnetic Resonance Imaging (MRI) or ultrasound imaging.
15
5. An imaging agent for use in a method according to any of the above claims, wherein said selectin ligand is selected in the group consisting of anti-selectin antibodies, sialyl Lewis X analogs and fucoidans.
- 20 6. An imaging agent for use in a method according to any of the above claims wherein said selectin ligand is an antibody that recognizes P-selectin.
7. An imaging agent for use in a method according to any of the above claims wherein said imaging agent is selected in the group consisting of microparticles of iron oxide, iron-containing
25 colloidal particles and gadolinium chelates.
8. Use of P-selectin as a biomarker for multiple sclerosis.
9. A method for predicting the onset of neurological symptoms or the remission from
30 neurological symptoms in a patient suffering from remitting-relapsing multiple sclerosis, said method comprising the steps of:

- administering to said patient a detectable amount of an imaging agent comprising a selectin ligand,
- subjecting said patient to MRI,
- collecting the MRI signal,
- 5 - quantifying the percentage of signal void,
- comparing said percentage of signal void to a predetermined value,

wherein an increase in the percentage of signal void compared to the predetermined value is indicative of a risk of relapse in a patient who does not present any neurological symptoms,

and/or wherein a decrease in the percentage of signal void compared to the predetermined value
10 is indicative of a risk of remission in a patient who presents neurological symptoms.

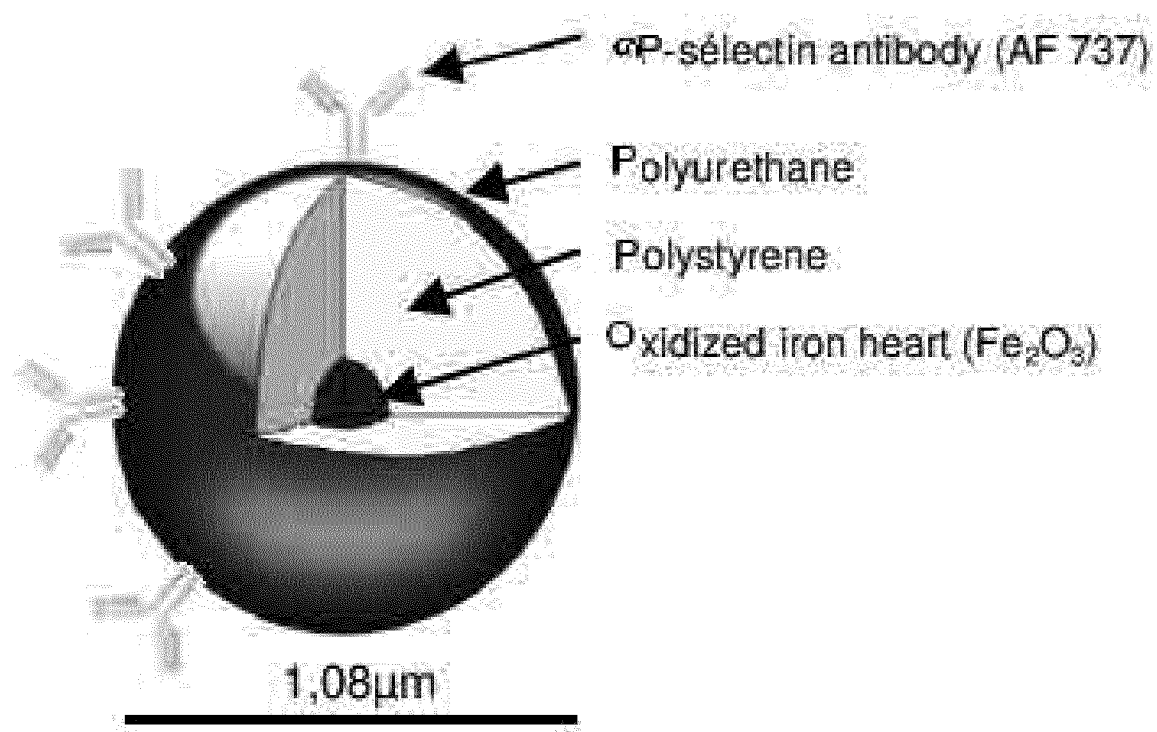


Figure 1

a

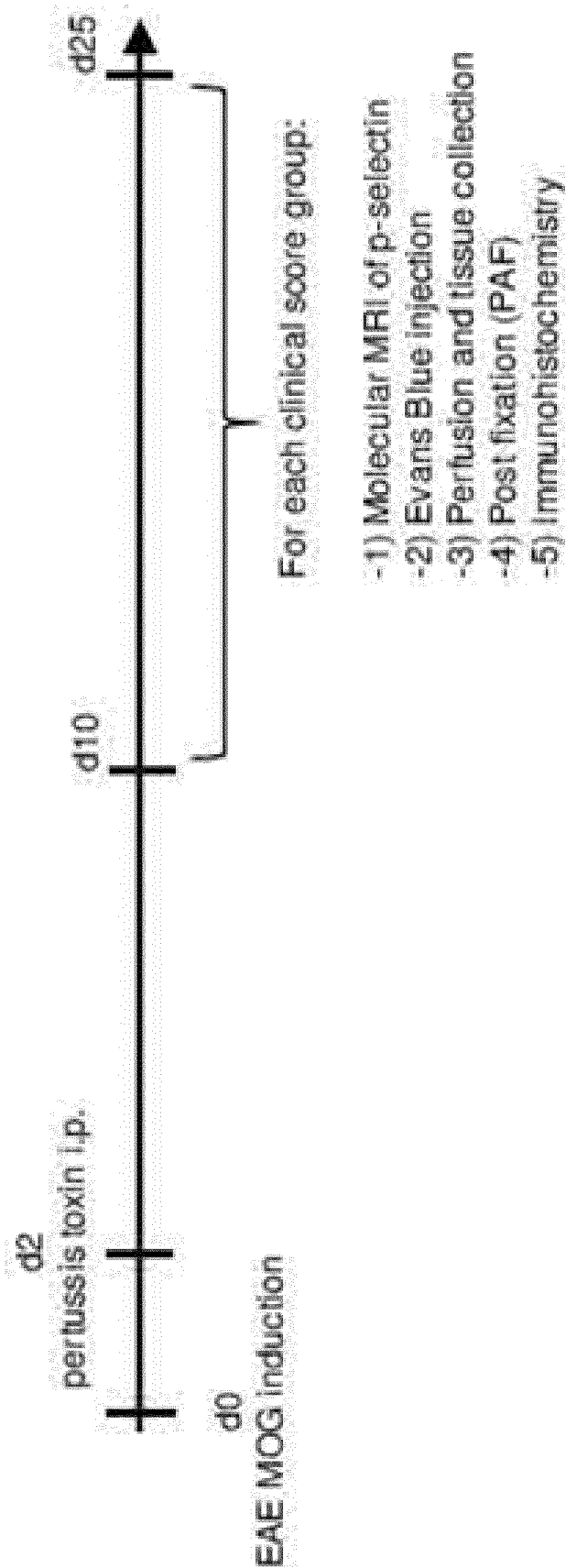


Figure 2

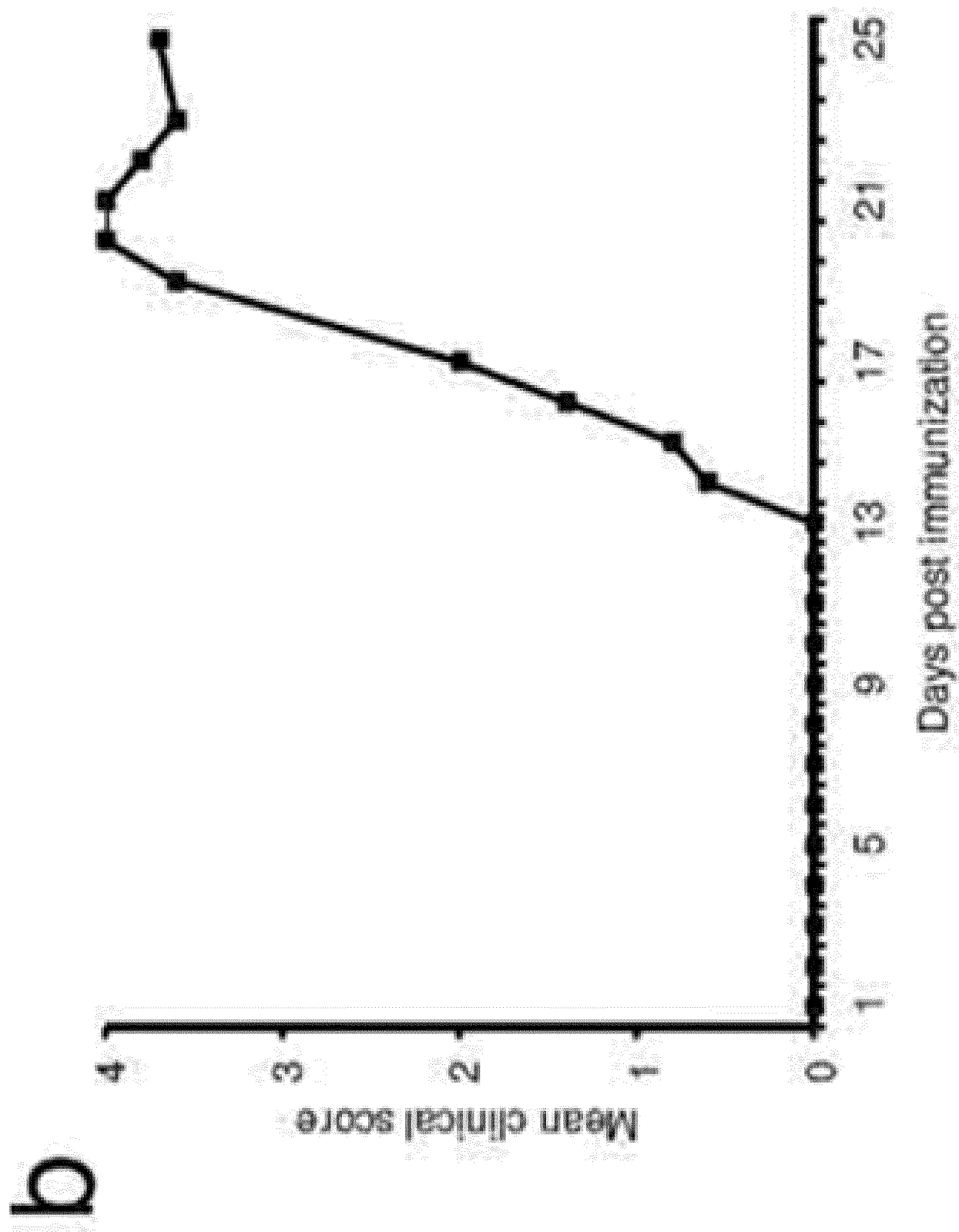
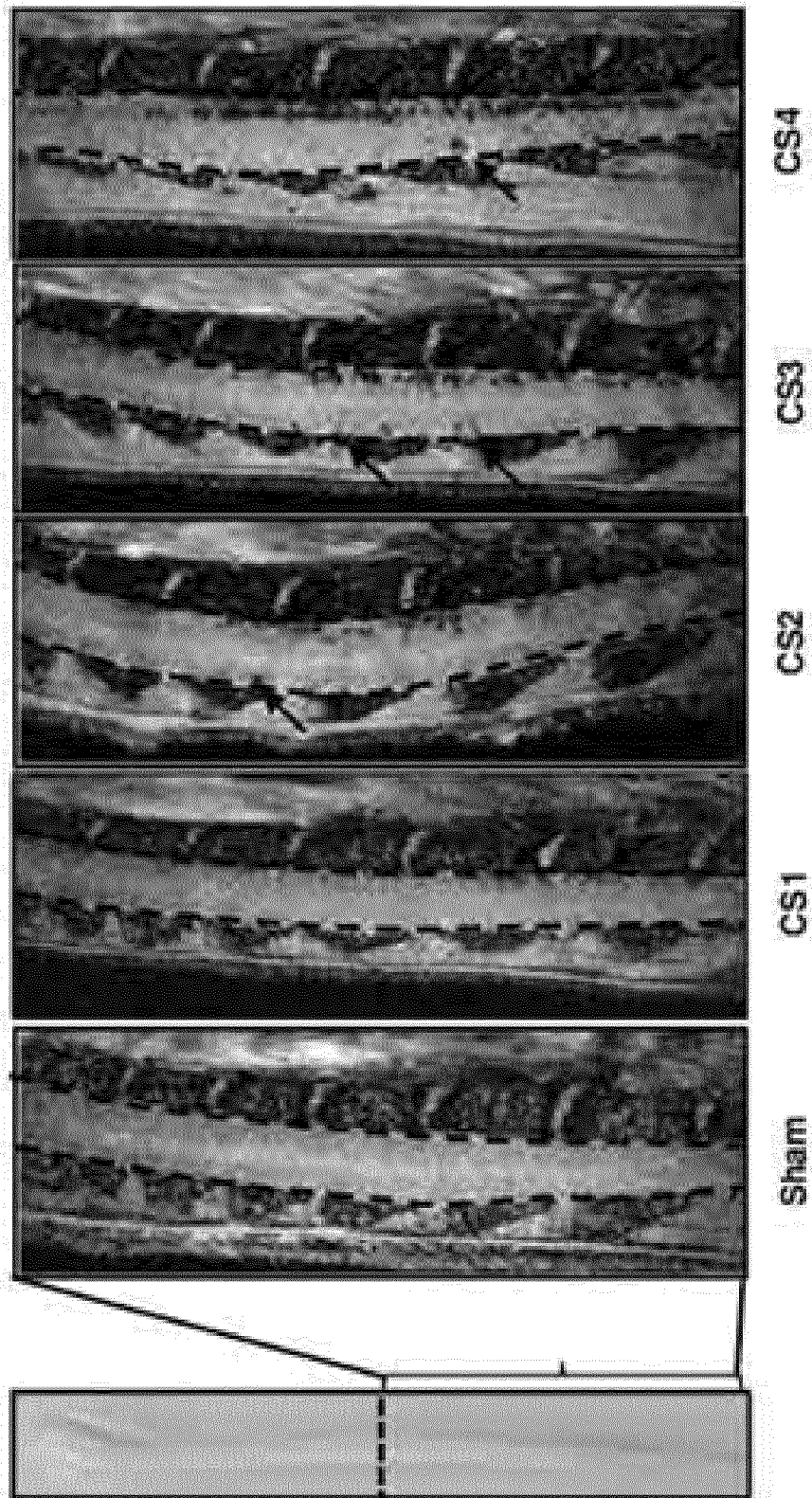


Figure 2 (continued)



C

Figure 2 (continued)
SUBSTITUTE SHEET (RULE 26)

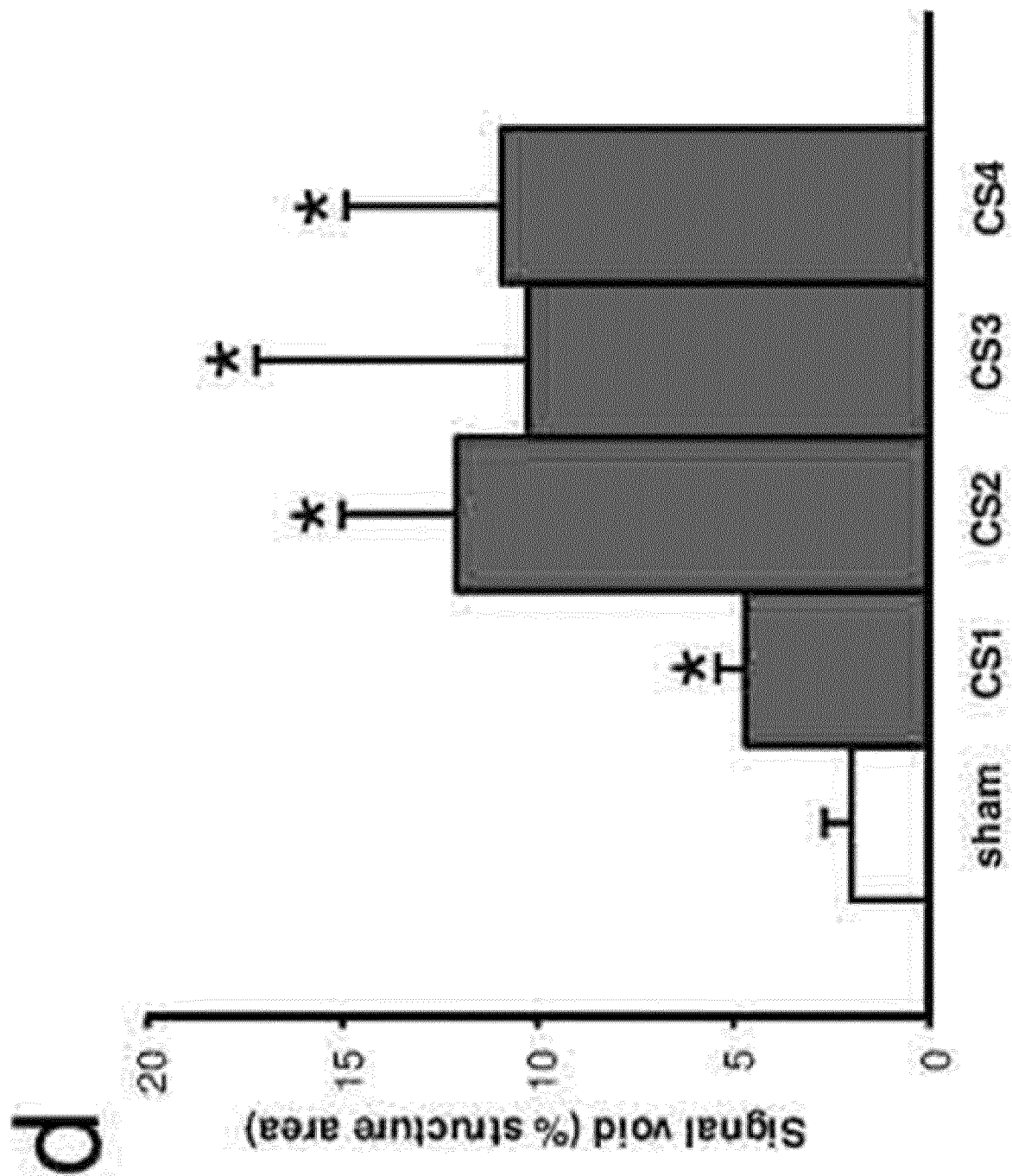
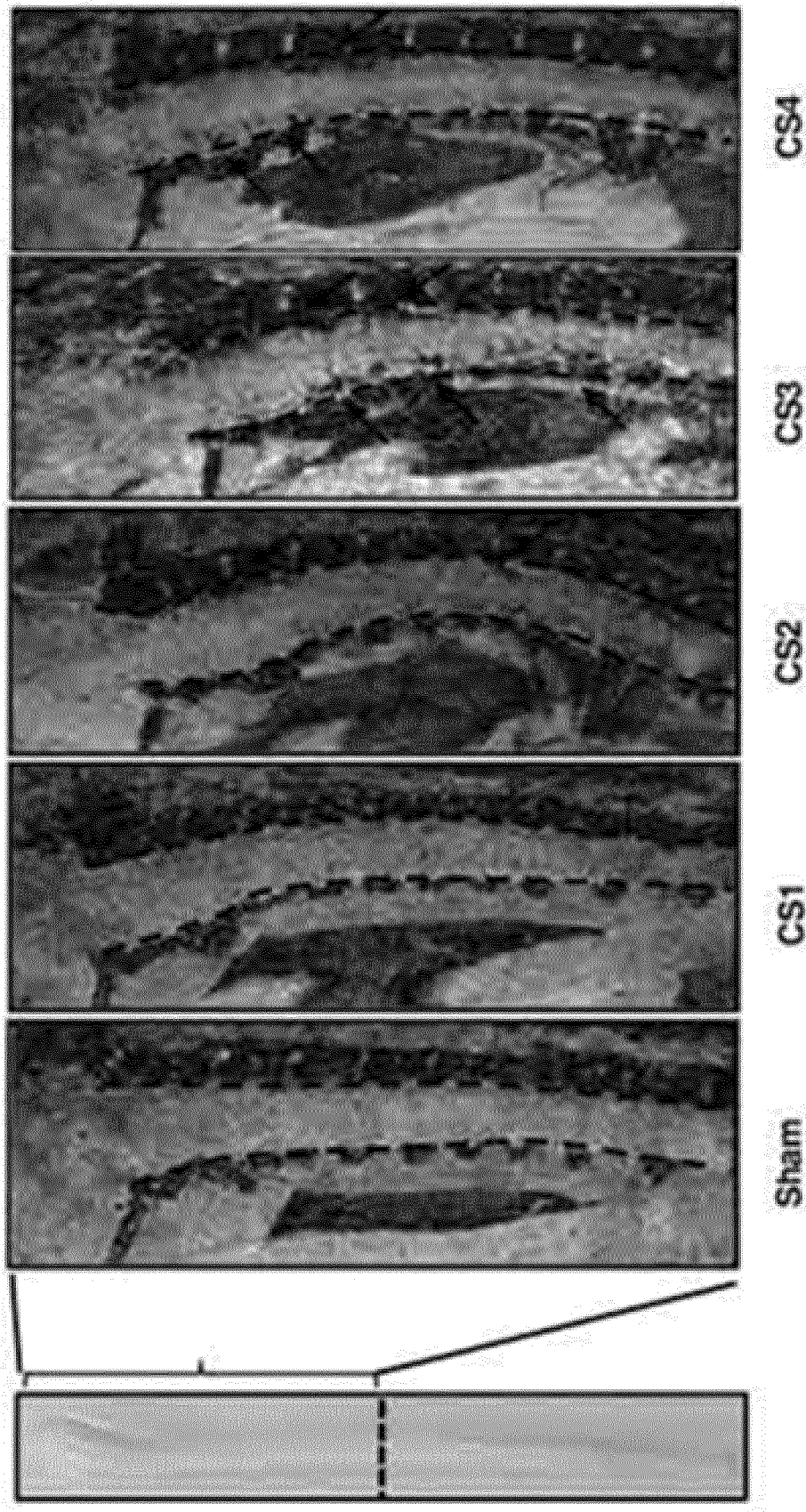


Figure 2 (continued)



e

Figure 2 (continued)
SUBSTITUTE SHEET (RULE 26)

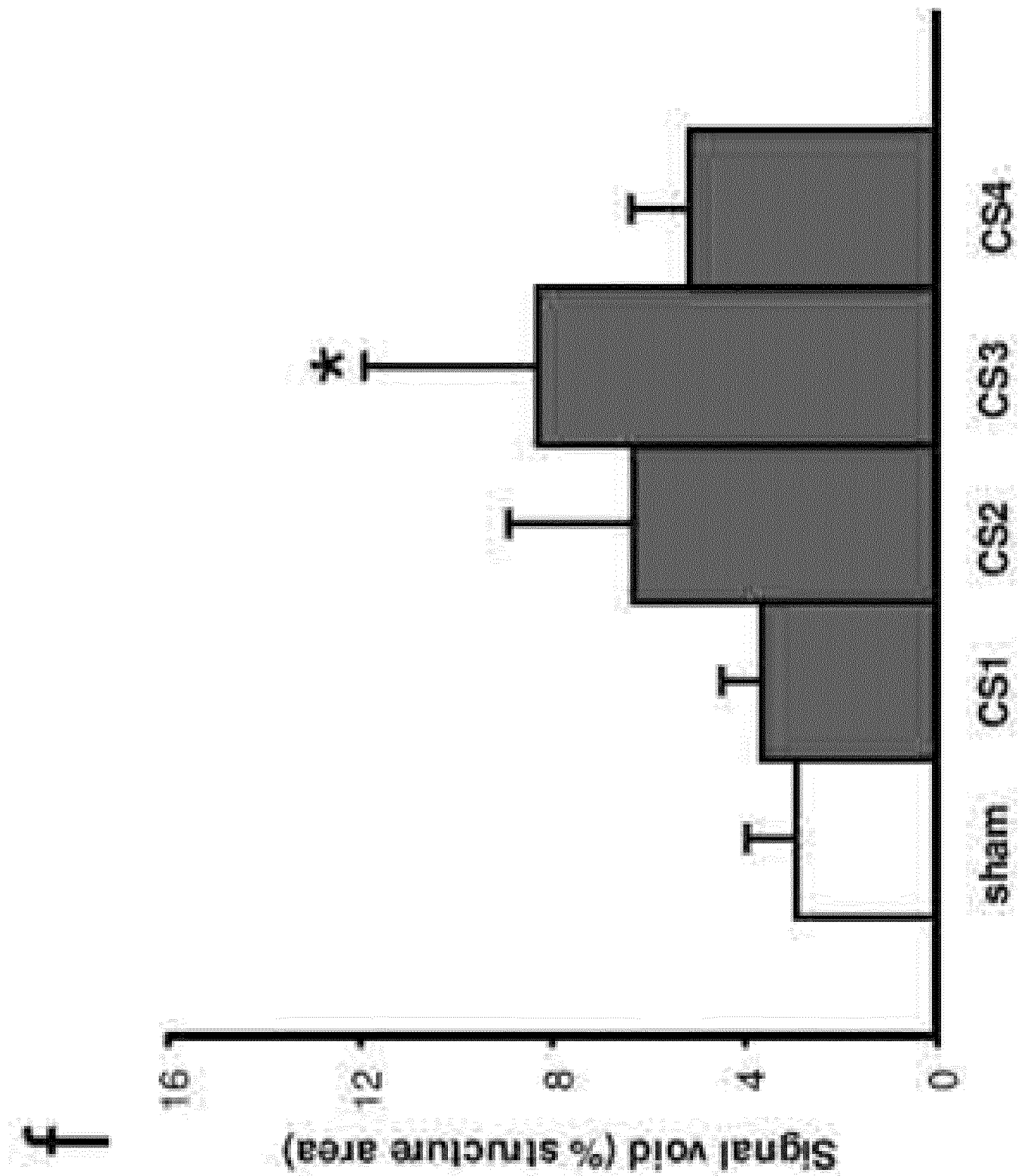
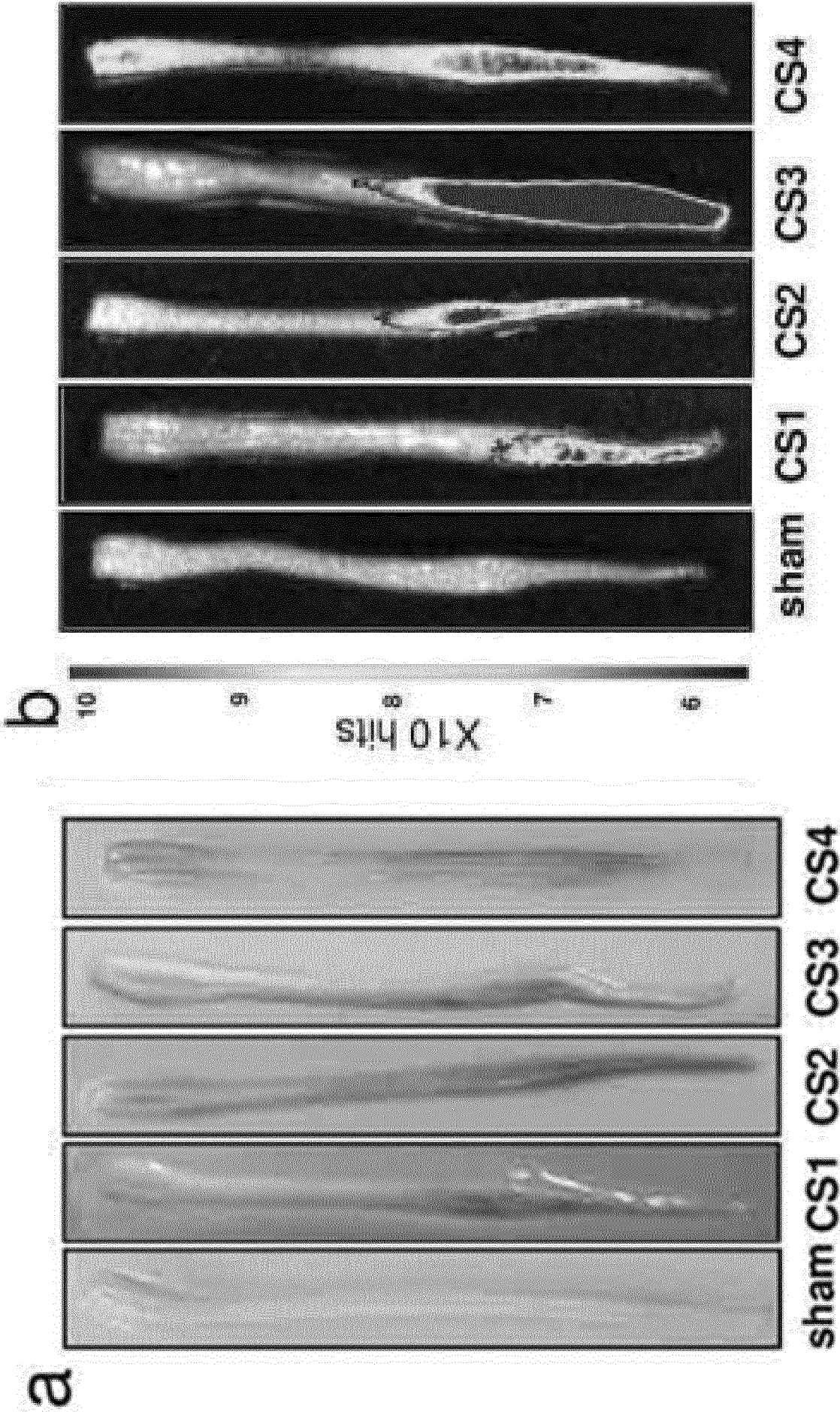


Figure 2 (continued)



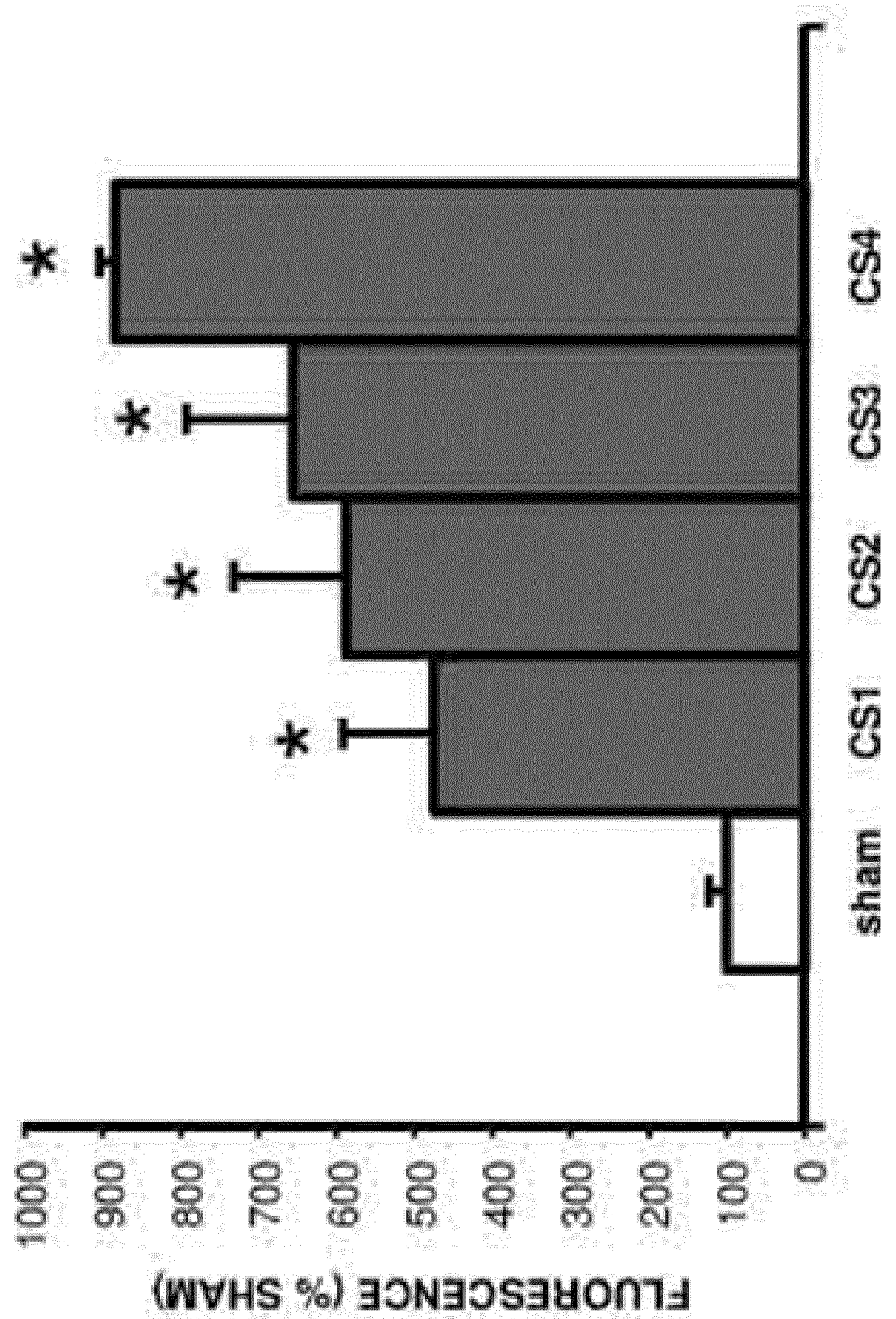


Figure 3 (continued)

a

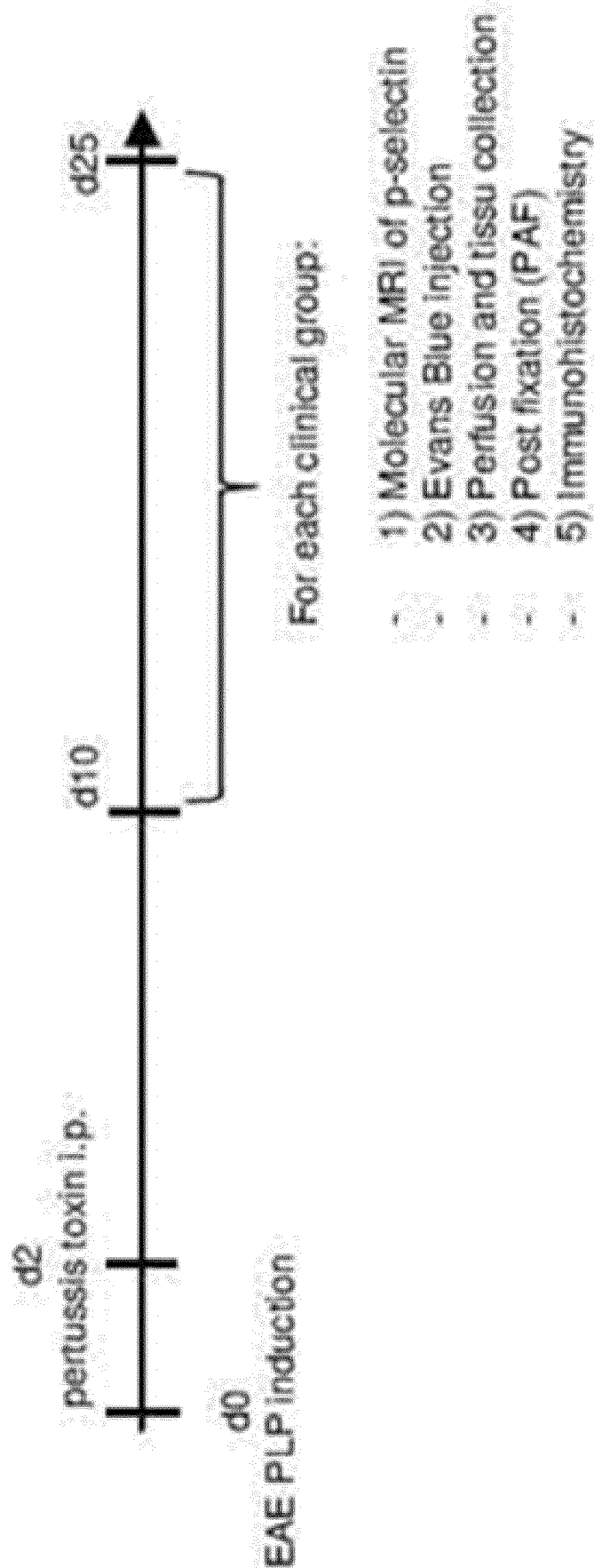


Figure 4

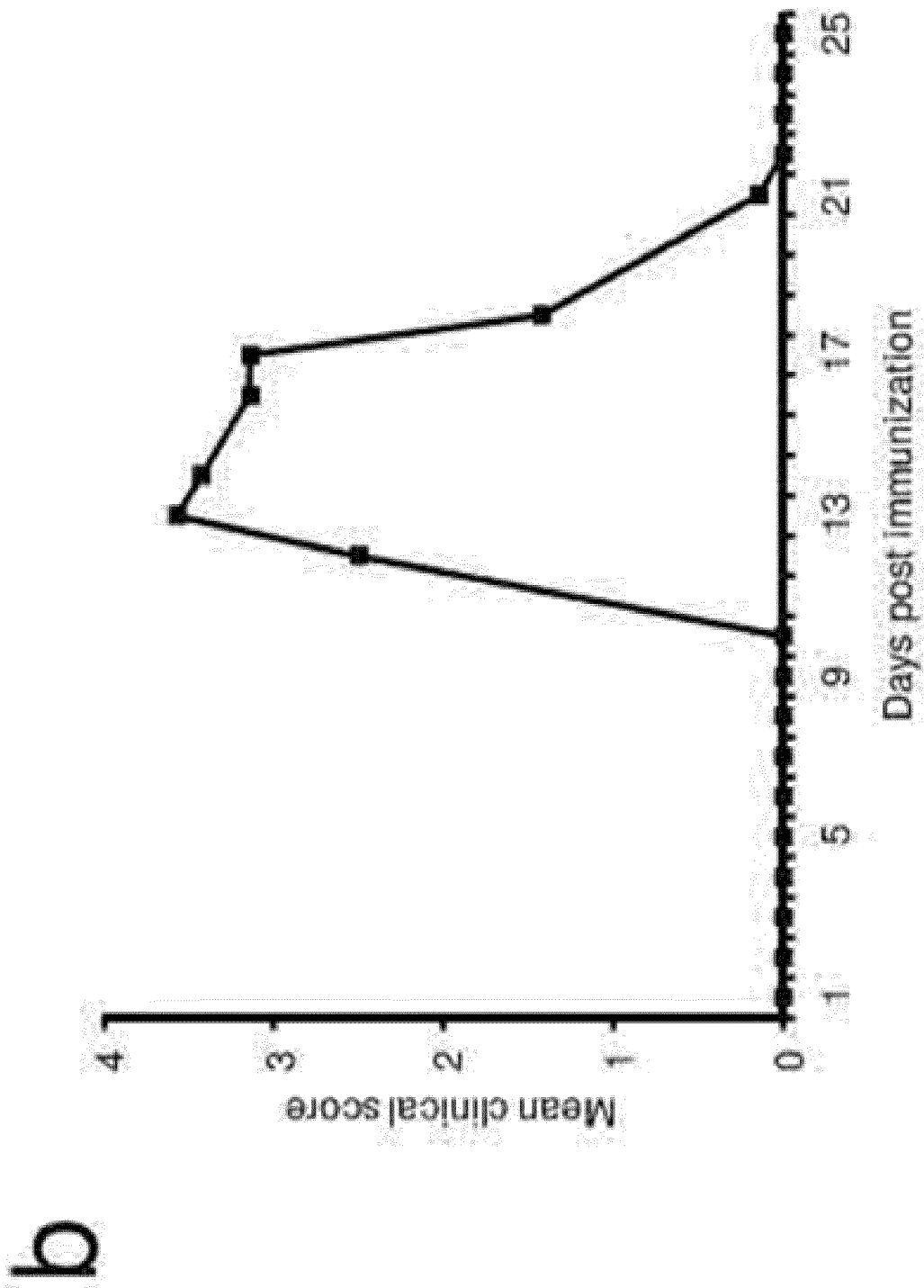


Figure 4 (continued)

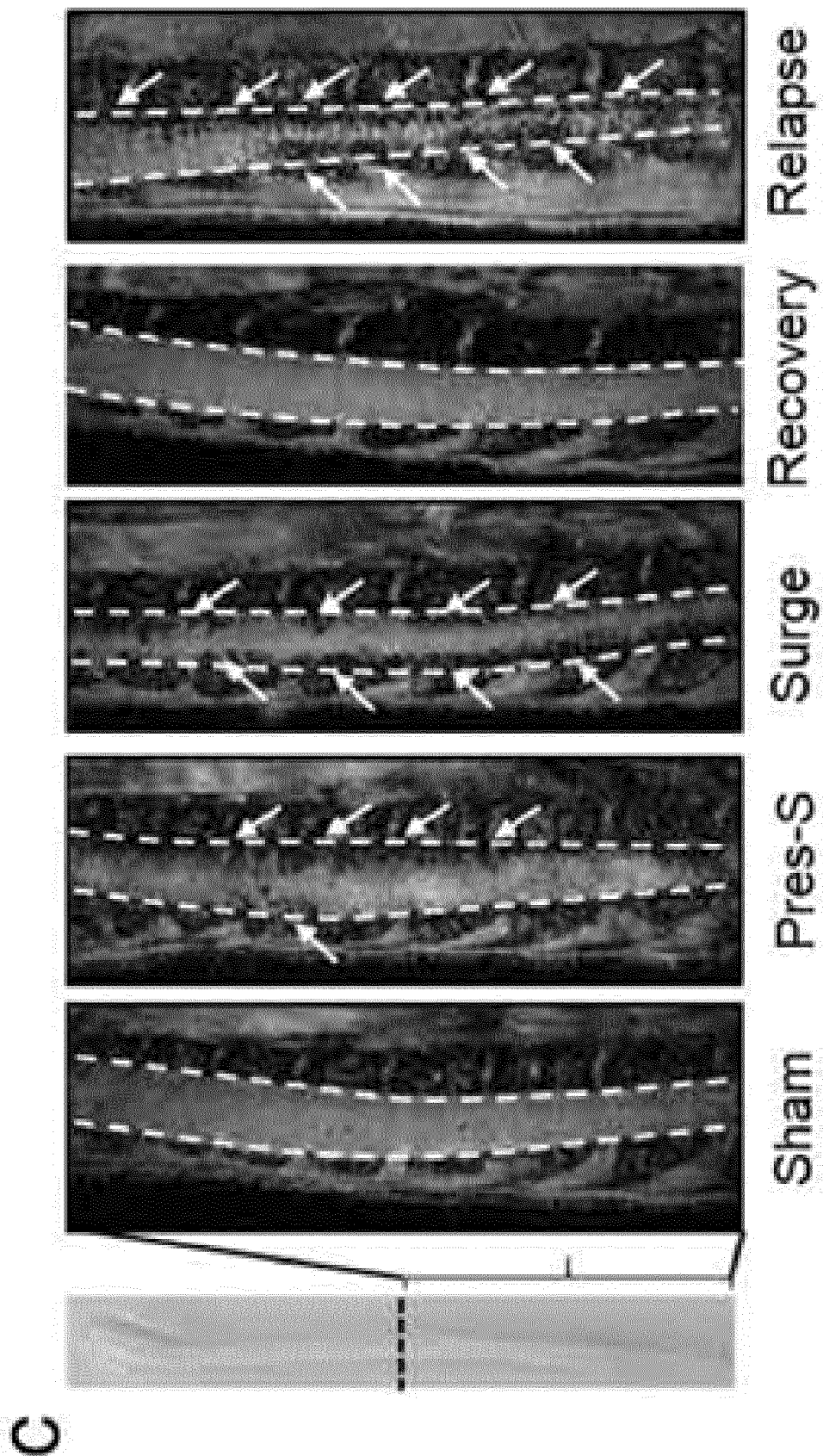


Figure 4 (continued)

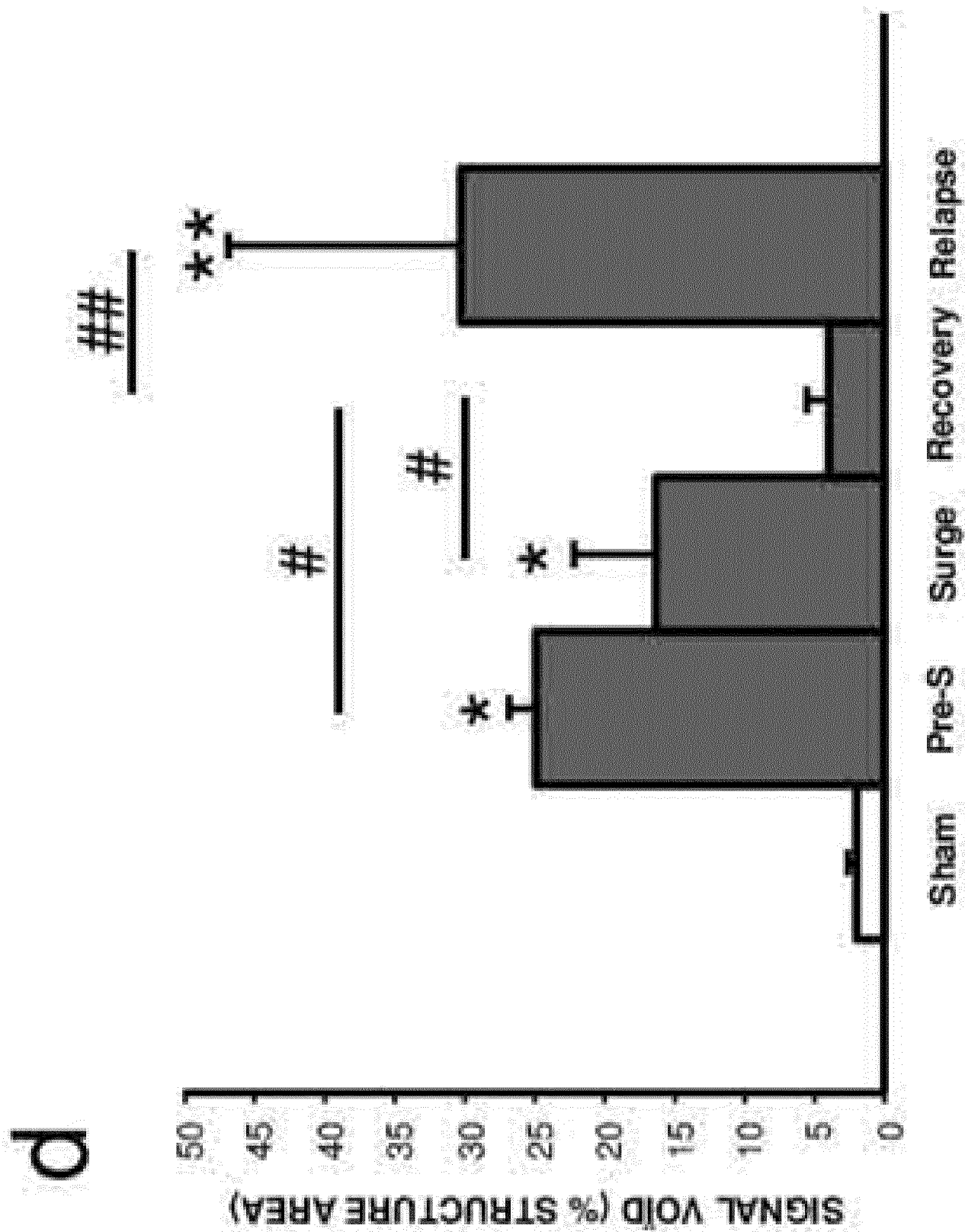


Figure 4 (continued)

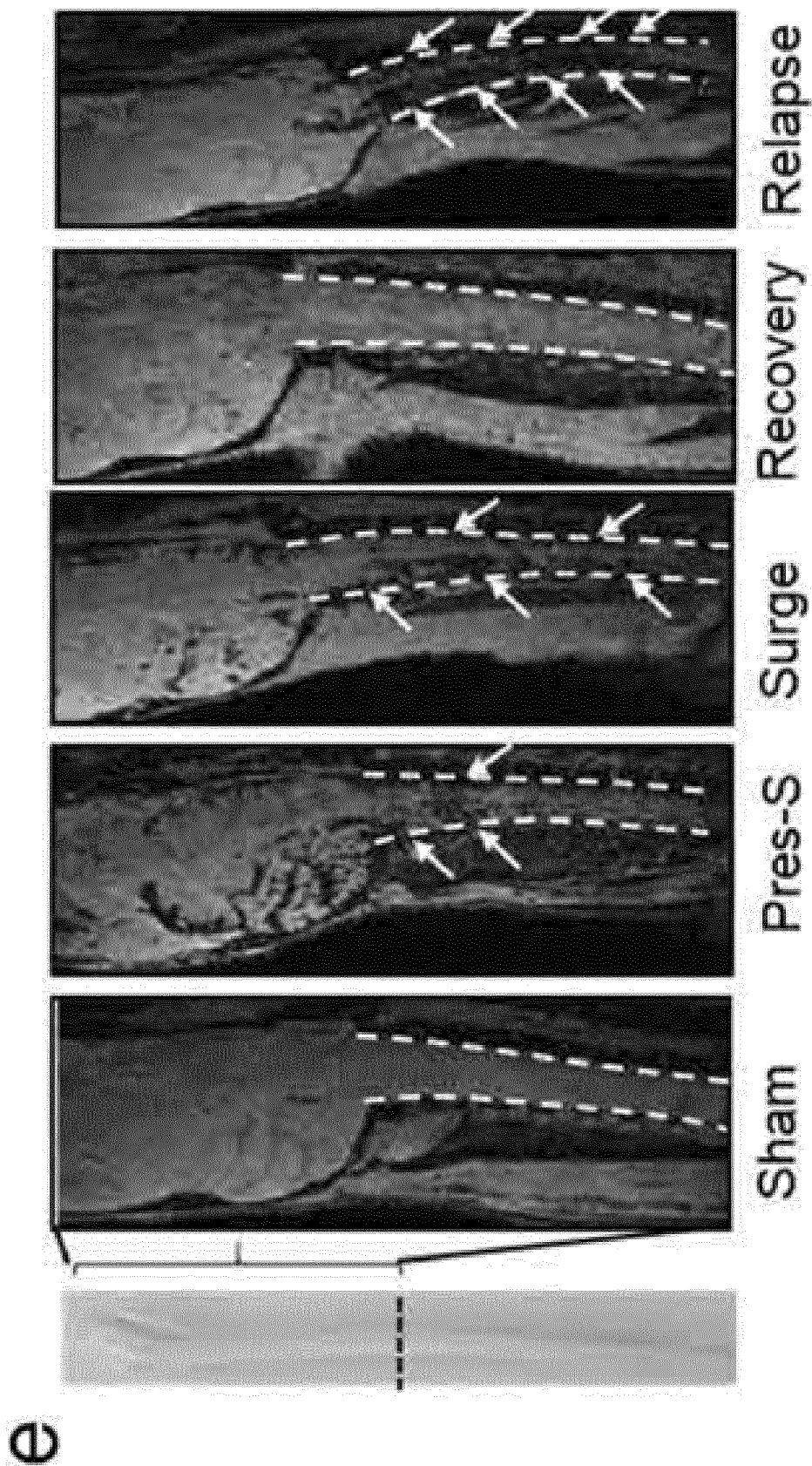


Figure 4 (continued)

f

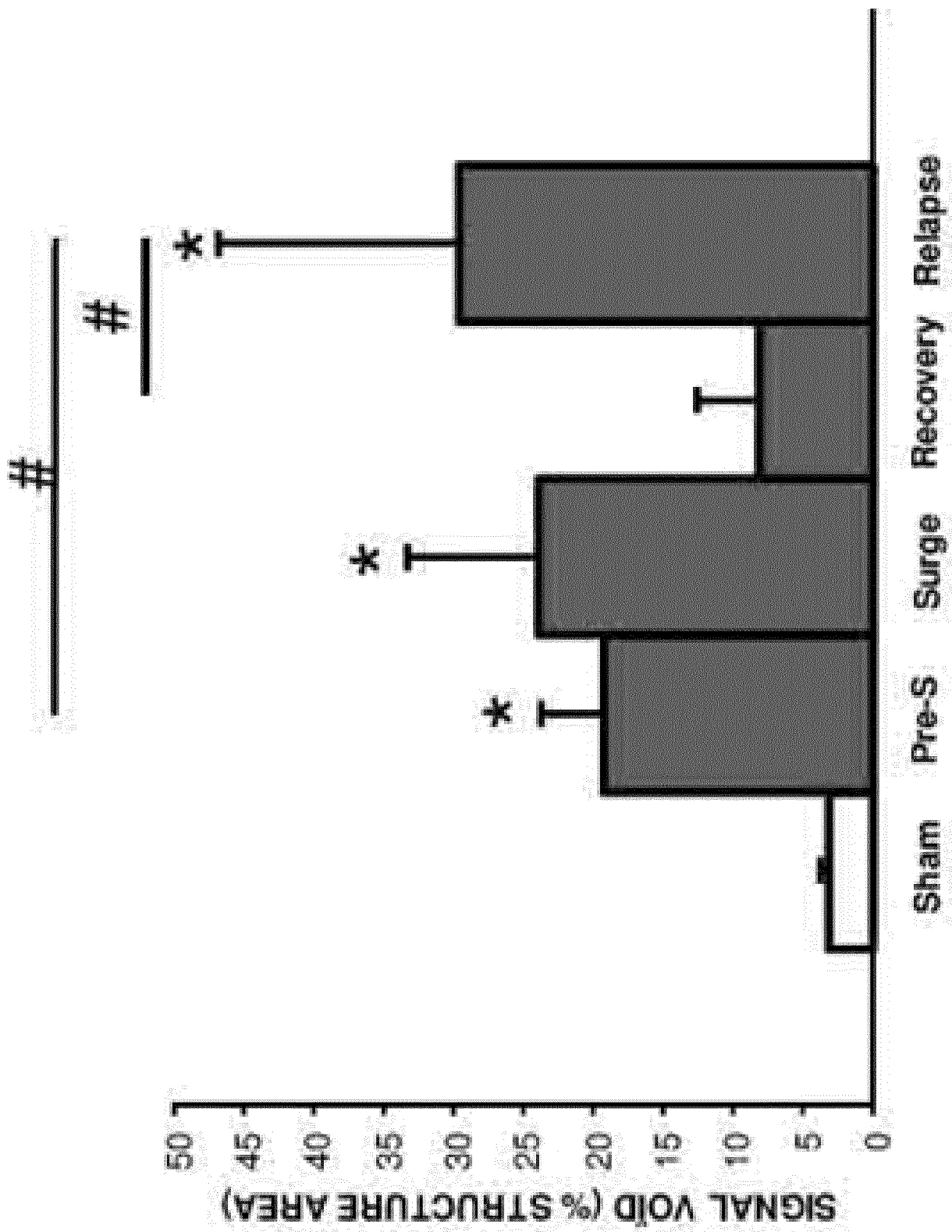
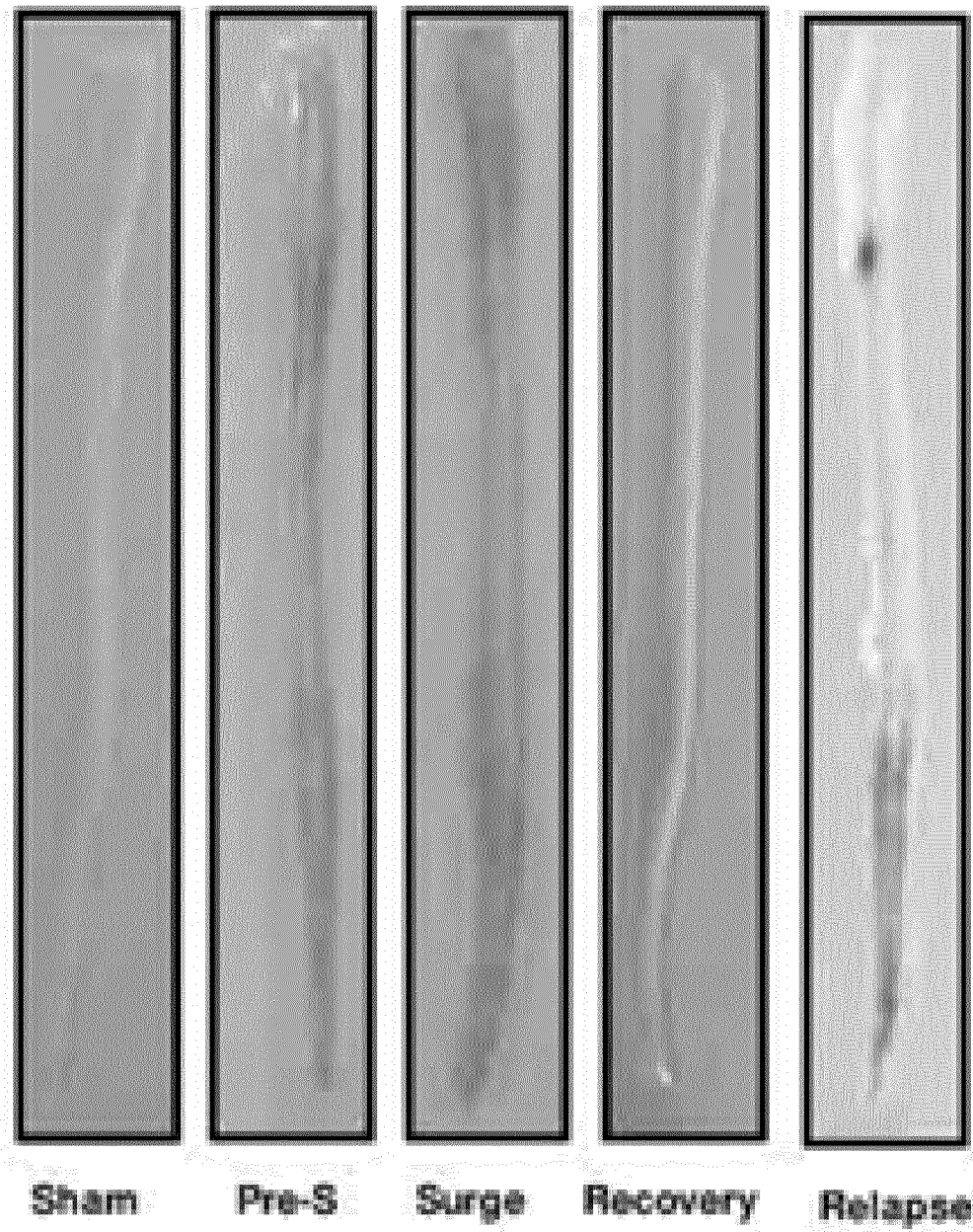
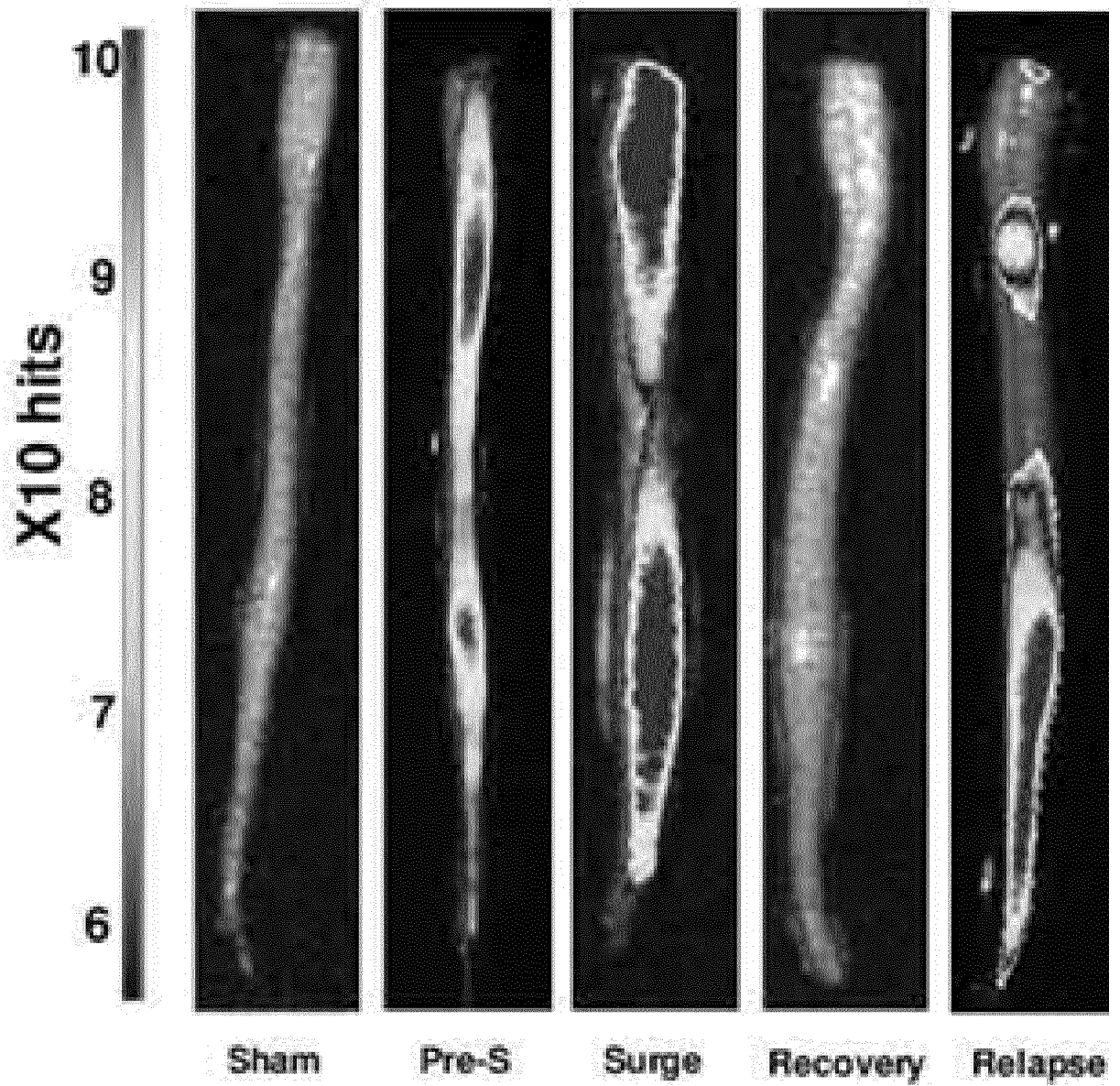


Figure 4 (continued)

a**Figure 5**

b**Figure 5 (continued)**

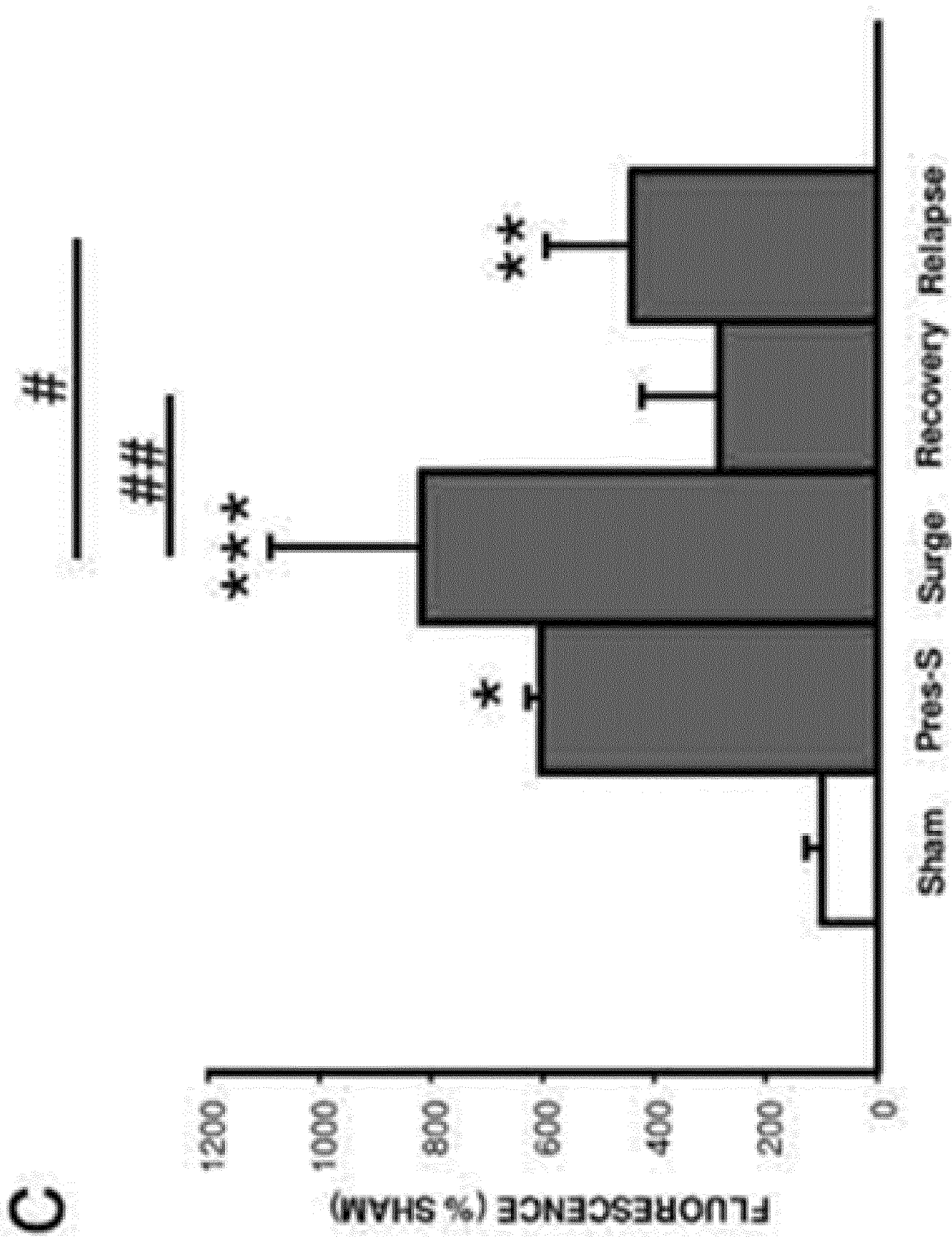


Figure 5 (continued)

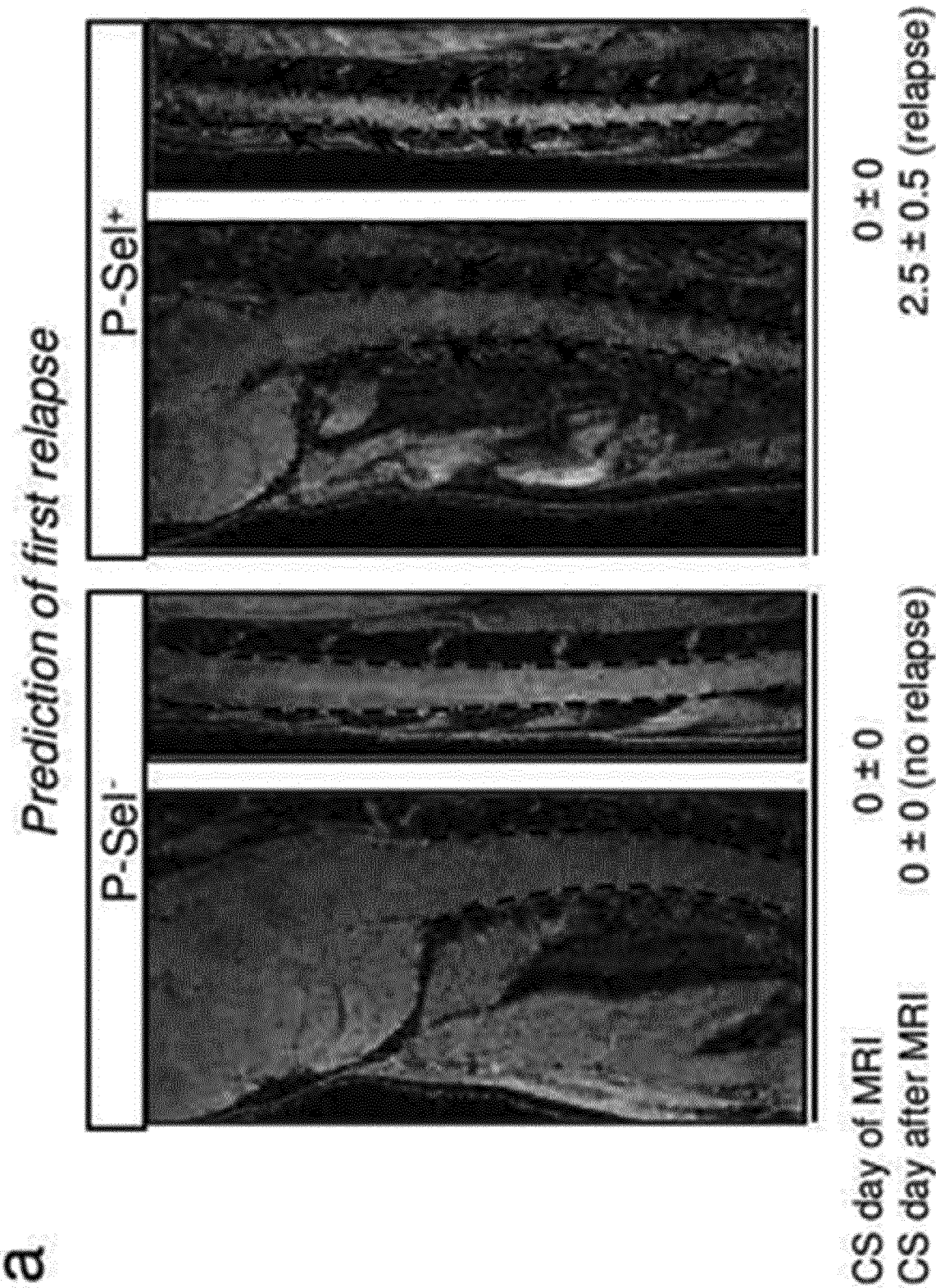


Figure 6
SUBSTITUTE SHEET (RULE 26)

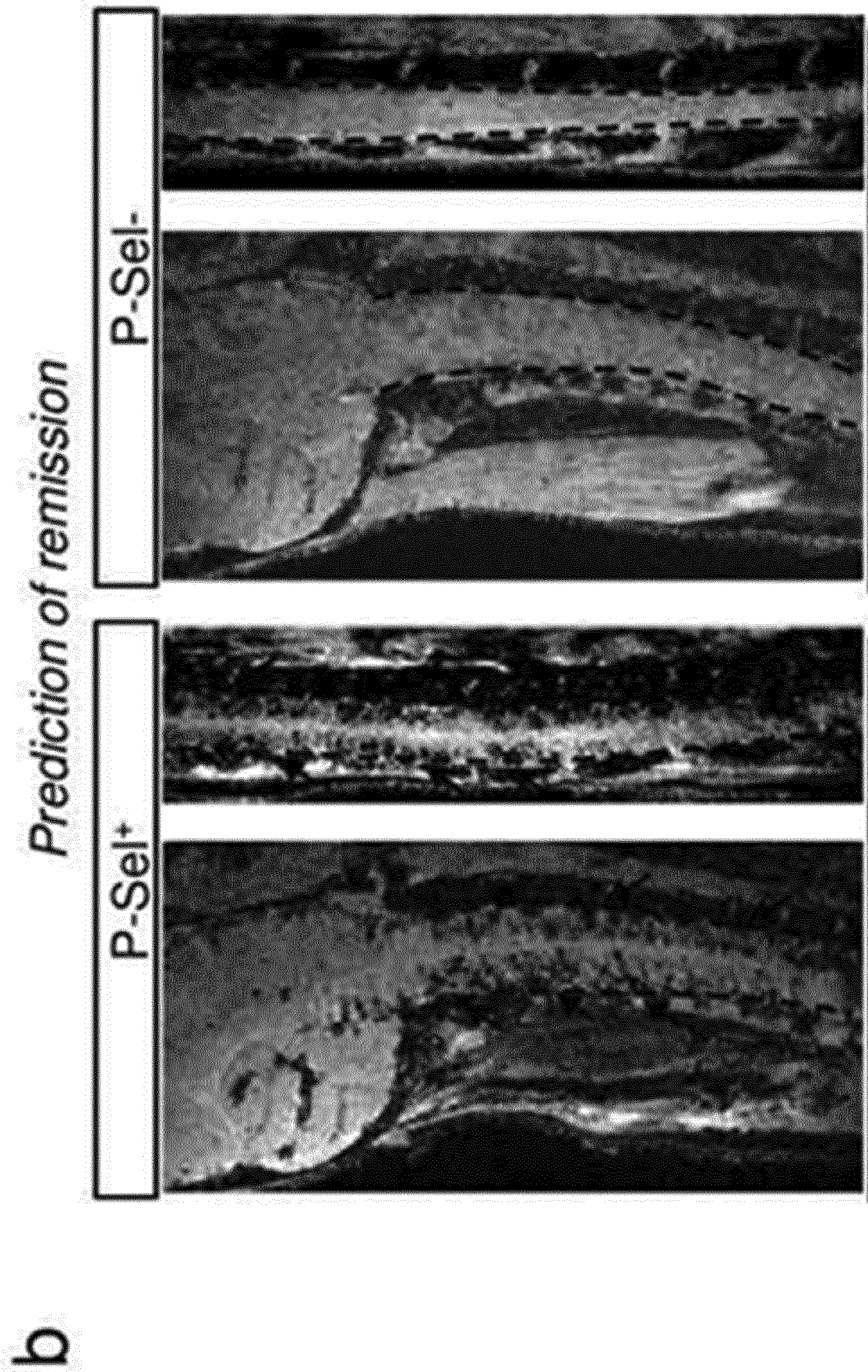


Figure 6 (continued)
SUBSTITUTE SHEET (RULE 26)

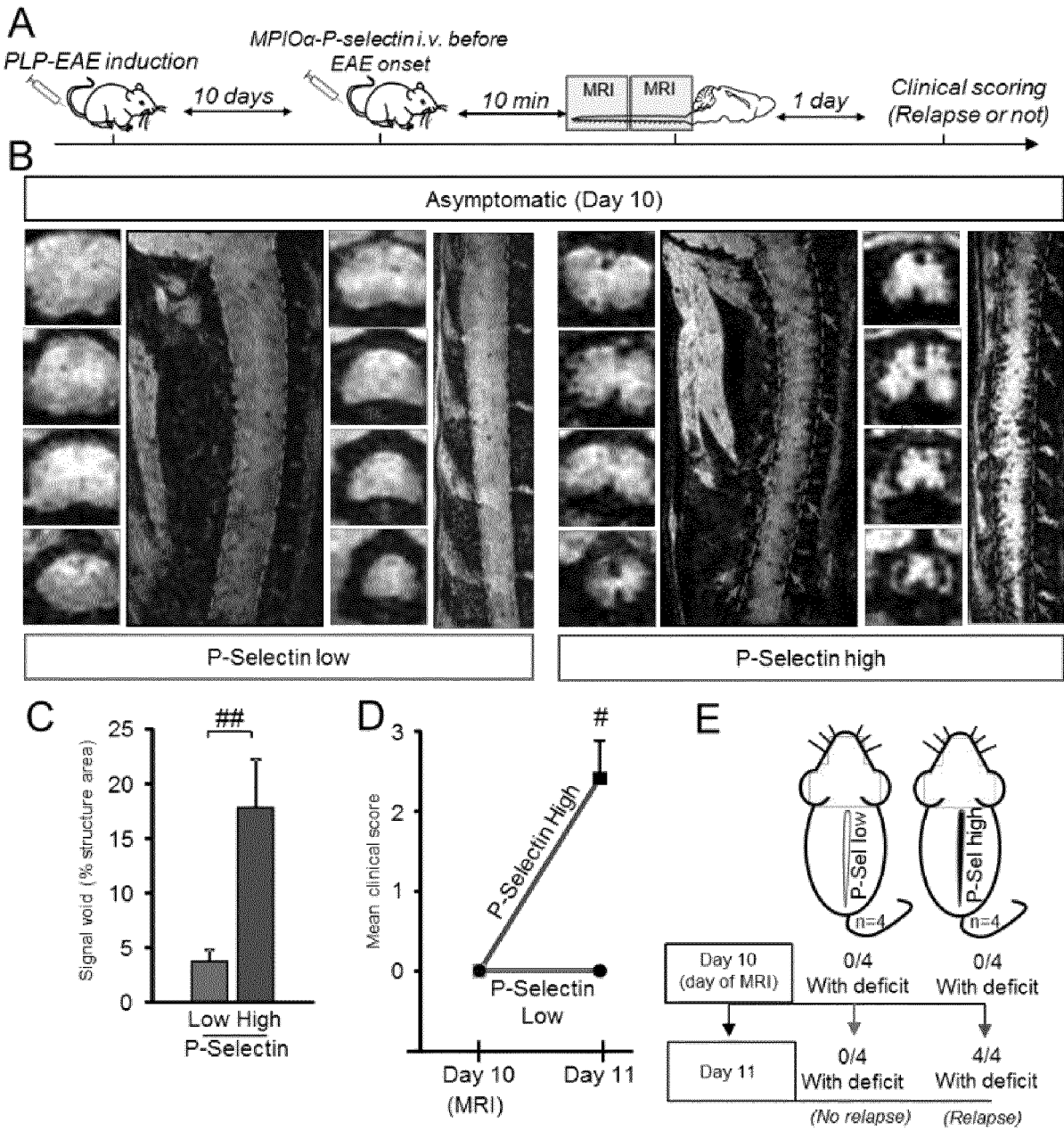


Figure 7

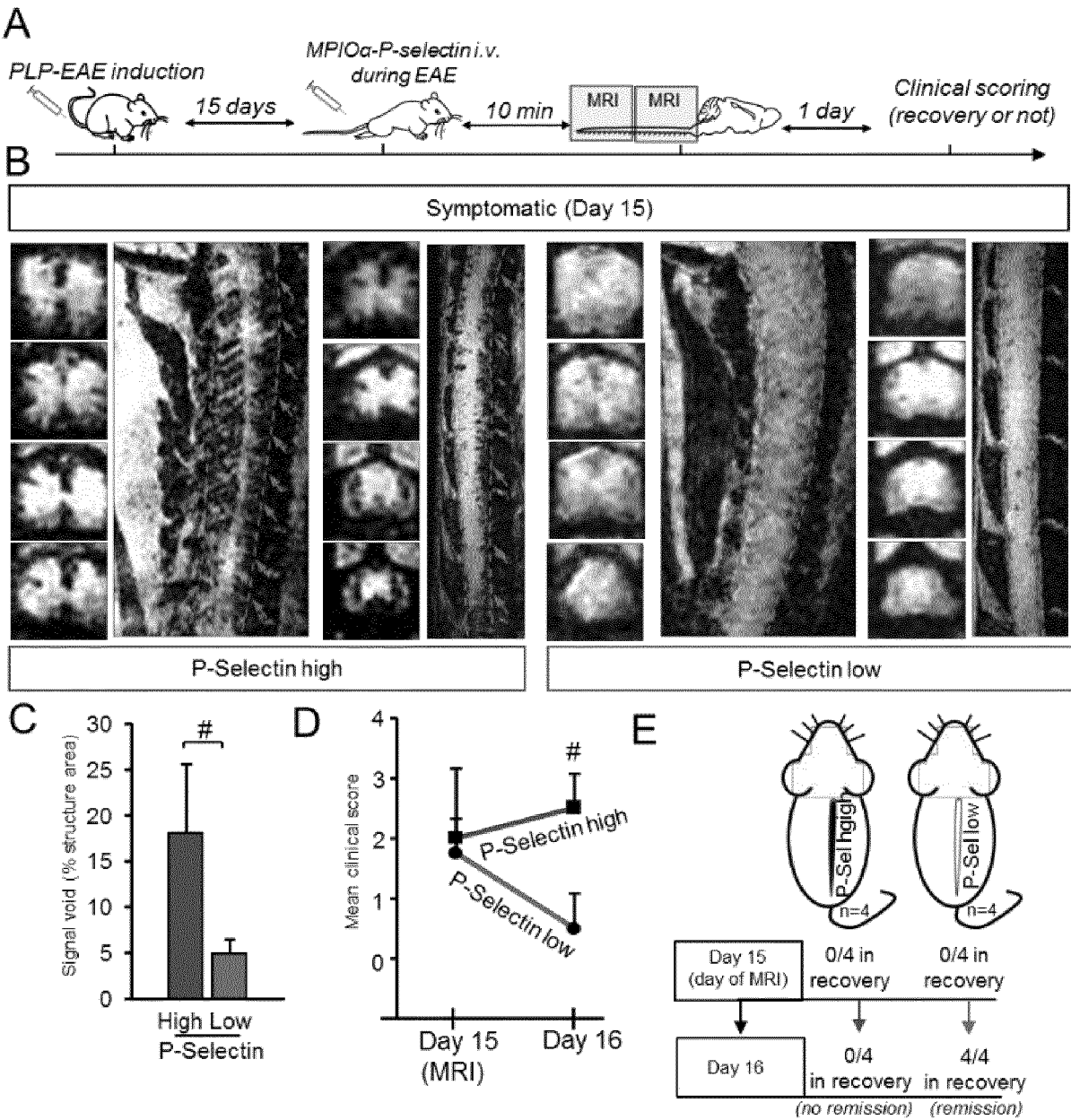


Figure 8

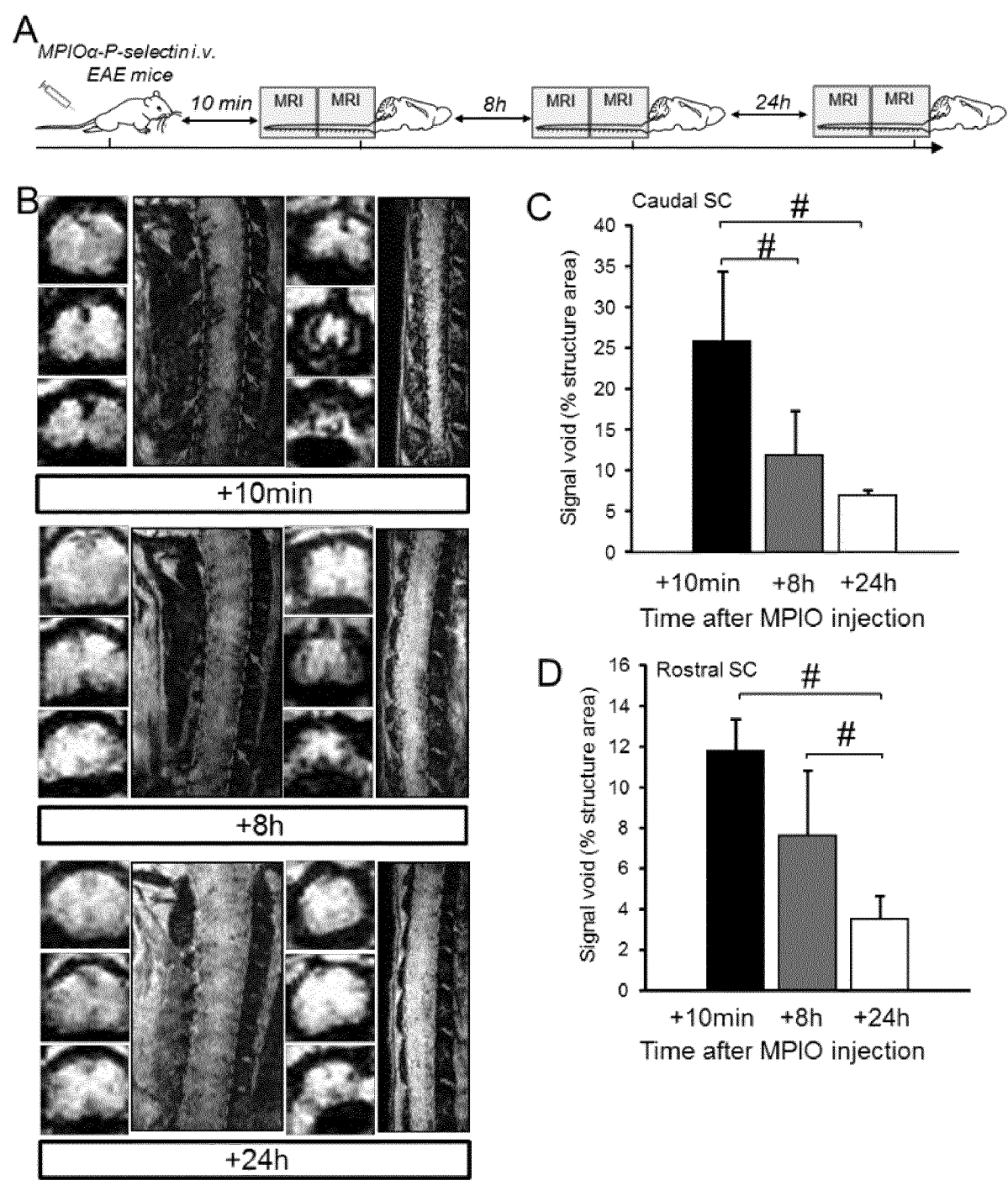


Figure 9

MOG-induced EAE

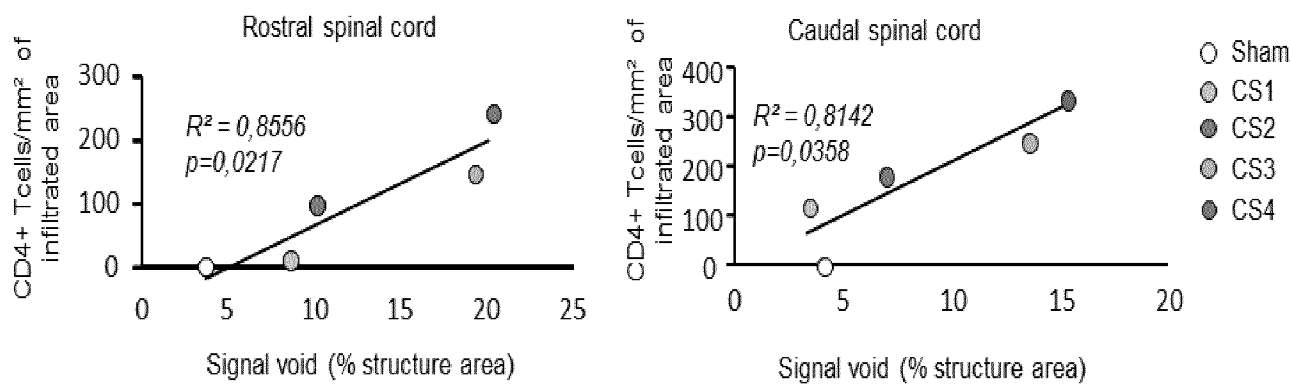


Figure 10

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/052284

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61K49/18 A61K49/22
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, EMBASE, CHEM ABS Data, DISSERTATION ABS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/020450 A2 (ISIS INNOVATION [GB]; DAVIS BENJAMIN GUY [GB]; VAN KASTEREN SANDER IZA) 22 February 2007 (2007-02-22) cited in the application page 8 claims 11-16 ----- -/--	1-5,7-9



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

11 April 2017

Date of mailing of the international search report

20/06/2017

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Dullaart, Anwyn

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/052284

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LUCA BATTISTINI ET AL: "CD8+ T cells from patients with acute multiple sclerosis display selective increase of adhesiveness in brain venules: a critical role for P-selectin glycoprotein ligand-1", BLOOD, vol. 101, no. 12, 15 June 2003 (2003-06-15), pages 4775-4782, XP002291380, ISSN: 0006-4971, DOI: 10.1182/BLOOD-2002-10-3309 abstract page 4781 -----	1-9
X	L. PICCIO ET AL: "Molecular Mechanisms Involved in Lymphocyte Recruitment in Inflamed Brain Microvessels: Critical Roles for P-Selectin Glycoprotein Ligand-1 and Heterotrimeric Gi-Linked Receptors", THE JOURNAL OF IMMUNOLOGY, vol. 168, no. 4, 15 February 2002 (2002-02-15), pages 1940-1949, XP055290778, ISSN: 0022-1767, DOI: 10.4049/jimmunol.168.4.1940 page 1945 - page 1946 figure 8 page 1948, right-hand column -----	1-9
X	SHEREMATA WILLIAM A ET AL: "Evidence of platelet activation in multiple sclerosis", JOURNAL OF NEUROINFLAMMATION, vol. 5, no. 1, 27 June 2008 (2008-06-27), page 27, XP021037938, ISSN: 1742-2094	8
Y	abstract page 2 of 6 figure 1B	1-7,9
X	----- L. PICCIO ET AL: "Efficient Recruitment of Lymphocytes in Inflamed Brain Venules Requires Expression of Cutaneous Lymphocyte Antigen and Fucosyltransferase-VII", THE JOURNAL OF IMMUNOLOGY, vol. 174, no. 9, 20 April 2005 (2005-04-20), pages 5805-5813, XP055290817, ISSN: 0022-1767, DOI: 10.4049/jimmunol.174.9.5805 abstract figure 3A page 5808, right-hand column page 5811, left-hand column -----	1-9
	-----	-/--

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/052284

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95/34324 A1 (PROTEIN DESIGN LABS INC [US]; BERG ELLEN L [US]) 21 December 1995 (1995-12-21) page 30 page 34 example 4	1-9
X	----- WO 2015/136468 A1 (PROTHENA BIOSCIENCES LTD [IE]; UNIV MUNSTER [DE]) 17 September 2015 (2015-09-17) examples 3,5 figure 5	1-9
X	----- WO 2010/116209 A1 (INST NAT SANTE RECH MED [FR]; MICHEL JEAN-BAPTISTE [FR]; LETOURNEUR DI) 14 October 2010 (2010-10-14) cited in the application page 9 examples 1-3	1-9
Y	----- F. KIESSLING ET AL: "Functional and Molecular Ultrasound Imaging: Concepts and Contrast Agents", CURRENT MEDICINAL CHEMISTRY-CARDIOVASCULAR & HEMATOLOGICAL AGENTS, vol. 16, no. 5, February 2009 (2009-02), pages 627-642, XP055112974, ISSN: 0929-8673, DOI: 10.2174/092986709787458470 abstract page 638, left-hand column	1-7,9
Y	----- BOUTRY SÉBASTIEN ET AL: "Specific E-selectin targeting with a superparamagnetic MRI contrast agent.", CONTRAST MEDIA & MOLECULAR IMAGING, vol. 1, no. 1, January 2006 (2006-01), pages 15-22, XP002760253, ISSN: 1555-4317 cited in the application the whole document	1-7,9
Y	----- NORMAN KEITH E ET AL: "P-selectin glycoprotein ligand-1 supports rolling on E- and P-selectin in vivo", BLOOD, vol. 96, no. 10, 15 November 2000 (2000-11-15), pages 3585-3591, XP055290777, ISSN: 0006-4971 abstract page 3586 ----- -/--	1-7,9

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/052284

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/057092 A1 (UNIV MUENSTER WILHELMS [DE]) 25 April 2013 (2013-04-25) examples claims -----	1-5,7,9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2017/052284

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

8, 9(completely); 1-7(partially)

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 8, 9(completely); 1-7(partially)

Imaging agent for use as defined in claims 1-7, and a method for predicting as defined in claim 9, where the imaging is performed using MRI; use of P-selectin as a biomarker for MS

1.1. claims: 9(completely); 1-7(partially)

Imaging agent for use as defined in claims 1-7, and a method for predicting as defined in claim 9, where the imaging is performed using MRI

1.2. claim: 8

Use of P-selectin as a biomarker for MS

2. claims: 1-7(partially)

Imaging agent for use as defined in claims 1-7, where the imaging is performed using ultrasound imaging

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2017/052284

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2007020450 A2	22-02-2007	AT 470455 T EP 1937315 A2 US 2008311048 A1 WO 2007020450 A2	15-06-2010 02-07-2008 18-12-2008 22-02-2007
WO 9534324 A1	21-12-1995	AT 248605 T AU 2821195 A CA 2191870 A1 DE 69531679 D1 DE 69531679 T2 EP 0765172 A1 HK 1016018 A1 JP H10502168 A US 5622701 A US 6210670 B1 WO 9534324 A1	15-09-2003 05-01-1996 21-12-1995 09-10-2003 08-07-2004 02-04-1997 14-05-2004 24-02-1998 22-04-1997 03-04-2001 21-12-1995
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