Zinc for GNAO1 encephalopathy: preclinical profiling and a clinical case

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Abstract

De novo mutations in *GNAO1* – the gene encoding the major neuronal G-protein Gαo – cause pediatric encephalopathies largely refractory to available therapies. Zn^{2+} emerged to restore GTP hydrolysis and cellular interactions of pathogenic Gαo; dietary Zn^{2+} supplementation improves lifespan and motoric function in a *Drosophila* disease model. Here we show that 16 different pathogenic missense mutations cluster in three distinct groups in their responsiveness to Zn^{2+}, and provide the safety study in a mouse disease model. We further describe treatment of a 3 years-old patient with a common *GNAO1* mutation c607G > A, p.Gly203Arg with oral 50mg Zn^{2+} daily, as applied in Wilson's disease. During 11 months of treatment, the patient shows cessation of daily hyperkinetic crises, improved Burke-Fahn Marsden Dystonia Rating Scale movement score and general well-being, and an excellent safety profile. Our findings warrant a large-scale clinical trial and might set the new standard of care for *GNAO1* encephalopathy.

Full Text

First identified in 2013, *GNAO1*-related neurodevelopmental disorders are caused by mutations in the *GNAO1* gene encoding the major neuronal G protein, Gαo[1]. Gαo is one of 16 human Gα-subunits that, together with Gβγ, form heterotrimeric G-protein complexes that are the primary transducers of G protein-coupled receptors (GPCRs). Ligand-activated GPCRs facilitate the exchange of GDP for GTP in Gα, thereby promoting the dissociation of Gα-GTP from Gβγ and the receptor[2]. Gα-GTP and Gβγ can then engage with distinct downstream effectors to transmit the GPCR signal inside the cell. With time, Gα hydrolyzes its GTP back to GDP, and this GTPase activity can be accelerated by the Regulator of G-protein Signaling (RGS) proteins[3].

As of today, about 200 patients worldwide are known to be affected by *GNAO1* encephalopathy[4], identifying it as an ultrarare disease. However, this number is expected to grow with the wider availability of whole exome / genome sequencing in undiagnosed patients. Patients demonstrate a broad range of symptoms, including epilepsy, movement disorders, hypotonia, developmental delay, and brain atrophy[4–7]. Current treatments attempt to alleviate the epileptic and movement disorder symptoms, but patients are largely refractory to the available treatments[4,8]. With more than 80 pathogenic (mostly missense) variants of Gαo identified to date (ncbi.nlm.nih.gov/clinvar/?term = gnao1%5Bgene%5D&redir = gene), in-depth molecular characterization is crucial to understand the etiology of the disease caused by the individual variants[9]. Earlier studies have revealed that different pathogenic Gαo exhibit a variety of molecular defects, such as accelerated GTP uptake / defective GTP hydrolysis that lead to constitutive GTP loading of Gαo, structural defects that lead to abnormal interaction with RGS proteins and Gβγ, decreased plasma membrane localization, defective GPCR coupling, and neomorphic binding to Ric8A/B proteins[10–17].

Powered by the understanding of the molecular defects of pathogenic Gαo, we have identified zinc salts as a potential therapy for patients with the most common variants of *GNAO1* encephalopathies: G203R, R209C, and E246K[12]. Replacing Mg^{2+} in the active center and inducing structural rearrangements, Zn^{2+} restores the GTPase activities of the mutants and their cellular interactions, without influencing wild-type Gαo[12]. Zinc supplementation improves motor function and longevity in a *Drosophila* model of *GNAO1* encephalopathy carrying the G203R variant; our studies using this model further indicate the need for continuous dietary zinc supplementation to achieve the therapeutic effect[12].

We reveal that Zn^{2+} counteracts the constitutive GTP binding of pathogenic Gαo variants by different mechanisms. Testing the biochemical properties of 16 variants (Supplementary TableS1), we find that most mutants have increased GTP binding rates (k_{bind}) and defective GTP hydrolysis (reduced k_{hydr}) in comparison to wild-type Gαo (Fig. 1A,B and Supplementary Fig.S1), agreeing with our previous studies[12–15,18]. Evaluation of the effects of increasing concentrations of $ZnCl_2$ on the GTP binding/hydrolysis permits to group the variants into three classes. The first includes the variants that are, like Gαo wild-type, unaffected by Zn^{2+} in GTP binding / hydrolysis: L23P, C215Y, and I344del, adding up to the T241_N242insPQ variant we identified previously[13] (Supplementary Fig.S2 and Fig. 1C). The second class represents the variants whose GTP binding remains unaffected, but whose GTP hydrolysis is restored by Zn^{2+}, adding K46R to the G203R, R209C, and E246K mutants we studied earlier[12] (Supplementary Fig.S3 and Fig. 1D). And the third category emerges to be the most populated and includes K46N, H57P, T182I, R209H, Y231C, E237K, and Y291N, in addition to P170R studied earlier[15]. Zn^{2+} reduces GTP uptake by these variants in both GTP binding and hydrolysis assays, the effect mediated by the > 3-fold reduction in the affinity of Gαo to GTP (Supplementary Fig.S4 and Fig. 1E,F)[15].

Interestingly, this stratification of pathogenic *GNAO1* mutations correlates with the clinical severity of the disease manifestations. Analysis of 26 class I, 63 class II, and 40 class III patients (Fig. 1F and Supplementary TableS1) reveals that the onset of disease differs strongly and significantly among the classes, class II being the most severe (average onset of 6.8 months) and class I – the least severe (average onset of 4.3 years), class III being the intermediate (6.8 years, Fig. 1G). Noteworthy, the biochemical perturbations of the class I mutants, which are biochemically unresponsive to zinc, are also milder (Fig. 1A,B). In contrast, for the pathogenic variants leading to more severe clinical phenotypes underlined by the biochemical severity, Zn^{2+} alleviates their constitutive GTP binding through one of the two mechanisms, both expected to bring clinical benefit: reduction in the GTP uptake or restoration of GTP hydrolysis.

These findings lay the basis for the patient stratification in the clinical applications of zinc. Zinc has been approved for treatment of diverse disorders including Wilson's disease and various neurological conditions[19,20], with the daily dose of elemental zinc in Wilson's disease treatment of 50mg daily for children under 6 years of age[21]. We argued that a similar dose should be applied for the treatment of *GNAO1* patients. Prior to the off-label clinical applications, we aimed at assessing the safety profile of

zinc in a mouse model of the disease. Zinc toxicity has not been evaluated in neonates and young pups, whereas zinc supplementation has been well-studied in adult mice. For example, maintenance of adult mice for up to 14 months on water supplemented with 0.5g/L elemental zinc in the form of $ZnSO_4$ resulted in no adverse effects in the animals[22]; the maximum tolerated dose (MTD) for adult mice is reported as 75mM (12.11g/L) $ZnSO_4$ in drinking water[23]. We thus first tested the near-MTD doses of $ZnSO_4$ supplied in drinking water to C57BL/6 mice all the way from birth to adulthood: 4-8g/L (ca. 1000-2000mg/kg/day of $ZnSO_4$; the human equivalent dose can be estimated (following fda.gov/media/72309/download) as 81-162mg/kg/day. The presence of $ZnSO_4$ in the drinking water of lactating dams resulted in a transient delay in the body weight gain of the pups with onset at post-natal day (PND)7 and complete resolution by ca. PND17 (Supplementary Fig.S5A). This delay is fully compensated and is not reflected in the body weights of adult males and females by the end of the 3-month measurement period (Fig. 2A,B). No differences were observed in the appearance of animals treated with the highest dose of $ZnSO_4$ (8g/L, Supplementary Fig.S6). Finally, after 3 months of this treatment, the animals were sacrificed and the weight and appearance of major organs were evaluated at necropsy (Supplementary Figs S5B and S7). No difference in organ weight or appearance was observed. Taken together, these results indicate only a low and transient toxicity of the near-MTD doses of Zn^{2+} and their suitability for long-term treatment.

Only three mouse strains with pathogenic *GNAO1* mutations have been described: R209H, C215Y, and G203R[18,24]. Of note, *G203R/+* mice die neonatally[24]. We thus used the C215Y mouse line available to us, in which behavioral disturbances in the form of hyperactive behavior have been described[24]. As the C215Y mutant was not responsive to Zn^{2+} in our *in vitro* biochemical assays (Fig. 1C,F), we did not expect to observe an improvement in the behavior of the mutant mice upon chronic $ZnSO_4$ administration. However, we argued that this mouse disease model could be a precious model to assess the safety profile of zinc supplementation in a *GNAO1* disease condition. We find that continuous supplementation of $ZnSO_4$ (2g/L in the drinking water) leads to no behavioral disturbances. Intriguingly, we see a noticeable improvement in the mouse performance in the rotarod test that can be observed in the three genotypes: *C215Y/+*, *C215Y/C215Y*, and the control wild-type littermates (Fig. 2C). These results indicate that $ZnSO_4$ supplementation can improve motor skills in mice regardless of their genetic background. Given the known association between mutations in the *GNAO1* gene and cognitive impairment in patients, the mice's exploratory behavior was also assessed using the novel object recognition test. In this test, all groups of mice, regardless of genotype, showed increased exploratory activity on the second day of the test. This was manifested by a significant increase in the time spent exploring objects (Fig. 2D and Supplementary Fig.S8A). In the open field test, where *C215Y/+* and *C215Y/C215Y* mice reveal the hyperkinetic activity[24], zinc supplementation did not change the behavioral readouts for either mutant genotype nor wild-type littermates (Supplementary Fig.S8B-D). Altogether, we conclude that zinc supplementation did not lead to any deterioration in the mouse model of *GNAO1* encephalopathy; a general, genotype-independent improvement in motor and cognitive skills was achieved by the treatment.

With this background, we applied zinc supplementation therapy to a 3.4 years-old patient with the c607G > A, p.Gly203Arg mutation (see online Methods for additional description of the patient). During the first year of life, the boy presented a severe epileptic encephalopathy without any head control nor achievement of any motoric milestones. Severe dystonia, choreoathetosis and repetitive hyperkinetic crises were prominent during the first months. Orofacial dystonia with tongue involvement caused severe feeding difficulties requiring percutaneous endoscopic gastrostomy (PEG) at the age of 12 months. A severe sleep disorder was present as well. The epilepsy was resistant to multiple antiseizure drugs. The disease progressed and daily hyperkinetic crises were the major cause of morbidity. Pharmacotherapy of the movement disorder comprised benzodiazepines, gabapentin, baclofen, clonidine and cannabinoids, all with minor or only short-lasting effects.

In the course of a severe bacterial infection with respiratory impairment and ascites, the boy showed acute deterioration of his global condition with severe disease progression. He was administered to a hospice at the age of 3.4 years. With the family's agreement we started an off-label zinc therapy. The initial dose was 40mg Zn^{2+} daily (2.7mg/kg/day) administered as zinc gluconate which was increased to 50mg Zn^{2+} daily (3.5mg/kg/day) administered as zinc acetate dihydrate. To date, the boy has been on zinc for 11 months. After grinding, the medication is provided through PEG as a suspension in water. The medication is well tolerated and no adverse events have been reported to date. Hemoglobin and copper serum levels were stable while the zinc serum level increased but did not exceed the upper limit (Fig. 2E). The patient's condition has stabilized and an improvement in quality of life is reported by the parents: the daily hyperkinetic crises stopped and the patient started to smile again. The application of emergency medication against hyperkinetic exacerbations could be reduced. The patient shows longer periods of awareness during daytime and the night sleep could be improved. The parents report that their son now enjoys their interaction without any distraction by seizures or involuntary movements after many months of the severe impairment. The Burke-Fahn Marsden Dystonia Rating Scale movement score (BFMDRS-M) improved by 46,5 eight months after the start of the medication (Fig. 2E). The Gross Motor Function measure-66 (GMFM-66) did not show any changes. Morphine was the only new concomitant medication given to reduce agitation due to shortness of breath in the palliative setting.

This is the first *GNAO1* patient who receives zinc in high dosages equal to the dosages recommended in Wilson's disease under controlled settings of a natural history study. This treatment trial has been supported by an extensive preclinical assessment, *in vitro* and in animal models, of the efficacy, mechanism of action, and safety of zinc administration in order to cure the molecular defects caused by pathogenic *GNAO1* mutations. The therapy has been feasible and no adverse events have been reported so far. It is possible that the beneficial effects cannot be attributed exclusively to the zinc treatment, but apart from morphine, which was started in the hospice setting, the patient did not receive any new therapies other than zinc during the treatment period of eight months. We conclude that further studies on the feasibility and safety of zinc in larger cohorts of patients with *GNAO1*-associated disorders are now required.

Declarations

Acknowledgments

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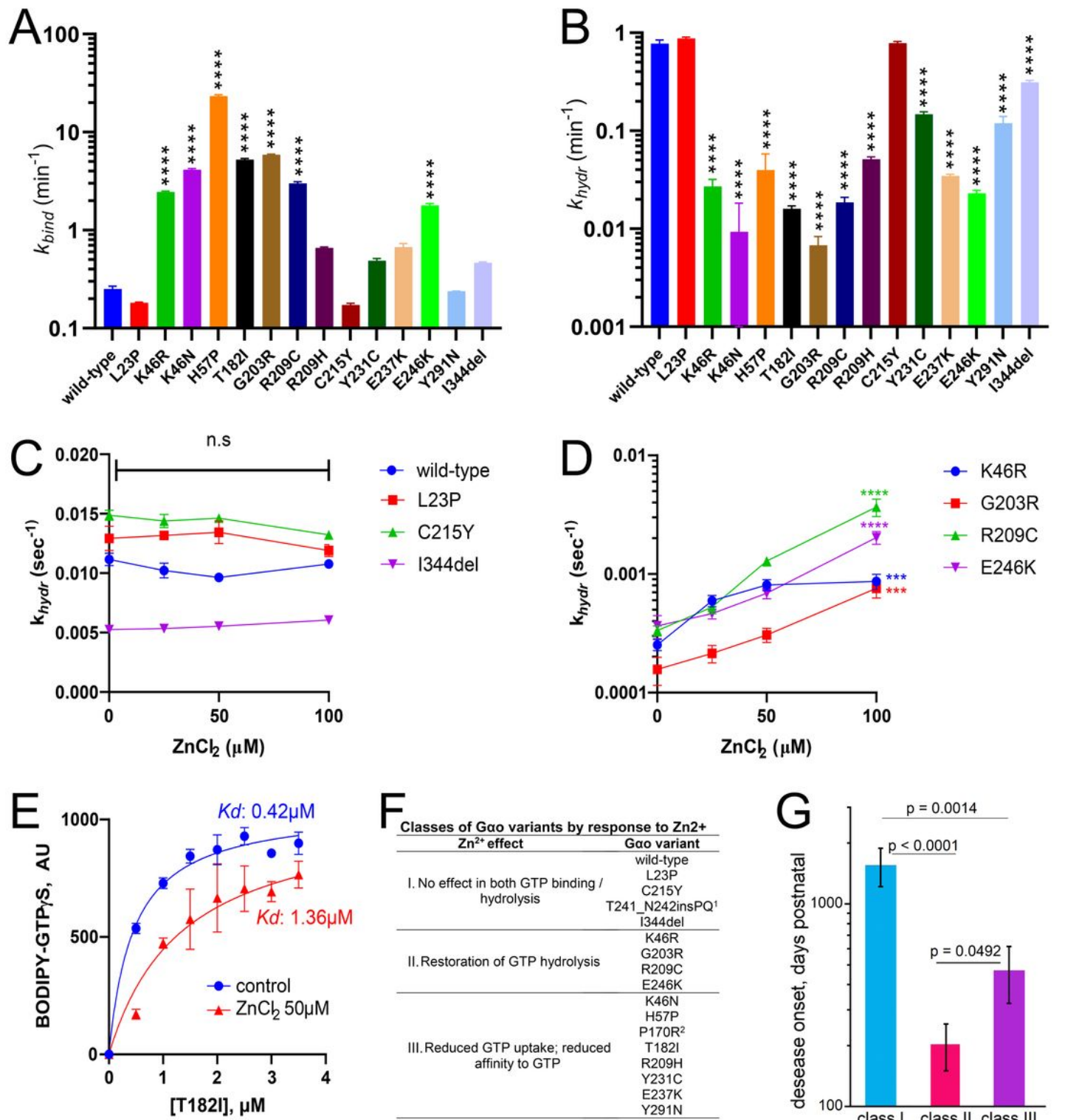
Figures

Figure 1

Three classes of pathogenic Gαo mutations by their sensitivity to Zn^{2+}.

(**A, B**) GTP binding (k_{bind}, A) and GTP hydrolysis (k_{hydr}, B) rates of Gαo: wild-type and 14 pathogenic mutants. (**C**) Class I pathogenic Gαo mutants, like wild-type Gαo, do not display any changes in GTP hydrolysis upon adding increasing concentrations of ZnCl$_2$. (**D**) Class II pathogenic Gαo mutants restore

their GTP hydrolysis capacity upon addition of $ZnCl_2$, in a dose-dependent manner. (**E**) As an example of class III pathogenic Gαo mutants, T182I displays reduced affinity to GTP upon addition of $ZnCl_2$. 1µM BODIPY-GTPγS was titrated with recombinant Gαo[T182I] in the absence or presence of 50µM $ZnCl_2$. The maximum values of BODIPY-GTPγS fluorescence (60sec after addition of T182I) were plotted against the concentration of recombinant Gαo[T182I] to calculate the K_d of BODIPY-GTPγS to Gαo[T182I]. Data in (A-E) are mean ± SEM (n≥3); statistical analysis was performed by one-way ANOVA followed by Holm-Sidak test, significance is shown as **** p<0.0001; n.s.: not significant. (**F**) List of mutants analyzed in this study (14 mutations) and in two previous publications (2 mutations) categorized into 3 classes by the responsiveness to Zn^{2+}. (**G**) The 3 classes of pathogenic Gαo mutants differ by the severity of disease they cause, measured as the individual patients' disease onset (in days). Data are mean ± SEM (n=26 to 63, see Supplementary TableS1); statistical significance by t-test is shown.

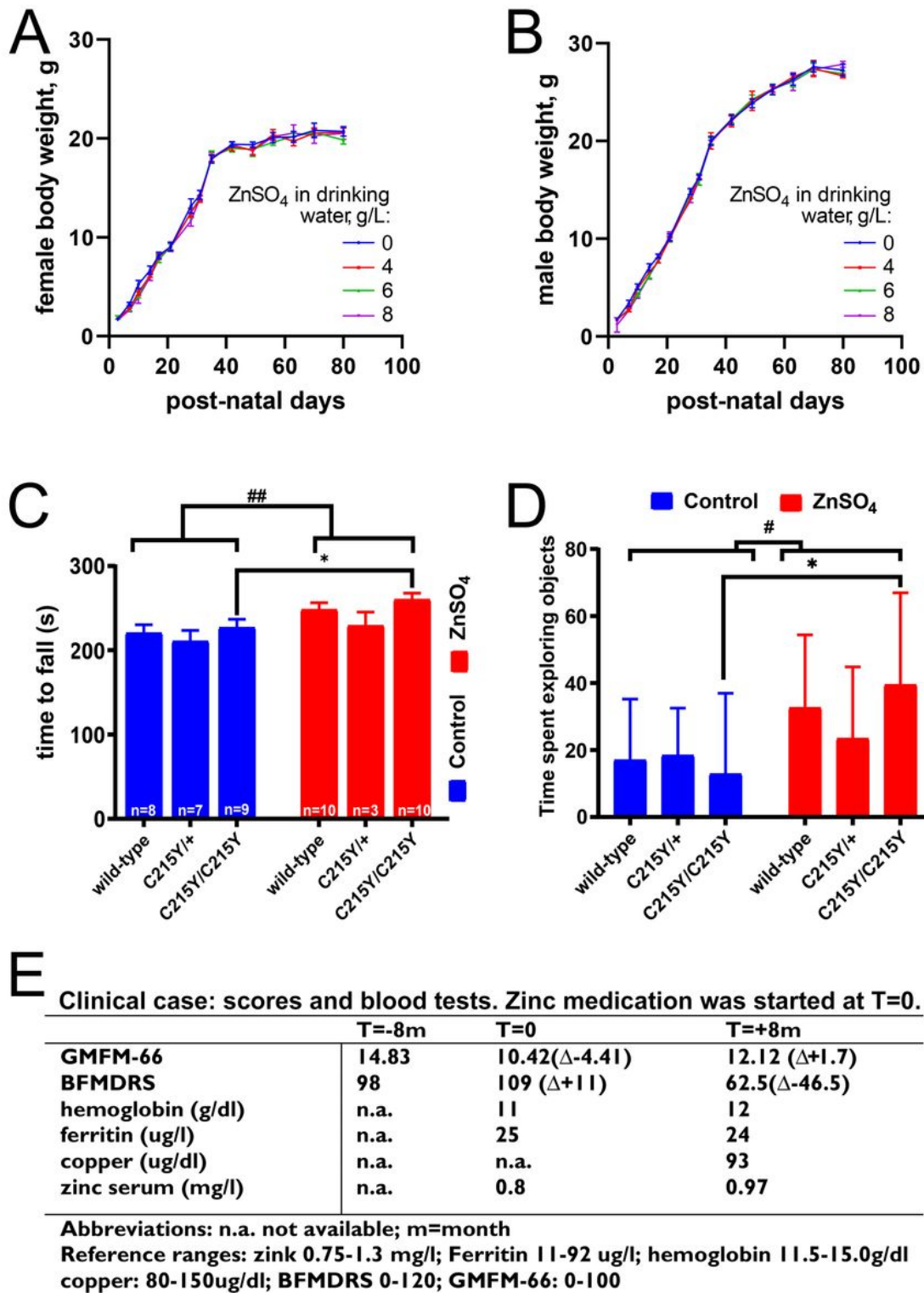**Figure 2**

Preclinical and clinical assessment of zinc supplementation.

(**A, B**) Body weight monitoring for continued treatment of C57BL/6 mice with the indicated concentrations of $ZnSO_4$ added to the drinking water shown separately for females (A) and males (B) reveals no significant changes between the control and $ZnSO_4$-treated groups. (**C**) In the rotarod test, $ZnSO_4$-treated

mice showed an improvement in motor skills, irrespective of the C215Y/+, C215Y/C215Y, or +/+ genotype. A quantitative assessment of the falling time parameter is shown as an average for 4 test days. The number of animals in each experimental group is shown on the bars. (**D**) Treatment with ZnSO$_4$ increased the exploratory activity of mice, regardless of the C215Y/+, C215Y/C215Y, or +/+ genotype, in the recognition task test. Day 2 performance is shown; data for day 1 are provided in Supplementary Fig.S8A. Data in (A-D) are shown as mean ± SD. #p < 0.05, ##p < 0.01 and *p < 0.05 as determined by two-way ANOVA with Sidak's multiple comparisons test. (**E**) Clinical characteristics of the *GNAO1* patient before and after continuous treatment with zinc.

Supplementary Files

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