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(71) Applicant(s):
Stefan L Marklund
Törnskatevägen 26, Umeå 906 51, Sweden

Peter M Andersen
Östermalmsgatan 22 C, Umeå 903 32, Sweden

Thomas Brännström
Grästensvägen 20, Umeå 907 42, Sweden

Mikael Oliveberg
Skaldevägen 20, Bromma 167 71, Sweden

(72) Inventor(s):
Stefan L Marklund
Peter M Andersen
Thomas Brännström
Mikael Oliveberg

(74) Agent and/or Address for Service:
Potter Clarkson LLP
The Belgrave Centre, Talbot Street, NOTTINGHAM, NG1 5GG, United Kingdom

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(58) Field of Search:
Other: **WPI, EPODOC, BIOSIS, MEDLINE**

(54) Title of the Invention: **Aggregates of superoxide dismutase**
Abstract Title: **Aggregates of superoxide dismutase**

(57) A method for typing an aggregate (including a fibril) of a polypeptide in a test sample, such as human superoxide dismutase (hS0D1), wherein the polypeptide is capable of forming at least two structurally distinct types of aggregates, said method comprising (i) determining the reactivity of the aggregate in the test sample with one or more different antibody preparations in a panel of antibody preparations, wherein each antibody preparation in the panel has binding specificity to a peptide sequence derived from the amino acid sequence of the polypeptide, such that different antibody preparations in the panel have binding specificity to different peptides, and wherein at least one of the antibody preparations of the panel, for which reactivity to the aggregate is determined in step (i), displays differential reactivity to at least two structurally distinct types of the aggregate; and (ii) attributing a type to the aggregate in the test sample, based on the determined level(s) of reactivity with the one or more antibody preparations in the panel as determined in step (i). Also claimed are preparations comprising aggregates identified by the above methods; methods for generating antibodies against aggregates; pharmaceutical compositions comprising peptides or antibodies for treating ALS and a method for the identification of an agent that binds to, interferes with, or promotes the degradation of, aggregates of hSOD1.

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Figure 1

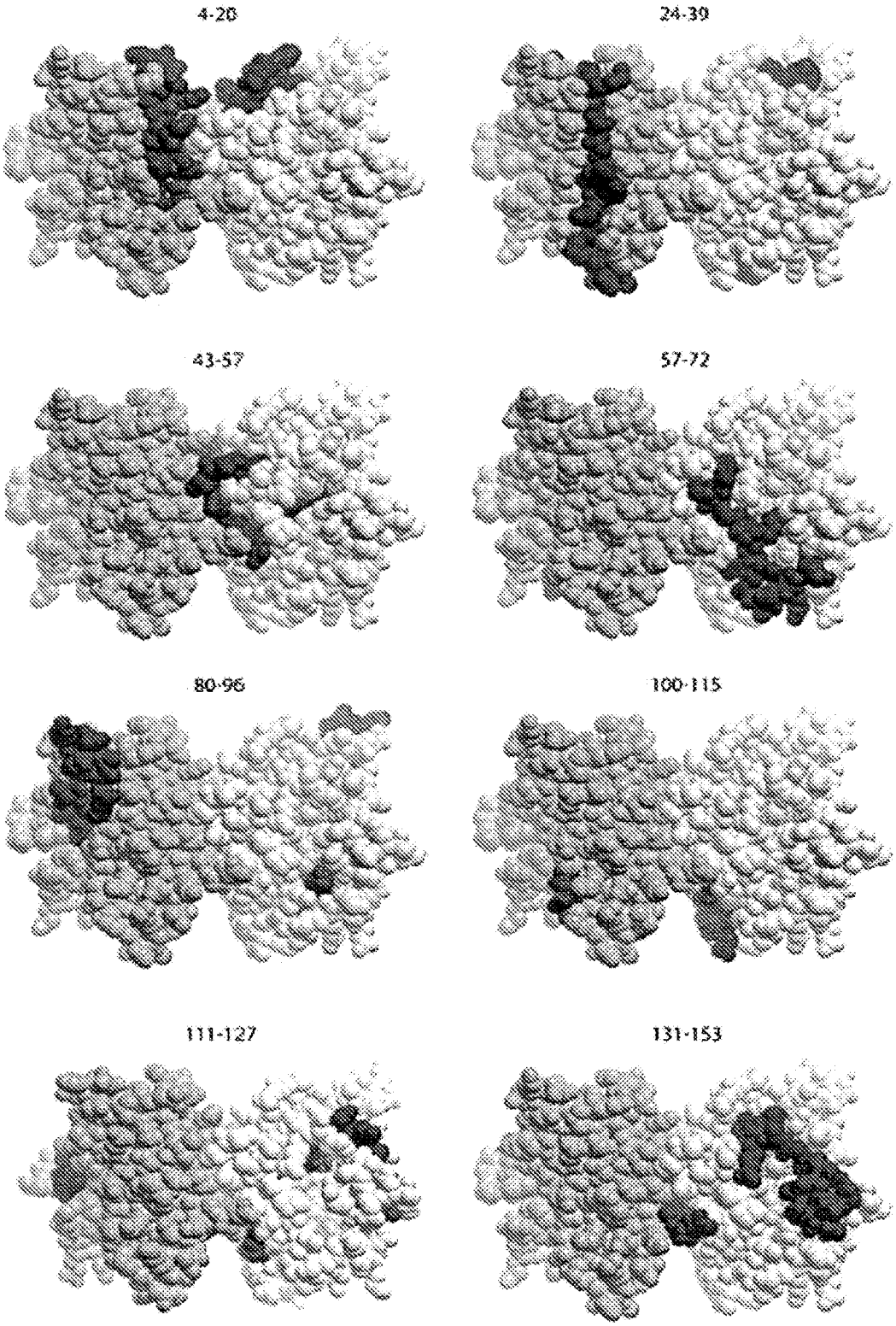
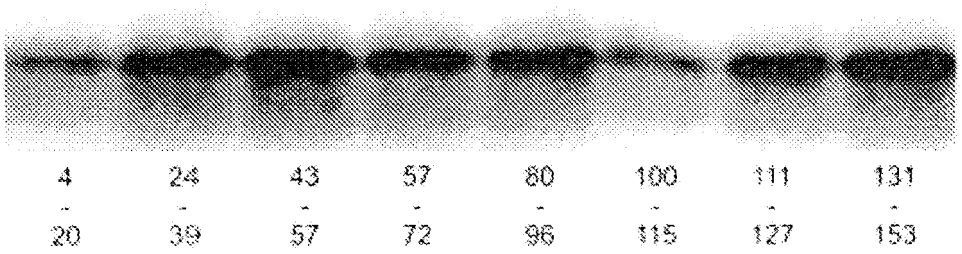


Figure 2

A


4 - 20
24 - 39
43 - 57
57 - 72
80 - 96
100 - 115
111 - 127
131 - 153

B

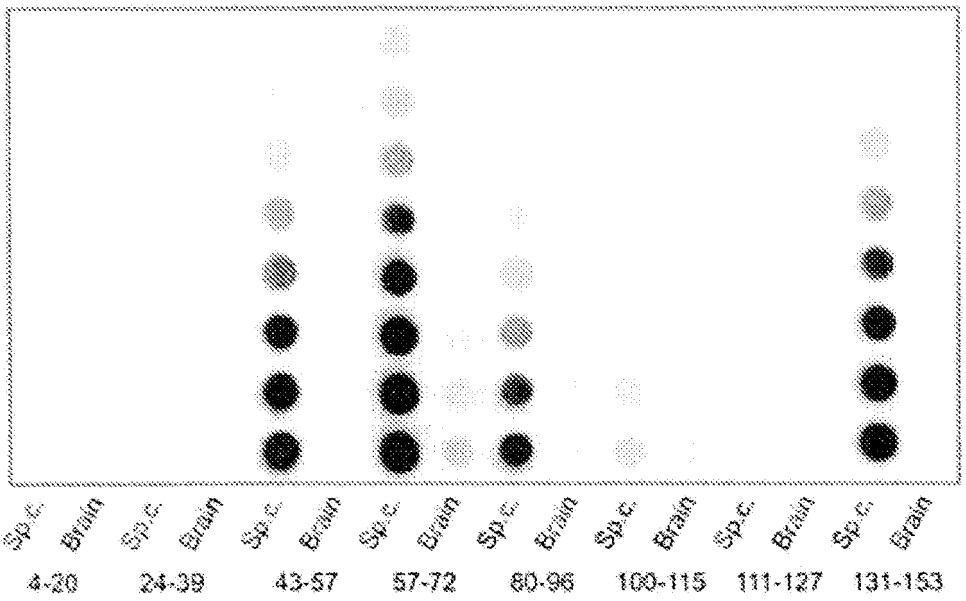
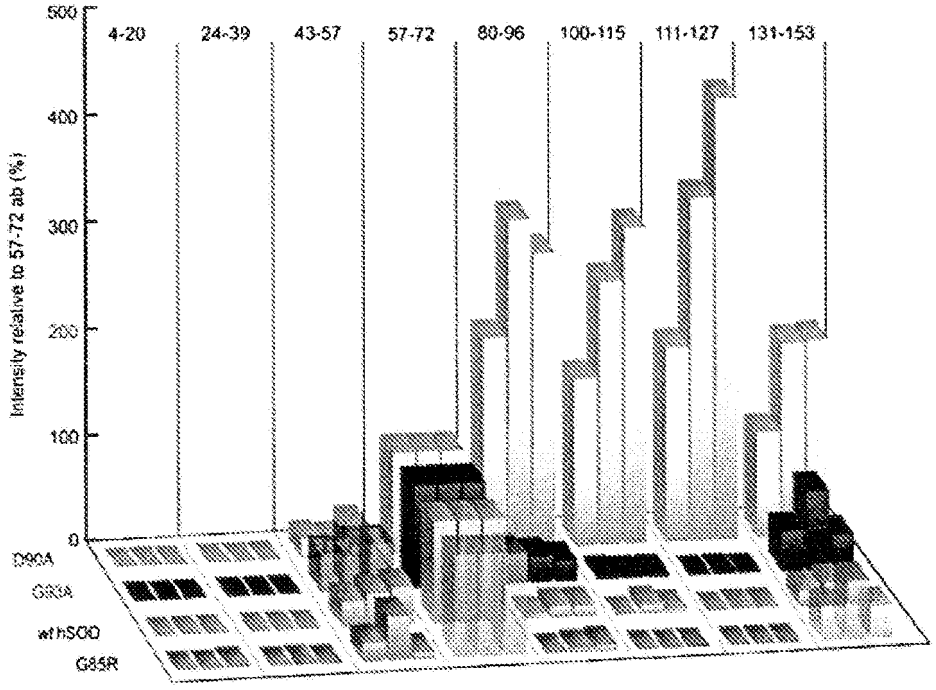

Sp.c.
Brain
4-20
24-39
43-57
57-72
80-96
100-115
111-127
131-153

Figure 2 (cont)

C



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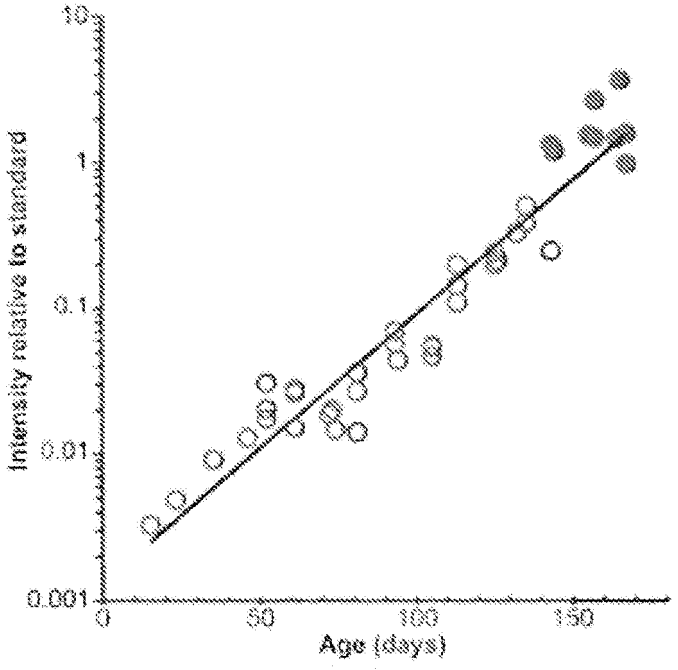


Figure 3

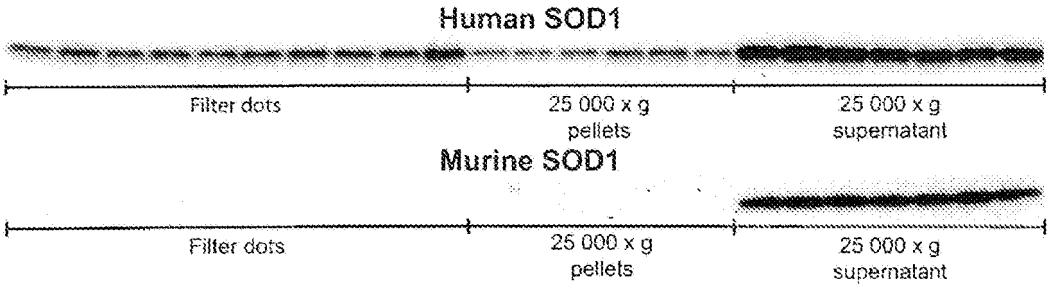


Figure 4

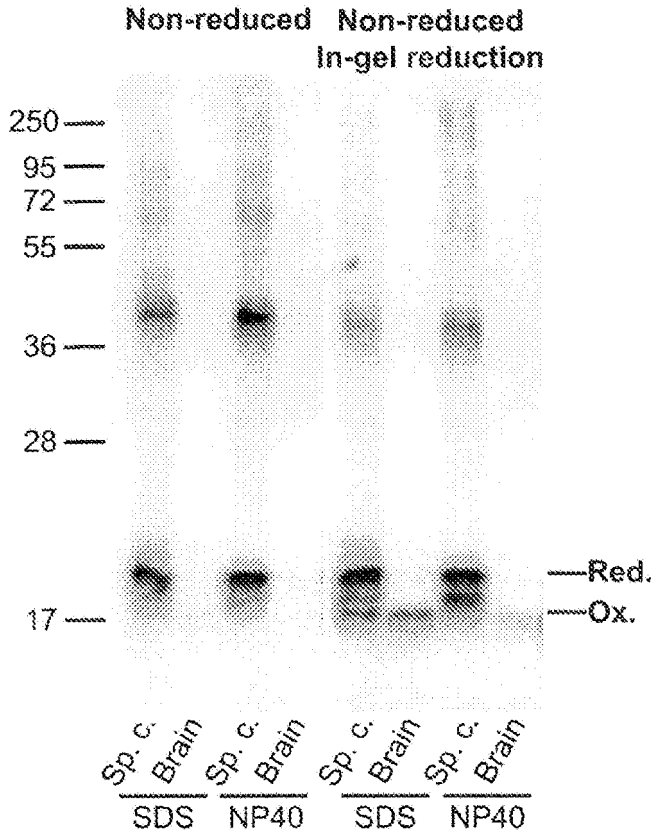
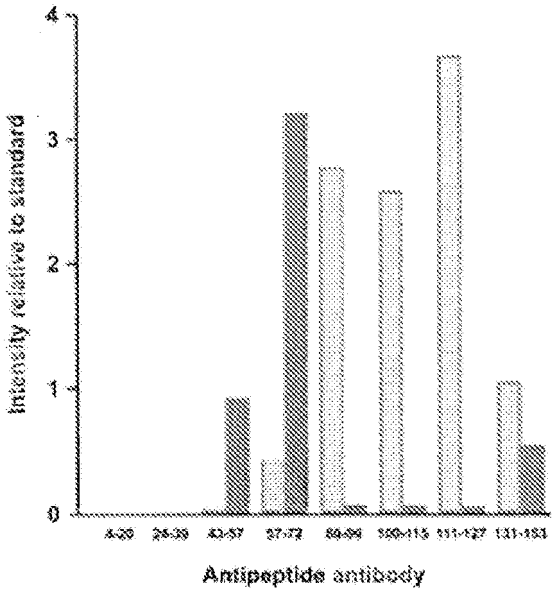
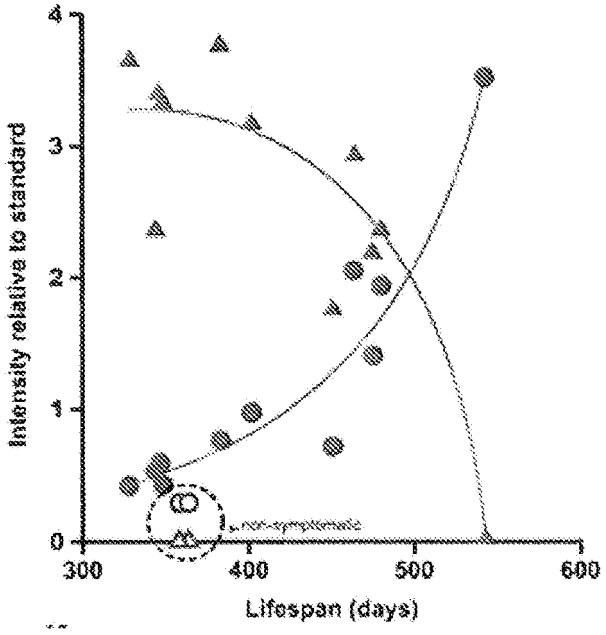


Figure 5

A


Intensity relative to standard
0
1
2
3
4
Antipeptide antibody

B


Intensity relative to standard
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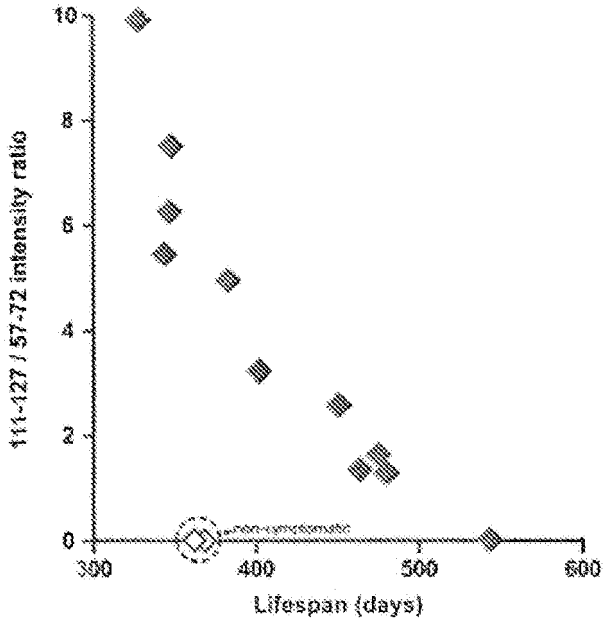
1
2
3
4
300
400
500
600
Lifespan (days)

Figure 5 (cont)

C



D

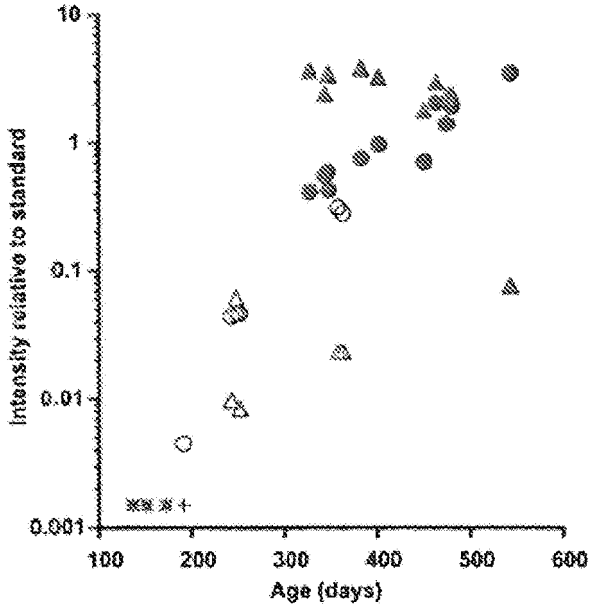
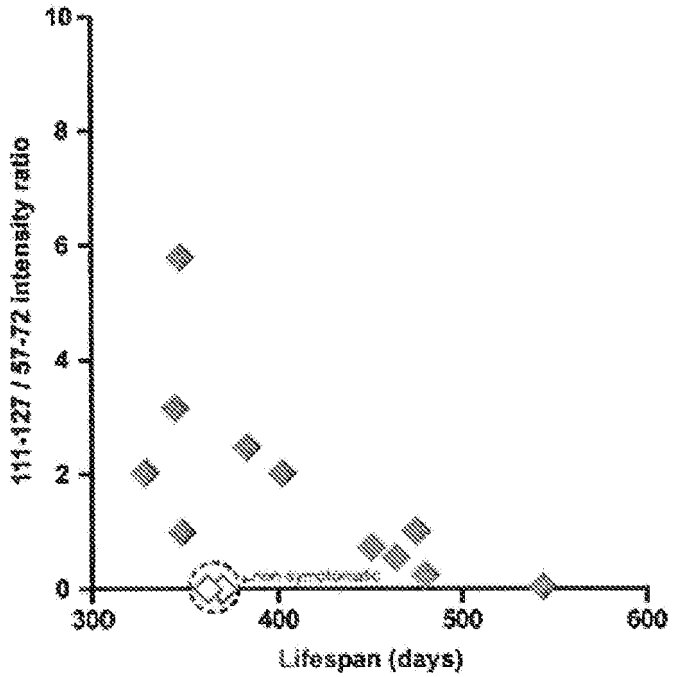


Figure 5 (cont)

E



F

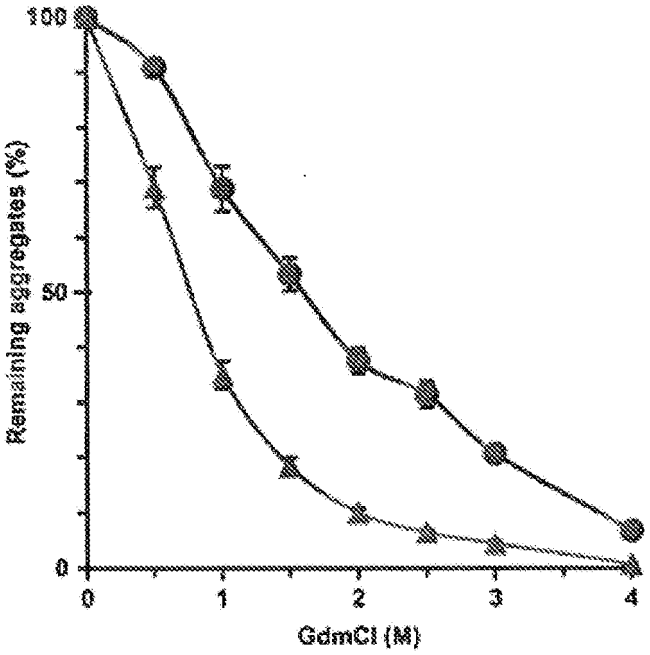


Figure 5 (cont)

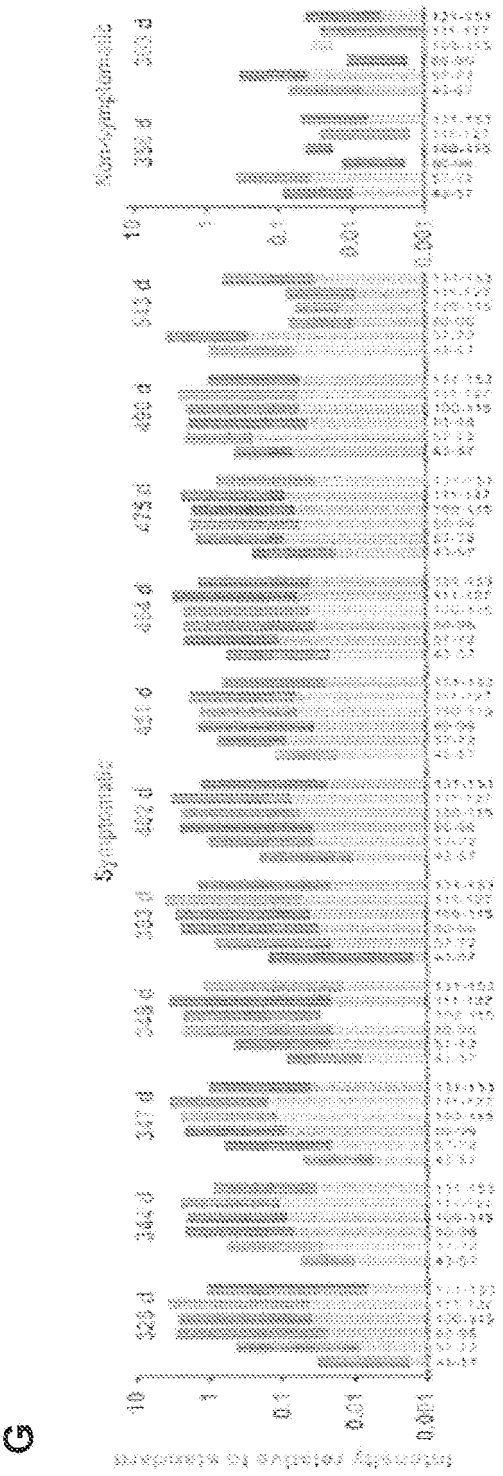


Figure 6

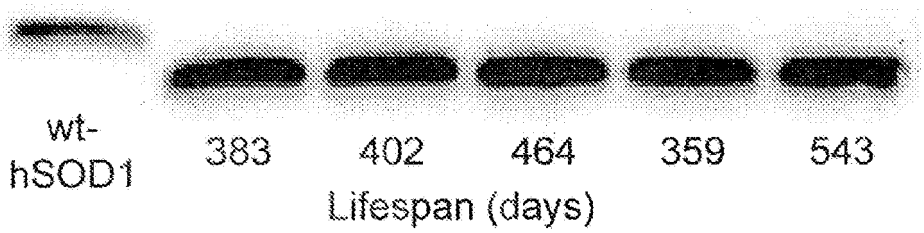


Figure 7

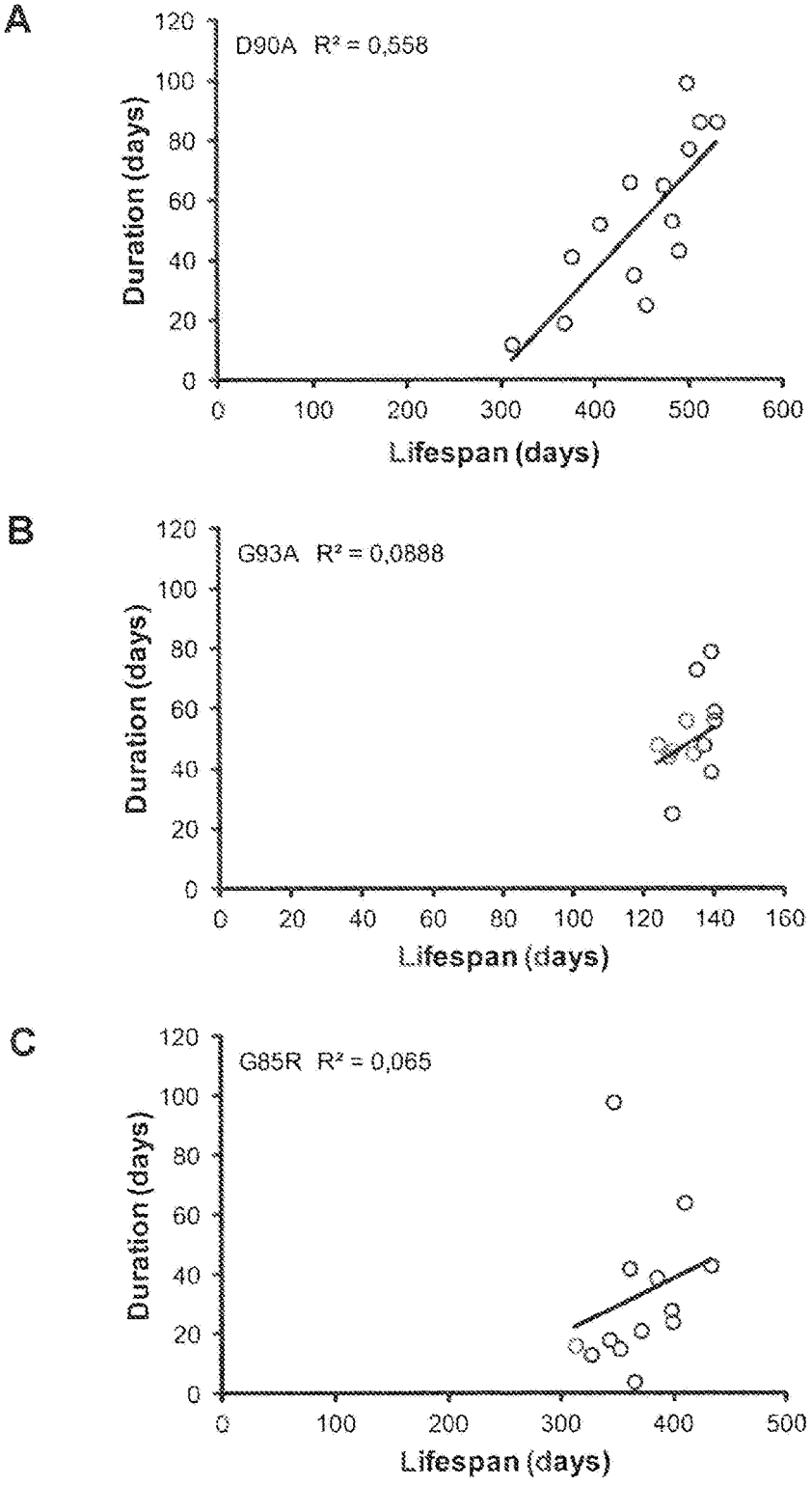
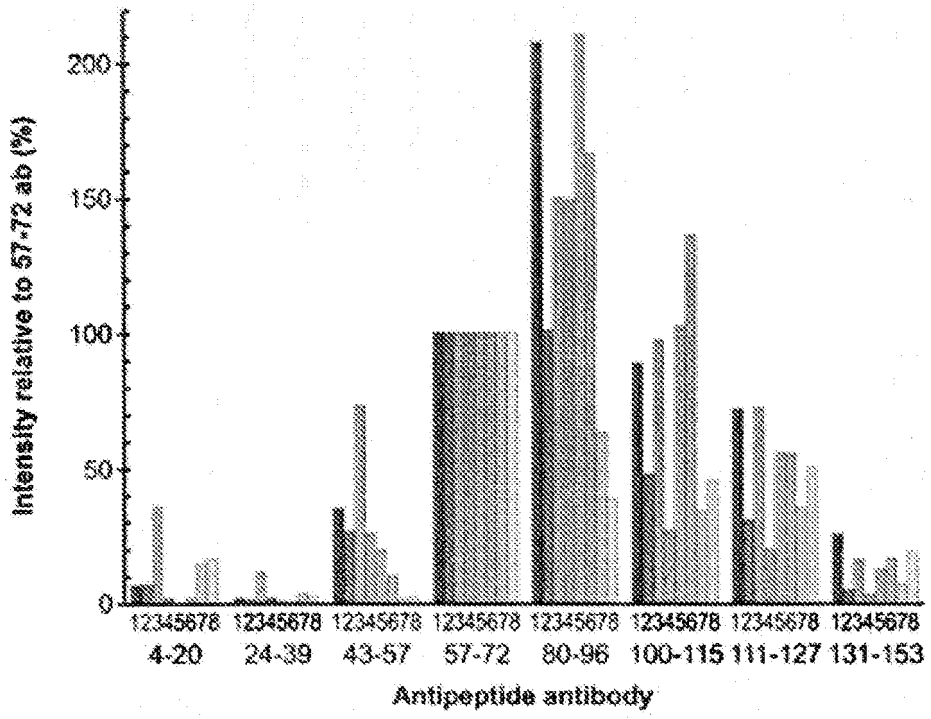
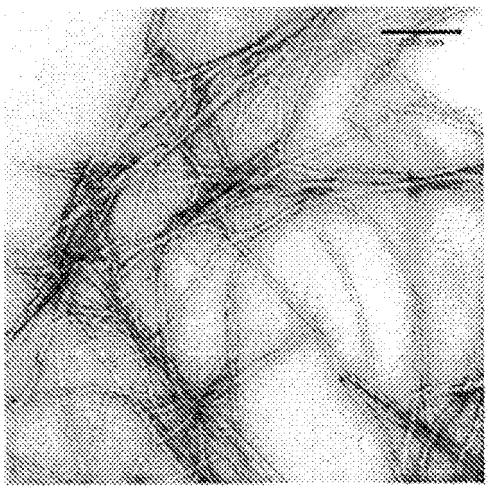

A
D90A R² = 0,558
Duration (days)
Lifespan (days)
0
20
40
60
80
100
120
0
100
200
300
400
500
600
B
G93A R² = 0,0888
Duration (days)
Lifespan (days)
0
20
40
60
80
100
120
140
160
C
G85R R² = 0,065
Duration (days)
Lifespan (days)
0
20
40
60
80
100
120
0
100
200
300
400
500

Figure 8

A


Intensity relative to 57-72 ab (%)
200
150
100
50
0
12345678 12345678 12345678 12345678 12345678 12345678 12345678 12345678
4-20 24-39 43-57 57-72 80-96 100-115 111-127 131-153
Antipeptide antibody

B



C

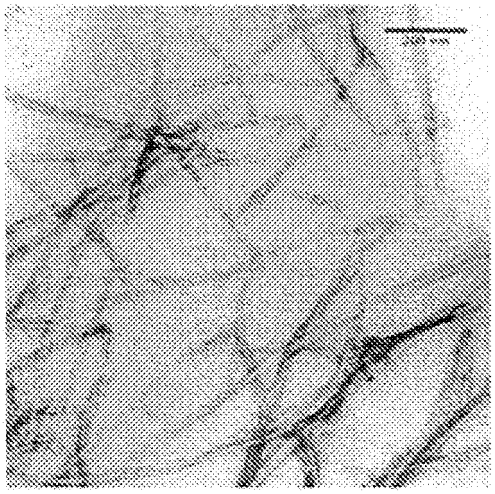
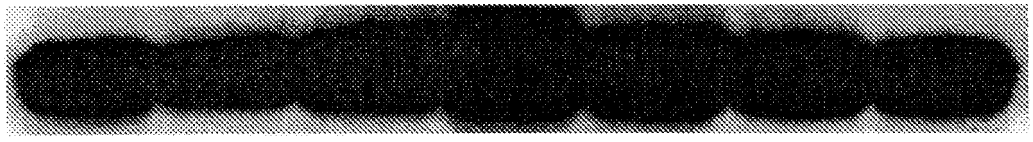


Figure 9

A

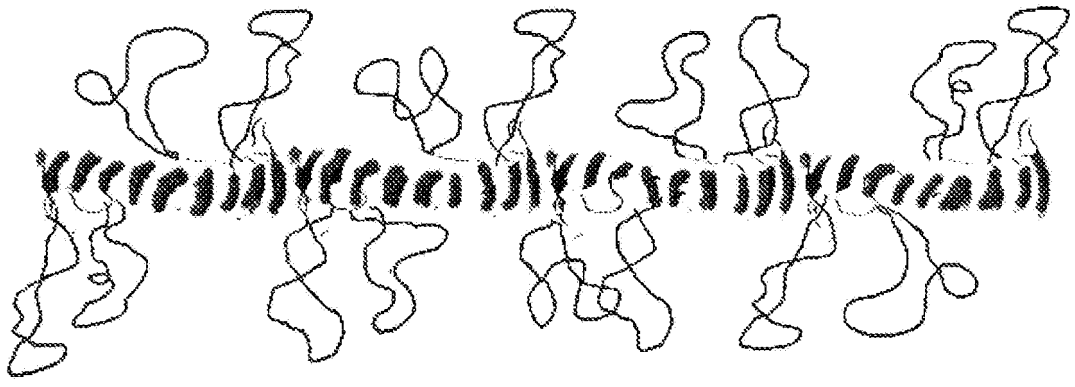
3-20 24-39 131-153 43-57 58-72 80-96 100-115

B



3-20 24-39 131-153 43-57 58-72 80-96 100-115

C



AGGREGATES OF SUPEROXIDE DISMUTASE

FIELD OF THE INVENTION

The present invention relates to aggregates of human superoxide dismutase (hSOD1), and their use in diagnosis and in methods for the identification and development of new therapeutical agents for use in treatment for amyotrophic lateral sclerosis (ALS)

BACKGROUND

Rational intervention strategies in neurodegenerative disease are hindered by poor understanding of protein misfolding and aggregation phenomena inside living cells. Despite decades of intense research the outstanding questions remain only partly answered: what triggers protein deposition *in vivo,* and what are the key mechanisms of cytotoxicity that should be the target(s) of intervention for successful treatment? Thus, it is not known whether proteinaceous deposits are causative of disease, downstream terminal markers, or even represent protective scavenging of noxious precursors under conditions of compromised proteostasis.

ALS is a fatal neurodegenerative syndrome characterized by progressive loss of motor neurons in the cortex, brainstem and spinal cord. The clinical hallmark is focal onset dysfunction of motor neurons resulting in paresis and wasting of skeletal muscles, which then spreads to become generalized, including invasion of other areas of CNS. The median age of onset is 58 years, the median survival time is only 26 months and the lifetime risk for developing ALS is about 1:400. Like in other protein-misfolding diseases, no effective treatment is available. Compared to the other diseases ALS offers some advantages for research: (i) A common cause is mutations in SOD1 (Rosen et al., 1993; Andersen et al., 2003) and currently >170 of such ALS-associated mutations have been found, which aid mechanistic studies; (ii) there are multiple, reliable mutant SOD1-expressing transgenic models, e.g. the G93A, D90A, G85R, G127instggg forms of human SOD1 (hSOD1), (Gurney et al., 1994; Jonsson et al. 2006a, 2006b), displaying variations in disease pathogenesis that mimic the spectrum of human disease; (iii) intracellular SOD1 deposits appear; and (iv) unlike most other precursors for neurodegenerative diseases, SOD1 is a globular enzyme that is amenable to atomic-resolution structural analysis (Nordlund et al., 2009; Teilum et al., 2009) and has well characterised folding and unfolding pathways (Nordlund and Oliverberg, 2006; Leinartaite et al., 2010).

SOD1 is a ubiquitously expressed antioxidant enzyme, which is primarily is located to the cytosol, but is also found in the nucleus and the inter-membrane space of mitochondria. The reasons behind the selective vulnerability of motor areas to SOD1 toxicity are not understood and are not related to high levels. The enzyme is more abundant in several other tissues in the body (Jonsson et al., 2009). SOD1 is composed of two equal 153 amino acids long non-covalently bound subunits. Each subunit contains a Cu ion which confers the enzymatic activity and a Zn ion which is important for the stability. There are 4 cysteine residues (C6, C57, C111 and C146) and two of them (C57-C146) form an intrasubunit disulphide bond. Structural disulphide bonds are rare in the strongly reducing cytosol, and the bond could be an Achilles heel of the protein. While holoSOD1 is superstable, loss of posttranslational modifications such as the disulphide bond and the bound metal ions leads to severe destabilization. Such immature forms are likely the primary source of the neurotoxicity. SOD1 aggregates that are formed in vivo are composed of subunits lacking the disulphide bond (Karch et al., 2009; Bergemalm et al., 2010).

DESCRIPTION OF THE INVENTION

The present inventors have, using an assay based on eight anti-hSOD1 peptide antibodies, identified two varieties of aggregates of hSOD1 with distinct molecular structures: type A and type B. The key feature of type A and type B aggregates of hSOD1 is that they are associated with different characters and severities of disease progression. The type A and type B aggregates of hSOD1 according to the invention are different from the hSOD1 aggregates that can be induced to form *in vitro*.

Accordingly, one aspect of the present invention provides methods for identifying and characterizing aggregates of aggregate forming polypeptides, said method comprising analysing the reactivity of said aggregate forming polypeptide with one or more of a panel of antibodies directed to short peptide sequences derived from the amino acid sequence of said aggregate forming polypeptide.

More particularly, an aspect of the present invention provides a method for typing an aggregate (including a fibril) of a polypeptide in a test sample, typically wherein the polypeptide is capable of forming at least two structurally distinct types of aggregates, said method comprising -

(i) determining the reactivity of the aggregate in the test sample with one or more, such as 2, 3, 4, 5, 6, 7, 8 or more, different antibody preparations in a panel of antibody preparations,

wherein each antibody preparation in the panel has binding specificity to a peptide sequence derived from the amino acid sequence of the polypeptide, such that different antibody preparations in the panel have binding specificity to different peptides, and

wherein at least one of the antibody preparations of the panel, for which reactivity to the aggregate is determined in step (i), displays differential reactivity to at least two structurally distinct types of the aggregate; and

(ii) attributing a type to the aggregate in the test sample, based on the determined level(s) of reactivity with the one or more antibody preparations in the panel as determined in step (i).

Furthermore, where the method of the invention has been used to identify and isolate structurally distinct types of aggregates of a polypeptide, then (as discussed further below) it will be possible to generate antibodies, preferably monoclonal antibodies, which bind specifically to a specific conformational epitope for each aggregate type, preferably wherein the generated antibodies (i) lack of reactivity with soluble native or denatured polypeptide, (ii) lack of reactivity with peptide sequences derived from the amino acid sequence of the polypeptide; and/or (iii) lack of reactivity with at least one alternative structurally distinct type of aggregates of a polypeptide. Such antibodies may be used in the above-mentioned method of typing in addition to, or in place of, any one or more (or even all) of the one or more antibody preparation in the panel which have binding specificity to a peptide sequence derived from the amino acid sequence of the polypeptide.

In the context of the present invention, an aggregate is a structurally ordered assembly of protein polypeptides, where the repetitive units are structurally different from the native state, either in terms of sequence connectivity (domain swapped-based aggregates), in terms of unit-unit interface (aggregates based on native-like units), or in terms of tertiary structure (amyloid-type of aggregates). The aggregates can also be mixtures of these basic structural architectures. In terms of the repetitive units, the aggregates can be uniformly

repetitive, i.e. with structurally identical, monomeric subunits (first-order subunits), or being composed of more complex repetitive units containing several protein polypeptides with identical or diverse structures following the basic architectures outlined above (high-order subunits). In one embodiment, the aggregate may comprise a fibril-like spine of stacked β sheet structures, optionally with domain-swapped material based on inter-molecular native contacts (a simplified cartoon in Figure 9C), wherein the sequence regions outside the ordered aggregate core, which will have lost their native contacts, form disordered fringes.

Although the number of repetitive units in an aggregate could be from 2 to infinite, and so is intended to exclude monomers, the term "aggregates" is used herein preferably to exclude dimers, and optionally (although not necessarily) to further exclude low-level oligomers of the polypeptide. Low-level oligomers are intended to include oligomers containing less than, for example, about 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, or 50 (such as 3-20, 3-15 or 3-10) polypeptide molecules. Generally, aggregates are non-soluble or substantially more resistant to solubilisation than monomers and dimers, and optionally also low-level oligomers. Moreover, where a sample contains an aggregate, the aggregate may be visible by light microscopy when viewed at a magnification with the range of 12.5 to 1000 times, whereas non-aggregated polypeptide would typically not be visible within this range of magnification. Additionally or alternatively, aggregates may possess a dimension of at least 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, 500 nm or more, in one or more dimensions, whereas non-aggregated polypeptide would typically not possess such dimensions. Further, additionally or alternatively, an aggregate present in a tissue homogenate (e.g. in APBS (see below) supplemented by 1.8 mM EDTA, 1mM DTT and 1% (w/v) of the detergent NP40) may form a pellet under various conditions from when it is left standing in a suspension to when it is centrifuged at 200,000 x g. A non-aggregated polypeptide typically would not form a pellet under such conditions. However, it should be appreciated that, in one embodiment of the present invention, the methods may be practiced on oligomers, such as low-level oligomers as described above, and, in that embodiment, the term aggregate is intended to include oligomers, such as oligomers containing at least 5, 10, 15, 20, 25, 30, 40, 50 60 or more polypeptide molecules. In one embodiment, the aggregate may be a fibril.

In the context of the present invention, the term "typing" is intended to include the meaning of discriminating between structurally distinct forms of aggregates of the same polypeptide. In one embodiment, the term "typing" can refer to discriminating between structurally distinct forms of aggregates of the same polypeptide wherein the structurally distinct forms each comprise a fibril-like spine of stacked β sheet structures, optionally with domain-swapped material based on inter-molecular native contacts (a simplified cartoon in Figure 9C), wherein the sequence regions outside the ordered aggregate core, which will have lost their native contacts, form disordered fringes.

Aggregates for typing according to the present invention may be obtained from biological sources or created *in vitro*.

In a preferred embodiment, the aggregates are obtained from biological sources, such as from a human or animal ALS patient (optionally wherein the ALS is sporadic or familial), a human or animal diagnosed as possessing a mutated SOD1 gene, or a transgenic animal carrying a wild-type or mutated hSOD1 gene. Suitable tissue sources from which aggregates may be obtained from such humans or animals include the spinal cord (such as the cervical spinal cord, the thoracic spinal cord, and/ or the lumbar spinal cord), the brain (such as the hippocampus, the ventral cingulate gyrus, the frontal cortex, the middle and/or superior temporal gyrus, the striatum, and/or the mesencephalon), the liver, the kidney, the blood, plasma, serum, cerebrospinal fluid, or isolated cells, such as leukocytes or lymphocytes. The human or animal from which the aggregate sample is taken may be living, or deceased, at the time of obtaining the sample. In the case of a human source of aggregate, the human may be aged up to 20 years, or at least 20, 30 40, 50, 60, 70, 75, 80, 85, 90, 95 or 100 years of age at the time of taking of the sample (or at the time of death, in the case of a sample from a deceased human). In the case of an animal source of aggregate, the animal may be aged up to 20 % of the mean lifespan of that species or strain of animal, or at least 5, 10, 20, 30, 40, 50, 60, 70, 75, 80, 85, 90, 95 or 100 % of the mean lifespan at the time of taking of the sample (or at the time of death, in the case of a sample from a deceased human).

The aggregate sample is preferably obtained from its source, and maintained for the purposes of typing, under conditions that do not substantially contribute to a change in the conformation of the aggregate present therein. Thus, for example, the sample should not

be exposed to extremes of pH (generally, operating within the range of pH 5.5 - 9 will be acceptable, and preferably pH values below 5.5, 5.0. 4.5, or 4.0 should be avoided; likewise pH values above 9.0, 9.5, 10.0, or 10.5 should preferably be avoided);; conditions that contribute to the proteolysis of the protein in the aggregate; and/or denaturing agents (such as trichloroacetic acid or the like) at levels that would contribute to a change in the conformation of the aggregate present therein.

Prior to typing, the test sample may, or may not, be subjected to purification step to render it substantially free of non-aggregated forms of the polypeptide, including monomeric forms and dimeric forms, denatured forms and/or misfolded forms of the polypeptide, and optionally low-level oligomeric forms. Such pre-typing purification is, however, not necessary for the practice of the present invention. Nevertheless, such forms can typically be separated from aggregated forms of the polypeptide by techniques well known in the art, such as centrifugation, as discussed in the examples. In the case of hSOD1, an antibody preparation having binding specificity for peptides consisting of the sequence of SEQ ID NO: 1 or 2 will bind to at least monomeric forms, misfolded dimeric forms, and/or denatured forms of hSOD1, but not to type A or type B aggregates, and so can be used to assess whether samples of type A or type B aggregates are free of such species.

The methods of the present invention can be used for identifying epitopes and peptide sequences of the aggregate forming polypeptide, which are specifically exposed in pathological aggregates of said aggregate forming polypeptide, said epitopes being useful as vaccine candidates and as target for identifying therapeutical antibodies and other types of binding molecules, including small molecules, which potentially can be used in the treatment of the related diseases.

The present invention employs one or more different antibody preparations in a panel of antibody preparations, wherein each antibody preparation in the panel has binding specificity to a peptide sequence derived from the amino acid sequence of the polypeptide, such that different antibody preparations in the panel have binding specificity to different peptides.

Typically, the, or each, peptide sequence derived from the amino acid sequence of the polypeptide contains no more than 25, 20, 15, 10 or fewer contiguous amino acids from the

amino acid sequence of the polypeptide. In any case, peptides contain less then the full polypeptide, such as the full naturally occurring protein (e.g. is smaller than hSOD1 in embodiments intended to type aggregates of hSOD1). The, or each, peptide sequence may optionally comprise one or more additional amino acids at the N-terminus or C-terminus, typically in which the additional amino acids do not match the amino acids found at a corresponding position in the sequence of the polypeptide. There may, for example, be up to 5, 4, 3, 2, 1 or optionally no additional amino acids at the N-terminus of the, or each, peptide. There may, for example, be up to 5, 4, 3, 2, 1 or optionally no additional amino acids at the C-terminus of the, or each, peptide. In total, such peptides may be less than 40, 35, 30, 25, 20, 19, 18, 17, 16, or 15 in length, and optionally contain no more than 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6 or 5 contiguous amino acids from the amino acid sequence of the polypeptide. Particularly preferred peptides may be 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids in length. In one embodiment, the peptide consists of no more than 25, 20, 15, 10 or fewer amino acids, which are contiguous amino acids from the amino acid sequence of the polypeptide.

In this context, "binding specificity" includes the meaning that there is no cross-reactivity between the peptide sequences targeted by the different antibody preparations in the panel. Thus, the different antibody preparations in the panel each bind to different peptide sequences derived from the amino acid sequence of the polypeptide.

In one embodiment, an antibody preparation can be said to bind specifically to a peptide (a 'target' peptide) sequence if a binding reaction can be seen in a dot blot assay (such as described in the Examples) between the antibody preparation and the target peptide that is at least 3, 4, 5, 6, 7, 8, 9, 10 or more times higher than –

the background reaction;

the reaction between the antibody preparation and other peptides targeted by other antibody preparations in the panel; and/or

any other peptide of 10-15 amino acids in length, derived from the amino acid sequence of the polypeptide of interest without overlapping with the target peptide.

Additionally, at least one of the antibody preparations of the panel, for which reactivity to the aggregate is determined in step (i), displays differential reactivity to at least two structurally distinct types of the aggregate. This may, for example, be adjudged with

reference to an internal control, such as the level of binding reactivity to another antibody preparation in the panel. For example, as shown in the following examples, where the level of binding reactivity to an antibody preparation with binding specificity to a peptide consisting of the sequence of SEQ ID NO: 4 is used as an internal control, then antibody preparations with binding specificity to peptides consisting of the sequence of SEQ ID NOs: 5, 6 and 7, respectively, display differential reactivity to at least two structurally distinct types of hSOD1 aggregates.

The antibodies present in an antibody preparation within the panel can be polyclonal or monoclonal. Polyclonal antibody preparations may be preferred in some instances, since this may render the panels more resilient to the existence of unanticipated point mutations in the polypeptides under assessment, and continue to permit the assessment of structural variations in the aggregates of such mutants. On the other hand, monoclonal antibody preparations may be preferred in other instances, in particular since this may permit further selectivity between polypeptide mutants as well as assessment of structural variations in the aggregates.

Therefore, according to one preferred embodiment the antibody preparations are peptide specific polyclonal sera, optionally generated in an animal model selected from rabbits, chicken, mice, goat, sheep, donkey, camelids, or other animals which are available for antibody generation. The peptide specific polyclonal sera may, for example, be generated by immunization of the animals with a peptide having a specific amino acid sequence, preferably a peptide having an amino acid sequence selected from the amino acid sequences SEQ ID NO:1-15, the obtained polyclonal sera purified by affinity chromatography using the same peptide as used as immunogen, resulting in a peptide specific polyclonal sera. For the purposes of immunization to generate such polyclonal antibody preparations, the peptides may be linked, directly or indirectly, to a carrier. Any suitable carrier may be used, including well-known carriers such as keyhole-limpet haemocynanin (KLH) or an albumin, such as human serum albumin (HSA) or bovine serum albumin (BSA). According to current immunological theories, a carrier function should be present in any immunogenic formulation in order to stimulate, or enhance stimulation of, the immune system. It is thought that the best carriers embody (or, together with the antigen, create) a T-cell epitope. The peptides may be associated, for example by cross-linking, with a separate carrier, such as serum albumins, myoglobins, bacterial

toxoids and keyhole limpet haemocyanin. More recently developed carriers which induce T-cell help in the immune response include the hepatitis-B core antigen (also called the nucleocapsid protein), presumed T-cell epitopes such as Thr-Ala-Ser-Gly-Val-Ala-Glu-Thr-Thr-Asn-Cys, beta-galactosidase and the 163-171 peptide of interleukin-1. The latter compound may variously be regarded as a carrier or as an adjuvant or as both. Methods of linking peptide to such carriers are well known in the art. One suitable manner may be by disulphide linkage, and this may be suitable where the peptide in question comprises a cysteine residue. Thus, sequence of the polypeptide form which the peptide is derived may be a cysteine-containing sequence, or the peptide may be engineered to comprise, consist essentially of, or consist of, the peptide sequence from the polypeptide of interest plus an additional cysteine (which most preferably will be a terminal cysteine). Other suitable cross-linking agents include those listed as such in the Sigma and Pierce catalogues, for example glutaraldehyde, carbodiimide and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, the latter agent exploiting the -SH group on the C-terminal cysteine residue (if present).

An important aspect of the practice of the present invention is that the each antibody preparation in the panel has binding specificity to a peptide sequence derived from the amino acid sequence of the polypeptide. The peptides should be short, typically less than 30, 25, 20, 19, 18, 17, 16, or 15 in length. Particularly preferred peptides may be 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acids in length.

When such short antibodies are used to raise antibodies, such as in a method as described above for obtaining polyclonal sera, the peptides will typically have no fixed conformation at the point of immunization (and/or if there is a conformation it is most likely different from the conformation of the corresponding segment in the native protein). Thus, peptides may, in one embodiment, be characterized as "short" if they can be used to raise antibodies that react with a non-native (such as denatured) conformation of the full polypeptide and not with the full polypeptide in native conformation.

In one option, the panel contains multiple different preparations of antibodies, wherein the different antibody preparations bind specifically to different peptides derived from the full polypeptide, such that the whole panel is capable of binding to locations on the full

polypeptide that, in sum, are greater than 50%, 60%, 70%, 80%, 85%, 90%, 95% or more, such as substantially 100%, of the full polypeptide.

In some embodiments, in addition to the at least one antibody preparation of the panel which displays differential reactivity to at least two structurally distinct types of the aggregate, the panel further comprises one or more antibody preparations that distinguish the polypeptide when in aggregated form from the polypeptide when in monomeric, dimeric, denatured and/or misfolded form. Thus, for example, the panel may comprise one or more antibody preparations that have binding specificity for the polypeptide when in monomeric, dimeric, denatured and/or misfolded form, but not in aggregated form. In that case, the absence of reactivity of such antibody preparations with the aggregate in the sample can be taken into account in confirming that the aggregate in the sample is, indeed, in aggregated form and/or that the sample is clear of non-aggregated polypeptide.

By taking this approach to generating antibody panels, i.e. by using short peptides to raise each antibody preparation, and extensive coverage of the full polypeptide over the whole panel, it is possible to produce a panels of multiple antibody preparations that, when used together, can effectively discriminate between structurally different types of aggregates of a polypeptide under non-denaturing conditions, as well as between aggregates and non-aggregated forms of the polypeptide.

This follows from the applicants' new understanding of aggregate structure. If not amorphous, protein aggregates are typically composed of a fibril-like spine of stacked β sheet structures, which is sometimes decorated with domain-swapped material based on inter-molecular native contacts (a simplified cartoon in Figure 9C). The sequence regions outside this ordered aggregate core, which will have lost their native contacts, form disordered fringes.

The present invention takes advantage of this knowledge to use an epitope-mapping type assay to type different aggregate structures, since it appears that antibody preparations with binding specificity to peptide sequences that are found in the aggregate core have no reactivity with the aggregate, because of the ordered structure and perhaps also because of steric hindrance, whereas antibody preparations with binding specificity to short peptides derived from sequences found in the disordered fringes in the aggregate fibrils should be

reactive with the aggregate. As discussed above, it is important that short peptides have been used, since short peptides can raise antibodies that react with a non-native conformation of the full polypeptide, and not with the full polypeptide in native conformation, and so these antibodies can bind to sequence present in the disordered fringes of the aggregated polypeptide, but typically not to the same sequence in the native polypeptide.

Furthermore, where the method of the invention has been used to identify and isolate structurally distinct types of aggregates of a polypeptide, then (as discussed further below) it will be possible to generate antibodies, preferably monoclonal antibodies, which bind specifically to a specific conformational epitope for each aggregate type, preferably wherein the generated antibodies (i) lack of reactivity with soluble native or denatured polypeptide, (ii) lack of reactivity with peptide sequences derived from the amino acid sequence of the polypeptide; and/or (iii) lack of reactivity with at least one alternative structurally distinct type of aggregates of a polypeptide. Such antibodies may be used in the above-mentioned method of typing in addition to, or in place of, any one or more (or even all) of the one or more antibody preparation in the panel which have binding specificity to a peptide sequence derived from the amino acid sequence of the polypeptide.

The aggregate forming polypeptide can, in a preferred embodiment, be superoxide dismutase (SOD), such as a human superoxide dismutase (hSOD), preferably human superoxide dismutase 1 (hSOD1). In this context, hSOD1 may be wild-type, or may be a mutant hSOD1, such as a mutant SOD1 that is associated with ALS, such as the D90A (i.e. substitution of D → A at position 90) hSOD1 mutant, G93A and/or G85R. Many other mutant hSOD1 proteins associated with ALS and/or altered aggregate formation are known in the art, and may be useful in the practice of the present invention, including any one or more of the following mutations: A4S, A4T, A4V, C6F, C6G, C6S, V7E, L8Q, L8V, G10R, G10V, G12R, V14G, V14M, G16A, G16S, N19S, F20C, E21K, E21G, Q22L, Q22R, G27delGP, V29A, G37R, G37V, L38R, L38V, G41D, G41S, H43R, F45C, H46D, H46R, V47A, V47F, H48R, H48Q, E49K, T54R, S59I, G61R, N65S, P66R, L67P, L67R, G72C, G72S, D76V, D76Y, E78insSI, H80R, L84F, L84V, G85R, N86I, N86K, N86S, V87A, V87M, T88delTAD, A89T, A89V, D90A, D90N, D90V, G93A, G93C, G93D, G93R, G93S, G93V, A95T, A95V, D96N, V97L, V97M, E100G, E100K, D101G, D101H, D101N, D101Y, I104F, S105deltaSL, S105L, L106P, L106V, G108V, D109Y, C111Y,

I112M, I112T, I113T, R115C, R115G, V118KTGPX, V118L, V118L, V118insQLKKLPKX, D124G, D124V, D125H, L126GQRWKX, L126S, L126X, G127R, G127GGQRWKX, E132DX, E133deltaE, E133V, S134N, S134T, K136X, T137R, G138E, N139D, N139H, N139K, A140G, G141E, G141X, L144F, L144S, L144FVX, A145T, C146R, G147D, G147R, G147S, V148G, V148I, I149T, I151S, I151T. A full list of mutant hSOD1 proteins associated with ALS and/or altered aggregate formation, currently in excess of 170, are known in the art, a list of which can be found in http://alsod.iop.kcl.ac.uk/, the contents of which are incorporated herein by reference. Note that aggregates/inclusions of SOD1 in motor neurons have also been reported also in ALS patients that lack SOD1 mutations (Forsberg *et al*, 2010; Forsberg *et al*, 2011; Bosco *et al,* 2010).

One particularly preferred embodiment provides methods for identifying and characterizing aggregates of hSOD1 with distinct molecular structures, said method comprising analysing the reactivity of a hSOD1 aggregate with one or more of a panel of antibodies directed to short peptide sequences derived from the amino acid sequence of hSOD1, and/or comprising analysing the reactivity of a hSOD1 aggregate with one or more antibodies as defined by claims 26 or 27, below.

In other words, the particularly preferred embodiment provides a method for typing an hSOD1 aggregate (including a fibril) in a test sample, said method comprising -

(i) determining the reactivity of the hSOD1 aggregate in the test sample with one or more, such as 2, 3, 4, 5, 6, 7, 8 or more, different antibody preparations in a panel of antibody preparations,

wherein each antibody preparation in the panel has binding specificity to a peptide sequence derived from the amino acid sequence of hSOD1, such that different antibody preparations in the panel have binding specificity to different peptides, and

wherein at least one of the antibody preparations of the panel, for which reactivity to the aggregate is determined in step (i), displays differential reactivity to at least two structurally distinct types of hSOD1 aggregate; and

(ii) attributing a type to the hSOD1 aggregate in the test sample, based on the determined level(s) of reactivity with the one or more antibody preparations in the panel as determined in step (i).

In an alternative to the method in the preceding paragraph, step (i) may comprise determining the reactivity of the hSOD1 aggregate in the test sample with one or more antibodies as defined by claims 26 or 27, below, and step (ii) may comprise attributing a type to the hSOD1 aggregate in the test sample, based on the determined level(s) of reactivity with the one or more antibodies as determined in step (i). The skilled person will appreciate that this alternative may also be used in combination with the method described in the preceding paragraph.

The present application may also be practiced on other aggregate forming polypeptides which may, for example, include Alpha-Synuclein/Parkinson's disease, Beta-amyloid/Alzheimer's disease, TAU/Alzheimer's disease, IAPP (Amylin)/Diabetes mellitus type 2, Serum Amyloid A/Rheumatoid arthritis, PrP/Transmissible spongiform encephalopathy, Transthyretin/Familial amyloid polyneuropathy, Huntingtin/Huntington's disease.

Having identified that a polypeptide can exist in at least two structurally distinct types of aggregates, it is then possible to type further aggregates of that protein and/or variants thereof. For example, some mutants of hSOD1 (e.g. D90A) are capable of existing in two structurally distinct types of aggregates, whereas other mutants may not be. The skilled person will appreciate that they can use the results from typing a first protein, which is capable of existing in two structurally distinct types of aggregates, to type a second related protein whether or not that second protein can exist in two or more structurally distinct types of aggregates. Thus, for example, the present invention also provides a method of typing an aggregate of human superoxide dismutase 1 (hSOD1) in a test sample, said method comprising -

(i) determining the reactivity of the hSOD1 aggregate in the test sample with one or more, such as 2, 3, 4, 5, 6, 7, 8 or more, different antibody preparations in a panel of antibody preparations,

wherein each antibody preparation in the panel has binding specificity to a peptide sequence derived from the amino acid sequence of hSOD1, such that different antibody preparations in the panel have binding specificity to different peptides, and

wherein at least one of the antibody preparations of the panel, for which reactivity to the hSOD1 aggregate is determined in step (i), displays differential reactivity to at least two structurally distinct types of aggregate of the hSOD1 D90A mutant; and

(ii) attributing a type to the aggregate in the test sample, based on the determined level(s) of reactivity with the one or more antibody preparations of the panel as determined in step (i).

In an alternative to the method in the preceding paragraph, step (i) may comprise determining the reactivity of the hSOD1 aggregate in the test sample with one or more antibodies as defined by claims 26 or 27, below, and step (ii) may comprise attributing a type to the hSOD1 aggregate in the test sample, based on the determined level(s) of reactivity with the one or more antibodies as determined in step (i). The skilled person will appreciate that this alternative may also be used in combination with the method described in the preceding paragraph.

In this context, hSOD1 polypeptide in the hSOD1 aggregate may be wild-type, or may be a mutant hSOD1, such as a mutant SOD1 that is associated with ALS, including any one or more of the following mutations: A4S, A4T, A4V, C6F, C6G, C6S, V7E, L8Q, L8V, G10R, G10V, G12R, V14G, V14M, G16A, G16S, N19S, F20C, E21K, E21G, Q22L, Q22R, G27delGP, V29A, G37R, G37V, L38R, L38V, G41D, G41S, H43R, F45C, H46D, H46R, V47A, V47F, H48R, H48Q, E49K, T54R, S59I, G61R, N65S, P66R, L67P, L67R, G72C, G72S, D76V, D76Y, E78insSI, H80R, L84F, L84V, G85R, N86I, N86K, N86S, V87A, V87M, T88delTAD, A89T, A89V, D90A, D90N, D90V, G93A, G93C, G93D, G93R, G93S, G93V, A95T, A95V, D96N, V97L, V97M, E100G, E100K, D101G, D101H, D101N, D101Y, I104F, S105deltaSL, S105L, L106P, L106V, G108V, D109Y, C111Y, I112M, I112T, I113T, R115C, R115G, V118KTGPX, V118L, V118L, V118insQLKKLPKX, D124G, D124V, D125H, L126GQRWKX, L126S, L126X, G127R, G127GGQRWKX, E132DX, E133deltaE, E133V, S134N, S134T, K136X, T137R, G138E, N139D, N139H, N139K, A140G, G141E, G141X, L144F, L144S, L144FVX, A145T, C146R, G147D, G147R, G147S, V148G, V148I, I149T, I151S, I151T.

The foregoing methods of the present invention can also be used for identifying epitopes and peptide sequences of aggregates of hSOD1, which are specifically exposed in aggregates of hSOD1, particularly pathological aggregates of hSOD1, said epitopes and peptide sequences being useful as vaccine candidates and as target for identifying therapeutical antibodies and other types of binding molecules, including small molecules, which potentially can be used in the treatment of ALS.

In the case of methods of the invention intended for typing hSOD1 aggregates, it may be preferred that the panel of antibody preparations comprises or consists of one or more antibody preparations selected from

a) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:1 or equivalent thereof,

b) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:2 or equivalent thereof,

c) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:3 or equivalent thereof,

d) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4 or equivalent thereof,

e) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:5 or equivalent thereof,

f) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:6 or equivalent thereof,

g) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:7 or equivalent thereof,

h) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:8 or equivalent thereof,

i) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:9 or equivalent thereof,

j) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:10 or equivalent thereof,

k) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:11 or equivalent thereof,

l) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:12 or equivalent thereof,

m) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:13 or equivalent thereof,

n) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:14 or equivalent thereof, and

o) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:15 or equivalent thereof,

wherein SEQ ID NOs: 1-15 are shown below in Table 1.

Table 1.

SEQ ID NO:	Amino acids sequence	aa of hSOD
1	Ala-Val-Cys-Val-Leu-Lys-Gly-Asp-Gly-Pro-Val-Gln-Gly-Ile-Ile-Asn-Phe	4-20
2	Glu-Ser-Asn-Gly-Pro-Val-Lys-Val-Trp-Gly-Ser-Ile-Lys-Gly-Leu-Thr	24-39
3	His-Gly-Phe-His-Val-His-Glu-Phe-Gly-Asp-Asn-Thr-Ala-Gly-Cys	43-57
4	Cys-Thr-Ser-Ala-Gly-Pro-His-Phe-Asn-Pro-Leu-Ser-Arg-Lys-His-Gly	57-72
5	His-Val-Gly-Asp-Leu-Gly-Asn-Val-Thr-Ala-Asp-Lys-Asp-Gly-Val-Ala-Asp	80-96
6	Glu-Asp-Ser-Val-Ile-Ser-Leu-Ser-Gly-Asp-His-Cys-Ile-Ile-Gly-Arg	100-115
7	Cys-Ile-Ile-Gly-Arg-Thr-Leu-Val-Val-His-Glu-Lys-Ala-Asp-Asp-Leu-Gly	111-127
8	Asn-Glu-Glu-Ser-Thr-Lys-Thr-Gly-Asn-Ala-Gly-Ser-Arg-Leu-Ala-Cys-Gly-Val-Ile-Gly-Ile-Ala-Gln	131-153
9	Gly-Leu-His-Gly-Phe-His-Val-His-Glu	41-49
10	Glu-Phe-Gly-Asp-Asn-Thr-Ala-Gly-Cys	49-57
11	Asp-Leu-Gly-Asn-Val-Thr-Ala-Asp-Lys	83-91
12	Val-Ala-Asp-Val-Ser-Ile-Glu-Asp-Ser	94-102
13	Arg-Thr-Leu-Val-Val-His-Glu	115-121
14	Glu-Glu-Ser-Thr-Lys-Thr-Gly-Asn-Ala	132-140
15	Arg-Leu-Ala-Cys-Gly-Val-Ile-Gly-Ile	143-151

Preferably the whole panel of antibody preparations for use in the method of the present invention has binding specificity peptides that correspond to locations on the full hSOD1 polypeptide that, in sum, is greater than 50%, 60%, 70%, 80%, 85%, 90%, 95% or more, such as substantially 100%, of the full hSOD1 polypeptide

In an alternative to the method in the preceding paragraphs, the antibody panel may comprise or consist of antibodies as defined by claims 26 or 27, below. The skilled person will appreciate that this alternative may also be used in combination with the method described in the preceding paragraph.

In one particularly preferred embodiment, the panel of antibody preparations comprises, or consists of, two or more (such as 3, 4, 5, 6, 7, 8 or more) different antibody preparations, and wherein –

at least one antibody preparation in the panel serves as a control and has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 4 or an equivalent thereof, and

one or more other antibody preparations in the panel display differential reactivity (relative to the control) to at least two structurally distinct types of hSOD1 aggregate, and the one or more other antibody preparations in the panel comprise, consist essentially of, or consist of -

(a) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 5, or an equivalent thereof;

(b) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 6, or an equivalent thereof;

(c) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 7, or an equivalent thereof;

(d) the antibody preparation of (a) and the antibody preparation of (b);

(e) the antibody preparation of (a) and the antibody preparation of (c);

(f) the antibody preparation of (b) and the antibody preparation of (c);

(g) the antibody preparations of each of (a), (b) and (c).

In one option, the binding specificity of preparation(s) with differential reactivity in the panel further comprises an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 8 or an equivalent thereof.

Further additionally or alternatively, the method further comprises determining the reactivity of the aggregate in the test sample with an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 3 or an equivalent thereof.

Yet further additionally or alternatively, the method further comprises determining the reactivity of the aggregate in the test sample with –

(a) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 1, or an equivalent thereof, and/or

(b) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 2, or an equivalent thereof.

In the foregoing methods, the hSOD1 aggregate may be identified as Type A, if the measured level of reactivity of the aggregate with the control preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4 or an equivalent thereof is greater than the level of reactivity of the aggregate with the or each of the antibody preparations in the panel having differential reactivity and which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 5, 6 and/or 7 (and optionally, also SEQ ID NO: 8), or an equivalents thereof, respectively.

As a more stringent check, the hSOD1 aggregate may be identified as Type A, if the measured level of reactivity of the aggregate is highest for X, lower than X for Y, and lower than Y and optionally not detectable for Z, wherein -

X is the measured level of reactivity of the aggregate to the control antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4 or an equivalent thereof,

Y is the measured levels of reactivity of the aggregate to antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 3, 5 and 8, or equivalents thereof, respectively, and

Z is the measured levels of reactivity of the aggregate to antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 1, 2, 6, and 7, or equivalents thereof, respectively.

In the foregoing methods, the hSOD1 aggregate may be identified as Type B, if the measured level of reactivity of the aggregate with the control preparation is less than the level of reactivity of the aggregate with the or each of the antibody preparations in the panel having differential reactivity and which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 5, 6 and/or 7 (and optionally, also SEQ ID NO: 8), or equivalents thereof, respectively.

As a more stringent check, the hSOD1 aggregate may be identified as Type B, if the measured level of reactivity of the aggregate is highest for X, lower than X for Y, and lower than Y and optionally not detectable for Z, wherein -

X is the measured levels of reactivity of the aggregate to antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 5, 6 and 7, or equivalents thereof, respectively,

Y is the measured level of reactivity of the aggregate to the control antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4 or an equivalent thereof and/or the measured level of reactivity of the aggregate to the antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:8 or an equivalent thereof, and

Z is the measured level of reactivity of the aggregate to antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs:1 and 2, or equivalents thereof, respectively.

Thus, in one preferred embodiment, the panel of antibody preparations for use in the methods of the invention comprises an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4 or an equivalent thereof; and one, two, or all three of –

- an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:5 or an equivalent thereof;
- an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:6 or an equivalent thereof; and/or
- an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:7 or an equivalent thereof;

and further optionally, comprises an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:8 or an equivalent thereof.

Thus, in another preferred embodiment, the panel of antibody preparations for use in the methods of the invention comprises eight different antibody preparations which, respectively, have binding specificity to a peptide consisting of the amino acid sequence of each of SEQ ID NO:1, 2, 3, 4, 5, 6, 7 and 8, or equivalents thereof.

In the foregoing discussion, an "equivalent" of a defined SEQ ID NO is intended to refer to a sequence that comprises, consists essentially of, or consists of, a portion of the primary amino acid sequence of hSOD1, wherein the portion is typically 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 or more amino acids in length, and wherein the portion

includes 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 or more amino acids of the defined SEQ ID NO.

In certain embodiments of any of the aspects of the present invention, peptides comprising the sequences of any of SEQ ID Nos: 1, 2, 3, 4,5 ,6, 7, 8, 9, 10,11, 12, 13, 14 and/or 15, and the "equivalents" thereof, may optionally exclude peptides which comprise, or consist, any of the sequences DLGKGGNEESTKTGNAGS (optionally, with N-terminal truncation of D, DL, DLG or DLGK, an/or C-terminal truncation of S, GS, AGS or NAGS); NPLSRKHGGPKDEE (optionally with N-terminal truncation of N, NP or NPL and/or C-terminal truncation of E, EE or DE); IKGLTEGLHGF; HCIIGRTLVVH (optionally with N-terminal truncation of H or HC and/or C-terminal truncation of H or VH; GLHGFHVH; RLACGVIGI (optionally with N-terminal truncation of R or RL); and/or KAVCVLK.

In one option, the "equivalent" of a defined SEQ ID NO does not overlap with any other defined SEQ ID NO in these claims by more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acids, and preferably wherein the equivalent does not include any overlap with any of the other defined SEQ ID NO in these claims. This is particularly the case where the presently defined SEQ ID NOs do not overlap with each other. Thus, for example SEQ ID NO: 3 overlaps with SEQ ID NOs 9 and 10, but not with SEQ ID NO: 2, and so a greater level of overlap may be tolerated between equivalents of SEQ ID NO 3 and either or both of SEQ ID NOs 9 and 10, than with SEQ ID NO:2. The same applies *mutatis mutandis* to other defined SEQ ID NOs.

In a further option, an antibody preparation which has binding specificity to the "equivalent" of a defined SEQ ID NO, may also possesses binding specificity to a peptide consisting of the defined SEQ ID NO.

In a further option, a peptide comprising the "equivalent" of a defined SEQ ID NO may be capable of competitively inhibiting the binding of a peptide consisting of the defined SEQ ID NO to an antibody preparation raised against the defined SEQ ID NO.

In a further option, an antibody preparation which binds specifically to a defined SEQ ID NO, for use in the methods of the present invention, may bind specifically to a peptide consisting of that defined SEQ ID NO in preference to (such as, with at least 2, 3, 4, 5, 10,

15, 20, 25, 30, 40 or 50 times greater reactivity, relative to background binding in a dot blot assay as described in the present examples, than to) a peptide which consists of any of the sequences DLGKGGNEESTKTGNAGS (optionally, with N-terminal truncation of D, DL, DLG or DLGK, an/or C-terminal truncation of S, GS, AGS or NAGS); NPLSRKHGGPKDEE (optionally with N-terminal truncation of N, NP or NPL and/or C-terminal truncation of E, EE or DE); IKGLTEGLHGF; HCIIGRTLVVH (optionally with N-terminal truncation of H or HC and/or C-terminal truncation of H or VH; GLHGFHVH; RLACGVIGI (optionally with N-terminal truncation of R or RL); and/or KAVCVLK.

Since the present invention is the first to recognise the existence of structurally different forms of protein aggregates formed *in vivo*, it also provides new ways to recognise and isolate such aggregates. Accordingly, another aspect of the present invention provides isolated aggregates of hSOD1, and preparations comprising the same.

In this context, "isolated" can include the meaning that the aggregate is substantially free of structurally different forms of aggregates of the same polypeptide. In this context, substantially free may include the meaning of no more than 50%, 40%, 30%, 20%, 15%, 10%, 5%, 4%, 3%, 2%, 1% or less (by weight) of a preparation comprising the isolated aggregate possesses a structurally different form of aggregate from the predominating aggregate form in the preparation.

Accordingly, in another aspect of the present invention, there is provided a preparation comprising, consisting essentially or, or consisting of, an isolated aggregate of hSOD1, wherein the isolated aggregate is characterized by:

reacting with an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4 at a level that is greater than the level at which the aggregate reacts with the or each of antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 5, 6 and/or 7 (and optionally, also SEQ ID NO: 8), respectively.

More preferably, this isolated aggregate is characterized by:

reacting with antibody preparations which have binding specificity to peptides consisting of the amino acid sequences SEQ ID NO: 3 and SEQ ID NO: 4, SEQ ID NO:5 and SEQ ID NO:8, respectively, and of them most strongly with the antibody preparation

with binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 4; and

not reacting with antibody preparations which have binding specificity to peptides consisting of the amino acid sequences SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO: 6, and SEQ ID NO: 7, respectively.

Accordingly, in one embodiment the aggregates are of type A. Type A aggregates are characterized by:

reacting with antibodies directed to the amino acid sequence SEQ ID NO: 3 and SEQ ID NO: 4, SEQ ID NO:5 and SEQ ID NO:8, and of them most strongly with antibodies directed to the amino acid sequence SEQ ID NO: 4; and

not reacting with antibodies directed to the amino acid sequence SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO: 6, and SEQ ID NO: 7.

Type A aggregates may optionally be further characterized by:

reacting with antibodies directed to the amino acid sequence SEQ ID NO: 10, and SEQ ID NO:14; and

not reacting with antibodies directed to the amino acid sequence SEQ ID NO:9, SEQ ID NO:11, SEQ ID NO: 12, and SEQ ID NO: 13.

In another aspect of the present invention, there is provided a preparation comprising, consisting essentially or, or consisting of, an isolated aggregate of hSOD1, wherein the isolated aggregate is characterized by:

reacting with an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4 at a level that is lower than the level at which the aggregate reacts with the or each of antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 5, 6 and/or 7 (and optionally, also SEQ ID NO: 8), respectively.

More preferably, this isolated aggregate is characterized by:

reacting with antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of SEQ ID NOs: 4, 5, 6, 7 and 8, respectively, and of them more strongly with the antibody preparations which have binding specificity to peptides consisting of the amino acid sequences SEQ ID NO: 5, 6 and 7, respectively, than

with the antibody preparations which have binding specificity to peptides consisting of the amino acid sequences SEQ ID NO: 4 and 8, respectively; and

not reacting with antibody preparations which have binding specificity to peptides consisting of the amino acid sequence SEQ ID NO:1, and SEQ ID NO:2, respectively.

Accordingly, this other embodiment the aggregates are of type B. Type B aggregates may be characterized by:

reacting with antibodies directed to the amino acid sequence SEQ ID NO: 4, SEQ ID NO:5, SEQ ID NO: 6, SEQ ID NO:7; and SEQ ID NO: 8, and of them most strongly with antibodies directed to the amino acid sequences SEQ ID NO:5, SEQ ID NO: 6, and SEQ ID NO:7, and

not reacting with antibodies directed to the amino acid sequence SEQ ID NO:1, and SEQ ID NO:2.

Type B aggregates may optionally be further characterized by:

reacting with antibodies directed to the amino acid sequence SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, and SEQ ID NO:14; and

not reacting with antibodies directed to the amino acid sequence SEQ ID NO:9.

A specific aggregate may be said to be reacting with an antibody, or antibody preparation, if a substantial reaction can be seen between the aggregate and antibody / antibody preparation in a dot blot assay (such as the assay as described in the Examples), wherein a substantial reaction can be a reaction that is least 3, 4, 5, 6, 7, 8, 9, 10 or more times higher than –

- the background reaction; and/or
- any other peptide of 10-15 amino acids in length, consisting of an amino acid sequence which is not found in the polypeptide of interest.

A specific aggregate is said to not be reacting with an antibody if no substantial reaction can be seen in a dot blot assay as described in the Examples.

As shown in the present application, Type A aggregates of hSOD1 form in the spinal cord and brain and can be isolated from transgenic mice expressing the G93A, G85R and wild-

type hSOD1. Both Type A and Type B aggregates form in the spinal cord and brain of and can be isolated from transgenic mice expressing the D90A form of hSOD1.

As also shown in the present application, Type B aggregates of hSOD1 are more neurotoxic than type A aggregates, and give rise to accelerated neurodegeneration.

Accordingly, methods of the present invention for typing aggregates of polypeptides, where the aggregate sample(s) as assessed is obtained from a subject, may further comprise the step of attributing a diagnosis, prognosis or disease-related prediction to the subject.

In certain embodiments, the hSOD1 aggregates according to the invention can be isolated from spinal cord or brain of transgenic mice expressing wild-type hSOD1 or mutant forms of hSOD1.

The hSOD1 aggregates according to the invention are different from previously reported hSOD1 aggregates formed *in vitro*.

Larger amounts of hSOD1 aggregates according to the invention can be generated and isolated *in vitro* by methods comprising seeding solutions of hSOD1 with hSOD1 aggregates according to the invention, allowing hSOD1 aggregates to be formed and isolating the formed aggregates.

Accordingly, the present invention also provides a method of producing Type A hSOD1 aggregate, the method comprising seeding a solution of hSOD1 with a Type A hSOD1 aggregate (optionally, wherein the Type A hSOD1 aggregate has been identified as such by a method of the present invention), and allowing further hSOD1 aggregates to be formed in the solution. The method may comprise the further step of typing the thus-formed hSOD1 aggregates by a typing method of the present invention. Additionally, or alternatively, the method may further comprise the step of isolating the thus-formed aggregate, and optionally formulating the aggregate in a composition with carriers or diluents.

The present invention also provides a method of producing Type B hSOD1 aggregate, the method comprising seeding a solution of hSOD1 with a Type B hSOD1 aggregate (optionally, wherein the Type A hSOD1 aggregate has been identified as such by a method

of the present invention), and allowing further hSOD1 aggregates to be formed in the solution. The method may comprise the further step of typing the thus-formed hSOD1 aggregates by a typing method of the present invention. Additionally, or alternatively, the method may further comprise the step of isolating the thus-formed aggregate, and optionally formulating the aggregate in a composition with carriers or diluents.

Suitable carriers or diluents according to the present application must be "acceptable" in the sense of being compatible with the other components in the compositions of the invention and not deleterious to the recipients thereof. Typically, the carriers may be water or saline which will be sterile and pyrogen free.

The hSOD1 aggregates according to the invention can be used to generate aggregate specific antibodies. Accordingly, another aspect of the present invention provides methods for generation of antibodies specific for pathological aggregates of hSOD1, said methods comprising the use of isolated hSOD1 aggregates according to the present invention, such as in the immunization of animals and collection of polyclonal sera, or in panning antibody libraries for specifically binding antibodies.

Another aspect of the present invention provides methods for the generation of monoclonal antibodies using isolated type A or type B hSOD1 aggregates of the present invention. The type A and type B aggregates should each contain specific conformational epitopes that are not bound by the anti-peptide antibodies discussed above. Such specific conformational epitopes may, for example, be present in ordered parts of the aggregates. Optionally, the isolated type A or type B hSOD1 aggregates used for the generation of such monoclonal antibodies may be generated by seeding solution of hSOD1 *in vitro* with type A or type B, as described above. Such aggregates that are generated by *in vitro* seeding may be preferred for this purpose, since their level of purity may be higher than type A or type B aggregates that have been purified from biological sources. In the event that aggregates that are generated by *in vitro* seeding, their type should be confirmed, for example, using the dot-blot assay described in the present examples. Resulting monoclonal antibodies made by this approach can also then be tested in the dot-blot assay to ascertain that they react with type A and or B aggregates. In a preferred embodiment, such monoclonal antibodies are further screened to identify those demonstrating a lack of reactivity with soluble native or denatured SOD1 (e.g. tested with antibodies immobilized to Sepharose

beads as described below). In another preferred embodiment, such monoclonal antibodies are further screened to identify those demonstrating a lack of reactivity with any one or more, such as all eight, of the peptides consisting of the sequences defined by SE ID NOs: 1, 2, 3, 4 ,5, 6, 7, and 8. Monoclonal antibodies fitting such binding criteria may be a preferred form to use therapeutically, since they would not react with mature and immature soluble forms of SOD1 in the tissues.

Another aspect of the present invention provides antibodies directed to the hSOD1 aggregates according to the present invention. Such antibodies may be useful in the treatment, monitoring and diagnosis of ALS. The antibodies may have binding specificity to a peptide that comprises, consists essentially of, or consists of any of SEQ ID NOs: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or an equivalent of any one thereof and/or monoclonal antibodies obtained using isolated type A or type B hSOD1 aggregates of the present invention as discussed above (and/or as defined in claims 26 or 27 below).

Given the observed binding reactivities, antibodies having binding specificity to a peptide that comprises, consists essentially of, or consists of any of SEQ ID NOs: 3, 4, 5, 8, 10, 11, 12, 14, or an equivalent of any one thereof, and/or monoclonal antibodies obtained using isolated type A or type B hSOD1 aggregates of the present invention as discussed above (and/or as defined in claims 26 or 27 below), are of greatest interest for providing direct therapeutic effects for ALS.

Accordingly, in another embodiment, the present invention provides a pharmaceutically acceptable composition, comprising a peptide corresponding to amino acid sequences specifically exposed on pathological aggregates of hSOD1, and preferably selected from peptides comprising, consisting essentially of, or consisting of

a) the amino acid sequence SEQ ID NO: 3 or an equivalent thereof,
b) the amino acid sequence SEQ ID NO: 4 or an equivalent thereof,
c) the amino acid sequence SEQ ID NO: 5 or an equivalent thereof,
d) the amino acid sequence SEQ ID NO: 8 or an equivalent thereof,
e) the amino acid sequence SEQ ID NO: 10 or an equivalent thereof,
f) the amino acid sequence SEQ ID NO: 11 or an equivalent thereof,
g) the amino acid sequence SEQ ID NO: 12 or an equivalent thereof, or
h) the amino acid sequence SEQ ID NO: 14 or an equivalent thereof,

optionally, wherein the peptide is linked, directly or indirectly, to another entity, such as a carrier; and

further optionally wherein the formulation further comprises one or more additional components, such as components selected from the group consisting of pharmaceutically acceptable carriers, diluents, adjuvants, delivery agents or the like.

Also provided is a method for the treatment and/or prophylaxis of ALS, including the delay of onset, reduction in the speed of progression and/or reduction in the symptoms of ALS, in a subject, the method comprising the active immunization of the subject by administration of the pharmaceutically acceptable composition.

Also provided is the foregoing pharmaceutically acceptable composition for use in medicine.

Also provided is the foregoing pharmaceutically acceptable for use in the treatment and/or prophylaxis of ALS, including the delay of onset, reduction in the speed of progression and/or reduction in the symptoms of ALS.

In another embodiment, the present invention provides a pharmaceutically acceptable composition, the composition comprising, consisting essentially of, or consisting of, one or more antibody preparations, wherein the or each antibody preparation has binding specificity to a peptide corresponding to amino acid sequences specifically exposed on pathological aggregates of hSOD1, such as a peptide that comprises, consists essentially of, or consists of –

a) the amino acid sequence SEQ ID NO: 3 or an equivalent thereof,
b) the amino acid sequence SEQ ID NO: 4 or an equivalent thereof,
c) the amino acid sequence SEQ ID NO: 5 or an equivalent thereof,
d) the amino acid sequence SEQ ID NO: 8 or an equivalent thereof,
e) the amino acid sequence SEQ ID NO: 10 or an equivalent thereof,
f) the amino acid sequence SEQ ID NO: 11 or an equivalent thereof,
g) the amino acid sequence SEQ ID NO: 12 or an equivalent thereof,
h) the amino acid sequence SEQ ID NO: 14 or an equivalent thereof, or
i) monoclonal antibodies obtained using isolated type A or type B hSOD1 aggregates of the present invention as discussed above,

optionally, wherein the peptide is linked, directly or indirectly, to another entity, such as a carrier; and

optionally wherein the composition further comprises one or more additional components, such as components selected from the group consisting of pharmaceutically acceptable carriers, diluents, delivery agents or the like.

Unless otherwise stated above, the antibodies in the antibody preparations may be polyclonal or monoclonal. The antibodies are preferably monoclonal antibodies.

The antibodies may be human or non-human. Preferably, where the antibodies are intended for administration to humans, then they will be human (e.g. human polyclonal or human monoclonal) or will be humanised.

Antibodies for use in the present invention includes antibody fragments. Antibody fragments will retain the binding specificity of the parent antibody. The antibody fragments can be generated by standard molecular biology techniques or by cleavage of purified antibodies using enzymes (e.g. pepsin or papain) that generates these fragments. Such antibody fragments according to the invention are exemplified, but not limited to, single chain antibodies, Fv, scFv, Fab, F(ab')2, Fab', Fd, dAb, CDR, or scFv-Fc fragments or nanobodies, and diabodies, or any fragment that may have been stabilized by e.g. PEGylation.

The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Effector functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and secreted from E. coli, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies, and F(ab')2 fragments are "bivalent". By "bivalent" we mean that the said antibodies and F(ab')2 fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.

In one option, antibodies for use in the present invention are antibodies preparation which binds specifically to a defined SEQ ID NO an/or to isolated type A or type B hSOD1 aggregates in preference to (such as, with at least 2, 3, 4, 5, 10, 15, 20, 25, 30, 40 or 50 times greater reactivity, relative to background binding in a dot blot assay as described in the present examples, than to) a peptide which consists of any of the sequences DLGKGGNEESTKTGNAGS (optionally, with N-terminal truncation of D, DL, DLG or DLGK, an/or C-terminal truncation of S, GS, AGS or NAGS); NPLSRKHGGPKDEE (optionally with N-terminal truncation of N, NP or NPL and/or C-terminal truncation of E, EE or DE); IKGLTEGLHGF; HCIIGRTLVVH (optionally with N-terminal truncation of H or HC and/or C-terminal truncation of H or VH; GLHGFHVH; RLACGVIGI (optionally with N-terminal truncation of R or RL); and/or KAVCVLK.

Also provided is a method for the treatment and/or prophylaxis of ALS, including the delay of onset, reduction in the speed of progression and/or reduction in the symptoms of ALS, in a subject, the method comprising the passive immunization of the subject by administration of the foregoing pharmaceutically acceptable composition.

Also provided is the foregoing pharmaceutically acceptable composition for use in medicine.

Also provided is the foregoing pharmaceutically acceptable for use in the treatment and/or prophylaxis of ALS, including the delay of onset, reduction in the speed of progression and/or reduction in the symptoms of ALS.

The foregoing pharmaceutical compositions may be given by any suitable means of administration, typically parenterally. In the case of pharmaceutical compositions which comprise peptides, the composition is preferably given by injection, but can in practice be administered by any suitable means that allows the peptide to provoke an immune response in the subject to which it is administered.

Thus, in one embodiment, the pharmaceutical compositions of the invention can be administered to a subject parenterally, for example, intravenously, intra-arterially, intraperitoneally, intrathecally, intraventricularly, intrasternally, intracranially, intra-muscularly or subcutaneously, or they may be administered by infusion techniques. They

may be best used in the form of a sterile aqueous solution which may contain other substances, for example, enough salts or glucose to make the solution isotonic with blood. The aqueous solutions should be suitably buffered (preferably to a pH of from 3 to 9), if necessary. The preparation of suitable parenteral formulations under sterile conditions is readily accomplished by standard pharmaceutical techniques well-known to those skilled in the art.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

For oral and parenteral administration to human patients, the daily dosage level of the peptides or antibodies will usually be from 1 to 1000 mg per adult (*i.e.* from about 0.015 to 15 mg/kg), administered in single or divided doses.

The physician in any event will determine the actual dosage which will be most suitable for any individual patient and it will vary with the age, weight and response of the particular patient. The above dosages are exemplary of the average case. There can, of course, be individual instances where higher or lower dosage ranges are merited and such are within the scope of this invention.

For veterinary use, a pharmaceutical compositions of the invention is administered as a suitably acceptable formulation in accordance with normal veterinary practice and the veterinary surgeon will determine the dosing regimen and route of administration which will be most appropriate for a particular animal.

A subject for the purposes of the present invention can be any human or animal, such as a mouse or a dog. In one embodiment, the subject may have been diagnosed with ALS, or at risk of ALS (such as having a family history of ALS). The subject may be homozygous or hemizygous for a mutant SOD1 gene, such as a mutant hSOD1, or be homozygous for wildtype SOD1. In the case that the subject is an animal, it may be a transgenic animal that carries a wild-type and/or mutant hSOD1 gene and thus, for example, an animal that is the subject of the present invention may be a animal model of ALS.

Accordingly, the present invention also includes a method of assessing the therapeutic potential of a test treatment for ALS, the method comprising using the foregoing methods of the present invention to assess the ability of test therapy to modulate the type, amount, rate of formation or destruction, and/or tissue distribution of hSOD1 aggregate. Such a method may comprise assessing the type, amount, rate of formation or destruction, and/or tissue distribution of hSOD1 aggregate in cohorts of test subjects, wherein at least one cohort receives the test therapy, and another cohort receives a placebo or alternative therapy.

Yet another aspect of the present invention provides antibodies or antibody fragments directed to epitopes specifically exposed on pathological aggregates of hSOD1, such as on a type A aggregate or a type B aggregate as defined herein, for passive immunization for the treatment and prophylaxis of ALS, preferably the antibodies and antibody fragments are selected from antibodies and antibody fragments specific for an aggregate of hSOD1 according to the invention, more preferably the antibodies and antibody fragments are selected from

a) antibodies and antibody fragments directed to the amino acid sequence SEQ ID NO:3 or an equivalent thereof,
b) antibodies and antibody fragments directed to the amino acid sequence SEQ ID NO:4 or an equivalent thereof,
c) antibodies and antibody fragments directed to the amino acid sequence SEQ ID NO:5 or an equivalent thereof,
d) antibodies and antibody fragments directed to the amino acid sequence SEQ ID NO:6 or an equivalent thereof,
e) antibodies and antibody fragments directed to the amino acid sequence SEQ ID NO:7 or an equivalent thereof,

f) antibodies and antibody fragments directed to the amino acid sequence SEQ ID NO:8 or an equivalent thereof,

g) antibodies and antibody fragments directed to the amino acid sequence SEQ ID NO:10 or an equivalent thereof,

h) antibodies and antibody fragments directed to the amino acid sequence SEQ ID NO:11 or an equivalent thereof,

i) antibodies and antibody fragments directed to the amino acid sequence SEQ ID NO:12 or an equivalent thereof,

j) antibodies and antibody fragments directed to the amino acid sequence SEQ ID NO:13 or an equivalent thereof, and

k) antibodies and antibody fragments directed to the amino acid sequence SEQ ID NO:14 or an equivalent thereof; and

l) monoclonal antibodies obtained using isolated type A or type B hSOD1 aggregates of the present invention as discussed above.

Unless specifically stated above, the antibodies and/or antibody fragments may be polyclonal or monoclonal. The antibodies and/or antibody fragments are preferably monoclonal antibodies.

The antibodies and/or antibody fragments may be human or non-human. Preferably, where the antibodies and/or antibody fragments are intended for administration to humans, then they will be human (e.g. human polyclonal or human monoclonal) or will be humanised. Antibody fragments will retain the binding specificity of the parent antibody. The antibody fragments can be generated by standard molecular biology techniques or by cleavage of purified antibodies using enzymes (e.g. pepsin or papain) that generates these fragments. Such antibody fragments according to the invention are exemplified, but not limited to, single chain antibodies, Fv, scFv, Fab, F(ab')2, Fab', Fd, dAb, CDR, or scFv-Fc fragments or nanobodies, and diabodies, or any fragment that may have been stabilized by e.g. PEGylation.

The advantages of using antibody fragments, rather than whole antibodies, are several-fold. The smaller size of the fragments may lead to improved pharmacological properties, such as better penetration of solid tissue. Effector functions of whole antibodies, such as complement binding, are removed. Fab, Fv, ScFv and dAb antibody fragments can all be

expressed in and secreted from E. coli, thus allowing the facile production of large amounts of the said fragments.

Whole antibodies, and F(ab')2 fragments are "bivalent". By "bivalent" we mean that the said antibodies and F(ab')2 fragments have two antigen combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are monovalent, having only one antigen combining sites.

In one option, antibodies and/or antibody fragments for use in the present invention binds specifically to a defined SEQ ID NO an/or to isolated type A or type B hSOD1 aggregates in preference to (such as, with at least 2, 3, 4, 5, 10, 15, 20, 25, 30, 40 or 50 times greater reactivity, relative to background binding in a dot blot assay as described in the present examples, than to) a peptide which consists of any of the sequences DLGKGGNEESTKTGNAGS (optionally, with N-terminal truncation of D, DL, DLG or DLGK, an/or C-terminal truncation of S, GS, AGS or NAGS); NPLSRKHGGPKDEE (optionally with N-terminal truncation of N, NP or NPL and/or C-terminal truncation of E, EE or DE); IKGLTEGLHGF; HCIIGRTLVVH (optionally with N-terminal truncation of H or HC and/or C-terminal truncation of H or VH; GLHGFHVH; RLACGVIGI (optionally with N-terminal truncation of R or RL); and/or KAVCVLK.

Another aspect of the present invention provides methods for the identification and characterization of substances or compounds that bind to, interfere with the formation of, and/or promotes the degradation of pathological aggregates of hSOD1 according to the present invention. Substance or compounds that bind to, interfere with the formation of, and/or promote the degradation of aggregates of hSOD1 are potentially useful for the treatment and/or prevention of ALS.

Accordingly, the present invention also provides a method for the identification of a substance or compound that binds to, interfere with the formation of, and/or promotes the degradation of pathological aggregates of hSOD1, said method comprising one or more of the steps of:

a) providing a test substance or compound,
b) contacting said test substance or compound with an aggregate of hSOD1 as defined above,

c) determining if the test substance or compound binds to the aggregate of hSOD1, inhibits the formation of aggregates of hSOD1 and/or promotes the degradation of aggregates of hSOD1,

d) identifying said compound as potentially suitable for the treatment of ALS.

The method may further comprise the step of determining if the test substance or compound alters a neurotoxic and/or neurodegenerating effect of the aggregates of hSOD1.

The method may further comprising the step of formulating the substance or compound in a pharmaceutically acceptable composition, optionally wherein the composition further comprises one or more additional components, such as components selected from the group consisting of pharmaceutically acceptable carriers, diluents, delivery agents or the like.

The present invention also provides a method for the treatment and/or prophylaxis of ALS, including the delay of onset, reduction in the speed of progression and/or reduction in the symptoms of ALS, in a subject, the method comprising the administration of a pharmaceutically acceptable composition to the subject, wherein the pharmaceutical composition comprises the substance or compound that has been as potentially suitable for the treatment of ALS.

Candidate compounds which may be tested in the methods according to the invention include simple organic molecules, commonly known as "small molecules", for example those having a molecular weight of less than 2000 Daltons. The methods may also be used to screen compound libraries such as peptide libraries, including synthetic peptide libraries and peptide phage libraries. Once an inhibitor or stimulator of aggregate activity is identified then medicinal chemistry techniques can be applied to further refine its properties, for example to enhance efficacy and/or reduce side effects. Candidate compounds which may be tested in the methods according to the invention further include antibodies, antibody fragments, such as a Fab fragment, a (Fab)2 fragment, a single chain Fab fragment, a single chain Fv fragment, or a single chain Fv dimer. The compound to be tested can be selected from engineered non-natural receptor derivatives, such as derivatives of the anticalin, Affibody, FNfn10, neocarzinostatin, ankyrin repeat protein, PDH finger, CDR3 grafted green fluorescent protein, and E. coli periplasmic binding protein scaffolds.

The determination of to what degree the test compound binds to the aggregate of hSOD1, inhibits the formation of aggregates of hSOD1 and/or promotes the degradation of aggregates of hSOD1, can preferably be performed using antibodies directed to peptide sequences derived from the amino acid sequence of hSOD1.

Preferably the antibodies are selected from

a) antibodies directed to the amino acid sequence SEQ ID NO:1,
b) antibodies directed to the amino acid sequence SEQ ID NO:2,
c) antibodies directed to the amino acid sequence SEQ ID NO:3,
d) antibodies directed to the amino acid sequence SEQ ID NO:4
e) antibodies directed to the amino acid sequence SEQ ID NO:5
f) antibodies directed to the amino acid sequence SEQ ID NO:6,
g) antibodies directed to the amino acid sequence SEQ ID NO:7,
h) antibodies directed to the amino acid sequence SEQ ID NO:8,
i) antibodies directed to the amino acid sequence SEQ ID NO:9,
j) antibodies directed to the amino acid sequence SEQ ID NO:10,
k) antibodies directed to the amino acid sequence SEQ ID NO:11,
l) antibodies directed to the amino acid sequence SEQ ID NO:12,
m) antibodies directed to the amino acid sequence SEQ ID NO:13,
n) antibodies directed to the amino acid sequence SEQ ID NO:14, and
o) antibodies directed to the amino acid sequence SEQ ID NO:15.

More preferably the antibodies to be used are selected from

a) antibodies directed to the amino acid sequence SEQ ID NO:3,
b) antibodies directed to the amino acid sequence SEQ ID NO:4
c) antibodies directed to the amino acid sequence SEQ ID NO:5
d) antibodies directed to the amino acid sequence SEQ ID NO:6,
e) antibodies directed to the amino acid sequence SEQ ID NO:7, and
f) antibodies directed to the amino acid sequence SEQ ID NO:8.

Most preferably the antibodies to be used are antibodies directed to the amino acid sequences SEQ ID NO: 4.

Another aspect of the present invention provides methods for identifying and/or quantifying the amount of hSOD1 aggregates and/or hSOD1 fibrils present in a biological sample obtained from a subject. The biological sample can be blood, plasma, serum, cerebrospinal fluid, or isolated cells, preferably leukocytes or lymphocytes. Such methods are potentially useful for diagnosis and monitoring of ALS:

The method may comprise the use of antibodies directed to peptide sequences derived from the amino acid sequence of hSOD1.

Accordingly, the present invention also provides a method for identifying and/or quantifying the amount of hSOD1 aggregates (including hSOD1 fibrils) present in a biological sample obtained from a subject, said method comprising the use of a panel comprising, consisting essentially of, or consisting of, one or more (such as 2, 3, 4, 5, 6, 7, 8 or more) antibody preparations selected from

a) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 1, or an equivalent thereof,
b) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:2, or an equivalent thereof,
c) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:3, or an equivalent thereof,
d) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4, or an equivalent thereof,
e) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:5, or an equivalent thereof,
f) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:6, or an equivalent thereof,
g) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:7, or an equivalent thereof,
h) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:8, or an equivalent thereof.
i) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:9, or an equivalent thereof,
j) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:10, or an equivalent thereof,

k) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:11, or an equivalent thereof,

l) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:12, or an equivalent thereof,

m) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:13, or an equivalent thereof,

n) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:14, or an equivalent thereof, and/or

o) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:15, or an equivalent thereof.

More preferably the antibodies to be used are selected from

a) antibodies directed to the amino acid sequence SEQ ID NO:3 or an equivalent thereof,

b) antibodies directed to the amino acid sequence SEQ ID NO:4 or an equivalent thereof,

c) antibodies directed to the amino acid sequence SEQ ID NO:5 or an equivalent thereof,

d) antibodies directed to the amino acid sequence SEQ ID NO:6 or an equivalent thereof,

e) antibodies directed to the amino acid sequence SEQ ID NO:7 or an equivalent thereof, and/or

f) antibodies directed to the amino acid sequence SEQ ID NO:8 or an equivalent thereof.

Most preferably the antibodies to be used are antibodies directed to the amino acid sequences SEQ ID NO: 4 or an equivalent thereof.

In an alternative to the method for identifying and/or quantifying the amount of hSOD1 aggregates (including hSOD1 fibrils) present in a biological sample, as described in the preceding paragraphs, the method may comprise the use of one or more antibodies as defined by claims 26 or 27, below. The skilled person will appreciate that this alternative may also be used in combination with the method described in the preceding paragraphs.

The antibodies can be labeled with a fluorescent label. In one preferred embodiment two different antibodies directed to the same amino acid sequence are used. The two antibodies are each labeled with a different fluorescent label, the fluorescent labels being able to undergo fluorescent resonance energy transfer (FRET). In this case a positive FRET signal due to a close proximity of multiple copies of hSOD1 would indicate the presence of aggregates of hSOD1.

Most preferably the same antibody is used, of which two samples are each labeled with a different fluorescent label, the fluorescent labels being able to undergo fluorescent resonance energy transfer (FRET).

Accordingly, the method for identifying and/or quantifying the amount of hSOD1 aggregates (including hSOD1 fibrils) present in a biological sample obtained from a subject, may comprising the use of a first and a second antibody preparation,

wherein the first antibody preparation has binding specificity to an amino acid sequence which is the same as, or equivalent to, an amino acid sequence to which the second antibody preparation has binding specificity, and wherein

the antibodies in the first preparation are labeled with a first fluorescent label,

the antibodies in the second preparation are labeled with a second fluorescent label, and

the first and second fluorescent labels are different and, together, are able to undergo fluorescent resonance energy transfer (FRET).

In one embodiment, the first antibody preparation has binding specificity to an amino acid sequence which is equivalent to an amino acid sequence to which the second antibody preparation has binding specificity, if the first antibody preparation can competitively inhibit the binding of the second antibody preparation to a peptide consisting of the amino acid sequence to which it binds.

The skilled person will also appreciate that FRET is not the only way to detect oligomers and aggregates using antibodies reacting with the same epitope or equivalent epitopes. Alternatively, the method may, for example, employ the same antibody labeled with two different markers. The point would be that each monomeric disordered SOD1 molecule would react with only one antibody. In contrast, polymers and aggregates can react with

both, and there are numerous ways known it the art of detecting the binding of two different markers compared to one only. Accordingly, other techniques that could be used include ELISA, DELFIA and other immunological techniques.

FIGURES

Figure 1. Location of peptides used for immunization in the human SOD1 structure.
The figure shows the two non-covalently associated hSOD1 subunits in different colors. The segments corresponding to the peptides used for immunization are black. Note that several of the peptide segments are well exposed on the surface. Still none of the anti-peptide antibodies reacts with native hSOD1, and all with misfolded hSOD1 (cf. Figure 2A).

Figure 2. Epitope mapping of aggregates from transgenic mice.
(**A**) Capacity of antipeptide antibodies to bind soluble misfolded hSOD1 in 25,000 x g spinal cord extract from a 100-day-old G93A mouse. The antibodies were coupled to Protein A-Sepharose beads in equal amounts. The washing of the gel beads was done to mimic the washing of dot-blot filters following incubation with the antibodies in the epitope-mapping. Bound hSOD1 was assayed by western immunoblotting.
(**B**) Example of antibody-developed filters with homogenates of spinal cord and brain from a terminal G93A mouse applied.
(**C**) Intensities of antibody staining in the assay of spinal cord aggregates from 3 terminally ill mice of different transgenic models. To facilitate comparison of patterns, all results were normalized against the intensity of staining with the 57-72 antibody (100%).
(**D**) Homogenates of spinal cords from G93A mice of different ages were assayed in the epitope-mapping assay and data for the 57-72 antibody are presented. The full circles represent terminally ill mice. The regression line has an R^2 of 0.922.

Figure 3. Estimation of the proportion of aggregates captured in filter dots.
A 200 x g supernatant of a spinal cord homogenate from a terminally ill G93A mouse was captured in filter dots in 10 wells, as described under Methods. Human SOD1 captured in the dots was dissolved by boiling in sample buffer. The 200 x *g* supernatant was also added to 6 replicate tubes and centrifuged at 25,000 x g for 30 min. The supernatants and washed pellets were collected. In the immunoblot, the volumes applied resulted in the pellets being

4 times more diluted and the 25,000 x *g* supernatants 10 times more diluted than the filter dot samples. A replicate immunoblot was run with the murine SOD1-specific antibody to assay for the presence of aggregated murine SOD1 in the homogenate.

Figure 4. Development of the assay: analysis of hSOD1 captured in cellulose acetate filters.

Non-reduced immunoblots of hSOD1 captured on filters from homogenates of spinal cord and brain from a terminally ill G93A mouse. The homogenates were sonicated in buffer containing either 1% NP-40 or 0.1% SDS. The filter dots were punched out and extracted in sample buffer. Following SDS-PAGE, one gel was subjected to in-gel reduction, to ensure that there was equal representation of reduced and oxidized hSOD1 and hSOD1 disulfide-coupled to other proteins [21]. The major proportion of trapped hSOD1 was subunits lacking the disulfide bond and a species with intermediate mobility perhaps representing subunits with non-native disulfide bond. High molecular-weight disulfide-linked species were also found, the material captured in the filter will probably also contain some hSOD1 present in debris in the homogenate. The extent of contamination can be estimated from the content of disulfide-oxidized subunits. In this case the oxidized band accounted for around 4% of all hSOD1 in the NP40-extracted spinal cord.

Figure 5. Epitope mapping of aggregates in spinal cord and brain from D90A mice.

(**A**) Different epitope-mapping staining patterns in terminally ill D90A mice; Filled bars a 543-day-old mouse, and grey bars a 328-day-old mouse.

(**B**) Type A-like (staining with 57-72 antibody, ●) and type B-like (staining with 111-127 antibody, ▲) staining of spinal cord aggregates from terminally ill D90A mice plotted against lifespan.

(**C**) and (**E**) Intensity ratios between the staining with the 111-127 and 57-72 antibodies plotted against lifespan for spinal cord and brain homogenates, respectively. Open symbols in (**B**), (**C**) and (**E**): two non-symptomatic 358- and 363-day-old D90A mice.

(**D**) Type A and type B aggregates in non-symptomatic and terminal D90A mice. Spinal cord homogenates from non-symptomatic and terminal D90A mice were analyzed for type A (○, ●) and type B (△, ▲) aggregates using the 57-72 and 111-127 antibodies, respectively. Open symbols represent non-symptomatic and full symbols terminal mice. ×, +, the results with the 57 – 72 and 111 – 127 antibodies, respectively, for these mice were

below the mean + 2 SD values for non-transgenic controls (0.0026 and 0.0048, respectively, cf. Table 2).

(**F**) Fragility of type A and B aggregates in D90A spinal cord. Spinal cord homogenates from 4 end-stage D90A mice were incubated in GdmCl for 10 min at 23°C followed by 90 s sonication and subsequent analysis of type A and type B aggregates using the 57-72 (●) and 111-127 (▲) antibodies, respectively, as described in Methods. The data are presented as mean ± SEM.

(**G**) Comparison of antibody staining patterns of aggregates in spinal cord (black bars) and brain (grey bars) in the D90A mice presented in panel (**B**). With some of the antibodies, the staining of brain homogenates were very low, and results below the mean + 2 SD of the non-transgenic control mice (cf. Table 2) are here presented in lighter shades. Spinal cords and brains dissected free from brainstem and cerebellum were examined.

Figure 6. Western immunoblots of hSOD1 in spinal cords of terminal D90A mice.

Homogenates of spinal cords from 359-, 383-, 402-, 464-, and 543-day-old terminally ill D90A mice were analyzed with western immunoblots. The intensities of staining were 96%, 92%, 100%, 105% and 106% of the means of the 5 mice, respectively. The amount of hSOD1 in the 543-day-old mouse was thus equal to that in the younger mice. Note also the higher mobility of D90A hSOD1 as compared to wt-hSOD1.

Figure 7. Relation between lifespan and disease duration in transgenic mice expressing different mutant hSOD1s.

The mice were examined twice a weak. Criteria for onset were reduced ability to extend hind legs or tremor when held by the tails, or gait disturbances, or foreleg weakness (in particular G85R mice). The latter was evaluated by letting the mouse grip the wire grid of the cage, while being slightly pulled from behind. Gait was evaluated by watching the mouse walk on a smooth surface. In the duration study, the mice were deemed terminally ill and sacrificed when they no longer could reach the food in the cages. The investigation of the mouse strains was conducted during 2004, 2005 and 2006 when the lifespans, in particular for G93A mice, were shorter than is currently observed.

Figure 8. Epitope mapping of hSOD1 aggregated *in vitro.*

(**A**) Human SOD1 was induced to aggregate under 8 different conditions *in vitro* as described in Methods. All results were normalized against the 57-72 antibody (100%).

(**B**) and (**C**), electron microscopy micrographs of aggregate preparations number 7 and 8 respectively

Figure 9. Antibody reaction with native hSOD1.

(A) Antibodies raised against short peptides in hSOD1 do not react with native hSOD1, only with denatured forms of the protein (B).

Antibodies raised against short peptides in hSOD1 do not react with native hSOD1, only with disordered forms of the protein: background to the principles behind the epitope-mapping assay.

Peptides corresponding to amino acids 3-20, 24-39, 43-57, 57-72, 80-96, 100-115, and 131-153 in hSOD1 were coupled to keyhole limpet hemocyanin and used to raise antibodies in rabbits. The antibodies were purified with Protein A-Sepharose (GE Healthcare, Uppsala, Sweden) followed by Sulfolink gel with the respective peptides coupled (Pierce, Rockford, IL). The antibodies were then coupled to Protein A-Sepharose and the resulting beads incubated in pH 7 PBS containing 5 μg/ml hSOD1 that was either native (Figure 9A) or had been denatured by exposure to guanidinium chloride and a chelator followed by dialysis (Figure 9B). Following washes, bound hSOD1 was analyzed by western immunoblots. The native hSOD1 solutions were incubated twice with the antibody beads, with the intention to capture any traces of denatured hSOD1 in the first, to make the second more representative for the reaction of the peptide antibodies with native hSOD1. Note that the "native" blot (Figure 9A) was exposed for 2 min and the denatured blot (Figure 9B) for 5 sec to the film.

The lack of reactivity is not explained by exposure or concealment of the peptides in the native hSOD1 structure (Figure 1), since several of the corresponding sequence elements are well exposed on the surface.

The explanation lies in the mode of antibody production and the properties of the native hSOD1 molecule. Short peptides, such as those used here, have generally no ordered structure, and if so not necessarily the same structure as in native hSOD1. The resulting antibodies will not bind to any specific structure in the rigid native hSOD1 molecule, but only to flexible segments that can adapt to the antigen-binding sites.

If not amorphous, protein aggregates are typically composed of a fibril-like spine of stacked β sheet structures, which is sometimes decorated with domain-swapped material based on inter-molecular native contacts (a simplified cartoon in Figure 9C). The sequence regions outside this ordered aggregate core, which will have lost their native contacts, form disordered fringes. In the epitope-mapping assay we assume that the aggregate core has no reactivity to the antibodies, because of the ordered structure and perhaps also because of steric hindrance. In contrast the disordered fringes in the aggregate fibrils should be reactive.

EXAMPLES

EXPERIMENTAL PROCEDURES

Mice

In this study the following transgenic mouse models were used: hemizygous G93AGur (G1), homozygous line 134 D90A mice (Jonsson et al., 2006b), and hemizygous G85R (Bruijin et al., 1997) and homozygous wild-type hSOD1 (wt-hSOD1) transgenic mice (Graffmo et al., 2012). The mean lifespans of the mouse lines are 155 ± 9 (n=170), 216 ±-15 (n=14), 424 ± 60 (n=139), 370 ± 34 (n=13) and 367 ± 56 (n=56) days respectively. The lines that carried mutant hSOD1s were backcrossed 10-30 generations in C57Bl/6 mice, whereas the homozygous wt-hSOD1 mice were in CBA background. For control purposes C57Bl/6 and SOD1 knockout mice (Reaume et al., 1996) were used. The mice were considered terminally ill if they could not right themselves within 5 seconds after being put on their side. The use and maintenance of the mice and the experimental protocol described in this article were approved by the Ethics Committee for Animal Research at Umeå University.

Antibodies

Antibodies to peptides corresponding to amino acids 4-20, 24-39, 43-57, 57-72, 80-96, 100-115, and 131-153 in hSOD1 were coupled to keyhole limpet hemocyanin and raised in rabbits as previously described (Jonsson et al., 2004). The antibodies were purified with Protein A-Sepharose (GE Healthcare, Uppsala, Sweden) followed by Sulfolink gel with the respective peptides coupled (Pierce, Rockford, IL). Antibodies to amino acids 111-132 and 123-132 in G127X mutant hSOD1 were similarly prepared. Amino acids 128-132 represent a neosequence in the mutant, which is why the 111-132 antibody only can react

with epitopes among amino acids 111-127 in full-length hSOD1. It is therefore designated so in assays involving full-length hSOD1s. The 123-132 antibody shows no reactivity with full-length hSOD1 (Jonsson et al., 2004). For analysis of murine SOD1 a specific antibody raised against a peptide corresponding to amino acids 24-36 in the sequence was used.

Tissue homogenization and epitope-mapping assay for SOD1 aggregate structure

Mice were killed by intraperitoneal injection of pentobarbital. The whole spinal cord, and brain dissected free from midbrain, pons, medulla and cerebellum were mostly examined. The dissected tissues were homogenized with an Ultraturrax apparatus (IKA, Staufen, Germany) for 30 s and sonication for 1 min in 25 volumes of ice-cold APBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na2HPO4, 1.4 mM KH2PO4, pH 7.0) supplemented with 1.8 mM EDTA, 1 mM dithithreitol (DTT), and the antiproteolytic cocktail Complete® without EDTA (Roche Diagnostics, Basel, Switzerland). The tissue homogenates were added to 20 volumes of the APBS with DTT and EDTA containing 1% of the detergent NP40, sonicated for 30 s and then centrifuged at 200 x g for 10 min. The supernatants were step-wise diluted 1+1 in the APBS, and 100 µl captured on 0.22 µm cellulose acetate filters in a 96-well dot-blot apparatus (Whatman GmbH, Dassel, Germany). The wells were then washed with 3 x 300 µl of the APBS without NP40. Following blocking in TBS with 5% dry milk and 0.1% Tween 20 for 1 hour the filters were cut and incubated overnight at 4°C with the anti-hSOD1 peptide antibodies at 0.01 µg/ml (the 4-20 and 100-115 antibodies at 0.02 µg/ml) dissolved in the blocking buffer. After washing, the blots were thereafter developed with HRP-substituted goat anti-rabbit Ig antibodies (Dako 1/42000) and ECL Advance (GE Healthcare), recorded in a Chemidoc apparatus and evaluated with Quantity One software (BioRad).

To allow comparison and quantification, one homogenate of a spinal cord from a terminal G93A mouse was designated as a standard (set to 1) and kept in multiple aliquots at -80 °C. Dilutions series (1 + 1) of this standard were run in 1 or 2 lanes on all filters and were stained with the 57-72 antibody. All blots of all homogenates with all antibodies were quantified against this standard. To facilitate comparison of staining patterns, in some cases the staining intensities of the 8 antibodies of individual homogenates were normalized against the staining of the homogenate with the 57 – 72 antibody (taken as 100%).

Fragilities of types A and B aggregates.

Spinal cord homogenates (1 + 25, see above) from 4 end-stage D90A mice were mixed with equal volumes of PBS containing 2% NP40, followed by sonication. The supernatants after 200 x g centrifugation were added to PBS containing various concentrations of GdmCl and incubated with shaking for 10 min at 23°C. The mixtures were then sonicated for 90 s, and thereafter diluted 120-fold in PBS. Diluted homogenates were finally captured on filters in the dot-blot apparatus, and type A and type B aggregates were analyzed using the 57-72 and 111-127 antibodies, respectively.

Assay of hSOD1 aggregates formed *in vitro*

Human SOD1 was induced to aggregate *in vitro* under 8 different conditions where the first 6 aggregations were carried out with 100 μM (subunits) wild-type hSOD1, were monitored by eye and generally required 3 – 5 days under shaking to develop.

(1) Human SOD1 was rendered in apo form by incubation in 3 M guanidinium chloride and EDTA. The apo hSOD1 was then dialyzed and incubated at 37°C in 100 mM Na phosphate, pH 7.0, and 20 mM dithiothreitol (DTT) to reduce the C57-C146 disulfide bond.

(2) The hSOD1 was incubated in 3 M guanidinium chloride, 10 mM EDTA and 20 mM DTT in 100 mM Na phosphate, pH 7.0 at 37°C.

(3) Reduced apo hSOD1 in 39 mM Na cacodylate, pH 6.0, and 20 mM DTT was precipitated overnight at 4°C in 10% TCA.

(4) HSOD1 in 39 mM Na cacodylate, pH 6.0, 10 mM EDTA and 20 mM DTT was incubated at 70°C.

(5) As in (3) but without disulfide bond reduction by DTT.

(6) Apo hSOD1 was incubated in 1 M guanidinium chloride in 100 mM Na phosphate, pH 7.0 at 37°C.

For (7) and (8) aggregates, 25 μM C6A/F50E/G51E/C111A and C6A/F50E/G51E/C111A/D90A mutant hSOD1s respectively, were rendered in apo form by incubation in guanidinium chloride and EDTA and following dialysis agitated at 1800 rpm by a small Teflon magnetic bar at 37°C in 10 mM BisTris, pH 6.3 containing 0.5 mM of the reductant tris(2-carboxyethyl)phosphine. The aggregations were monitored with thioflavine T fluorescence.

The aggregates were collected by centrifugation at 20,000 x g for 15 min, washed 5- times with PBS and kept as pellets at -80 °C. The pellets were then suspended by sonication for 2 x 15 s in 1+25 homogenates of brain from SOD1 knockout mice, made in a similar way to the tissue homogenates in the epitope-mapping assay. The resulting aggregate suspensions were then analyzed by the epitope-mapping assay similar to the tissue extracts. The suspensions were diluted to varying extents with PBS, 1.8 mM EDTA, 1 mM DTT and Complete to produce intensities in the assay that were similar to those from spinal cord homogenates.

Electron microscopy

Carbon-coated 200 mesh copper grids were applied on top of 20 µl droplets of aggregate preparations 7 and 8 and blotted for 5 min. The grid was stained with 1% (w/v) uranyl acetate and allowed to dry in air. Images of 18,000-fold magnification were recorded in a Tecnai G2 Spirit BioTWIN microscope with a tungsten filament operating at 80 kV.

Immunoblots and quantifications

The western immunoblots were generally carried out as previously described (Jonsson et al., 2004) using an antibody raised in chicken against a peptide corresponding to amino acids 57-72 of the hSOD1 sequence. The chemiluminescence of the blots was recorded in a ChemiDoc apparatus and analyzed with Quantity One software (BioRad). For analysis of C57-C146 oxidized and reduced hSOD1, 40 mM iodoacetamide (IAM) (and no DTT) was added to the homogenization buffer to block free sulfhydryl groups. The SDS-PAGE separations were then carried out without reductant but with 100 mM IAM in the sample buffer (non-reduced immunoblot).

Reduced hSOD1 in such blots has an immunoreactivity that is 20-fold higher than that of disulfide-oxidized subunits (Jonsson et al., 2006a; Zetterstrom et al. 2007b). This is advantageous for demonstration of small proportions of reduced subunits. To achieve equal representation of disulfide-reduced and oxidized hSOD1 as well as hSOD1 disulfide-coupled to other proteins, the SDS-PAGE gels were in some cases soaked in 2% mercaptoethanol for 10 min prior to electroblotting to the membrane (in-gel reduction).

RESULTS

Development and properties of the epitope-mapping assay

The assay employed 8 polyclonal rabbit anti-hSOD1 peptide antibody preparations, raised to a panel of 8 peptides that, between them, cover over 90% of the hSOD1 sequence. The peptide antibodies are all specific for misfolded/unfolded/non-native/disordered (in the following text the term misfolded will be used) hSOD1 species; there is no reaction with natively folded hSOD1 (Forsberg et al., PLos ONE 2010). This lack of reactivity is not simply explained by exposure or concealment of the peptides in the native hSOD1 structure, since several of the sequences are well exposed on the surface. The explanation rather lies in the mode of antibody production and properties of the hSOD1 molecule. Short peptides, such as those used here as antigens have generally no ordered structure, and if so, not necessarily the same as in native hSOD1. The resulting antibodies will not bind to any specific structure in the rigid native hSOD1 molecule, but only to flexible segments that can adapt to the antigen-binding sites.

For analysis, the hSOD1 aggregates were captured on 0.2 μm cellulose acetate filters in a 96-well dot-blot apparatus. The filters were then stained similar to western immunoblots using the 8 anti-hSOD1 peptide antibodies (Figure 2B). Antibody concentrations estimated to bind equally well to soluble misfolded hSOD1 species present in spinal cord extracts were used. Around 3% of the soluble hSOD1 in G93A spinal cords is misfolded (Zetterstrom et al., 2007). When equal amounts of peptide antibodies immobilized on Sepharose were incubated with a G93A spinal cord extract, they were found to capture similar amounts of such hSOD1 from an extract (Figure 2A). The 4-20 and 100-115 antibodies bound about half as much as the others and they were used in double concentration in the dot-blot assay. There is a close to linear relationship between staining intensity and antibody concentration in the dot-blot assay (not shown).

If not amorphous, protein aggregates are typically composed of a fibril-like spine of stacked β sheet structures, which is sometimes decorated with domain-swapped material based on inter-molecular native contacts (Eisenberg and Jucker, 2012). The sequence regions outside this ordered aggregate core form disordered fringes. In the assay it was assumed that this aggregate core has no reactivity to the antibodies, because of the ordered structure and perhaps also because of steric hindrance. In contrast the disordered fringes should be reactive.

Two structural varieties of hSOD1 aggregates are formed in transgenic ALS model mice

Figure 2B shows epitope-mapping assay patterns for the spinal cord and brain of a terminally ill G93A mouse. The patterns were distinctly different from the patterns of soluble misfolded G93A hSOD1 from the spinal cord extract of 100-day-old G93A mouse, as captured by the same antibodies (cf. Figure 2A). The results show that aggregates formed in vivo are not amorphous, but have well-defined and clearly distinguishable structures.

In what follows, all data for staining with the antibodies are presented as results of quantifications against a standard, as described in above, in the Experimental Procedures section.

Four ALS mouse models were analyzed in the assay. The aggregates in terminally ill G85R mice and homozygous wt-hSOD1 mice showed a high similarity with those in G93A mice (Figure 2C, Table 2): a structure that was denoted as type A. In clear contrast, the hSOD1 aggregates seen in most terminal D90A mice were different (Figure 2C) and denoted type B. Both types of aggregates lacked reactivity with the 4-20 and 24-39 antibodies, there appeared to be similar reactivities with the 43-57 and 57-72 antibodies, but from there on until the C-terminal there were large differences.

Table 2. Human SOD1 aggregate levels in terminally ill mice of different transgenic models as analyzed with the epitope-mapping assay.

	43-57	57-72	80-96	100-115	111-127	131-153
	Absolute	Absolute	Absolute	Absolute	Absolute	Absolute
	Relative	Relative	Relative	Relative	Relative	Relative
G93A	0.444 ± 0.233	1.241 ± 0.532	0.184 ± 0.104	0.022 ± 0.006	0.012 ± 0.009	0.513 ± 0.218
(n = 6)	38.3 ± 13.0	100	18.0 ± 9.9	2.4 ± 1.3	0.88 ± 0.45	43.0 ± 11.6
G85R	0.255 ± 0.184	1.575 ± 0.697	0.061 ± 0.033	0.007 ± 0.005	0.001 ± 0.001	0.502 ± 0.165
(n = 5)	16.6 ± 11.8	100	3.4 ± 2.6	0.53 ± 0.5	0.09 ± 0.2	34.3 ± 3.6
***wt*-hSOD1**	0.206 ± 0.087	0.678 ± 0.345	0.046 ± 0.018	0.017 ± 0.008	0.005 ± 0.004	0.113 ± 0.040
(n = 6)	31.2 ± 3.4	100	7.5 ± 3.3	3.3 ± 2.9	0.89 ± 0.76	17.4 ± 4.6
Non-tg controls	0.00155 ±	0.00155 ±	0.00319 ±	0.01731 ±	0.00341 ±	0.00122 ±
(n = 8)	0.00048	0.00054	0.00126	0.00789	0.00068	0.00023

The upper data set represents absolute results compared with the results for the G93A standard with the 57-72 antibody (set to 1). The lower set shows means for the groups with

the results for the individual mice with the different antibodies calculated relative to the 57-72 antibody (set to 100%). For D90A, see Figure 5G. The lowest row presents blank reactions given by 4 C57Bl/6 and 4 SOD1 knockout mice (only absolute results). There were no systematic differences between the 2 groups and these non-transgenic controls are therefore presented together. All data are given as mean ± SD.

Time-course of aggregate formation in G93A spinal cords

The G93A model has been extensively examined. Paralytic symptoms are seen from around 110 days, but there is both functional and histopathological evidence for injury to the motor system from around 40 days (Eisenberg & Jucker, 2012). Therefore the time-course of hSOD1 aggregation in spinal cords of G93A mice using the epitope-mapping assay was estimated (Figure 2D). Aggregates could be detected from 14 days of age. Moreover, there was an exponential build-up of aggregate levels with a doubling time of around 14 days. There was no evidence for a lag period or any surge in aggregation at the terminal phase.

Coupling between aggregate structure and disease progression in D90A mice

The structures of aggregates in terminal D90A mice were found to vary considerably in the epitope-mapping assay (Figure 2C), which prompted examination of a large number of such mice. One mouse was found to carry only type A aggregates (Figure 5A). The lifespans of D90A mice show very wide variation (424 ± 60 days, mean ± SD), and this mouse had survived unusually long: 543 days. To check for the possibility of mislabeling of the sample, or a change in mutant hSOD1 expression in this mouse, the spinal cord homogenate was examined by western immunoblotting (Figure. 6). Both the higher mobility and the content of D90A mutant hSOD1 were similar to those in younger D90A mice containing mainly type B aggregates.

Based on this indication of a link between aggregate structure and lifespan of the mice, type A and B patterns (staining intensities with the 57-72 and 111-127 antibodies, as well as ratios between the staining intensities, respectively, cf. Figure 5A) of the individual mice were plotted against lifespan (Figure 5B, Figure 5C). Type A and type B aggregates appear to coexist in D90A mice, and the longer the lifespan the more the type A aggregates predominate.

As a control, non-symptomatic mice within the same age-span were examined (Figure 5B, Figure 5C, open symbols). These mice contained mainly type A aggregates, but apparently less than mice that had become terminally ill around the same age. Because the type B aggregates also appeared to show some reactivity with the 57-72 antibody (Figure 5A), the type A aggregation in the non-symptomatic mice appeared to fit the trajectory of type A aggregation seen in the terminally ill mice.

The study was extended to include also younger non-symptomatic D90A mice (Figure 5D). The data show that the time-course of type A aggregation is relatively similar between individual D90A mice. In contrast, the levels of type B aggregates in non-symptomatic mice were low but highly variable, and there was a precipitous jump to the levels seen in terminal D90A mice.

On this basis, it was concluded that type B aggregation can occur in D90A mice, and when it is initiated, seemingly for stochastic reasons, there is rapid spread of the aggregation and neurodegeneration. If type B aggregation is not initiated, the mice have a long lifespan and eventually die bearing only type A aggregates. The type A aggregate level in the oldest D90A mouse was within the range seen in terminally ill G93A mice (Figure 2D, Figure 5B). The relationships between epitope-mapping patterns and lifespans of individual G93A, G85R, and wt-hSOD1 mice were also explored, but no age-related differences were found.

If D90A mice with short lifespans are primarily killed by rapidly evolving type B aggregation, their disease durations would be expected to be short. Conversely, mice with long lifespans should first be affected by slowly evolving type A aggregation, and then after various times also type B aggregation, resulting in longer durations. To explore this notion the lifespans of mice of the different transgenic models were plotted against the disease durations (Figure 7). A distinct positive correlation was found in the D90A mice, but less so in the other examined models.

Comparison of spinal cord and brain in D90A mice, evidence for spread of aggregation

The link between aggregate structure and lifespan in D90A mice points to the existence of cell-to-cell transmission by template-directed aggregation (Figure 5B, Figure 5C). To

examine this possibility further, aggregate patterns in spinal cord were compared with those in brains of D90A mice (Figure 5G). The data show that overall, the amounts of type A and B aggregates are smaller in the brain than in the spinal cord, but the patterns show distinct similarities. In the non-symptomatic mice, mainly small amounts of type A aggregates were found in spinal cord and even less in brain. The ratios between staining with the 111-127 and 57-72 antibodies plotted against lifespan also showed similarities between spinal cords (Figure 5C) and brains (Figure 5E). The data suggest that the hSOD1 aggregations are not cell-autonomous independent events, but that they are initiated locally and then transmitted in a templated manner to other parts of the CNS.

Fragilities of type A and B aggregates in D90A mice

There is evidence for a positive correlation between aggregate fragility and both neuroinvasiveness and *in vitro* toxicity of aggregates formed from other proteins 24-27. The epitope-mapping patterns suggest that greater proportions of hSOD1 subunits are engaged in fibril core contacts in type A aggregates than in type B aggregates (Figure 2C, Figure 5A). This difference in structural composition could also indicate that the type A and B aggregates are differently fragile. To test this idea, spinal-cord homogenates from 4 terminal D90A mice were added to PBS containing various concentrations of the strong chaotrope guanidinium chloride (GdmCl) and sheared by sonication. After dilution, the mixtures were analyzed for type A and type B aggregates with the epitope-mapping assay (Figure 5F). The type B aggregates were clearly more fragile than the type A aggregates, which is consistent with the type B aggregates causing the most rapid disease progression.

Aggregates in other parts of the CNS and peripheral tissues

In most assays of end-stage mice, brain was examined in parallel with spinal cord (cf. Figure 2B). Aggregates were found in all cases and the patterns appeared to be similar in brains and spinal cords. Even so, overall, the amounts of hSOD1 aggregates were smaller in the brain: on average 4%, 4%, and 40% of the amounts found in terminal G93A, G85R, and wt-hSOD1 spinal cords, respectively. For D90A mice, see Figure 5G. Liver, kidney, heart and skeletal muscle were also analyzed in terminally ill mice of all the models, and in all cases they were found to lack aggregates (not shown).

Human SOD1 aggregates produced *in vitro* are different from those formed in the CNS

Human SOD1 variants were subjected to a variety of conditions that induce aggregation. The aggregate preparations were added to homogenates of brain from SOD1 knockout mice and then examined with the epitope-mapping assay (Figure 8A). A variety of patterns appeared which were generally more similar to each other than to the aggregates formed *in vivo* (cf. Figure 2C). The single similarity was the limited or sometimes absent staining with the 4-20 and 24-39 antibodies. In two cases, numbers (7) and (8), the resulting aggregates were examined by electron microscopy. The micrographs showed similar patterns of fibrils with diameters of around 10 nm, which were occasionally double and apparently twisted (Figure 8B, Figure 8C), resembling appearances of some previously reported hSOD1 aggregate preparations (Chia et al., 2010, DiDonato et al., 2003; Furukawa et al., 2010; Stathopulos et al., 2003).

Taken together , these structural differences between *in vivo* and *in vitro* aggregates show that the hSOD1 aggregation process is malleable and critically sensitive to environmental conditions.

DISCUSSION

Human SOD1 aggregation is malleable, and controlled by the CNS environment

The aim of this study was to gain insight into the formation, structure, and role of hSOD1 aggregates in ALS. Our epitope-mapping assays showed that the hSOD1 aggregates in the transgenic models gave rise to distinct patterns of reactivities with the different antibodies, suggesting that they are not amorphous but have well-ordered molecular structures. Moreover, the precise structures of these aggregates were variable and depended on the identity of the hSOD1 mutation. Similarly, hSOD1 aggregates produced *in vitro* under a variety of conditions also displayed different reactivities with the antibodies, albeit that in all cases these were different from those formed *in vivo*. From this, it was concluded that the aggregation process of hSOD1 is plastic and depends on both mutation and environmental conditions. Our observations are therefore fully consistent with the general notion that protein aggregation, typically in the form of fibrillation, is a generic property of proteins that can occur through multiple pathways: the aggregation energy landscape is malleable and responds both to environmental conditions, protein concentration, and the specific sequence details of the aggregating polypeptide chain (Eichner & Radford 2011;

Eisenberg. & Jucker 2012). Here, it is of particular interest that the cellular environment in the CNS appears to play a critical role in controlling the hSOD1 aggregation pathways. In transgenic mice, this control appears to result in formation of at least two types of aggregate structures. Type A aggregates are found in the G93A, G85R, wt-hSOD1 models, and variable mixtures of type A and type B are found in D90A mice.

A remaining question is why hSOD1 primarily aggregates in the spinal cord, when the level of the protein is similar to that in the brain (Graffmo et al., 2013; Jonsson et al., 2006). One explanation could be that unfolded hSOD1 monomers that lack the stabilizing C57-C146 disulfide bond are present at higher levels in the spinal cord than in brain and other tissues in the transgenic mice (Zetterstrom et al., 2007; Zetterstrom et al., 2013), and that such species are the primary precursors for aggregation. Consistently, the aggregates in transgenic mice are mostly composed of disulfide-reduced monomers (Karch et al., 2009; Bergemalm et al., 2010), and loss of the disulfide bond promotes *in vitro* aggregation of hSOD1 (Chattopadhyay et al., 2008; Furukawa et al., 2008). Moreover, agitation-induced fibrillation of recombinant hSOD1 in vitro has been found to progress by fragmentation-limited kinetics from globally unfolded monomers (Lang et al., 2012).

The observation that *in vivo* and *in vitro* conditions can yield different aggregate structures is not unique to hSOD1; this has also been observed for precursor proteins of other neurodegenerative diseases. For example, many *in vitro*-formed prion protein aggregate preparations have been unable to infect upon inoculation into the CNS, although infective species have also been reported recently (Colby & Prusiner 2011). Intracerebral injection of amyloid-containing extracts from both human Alzheimer patients and human APP transgenic mice can induce cerebral amyloidosis in mice that express a human APP. In contrast, *in vitro*-produced Aβ fibril preparations have either failed (Meyer-Luehmann, et al., 2006) or produced amyloidosis with much lower potencies (Stohr et al., 2012). It is, however, not yet known whether these synthetic aggregate preparations induce formation of aggregates in the CNS with the same molecular structure as those that arise spontaneously. Of note, the amyloidosis induced by the synthetic Aβ fibril preparations showed a distribution in the brain that differed from that caused by inoculation of *in vivo*-formed fibrils (Stohr et al., 2012).

Correlation between aggregate structure and disease phenotype, evidence for spread

D90A mice show large variations in lifespan, and type A and type B aggregates coexisted in most of them (Figure 5B, Figure 5D). The longer the lifespan, the more type A aggregates predominate. Mice within the age range of terminal disease, but which still lacked symptoms, contained mainly type A aggregates. The findings suggest that type A aggregation is a default for the 4 hSOD1 variants examined in this study. The time-course of type A aggregation did not appear to differ much between individual D90A mice, but became detectable much later than in G93A mice (Figure 2D, Figure 5D). One explanation for these differences could be that the levels of unfolded disulfide-reduced monomers are low in young D90A mice and rise markedly with age, whereas the levels are uniformly high in G93A mice of different ages (Zetterstrom et al., 2007).

The levels of type B aggregates were very low in non-symptomatic D90A mice and large differences between individual mice were seen (Figure 5D). Moreover, the rise to the levels found in terminal D90A mice seems to be abrupt. This suggests that when type B aggregation is initiated in a D90A mouse, it spreads rapidly causing acceleration in the neurodegeneration. The later this happens the longer the lifespan, and D90A mice can eventually die bearing only type A aggregates. The notion of a rapid spread of type B aggregation is also supported by the correlation between lifespan and disease duration in the D90A mice (Figure 7). The similarities in patterns between spinal cord and brain in individual mice argue against cell-autonomous aggregation and suggest that a prion-like templated spread might occur (Figure 5G). Supporting this idea, transmission of hSOD1 aggregation has recently been demonstrated in cell cultures (Grad et al., 2011; Munch et al., 2011), and CNS extracts from terminally ill G93A mice have been shown to seed hSOD1 aggregation *in vitro* (Chia et al., 2010).

Primary fibril nucleation events are probably rare since they are energetically unfavorable, and secondary nucleation via fragmentation of fibrils seems to be the principal driver of fibril formation (Lang et al., 2012; Knowles et al., 2009). Moreover, there is evidence for a positive correlation between fragilities of protein aggregates and both their neuroinvasiveness and *in vitro* toxicity (Colby & Prusiner 2011; Xue et al., 2010; Lee et al., 2007; Bett et al., 2012). As could be predicted from the limited fibril core contacts in type B aggregates as compared to type A aggregates, the former are clearly more fragile

than the latter (Figure 5F). This property of type B aggregates might explain their apparently rapid propagation and high neurotoxicity.

The observations in D90A mice naturally draw attention to further analogies with the prion protein diseases, where the disease manifestations are variable and linked to different strains of prions (Colby & Prusiner 2011). As a rule, the D90A mutation causes ALS with a phenotype that deviates from ALS in general: there are commonly both sensory symptoms and bladder control disturbances (Andersen et al., 1996). D90A mice also show bladder control problems, which seldom appear in other hSOD1 transgenic model mice (Jonsson et al., 2006). Perhaps these phenotype deviations are related to the occurrence of type B aggregates.

Role of human SOD1 aggregation in ALS neuropathology

The role of proteinaceous aggregates in the pathogenesis of neurodegenerative diseases is disputed: are the aggregates the primary cause of disease, or are they formed in cells that are terminally compromised by other effects of disordered proteins? The findings in the D90A model provide strong evidence for the former interpretation: the stochastic initiations of types A and B aggregations determine the progression of the disease in individual mice. More indirect evidence is given by the fact that the levels of aggregates in end-stage mice of the different models are similar (Figure 2D, Figure 5B, Table 2), and that despite 50-fold differences in total content of the hSOD1 variants in the spinal cord (Graffmo et al., 2013, Jonsson et al. 2006). Moreover, aggregates can be demonstrated in the G93A model long before any appearance of injury (Figure 2D) (Kanning et al., 2010).

Yet, the identities of the major harmful molecular species formed in the aggregations are still open to conjecture. There is likely a continuum of aggregate species from small fibril fragments to large aggregates. The latter are gradually sequestered into inclusion bodies, and in that form most likely rendered innocuous (Chen et al., 2011). Oligomers, with a structure different from A or B fibrils containing a few hSOD1 monomers, might conceivably correlate with the content of aggregates. However, no such hSOD1 species have with certainty been identified so far. More likely are small fibril fragments, which would more directly correlate with the aggregate load. They are known to be more cytotoxic than larger aggregates (Xue et al., 2010), and the smaller size would facilitate spread both within cells including neurites, and in the interstitial space of the CNS. The

mechanisms by which fibrils are fragmented in the cells are not well understood, but could involve thermal and mechanical impacts at a molecular length scale, the action of various classes of chaperones (sun et al., 2008; Winkler et al., 2012), and the impact of pulse waves.

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CLAIMS

1. A method for typing an aggregate (including a fibril) of a polypeptide in a test sample, wherein the polypeptide is capable of forming at least two structurally distinct types of aggregates, said method comprising -

(i) determining the reactivity of the aggregate in the test sample with one or more, such as 2, 3, 4, 5, 6, 7, 8 or more, different antibody preparations in a panel of antibody preparations,

wherein each antibody preparation in the panel has binding specificity to a peptide sequence derived from the amino acid sequence of the polypeptide, such that different antibody preparations in the panel have binding specificity to different peptides, and

wherein at least one of the antibody preparations of the panel, for which reactivity to the aggregate is determined in step (i), displays differential reactivity to at least two structurally distinct types of the aggregate; and

(ii) attributing a type to the aggregate in the test sample, based on the determined level(s) of reactivity with the one or more antibody preparations in the panel as determined in step (i).

2. The method according to claim 1, wherein the aggregate is formed from a polypeptide which is a superoxide dismutase (SOD), such as a human superoxide dismutase (hSOD), preferably human superoxide dismutase 1 (hSOD1).

3. The method of claim 1, wherein the hSOD1 polypeptide is a mutant hSOD1 that is associated with ALS, such as the D90A hSOD1 mutant.

4. A method for typing an aggregate of human superoxide dismutase 1 (hSOD1) in a test sample, said method comprising -

(i) determining the reactivity of the hSOD1 aggregate in the test sample with one or more, such as 2, 3, 4, 5, 6, 7, 8 or more, different antibody preparations in a panel of antibody preparations,

wherein each antibody preparation in the panel has binding specificity to a peptide sequence derived from the amino acid sequence of hSOD1, such that different antibody preparations in the panel have binding specificity to different peptides, and

wherein at least one of the antibody preparations of the panel, for which reactivity to the hSOD1 aggregate is determined in step (i), displays differential reactivity to at least two structurally distinct types of aggregate of the hSOD1 D90A mutant; and

(ii) attributing a type to the aggregate in the test sample, based on the determined level(s) of reactivity with the one or more antibody preparations of the panel as determined in step (i).

5. The method of claim 4, wherein hSOD1 polypeptide in the aggregate comprises mutant hSOD1 that is associated with ALS, such as the D90A hSOD1 mutant.

6. The method of any of claims 2 to 5, wherein the polypeptide is hSOD1, and the panel of antibody preparations comprises or consists of one or more antibody preparations selected from

a) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:1 or equivalent thereof,

b) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:2 or equivalent thereof,

c) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:3 or equivalent thereof,

d) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4 or equivalent thereof,

e) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:5 or equivalent thereof,

f) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:6 or equivalent thereof,

g) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:7 or equivalent thereof,

h) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:8 or equivalent thereof,

i) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:9 or equivalent thereof,

j) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:10 or equivalent thereof,

k) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:11 or equivalent thereof,

l) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:12 or equivalent thereof,

m) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:13 or equivalent thereof,

n) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:14 or equivalent thereof, and

o) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:15 or equivalent thereof.

7. The method of claim 6, wherein the panel of antibody preparations comprises, or consists of, two or more (such as 3, 4, 5, 6, 7, 8 or more) different antibody preparations, and wherein –

at least one antibody preparation in the panel serves as a control and has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 4 or an equivalent thereof, and

one or more other antibody preparations in the panel display differential reactivity (relative to the control) to at least two structurally distinct types of hSOD1 aggregate, and the one or more other antibody preparations in the panel comprise, consist essentially of, or consist of -

(a) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 5, or an equivalent thereof;

(b) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 6, or an equivalent thereof;

(c) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 7, or an equivalent thereof;

(d) the antibody preparation of (a) and the antibody preparation of (b);

(e) the antibody preparation of (a) and the antibody preparation of (c);

(f) the antibody preparation of (b) and the antibody preparation of (c);

(g) the antibody preparations of each of (a), (b) and (c).

8. The method of Claim 7 (a), (b), (c), (d), (e), (f) and/or (g) wherein the binding specificity of preparation(s) with differential reactivity in the panel further comprises an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 8 or an equivalent thereof.

9. The method of Claim 7 (a), (b), (c), (d), (e), (f) and/or (g), or Claim 8, wherein the method further comprises determining the reactivity of the aggregate in the test sample with an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 3 or an equivalent thereof.

10. The method of Claim 7 (a), (b), (c), (d), (e), (f) and/or (g), or Claim 8, or Claim 9, wherein the method further comprises determining the reactivity of the aggregate in the test sample with –

(a) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 1, or an equivalent thereof, and/or

(b) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 2, or an equivalent thereof.

11. The method of Claim 7 or 8 wherein the hSOD1 aggregate is identified as Type A, if the measured level of reactivity of the aggregate with the control preparation is greater than the level of reactivity of the aggregate with the or each of the antibody preparations in the panel having differential reactivity and which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 5, 6 and/or 7 (and optionally, also SEQ ID NO: 8), or an equivalents thereof, respectively.

12. The method of Claim 10 or 11, wherein the hSOD1 aggregate is identified as Type A, if the measured level of reactivity of the aggregate is highest for X, lower than X for Y, and lower than Y and optionally not detectable for Z, wherein -

X is the measured level of reactivity of the aggregate to the control antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4 or an equivalent thereof,

Y is the measured levels of reactivity of the aggregate to antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 3, 5 and 8, or equivalents thereof, respectively, and

Z is the measured levels of reactivity of the aggregate to antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 1, 2, 6, and 7, or equivalents thereof, respectively.

13. The method of Claim 7 or 8 wherein the hSOD1 aggregate is identified as Type B, if the measured level of reactivity of the aggregate with the control preparation is less than the level of reactivity of the aggregate with the or each of the antibody preparations in the panel having differential reactivity and which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 5, 6 and/or 7 (and optionally, also SEQ ID NO: 8), or equivalents thereof, respectively.

14. The method of Claim 10 or 13, wherein the hSOD1 aggregate is identified as Type B, if the measured level of reactivity of the aggregate is highest for X, lower than X for Y, and lower than Y and optionally not detectable for Z, wherein -

X is the measured levels of reactivity of the aggregate to antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 5, 6 and 7, or equivalents thereof, respectively,

Y is the measured level of reactivity of the aggregate to the control antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4 or an equivalent thereof and/or the measured level of reactivity of the aggregate to the antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:8 or an equivalent thereof, and

Z is the measured level of reactivity of the aggregate to antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs:1 and 2, or equivalents thereof, respectively.

15. The method of any of claims 6-14, wherein the equivalent of a defined SEQ ID NO is a sequence that comprises, consists essentially of, or consists of, a portion of the primary amino acid sequence of hSOD1, wherein the portion is typically 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 or more amino acids in length, and wherein the portion includes 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 or more amino acids of the defined SEQ ID NO.

16. The method of claim 15, wherein the equivalent of a defined SEQ ID NO does not overlap with any other defined SEQ ID NO in these claims by more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acids, and preferably wherein the equivalent does not include any overlap with any of the other defined SEQ ID NO in these claims.

17. The method of claim 15 or 16, wherein –

(a) an antibody preparations which has binding specificity to the equivalent of a defined SEQ ID NO, also possesses binding specificity to a peptide consisting of the defined SEQ ID NO; and/or

(b) wherein the equivalent of a defined SEQ ID NO will competitively inhibit the binding of a peptide consisting of the defined SEQ ID NO to an antibody preparation raised against the defined SEQ ID NO.

18. A preparation comprising, consisting essentially or, or consisting of, an isolated aggregate of hSOD1, wherein the isolated aggregate is characterized by:

reacting with an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4 at a level that is greater than the level at which the aggregate reacts with the or each of antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 5, 6 and/or 7 (and optionally, also SEQ ID NO: 8), respectively.

19. The preparation of claim 18, wherein the isolated aggregate is characterized by:

reacting with antibody preparations which have binding specificity to peptides consisting of the amino acid sequences SEQ ID NO: 3 and SEQ ID NO: 4, SEQ ID NO:5 and SEQ ID NO:8, respectively, and of them most strongly with the antibody preparation with binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 4; and

not reacting with antibody preparations which have binding specificity to peptides consisting of the amino acid sequences SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO: 6, and SEQ ID NO: 7, respectively.

20. A preparation comprising, consisting essentially or, or consisting of, an isolated aggregate of hSOD1, wherein the isolated aggregate is characterized by:

reacting with an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4 at a level that is lower than the level at which the aggregate reacts with the or each of antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of each of SEQ ID NOs: 5, 6 and/or 7 (and optionally, also SEQ ID NO: 8), respectively.

21. The preparation of claim 20, wherein the isolated aggregate is characterized by:

reacting with antibody preparations which have binding specificity to peptides consisting of the amino acid sequences of SEQ ID NOs: 4, 5, 6, 7 and 8, respectively, and of them more strongly with the antibody preparations which have binding specificity to peptides consisting of the amino acid sequences SEQ ID NO: 5, 6 and 7, respectively, than with the antibody preparations which have binding specificity to peptides consisting of the amino acid sequences SEQ ID NO: 4 and 8, respectively; and

not reacting with antibody preparations which have binding specificity to peptides consisting of the amino acid sequence SEQ ID NO:1, and SEQ ID NO:2, respectively.

22. A method for the generation of antibodies having binding specificity for an isolated aggregate as defined by (a) claim 18 or 19; or (b) claim 20 or 21, the method comprising –

immunization of an animal with an isolated aggregate as defined by (a) claim 18 or 19; or (b) claim 20 or 21, and/or panning an antibody library with an isolated aggregate as defined (a) claim 18 or 19; or (b) claim 20 or 21,

identifying antibodies having binding specificity for an isolated aggregate as defined by (a) claim 18 or 19; or (b) claim 20 or 21, and

optionally isolating a cell that produces the thus-identified antibodies and/or one or more nucleic sequences that encode the thus-identified antibodies.

23. The method of claim 22, wherein the method comprises the production of a monoclonal antibody that is identified as having binding specificity to an isolated aggregate as defined by (a) claim 18 or 19; or (b) claim 20 or 21.

24. The method of claim 22 or 23, wherein the isolated aggregate is generated by a method comprising the step of –

(a) seeding a solution of hSOD1 *in vitro* with an isolated aggregate as defined by claim 18 or 19, thereby to produce further isolated aggregate having the characteristics defined by claim 18 or 19; or

(b) seeding a solution of hSOD1 *in vitro* with an isolated aggregate as defined by claim 20 or 21, thereby to produce further isolated aggregate having the characteristics defined by claim 20 or 21.

25. The method of any of claims 22-24, wherein the or each of the generated antibodies are further screened to identify one or more antibodies demonstrating at least one, at least two, or all three of –

(i) a lack of reactivity with soluble native or denatured SOD1,

(ii) a lack of reactivity with any one or more, such as all eight, of the peptides consisting of the sequences defined by SEQ ID NOs: 1, 2, 3, 4 ,5, 6, 7, and 8; and/or

(iii) a lack of reactivity with an isolated aggregate having the characteristics defined by claim 20 or 21 in the case antibodies generated with an isolated aggregate as defined by claim 18 or 19, or a lack of reactivity with an isolated aggregate having the characteristics defined by claim 18 or 19 in the case antibodies generated with an isolated aggregate as defined by claim 20 or 21.

26. An antibody obtained or obtainable by the method of any of claims 22-25, a cell expressing said antibody, or a nucleic acid sequence encoding said antibody.

27. A monoclonal antibody, or fragment thereof, which has binding specificity to an isolated aggregate as defined by any of claims 18-21, and preferably lacks reactivity with soluble native or denatured SOD1 and/or with any one or more, such as all eight, of the peptides consisting of the sequences defined by SE ID NOs: 1, 2, 3, 4 ,5, 6, 7, and 8.

28. A pharmaceutically acceptable composition, comprising a peptide selected from peptides comprising, consisting essentially of, or consisting of

a) the amino acid sequence SEQ ID NO: 3 or an equivalent thereof,
b) the amino acid sequence SEQ ID NO: 4 or an equivalent thereof,
c) the amino acid sequence SEQ ID NO: 5 or an equivalent thereof,
d) the amino acid sequence SEQ ID NO: 8 or an equivalent thereof,
e) the amino acid sequence SEQ ID NO: 10 or an equivalent thereof,
f) the amino acid sequence SEQ ID NO: 11 or an equivalent thereof,
g) the amino acid sequence SEQ ID NO: 12 or an equivalent thereof, or
h) the amino acid sequence SEQ ID NO: 14 or an equivalent thereof,

optionally, wherein the peptide is linked, directly or indirectly, to another entity, such as a carrier; and

further optionally wherein the formulation further comprises one or more additional components, such as components selected from the group consisting of pharmaceutically acceptable carriers, diluents, adjuvants, delivery agents or the like.

29. A pharmaceutically acceptable composition, the composition comprising, consisting essentially of, or consisting of, one or more antibody preparations, wherein the or each antibody preparation has binding specificity to a peptide that comprises, consists essentially of, or consists of –

a) the amino acid sequence SEQ ID NO: 3 or an equivalent thereof,
b) the amino acid sequence SEQ ID NO: 4 or an equivalent thereof,
c) the amino acid sequence SEQ ID NO: 5 or an equivalent thereof,
d) the amino acid sequence SEQ ID NO: 8 or an equivalent thereof,
e) the amino acid sequence SEQ ID NO: 10 or an equivalent thereof,

f) the amino acid sequence SEQ ID NO: 11 or an equivalent thereof,

g) the amino acid sequence SEQ ID NO: 12 or an equivalent thereof, or

h) the amino acid sequence SEQ ID NO: 14 or an equivalent thereof,

optionally, wherein the peptide is linked, directly or indirectly, to another entity, such as a carrier; and

optionally wherein the composition further comprises one or more additional components, such as components selected from the group consisting of pharmaceutically acceptable carriers, diluents, delivery agents or the like.

30. The pharmaceutically acceptable composition of claim 23, wherein the, more than one, or each of the one or more antibody preparations is/are monoclonal antibody preparation(s).

31. A pharmaceutically acceptable composition, the composition comprising, consisting essentially of, or consisting of, antibodies as defined by claim 26 or 27, optionally wherein the composition further comprises one or more additional components, such as components selected from the group consisting of pharmaceutically acceptable carriers, diluents, delivery agents or the like.

32. A method for the treatment and/or prophylaxis of ALS, including the delay of onset, reduction in the speed of progression and/or reduction in the symptoms of ALS, in a subject, the method comprising the active immunization of the subject by administration of the pharmaceutically acceptable composition of claim 28, or the passive immunization of the subject by administration of the pharmaceutically acceptable composition of claims 29, 30 or 31.

33. The pharmaceutically acceptable composition of claim 28, 29, 30 or 31 for use in medicine.

34. The pharmaceutically acceptable composition of claim 28, 29, 30 or 31 for use in the treatment and/or prophylaxis of ALS, including the delay of onset, reduction in the speed of progression and/or reduction in the symptoms of ALS.

35. The use of a peptide in the manufacture of a medicament for the treatment and/or prophylaxis of ALS, including the delay of onset, reduction in the speed of progression and/or reduction in the symptoms of ALS, by active immunization of a subject, wherein the peptide is selected from peptides comprising, consisting essentially of, or consisting of

a) the amino acid sequence SEQ ID NO: 3 or an equivalent thereof,
b) the amino acid sequence SEQ ID NO: 4 or an equivalent thereof,
c) the amino acid sequence SEQ ID NO: 5 or an equivalent thereof,
d) the amino acid sequence SEQ ID NO: 8 or an equivalent thereof,
e) the amino acid sequence SEQ ID NO: 10 or an equivalent thereof,
f) the amino acid sequence SEQ ID NO: 11 or an equivalent thereof,
g) the amino acid sequence SEQ ID NO: 12 or an equivalent thereof, or
h) the amino acid sequence SEQ ID NO: 14 or an equivalent thereof,

optionally, wherein the peptide is linked, directly or indirectly, to another entity, such as a carrier.

36. The use of one or more antibody preparations, in the manufacture of a medicament for the treatment and/or prophylaxis of ALS, including the delay of onset, reduction in the speed of progression and/or reduction in the symptoms of ALS, by passive immunization of a subject, wherein the or each antibody preparation has binding specificity to a peptide that comprises, consists essentially of, or consists of –

a) the amino acid sequence SEQ ID NO: 3 or an equivalent thereof,
b) the amino acid sequence SEQ ID NO: 4 or an equivalent thereof,
c) the amino acid sequence SEQ ID NO: 5 or an equivalent thereof,
d) the amino acid sequence SEQ ID NO: 8 or an equivalent thereof,
e) the amino acid sequence SEQ ID NO: 10 or an equivalent thereof,
f) the amino acid sequence SEQ ID NO: 11 or an equivalent thereof,
g) the amino acid sequence SEQ ID NO: 12 or an equivalent thereof, or
h) the amino acid sequence SEQ ID NO: 14 or an equivalent thereof,

optionally, wherein the peptide is linked, directly or indirectly, to another entity, such as a carrier.

37. The use of claim 29 wherein the, more than one, or each of the one or more antibody preparations is/are monoclonal antibody preparation(s).

38. The use of antibodies as defined by claim 26 or 27 in the manufacture of a medicament for the treatment and/or prophylaxis of ALS, including the delay of onset, reduction in the speed of progression and/or reduction in the symptoms of ALS, by passive immunization of a subject.

39. The pharmaceutically acceptable composition of any of claims 28, 29, 30, 33 or 34, the method of claim 32, or the use of any of claims 35, 36 or 37, wherein the peptide comprises, consists essentially of, or consists of, the amino acid sequence SEQ ID NO: 4, or an equivalent thereof, and is optionally linked, directly or indirectly, to another entity, such as a carrier.

40. The pharmaceutically acceptable composition of any of claims 28, 29, 30, 33, 34 or 39, the method of claim 32 or 39, or the use of any of claims 35, 36, 37 or 39, wherein the equivalent of a defined SEQ ID NO is an equivalent as defined by any of claims 15, 16 and/or 17.

41. A method for identifying and/or quantifying the amount of hSOD1 aggregates (including hSOD1 fibrils) present in a biological sample obtained from a subject, said method comprising the use of a panel comprising, consisting essentially of, or consisting of, one or more (such as 2, 3, 4, 5, 6, 7, 8 or more) antibody preparations selected from

a) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO: 1, or an equivalent thereof,
b) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:2, or an equivalent thereof,
c) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:3, or an equivalent thereof,
d) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:4, or an equivalent thereof,
e) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:5, or an equivalent thereof,
f) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:6, or an equivalent thereof,

g) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:7, or an equivalent thereof,

h) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:8, or an equivalent thereof.

i) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:9, or an equivalent thereof,

j) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:10, or an equivalent thereof,

k) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:11, or an equivalent thereof,

l) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:12, or an equivalent thereof,

m) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:13, or an equivalent thereof,

n) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:14, or an equivalent thereof, and

o) an antibody preparation which has binding specificity to a peptide consisting of the amino acid sequence SEQ ID NO:15, or an equivalent thereof.

42. The method according to claim 41 comprising the use of a first and a second antibody preparation,

wherein the first antibody preparation has binding specificity to an amino acid sequence which is the same as, or equivalent to, an amino acid sequence to which the second antibody preparation has binding specificity, and wherein

the antibodies in the first preparation are labeled with a first fluorescent label,

the antibodies in the second preparation are labeled with a second fluorescent label, and

the first and second fluorescent labels are different and, together, are able to undergo fluorescent resonance energy transfer (FRET).

43. The method of claim 42, wherein the first antibody preparation has binding specificity to an amino acid sequence which is equivalent to an amino acid sequence to which the second antibody preparation has binding specificity, if the first antibody

preparation can competitively inhibit the binding of the second antibody preparation to a peptide consisting of the amino acid sequence to which it binds.

44. A method for the identification of a substance or compound that binds to, interfere with the formation of, and/or promotes the degradation of pathological aggregates of hSOD1, said method comprising one or more of the steps of:
    a) providing a test substance or compound,
    b) contacting said test substance or compound with an aggregate of hSOD1 as defined by any of claims 18, 19, 20 or 21,
    c) determining if the test substance or compound binds to the aggregate of hSOD1, inhibits the formation of aggregates of hSOD1 and/or promotes the degradation of aggregates of hSOD1,
    d) identifying said compound as potentially suitable for the treatment of ALS.

45. The method of claim 44 further comprising the step of determining if the test substance or compound alters a neurotoxic and/or neurodegenerating effect of the of aggregates of hSOD1.

46. The method of claim 44 or 45, further comprising the step of formulating the substance or compound in a pharmaceutically acceptable composition, optionally wherein the composition further comprises one or more additional components, such as components selected from the group consisting of pharmaceutically acceptable carriers, diluents, delivery agents or the like.

47. A method for the treatment and/or prophylaxis of ALS, including the delay of onset, reduction in the speed of progression and/or reduction in the symptoms of ALS, in a subject, the method comprising the administration of a pharmaceutically acceptable composition as defined by claim 46 to the subject.

48. The pharmaceutically acceptable composition of claim 46 for use in medicine.

49. The pharmaceutically acceptable composition of claim 46 for use in the treatment and/or prophylaxis of ALS, including the delay of onset, reduction in the speed of progression and/or reduction in the symptoms of ALS.

Application No:	GB1309329.9	**Examiner:**	Dr Jeremy Kaye
Claims searched:	1-17 & 41-43	**Date of search:**	22 November 2013

Patents Act 1977: Search Report under Section 17

Documents considered to be relevant:

Category	Relevant to claims	Identity of document and passage or figure of particular relevance
X	1-17 & 41-43	Acta Neuropathol., Vol.121, 2011, Forsberg, K. et al., "Glial nuclear aggregates of superoxide dismutase-1...", pp.623-634 Available online: http://rd.springer.com/article/10.1007%2Fs00401-011-0805-3
X	1-17 & 41-43	Mol. Neurodegeneration, Vol.6, 2011, Prudencio, M & Borchelt, D. R., "Superoxide dismutase 1 encoding mutations...", p.77 Available online: http://www.molecularneurodegeneration.com/content/6/1/77
X	1-17 & 41-43	US2008/0070233 A1 (BIESCHKE ET AL.) see paras.[0004]-[0012]; [0041]-[0044]; [0050]; [0157]-[0159]
X	1-17 & 41-43	FEBS Letts., Vol.583, 2009, Peter, K. & Nilsson, R., "Small organic probes as amyloid specific ligands...", pp.2593-2599 see esp. pp.2586-2598
A	-	Neuromol. Med., Vol.15, 2013, Zetterstrom, P. et al., "Composition of soulble misfolded...", pp.147-158
A	-	PLoS one, Vol. 5, 2010, Forsberg, K. et al., "Novel antibodies reveal...", p.e11552 Available online: http://www.plosone.org/article/info%3Adoi%2F10.1371%2Fjournal.pone.0011552
A	-	WO 2009/098607 A1 (AMORFIX LIFE SCI. LTD.) see whole document

Categories:

X	Document indicating lack of novelty or inventive step	A	Document indicating technological background and/or state of the art.
Y	Document indicating lack of inventive step if combined with one or more other documents of same category.	P	Document published on or after the declared priority date but before the filing date of this invention.
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Field of Search:

Search of GB, EP, WO & US patent documents classified in the following areas of the UKC^{X} :

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The following online and other databases have been used in the preparation of this search report
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Subclass	Subgroup	Valid From
None		