ALS patients with mutations in the SOD1 gene have an unique metabolomic profile in the cerebrospinal fluid compared with ALS patients without mutations

Anna Wuolikainen a,b, Peter M. Andersen a, Thomas Moritz c, Stefan L. Marklund d, Henrik Antti b,*

Abstract
A specific biochemical marker for early diagnosing and for monitoring disease progression in amyotrophic lateral sclerosis (ALS) will have important clinical applications. ALS is a heterogeneous syndrome with multiple subtypes with ill-defined borders. A minority of patients carries mutations in the Cu/Zn-superoxide dismutase (SOD1) gene but the disease mechanism remains unknown for all types of ALS. Using a GC-TOFMS platform we studied the cerebrospinal fluid (CSF) metabolome in 16 ALS patients with six different mutations in the SOD1 gene and compared with ALS-patients without such mutations. OPLS-DA was used for classification modeling. We find that patients with a SOD1 mutation have a distinct metabolic profile in the CSF. In particular, the eight patients homozygous for the D90A SOD1 mutation showed a distinctively different signature when modeled against ALS patients with other SOD1 mutations and sporadic and familial ALS patients without a SOD1 gene mutation. This was found irrespective of medication with riluzole and survival time. Among the metabolites that contributed most to the CSF signature were arginine, lysine, ornithine, serine, threonine and pyroglutamic acid, all found to be reduced in patients carrying a D90A SOD1 mutation. ALS-patients with a SOD1 gene mutation appear as a distinct metabolic entity in the CSF, in particular in patients with the D90A mutation, the most frequently identified cause of ALS. The findings suggest that metabolomic profiling using GC-TOFMS and multivariate data analysis may be a future tool for diagnosing and monitoring disease progression, and may cast light on the disease mechanisms in ALS.

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1. Introduction
Since the discovery in 1993 of mutations in the gene encoding the ubiquitously expressed Cu/Zn-superoxide dismutase (SOD1) as a cause of 12–23% of familial ALS (FALS) and 1–7% of sporadic ALS (SALS), 164 mutations have been found in the gene in ALS patients. It is at present unclear whether or not all mutations are pathogenic [11]. A large number of studies have been performed to try to find a common denominator for the mutants and to elucidate the disease mechanism in ALS. The collective evidence suggests the gain of a novel cytotoxic function by the mutant protein or part of it. Phenotypically, patients with SOD1 mutations have been reported to be clinically similar to patients without such mutations, but no blinded comparative study exists [6].

Patients with SOD1 mutations have been reported to present with almost any subtype within the ALS syndrome including cases with Vulpian-Bernharts (Flail-Arm Syndrome or Dead Arm Syndrome) ALS-variant, cases with onset in the diaphragma or the muscles of the vocal cords [4,6]. However, the dominating feature is that of spinal onset of a predominantly lower-motor neuron disorder initially affecting the myotomes of the limbs. No SOD1 mutation has been associated with a predominantly upper-motor neuron phenotype or primary lateral sclerosis phenotype. The post-mortem pathological hallmark of ALS with SOD1 mutations is loss of anterior horn cells with astrogliosis, and in the few remaining motor neurons Bunina bodies and ubiquitinated skein-like cytoplasmic inclusions. It has been claimed that the nuclear factor TAR DNA-binding protein (TDP-43) is a major component of these inclusions in patients without SOD1 gene mutations but not in patients with a SOD1 gene mutation [18,20]. The reported absence of TDP-43 positive cytoplasmic inclusions in ALS associated with SOD1 mutations is the only reported biochemical difference

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**Abbreviations:** ALS, amyotrophic lateral sclerosis; FTD, fronto-temporal dementia; NPH, normal pressure hydrocephalus; PBP, progressive bulbar palsy; SBMA, spinal bulbar muscular atrophy; SALS, sporadic ALS; FALS, familial ALS; wt, wild-type SOD1 genotype; ALSFRS, ALS Functioning Rating Scale; D90A/D90A, homozygous for the D90A SOD1 allele (mc); D90A/wt, : heterozygosity for the D90A SOD1 allele (mc); CSF, cerebrospinal fluid; GC-TOFMS, gas chromatography-time of flight mass spectrometry; HMCR, hierarchical multivariate curve resolution; OPLS-DA, orthogonal partial least squares-discriminant analysis.

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between patients with or without SOD1 gene mutations. Metabolomics has the potential to act as a link between genetic variation and biochemical pathway information. Thus detected disease-causing mutations could be mechanistically interpreted, which could potentially lead to an increased understanding of diseases and disease subtypes as well as revealing new therapeutic targets. We here report, as a proof of principle, how metabolomics can be used to reveal metabolic patterns associated with different genetic mutations known to cause ALS. In summary we found significant differences in the cerebrospinal fluid (CSF) metabolome of ALS patients with different SOD1 gene mutations as well as between patients with and patients without mutations in the SOD1 gene. Inclusions containing misfolded SOD1 are, however, found both in patients with (S. Kato, M. Takikawa, K. Nakashima, A. Hirano, D.W. Cleveland, H. Kusaka, N. Shibata, M. Kato, I. Nakano, E. Ohama, New consensus research on neuropathological aspects of familial amyotrophic lateral sclerosis with superoxide dismutase 1 (SOD1) gene mutations: Inclusions containing SOD1 in neurons and astrocytes, Amyotrophic Lateral Sclerosis, 1 (2000) 163–184.) and without (K. Forsberg, P.A. Jonsson, P.M. Andersen, D. Bergemalm, K.S. Graffmo, M. Hultdin, J. Jacobsson, R. Rosquist, S.L. Marklund, T. Brannstrom, Novel antibodies reveal inclusions containing non-native SOD1 in sporadic ALS patients, PloS ONE, 5(7) (2010) e11552. doi:10.1371/journal.pone.0011552; K. Forsberg, P.M. Andersen, S.L. Marklund, T. Brannstrom, Gial nuclear aggregates containing misfolded SOD1 are regularly present in patients with amyotrophic lateral sclerosis, Acta Neuropathol 121 (2011) 623–634. SOD1 mutations.

2. Materials and methods

2.1. Sample collection, handling and storage

The study was performed in accordance with the Declaration of Helsinki (World Medical Association link: http://www.wma.net/en/30publications/10policies/b3/, 1964) and approved by the medical ethical review board in Umeå, Sweden. With informed consent CSF was collected from ALS patients and controls (healthy subjects and subjects with other neurological conditions). The ALS patients were diagnosed according to standard criteria[5]. In some, the CSF samples were collected during second opinion investigations. Patients with and without riluzole treatment were thus included in the study. Based on familial disposition for ALS (FALS/SALS), the results of genetic analysis (see below), gender, age and storage time, CSF samples from ALS patients were selected for inclusion the study. A matched control subject was carefully assigned to each patient to account for variations due to age, sex and the samples time in storage. Details of the test subjects are summarized in supplemental Table 1 online. In all subjects, the spinal tap was performed at the L4–L5 or L5–S1 levels with the individual lying in a horizontal resting fetal position (right side down). The spinal taps were performed non-traumatically without hemorrhage using a 20 G Spinocan® cannula. The CSF samples used for this study were collected among the first 4 ml. None of the patients were fasting at the time of CSF tapping. After collection the CSF was immediately frozen in 1 ml polystyrene tubes to −80 °C and stored [26].

2.2. Genetic analysis

All patients were screened for DNA-mutations in the following genes: SOD1, VAPB, TARDBP, FUS, progranulin and angiogenin using procedures as described [1,11,12,24]. Only patients with a SOD1 gene mutation or patients without a mutation in any of the analyzed genes were included in this study (Table 1).

2.3. Metabolomic analysis

Chemical analysis and practices related to the analysis of the metabolome was performed in accordance with the standards proposed by the Chemical Analysis Working Group and Metabolomics Standards Initiative [22]. The CSF samples were analyzed using gas chromatography-time of flight mass spectrometry (GC-TOFMS) in two separate batches (sets) including randomly assigned replicate samples. Set I consisted of 30 SALS and 9 FALS; 8 with bulbar onset, 31 with spinal onset. 19 were male subjects and 20 were female subjects. Set II consisted of 25 SALS and 14 FALS; 9 with bulbar onset, 30 with spinal onset; 20 of the subjects were males and 19 were females. The GC-TOFMS runorder was randomized within each set. Extraction, derivatization, GC-TOFMS analysis, data pre-processing and metabolite identification of CSF samples were performed as described [27]. The raw GC-TOFMS data from set II were used for pre-processing using hierarchical multivariate curve resolution (HMCR) [16]. Set I was then predictively resolved [17] using the same settings as set II, in order to extend the dataset to include all subjects. Prior to multivariate analysis the

Table 1

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<th>Sex</th>
<th>Age</th>
<th>Site of onset</th>
<th>First symptom</th>
<th>Spinal tap</th>
<th>Deceased</th>
<th>Survival time</th>
<th>Symptom onset to spinal tap</th>
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<td>7</td>
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</tr>
<tr>
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<td>I113F/wt</td>
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<td>both legs</td>
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<td>alive</td>
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<td>7</td>
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</tbody>
</table>

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a yyyy/mm.

b Months from onset of first symptom.

c Months.

d At spinal tap.
matched control data were subtracted from the data of each paired ALS subject.

2.4. Data analysis

Orthogonal partial least squares discriminant analysis (OPLS-DA) [7] was used for multivariate statistical classification modeling. Orthogonal partial least squares (OPLS) [23] is a multivariate regression method developed for extracting systematic variation among many variables related to a certain response (for example disease), in the presence of systematic variation of multiple origins unrelated to the response (runorder, drift in system, etc.). The systematic variation related to the response of interest is modeled in a predictive model component while the variation unrelated to the response of interest is modeled separately in orthogonal model components. This facilitates model interpretation and visualization of the different sources for variation. OPLS-DA is a special case of OPLS where the response is constructed as dummy variables holding information about the sample class [7]. Validation of models was performed using full cross validation (CV) for estimating the number of components in the model and CV-ANOVA [9] to obtain a p-value for the detected group separations. Models were calculated for the following sample classifications: i) A five class model for a complete overview, i.e. the five classes being SALS, FALS, subjects carrying a D90A mutation (homozygous) in the SOD1 gene, subjects carrying a D90A mutation (heterozygous) in the SOD1 gene and carriers of a mutation in the SOD1 gene other than D90A; ii) Carriers of a mutation in the SOD1 gene versus non-carriers (ALS, SALS and FALS); iii) Homozygous carriers of the SOD1 mutation D90A versus non-carriers of a SOD1 mutation (SALS and FALS respectively); iv) Carriers of a mutation (other than D90A) versus non-carriers (SALS and FALS respectively); and v) Homozygous carriers of D90A SOD1 mutation versus heterozygous carriers. All multivariate statistical modeling was performed in SIMCA P + 12.0 (Umetrics, Umeå, Sweden). Model plots were created using Evince 2.3.1 (UmBio AB, Umeå, Sweden).

3. Results

To overview potential sub-grouping of ALS cases, classification modelling by means of OPLS-DA was performed and three significant components (p<0.0001) were obtained based on cross validation (Fig. 1). Separation of all pre-defined classes was detected except for the small group comprising of three subjects with a heterozygous D90A mutation. A significant separation (p<0.0001) was also obtained between subjects carrying a mutation in the SOD1 gene versus subjects not carrying a mutation in the SOD1 gene (including both SALS and FALS cases; Fig. 2).

Furthermore, when performing comparisons between subjects carrying a mutation in the SOD1 gene versus SALS and FALS respectively, significant separations were seen for all SOD1 mutations, SOD1 mutations not including D90A and D90A (homozygous) versus SALS and FALS respectively (Fig. 3).

![Fig. 1. Mapping of the included ALS subjects based on the CSF metabolomics data using OPLS-DA. OPLS-DA score plots are shown of the two first predictive components (t1P and t2P; left), and the second and third predictive components (t2P and t3P; right). The samples were assigned to subgroups defined as SALS (dark grey dots), FALS (light grey dots), subjects carrying a D90A mutation (homozygous) in the SOD1 gene (light grey crosses), subjects carrying a D90A mutation (heterozygous) in the SOD1 gene (light grey stars) and carriers of a mutation in the SOD1 gene other than D90A (red crosses).](image1)

![Fig. 2. OPLS-DA scores plot based on CSF metabolomics data of subjects carrying a mutation in the SOD1 gene (red crosses) modelled against subjects not carrying a mutation in the SOD1 gene (SALS and FALS, shown as dark grey dots). The first predictive component (t1P) is plotted against the subject number.](image2)
Fig. 3. (top) OPLS-DA score plots from classification modeling of subjects carrying a mutation in the SOD1 gene (red crosses) against SALS (left) and FALS (right). (middle) OPLS-DA score plots from classification modeling of subjects carrying a mutation (other than D90A) in the SOD1 gene (red crosses) against SALS (left) and FALS (right). (bottom) OPLS-DA score plots from classification modeling of subjects carrying a D90A mutation (homozygous) in the SOD1 gene (grey crosses) against SALS (left) and FALS (right). In all figures the first predictive component (t1P) is plotted against the subject number.
Notably, no significant models could be obtained for the difference between the group of subjects carrying a D90A mutation (heterozygous) in SOD1 in relation to SALS and FALS respectively. This was also the case for the comparison of homozygous carriers versus heterozygous carriers of a D90A mutation in the SOD1 gene ($p = 0.318$) and for the heterozygous carriers of a D90A mutation in the SOD1 versus the carriers of a mutation in the SOD1 gene other than D90A (no model obtained).

Since no significant difference was found between the homozygous and the heterozygous D90A SOD1 gene mutation carriers these subjects were combined into one group and compared to subjects carrying a different mutation (other than D90A) in the SOD1 gene, FALS and SALS both in an overview model including all four classes and in separate pair-wise comparisons (Fig. 4). From the overview model based on three significant components ($p < 0.0001$) it was clear that metabolite differences existed between the sample groups. The unique metabolite signature of D90A mutation was further emphasized by the significant separations found when compared against SALS ($p = 0.029$), FALS ($p = 0.05$) and subjects carrying a mutation other than D90A in the SOD1 gene ($p = 0.017$). The metabolites contributing most to the differences illustrated in Fig. 4 are summarized in Table 2.

4. Discussion

The most frequent SOD1 gene mutation is D90A which in many European countries causes ALS inherited as a recessive trait with a characteristic uniform and slowly progressing lower-limb onset phenotype with a mean survival time of 14 years from onset [2]. An unusual feature in D90A-homozygous ALS which may partly explain the long survival time of these patients is the pronounced early appearance of lesion to pyramidal tracts as shown by pathological Babinski signs, very brisk deep-tendon stretch-reflexes and delayed central cortical latency upon transcranial magnetic stimulation of the motor cortex [2,25]. ALS patients homozygous for the D90A mutation have been found in all countries where a larger number of patients have been studied, except for Iceland, Ireland, Japan and China. Irrespective of population studied, in all pedigrees with ALS

![Fig. 4. OPLS-DA mapping (t2P/t1P) of the ALS subjects based on the CSF metabolomics data using OPLS-DA (top left). The samples were assigned to subgroups defined as SALS (dark grey dots), FALS (light grey dots), subjects carrying a D90A mutation (light grey crosses) and carriers of a mutation in the SOD1 gene other than D90A (red crosses). OPLS-DA score plots from classification modeling of subjects carrying a D90A mutation in the SOD1 gene versus SALS (t1P; top right), FALS (t1P; bottom left) and subjects carrying a mutation other than D90A in the SOD1 gene (t1P; bottom right).](image-url)
caused by homozygosity for the D90A mutation, D90A heterozygous individuals have been without symptoms of ALS [3,8]. However, a few rare pedigrees where the patients have been found to be heterozygous for the D90A mutation have also been reported [1,4,10,13,21]. Clinically, these rare patients can be divided into two groups, a group with symptoms and signs similar to the uniform phenotype of the D90A homozygous patients, and a group with a very variable phenotype with either spinal or bulbar onset and usually an aggressive disease course with a survival time of less than two years [1,10,13].

The phenotype of these D90A heterozygous cases is more in line with what have been reported for patients with most other SOD1 mutations, including the five included in this study. The three D90A heterozygous ALS patients included in this study all show slow disease progression, two of them with a phenotype reminiscent of the uniform phenotype seen in the D90A homozygous ALS patients. The third patient presented with the flail-arm variant of ALS with symmetrical onset in the upper extremities had, when the spinal tap was performed, no signs or symptoms from the lower extremities or bulbar innervated muscles. An international study of the haplotype of patients homozygous or heterozygous for the D90A SOD1 mutation has shown that all patients have a common ancestor some 18000 years ago, with the homozygous individuals later forming a separate subgroup [19].

It is at present not clear whether or not a single D90A allele is sufficient to cause symptomatic ALS, or whether concomitant mutations in other genes are involved in D90A heterozygous individuals who develop ALS [10]. We therefore screened our patients for other ALS-causing genes, finding nothing. There is no evidence for different expression from the D90A mutant allele in recessive and dominant cases [29–30]. Expression of D90A mutant human SOD1 in transgenic mice results in a phenotype similar to that in D90A homzygous ALS cases: slow progression and bladder disturbances [15,17].

Analysis of the neurofilament light chain in the CSF suggests that patients with SOD1 gene mutations constitute a distinct subgroup within the ALS-syndrome, and more so patients homozygous for the D90A SOD1 mutation [28].

In the present study we found that ALS patients carrying a mutation in the gene encoding SOD1 have characteristic patterns in the CSF metabolome that are statistically different from ALS patients not carrying a SOD1 mutation.

ALS subjects carrying a D90A mutation show significant differences compared to carriers of other mutations in the SOD1 gene, SALS and FALS. A characteristic finding is a reduction in the content of primarily amino acids in the CSF. Contributing significantly to the profile is also a number of other low molecular weight compounds that remain to be identified. The D90A-homozygous carriers do not show significant differences in the CSF metabolome compared to D90A-heterozygous carriers, although the study concerns a limited number of heterozygous carriers and follow up studies are needed for validation. An interpretation of our results is that the detected metabolomic signature simply reflects that all D90A patients are closely related individuals. However, the D90A subjects included in this study were from widely separate parts of the Scandinavian peninsula, and both the patients with the D101G and I113F mutations came from geographic places where other ALS patients have been found to carry the D90A SOD1 allele, making consanguinity a less likely explanation for the uniform metabolic signature of the D90A patients. Caveats of the study are that most of the patients with a SOD1 mutation had a slowly progressing disease course and only one patient with a SOD1 mutation and fast progression was included. Further validation in larger number of patients with different disease progression rates and mutations in the SOD1 gene as well as comparison with patients with mutations in other genes will be of great interest. For this the use of metabolomics as the link to pathway information in genome-wide association studies could be of great interest for mapping and interpreting the effects of different mutations and even combinations of mutations in relation to ALS subtypes [14]. We thus foresee that metabolomics will contribute to deciphering the complex interactions behind ALS.

Supplementary materials related to this article can be found online at doi:10.1016/j.ymgme.2011.11.201.

### 5. Conflict of interest statement

None of the authors report conflicting interests.

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References


