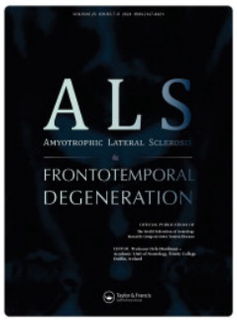

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RESEARCH ARTICLE

Serum heat shock protein concentrations are not associated with amyotrophic lateral sclerosis risk or survival in three European populations

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Abstract

Introduction: Serum heat shock protein (HSP) concentrations have been reported as potential biomarkers for amyotrophic lateral sclerosis (ALS). Here, we investigate the role of serum HSP70, HSP90, and DNAJC7 as biomarkers for ALS. *Methods*: Serum samples were collected from ALS patients and volunteer controls from three different clinical cohorts (in Germany, Ireland, and Italy). Serum HSP concentrations were determined using enzyme-linked immunosorbent assay. Descriptive statistics, generalized logistic regression, and Cox proportional hazards models were used to model associations between log serum HSP concentrations and ALS risk. *Results*: In total, 251 ALS patients and 184 healthy volunteers were included. Logistic regression models failed to find associations between ALS risk and log serum concentration of HSP70 (OR 0.43, 95% CI: 0.10–1.78, $p = 0.242$), HSP90 (OR 0.95, 95% CI: 0.39–2.37, $p = 0.904$), or DNAJC7 (OR 1.55, 95% CI: 0.90–2.68, $p = 0.118$). Survival of ALS patients was not associated with log serum concentration of HSP HSP70 (HR1.06, 95% CI: 0.36–3.14, $p = 0.916$), HSP90 (HR 1.17, 95% CI: 0.67–2.02, $p = 0.584$), or DNAJC7 (HR 0.83, 95% CI: 0.57–1.21, $p = 0.337$). *Discussion*: We did not replicate previous findings that serum HSP70 and HSP90 concentrations were associated with risk of ALS. DNAJC7 was not associated with ALS risk, and there were no obvious longitudinal patterns in log serum concentrations of HSP70, HSP90, or DNAJC7. In addition, serum HSP concentrations were not associated with ALS survival.

Keywords: *Biomarker, heat shock proteins, risk, survival*

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by loss of motor neurons and neuromuscular weakness, with a heterogeneous presentation and a generally poor overall prognosis. Approximately, 10% of cases are known to be of genetic etiology with a large number of genes implicated; however, the cause(s) of the remaining cases are currently unknown, with a complex interaction of genetic and environmental causes thought to be responsible (1).

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Heat shock proteins (HSPs) are a large family of proteins that are highly conserved across species (2,3). HSPs are considered part of the chaperone protein system and fill a wide variety of functions relating to protein folding, protein unfolding, remodeling of protein complexes, targeting of proteins for degradation, disaggregation of protein aggregates, and other related functions (2,3). Dysfunction of the HSP system or individual HSPs have been implicated in a number of diseases affecting numerous body systems, including a number of neurological diseases such as spinal muscular atrophy, Parkinson's disease, ALS, and others (2–4). With respect to ALS, recently a mutation of DNAJC7 has been identified as a rare cause of ALS (4). DNAJC7 is a member of the HSP40 family that is highly expressed in brain tissue (2). The HSP40 proteins are considered to be co-chaperones to the HSP70 family and are thought to mediate the target molecule specificity of HSP70 actions—a central mechanism of the body's protein quality control network (2). However, DNAJC7 is responsible for only a small number of ALS cases (4,5). Beyond DNAJC7, other evidence has implicated the HSP system in the etiology of ALS. The protein aggregate TAR DNA-binding protein 43 (TDP-43) is found in 97% of ALS cases (6–8), while aggregation of TDP-43, FUS, or SOD1 insoluble proteins has been identified as a common feature of ALS (9). It is known that the heat shock response is important in TDP-43 clearance in ALS (10).

Nevertheless, HSPs as biomarkers for ALS are largely underexplored in the literature. However, serum HSP70 and HSP90 were significantly elevated in 58 Japanese ALS patients compared to 85 healthy controls (11). The aim of the current study is to compare concentrations of HSP70, HSP90, and DNAJC7 in serum collected from ALS patients and healthy control volunteers from European countries, including Ireland, Italy, and Germany, at both single timepoints and for repeated timepoints where samples are available. In addition, we aim to characterize the correlations between HSPs measured both in cases and controls.

Methods

Study population

This study includes samples from three different source studies which will be described in brief. The EuroMOTOR study had been well described previously (12–18). EuroMOTOR was an international, prospective, case-control study designed to test a wide range of purported environmental risk factors for ALS (12). Patients and healthy controls were recruited in Ireland, Italy, and the Netherlands with matching by age, sex, and location, and asked to complete a detailed questionnaire regarding occupation, lifestyle, medical history, family history, and more, while blood and urine samples were collected for proteomic analysis (19). The Irish cohort included originally 148 cases and 273 controls, while the Turin cohort included 262 case and 290 controls (12). Samples remaining after use in previous studies from the Irish EuroMOTOR participants, and 80 cases and 80 controls from the Italian (Turin only) EuroMOTOR participants were included in this study. The MetALS study was established to recruit additional samples in Ireland. It aimed to recruit an additional 100 patients and 100 controls to provide longitudinal samples; however, recruitment was disrupted by the COVID 19 pandemic. As part of the MetALS study, participants were asked to provide repeat samples with an aim to gather at least three longitudinal timepoints per individual. MND-Net (https://mnd-net.de/de/) is the German ALS research network which is made up of ALS research groups from multiple locations throughout Germany. The network maintains its own biobank of samples obtained from ALS patients. Longitudinal samples from the ALS centers from the University Hospitals at Ulm and Munich were available for the current study.

Sample collection

Samples were stored at −80 °C. Blood (and urine where applicable) samples were collected at enrollment. Samples were stored on ice for same-day transport to the laboratory, whereupon samples were centrifuged. Serum and plasma samples were decanted prior to storage in 2 ml cryovials. Samples were stored at −80 °C.

Laboratory analysis

Serum HSPs were measured using commercially available enzyme-linked immunosorbent assay (ELISA) kits. HSP70 and HSP90 were measured using kits SKT-108-96 and SKT-107-96 from StressMarq (Victoria, Canada), while DNAJC7 was measured using kit MBS9340859-96 from MyBioSource following the manufacturers' instructions. Each ELISA kit was run with a proportionate mixture of samples from ALS patients and volunteer controls to minimize any potential kit-to-fit bias on results. All samples were measured in duplicate and read using a microplate spectrophotometer (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA). Measurements from individual kits were considered valid if intra-assay coefficients of variation were <20%. Due to frequent unreliability of the HSP90 kits, HSP90 samples were only measured in the Irish samples. As a number of samples were measured in more than one kit, a repeated measures mixed model was used to calculate mean HSP concentrations.

Table 1. Baseline demographics and clinical data of study participants.

	Ireland (EuroMOTOR)		Ireland (MetALS)		Italy (EuroMOTOR)		Germany (MND-Net)		Total	
	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls	Cases	Controls
N	47	95	45	12	81	77	78	0	251	184
Number with longitudinal serum samples	0	0	12	4	0	0	72	0	84	4
Mean age at survey in years (SD)	65.7 (12.1)	64.9 (11.3)	64.9 (10.0)	32.8 (12.0)	66.4 (9.8)	64.4 (11.6)	61.9 (10.8)	–	64.6 (10.7)	62.6 (13.9)
Female, *N* (%)	18 (38.3)	33 (34.7)	21 (46.7)	9 (75.0)	35 (46.1)	37 (48.1)	33 (42.3)	–	107 (43.5)	79 (42.9)
Height in meters, mean (SD)	170.1 (9.1)	170.5 (9.1)	169.6 (9.0)	169.0 (9.7)	165.8 (9.1)	167.0 (8.9)	170.7 (8.1)	–	168.9 (9.0)	168.9 (9.2)
Weight in kg, mean (SD)	73.9 (13.7)	79.2 (15.5)	73.1 (14.9)	67.2 (14.7)	66.6 (12.6)	71.4 (13.1)	73.2 (13.4)	–	71.3 (13.8)	75.2 (15.0)
BMI	25.7 (4.2)	27.1 (4.4)	25.5 (5.0)	23.6 (5.2)	24.2 (4.1)	25.6 (4.3)	25.1 (4.1)		25.0 (4.3)	26.2 (4.49)
Mean age at ALS onset in years (SD)	64.1 (11.9)	–	63.9 (9.4)	–	64.6 (9.8)	–	59.1 (12.0)	–	62.6 (11.2)	–
Mean age at ALS diagnosis in years (SD)	65.2 (12.1)	–	65.1 (9.4)	–	66.04 (9.8)	–	61.1 (11.0)	–	64.1 (10.8)	–
Median diagnostic delay in months[a] [IQR]	11.3 [8.0, 13.4]	–	11.1 [5.8, 15.3]	–	9.9 [5.0, 16.4]	–	10.4 [6.0, 18.3]	–	10.9 [6.0, 16.4]	–
Bulbar onset ALS, *n* (%)	9 (19.1)	–	14 (32.6)	–	29 (38.2)	–	20 (26.7)	–	72 (29.9)	–
El Escorial category										
Probable	13 (28.3)	–	12 (34.3)	–	23 (30.3)	–	16 (20.5)	–	64 (27.2)	–
Probable lab supported	0 (0.0)	–	4 (11.4)	–	18 (23.7)	–	6 (7.7)	–	28 (11.9)	–
Definite	23 (50.0)	–	13 (37.1)	–	17 (22.4)	–	32 (41.0)	–	85 (36.2)	–
Possible	10 (21.7)	–	5 (14.3)	–	18 (23.7)	–	11 (14.1)	–	44 (18.7)	–
Suspected	0 (0.0)	–	1 (2.9)	–	0 (0.0)	–	13 (16.7)	–	14 (6.0)	–
Baseline total ALSFRS-r median [IQR]	41.0 [34.5, 43.0]	–	42.0 [36.5, 45.0]	–	36.0 [29.0, 42.0]	–	40.0 [36.0, 44.0]	–	40.0 [35.0, 44.0]	–

[a]Diagnostic delay: time from disease onset to diagnosis; BMI: body mass index; IQR: inter-quartile range.

Statistical analysis

Demographic, clinical, and laboratory variables were summarized descriptively. Geometric and arithmetic means and percentiles (5%, 25%, 50%, 75%, and 95%) of HSPs were tabulated by cohort. Next, for comparison of baseline HSP's levels between cases and controls, data from Ireland and Italy were included and compared graphically and by *t*-test of log transformed HSP concentrations. Crude correlations of HSPs were visualized via correlogram for ALS patients and controls separately. The relationships between HSPs were visualized using scatterplots of log transformed HSP concentrations and by comparing ratios of HSPs stratified by case/control status. In addition, longitudinal trends of HSPs were examined by plotting log concentration of HSPs vs. disease duration from onset for ALS patients. The relationship between total ALSFRS-r and HSPs was also examined graphically. Generalized logistic regression models were fit for case-control status as outcome as a function the log concentration of each HSP in turn adjusted for age, and sex as fixed effects, with cohort as a random effect. Finally, Cox proportional hazard models were fit to identify any potential associations of log HSP concentration with ALS survival from the date of blood sampling to the date of death or tracheotomy, or to the last known date of being alive for censored cases. Models included log HSP concentrations at baseline and were adjusted for the known ALS prognostic factors, age at ALS onset, site of ALS onset and diagnostic delay time. For any model with statistically significant results ($p < 0.05$) for HSP concentration parameters, a sensitivity analysis was performed excluding any serum HSP concentration more the 4 SD's from the mean (after log transformation). All statistical analyses were carried out using R Statistical Software (20) with additional R packages (R Foundation for Statistical Computing, Vienna, Austria) (21–29).

Results

Table 1 summarizes the baseline characteristics of participants stratified by cohort. There were a total of 251 ALS patients, and 184 healthy volunteers included. Of those, 84 ALS patients and four controls had longitudinal serum samples available. Due to the Covid19 pandemic, recruitment for the MetALS cohort was interrupted by the reduction in face to face visits from April 2020 onwards. Recruitment of volunteer controls was restricted for a longer period, and controls were therefore recruited among the healthcare staff. For this reason, the mean age for the low number ($n = 12$) of MetALS controls (32.8 years) was lower than that of MetALS cases (64.9 years), and more controls were female (75%) compared to patients (46.7%). Nevertheless, age and percentage female were similar for total cases vs. total controls (Table 1). Mean age of patients at onset was 64.1 years and 64.6 years in the Irish and Italian EuroMOTOR cohorts, respectively, while the MetALS (mean age 63.9 years) and MND-Net cohorts (mean age 59.1 years) were somewhat younger. Overall 29.9% of cases had bulbar onset ALS.

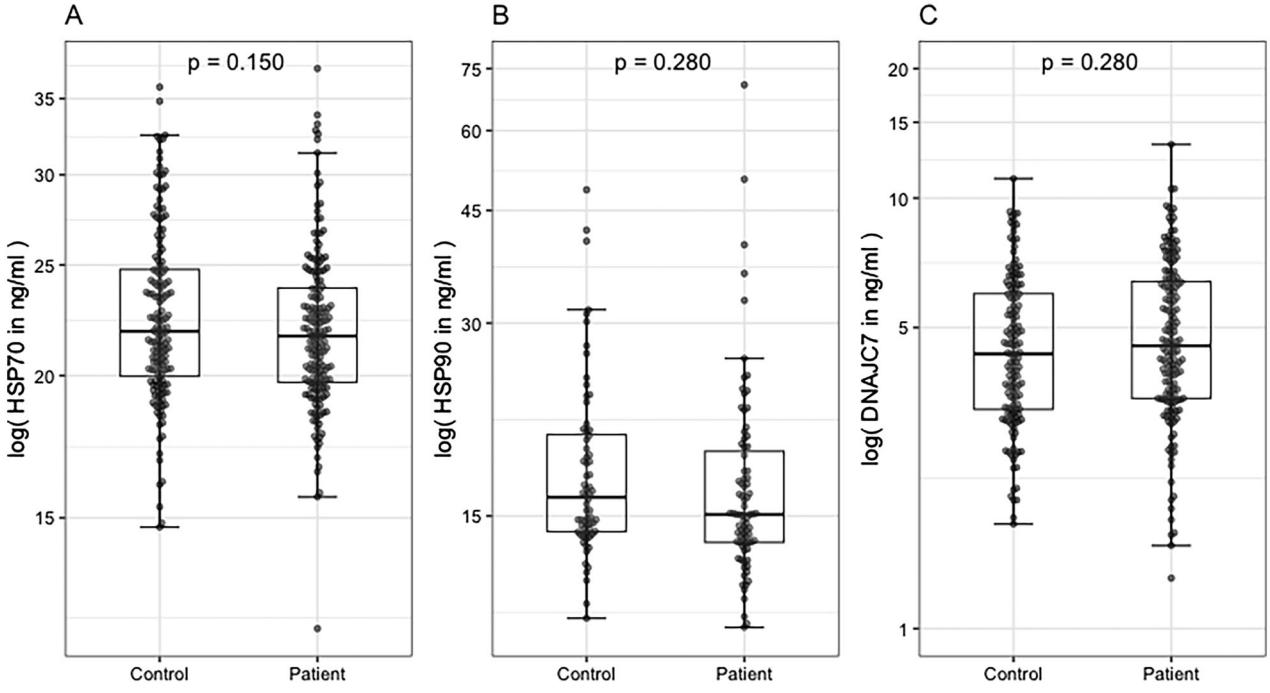

Figure 1. Log of serum concentrations of heat shock proteins in ALS patients vs. healthy volunteers. Baseline data from Irish and Italian cohorts were included. German patients were not included in these comparisons due to the lack of comparative control data. *p* Values were calculated using *t*-tests of log transformed HSP concentrations.

Figure 1 shows serum log concentrations of HSP70, HSP90, and DNAJC7 for baseline cases vs. controls (excluding German cases). Boxplots and p values demonstrated no differences in serum HSP concentrations between patients and controls without adjustment for any other variables (including cohort). Descriptive statistics for all serum biochemicals are shown in Supplementary Table 1, while serum log concentrations of HSP70, HSP90, and DNAJC7 for baseline cases vs. controls stratified by cohort are shown in Supplementary Figure 1. Supplementary Figure 2 shows crude correlations between serum HSPs, height, weight, and age for ALS cases and controls including all timepoints. Figure 2 displays the relationships between log concentrations of HSPs. Log DNAJC7 concentration was linearly correlated with log HSP70 concentration in cases and controls. Figure 2 also shows that log HSP90 concentration and log HSP70 concentration were correlated in patients but not controls; however, the ratio of log HSP90 vs. log HSP70 did not differ between patients and controls (Supplementary Figure 2). Figure 3 displays serum HSP concentrations vs. time from disease onset in ALS patients. No clear increasing or decreasing pattern of any HSP was observed with increasing time from onset. Similarly, Supplementary Figure 3 shows there is no clear relationship between log of serum HSP concentrations and the total ALSFRS-r. Results of logistic regression models are shown in Table 2. After adjustment for age, sex, and cohort, none of the individual HSPs were significantly associated with the risk of ALS. A further model was built including both log

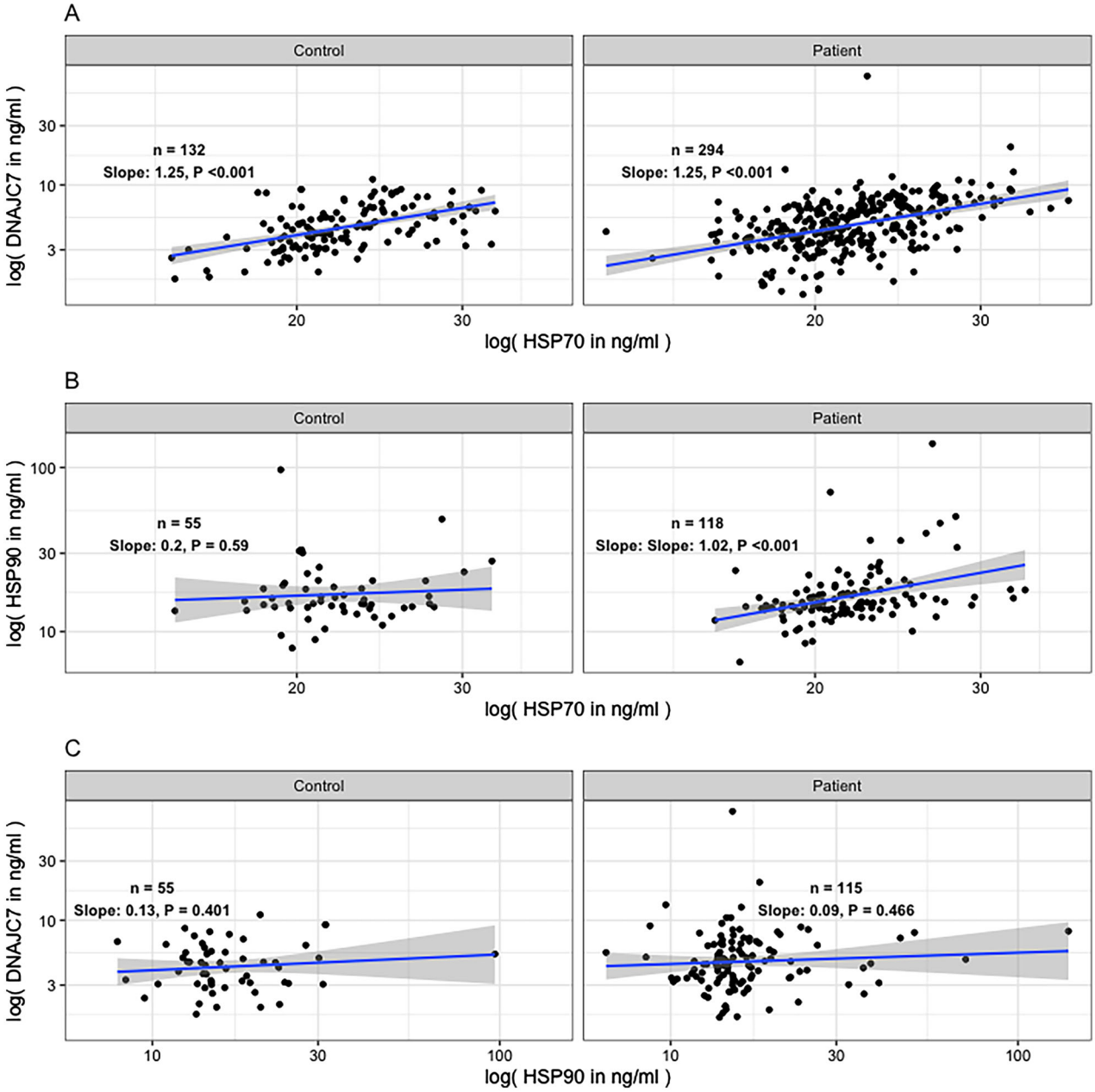

Figure 2. Scatterplots showing log concentrations of HSP70, HSP90, and DNAJC7.

HSP70 and DNAJC7 concentrations (HSP90 was not included as it was not measured in the Italian patients). In this model, odd ratios for both log HSP70 and log DNAJC7 deviated further from 1.0 relative to models of either molecule alone, and were significant at $p < 0.05$. However, the sensitivity analysis revealed that removing one individual with an extremely high serum DNAJC7 concentration (75.5 ng/ml) yielded results that were no longer significant (Table 2). Cox proportional hazards models similarly did not find any significant associations with log serum HSP concentrations (Table 2).

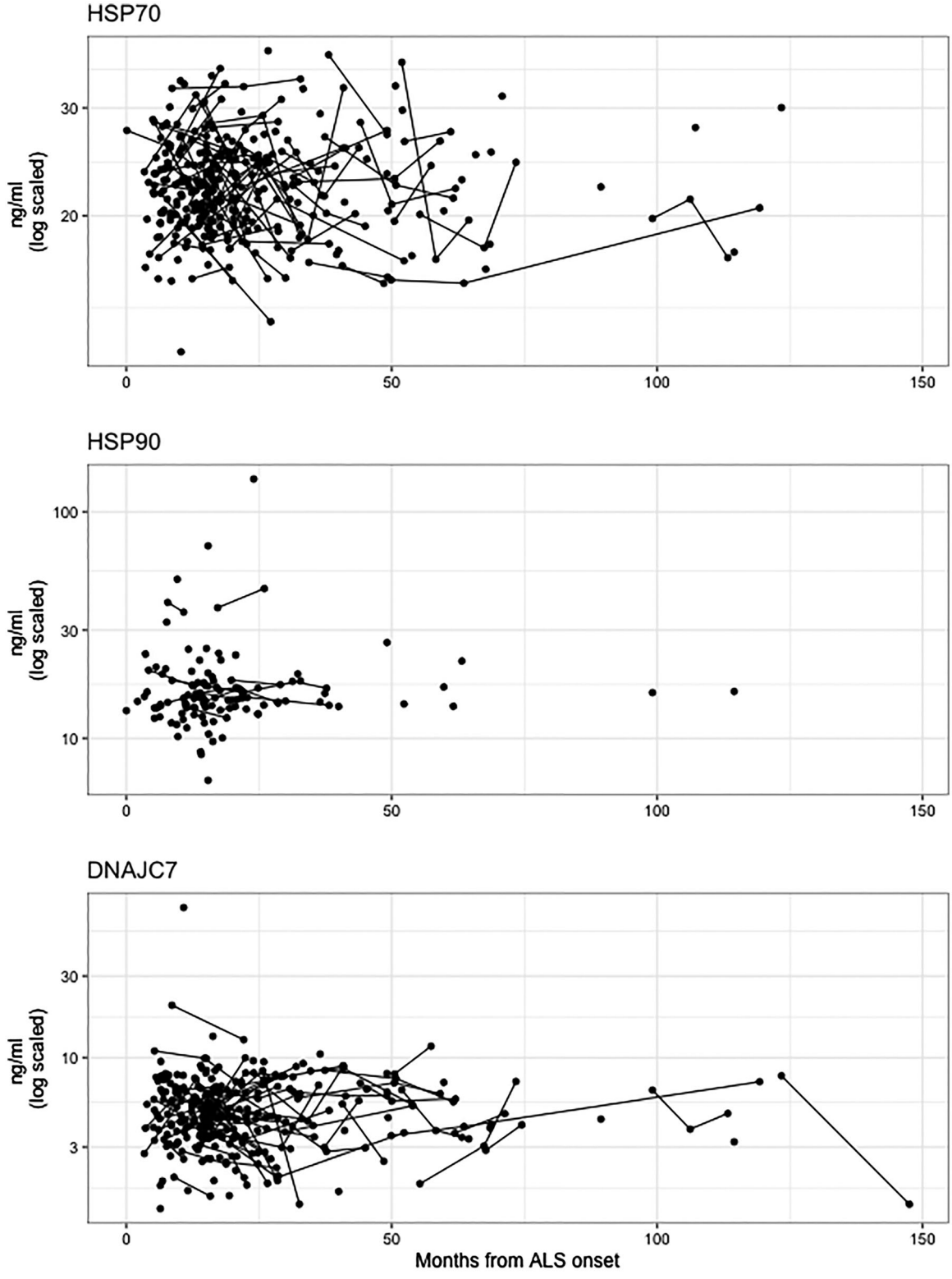

Figure 3. Longitudinal trends of HSPs vs. disease duration from onset for ALS patients.

Table 2. Results of regression models.

Generalized logistic regression modelsa

Model	*n* patient	*n* control	Variable	Odds ratio	95% CI's	*p* Value
1	167	154	log (HSP70 ng/ml)	0.43	0.10–1.78	0.242
2	88	72	log (HSP90 ng/ml)	0.95	0.39–2.37	0.904
3	152	139	log (DNAJC7 ng/ml)	1.55	0.90–2.68	0.118
4	152	121	log (HSP70 ng/ml)	0.16	0.03–0.95	**0.044**
			log (DNAJC7 ng/ml)	1.89	1.01–3.53	**0.047**
4 (sensitivity analysis)[c]	151	121	log (HSP70 ng/ml)	0.17	0.03–1.03	0.054
	151	121	log (DNAJC7 ng/ml)	1.75	0.90–3.43	0.101

Cox proportional hazards models[b] (patients only)

Model[a]	*n* patient	*n* events	Variable	Hazard ratio	95% CI's	*p* Value
1	157	141	log (HSP70 ng/ml)	1.06	0.36–3.14	0.916
2	85	70	log (HSP90 ng/ml)	1.17	0.67–2.02	0.584
3	144	129	log (DNAJC7 ng/ml)	0.83	0.57–1.21	0.337
4	144	129	log (HSP70 ng/ml)	2.13	0.56–8.13	0.270
	144	129	log (DNAJC7 ng/ml)	0.77	0.51–1.15	0.202

[a]Generalized logistic regression model adjusted for age at baseline and sex as fixed effects, and cohort (Ireland—EuroMOTOR, Ireland—MetALS or Italy) as a random effect.
[b]Cox proportional hazards models adjusted for age at onset, site of onset, diagnostic delay, and cohort.
[c]For the model 4 sensitivity analysis, one patient with an outlier serum DNAJC7 concentration of 75.5 ng/ml was excluded from the model.
The bold numbers indicate a *p* value 0.05.

Discussion

In this study which included participants from three European countries, we did not find a relationship between serum HSPs and ALS risk. This stands in contrast to previous findings from Miyazaki *et al.*, who reported that serum HSP70 and HSP90 had significantly raised concentrations in ALS cases vs. controls (11). Indeed, in our results, the concentrations of HSP70 and HSP90 are lower in patients compared to controls, although not significantly. Additionally, visualization of longitudinal HSP concentrations in ALS cases failed to demonstrate any increasing or decreasing pattern with time from disease onset. However, despite above findings, a linear relationship between log serum HSP70 concentration and log serum DNAJC7 concentration was noted and mutual adjustment for log HSP70 concentration and log DNAJC7 concentration via multivariate regression did lead to larger parameter estimates.

Although our results contradict those of Miyazaki *et al.*, it is possible that differences in study design could be a factor, e.g. differences in freezer delay from date of sample to measurement, or differences in number of freeze–thaw cycles. Alternatively, differences in other factors between the Japanese and European cohorts which can trigger the heat shock response could be present. However, we note that in the recent phase 3 clinical trial in SOD1 ALS for the heat shock response inducer, arimoclomol, no beneficial effect was found on the main outcomes (30), which is congruent with our results.

The linear correlation between log HSP70 and log DNAJC7 concentrations seems unlikely to be related to the ALS disease process since it is present in both patients and controls. DNAJC7 is thought to provide control mechanism for glucocorticoid and progesterone receptors via regulation of the flow of substrates from HSP70 to HSP90 (3). DNAJC7 is known to physically interact with a number of HSP70s, stimulating the ATPase function of HSP70 and thereby promoting polypeptide binging and protein folding (3). Therefore, the linear correlation we have observed between these proteins, may simply reflect normal expression and regulation of these molecules in healthy individuals. DNAJC7 is thought to disrupt the interactions of HSP90 and its substrates in normal function which may affect efficiency of protein folding (3). A linear relationship was not found between log DNAJC7 and log HSP90 concentrations, although this may be because of the lower number of individuals who had HSP90 concentration measurements.

Our study benefits compared to the previous study by having a large sample size, and by drawing patient and controls from multiple populations and time periods. However, the older samples have had long storage times (up to 10 years) and have undergone multiple freeze–thaw cycles prior to measurement which may have affected the results. It is also possible that unmeasured confounding factors may have affected HSP concentrations. In addition, we cannot out-rule bias affecting our results, since missing values are relatively high.

In summary, log concentrations of HSP70, HSP90, and DNAJC7 were not associated with risk of ALS when compared between 251 ALS patient and 184 control participants. Similarly, there was no clear trend in longitudinal concentrations of these three molecules in ALS patients. A linear relationship between log DNAJC7 concentration and log HSP70 concentration was found, although this had a similar slope in ALS patient and control participants.

Ethics statement

The EuroMOTOR project received ethical approval from Beaumont Hospital Ethics Committee (05/49). Subsequent approval for measurement of metals in samples collected as part of the EuroMOTOR project was obtained from Beaumont Hospital Ethics Committee (19/82) and from Ethics Commission at Ludwig-Maximilians-Universität München (19-855). The study was approved by the "A.O.U. Città della Salute e della Scienza of Torino" ethical committee. Databases were treated according to Italian privacy regulations. The study was approved by the local ethics committees (application number 19/12).

Consent form

Written consent, or in cases where the disease process has affected the patient's ability to write, oral consent, was obtained from all participants, and documentation of consent is kept on file at the Academic Unit of Neurology, Trinity College Dublin. All Italian patients and controls signed written informed consent. Biosamples were collected via participation in the MND-Net cohort study, for which a separate declaration of consent was obtained.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

Code availability statement

Analysis code is archived at: https://doi.org/10.5281/zenodo.11068452.

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Data availability statement

The data that support the findings of this study are available on request from Orla Hardiman.

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