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ORIGINAL ARTICLE

Critical issues in ALS case-control studies: the case of the Euro-MOTOR study

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Abstract

Backround: Political and sociocultural differences between countries can affect the outcome of clinical and epidemiological studies in ALS. Cross-national studies represent the ideal process by which risk factors can be assessed using the same methodology in different geographical areas. *Methods*: A survey of three European countries (The Netherlands, Ireland and Italy) has been conducted in which incident ALS patients and matched controls were recruited in a population-based study based on age, gender and area of residency, under the Euro-MOTOR systems biology programme of research. *Findings*: We have identified strengths and limitations during the trajectory of the Euro-MOTOR study, from the research design to data analysis. We have analysed the implications of factors including cross-national differences in healthcare systems, sample size, types of matching, the definition of exposures and statistical analysis. *Conclusions*: Addressing critical methodological aspects of the design of the Euro-MOTOR project minimises bias and will facilitate scientific assessment of the independent role of well-defined exposures.

Keywords: *Epidemiology*, *case-control study*, *risk*, *criticisms*, *genetics*

Introduction

Amyotrophic lateral sclerosis (ALS) is a rare neurodegenerative disease of unknown aetiology for which no definite risk factors have been identified with certainty, with the notable exception of genetic factors, which account for up to 10–20% of cases (1,2). Identification of environmental risk factors of ALS can be achieved using various observational designs, such as cohort studies and case-control studies. Although cohort studies may be preferred, they are limited in power due to the low incidence of ALS and the long and unknown interval between exposure and disease onset due to misclassification of the disease. For this reason, case-control studies remain the most informative study design in ALS research.

The failure to identify potentially modifiable risk factors can be explained by several factors: first, the heterogeneity of the disease; secondly, the relative rarity of ALS, making it difficult to collect a large enough number of patients to reach adequate study power; and thirdly, the methodological limitations of many published reports, including poor representativeness of the source population, small sample size, incorrect and/or varying definitions of exposures and inadequate control of confounders (3).

Studies of non-representative samples of patients are likely to confound results and to prevent comparisons across different populations.

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Small samples are unlikely to provide precise estimates of the risks and increase heterogeneity in published results. The use of subjective (often unmeasurable) definitions of exposures prevents correct estimates of the risks and comparisons across studies.

Accordingly, there is a clear need for large studies using a standardised protocol. An international multicentre study offers a unique opportunity to examine several risk factors at the same time and with the same methodology in different geographical areas, while aiming to obtain a truly representative sample of ALS patients in a population based design. This approach also explores the possibility that the same risk factor may not have the same effect in different populations. Another possibility is that different populations may exhibit subtle differences in phenotypical characteristics (4). All of these issues have been addressed in a case-control study by the Euro-MOTOR consortium, which has collected clinical and biological variables in a large sample of patients with ALS and representative controls across Europe, using a population based design to investigate the aetiology and mechanisms of the disease. Our previous experience with the investigation of the role of traumatic events (5) and physical exercise (6) was helpful to identify the weaknesses of a case-control study and served to improve the study design with particular reference to sample size and criteria used for data collection. This provided sufficient power to investigate research hypotheses regarding previous disputed environmental risk factors for ALS.

The purpose of this report is to describe the study design and methods, highlighting the strengths and limitations of the basic structure of the project.

Aims of Euro-MOTOR project

The main objective of Euro-MOTOR is to discover new causative and disease-modifying pathways to pave the way for novel therapies. The epidemiological component of Euro-MOTOR is a pooled case-control study, which consists of a paper and pencil survey through face-to-face interviews undertaken in three European countries (The Netherlands, Ireland and Italy), where incident ALS patients and matched controls were recruited in a population based design based on age, gender and area of residency. Core demographic and ALS-related clinical data were collected by trained investigators. The information was obtained from the patient/control or, for cognitively impaired individuals, from an informed caregiver. The data were entered into a centralized online database, along with biological samples collected to investigate genetic and biochemical profiles of cases and controls.

The recruitment of ALS cases and controls spanned from February 2011 to January 2014.

Representativeness of the study population

In the participating population based registers, several sources are periodically interrogated to ensure full case ascertainment. These include neurologists, neurophysiologists, and pulmonologists with interest in ALS, riluzole pharmacy records, lay association archives, general practitioners' records, and administrative sources (hospital discharge records, disability lists, etc.). The validity of these sources has been documented in published reports (7–11). In addition, when comparing the study sample with the original ALS population from each country, no relevant differences in the main demographic and clinical indicators were detected, with the exception of patients with bulbar site disease from the Irish cohort (23.7% vs. 35.3% in the registry) (Table 1).

The underlying populations have intrinsic differences in terms of education and clinical characteristics of the disease at enrolment. Since the classification of education in Italy did not take into account the attendance at primary and secondary school separately, and a national reform occurred during the 1960s, and to make education comparable across countries, the six categories were aggregated into two: low level (none, grade school, high school and technical or trade school) and high

Table 1. Annual incidences per 100,000 person-years and comparison of gender, age and bulbar site of onset between local registries and the case-control study by cohort.

	Netherlands	Ireland	Apulia	Lombardy	Piedmont and Valle d'Aosta
Registry ALS population					
Annual incidence per 100,000 person-years	2.8	2.0	1.6	2.1	2.9
Male:female ratio	1.5	1.4	1.6	1.3	1.2
Age at onset	63.0 (median)	66.0 (median)	64.6 (median)	63.6 (mean)	64.8 (mean)
Bulbar site onset (%)	30	35.3	21	35.2	37.5
Case-control study ALS population					
Male:female ratio	1.5	1.5	1.3	1.2	1.2
Age at onset	64.1 (median)	64.4 (median)	62.6 (median)	63.6 (mean)	64.2 (mean)
Bulbar site onset (%)	35.4	23.7	26.5	29.6	37.0

Data acquired from published studies (7–11).

Table 2. Frequency distributions of level of educational attainment by country in the case-control study and in the official EUROSTAT statistics on education, 2014 (%).

	EUROMOTOR CASE-CONTROL STUDY									EUROSTAT STATISTICS 2014		
	The Netherlands			Ireland			Italy			NL	IRE	ITA
	Cases	Controls	Total	Cases	Controls	Total	Cases	Controls	Total	Total	Total	Total
Low level	583 (74.0)	1,348 (71.7)	1,931 (72.4)	148 (83.6)	273 (78.2)	421 (80.0)	526 (92.4)	552 (80.5)	1,078 (85.9)	1,257 (81.9)	2,173 (74.5)	3,430 (77.1)	8,943 (71.5)	2,146 (66.1)	39,271 (86.1)
High level	205 (26.0)	532 (28.3)	737 (27.6)	29 (16.4)	76 (21.8)	105 (20.0)	43 (7.6)	134 (19.5)	177 (14.1)	277 (18.1)	742 (25.5)	1,019 (22.9)	3,563 (28.5)	1,102 (33.9)	6,355 (13.9)
Total	788 (100.0)	1,880 (100.0)	2,668 (100.0)	177 (100.0)	349 (100.0)	526 (100.0)	569 (100.0)	686 (100.0)	1,255 (100.0)	1,534 (100.0)	2,915 (100.0)	4,449 (100.0)	12,506 (100.0)	3,248 (100.0)	45,626 (100.0)

Education was classified into two levels, following the International Standard classification of Education (ISCED): low level (ISCED 0-4) and high level (ISCED 5-8); Statistics on education of population 15–75 years old resident in the three countries were obtained from the following source: http://ec.europa.eu/eurostat/web/education-and-training/data/main-tables

level (university and graduate school). Comparing education across all participants (Table 2), the majority of subjects reported low educational levels (International Standard Classification of Education (ISCED) 0-4) (77.1%). The educational levels were highest in the Netherlands and lowest in Italy.

Descriptive statistics stratified by country revealed that significant differences between cases and controls in the two educational levels were detected only in Italy ($p<0.001$). The better educational level of controls is generally consistent across countries and is to be expected, as educated healthy individuals are more likely to participate in epidemiological studies.

Disease phenotype

The site of ALS onset of patients was mainly spinal (64%), followed by bulbar (33%) and thoracic-respiratory (3%). Bulbar-onset ALS is usually considered to account for up to one-third of cases. However, the proportion varies across populations (3). In the present study, significant differences across countries were detected ($p<0.01$), especially regarding Ireland where bulbar-onset patients (24%) were fewer than in Italy (32%) and the Netherlands (35%) (Table 3). These differences are partly explained by the interval between onset of symptoms and diagnostic assessment (medians were 9.2 (IQR =9) months in the Netherlands, 9.8 (IQR =9) in Italy and 11.4 (IQR =9.5) in Ireland). In this regard, patients with bulbar onset – who have a worse prognosis than patients with spinal onset – are more likely to have declined participation in the Irish cohort due to the severity of their illness.

With reference to the El Escorial classification, almost half of cases were classified as probable ALS at diagnosis (45%), of whom 18% were laboratory supported, but frequency distributions of El Escorial categories were significantly different across countries ($p<0.001$). In Ireland, the term laboratory supported probable ALS was infrequently applied whereas 52% of cases fulfilled the clinical criteria for definite ALS at the time of categorization. This compared to 19% in the Netherlands and 26% in Italy, respectively. These differences cannot be explained by discordance in the diagnosis of ALS among local investigators because inter-rater agreement was satisfactory ($K=0.83$; $p<0.0001$), as measured by the Kendall (K) Coefficient of Concordance, a non-parametric statistic assessing agreement among raters and ranging from 0 (no agreement) to 1 (complete agreement). The agreement was tested among nine raters (from the Euro-MOTOR consortium) on 52 patients with ALS or other clinical conditions. ALS patients were classified according to the revised El Escorial categories (12). These differences most likely reflect differences in methods of case ascertainment, subtle differences in the

Table 3. Frequency distributions of site of onset and El Escorial categories by country.

	NL		IRE		ITA		Total	
	n	%	*n*	%	*n*	%	*n*	%
Site of onset								
Bulbar	275	35.3	42	23.7	188	32.2	505	32.8
Spinal	476	61.2	132	74.6	385	65.9	993	64.5
Thoracic/respiratory	27	3.5	3	1.7	11	1.9	41	2.7
Total	778	100.0	177	100.0	584	100.0	1,539	100.0
Missing	13		0		5		18	
El Escorial								
Definite ALS	144	18.7	88	52.4	155	26.5	387	25.4
Possible ALS	116	15.1	32	19.0	148	25.3	296	19.4
Probable ALS	316	41.2	48	28.6	202	34.5	566	37.3
Probable ALS (lab. supported)	192	25.0	0	0.0	80	13.7	272	17.9
Total	768	100.0	168	100.0	585	100.0	1,521	100.0
Missing	23		9		4		36	
Total	791		177		589		1,557	

timing and method of assignment of diagnostic category (e.g. assignment at a specialist clinic, or by chart review or at the time of clinical evaluation): differences in practice with respect to when patients receive a definitive diagnosis of ALS (in Ireland this frequently takes place at the time of evaluation at the specialist clinic, rather than at the time of first encounter with a neurologist).

Matching

In a case-control study, matching is done on one or more variables. Matching may be performed on an individual basis (individual matching) or for groups of subjects (frequency matching). Individual matching implies the identification of one or more controls matched to each case for the values of the matching factor. Frequency matching involves the selection of an entire stratum of reference subjects with value(s) of the matching factor(s) equal to the value(s) of the stratum of the index case. Matching can be justified by the need to adjust for variables known to be potential confounders and provide more efficient stratified analyses. However, matching on factors that do not affect the risk of disease but with effects on exposure may bias the results of the study. In the Euro-MOTOR study, both individual and frequency matching were used in different countries. Matching variables included age, gender and area of residency. Italy and Ireland adopted individual matching while the Netherlands adopted a frequency matching approach. Irish cases were matched a priori by age (±5 years), gender and location (general practitioner practice when possible, otherwise local area), with a 1:2 case-control ratio. The same criterion was followed by the three Italian centres, but location was here represented by local area (i.e. distance in kilometres and urban/rural setting), and the case-control ratio was slightly greater than 1:1. Cases in the Netherlands were frequency matched using general practitioners' registries, in order to select a representative population list by age (±5 years), gender and location (based on general practitioners' area), to achieve a 1:2 case-control ratio.

Environmental exposures

Environmental epidemiology attempts to characterise the health effects of known environmental exposures. The quality of exposure measurement will determine the validity of the results. A crude binary variable is necessary, but is not sufficient to determine a dose-response relationship and therefore ordinal categories are needed to increase the sensitivity of the study. It is important to distinguish between exposure setting, a complex mixture, and a single agent. Exposure to environmental agents can be assessed using different instruments, including interviews, structured diaries, measurements in external media, measurements of concentrations in human tissues, and markers of physiological effects. Several methods for assessment of exposure are available. These include, among others, Job Exposure Matrices (JEMs), a system linking occupations to profiles of environmental exposures by providing (semi-)quantitative assessments of exogenous exposures for each occupation. JEMs are dependent on quality of job history and assignment at job level and may result in exposure misclassification for an individual subject. In addition, occupational studies using job classifications may have large within-category variability. Moreover, there may also be some intra-individual variability. Other exposure measures may have even greater limitations: concentrations in tissues may reflect primarily or exclusively recent exposure; use of concentrations in external media may result in misclassification for individuals; markers of physiological effects may be non-specific.

Research on environmental exposures for ALS has produced a large number of studies, although findings are conflicting. A significant excess of deaths for ALS has been reported in soccer players

Table 4. Distributions of cases and controls by country and cohort.

		Cases		Controls		Total		
	Source population (million)	*n*	%	*n*	%	*n*	%	Ratio
Netherlands	16.0	791	48,5%	1,880	64,3%	2671	58,7%	1:2.4
Ireland	4.5	177	10,9%	349	11,9%	526	11,6%	1:2
Italy	13.6	589	36,1%	693	23,7%	1282	28,2%	1:1.2
Apulia	4.1	141	8,6%	213	7,3%	354	7,8%	1:1.5
Lombardy	5.0	186	11,4%	190	6,5%	376	8,3%	1:1
Piedmont and Valle d'Aosta	4.5	262	16,1%	290	9,9%	552	12,1%	1:1.1
Total	34.1	1,557	100.0	2,922	100.0	4,479	100.0	1:1.9

(13,14). A history of repeated (head) injuries has been reported more frequently by ALS patients than by individuals with other clinical conditions (5). An association between exercise and ALS has also been suggested (6,15) and dietary supplements, including branched-chain amino acids, have been also implicated (16). Recent reviews have also concluded that smoking should be considered a risk factor for sporadic ALS (17,18). Industrial and agricultural chemicals, such as pesticides, solvents and heavy metals, have also been reported as risk factors of ALS, but inconsistency of findings and the poor methodological quality of most studies in this field, in particular regarding exposure assessment, has limited the potential to establish a causal relationship with ALS (19).

Based on existing preliminary data, numbers of environmental exposures have been considered in the Euro-MOTOR study (Supplementary data). We attempted to minimise recall bias by using a very detailed questionnaire that included focused questions on the history of selected exposures (i.e. occupations, sports, hobbies, traumatic events, places of residence, familial diseases, drugs intake, smoking and alcohol habits).

Definition and classification of exposures

The English version of the study protocol is reported in the Web Appendix. Each exposure was defined using conventional definitions or, where not available, using pragmatic definitions that were accepted by consensus among the principal investigators. Where possible, a given exposure was graded to capture a gradient. Definitions and classifications are available as Supplementary data.

Two neuro-psychological tests were also used to identify cognitive impairment: the Frontal Assessment Battery (FAB) and the Verbal Fluency Index (VFI), administered to both cases and controls.

Genetic classification

A hexanucleotide (G_4C_2) repeat expansion in the 5' non-coding region, C9orf72, is the most common genetic cause of amyotrophic lateral sclerosis and frontotemporal lobar degeneration (FTLD) (20,21). A total of 1257 (77%) patients underwent a genetic analysis to identify the presence of the GGGGCC repeat expansion in the first intron of the gene *C9orf72*, according to the methodology described elsewhere (22,23). Recently, a survival study of the *C9orf72* expansion that included Euro-MOTOR patients from Ireland, the Netherlands and Italy (Piedmont and Val d'Aosta regions) was published (24).

Population at risk

The total population at risk in the Euro-MOTOR project was 34.1 million, distributed over three countries and five cohorts: The Netherlands, 16 million; Ireland, 4.5 million; Italy, 13.6 million (Piedmont and Valle d'Aosta, 4.5 million; Lombardy, 5 million; Apulia, 4.1 million) (Table 4).

On this basis, and considering country-specific annual incidence rates, as shown in Table 1, a conservative estimate of the number of patients with ALS to be recruited was 838, but only 626 per year (or 1.84 per 100,000 per year) should be considered assuming that approximately 75% of patients would agree to participate. The Euro-MOTOR study enrolled 519 cases per year (83% of total cases estimated) and 1.9 controls per case, achieving a power of 76% powered to detect an odds ratio (OR) of 2, with a 1% level of exposure in the controls and a 2% level of exposure in the cases. Our preliminary results indicated that, with these numbers, the exposures we investigated can be evaluated with sufficient statistical power.

The population considered in the analyses (4479) was represented by 1557 cases and 2922 controls. The distribution of definite and probable ALS cases and controls included from the three countries and five cohorts is given in Table 4.

Statistical analysis

The statistical analysis of data collected from case-control studies is performed under the conventional assumptions of independence and identical

exposure in patients with and without the disease of interest. Parametric and non-parametric tests are used after verification of the distribution of each variable's values. In the Euro-MOTOR study, the Student's *t*-test (or the Wilcoxon test when a non-parametric equivalent is required) was used to test height, weight, BMI, MET, duration of occupation, duration of physical exercise, number of traumatic events, duration of drug and toxin exposure. The χ^2 test was used to test all categorical variables, as centre or demographics. Generalized logistic regressions models were used with additional use of splines to investigate continuous exposures, and stratification/mixed-models/forest plots to investigate countrywise variations. To account for the matching conditions, models were at a minimum corrected for age and gender before addition of further covariates (25). Therefore, any stratification that was finer than the original matching criteria was potentially biased and such fine stratification was avoided.

Two sensitivity models were used: the first adjusted for age and gender, the second adjusted for age, gender, education, smoking and METs. Analysis of repeated measures per patient such as BMI at different ages used generalized linear mixed models by default. In addition, sensitivity analyses were performed with and without imputation of factors of interest. Imputation was carried out using multiple iterations ($n = 100$) of predictive mean matching with optional weighted probability sampling of the other variables.

The primary aim for each risk factor is to identify an overall exposure response curve, while the secondary aim is to identify a temporal relationship between the exposure time to the given risk factor and development of the disease. It is also important to compare different exposure (induction) periods. Analysis of exposures at different times was carried out in the case-control study. All exposures were truncated at three years before dates of survey, a threshold below which all intervals between dates of onset and dates of survey, up to the third quartile and of all cohorts, were calculated (Figure 1). This operation was performed for both cases and controls, because failure to adjust cases and controls for timing and extent would introduce a systematic bias.

Discussion

A case-control design is the best compromise to reconcile the low incidence of ALS in a population at risk and the time-lapse between any given exposure and the onset of the disease. Our study is population based and the ALS sample largely reflects the origin populations in each country. Our data suggest that comparison of patients from different countries has the potential to introduce bias including the time-lapse between onset of symptoms and enrolment of patients, and variance in the methods used to classify patients. It is also possible that the disease presents with different phenotypes as shown when comparing published reports from different countries (26).

In the present study all exposures have been defined according to standard and/or objective definitions. The use of exposures with the same definition in a large multi-institutional cohort is

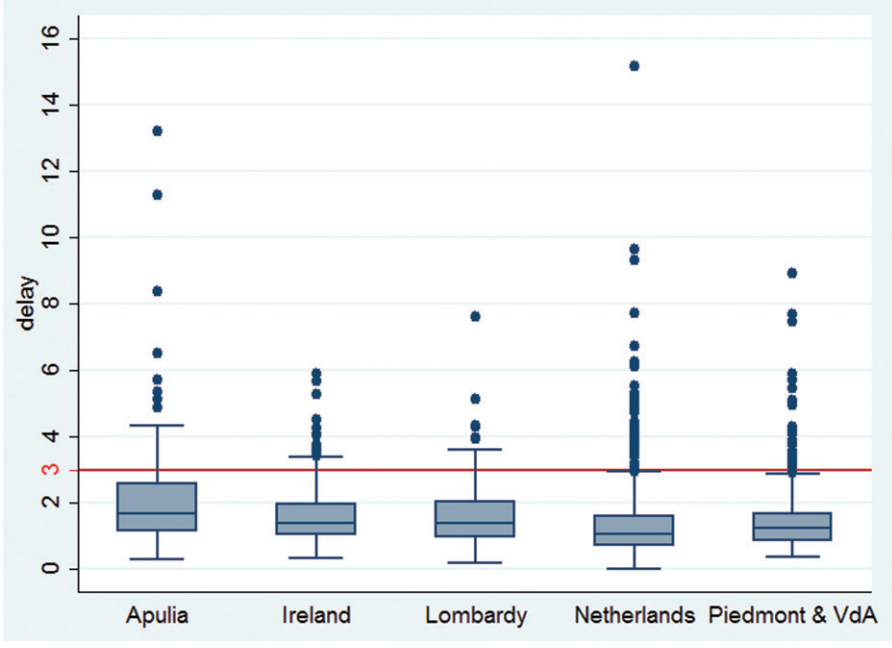

Figure 1. Number of years between date of onset and date of survey by cohort. The plot shows the delay in years from disease onset to survey for ALS cases by cohort. Over 95% of all participants have a delay of less than 3 years.

advantageous, within the limits of a retrospective design, as several factors (and combinations of factors) have been assessed using the same methodology.

Some exposures (e.g. the occupations) are inherently poor surrogate markers of individual risk factors, even if models quantifying dose-response relationships are available and have been used in the project. In this regard, the duration of the induction periods and the intervals between the end of the exposures and the onset of symptoms may be used to provide quantitative measures of exposure.

In this complex scenario, exposures were compared within differing strata of cases and controls and differing regression models, in order to test the consistency of the results on all variables and relevant variable combinations.

Our study design is limited by different socio-cultural, economic and institutional systems among the three countries, which could play an important role in defining, systematizing and interpreting both questionnaires and results. Additionally, the level of education varies across countries and, on average, controls are better educated than the cases. For these reasons, we can conclude that the results are generalizable to populations having similar characteristics to those participating in this study. Sensitivity analyses are, however, necessary to verify the consistency of the study findings even though some effects on the dimension of the risk can still be present.

Conclusions

The aetiology of ALS is still unknown. The research on genetic factors, which affect up to 10–20% of total ALS patients, has moved forward but no definite environmental risk factors have been defined yet.

Identifying and studying environmental and epigenetic factors can improve our knowledge of the ALS aetiology, but methodological limitations can confound analyses. The representativeness and comprehensiveness of population based registers in the Netherlands, Ireland and Italy represent a great opportunity for studying in a large scale exposure and incidence of ALS, stratified by country, single cohort, phenotype and genetic mutation. This cross-national study has identified methodological challenges that must be recognized and addressed to minimise bias and assess the independent role of well-defined exposures.

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