Role of the Specialized Proresolving Mediator in Amyotrophic lateral sclerosis

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Research Article

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Abstract

Amyotrophic Lateral Sclerosis (ALS) is a devastating progressive neurodegenerative disease that affects motor neurons, leading to a relentless paralysis of skeletal muscles and eventual respiratory failure. Neuroinflammation is one of the hallmarks of ALS and may contribute to motoneurons degeneration. Accumulating evidence suggests that chronic inflammation resulting from insufficient resolution, a process operated by specialized pro-resolving mediators (SPMs). However, whether resolution of inflammation is impaired in ALS is not known. The objective of this study was to investigate the levels of SPMs in the serum and cerebrospinal fluid (CSF) of ALS patients, and to explore the roles of SPMs in clinical features of ALS. Forty-one patients with ALS, and 73 patients with non-inflammatory neurological diseases were enrolled in this study. Pro-resolving mediators including resolvin D1 (RvD1), maresin 1 (MaR1) and lipoxin A4 (LxA4) levels were analyzed using enzyme-linked immunosorbent assay. Pro- and anti-inflammatory cytokines levels were analyzed by cytometric beads array (CBA). Our results showed serum RvD1 levels were decreased, whereas CSF RvD1 levels were significantly increased in ALS patients compared to controls. Serum RvD1 was also negatively correlated to disease progression rate (DPR), and positively correlated with forced vital capacity (FVC). The present preliminary study allows hypothesizing impaired resolution of inflammation in patients with ALS, confirming the emerging role of bioactive lipids in this disease.

Background

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by the selective loss of upper and lower motoneurons, which leads to muscle atrophy, paralysis, respiratory failure, and eventual death usually within 2–4 years after clinical onset (Hardiman et al. 2017). ALS is a heterogeneous and multifactorial disease triggered by a complex interaction of environmental factors and potentially susceptible gene alterations. The key mechanisms of ALS pathogenesis include glutamate induced excitotoxicity (Van Damme et al. 2005), oxidative stress, mitochondria dysfunction (Obrador et al. 2020), cytoskeleton alterations, axonal transport dysregulation (De Vos et al. 2008), and neuroinflammation (Thompson and Turner 2019).

ALS-related neuroinflammation classically features microglia and astrocytes activation, moderate infiltration of peripheral immune cells, as well as elevated levels of inflammatory mediators, affects motor regions of the central nervous system (CNS) (Liu and Wang 2017). Numerous studies have reported neuroinflammation could be a consequence of failure to resolve inflammation and to return to homeostasis (Li et al. 2020; Shang et al. 2019). The resolution of inflammation is an active process regulated by specialized pro-resolving lipid mediators (SPMs), a superfamily of pro-resolving lipids that derive metabolically from ω-3 and ω-6 essential fatty acids (Chiurchiu et al. 2018; Serhan 2014; Serhan et al. 2000). These include lipoxins (LX), resolvins (Rv), protectins, and maresins (MaR), which are derived from arachidonic acid (AA), docosahexanoic acid (DHA), and eicosapentanoic acid (EPA)(Serhan et al. 2014). It is a new field of research, relatively little is known about the role of SPMs in neurological diseases. In a previous work, our lab has shown that lower levels of pro-resolving SPMs in neuromyelitis
optica spectrum disorders than healthy controls (Wang et al. 2019). Chronic and early resolvins D1(RvD1) administration in Parkinson's disease rats model prevents central and peripheral inflammation, as well as neuronal dysfunction and motor deficits (Krashia et al. 2019). In vitro studies demonstrate that RvD1 also reduces inflammatory mediators and oxidative stress in ALS macrophages (Liu et al. 2012). To pave the way for future treatments and biomarkers based on the resolution of inflammation, we aimed at analyzing plasma and cerebrospinal fluid (CSF) levels of SPMs in patients with ALS and healthy individuals, testing their biomarker potential for ALS and assessing its relationship with disease activity and laboratory parameters.

**Materials And Methods**

**Patient sample collection**

We analyzed serum, CSF and muscle samples in a total group of 41 patients with sporadic ALS according to the revised El Escorial criteria (Wilbourn 1998), which provided by Tissue Bank of the First Affiliated Hospital of Jilin University. Among the 41 patients, 7 without CSF samples, thus 34 CSF samples and 41 serum samples from 41 patients were used to analyze SPMs levels. CSF from 36 aged and gender-matched patients with other non-inflammatory neurological diseases was collected in the Department of Neurology as controls. The serum of 37 healthy subjects was collected from the Physical Examination Center in the same hospital as controls. The serum and CSF samples were stored at −80°C until analysis. Additionally we collected clinical data, including age, gender, disease subtype at onset, forced vital capacity (FVC), disease duration (time between symptom onset and the date of sample collection), the ALS Functional Rating Scale revised (ALSFRS-R), the disease progression rate (DPR) was calculated as follows: (48−ALSFRS-R score at date of sample collection)/disease duration (months). To check for differences between fast and slow progressors, we defined fast progressors with a DPR ≥0.5/month and slow progressors with a DPR <0.5/month according to Ludolph et al. (Ludolph et al. 2018). All participants had not received therapy (Riluzole and edaravone). Ethical approval was obtained from the Ethics Committee from the First Hospital of Jilin University, Changchun, China. The study was carried out following the ethical principals described in the Declaration of Helsinki.

**Enzyme-linked immunosorbent assay (ELISA) of RvD1, maresins R1(MaR1) and lipoxins A4(LxA4) measurement**

The levels of RvD1, MaR1 and LxA4 in the serum and CSF of patients with ALS and those of controls were analyzed by human resolvin D1 ELISA kit, human maresin 1 ELISA kit, and human LxA4 ELISA kit (Cayman Chemical Company, Ann Arbor, USA) according to the manufacturer's instructions.

**Cytometric Beads Array (CBA) for cytokine measurement**

The frozen CSF supernatants and serum samples were thawing one hour before the CBA measuring was performed. The levels of seven cytokines (including IL-2, IL-4, IL-6, IL-10, IL-17A, IFN-γ and TNF-α) in the
serum and CSF of patients with ALS and controls were tested by CBA Flex Set (BD Biosciences), and determined according to the manufacturer's instructions.

Disease severity assessment

The symptoms of all patients were evaluated according to the clinical manifestation. The ALSFRS-R was assessed according to Cedarbaum's standard (Cedarbaum et al., 1999) on the basis of medical journal records in the First Hospital of Jilin University. Muscle sections were stained according to standard histological and enzyme histochemical procedures with hematoxylin and eosin (H&E), modified Gomori trichrome (MGT), periodic acidic Schiff (PAS), oil red O (ORO), nicotinamide adenine dinucleotide tetrazolium reductase (NADH-TR), succinate dehydrogenase (SDH), and cytochrome c oxidase (COX) stain.

Statistical Analysis

SPMs levels between patients with ALS and controls as well as demographic and laboratory parameters have been compared using Mann–Whitney U test. Receiver operating characteristic (ROC) analysis was used to define the ability of SPMs to differentiate patients with ALS and controls; the optimum cutoff value has been identified from the highest Youden's index. Spearman correlation was used to find possible correlation between SPMs level and disease severity on one hand and cytokines on the other hand. A P-value of < 0.05 was considered statistically significant. Statistical analysis was performed using the GraphPad Prism 8 and Service Solutions (SPSS) version 26 (IBM, USA).

Results

Patient information

Clinical data and bio-samples were collected from 41 ALS (41 serum/34 CSF) patients and 73 patients with non-inflammatory neurological diseases (37 serum/36 CSF). The basic information of patients with ALS is displayed in Table 1. No significant differences were observed regarding sex or age among ALS and control groups. Disease duration in ALS patients was 8 (5–15) months, mean ALSFRS-R was 43 (37–46) points and 90% of all cases were classified as spinal onset and 10% as bulbar onset.
Table 1
Patients’ characteristics at enrollment

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ALS (n = 41)</th>
<th>Serum control (n = 37)</th>
<th>CSF control (n = 36)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex ratio (female/male)</td>
<td>12/29</td>
<td>10/27</td>
<td>9/27</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55(49–65)</td>
<td>53(47–67)</td>
<td>52(46–60)</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>8(5–15)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Onset (bulbar/limb)</td>
<td>4/37</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>ALS-FRS score</td>
<td>43(37–46)</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

NA: not applicable; ns: not significant.

Changes in resolution markers of patients with ALS
To evaluate the resolution function in patients with ALS, the levels of MaR1, RvD1, and LxA4 were analyzed. Levels of CSF-RvD1 in the ALS group were significantly higher than in the control group (P < 0.01; Fig. 1a). The median RvD1 level in the NALS-CSF was 8.85(7.68–9.98) pg/ml (n = 36), whereas in the ALS-CSF the median was 11.53(9.98–13.85) pg/ml (n = 34). In contrast, the serum RvD1(S-RvD1) levels in the ALS patients were lower than those in the control group (P < 0.001; Fig. 1b). The median RvD1 level in the NALS-serum was 130.31(84.65-189.31) pg/ml (n = 37), whereas in the ALS-serum the median was 47.21(42.58–50.72) pg/ml (n = 41). Other specialized pro-resolving lipid mediators – MaR1 and LxA4 – were not detected (data not shown). In addition, S-RvD1 were decreased in patients since the 6 months, the fluctuations of S-RvD1 levels throughout the entire disease course is similar with the CSF-RvD1(Fig. S1).

The area under the ROC curve (AUC) for CSF-RvD1 and S-RvD1 were 0.738 (95%CI, 0.612–0.864) (P < 0.01) and 0.768 (95% CI, 0.627–0.909) (P < 0.001), respectively. The optimal cut-off values to discriminate between ALS and controls were calculated at 9.39 pg/ml CSF-RvD1 (a sensitivity of 73.1% and specificity of 64.7%) and 67.39 pg/ml S-RvD1 (a sensitivity of 73.9% and specificity of 90.2%) in ROC curves (Fig. 2).

Among different subgroups based on median values, the S-RvD1 level was higher in bulbar onset (72.15 pg/mL) than in limb onset (41.68 pg/mL) (P < 0.05), and in slowly progressive group (49.18 pg/mL) than in rapidly progressive group (39.54 pg/mL) (P < 0.05). The CSF-RvD1 level was higher in female (11.34 pg/mL) than in male (9.72 pg/mL) (P < 0.05) (Table S2). There were no significant differences for the age and duration between both groups.

Changes in inflammatory markers in patients with ALS
In order to study the cytokine profile in ALS, we analyzed pro-inflammatory cytokines (IL-2, IL-6, TNF-α), the anti-inflammatory cytokine (IL-4 and IL-10) as well as cytokines that characterize specific T cell responses (IFN-γ and IL-17A). In the CSF of ALS group, the level of IL-17A ($P < 0.001$), IL-6 ($P < 0.001$) and TNF-α ($P < 0.05$) were significantly higher in comparison to controls whereas the anti-inflammatory cytokine IL-10 ($P < 0.001$) and IL-4 ($P < 0.01$) were significantly decreased. There were no differences in cytokine expression of IL-2 and IFN-γ between ALS and controls (Fig. S2a). In the serum of ALS group, the level of IL-6 ($P < 0.01$) and TNF-α ($P < 0.01$) were significantly higher in comparison to controls whereas the anti-inflammatory cytokine IL-10 ($P < 0.05$) was significantly decreased. There were no differences in serum cytokine expression of IL-4, IL-2 and IFN-γ (Fig. S2b).

**Correlations between resolution markers, cytokines, and disease severity in patients with ALS**

To further evaluate coherences to further clinical and demographic parameters (ALSFRS-R, DPR, disease duration and FVC), we calculated Spearmen rank correlation coefficients. In the ALS cohort, S-RvD1 negatively correlated to DPR ($\rho = -0.331; P = 0.034$) and positively with FVC ($\rho = 0.325; P < 0.05$), indicating that patients with high disease severity have low level of RvD1 (Fig. S3). In addition, RvD1 did not correlate to cytokines, except for IL-2 in the CSF of patients with ALS ($\rho = -0.446, P = 0.008$) (Fig. S4). Nonetheless, no relation has been demonstrated between RvD1 levels and ALSFRS-R or disease duration.

**Effect of RvD1 on Metabolism**

Histological and histochemical staining was performed on muscle samples of ALS patients. The pathological changes of biopsied muscle from a patient presented grouping of small angular atrophic fibers. ORO staining revealed the accumulation of lipid droplets in muscle fibers. SDH staining reveals dark angular fibers (Figure S5). Remarkably, low S-RvD1 levels (32.23 pg/mL) was observed in this patient. We further examined the correlation between the S-RvD1 and triglycerides, total cholesterol, and found that S-RvD1 was negatively associated with total cholesterol levels, suggesting RvD1 might inhibit metabolic alterations occurred in muscle degeneration (data not shown).

**Discussion**

SPMs, including RvD1, play an important role in the regulation of inflammation. Accumulating evidence has revealed that neuroinflammation is involved in the degenerative process of motoneurons in ALS, indicating a role of the SPMs in the pathophysiology of ALS. However, there is no study regarding the impact of the SPMs in ALS. In this report, we measured levels of resolution and inflammatory markers in the serum and CSF of ALS patients and controls, and determined whether these changes correlate with disease-related markers/phenotypes.

Analysis of the entire cohort showed that the levels of S-RvD1 of ALS patients were substantially decreased compared with the control group, which was consistent with the results of other neurological diseases (Krashia et al. 2019; Wang et al. 2019; Zhu et al. 2016). In addition, we found that the CSF levels of RvD1, were higher in ALS compared to controls, like in 4-month-old Syn animals (Parkinson's disease...
Further analysis of the time-course of changes in CSF-RvD1 levels in our ALS cohort suggests that the RvD1 recurrent boost in the CSF could reflect an attempt to counteract the inflammation at initial stages, being a molecule of early intervention against inflammation. With respect to the RvD1 levels fluctuate so drastically in disease stages, there might be several factors. Firstly, the possibility of TDP43 aggregation overexpression interfering with RvD1 synthesis might cause reduction of RvD1. Secondly, the drop of RvD1 might be due to overall failure of the immune system to chronically sustain high RvD1 levels. Interestingly, the trend in serum RvD1 levels is illuminated to be similar with the CSF-RvD1 led us to assume that an attempt of the CNS to recruit RvD1 from the periphery for the CNS inflammation process.

Subtype analysis revealed relative higher amounts of RvD1 in female with ALS. Several factors may influence the levels of RvD1 and give rise to the discrepant results seen in male and female, including age, sex hormones, diet, and the ability to synthesize lipids. Actually, conversion rate of α-linoleic acid (ALA) to DHA, a precursor in the synthesis of RvD1, was shown to be higher for female than male (Burdge and Wootton 2002). Earlier researches suggest that longer endogenous estrogen exposure has a neuroprotective effect in ALS mice and patients (de Jong et al. 2013; Kim et al. 2013). Furthermore, we analyzed differences in the inflammatory response between rapid and slow sporadic ALS. Except for IL-10, which was significantly higher in slow ALS cases, we found no significant differences between slow and rapid progressive ALS regarding RvD1 and cytokine levels (data not shown).

The changes of the cytokine levels in ALS patients likewise lead to the conclusion of an impaired balance of immune system. IL-10, an anti-inflammatory cytokine, was significantly decreased in ALS patients. In contrast, the levels of inflammatory cytokines IL-6 and TNF-α, were identified in abnormally high levels in ALS patients, agree with previous studies (Chen et al. 2018; Michaelson et al. 2017). For IFN-γ, there was not a significant increase, which is different from recent studies (Jin et al. 2020). In addition, T helper 17 (Th17) cells has been thought to play crucial role in the pathogenesis of ALS and IL-17A serum concentrations in ALS patients have been reported significantly higher than control subjects without autoimmune disorders (Fiala et al. 2010; Rentzos et al. 2010). However, we found decreased levels of IL-17A in the serum and increased levels of IL-17A in the CSF of ALS patients, indicating that IL-17A levels may fluctuate at different phase of disease.

One key molecular mechanism by which resolvins function is through blunting production of proinflammatory cytokines (Serhan et al. 2002). Evidence from the literature has indicated that RvD1 inhibited IL-6 and TNF-α production (Liu et al. 2012). Another recent study demonstrated RvD1 control CD4+ T cell differentiation into Th1 and Th17 effectors, with decreased production of IFN-γ and IL-17(Serhan and Levy 2018). In contrast, we found no associations between RvD1 levels and cytokines except a weak correlation between CSF-RvD1 and IL-2, suggesting RvD1 might inhibit the release of IL-2 in CNS.

The immune system in ALS shows a neuroprotective phase with increased anti-inflammatory cytokine in very early or presymptomatic ALS disease. In summary of all variations in RvD1 and cytokines we found,
a proinflammatory shift of the immune components in ALS patients becomes obvious. This might depend on the fact, that the median ALSFRS-R in our group was 43 points, which means our patients were mainly in a progressed stage. In conclude, the evidence that RvD1 changes and cytokines variations presented in the blood and CSF indicates that they could be the result of complex crosstalk between peripheral and central immune responses.

As demonstrated in our study, levels of RvD1 levels in serum and CSF were negative correlated with DPR, warranting further study for the use of RvD1 at an early stage as biomarker for predicting ALS disease progression. Most interestingly, we disclosed a weak correlation between S-RvD1 and the respiratory function index of FVC. Previous study manifested that FVC value may serve as a predictor of survival and disease-progression (Czaplinski et al. 2006), which is further demonstrated by our results here. Further studies with larger focus on the RvD1 are needed to clarify the influence on respiratory status in ALS disease. ROC curves revealed that S-RvD1 outperformed CSF-RvD1 in discriminating patients with ALS from controls. Given the suitable and easily accessible ways of serological specimens in contrast to lumbar puncture invasive procedure for CSF collection, S-RvD1 are thereby recommended as potential biomarker to evaluate the disease progression. Above all, blood-based measurement contributes to the conduction of a series of longitudinal studies.

The histopathologic findings of lipid droplets aggregate in the relatively hypertrophy fibers in a patient found in this study agree with two other studies available in the literature, reporting increased FUS mislocalization and mitochondrial damage that might cause muscle metabolic alterations (Yu et al. 2022; Zhou et al. 2020). Unfortunately, peripheral blood sample for genetic analysis is not available. To our surprise, we found lower levels of S-RvD1 in patient with lipid droplets in muscle. We retrospectively examined hospital records covering 6 months after the analysis to observe whether the S-RvD1 levels were associated with the development of lipid concentration in ALS. In patients with normal serum lipid profiles at the time of specimen collection, we found that 3 patients from high RvD1 group (3/15, 20% of patients) and 6 patients from low RvD1 group (6/15, 40% of patients) were diagnosed with hypercholesterolemia. This finding suggested that the low S-RvD1 may be a risk for the development of hyperlipidemia in the later period. Here, our study further supported the role of RvD1 in lipid metabolism.

Several shortcomings of this study should be considered. For the measurements of SPMs, we used ELISA method instead of the reference method (liquid chromatography with the tandem mass spectrometry). The sample size was relatively small, and further studies on a larger number of patients are required to better evaluate the role of RvD1 in ALS, especially its potential role as a biomarker. Furthermore, this study did not schedule a follow-up of patients and therefore could not ascertain the role RvD1 in predicting changes in disease activity, damage accrual, or laboratory parameters over time.

**Conclusion**

In summary, this study validated the existence of resolution of inflammation abnormalities in ALS patients and explored the association between RvD1 and the clinical characteristics (progression rate and
respiratory function) of ALS. In addition, our study provides additional evidence RvD1 as a key molecule for therapeutic intervention at the early stages of this crippling and fatal neurodegenerative disorder.

Declarations

Acknowledgments

We wish to thank the patients for their cooperation in this study. We acknowledge Tissue Bank of the First Affiliated Hospital of Jilin University, for providing serum, CSF and muscle samples.

Authors’ contributions

XJW contributed to conception of the study and contributed to design of the study. XXL enrolled patients with ALS, performed clinical evaluations and collected sample. HS and RQQ contributed to the acquisition and analysis of data. XFY contributed to drafting the manuscript and all authors assisted in reviewing manuscript. The authors read and approved the final manuscript.

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Date Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Serum, CSF and muscle samples were collected after approval of human ethics committee, and informed consent was taken prior to samples collection.

Consent for publication

Not applicable, as no individual participant’s data is presented.

Competing interests

The authors declare that they have no competing interests.

References


**Figures**
Figure 1

Levels of RvD1 in CSF and serum of Amyotrophic lateral sclerosis (ALS) group and controls. (a) CSF RvD1 levels were higher in ALS than in controls. (b) S-RvD1 levels were lower in ALS than in controls. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by Mann Whitney U test.

Figure 2
Receiver operating characteristic (ROC) curves of CSF-RvD1 (a) and S-RvD1 (b) for discrimination between ALS and disease controls. The corresponding AUCs for CSF-RvD1 and S-RvD1 were 0.738 (95% CI, 0.612-0.864) and 0.768 (95% CI, 0.627-0.909), respectively.

Supplementary Files

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- FigureS4.tif
- FigureS5.tif
- SupplementaryTable1.docx