IgGs from patients with amyotrophic lateral sclerosis and diabetes target CaV\(\alpha_2\delta1\) subunits impairing islet cell function and survival

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Patients with amyotrophic lateral sclerosis (ALS) often show hallmarks of type 2 diabetes mellitus (T2DM). However, the causal link between ALS and T2DM has remained a mystery. We now demonstrate that 60% of ALS patients with T2DM (ALS-T2DM) have sera that exaggerated K+–induced increases in cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) in mouse islet cells. The effect was attributed to the presence of pathogenic immunoglobulin Gs (IgGs) in ALS-T2DM sera. The pathogenic IgGs immunocaptured the voltage-dependent Ca\(^{2+}\) (Ca\(^{2+}\)) channel subunit Ca\(_{\alpha_2}\delta1\) in the plasma membrane enhancing Ca\(_{\alpha_2}\delta1\)-mediated Ca\(^{2+}\) influx and [Ca\(^{2+}\)]\(_i\), resulting in impaired mitochondrial function. Consequently, impairments in [Ca\(^{2+}\)]\(_i\) dynamics, insulin secretion, and cell viability occurred. These data reveal that patients with ALS-T2DM carry cytotoxic ALS-T2DM-IgG autoantibodies that serve as a causal link between ALS and T2DM by immunonaotcking Ca\(_{\alpha_2}\delta1\) subunits. Our findings may lay the foundation for a pharmacological treatment strategy for patients suffering from a combination of these diseases.

Significance

We provide evidence of a mechanistic link between ALS and T2DM. Our data show that a subgroup of ALS-T2DM patients have sera that enhance Ca\(_{\alpha_2}\delta1\)-mediated Ca\(^{2+}\) influx and exaggerate [Ca\(^{2+}\)]\(_i\). These effects occur because the sera accommodate cytotoxic IgG autoantibodies that immunocapture Ca\(_{\alpha_2}\delta1\) subunits. As a consequence, impairments in [Ca\(^{2+}\)]\(_i\) dynamics, mitochondrial function, insulin secretion, and cell viability appear. We could clarify not only the identity of this serum factor but also the molecular mechanisms underlying its effects on the islet cells. Our findings may lay the foundation for a treatment strategy for this complex and severe group of diabetic patients.

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in motor neurons, thereby damaging these cells in a Ca\(^{2+}\)-dependent manner (2–4). This prompted us to explore if ALS-T2DM serum drives similar pathological events in mouse islet cells. To implement such an exploration, we collected 4 types of sera from healthy human subjects (HSs) and patients with ALS, T2DM, and ALS-T2DM (SI Appendix, Table S1). During the course of the present study, 2 separate batches of sera were donated by 5 patients with ALS-T2DM and 6 T2MD patients (SI Appendix, Table S1) and 3 out of 5 ALS-T2DM sera gave rise to significant elevations in K\(^{+}\)-induced [Ca\(^{2+}\)]; responses in comparison to T2DM sera (Fig. 1 D and E). Moreover, the specific Ca\(_{\alpha 1}\) channel blocker nifedipine almost completely ablated K\(^{+}\)-induced [Ca\(^{2+}\)]; responses in mouse islet cells (SI Appendix, Fig. S1). In addition, there was no significant difference in basal [Ca\(^{2+}\)] between T2DM and ALS-T2DM groups (Fura-2 F340/F380 ratio for the T2DM group of 0.537 ± 0.028 vs. Fura-2 F340/F380 ratio for the ALS-T2DM group of 0.541 ± 0.017, P > 0.05). Taken together, 60% of ALS-T2DM patients have sera that authentically exaggerate K\(^{+}\)-induced [Ca\(^{2+}\)]; responses in islet cells. These ALS-T2DM sera were defined as positive ALS-T2DM sera and randomly chosen for subsequent experiments.

Pathogenic IgGs Present in Positive ALS-T2DM Sera Enhance K\(^{+}\)-Induced [Ca\(^{2+}\)]; Responses in Mouse Islet Cells. It has been demonstrated that pathogenic IgGs reside in sera of ALS patients and account for a great deal of Ca\(^{2+}\)-dependent destruction of motor neurons and skeletal muscle cells (4, 15–18). This raised the question of whether IgGs in positive ALS-T2DM sera (ALS-T2DM-IgGs) also serve as molecular pathogenic factors to impair islet cell function and survival by perturbing [Ca\(^{2+}\)]; homeostasis. To tackle this question, we purified IgGs from positive ALS-T2DM sera and T2DM sera. Thereafter, we measured the effect of IgGs purified from ALS-T2DM sera on [Ca\(^{2+}\)]; responses in islet cells. Incubation with individual ALS-T2DM-IgGs, in comparison to exposure to IgGs from T2DM sera (T2DM-IgGs) in mouse islet cells (Fig. 2 A and B). Furthermore, the effect of ALS-T2DM-IgGs was lost when boiled (Fig. 2 C). Cells exposed to either boiled ALS-T2DM-IgGs or T2DM-IgGs responded similarly to KCl stimulation with regard to increases in [Ca\(^{2+}\)]; (Fig. 2 C). These data demonstrate that ALS-T2DM-IgGs in ALS-T2DM sera enhances K\(^{+}\)-induced [Ca\(^{2+}\)]; responses.

Positive ALS-T2DM Sera Up-regulate Ca\(_{\alpha 1}\) Channels through Direct Interaction with Ca\(_{\alpha 1}\)-δ Subunits in Mouse Islet Cells. Autoantibodies against Ca\(_{\alpha 1}\),1, Ca\(_{\alpha 1}\),2, and Ca\(_{\alpha 1}\),2.2 subunits have been demonstrated to be present in ALS patients (15, 16). Importantly, these autoantibodies enhance Ca\(^{2+}\)-conductivity of these Ca\(_{\alpha}\)-conducting pores, resulting in excessively high [Ca\(^{2+}\)];, and, consequently, Ca\(^{2+}\)-dependent cytotoxicity in skeletal muscle cells and neurons (4, 15–19). Of particular importance is that selective Ca\(_{\alpha 1}\),1.1, Ca\(_{\alpha 1}\),2.1, and Ca\(_{\alpha 1}\),2.2 channel blockers substantially improve defects in neuromuscular activity and viability induced by IgGs from ALS patients (2, 18, 20–23). Our finding that both ALS-T2DM serum and ALS-T2DM-IgG promote K\(^{+}\)-evoked [Ca\(^{2+}\)]; responses suggests that Ca\(_{\alpha 1}\),2 channels might serve as downstream targets of ALS-T2DM serum and ALS-T2DM-IgG. It is well known that depolarization-evoked [Ca\(^{2+}\)];, responses in mouse islet cells primarily result from Ca\(^{2+}\)-influx through Ca\(_{\alpha 1}\),2 channels (24).

To clarify if ALS-T2DM serum affects β cell Ca\(_{\alpha 1}\) channels, we examined the effect of positive ALS-T2DM serum on β cell Ca\(_{\alpha 1}\) channel currents. Indeed, whole-cell patch-clamp analysis revealed that treatment with individual positive ALS-T2DM sera obtained in the first and second batches significantly elevated whole-cell Ca\(_{\alpha 1}\) channel currents in mouse β cells, as manifested by representative whole-cell Ca\(_{\alpha 1}\) current traces and average Ca\(_{\alpha 1}\) channel current density, in comparison to T2DM serum exposure (Fig. 3 A–C). These data verify that ALS-T2DM serum up-regulates Ca\(_{\alpha 1}\) channels, causing pathologically exaggerated [Ca\(^{2+}\)];, responses.

The up-regulation of Ca\(_{\alpha 1}\) channels by ALS-T2DM serum raises the possibility that IgGs in ALS-T2DM serum may target Ca\(_{\alpha 1}\) channel subunits in β cells. We chose the most important pore-forming subunit Ca\(_{\alpha 1}\),1.2 as a starting point. Immunoprecipitation assays followed by immunoblot analysis showed that antibodies against Ca\(_{\alpha 1}\),1.2 subunits efficiently pulled down Ca\(_{\alpha 1}\),2
subunits from the membrane fraction of insulin-secreting RINm5f cells (SI Appendix, Fig. S2A). However, neither ALS-T2DM-IgGs nor T2DM-IgGs could recognize the immunoprecipitated CaV1.2 subunits under denaturing or renaturing conditions (SI Appendix, Fig. S2 B and C). Furthermore, both ALS-T2DM-IgGs and T2DM-IgGs could not specifically fish out CaV1.2 subunits and additional proteins (SI Appendix, Fig. S2 D and E). These results show that ALS-T2DM-IgGs could not strongly bind to immunoprecipitated CaV1.2 subunits under such experimental conditions.

The CaVαδ1 subunit, an important constituent of CaV channel complexes, including the β cell CaV1.2 channel complex, is critical for the surface expression of functional CaV channels (12, 25–27). Moreover, the entire CaVαδ1 is exposed extracellularly (28). Among all β cell CaV1.2 channel components, they have the highest likelihood of serving as targets for factors, such as ALS-T2DM-IgGs, of positive ALS-T2DM sera. In addition, polyclonal anti-CaVαδ1 antibodies selectively recognize extracellular CaVαδ1 subunits associated with the plasma membrane of living cells bathed in a physiological solution (28, 29). This prompted us to clarify if ALS-T2DM-IgGs interacts with CaVαδ1 subunits under physiological conditions by using anti-CaVαδ1 antibodies. We carried out 4-(2-[6-(Diocytlylamo)-2-naphthalenyl]ethenyl)-1-(3-sulpropyl)pyridinium inner salt (di-8-ANEPPS) labeling of the plasma membrane and immunofluorescence staining of CaVαδ1 subunits in intact living mouse islet cells and tSA-201 cells stably expressing CaVαδ1 subunits co incubated with antibodies against CaVαδ1 subunits and ALS-T2DM-IgGs. CaVαδ1-specific immunofluorescence was clearly localized in the di-8-ANEPPS-labeled plasma membrane (Fig. 3 D–R and SI Appendix, Fig. S3). Interestingly, ALS-T2DM-IgGs effectively competed with the anti-CaVαδ1 antibodies for the extracellular CaVαδ1 subunits, resulting in a significant reduction in the immunofluorescence intensity of the anti-CaVαδ1 antibodies in comparison to HS-IgGs or T2DM-IgGs (Fig. 3 S and T). This verifies that ALS-T2DM-IgGs are capable of directly interacting with CaVαδ1 subunits in living cells in the absence of interferences from detergents, high ionic strengths, and substantial rinsing, which are unavoidable in immunoprecipitation and immunoblot analyses of association between ALS-T2DM-IgGs and CaV1.2 subunits.

Positive ALS-T2DM Sera Interfere with Mitochondrial Function in Mouse Islet Cells. Translation of an excessive elevation of [Ca2+]i into mitochondrial Ca2+ overload results in mitochondrial membrane depolarization, concomitant mitochondrial dysfunction, and eventual apoptosis, thus playing an important role in driving Ca2+-dependent cell death (30). This made us wonder whether such a mitochondrial mechanism is able to convert the ALS-T2DM serum-induced exaggeration of [Ca2+]i to mitochondrial dysfunction in mouse islet cells. Therefore, we measured mitochondrial membrane potential in mouse islet cells using rhodamine 123. As shown in Fig. 4 A–C, ALS-T2DM serum-treated cells displayed a significant decrease not only in basal fluorescence intensity of rhodamine 123 (Fig. 4 A and B) but also in the glucose-induced quenching of rhodamine 123 (Fig. 4 A and C), compared to those cells incubated with T2DM serum. The effects are attributed to reduced amounts of rhodamine 123 loaded into mitochondria due to less negative mitochondrial membrane potential, i.e., mitochondrial dysfunction, induced by ALS-T2DM serum treatment (31). Our results demonstrate that mouse islet cells insulted by exaggerated [Ca2+]i, resulting from exposure to positive ALS-T2DM sera undergo mitochondrial dysfunction and suggest that ALS-T2DM serum-induced mitochondrial dysfunction is most likely to drive islet cell death.

Positive ALS-T2DM Sera Impair [Ca2+]i Dynamics and Insulin Secretion in Mouse Islets. Normal glucose homeostasis critically relies on adequately functioning β cells (32). The function of β cells is under the control of exquisitely fine-tuned [Ca2+]i dynamics that serves as fingerprints for β cell well-being (12, 13, 24, 33, 34). This made us wonder if ALS-T2DM serum drives disorganized [Ca2+]i dynamics and impaired insulin secretion in islets, thereby accounting for aberrant glucose homeostasis often observed in ALS patients.

We characterized [Ca2+]i dynamics in β cells situated within intact islets during glucose stimulation. As shown in photomicrographs of Fura-2-loaded mouse islets, ALS-T2DM serum treatment made islets become irregular and disintegrated (Fig. 5 A, Lower). In striking contrast, incubation with T2DM serum did not alter the morphology of islets that were intact with spherical shapes and smooth boundaries (Fig. 5 A, Upper). Indeed, ALS-T2DM serum-treated islets showed chaotic [Ca2+]i dynamics manifested as a relatively steady increase in [Ca2+]i with tiny amplitude oscillations in response to 11.1 mM glucose (Fig. 5 B, Lower and SI Appendix, Fig. S4, Lower). However, islets exposed to T2DM serum displayed a normal [Ca2+]i profile, characterized by fast oscillations superimposed on slow oscillations, following stimulation with 11.1 mM glucose (Fig. 5 B, Upper and SI Appendix, Fig. S4, Upper). These results reveal that ALS-T2DM serum does indeed potentely derange [Ca2+]i handling in β cells.

The primary function of β cells is glucose-stimulated insulin secretion that crucially depends on CaV channel-mediated Ca2+ influx and complex [Ca2+]i dynamics (12, 13, 24, 33–35).
ALS-T2DM serum-induced defects in \([Ca^{2+}]_i\), dynamics should cause impaired glucose-stimulated insulin secretion. T2DM serum- and ALS-T2DM serum-treated islets released a similar amount of insulin following incubation with 11.1 mM glucose (Fig. 5 C and D). However, insulin secreted from T2DM serum-treated islets at 11.1 mM glucose was significantly greater than that at 3.3 mM glucose, whereas insulin released from ALS-T2DM serum-treated islets at 11.1 mM glucose did not significantly differ from that at 3.3 mM glucose due to increased basal insulin release (Fig. 5 C and D). In addition, the insulin content of ALS-T2DM serum-treated islets was significantly lower than that of islets exposed to T2DM serum (Fig. 5E). These data suggest that exposure to ALS-T2DM serum interferes with the ability of the β cell to maintain adequate insulin release.

**Positive ALS-T2DM Sera Reduce Mouse Islet Cell Viability in an IgG- and Ca\(\text{v}_1\) Channel-Dependent Manner.** The exaggerated Ca\(\text{v}_1\) channel-mediated Ca\(^{2+}\) influx, increased [Ca\(^{2+}\)], and disturbed [Ca\(^{2+}\)], dynamics in islet cells exposed to ALS-T2DM serum might explain the destructive action of this serum on islet integrity and islet insulin content. Therefore, we examined the possible effects of ALS-T2DM serum on islet cell survival. WST-1 assay showed that ALS-T2DM serum exposure significantly decreased islet cell viability, as reflected by significantly reduced WST-1 absorbance, in comparison to treatment with T2DM serum (Fig. 6A). Furthermore, cell death imaging with SYTOX Orange nucleic acid stain revealed that SYTOX Orange-positive profiles, representing dead nuclei, were significantly greater in dissociated islet cells incubated with ALS-T2DM serum compared to T2DM serum-treated ones (Fig. 6 B and C). Furthermore, the selective Ca\(\text{v}_1\) channel blocker nifedipine fully ablated ALS-T2DM serum-induced reduction of islet cell viability (Fig. 6D). In addition, treatment with ALS-T2DM-IgGs significantly reduced mouse islet cell viability in comparison to incubation with T2DM-IgGs. The effects of ALS-T2DM-IgG on islet cell viability were effectively ablated by boiling (Fig. 6D). The results demonstrate that positive ALS-T2DM sera interfere with islet cell survival in an IgG- and Ca\(\text{v}_1\) channel-dependent manner. Taken together, our results suggest that ALS-T2DM serum treatment destroys islet cells by excessively increasing Ca\(\text{v}_1\) channel-mediated Ca\(^{2+}\) influx and [Ca\(^{2+}\)], and then pathologically translating the exaggerated [Ca\(^{2+}\)], to eventual mitochondrial dysfunction.

**Discussion**

We have identified a subgroup of ALS-T2DM patients who have positive sera that exaggerate [Ca\(^{2+}\)], in pancreatic islet cells upon depolarization. This suggests that these positive sera are likely to interfere with Ca\(\text{v}\) channels via serum molecular constituent(s). Indeed, we demonstrate that pathogenic IgG is accommodated in the sera of this subgroup of ALS-T2DM patients. This not only establishes a mechanistic link between ALS and T2DM but also suggests a potential role of altered humoral immunity in the development of ALS-associated T2DM.

Importantly, we reveal that ALS-T2DM serum significantly increases whole-cell Ca\(^{2+}\) currents predominantly passing through Ca\(\text{v}_{1.2}\) channels in mouse β cells (24). This mechanistically explains how ALS-T2DM serum and ALS-T2DM-IgG promote K\(^{-}\)-evoked [Ca\(^{2+}\)], responses and pinpoints that Ca\(\text{v}_{1.2}\) channels most likely serve as downstream targets of ALS-T2DM serum and ALS-T2DM-IgG. This finding is intriguing since ALS-T2DM serum
is verified to functionally interfere with mouse β cell CaV1.2 channels, which almost exclusively mediate the nifedipine-sensitive Ca2+ currents (11–13, 36, 37).

Interestingly, we found that ALS-T2DM-IgGs are strong enough to compete with an IgG rabbit polyclonal antibody specific to the extracellular epitope of CaVα1.61 subunits in living islet cells and in tsA-201 cells stably expressing CaVα1.61 subunits. This is in accordance with the fact that the CaVα1.61 subunits are entirely exposed to the extracellular space and thereby the most accessible to serum components among all β cell CaV1.2 channel subunits. In addition, the CaVα1.61 subunit serves as an indispensable building element of the β cell CaV1.2 channel complex to up-regulate the conductivity and surface expression of functional CaV channels (12, 25–27). Based on our results, we propose that ALS-T2DM-IgGs serve as autoantibodies that immunocapture CaVα1.61 subunits in the plasma membrane, thereby enhancing CaV1 channel-mediated Ca2+ influx and [Ca2+]i in islet cells. This process likely occurs through allosteric activation and/or gradual accumulation of CaV1 channels in the β cell plasma membrane since antibodies in some cases activate and accumulate rather than inhibit and neutralize their binding partners (38, 39). This autoimmune mechanism is particularly interesting since ALS autoantibodies have been shown to target only CaV1.1, CaV2.1, and CaV2.2 channels prior to the present work (2, 18, 20–23). Now the immunocapture of β cell CaVα1.61 subunits by ALS-T2DM-IgGs and consequent up-regulation of β cell CaV1.2 channels come into the picture. Importantly, the present work reveals that humoral autoimmune reactions are as pathogenic machinery leading to this subset of diabetes. It is intriguing to explore if such a humoral mechanism not only operates in the pathogenesis of T2DM but also in that of type 1 diabetes in addition to a T cell–mediated autoimmune destruction of β cells (40). Moreover, our findings offer a causal link between ALS and T2DM and shed light on potential therapeutic targets for prevention and treatment of a subgroup of ALS-T2DM patients.

The mitochondrion is a vulnerable target downstream of excessive accumulation of [Ca2+]i to mediate Ca2+-dependent impairments in cell function and viability (30, 41). Consequently, islet cells exposed to positive ALS-T2DM sera not only display exaggerated [Ca2+]i, but also mitochondrial dysfunction. These findings provide a strong rationale for the ALS-T2DM serum-induced islet cell dysfunction and death. We found that positive ALS-T2DM sera render [Ca2+]i dynamics, impair insulin secretion, and drive islet cell death in a CaV1 channel- and IgG-dependent manner. The occurrence of these pathological phenotypes is well accounted for by impaired mitochondrial function. Our data thus suggest that IgG autoantibodies in ALS-T2DM sera immunocapture CaVα1.61 subunits in the plasma membrane, resulting in a destructive exaggeration of [Ca2+]i, followed by its pathological translation into mitochondrial dysfunction, subsequent impairment of insulin secretion, eventual islet cell death, and diabetes. Of note, in vivo ALS-T2DM-IgGs may target peripheral insulin-sensitive tissues of patients, leading to insulin resistance (8).

The exact reason why only a fraction of ALS patients develop diabetes is unclear but is most likely due to a heterogeneous β cell sensitivity to the evoked [Ca2+]i, challenges. In addition, both ALS and T2DM represent an etiologically heterogeneous group of disorders where patients are likely to experience particular environmental challenges on top of their specific genetic
In conclusion, the present work demonstrates that a subgroup of ALS-T2DM patients have sera that enhance CaV1 channel-mediated Ca\(^{2+}\) influx, resulting in exaggerated [Ca\(^{2+}\)]. These effects are attributed to the fact that the sera accommodate cytotoxic IgG autoantibodies that immunocapture CaV\(_{\alpha2\delta1}\) subunits. As a consequence, impairments in \([\text{Ca}^{2+}]\), dynamics, mitochondrial function, insulin secretion, and cell viability occur. This suggests that cytotoxic ALS-T2DM-IgG autoantibodies serve as a causal link between ALS and T2DM by interacting with and modulating the CaV\(_{\alpha2\delta1}\)–CaV1 channel complex, which may lay the foundation for a pharmacological treatment strategy for patients suffering from a combination of these severe diseases.

**Methods**

**Animals.** Male C57BL/6J mice aged from 8 to 10 wk were purchased from The Jackson Laboratory (Bar Harbor, ME). All animal experiments were conducted according to the guidelines of the Ethics Committee at Pohang University of Science and Technology (2011-0001) and the Animal Experiment Ethics Committee at Karolinska Institutet (N183/13).

**Enrollment of Patients.** Seventeen patients with ALS-T2DM (11 males and 6 females, age: 55.0 ± 2.1), 12 HCs, 9 patients with ALS, and 14 patients with T2DM (7 males and 7 females, age: 60.4 ± 2.5) were randomly selected (SI Appendix, Table S1). One ALS patient carrying a SOD1 mutation was excluded from the study (SI Appendix, Table S1). Ethical approval to use serum from patients was obtained from Hanyang University Hospital in Seoul, Korea (HYUH 2006-04-001-004). Blood samples were collected after obtaining written informed consent. Actual patients had no known family history of neuromuscular disease or wasting. All subjects diagnosed with T2DM were well controlled with hypoglycemic agents or insulin.

**Data Availability.** All of the data, associated protocols, code, and materials for this study are available within the paper and its SI Appendix.

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**Supplemental Data.** All of the data, associated protocols, code, and materials for this study are available within the paper and its SI Appendix.

**SI Appendix**

**Materials and Methods.**

**Fig. 6.** Effects of positive ALS-T2DM sera on mouse islet cell viability. (A) Mean WST-1 absorbance showing the viability of dissociated islet cells exposed to T2DM or ALS-T2DM serum. Experiments were done with 5 T2DM and 7 ALS-T2DM sera. **P < 0.01 vs. the T2DM group.** (B) Example SYTOX Orange fluorescence (Left), transmitted light (Middle), and their overlay images (Right) of dissociated islet cells incubated with T2DM (Upper) and ALS-T2DM serum (Lower) followed by exposure to SYTOX Orange nucleic acid stain. (Scale bar, 50 μm.) (C) Mean percentage of dead cells labeled with SYTOX Orange nucleic acid stain in T2DM and ALS-T2DM groups. Experiments were done with 3 T2DM and 3 ALS-T2DM sera in triplicate. **P < 0.05 vs. the T2DM group.** (D) Mean WST-1 absorbance showing the viability of dissociated islet cells exposed to 3 T2DM sera or 3 ALS-T2DM sera in the absence and presence of the CaV1 channel blocker nifedipine. **P < 0.01 vs. the T2DM group and P < 0.05 vs. the ALS-T2DM/nifedipine group.** (E) Mean WST-1 absorbance in islet cells following 24 h exposure to T2DM-IgG, ALS-T2DM-IgG, or boiled ALS-T2DM-IgG. Experiments were done with 3 T2DM-IgGs, 3 ALS-T2DM-IgGs, or 3 boiled ALS-T2DM-IgGs. **P < 0.01 vs. the T2DM-IgG group and **P < 0.01 vs. the boiled ALS-T2DM-IgG group. 1. B. R. Brooks, Natural history of ALS: Symptoms, strength, pulmonary function, and disability. Neurology 47 (suppl. 2), S171–S528 (1996).

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