



OPEN Genetic effects of circulating hormone and proteome on amyotrophic lateral sclerosis identified by Mendelian randomization

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Altered circulating hormones in ALS patients have been widely reported by previous observational studies, but whether these relationships are causal is unclear. Moreover, the potential therapeutic targets for ALS and the effects of plasma protein fluctuation on ALS progression are not fully understood. Therefore, we conducted a Mendelian randomization (MR) study to evaluate the causal role of 5 hormonal risk factors (insulin-like growth factor-1, IGF-1; sex hormone-binding globulin, SHBG; free testosterone, FT; total testosterone, TT; and estradiol) in ALS risk. Furthermore, we screened up to 90 circulating proteins including cytokines, chemokines, growth factors, and interferons, to identify potential therapeutic targets for ALS. Our MR analysis found genetically predicted higher level of FT was associated with a 23% lowered risk of ALS. Further screening of proteomic traits found that 12 plasma proteins were causally associated with ALS. These findings suggest that higher FT potentially exerts a protective effect on ALS risk. Several proteins may act as potential circulating biomarkers and therapeutic targets for ALS. In the future, high-throughput proteomic analyses and experimental explorations are likely needed to clarify the regulated role and mechanistic pathways.

Keywords Amyotrophic lateral sclerosis, Hormone, Free testosterone, Proteome, Cytokine, Inflammation, Mendelian randomization

Abbreviations

ALS	Amyotrophic lateral sclerosis
IGF-1	Insulin-like growth factor-1
MR	Mendelian Randomization
SHBG	Sex hormone-binding globulin
FT	Free testosterone
TT	Total testosterone
GWASs	Genome-wide association studies
SNPs	Single-nucleotide polymorphisms
IVW	Inverse-variance weighted
OR	Odds ratio
SD	Standard deviation
CI	Confidence interval

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder with a median survival of about 3 to 5 years after symptom onset. The causes of ALS remain far to be explored and appear heterogeneous. Hitherto, there is no cure or effective medical treatment for ALS, and the standard of disease management

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remains multidisciplinary care¹. Therefore, it is fundamental to identify modifiable risk factors and circulating biomarkers for ALS to develop effective diagnosis, prevention and treatment strategies.

Accumulating evidence has suggested that circulating hormones, including testosterone^{2,3}, estradiol⁴, and insulin-like growth factor-1 (IGF-1)^{5,6} may play a role in ALS progression. As a consequence, androgen and IGF-1 therapies were proposed as therapeutic candidates against ALS⁷. Although observational studies have evaluated the association between the concentrations of these hormones and ALS, the causality and the underlying molecular mechanism remain to be established. On the other hand, biological studies led to the opinion that inflammatory response contributes to the pathogenesis of ALS⁸. In addition, altered levels of peripheral cytokines and proteins were observed in ALS patients, but the results were inconsistent^{9,10}. Several attempts have been made to identify therapeutic targets; however, few clinical studies are available on human individuals. Thus, the causal nature of these risk factors, and their suitability as intervention targets for ALS prevention, are still unknown.

Observational studies are susceptible to residual confounding and reverse causality, which may bias the association estimates between modifiable risk factors and ALS. Mendelian Randomization (MR) overcomes these defects by using the genetic variants as instrumental variables to investigate the causal effect of exposure (risk factors) on a disease outcome¹¹. As this genetic-based analysis is compliant with Mendel's law of segregation and independent assortment, the estimates of MR are less likely to be affected by confounding factors and biased by reverse causation.

Given the unclear relevance of circulating hormones and plasma proteins in ALS etiology, we conducted multiple MR analyses to evaluate the causal role of the five known endogenous hormones, including insulin like growth factor (IGF-1), sex hormone-binding globulin (SHBG), free (bioavailable) testosterone (FT), total testosterone (TT), and estradiol, and up to 90 plasma proteins including cytokines, chemokines, growth factors and interferons, in ALS risk.

Materials and methods

The analytical strategy employed in this study is depicted in Fig. 1. In the initial phase of our study, we examined the causal relationships between five hormones (including IGF-1, FT, TT, SHBG, and estradiol) and the risk of developing ALS. On the other hand, we evaluated the causal effects of circulating proteins on ALS progression.

All analyses were based on summary-level data on measures of hormones, ALS, and circulating proteins from published genome-wide association studies (GWASs). Appropriate ethical approval and informed patient consent could be found in the original studies.

Growth and sex hormones instrument selection

Genetic instruments associated with IGF-1 were obtained from a GWAS including 358,072 European-descent participants of the UK Biobank¹². Single-nucleotide polymorphisms (SNPs) predicting levels of FT, TT, and

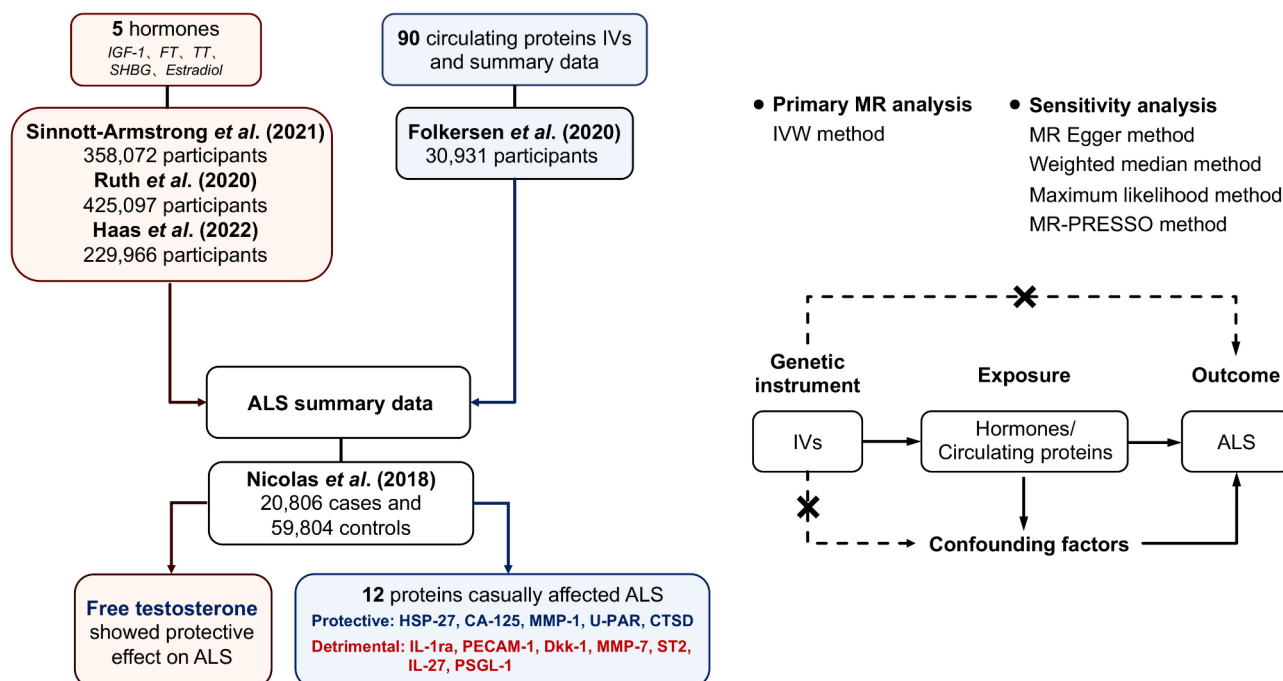


Fig. 1. The study design overview and the conceptual schematic of the Mendelian randomization. ALS, amyotrophic lateral sclerosis; FT, free testosterone; IVW, the inverse-variance weighted method; IGF-1, insulin-like growth factor 1; IVs, instrumental variables; MR, Mendelian randomization; MR-PRESSO, MR-pleiotropy residual sum and outlier; SHBG, sex hormone-binding globulin; TT, total testosterone.

SHBG were extracted from a GWAS conducted by Ruth et al. using UK Biobank data¹³, which included 425,097, 382,988, and 368,929 European-descent participants for FT, TT, and SHBG, respectively. Variants selection for estradiol levels was based on a GWAS including 229,966 women of European ancestry from the UK Biobank dataset¹⁴. To construct genetic instruments for growth and sex hormones, we obtained independent ($r^2 < 0.01$ and clump distance $> 10,000$ kb) SNPs that reached genome-wide significance ($P < 5 \times 10^{-8}$). Information on genetic instruments is provided in Table S1.

The data source for ALS

Summary genetic association data on ALS was obtained from a publicly available GWAS conducted by Nicolas et al.¹⁵. This large-scale GWAS involved 12,663 ALS patients and 53,439 controls. The data was then incorporated into a meta-analysis with a previous GWAS including 12,577 ALS patients and 23,475 controls¹⁶. After imputation and quality control measures, genetic variants from 20,806 cases and 59,804 controls in populations of European ancestry were available for association analyses. ALS patients were diagnosed by neurologists according to the EI Escorial criteria¹⁵. In the replication analyses, the summary data of ALS (GWAS ID: finn-b-G6_ALS) was extracted from FinnGen consortium and provided by the IEU database (<https://gwas.mrcieu.ac.uk/>), which included 219 cases and 111,621 controls.

Circulating proteins study population

The summary statistics and genetic instruments for circulating proteins were derived from a GWAS conducted by Folkersen et al.¹⁷. Ninety proteins, comprising cytokines, chemokines, growth factors, and interferons, in up to 30,931 European individuals from 13 cohorts passed quality control, were available for our hormone-protein MR analyses. Details of the included proteins are shown in Table S2. To select genetic instruments, we first extracted SNPs associated with circulating proteins at the genome-wide significance level ($P < 5 \times 10^{-8}$) and then pruned these SNPs according to linkage disequilibrium ($r^2 < 0.01$) based on the 1000 Genomics European reference panel. We excluded proteins that had no or limited (< 2) genetic instruments. After removing 14 proteins, 76 circulating proteins were included in the protein-ALS MR analyses. Detailed information on used SNPs is presented in Table S3.

Statistical analyses

In our primary analyses, we used the Wald ratio to generate effect estimates and used the delta method to approximate standard errors¹⁸. For circulating proteins instrumented by only two SNPs, inverse-variance weighted (IVW) fixed-effects models were used to estimate causal effects. For traits instrumented by three or more SNPs, IVW random-effects models were used¹⁸. To evaluate the presence of horizontal pleiotropy (i.e. the genetic variant influences an outcome through a biological pathway independent of the exposure), we conducted various sensitivity analyses, including MR Egger regression¹⁹, weighted median estimation²⁰, maximum likelihood estimation²¹, and MR Pleiotropy Residual Sum and Outlier (MR-PRESSO) estimation²². Each of these methods consists of different assumptions regarding the potential horizontal pleiotropy, thus conducting all of them can provide complementary support to the IVW method and examine the robustness of associations. These methods were not performed when instruments consisted of only two SNPs because of the reduced statistical power to detect horizontal pleiotropy in these circumstances.

All statistical analyses were conducted using R (Vienna, Austria) version 4.0.1 together with the R package TwoSampleMR (<https://github.com/MRCIEU/TwoSampleMR>) and MR-PRESSO (<https://github.com/rondolab/MR-PRESSO>).

Results

Causal estimates of the genetically predicted Circulating hormone in ALS

In the growth hormone analysis, IGF-1 was not associated with ALS (odds ratio (OR) per increase in standard deviation (SD) IGF-1, 1.00, 95% confidence interval (CI) 0.93–1.08, $P = 0.901$) (Fig. 2). As for sex hormones, a genetically predicted higher level of FT was associated with a 23% lowered risk of ALS (OR per increase in INT nmol/L FT 0.77, 95% CI 0.65–0.91, $P = 0.003$), corroborated by sensitivity estimates (Weighted median method, OR = 0.73, 95% CI 0.55–0.97, $P = 0.029$; MR Egger method, OR = 0.62, 95% CI 0.43–0.91, $P = 0.015$; Maximum Likelihood, OR = 0.77, 95% CI 0.65–0.91, $P = 0.002$). The MR PRESSO method showed no outliers in IVs. In addition, MR-Egger intercept was not significantly different from zero ($P = 0.218$). We found that no specific SNP had a decisive role regarding the association between FT and ALS by applying the leave-one-out analysis. Further validation analyses by using ALS data from FinnGen consortium yielded consistent negative association of FT with ALS risk through IVW method (OR = 0.30, 95% CI 0.11–0.80, $P = 0.016$) (Table S4).

In contrast, the IVW MR estimates for TT (OR per increase in inverse-normal transformed [INT] nmol/L TT 0.95, 95% CI 0.79 to 1.14, $P = 0.600$), SHBG (OR per increase in natural log transformed nmol/L SHBG 1.08, 95% CI 0.94 to 1.24, $P = 0.280$), and estradiol (OR per increase in pmol/L estradiol 1.02, 95% CI 0.60 to 1.72, $P = 0.953$) were not significant (Fig. 2).

Identification of causal risk proteins for ALS

After removing the instruments that did not satisfy the inclusion criteria, seventy-six plasma proteins were tested for the causal relationships with ALS (Table S5). The MR estimates identified twelve potential causal risk proteins for ALS: HSP-27, CA-125, MMP-1, U-PAR, CTSD, IL-1ra, PECAM-1, Dkk-1, MMP-7, ST2, IL-27, and PSGL-1. Among of which, Genetic predispositions to 1-SD increase in HSP-27 (OR 1.08, 95% CI 1.03 to 1.09, $P = 1.1 \times 10^{-4}$), CA-125 (1.06, 95% CI 1.02 to 1.11, $P = 0.007$), MMP-1 (1.09, 95% CI 1.01 to 1.17, $P = 0.020$), U-PAR (1.16, 95% CI 1.02 to 1.31, $P = 0.023$), and CTSD (1.06, 95% CI 1.00 to 1.13, $P = 0.049$) were associated with higher risk of ALS (Fig. 3). In contrast, the results from the IVW method suggested the protective effects

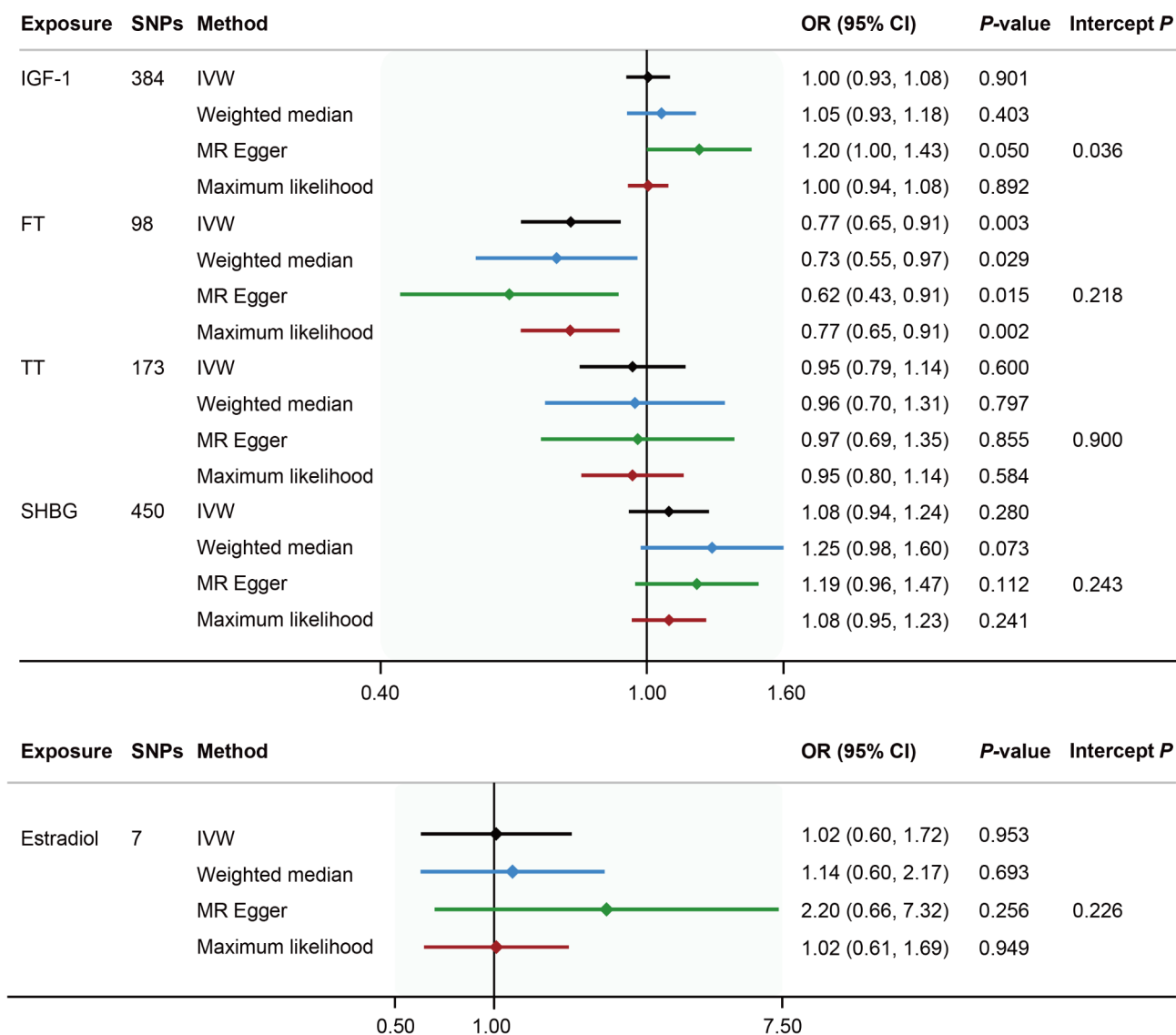


Fig. 2. Mendelian randomization association of genetically predicted growth and sex hormones with ALS. Odds ratios are scaled per genetically predicted standard deviation change in IGF-1, inverse-normal transformed nmol/L change in FT and TT, natural log transformed nmol/L change in SHBG, and pmol/L change in estradiol. ALS, amyotrophic lateral sclerosis; FT, free testosterone; IVW, the inverse-variance weighted method; IGF-1, insulin-like growth factor 1; MR, Mendelian randomization; OR, odds ratio; SHBG, sex hormone-binding globulin; TT, total testosterone.

of IL-1ra (0.92, 95% CI 0.87 to 0.97, $P=0.001$), PECAM-1 (0.92, 95% CI 0.86 to 0.97, $P=0.003$), Dkk-1 (0.91, 95% CI 0.84 to 0.98, $P=0.011$), MMP-7 (0.90, 95% CI 0.84 to 0.98, $P=0.014$), ST2 (0.96, 95% CI 0.93 to 0.99, $P=0.017$), IL-27 (0.93, 95% CI 0.88 to 0.99, $P=0.022$), and PSGL-1 (0.94, 95% CI 0.90 to 0.99, $P=0.022$) on ALS risk (Fig. 3). The causal estimates were broadly consistent, when using the additional methods for sensitivity analysis (Table S5). Horizontal pleiotropies were not observed in the MR-Egger intercept test.

Discussion

Since the incidence of ALS has been reported to be sex-related, there raised the hypothesis that sex hormones may be involved in the etiopathogenesis of this disorder²³. Our systematic MR analysis of endogenous hormones in 20,806 ALS cases and 59,804 controls provided genetic evidence for the protective role of free (bioavailable) testosterone (FT) in the risk of ALS. Although other hormones including IGF-1, SHBG, and total testosterone (TT) have been investigated in previous observational and laboratory studies, little evidence from our study suggested that they may be causally implicated in ALS risk. Moreover, our screening of molecular traits found that genetic predisposition to higher levels of IL27, IL-1ra, Dkk-1, PSGL-1, MMP7, PECAM-1, and ST2 were persistently associated with lower odds of ALS, whereas genetic liability to HSP-27, CA-125, cathepsin D, MMP1, and U-PAR were positively associated with ALS risk.

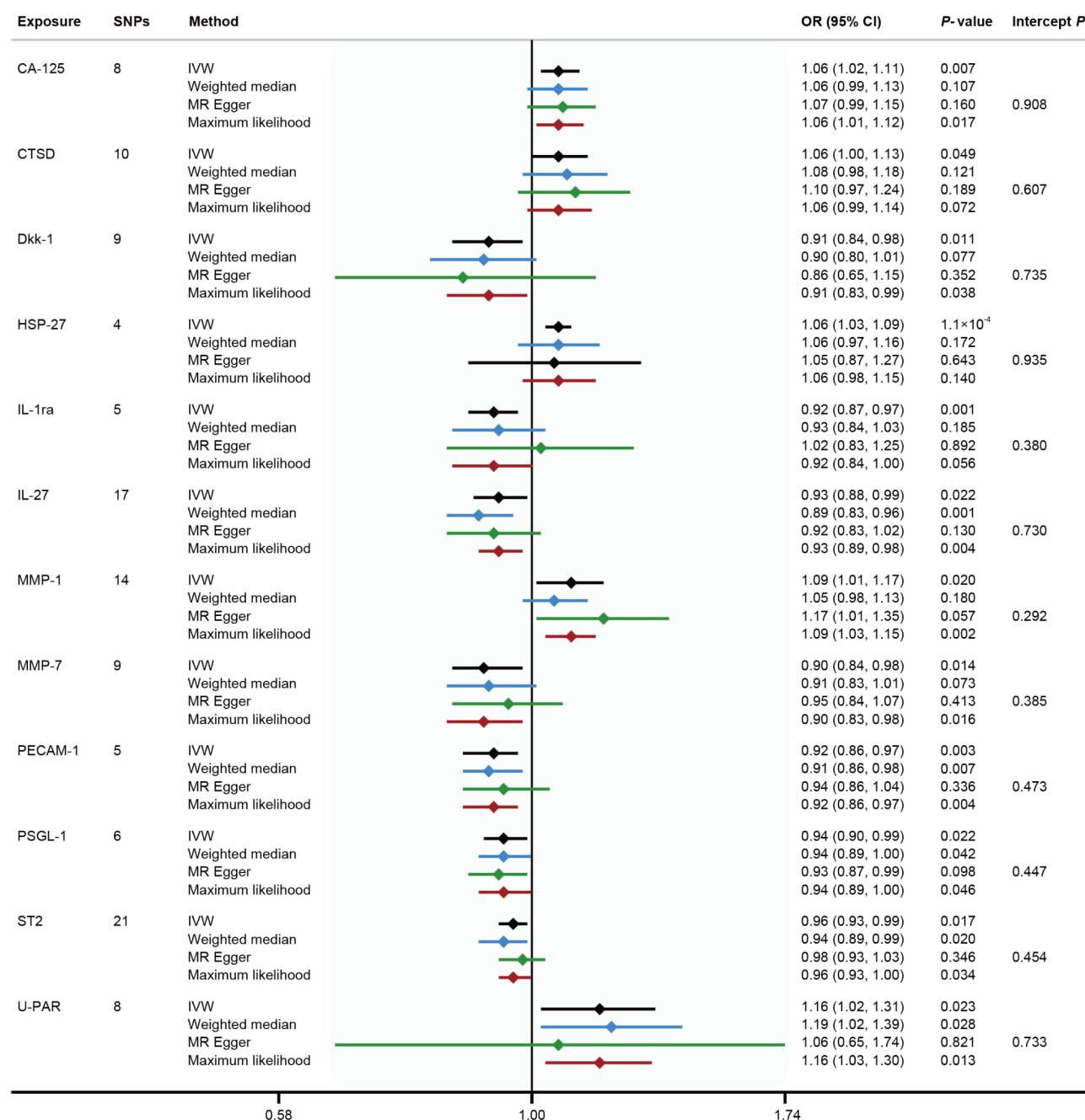


Fig. 3. Mendelian randomization association of genetically predicted circulating proteins with ALS. Odds ratios are scaled per increase in standard-deviations of proteins. Detailed information for the proteins are provided in Supplemental Material. ALS, amyotrophic lateral sclerosis; CI, confidence interval; IVW, the inverse-variance weighted method; MR, Mendelian randomization; OR, odds ratio; SNP, single nucleotide polymorphism.

Previously, the impact of androgens, including testosterone and its reduced metabolites, on the morphology and viability of spinal and brainstem motoneurons has been well-documented. Several studies have demonstrated that the administration of androgens, especially testosterone, promotes the restoration of motoneurons, leading to the regeneration of both axons and dendrites⁷. Nevertheless, despite the safety and low cost of androgen therapy, few clinical studies are available on human individuals. Although previous observational study observed that the serum level of FT was reduced in ALS patients, and significantly lowered CSF testosterone in ALS patients compared to healthy controls was found in a recent study^{3,24}, discrepancy was reported that female ALS patients showed higher testosterone levels and lower progesterone/FT ratio presented a more rapid worsening of the monthly forced vital capacity²⁵. Our primary and validated analyses in different ALS populations both revealed inverse association of genetically predicted plasma FT with ALS risk, suggested a potential protected effect of FT on ALS progression. Similarly, a previous study by using different dataset reported that bioavailable testosterone

was negatively associated with the risk of developing ALS²⁶, further supported the confidence of the findings. In contrast, we found no significant difference in the relationship between TT and ALS. It is accepted that the distribution of circulating testosterone is constituted by bound and unbound forms (the latter represents FT). 60–70% of circulating testosterone is tightly bound to SHBG, whereas the remaining 30–40% binds loosely to albumin; only 0.5–2% is free and in this form the hormone can cross the blood–brain barrier (BBB)²⁷. Given the BBB permeability of FT, we proposed that this form of hormone is potentially involved in the pathophysiology of ALS, which may partially explain the inconsistent effects of TT and SHBG. Notably, testosterone has also been reported to be negatively associated with other neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and cognitive functions^{26,28–30}, suggesting that the genetic effect and therapeutic potential of testosterone should be generalized to more neurodegenerative diseases not only for motor neuron dysfunctions. Although dysregulation of IGF-1 was suggested to play a significant role in the progression of ALS⁵, and elevated IGF-1 was reported to delay disease progression, extend survival time, and decrease disease progression in the ALS mouse model⁶, we found little evidence to support a protective role of IGF-1 in ALS risk.

Mechanistically, testosterone treatment of ALS mice demonstrated inhibition of astrogliosis in the spinal cord, leading to the reduction of proinflammatory factors². Moreover, testosterone increased the expression of TGFβ1, a factor well-known for its anti-inflammatory properties³¹. Besides, testosterone was reported to improve the survival of human neurons and astrocytes, acting directly on the mitochondrial membrane, and inhibiting the generation of reactive oxygen and nitrogen species, both of which are clear triggers for ALS³². On the other hand, since testosterone treatment demonstrated improvement on muscle atrophy and physical function of ALS mouse models², and clinical trials of testosterone therapy in low-testosterone-level populations exhibited benefit effects on muscle strength and recovery^{33–35}, it is reasonable to infer the possibility of increased muscle reserve as a potential driver of the protective effects for testosterone in ALS progression. This finding may redefine our understanding of the disease as it being a male-predominant disease and also affecting high-functioning athletes who presumably have higher levels of testosterone. Due to the etiological complexity and heterogeneity of the disease, causes of which including genetic, environment, and gene–environment interactions, disease genetics can help guide further insights that challenge our current understanding and provide starting point for targeted drug development and personalized medicine³⁶.

The screening of circulating proteome in our study identified several risk proteins for ALS. Similar to previously reported results, higher levels of IL-1ra were causally associated with lower odds of ALS, suggesting the robustness of our findings³⁷. Evidence is accumulating that IL-27 has neuroprotective activities as it promotes neuronal survival by regulating pro- and anti-inflammatory cytokines, neuroinflammatory pathways, oxidative stress, apoptosis, autophagy, and epigenetic modifications³⁸. We found a causal inverse association of IL-27 levels with ALS, suggesting a potential protective effect. Abnormal angiogenesis may occur in ALS due to increased microvascular density and accumulation of collagen fibers in the spinal cord perivascular space. Dkk-1 and U-PAR are pro-angiogenic factors, both of which have been demonstrated to increase in ALS patients from prior observational studies^{9,39}. However, our findings suggested inverse directions of these two proteins for ALS risk, the underlying mechanism needs further investigation. Previous studies on the association between HSP-27 and ALS have been contradictory, one study reported that HSP-27 from peripheral blood was elevated in patients¹⁰, whereas another one found serum levels of HSP-27 did not differ significantly between the patient and control groups⁴⁰. By conducting MR analysis, our result suggested that HSP-27 was causally and positively associated with ALS risk, supported by the experimental study that HSP-27 regulates p62 condensates and promotes lysophagy by forming platforms for autophagosome biogenesis at damaged lysosomes thus may contribute to neurodegeneration⁴¹. Cathepsins are intracellular proteases located within the lysosomes and are critical for normal protein turnover. The upregulation of cathepsin D was observed in the spinal cord of ALS mice, accompanied by alterations in the localization and associations of cathepsin D with lysosomes⁴². We further observed a negative association between cathepsin D and ALS, supporting its detrimental effect. MMPs are zinc-dependent enzymes that respond to inflammation, they are known to play a role in brain development and physiology⁴³. MMP1 was suggested as a disease progression marker for ALS before⁴⁴, which was also identified by our MR analysis. Apart from the above, the function of other potentially risk proteins identified by our study, including PSGL-1, MMP7, PECAM-1, ST2, and CA-125, need to be explored in future studies.

There are several limitations to our analysis. First, we could not completely rule out pleiotropy. However, several aspects of our results indicated the presence of pleiotropy was minimal, including little indication from the MR-Egger intercept analyses, no outlier detected in the MR-PRESSO analyses, and consistent results from sensitivity models. Second, although we have identified potential risk hormone and proteins for ALS, rigorous experimental verification is essential, and the underlying mechanisms demand further exploration. Third, this analysis was restricted to individuals of European ancestry to minimize bias from population stratification, which may constrain the generalizability of our findings to other ethnicities. Fourth, this study only looked at ALS risk, limiting the application of the results to other neurodegenerative diseases. Moreover, as a clinically, genetically and pathologically heterogeneous disease, the results may not be indicative of subgroups within the ALS risk group, which could explain heterogeneity within some of the data. Fifth, the dynamic changes in hormone levels during life stages may also exert an influence on ALS development. Future investigations could explore time-dependent effects with the availability of relevant GWAS data.

Conclusion

Our results based on genetic MR analyses implicated a protective role of circulating free testosterone in the development of ALS. Moreover, ALS progression was also affected by several plasma protein alterations. In further studies, high-throughput proteomic measurements are needed to clarify the underlying mediated and mechanistic pathways.

Data availability

Data generated or analysed during this study are included in this published article and its supplementary information files.

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Author contributions

LJ, KW, XYF, YTZ and FFZ were responsible for the writing of the original draft; LJ, KW, XYF, YTZ, FFZ, YX, QXD and STL for reviewing and editing the manuscript; LJ, KW, XYF, YTZ, FFZ, YX, QXD, STL, and YML participated in the revision of the manuscript; LJ, KW, XYF, YTZ, and FFZ for acquisition, analysis, or interpretation of data; LJ and KW for the conceptualization and statistical analysis of the study; and LJ for supervision. LJ and KW accessed the database and raw data.

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Competing interests

The authors declare no competing interests.

Additional information

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