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The Role of Nutrition and Other Lifestyle Habits in the Progression of Amyotrophic Lateral Sclerosis: A Multicentre Cross-Sectional Study

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List of Publications related to this thesis

The following original papers, which will be referred to by their Roman numerals:

Paper I. **Cucovici A** et al., Coffee and Tea Consumption Impact on Amyotrophic Lateral Sclerosis Progression: A Multicentre Cross-Sectional Study. Submitted to Frontiers Journal, Neurology 2020; December 4.

Paper II. **Cucovici A** et al., The Impact of lifetime Alcohol and Cigarette Smoking Loads on Severity of Amyotrophic Lateral Sclerosis: a cross-sectional Study. Submitted to Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration Journal 2021; January 1st.

Paper III. Maurizio A. Leone, Andrea Fontana, Andrei Ivashynka, **Aliona Cucovici**, Paola Naldi, Massimiliano Copetti, Nadia Barizzone, Sandra D'Alfonso. Impact of Lifetime Coffee and Tea Loads on Multiple Sclerosis Severity (manuscript in preparation).

Abstract

Introduction: Amyotrophic Lateral Sclerosis (ALS) is a devastating and still untreatable motor neuron disease. The causes of ALS are unknown, but nutritional and lifestyle factors such as coffee and tea consumption, alcohol drinking, and cigarette smoking may impact the rate of disease progression. However, the currently used research methods and outcomes (punctual and not cumulative evaluation of quantity/frequency) do not adequately assess the effect of coffee and tea consumption, and alcohol intake, and cigarette smoking. This is one of the reasons why the nutritional lifestyle factors analysis for people with ALS in different studies sometimes has conflicting results. This study used a new approach to assess the role of potentially modifiable risk factors on the ALS progression. This study used cumulative lifetime coffee and tea consumption, alcohol drinking, and cigarette smoking loads. Lifetime coffee and tea consumption, alcohol drinking, and cigarette smoking loads are applied in the practice of oncologists, dieticians, and other areas of medicine, but not in neurological practice. These values allow us to estimate the cumulative effect of coffee and tea consumption, alcohol drinking, and cigarette smoking on disease course, even for low to moderate doses.

A similar study was done for Multiple Sclerosis, another autoimmune and degenerative disease of the central nervous system. If some potentially modifiable lifestyle factors could impact on MS progression, possible interventions may be suggested, and possible clues to understand the pathogenesis of progression may be uncovered.

Objectives: This PhD thesis aimed to evaluate the role of coffee and tea consumption, alcohol drinking, and cigarette smoking as potentially modifiable risk factors on ALS progression rate. Additional goals were to assess a possible role of lifetime coffee and tea consumption on Multiple Sclerosis progression and severity and their possible

interaction with smoking and alcohol use; to investigate whether coffee and tea consumption interacts with HLA susceptibility risk genes in determining MS progression, as a comparative study.

Subjects and Methods: In this multicentre cross-sectional study were recruited 241 patients, 96 females and 145 males; the mean age at onset was 59.9±11.8 years. According to El Escorial criteria, 74 were definite ALS, 77 probable, 55 possible, and 35 suspected; 187 patients had spinal onset and 54 bulbar. The patients were categorized into three groups, according to ΔFS (derived from ALS Functional Rating Scale-Revised score and disease duration from onset): slow (81), intermediate (80), and fast progressors (80).

The design of the comparative study "The Impact of Lifetime Coffee and Tea Loads on Multiple Sclerosis Severity" was a cross-sectional study, 208 patients consecutively admitted to the Department of Neurology were asked to complete the "Questionnaire of Lifestyle" (part of the European Prospective Investigation into Cancer and Nutrition project). An estimation of the intensity of drinking (drinks/day) was calculated as the weighted sum of the mean number of standard cups drunk per day at different ages. A measure of lifetime load of the exposure was was expressed in terms of cups-year. Disease severity was estimated by the Multiple Sclerosis Severity Score (MSSS).

Results: Current coffee consumers were 179 (74.3%), 34 (14.1%) were non-consumers, 22 (9.1%) former consumers, whereas six (2.5%) consumed decaffeinate coffee only. The log-ΔFS was weakly correlated with the duration of coffee consumption ($p=0.034$), but not with the number of cups-year ($p=0.932$). Current tea consumers were 101 (41.9%), 6 (2.5%) were former-consumers, and 134 (55.6%) non-consumers. Among 107 current and former consumers, 27 (25.2%) consumed only green tea, 51 (47.7%) other types of tea, and 29 (27.1%) both. The log-ΔFS was weakly correlated with the consumption

duration of other tea types (p=0.028), but not with the number of cups-year. Current smokers were 44 (18.3%), 187 (77.6%) were non-smokers, and 10 (4.1%) former smokers. Age of ALS onset was lower in current smokers than non-smokers, and the ΔFS was slight, although not significantly, higher for smokers of >14 cigarettes/day. Current alcohol drinkers were 147 (61.0%), 5 patients (2.1%) were former-drinkers, and 89 (36.9%) non-drinkers. The log-ΔFS was weakly correlated with the duration of alcohol consumption (p=0.038), but not with the number of drinks-day or the drink-years.

In the study "The Impact of Lifetime Coffee and Tea Loads on Multiple Sclerosis Severity" we did not find any trend with the quantity of coffee drunk for both the intensity and cumulative exposures. The multivariable analysis did not show any association between coffee and tea consumption (cups/day) and MSSS. Regarding tea consumption, we found no correlation with Multiple Sclerosis severity, measured with the MSSS, age at onset, or clinical form. Compared to non-consumers, the ORs were 1.27 for coffee drinkers, and 0.68 for tea drinkers.

Conclusions: The study does not support the hypothesis that coffee or tea consumption is associated with ALS progression rate. The results of this cross-sectional multicenter study evidence a possible minor role for smoking, but not for alcohol drinking in worsening disease progression.

The results of the comparative study "The Impact of Lifetime Coffee and Tea Loads on Multiple Sclerosis Severity" do not support the hypothesis that coffee or tea intake is associated with a different severity or progression of MS, contrarily to other neurodegenerative diseases. However, we cannot exclude a possible effect of higher doses of coffee or tea or an effect on a subgroup of patients.

Keywords: Amyotrophic Lateral Sclerosis (ALS), coffee and tea consumption, alcohol drinking, cigarette smoking, risk factors, rate of disease progression, Multiple Sclerosis, Multiple Sclerosis Severity Score.

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List of Abbreviations

ALS	Amyotrophic Lateral Sclerosis
UMN	Upper motor neuron
LMN	Lower motor neuron
ROS	Reactive oxygen species
DNA	Deoxyribonucleic Acid
EPIC	European Prospective Investigation into Cancer and Nutrition
ALSFRS-R	ALS Functional Rating Scale-Revised
FVC	Forced volume vital capacity
PLS	Primary lateral sclerosis
PMA	Progressive muscular atrophy
ΔFS	Rate of disease progression
EMG	Electromyography
FTD	Frontotemporal dementia
MS	Multiple Sclerosis
HLA	Human leukocyte antigens
EDSS	Expanded Disability Status Scale
MSSS	Multiple Sclerosis Severity Score

Chapter 1: Introduction

1.1 Background and Aims

Amyotrophic Lateral Sclerosis (ALS) is an untreatable neurodegenerative disease characterized by progressive degeneration of upper (motor cortex) and lower (brainstem and spinal cord) motor neurons, resulting in progressive muscle weakness and paralysis.

The symptoms are progressive muscle atrophy and weakness, fatigue, bulbar symptoms, and eventually respiratory failure. Several heterogeneous clinical phenotypes can be distinguished: classical ALS presents as a mixture of upper and lower motor signs and is the most common form, its variants include predominantly upper motor neuron forms, i.e. primary lateral sclerosis (PLS) and predominantly lower motor neuron forms, i.e. progressive muscular atrophy (PMA), flail arm or flail leg syndrome, progressive bulbar palsy (Al-Chalabi *et al.*, 2016).

In Europe and the USA, there are 1 or 2 new cases of ALS per year per 100,000 people. The prevalence is 3 to 5 per 100,000 (Chiò *et al.*, 2013).

The clinical manifestation varies regarding the site of symptoms onset: for most the cases (65%) limb symptoms are initially experienced, followed by symptoms of bulbar dysfunction (i.e. dysarthria or dysphagia; for 30% of all ALS cases). In five per cent of ALS patients is reported respiratory onset (Hardiman *et al.*, 2011). Cognitive or behavioural changes have been repeatedly reported. Fifty per cent of patients suffer from cognitive impairment and up to 10% present with frank frontotemporal dementia (FTD) (Phukan *et al.*, 2012).

The average delay between first symptoms and formal clinical diagnosis of ALS is 9-16 months (Chiò et *al.*, 1999; Cellura et *al.*, 2012).

There is no definitive diagnostic test for ALS. The clinical diagnosis of ALS depends on the identification of upper and lower motor neuron signs within body regions defined as bulbar, cervical, thoracic, and lumbar, according to the El Escorial criteria (Brooks et *al.*, 1994), clinical progression and negative laboratory tests for ALS mimics.

Up to 10% of ALS cases have a strong family history suggesting familial ALS. The remaining 90% of cases appear sporadic, meaning they appear to occur randomly (Renton et *al.*, 2014). The clinical manifestation of familial ALS is very similar to sporadic ALS (Andersen & Al-Chalabi, 2011). Genetic studies have shown that C9orf72, SOD1, TARDBP and FUS are the most common mutated genes in amyotrophic lateral sclerosis (Zou et *al.*, 2017).

The main clinical predictors of progression are age and site of onset, diagnostic delay, and the Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised (ALSFRS-R) baseline score (Creemers *et al.*, 2015).

The role of lifestyle factors, such as physical activity, cigarette smoking, diet, alcohol drinking, coffee and tea consumption on ALS progression is unclear.

Like other neurodegenerative diseases, some potentially modifiable lifestyle factors could impact ALS progression, suggesting possible clues to understand its pathogenesis and possible interventions (Ivashynka *et al.*, 2019; Belvisi *et al.*, 2020; Korner *et al.*, 2019).

Multiple sclerosis is an autoimmune, degenerative disease of the central nervous system with a heterogeneous clinical course, that could be determined by the interaction between environmental and genetic factors.

The aims of the thesis were to determine the role of lifetime coffee and tea consumption, and to figure out the influence of cigarette smoking and alcohol drinking on ALS progression.

Additional goals were to assess a possible role of lifetime coffee and tea consumption on Multiple Sclerosis progression and severity and their possible interaction with smoking and alcohol use; to investigate whether coffee and tea consumption interacts with HLA susceptibility risk genes in determining MS progression, as a comparative study.

1.2 Thesis structure

Chapter 1 contains an introduction, background and aims, thesis structure. Chapter 2 includes a literature review, antioxidant and pro-oxidant role of lifestyle factors, the background of Paper I, Paper II and Paper III, summary and conclusions. Chapter 3 consists of the research design, subjects, and methods of the study. Chapter 4 includes the results of all three papers. Chapter 5 contains the study discussion of all three papers. Chapter 6 includes the conclusions of all three papers.

Chapter 2: Literature review

2.1 Background

ALS is a fatal adult-onset, progressive neurodegenerative disease that primarily affects the motor system, resulting in muscle weakness and paralysis. In 1869 ALS was described by the French neurologist Jean-Martin Charcot and hence is also known as Charcot disease. In 1939 ALS gained popular recognition and its best-known eponym in the USA after the baseball player Lou Gehrig, also is known as motor neuron disease (MND) (Charcot & Joffroy; Wijesekera *et al.*, 2009).

ALS incidence is 1 or 2 new cases of ALS per year per 100,000 people in Europe and the USA; the ALS prevalence is 3 to 5 per 100,000. The incidence and prevalence of ALS increase with age. In the USA and Europe, the cumulative lifetime risk of ALS is about 1 in 400; in the United States alone, 800,000 persons who are now alive are expected to die from ALS. The etiology of ALS is unknown for most of the patients. The mean age of onset of sporadic ALS patients is around 60 years, overall, the male to female ratio is around 1.5:1. The disease is rapidly progressing with a survival time since onset ranging from 24 to 48 months. Now, there is no known therapy capable of curing ALS (Chiò *et al.*, 2013; Petrov *et al.*, 2017). Up to 10% of ALS cases have a strong family history suggesting familial ALS. The remaining 90% of cases appear sporadic, meaning they appear to occur randomly (Renton et al., 2014). Genetic studies have shown that C9orf72, SOD1, TARDBP and FUS are the most common mutated genes in amyotrophic lateral sclerosis (Zou et *al.*, 2017).

The discovery of mutations in the SOD1 gene, responsible for an inherited form of ALS and the widespread use in the preclinical studies of mutant SOD1-G93A mouse

models allowed the identification of mechanisms plausibly implicated in the onset and progression of ALS, such as oxidative stress, glutamate excitotoxicity, neuroinflammation, mitochondrial dysfunction, protein aggregation, impaired axonal transport (Rothstein, 2009; D'Amico *et al.*, 2013).

Oxidative stress is one of the main mechanisms associated with the pathogenesis of ALS. The oxidative stress is an imbalance in the homeostasis of oxidation-reduction reactions and evolves as a result of increased reactive oxygen species (ROS) in excess of available antioxidants. Oxidative stress can cause cellular damage and ROS oxidize critical cellular components such as membrane lipids, proteins, and DNA, by inducing apoptosis and necrosis. The motor neurons seem to be particularly sensitive to these pathological effects and ROS (Chen *et al.*, 2012; Rojas *et al.*, 2015).

Studies on ALS cohorts have shown some predictive factors of disease progression, including age at onset, bulbar type at the onset, the interval between onset-diagnosis and severity score, respiratory function, and body mass index at diagnosis (Chiò *et al.*, 2009). In recent years epidemiological and experimental studies have focused on the oxidative stress as a predictive factor (D'Amico *et al.*, 2013).

Several nutrition factors and lifestyle habits may influence the oxidative balance, including smoking, alcoholic beverages (white and red wine and other alcoholics), coffee, tea, consumption of foods containing phenolic compounds. There are epidemiological studies that show a possible protective effect of the moderate consumption of alcohol on the susceptibility to ALS.

Other single studies or meta-analyses found an association of high and prolonged coffee intake, consumption of tea and of regular use of vitamin E supplements with a

lower risk of ALS (de Jong *et al.*, 2012; Creemers *et al.*, 2015; Huisman *et al.*, 2015; Ingre *et al.*, 2015).

On the contrary, cigarette smoking has been associated in some studies with an increased risk of developing ALS (Wang *et al.*, 2011).

MS is an autoimmune, degenerative disease of the central nervous system that could be determined by the interaction between environmental and genetic factors.

All these lifestyle factors with antioxidative and pro-oxidative role have been studied in humans so far, for their possibility to increase or decrease the susceptibility to the disease, but they have never been assessed as predictors of the course and progression of disease once it is already present.

2.2 Antioxidant and Pro-oxidant Role of Lifestyle Factors

Antioxidants and Pro-oxidants

Since the late 19th and early 20th century, chemists have studied antioxidants, a defined group of compounds characterized by their ability to be oxidized in place of other compounds present. The role of antioxidants in a physiological setting is to prevent ROS concentrations from reaching a high-enough level within a cell that damage may occur. Cellular antioxidants could be enzymatic (catalase, glutathione peroxidase, superoxide dismutase) or nonenzymatic (glutathione, thiols, some vitamins and metals, or phytochemicals such as isoflavones, polyphenols, and flavanoids) (Seifried *et al.*, 2007).

Flavonoids (catechins, in epigallocatechin gallate) and non-flavonoids (resveratrol) compounds of alcohol, coffee, tea, and foods have known antioxidant and anti-inflammatory properties (Maher, 2019).

Caffeine from coffee, tea, foods, beverages may be neuroprotective through inhibition of adenosine A2a receptors, which may modulate dopaminergic transmission and mitigate neurotoxicity (Kolahdouzan & Hamadeh, 2017).

Prooxidant corresponds to any endobiotic or xenobiotic that induces oxidative stress either by generation of ROS or by inhibiting antioxidant systems. It can include all reactive, free radical containing molecules in cells or tissues (Rahal *et al.*, 2014).

Potentially modifiable risk factors

Modifiable risk factors are behaviours and exposures that can raise or lower a person's risk of disease. They are modifiable because they can, in theory, be changed. Potentially modifiable risk factors are the subject of research into the causes of many neurological and neurodegenerative diseases (Gatz *et al.*, 2006; Østergaard *et al.*, 2015; Gallagher *et al.*, 2016; O'Donnell *et al.*, 2016; Larsson *et al.*, 2017; Hankey, 2020). The role of potentially modifiable risk factors in the progression of ALS disease has not been studied.

Coffee and Tea Consumption

Coffee and tea are the most consumed methylxanthine-containing beverages worldwide, and their effects on the nervous system have been widely explored (Srinivasan & Rajasekaran, 2017; De Luca *et al.*, 2018). Caffeine is a major active

principle in coffee and tea, antagonizing the adenosine A2A receptors in the brain and defending the motor neurons against excitotoxicity (Kolahdouzan & Hamadeh, 2017).

Alcohol Drinking (Red and White Wines)

Wine characteristics are determined by the combination and interaction of organic compounds from grapes, such as polysaccharides, acids, and phenolic compounds (flavonoids and non-flavonoids), and their changes during the winemaking process. Since the early 2000s, there are numerous reports in the literature of a reduced risk of neurodegenerative diseases associated with regular consumption of flavonoids. The main compounds of red wine that may have a protective role against the pathogenic mechanisms of Amyotrophic Lateral Sclerosis are catechins, epigallocatechin gallate, and resveratrol. These substances have known antioxidant and anti-inflammatory properties (Fernandes *et al.*, 2017; Maher, 2019).

Cigarette Smoking

Cigarette smoking might increase the risk of ALS through several mechanisms: inflammation, neurotoxicity, and oxidative stress caused by heavy metals and chemical compounds present in cigarette smoke (Alonso *et al.*, 2010). Neurotoxic effects have been ascribed to the particulates in cigarette smoke or their byproducts, which contain nitric oxide, lead, formaldehyde, and other chemicals that can lead to oxidative damage.

2.3 Paper I

Amyotrophic Lateral Sclerosis is a fatal adult-onset neurodegenerative disease characterized by progressive degeneration of motor neurons in the spinal cord and brain. The main clinical predictors of progression are age and site of onset, diagnostic delay, and the Amyotrophic Lateral Sclerosis Functional Rating Scale-Revised (ALSFRS-R) baseline score (Creemers *et al.*, 2015). The role of lifestyle factors, such as physical activity, smoking, diet, alcohol intake, coffee and tea consumption on ALS progression is unclear. Similar to other neurodegenerative diseases, some potentially modifiable lifestyle factors could impact ALS progression, suggesting possible clues to understand its pathogenesis and possible interventions (Ivashynka *et al.*, 2019; Belvisi *et al.*, 2020). Coffee and tea are the most consumed methylxanthine-containing beverages worldwide, and their effects on the nervous system have been widely explored (Srinivasan & Rajasekaran, 2017; De Luca *et al.*, 2018). Caffeine is a major active principle in coffee and tea, antagonizing the adenosine A2A receptors in the brain and defending the motor neurons against excitotoxicity (Kolahdouzan & Hamadeh, 2017). Coffee and tea consumption were studied for their possible impact on the risk of ALS onset, although most studies are negative (Fondell *et al.*, 2015; Petimar *et al.*, 2019). Still, there are no studies regarding the influence of coffee and tea consumption on ALS progression. Risk factors for progression may not be the same as for disease susceptibility; we aimed to assess a possible role of lifetime coffee and tea consumption on ALS progression.

2.4 Paper II

Amyotrophic lateral sclerosis is an untreatable neurodegenerative disease characterized by progressive degeneration of motor neurons. The main clinical predictors of progression are age, site of onset, diagnostic delay, and the ALS Functional Rating Scale-revised (ALSFRS-R) baseline score (Creemers *et al.*, 2015). The role of some potentially modifiable lifestyle factors, such as cigarette smoking and alcohol consumption on ALS has been studied so far in humans for their possible impact on the risk of developing ALS (susceptibility) (Belbasis *et al.*, 2016; Krewski *et al.*, 2017), but not as much for their possible impact on ALS progression. Cigarette smoking was found to increase the susceptibility to ALS in most studies (Kamel *et al.*, 1999; Gallo *et al.*, 2009; Wang *et al.*, 2017; Peters *et al.*, 2020), although some aspects are still unclear, such as the absence of a dose-dependency (Opie-Martin *et al.*, 2020). On the contrary, results for alcohol intake are more controversial, showing an increased (Yu *et al.*, 2020), or a reduced risk (E *et al.*, 2016), or no association (Ovidio *et al.*, 2019; Peng *et al.*, 2020). Since risk factors for progression may not necessarily match those for susceptibility to the disease (Waubant *et al.*, 2019), we aimed to assess a possible role of lifetime smoking and alcohol drinking on ALS progression (See Appendix D).

2.5 Paper III

Multiple sclerosis (MS) is an autoimmune disease of the central nervous system with a heterogeneous clinical course that could be determined by the interaction between environmental and genetic factors. The role of some lifestyle factors, such as smoking, diet, intake of alcohol, coffee and tea on MS has been studied so far for their possible impact on the risk of developing the diseases (susceptibility) (Koch *et al.*, 2013; Hedström *et al.*, 2014), but not as much for their possible impact on MS progression and severity (Marrie *et al.*, 2009; Hempel *et al.*, 2017a). Smoking worsens disease progression (Hedström *et al.*, 2014; Ivashynka *et al.*, 2019), whereas the role of alcohol is not clear (Hempel *et al.*, 2017b; Ivashynka *et al.*, 2019), and even less studied are coffee and tea.

Coffee and tea are the most consumed methylxanthine-containing beverages all over the world, and their biological effect have been linked to possible anti-inflammatory, immunosuppressive, or antioxidant properties (De Luca *et al.*, 2018). Also a neuroprotective effect by antagonizing the adenosine A2A receptors in the brain and defending the motor neurons against excitotoxicity has been studied (Kolahdouzan & Hamadeh, 2017), and may be the basis of the protective role of caffeine against neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases (Hernán *et al.*, 2002; Panza *et al.*, 2015).

The potential effect of coffee on MS has not been deeply explored. Some studies have suggested that coffee intake might be associated with decreased incidence of MS (Jahromi *et al.*, 2012; Hedström *et al.*, 2016; Al Wutayd *et al.*, 2018), whereas others did not show any significant association between coffee or caffeine intake and the risk of MS (Pekmezovic *et al.*, 2006; Massa *et al.*, 2013; Ponsonby *et al.*, 2013; Lu *et al.*,

2020), or found a higher intake of coffee among MS cases compared to controls (Tola *et al.*, 1994; Pekmezovic *et al.*, 2006). Tea consumption has also been evaluated in some of the studies on coffee without finding any association (Tola *et al.*, 1994; Hedström *et al.*, 2016).

These inconsistencies may be due to differences in the study population, small sample size, inclusion of different covariates, or differences in the preparation and dosages of the two beverages.

Only one study (D'Hooghe M *et al.*, 2012) evaluated the association of coffee and tea consumption with disease progression and found that coffee but not tea intake was protective towards reaching the score of 6 at the Expanded Disability Status Scale (EDSS) for relapsing-remitting MS patients. No association was found for progressive forms and for tea consumption.

Since risk factors for progression and severity may not necessarily match those for susceptibility to the disease (Waubant *et al.*, 2019), we aimed to assess a possible role of lifetime coffee and tea consumption on MS progression and severity and their possible interaction with smoking and alcohol use. Also, since human leukocyte antigens (HLA) haplotypes are strongly linked to the onset of MS (Jokubaitis *et al.*, 2018), we aimed to investigate whether coffee and tea consumption interacts with HLA susceptibility risk genes in determining MS progression.

2.6 Summary and Conclusions

The balance of pro-oxidants and antioxidants may be important in predicting a slower or faster progression of ALS. If some potentially modifiable lifestyle factors could

impact MS progression, possible interventions may be suggested, and possible clues to understanding the pathogenesis of progression may be uncovered.

Chapter 3: Research Design

3.1 Subjects and Methods

Paper I&II

The study was designed as a cross-sectional multicentre study. It was conducted in three Centres in Italy: San Giovanni Rotondo (SGR), Novara, and Modena, one in the Republic of Moldova (Chisinau), and one in Romania (Cluj-Napoca). The study was approved by the Institutional Review Boards of the coordinating Centre (N96/CE/2016) and the other four Centres. Written informed consent was obtained from all participants. Patients were recruited from March 2016 to January 2020, in different periods of time in each Centre.

Inclusion criteria were age more than 20 years old; clinical diagnosis of ALS according to the El Escorial criteria (Table 3.1) (Brooks et al., 1994); consecutive in- and out-patients with a new or already made diagnosis of ALS.

Exclusion criteria were patients with tracheostomy or receiving mechanical ventilation, with percutaneous endoscopic gastrostomy, who did not sign an informed consent and disagreed to participate in the study.

Table 3.1 The El Escorial criteria (1994)

Definite ALS*	UMN and LMN signs in three regions of the body#
Probable ALS*	UMN and LMN signs in at least two regions, with some UMN signs rostral to LMN signs
Possible ALS*	UMN and LMN signs in only one region, or UMN signs alone in two or more regions, or LMN signs rostral to UMN signs
Suspected ALS*	LMN signs only

LMN=lower motor neuron. UMN=upper motor neuron.

*Neuroimaging and clinical laboratory studies must be done to exclude ALS mimics.

#Regions: ***a)*** *bulbar;* ***b)*** *cervical* (neck, arm, hand, diaphragm, and cervical spinal cord-innervated muscles); ***c)*** *thoracic* (back and abdomen muscles), and ***d)*** *lumbar* (back, abdomen, leg, foot, and lumbosacral spinal cord-innervated muscles).

3.2 Data Collection and Disease Progression Assessment

Paper I&II

For each patient, we collected demographics (date of birth, gender, education, BMI) and clinical data (date of onset and diagnosis, site of onset, diagnostic category according to El Escorial criteria, FVC%, treatment). Disease severity was estimated through the ALSFRS-R (see Appendix B) through a 12-item questionnaire (E *et al.*,

2016). The ALSFRS-R examines four domains of the nine daily activities plus three respiratory functions and assigns scores from 0 (function absent) to 4 (function normal) – maximum score is 48 (normal function) (Bakker *et al.*, 2017). The rate of disease progression (ΔFS) at recruitment was calculated by dividing the ALSFRS-R total score by symptom duration applying the formula: ΔFS= 48-(total ALSFRS-R at visit)/symptom duration in months (Kimura *et al.*, 2006). The time of disease onset was determined on subjective complaints and information confirmed from relatives and clinical charts.

3.3 Exposure Assessment

Paper I&II

Cigarette smoking and alcohol consumption histories were evaluated with the "Questionnaire of Lifestyle," which is part of the European Prospective Investigation into Cancer and Nutrition project study (Riboli *et al.*, 2002; Ferrari *et al.*, 2007).

3.3.1 Cigarette Smoking

Paper I&II

Smoking status at recruitment was defined as never-smokers if they had smoked <100 cigarettes up to the time of the interview (Centers for Disease Control and Prevention, 2004); former smokers if they had smoked >100 cigarettes and had stopped smoking at least six months before the time of the interview; current smokers if they had smoked >100 cigarettes and were still smoking at the time of the interview.

3.3.2 Alcohol Drinking

Paper I&II

Alcohol drinking status was defined as never-drinkers if they had drunk less than one standard alcohol drink/month; former drinkers if they had drunk one or more standard alcohol drinks/month and had stopped drinking at least six months before the interview; current drinkers if they had consumed more than one standard alcohol drink/month for six months or longer and were still drinking at recruitment (Ivashynka *et al.*, 2019). A 'standard drink' (or 'unit of alcohol' in the UK) is a notional drink that contains a specified amount of pure alcohol (ethanol). It is usually expressed as a certain measure of beer, wine, or spirits. One standard drink always contains the same amount of alcohol regardless of the container size or the type of alcoholic beverage but does not necessarily correspond to the typical serving size in the country in which it is served.

3.3.3 Coffee and Tea Consumption

Paper I&II

Coffee and tea consumption histories were evaluated with a questionnaire built in analogy to the "Questionnaire of Lifestyle" asking patients whether they consumed or had consumed in the past coffee (regular or decaffeinated) or tea (green tea or other types of tea) and, if so, how many cups per day. One standard unit is equivalent to 1 cup of coffee or tea (about 30 ml and 170 ml, respectively) (Filiberti *et al.*, 2017). Detailed information was obtained regarding coffee and tea consumption during six age periods (20, 30, 40, 50, 60, 70, and over) up to the participants' current age. For

each beverage, we obtained age at onset of consumption and cessation (for former consumers). For coffee and tea consumption, we defined three categories of consuming status at recruitment: non-consumer (who had never consumed more than one unit/month or stopped drinking at least one year before disease onset); current consumer (who consumed beverages at least monthly for six months or longer and were still consuming at recruitment), and former consumer (who stopped intake coffee and tea after disease onset, but prior of recruitment). For each current or former consumer, a cumulative lifetime exposure load for each beverage was computed as the weighted sum of a number of cups consumed per year within each decade (six age periods), with weights equal to the number of years spent drinking in the decade (cup-year). This is the measure of the amount a person has consumed over a lifetime and was computed by dividing the cumulative lifetime exposure load by 365.25. The mean number of cups drunk per day during the lifetime was calculated as the cup-year divided by the number of years spent drinking during a lifetime (i.e., coffee or tea consumption duration in years).

3.4 Questionnaire

Paper I&II

The questionnaire included three parts: first part (smoking and drinking history) contains items from the European Prospective Investigation into Cancer and Nutrition (EPIC) questionnaire, the second part (current consumption of alcoholic beverages) contains items from the questionnaire of ALCE (Alcohol and Epilepsy) Study Group, the third part is an ad-hoc questionnaire collecting information about consumption of antioxidant-rich beverages and foods. The questionnaire was designed in Italian (See

Appendix A), then translated in Romanian by a mother language, and back-translated by an Italian mother language. Two raters, previously trained in using the questionnaire and blinded to the patients' clinical status, interviewed patients in a dedicated room. To evaluate the reliability of the questionnaire, two pairs of raters interviewed healthy subjects or patients with neurological diseases before the study start (40 in Chisinau and 25 in SGR). The sequence of interviews was randomized, and the randomization list was concealed. Each rater did the interviews on at least one day and no more than seven days apart; this was considered a sufficient time window for the subjects being unable to remember their answers and not to change their consumption habits. Agreement between two raters for consumption (yes/no) was calculated with Cohen's kappa statistics (Landis & Koch, 1977) and was 0.95/1.0 for coffee/tea in Chisinau and 0.90/0.95 in SGR. Agreement for continuous variables was determined with the intraclass correlation coefficient (Bartko, 1966) and was 0.99/1.0 for coffee/tea duration and 0.65/0.94 for coffee/tea cups-year (See Appendix C).

3.5 Statistical Analysis_Paper I

Patients' characteristics were reported as mean ± standard deviation, or median with Interquartile range (IQR), depending on their distribution, for continuous variables, and with absolute and relative frequencies (%) for categorical variables. The normality of continuous variables distribution was checked by the Q-Q plot and the Shapiro-Wilk test. In the presence of right-skewed continuous variables, statistical analyses were performed on log values. Comparisons between two categorical variables were assessed by Chi-Square or Fisher exact tests, whereas comparisons between a continuous and a categorical variable were assessed by univariable and multivariable

ANOVA models. Pairwise comparisons between groups of the categorical variables were performed and, if necessary, least-square means of the dependent variable (along with their 95% confidence interval) were estimated for each level of the categorical variable. The standardized mean difference was further reported to describe clinical characteristics and was computed as the average of all possible standardized mean differences across pairwise comparisons. Correlation between two continuous variables was assessed by Pearson correlation coefficient. To visually assess the relationship between drink dose (i.e., cups-year) and ΔFS or duration of drink consumption, boxplots and scatterplots with fitted regression line were depicted into a plot matrix. To detect all clinical, demographical, pathological, treatment, and lifestyle variables, which were mostly associated with (log-transformed) ΔFS, the conditional Random Forest (RF) algorithm (18) with 100'000 trees was performed. The RF is a popular machine learning tool that assesses the relationship between a dependent variable and a set of covariates in a (nonparametric) tree-based fashion. An important feature of RF is that it provides a rapidly computable internal measure of variable importance (VIMP) that can be used to rank variables. The VIMP produced by a conditional RF was not affected by the correlation structure of all the included covariates. Formally, a VIMP of a specific covariate is defined as the sum of the decrease in prediction error values when a tree of the forest splits by that covariate. The more a tree relies on a variable to make predictions, the more important it is for that tree. The relative importance is the VIMP divided by the highest VIMP value. A two-sided p-value < 0.05 was considered for statistical significance. All statistical analyses were performed using SAS Release 9.4 (SAS Institute, Cary, NC, USA). Conditional Random Forests and plots were performed using R Foundation for Statistical Computing (version 3.6, packages: party, GGall.

3.6 Statistical Analysis _Paper II

Patients' characteristics are reported as mean ± standard deviation, or median along with range, depending on their distribution, and with absolute and relative frequencies (percentages) for continuous and categorical variables, respectively. The normality of continuous variables distribution was checked by the Q-Q plot and the Shapiro-Wilk test. In the presence of right-skewed continuous variables, statistical analyses were performed on log values. Comparisons between two categorical variables were assessed by Chi-Square or Fisher exact tests (as appropriate), whereas comparisons between a continuous and a categorical variable were assessed by univariable and multivariable ANOVA models. Pairwise comparisons between groups of the categorical variables were performed (from ANOVA models), and, if necessary, least-square means of the dependent variable (along with their 95% confidence interval) were estimated for each level of the categorical variable. The standardized mean difference was further reported to quantify, from a clinical perspective, the difference of investigated variables between groups and was computed as the average of all possible standardized mean differences across pairwise comparisons. Correlation between two continuous variables was assessed by Pearson correlation coefficient. To visually assess the relationship between the measures of intensity (cigarettes or drinks per day) and of cumulative lifetime load (pack or drink/years), and ΔFS, and the duration of consumption, boxplots and scatterplots with fitted regression lines were depicted in a plot matrix. To detect all clinical, demographical, pathological, treatment, and lifestyle variables, which were mostly associated with ΔFS, the conditional Random Forest (RF) algorithm (Strobl *et al.*, 2008) with 100,000 trees was performed. The RF is a popular machine learning tool that assesses the relationship between a

dependent variable and a set of covariates in a (nonparametric) tree-based fashion. An important feature of RF is that it provides a rapidly computable internal measure of variable importance (VIMP) that can be used to rank variables. Moreover, the VIMP produced by a conditional RF was not affected by the correlation structure of all the included covariates. Formally, a VIMP of a specific covariate is defined as the sum of the decrease in prediction error values when a tree of the forest splits by that covariate. The more a tree relies on a variable to make predictions, the more important it is for that tree. The relative importance is the VIMP divided by the highest VIMP value. A two-sided p-value <0.05 was considered for statistical significance. All statistical analyses were performed using SAS Release 9.4 (SAS Institute, Cary, NC, USA). Conditional Random Forests and plots were performed using R Foundation for Statistical Computing (version 3.6, packages: party, GGally).

3.7 Paper III

Subjects and Methods

A sample of 356 patients followed at the MS Center of the Department of Neurology of the "Maggiore della Carità" University Hospital in Novara (Italy) were consecutively recruited between 2011 and 2012 for a cross-sectional study on lifestyle factors and progression (Ivashynka *et al.*, 2019). The study was approved by the Ethics Committee of the Hospital. Cases were diagnosed by neurologists according to the McDonald criteria (Polman *et al.*, 2011). More information on recruitment is reported

elsewhere (Ivashynka *et al.*, 2019). The last 208 patients of the sample were interviewed on their consumption of coffee and tea.

Disease severity examination

Disease severity was estimated through the Expanded Disability Status Scale (EDSS) and the Multiple Sclerosis Severity Score (MSSS). The MSSS corrects EDSS for disease duration, allowing us to compare an individual's disability with the distribution of scores in cases having similar EDSS scores. The MSSS score (range 0 - 9.9) was calculated according to Roxburgh et al. (Roxburgh *et al.*, 2005).

Exposure Assessment

Coffee and tea consumption histories were evaluated at recruitment with a questionnaire built in analogy to the "Questionnaire of Lifestyle", which is part of the European Prospective Investigation into Cancer and Nutrition project (EPIC) study (Riboli *et al.*, 2002; Ferrari *et al.*, 2007). Patients were asked whether they drank or had drunk in the past coffee (regular or decaffeinated) or tea (green tea or other types of tea) and, if so, how many cups per day. One standard unit is equivalent to 1 cup of coffee or tea (about 30 ml and 170 ml, respectively) (Filiberti *et al.*, 2017). For each beverage, we obtained the age at onset of consumption and of cessation (for former consumers).

For both coffee and tea consumption, we defined three categories of drinking status at recruitment: Current drinkers were those who had consumed more than one unit/month for six months or longer and were still consuming at recruitment. Former drinkers were

patients who had consumed more than one unit/month for six months or longer and stopped drinking at least six months before the time of the interview. Never-drinkers were patients who had never consumed coffee or tea or had consumed less than one unit/month.

All current and former drinkers were asked to quantify the number of units drunk per day during five age periods (20, 30, 40, 50, 60, and over) up to the participants' current age. For each age period, we calculated the mean number of units drunk per day based on the questionnaire information and the number of years spent drinking (i.e., drinking duration). The drinking duration (years) was calculated as the difference between age at recruitment or at drinking cessation and age at start drinking. We estimated a drinking intensity (cups-day) as the weighted mean of the number of cups drunk per day at different age periods, with weights equal to the drinking duration within each age period. Drink-years (a measure of the amount a person has drunk over lifetime load in analogy to the pack-years used for smoking) was calculated by multiplying the drinking intensity by drinking duration (in years). We also measured smoking and alcohol drinking status at recruitment as possible confounders or effect modifiers of the exposure to coffee or tea (Ivashynka *et al.*, 2019).

Genetics

HLA-DRB1*15 and HLA-A*02 genotyping were performed as previously described (Bergamaschi *et al.*, 2010).

Statistical Analysis

Patients' characteristics are reported as mean ± standard deviation, or median with Interquartile range (IQR) depending on their distribution, for continuous variables, and with absolute and relative frequencies (percentages) for categorical variables. The normality of continuous variables distribution was checked by the Q-Q plot and the Shapiro-Wilk test. MSSS was converted into a trichotomous variable, based on tertiles of the distribution: ≤1.53 (first tertile); 1.54 – 3.52 (second tertile); >3.52 (third tertile). The association between drinking habits and disease severity was evaluated using a univariable and multivariable logistic regression model that was adjusted for age, sex, and education. Logistic regression analyses were performed using MSSS 1.8 (first tertile) versus >3.9 (third tertile) as the outcome. Risks were reported as odds ratios (OR) along with their 95% confidence intervals (CI).

Comparisons between two categorical variables were assessed by Chi-Square or Fisher exact tests (as appropriate), whereas comparisons between a continuous and a categorical variable were assessed by univariable and multivariable ANOVA models. Student's T-test or Mann-Whitney and Kruskal-Wallis tests were used when appropriate. A two-sided p-value <0.05 was considered for statistical significance. Pairwise comparisons between groups of the categorical variables were performed (from ANOVA models), and, if necessary, least-square means of the dependent variable (along with their 95% confidence interval) were estimated for each level of the categorical variable.

The standardized mean difference was further reported to quantify, from a clinical perspective, the difference of investigated variables between groups and was computed

as the average of all possible standardized mean differences across pairwise comparisons. All statistical analyses were performed using SAS Release 9.4 (SAS Institute, Cary, NC, USA). Plots were performed using R Foundation for Statistical Computing (version 3.6, packages: party, GGally).

Chapter 4: Results

4.1 Demographic and Clinical Data

Paper I&II

We recruited 241 patients, 145 men, and 96 women, with a sex ratio of 1.5:1. Onset was in the spinal district in 187 (77.6%) and bulbar in 54 (22.4%). The mean age was 59.9±11.8 years at onset and 62.4±11.1 at recruitment. The median time elapsed between disease onset to recruitment was 20 months (range 1.7-273). According to El Escorial criteria, 74 (30.7%) patients were categorized as definite ALS, 77 (32.0%) as probable, 55 (22.8%) as possible, and 35 (14.5%) as suspected. Other demographic and clinical characteristics are shown in Table 4.1. Patients were categorized into tertiles according to the ΔFS distribution: a) ≤ 0.333 (slow progressors), b) 0.334-0.875 (intermediate progressors); c) >0.875 (fast progressors). ALSFRS-R score ranged from 10 to 48, with a mean of 34.9±8.3. DeltaFS score ranged from 0 to 5.3, with a median of 0.56 (IQR:0.25-1.05). Table 4.1 shows clinical characteristics according to ΔFS tertiles. Slow progressors were younger at disease onset and recruitment, had less frequently bulbar onset and diagnosis of definite ALS, and had better FVC% (Table 4.1).

Table 4.1 Clinical variables overall and according to the tertiles of ΔFS distribution

Variable	Category	All (N=241)	I: Slow progression rate of disease (N=81)	II: Intermediate progression rate of disease (N=80)	III: Fast progression rate of disease (N=80)	p-value	SMD
Country - N (%)	Italy	206 (85.5)	71 (87.7)	67 (83.8)	68 (85.0)	0.649	0.176
	Republic of Moldova	22 (9.1)	8 (9.9)	8 (10.0)	6 (7.5)		
	Romania	13 (5.4)	2 (2.5)	5 (6.2)	6 (7.5)		
Gender - N (%)	Males	145 (60.2)	53 (65.4)	44 (55.0)	48 (60.0)	0.401	0.143
	Females	96 (39.8)	28 (34.6)	36 (45.0)	32 (40.0)		
Age at recruitment (years)	Mean±SD	62.4 ± 11.0	59.8 ± 12.3	63.6 ± 10.4	63.9 ± 9.8	0.032	0.241
Age at onset (years)	Mean±SD	59.9 ± 11.8	54.6 ± 12.9	62.0 ± 10.5	63.2 ± 9.8	<0.001	0.502
Diagnostic delay (years)	Median (range)	0.9 (0.1-15.8)	1.7 (0.1-15.8)	0.8 (0.1-4.1)	0.5 (0.1-1.8)	<0.001*	1.020*
Education (years)	Mean±SD	10.4 ± 4.4	11.1 ± 4.4	10.6 ± 4.3	9.5 ± 4.2	0.058	0.248
Site of onset - N (%)	Spinal	187 (77.6)	71 (87.7)	53 (66.2)	63 (78.8)	0.005	0.349
	Bulbar	54 (22.4)	10 (12.3)	27 (33.8)	17 (21.2)		
El Escorial ALS - N (%)	Definite	74 (30.7)	16 (19.8)	25 (31.2)	33 (41.2)	0.014	0.460
	Possible	55 (22.8)	23 (28.4)	23 (28.7)	9 (11.2)		
	Probable	77 (32.0)	26 (32.1)	23 (28.7)	28 (35.0)		
	Suspected	35 (14.5)	16 (19.8)	9 (11.2)	10 (12.5)		
FVC - N (%)	<80%	88 (43.8)	20 (29.0)	32 (47.1)	36 (56.2)	0.005	0.379
	≥80%	113 (56.2)	49 (71.0)	36 (52.9)	28 (43.8)		
BMI - N (%)	<18.5	15 (6.2)	5 (6.2)	4 (5.0)	6 (7.5)	0.967	0.083
	18.5-24.9	121 (50.2)	42 (51.9)	40 (50.0)	39 (48.8)		
	≥25	105 (43.6)	34 (42.0)	36 (45.0)	35 (43.8)		
ALSFRS-R	Mean±SD	34.9 ± 8.3	38.8 ± 6.9	35.2 ± 7.5	30.6 ± 8.4	<0.001	0.713
Riluzole - N (%)	Yes	129 (53.5)	41 (50.6)	47 (58.8)	41 (51.2)	0.517	0.109
	No	112 (46.5)	40 (49.4)	33 (41.2)	39 (48.8)		

Coffee consumption status - N (%)	Decaffeinate only	6 (2.5)	1 (1.2)	2 (2.5)	3 (3.8)	0.929	0.147
	Current consumer	179 (74.3)	62 (76.5)	60 (75.0)	57 (71.2)		
	Former consumer	22 (9.1)	6 (7.4)	7 (8.8)	9 (11.2)		
	Non- consumer	34 (14.1)	12 (14.8)	11 (13.8)	11 (13.8)		
Tea consumption status - N (%)	Current consumer	101 (41.9)	32 (39.5)	36 (45.0)	33 (41.2)	0.970	0.075
	Former consumer	6 (2.5)	2 (2.5)	2 (2.5)	2 (2.5)		
	Non- consumer	134 (55.6)	47 (58.0)	42 (52.5)	45 (56.2)		
Alcoholic-drinking status- N (%)	Current drinker	157 (65.1)	52 (64.2)	59 (73.8)	46 (57.5)	0.054	0.321
	Former drinker	18 (7.5)	5 (6.2)	2 (2.5)	11 (13.8)		
	Non-drinker	66 (27.4)	24 (29.6)	19 (23.8)	23 (28.7)		
Cigarette smoking - N (%)	Current smoker	28 (11.6)	10 (12.3)	10 (12.5)	8 (10.0)	0.403	0.188
	Former smoker	93 (38.6)	28 (34.6)	27 (33.8)	38 (47.5)		
	Non-smoker	120 (49.8)	43 (53.1)	43 (53.8)	34 (42.5)		
Current consumers of both coffee and tea - N (%)	Yes	72 (29.9)	25 (30.9)	25 (31.2)	22 (27.5)	0.850	0.055
	No	169 (70.1)	56 (69.1)	55 (68.8)	58 (72.5)		

4.2 Coffee Consumption

Paper I

Current coffee consumers were 179 (74.3%), 34 (14.1%) were non-consumers, 22 (9.1%) former consumers whereas six patients (2.5%) consumed decaffeinate coffee only. No patients started consuming coffee after the ALS diagnosis. Table 4.2 shows unadjusted comparisons of clinical variables among non-consumers, former-consumers, and consumers of coffee according to the number of the mean daily cups during lifetime categories. Patients who consumed decaffeinate coffee only were excluded from the analysis because of their small number. The Median ΔFS score was similar among all categories. All clinical factors (age, gender, age at onset, BMI, FVC) were equally distributed across the categories. Pairwise associations between cup-years, duration of coffee consumption, and log-transformed ΔFS were assessed, and results are reported in Figure 4.1. The log-ΔFS was weakly correlated with the duration of coffee consumption ($r=0.15$, $p=0.034$), but not with the number of cups-year ($p=0.932$). The number of cups-year was associated with the duration ($p=0.002$).

Table 4.2 Clinical variables according to coffee consumption status (i.e. mean daily cups per day groups).

Variable	Category	Non-consumers (0 cups/day) (N=34)	Former coffee consumers		Current coffee consumers		Comparisons (p-values)			
			1-3 cups/day* (N=12)	4-8 cups/day* (N=10)	1-3 cups/day* (N=138)	4-8 cups/day* (N=41)	1-3 vs. 4-8 cups/day among former-consumers	1-3 vs. 4-8 cups/day among consumers	Former consumers vs. non-consumers	Current consumers vs. non-consumers
Country - N (%)	Italy	23 (67.6)	9 (75.0)	9 (90.0)	120 (87.0)	39 (95.1)	0.594	0.472	0.304	0.005
	Republic of Moldova	7 (20.6)	3 (25.0)	1 (10.0)	10 (7.2)	1 (2.4)				
	Romania	4 (11.8)	0 (0.0)	0 (0.0)	8 (5.8)	1 (2.4)				
Gender - N (%)	Males	18 (52.9)	7 (58.3)	6 (60.0)	86 (62.3)	26 (63.4)	1.000	1.000	0.785	0.339
	Females	16 (47.1)	5 (41.7)	4 (40.0)	52 (37.7)	15 (36.6)				
BMI - N (%)	<18.5	5 (14.7)	1 (8.3)	2 (20.0)	7 (5.1)	0 (0.0)	0.427	0.116	1.000	0.057
	18.5-24.9	15 (44.1)	7 (58.3)	3 (30.0)	76 (55.1)	18 (43.9)				
	≥25	14 (41.2)	4 (33.3)	5 (50.0)	55 (39.9)	23 (56.1)				
Age at recruitment (years)	Mean±SD	64.3 ± 11.3	64.6 ± 12.4	58.9 ± 10.1	62.5 ± 11.3	60.9 ± 9.3	0.227	0.415	0.403	0.232
Age at onset (years)	Mean±SD	60.5 ± 12.6	60.8 ± 15.8	56.1 ± 9.3	60.6 ± 12.0	57.8 ± 9.1	0.348	0.190	0.521	0.562
Diagnostic delay (years)#	Median (range)	0.8 [0.1-15.8]	1.0 [0.3-4.3]	0.6 [0.3-1.8]	0.9 [0.1-5.0]	0.9 [0.1-9.3]	0.077	0.614	0.920	0.977
Education (years)	Mean±SD	9.4 ± 4.5	12.2 ± 4.7	9.4 ± 3.9	10.3 ± 4.1	11.1 ± 5.2	0.142	0.290	0.236	0.105
Age at start coffee consumption (years)	Mean±SD	---	22.8 ± 12.2	20.0 ± 8.5	22.1 ± 9.1	18.1 ± 5.5	0.459	0.010	---	---
Duration of coffee consumption (years)	Mean±SD	---	39.8 ± 18.3	37.0 ± 13.9	40.4 ± 13.8	42.8 ± 10.5	0.634	0.318	---	---
Coffee cups-year#, §	Median (range)	---	84.9 [4.0-119.9]	159.9 [60.0-303.8]	81.9 [2.0-119.9]	187.9 [77.9-341.8]	<0.001	<0.001		
ΔFS#	Median (range)	0.6 [0.1-5.3]	0.6 [0.1-1.5]	1.1 [0.1-2.4]	0.6 [0.0-4.3]	0.4 [0.1-3.4]	0.267	0.646	0.746	0.716

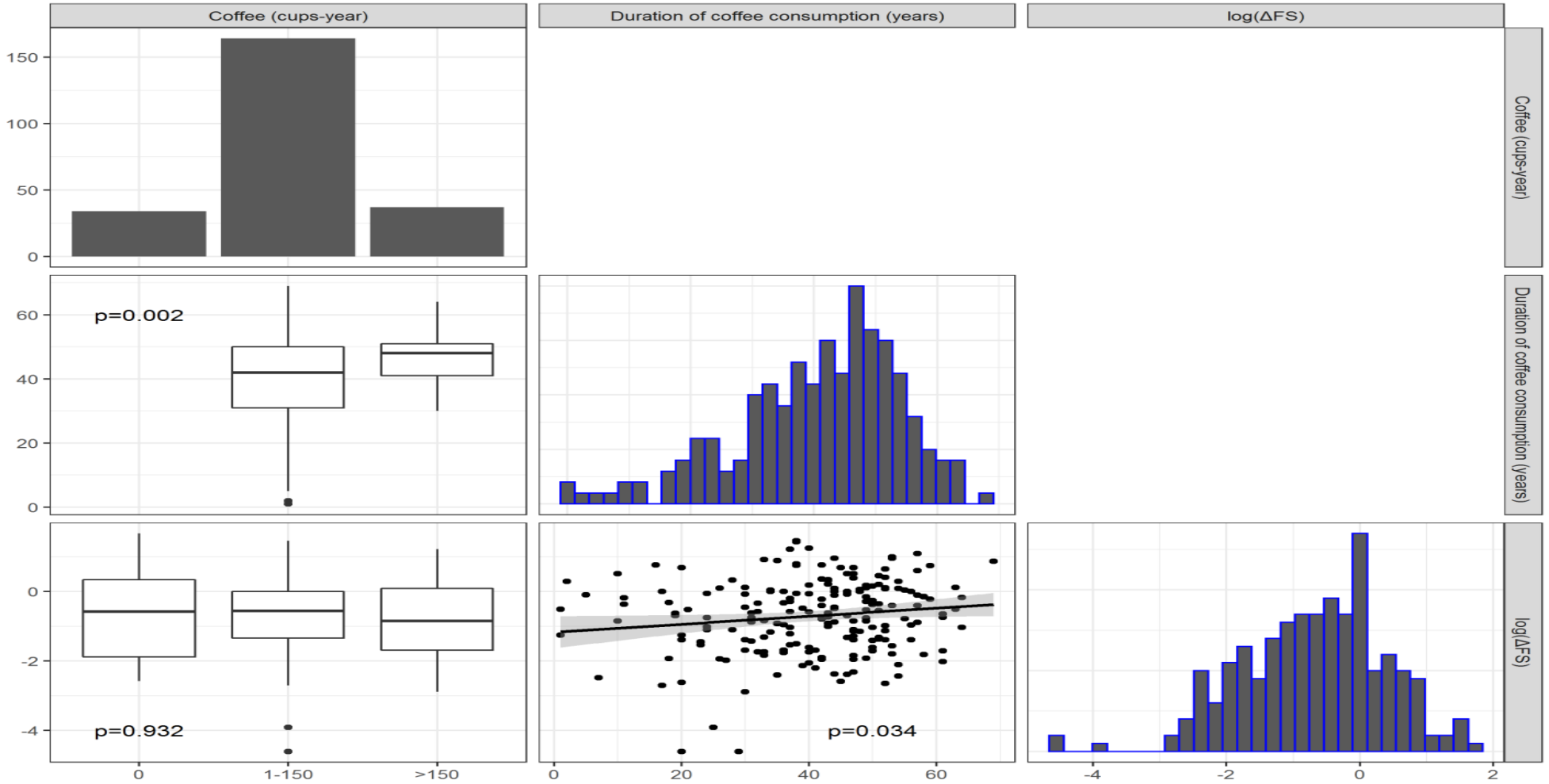

Figure 4.1 Plot matrix depicting pairwise associations between coffee consumption, duration of coffee consumption and log-transformed ΔFS (lower diagonal elements). Comparisons with coffee consumption (cups-year) are reported as boxplots, whereas the correlation between log-transformed ΔFS and duration of coffee consumption is reported as a scatterplot with fitted regression line. The distribution of each variable at issue is reported as bar chart or histograms in the diagonal. Only consumers and former consumers are considered. *Correlation between log-ΔFS and duration of coffee consumption: R= 0.15 (p=0.034).*

4.3 Tea Consumption

Paper I

Current tea consumers were 101 (41.9%), 6 (2.5%) patients were former-consumers, and 134 (55.6%) non-consumers. Among 107 current and former consumers, 27 (25.2%) consumed only green tea, 51 (47.7%) other types of tea, and 29 (27.1%) consumed both. No patients started consuming tea after the ALS diagnosis. Table 4.3 shows unadjusted comparisons of clinical variables among tea consumers, non-consumers, and former-consumers according to the number of the mean daily cups during lifetime categories. The Median ΔFS score was similar among all categories. We found no significant differences in the rate of disease progression between tea consumers and non-consumers. All clinical factors were equally distributed across the categories. Pairwise associations between cup-years, duration of tea consumption, and log-transformed ΔFS were assessed, and results are reported in Figure 4.2. Log-ΔFS was weakly correlated only with the duration of consumption of other types of tea ($r=0.25$, $p=0.028$). The number of cups-year was associated with the duration ($p<0.001$).

Table 4.3 Clinical variables according to tea consumption status

Variable	Category	Non-consumers (N=134)	Former tea consumers (N=6)	Current tea consumers (N=101)	Comparisons (p-values)			
					Overall	Former consumers vs. non-consumers	Current consumers vs. non-consumers	Current consumers vs. former-consumers
Country - N (%)	Italy Republic of Moldova Romania	128 (95.5) 2 (1.5) 4 (3.0)	6 (100.0) 0 (0.0) 0 (0.0)	72 (71.3) 20 (19.8) 9 (8.9)	<0.001	1.000	<0.001	0.616
Gender - N (%)	Males Females	87 (64.9) 47 (35.1)	2 (33.3) 4 (66.7)	56 (55.4) 45 (44.6)	0.154	0.190	0.177	0.409
BMI - N (%)	<18.5 18.5-24.9 ≥25	6 (4.5) 62 (46.3) 66 (49.3)	1 (16.7) 2 (33.3) 3 (50.0)	8 (7.9) 57 (56.4) 36 (35.6)	0.123	0.423	0.097	0.333
Age at recruitment (years)	Mean±SD	64.2 ± 10.5	55.7 ± 13.0	60.4 ± 11.1	0.009	0.058	0.007	0.300
Age at onset (years)	Mean±SD	61.5 ± 11.1	51.9 ± 14.1	58.3 ± 12.2	0.028	0.048	0.038	0.188
Diagnostic delay (years)[#]	Median (range)	0.9 [0.1-15.8]	0.8 [0.2-3.0]	0.8 [0.1-9.3]	0.588	0.657	0.326	0.895
Education (years)	Mean±SD	10.2 ± 4.7	11.7 ± 4.7	10.7 ± 3.9	0.521	0.419	0.362	0.605
Age at start tea consumption (years)	Mean±SD	---	24.8 ± 13.6	23.8 ± 18.5	0.893	---	---	0.893
Duration of (green tear or other types of tea) tea consumption (years)	Mean±SD	---	28.7 ± 15.0	36.6 ± 17.1	0.268	---	---	0.268
Other types of tea daily dose- N (%)	0 cups/day[*] 1-2 cups/day[*] >2 cups/day[*]	---	3 (50.0) 3 (50.0) 0 (0.0)	24 (23.8) 75 (74.3) 2 (2.0)	0.265	---	---	0.265

Green tea daily dose- N (%)	0 cups/day* 1-2 cups/day* >2 cups/day*	---	3 (50.0) 3 (50.0) 0 (0.0)	48 (47.5) 51 (50.5) 2 (2.0)	1.000	---	---	1.000
Other tea cups-year#, §	Median (range)	---	14.0 [0.0-58.0]	50.0 [0.0-187.9]	0.152	---	---	0.152
Green tea cups-year#, §	Median (range)	---	4.0 [0.0-89.9]	4.0 [0.0-187.9]	0.926	---	---	0.926
ΔFS#	Median (range)	0.6 [0.0-5.3]	0.5 [0.0-4.3]	0.6 [0.0-4.2]	0.639	0.356	0.931	0.345

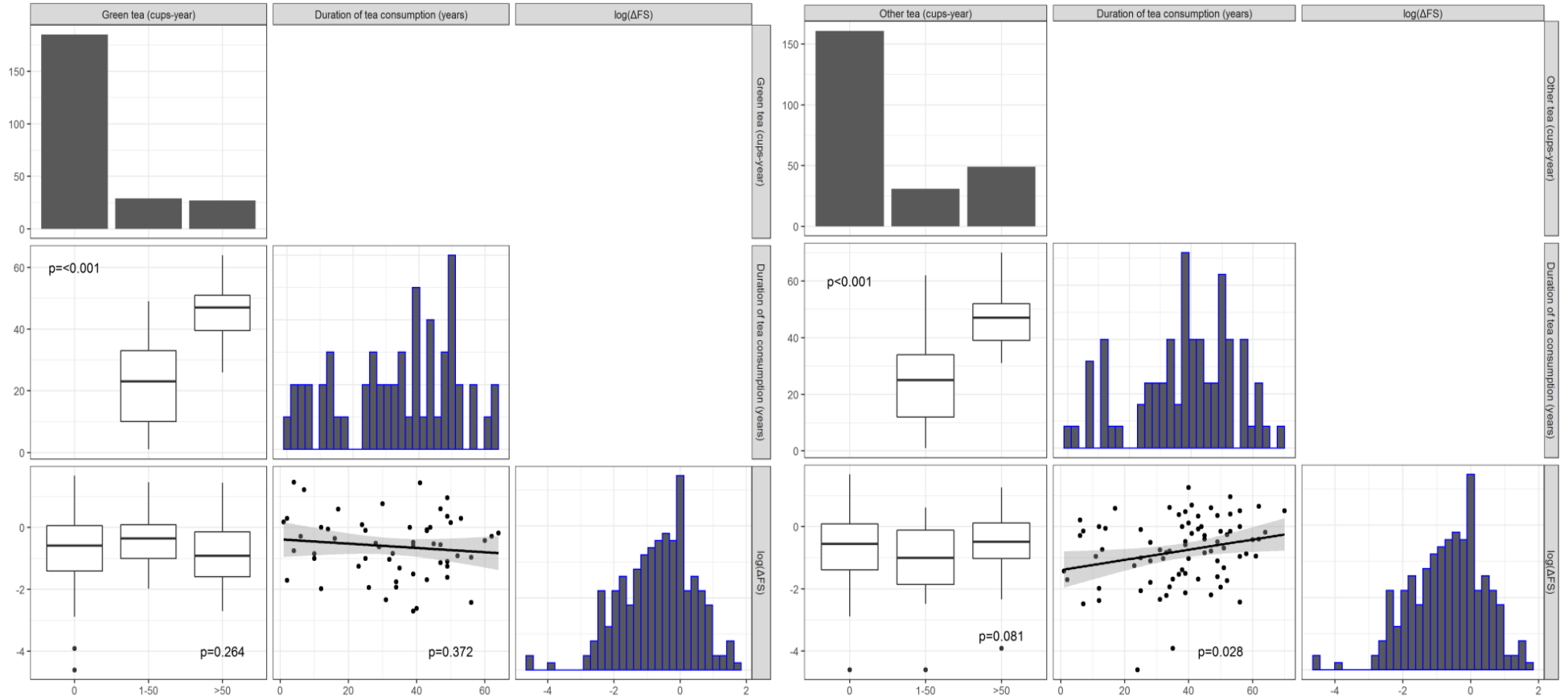

Figure 4.2 Plot matrices depicting pairwise associations between long life tea consumption (green tea and other types of tea in the left (A) and right (B) panels, respectively), duration of tea consumption and log-transformed ΔFS (lower diagonal elements). Comparisons with tea consumption (cups-year) are reported as boxplots whereas the association between log-transformed ΔFS and duration of tea consumption is reported as a scatterplot with fitted regression line. Distribution of each variable at issue is reported as bar chart or histograms in the diagonal. Only consumers and ex-consumers are considered. *Correlation between log-ΔFS and duration of tea consumption: A) green tea R= -0.12 (p=0.372); B) other types of tea R= 0.25 (p=0.028).*

4.4 Predictors of ΔFS_Paper I

The VIMP provided by the conditional RF algorithm that we used to detect the variables most associated with (log-transformed) ΔFS, suggested that diagnostic delay, age at onset, El Escorial category, and education were covariates, which explained the largest amount of the log-ΔFS variance (Table 4.4). Specifically, the diagnostic delay was the strongest predictor, achieving the highest VIMP of 0.61 at the top of VIMP list, whereas all the lifestyle variables (coffee, tea, alcohol drinking, and smoking status) were at the bottom of the list, with the only exception of duration of coffee consumption, although with a VIMP close to zero. Association between coffee and tea consumption (mean daily cups/day) on the log ΔFS was eventually assessed both in a univariable and multivariable analysis, adjusting ANOVA models for the four possible confounders (diagnostic delay, age at onset, El-Escorial criteria, education), alone or in combination. Results are reported in Table 4.5, log-ΔFS least-square means did not significantly vary across coffee and tea consumption groups.

Table 4.4 Variable importance (VIMP) and relative variable importance (RVIMP) values from conditional Random Forest algorithm.

Rank	Variable	VIMP	RVIMP
1	Diagnostic delay	0.6131	100.0%
2	Age at onset	0.1501	24.5%
3	Escorial ALS	0.0426	6.9%
4	Education	0.0303	4.9%
5	Site of onset	0.0077	1.3%
6	Coffee duration	0.0031	0.5%
7	Country	0.0020	0.3%
8	Other types of tea (cups/day)	0.0004	0.1%
9	Riluzole	0.0003	0.0%
10	Gender	0.0003	0.0%
11	BMI	0.0002	0.0%
12	Current alchool drinker	0.0001	0.0%
13	Tea duration	0.0000	0.0%
14	Green tea (cups/day)	0.0000	0.0%
15	Other types of tea (cups-year)	0.0000	0.0%
16	Green tea (cups-year)	0.0000	0.0%
17	Coffee (cups/day)	0.0000	0.0%
18	Tea consumption status	0.0000	0.0%
19	Current smokers	0.0000	0.0%
20	Coffee (cups-year)	0.0000	0.0%
21	Coffee consumption status	0.0000	0.0%

Random Forest algorithm (100'000 trees) of each candidate clinical, demographical, pathological, treatment and coffee/tea consumption variables in explaining the variability of the ΔFS (log values).

Table 4.5 Association between coffee and tea consumption (mean daily cups per day groups) on (log transformed) ΔFS.

		Least square means (95%CI)			
Exposure	**Confounders**	**Group 1**	**Group 2**	**Group 3**	**p-value**
Coffee (groups)	None (unadjusted)	-0.66 (-1.03,-0.30)	-0.69 (-0.86,-0.52)	-0.69 (-0.99,-0.39)	0.991
1: 0 cups/day	Diagnostic delay*	-0.64 (-0.94,-0.35)	-0.69 (-0.83,-0.55)	-0.70 (-0.94,-0.45)	0.986
2: 1-3 cups/day	Age at onset	-0.68 (-1.02,-0.34)	-0.71 (-0.87,-0.55)	-0.61 (-0.88,-0.33)	0.989
3:>3 cups/day	El Escorial ALS	-0.75 (-1.11,-0.38)	-0.74 (-0.92,-0.57)	-0.73 (-1.03,-0.44)	0.990
	Education	-0.71 (-1.07,-0.35)	-0.68 (-0.85,-0.52)	-0.67 (-0.97,-0.38)	0.990
	Diagnostic delay* + Age at onset	-0.66 (-0.94,-0.39)	-0.71 (-0.84,-0.58)	-0.62 (-0.85,-0.40)	0.984
	Diagnostic delay* + El Escorial ALS	-0.73 (-1.02,-0.44)	-0.74 (-0.88,-0.60)	-0.74 (-0.98,-0.51)	0.985
	Diagnostic delay* + Education	-0.70 (-0.99,-0.40)	-0.69 (-0.82,-0.55)	-0.68 (-0.92,-0.44)	0.985
	Diagnostic delay* + Age at onset + El Escorial ALS	-0.73 (-1.00,-0.46)	-0.75 (-0.88,-0.62)	-0.67 (-0.88,-0.45)	0.982
	Diagnostic delay* + Age at onset + El Escorial ALS + Education	-0.75 (-1.02,-0.48)	-0.74 (-0.87,-0.62)	-0.66 (-0.88,-0.45)	0.982
Green tea (groups)	None (unadjusted)	-0.71 (-0.86,-0.55)	-0.61 (-0.90,-0.33)	---	0.568
1: 0 cups/day	Diagnostic delay*	-0.69 (-0.81,-0.56)	-0.68 (-0.92,-0.45)	---	0.483
2: 1-2 cups/day	Age at onset	-0.73 (-0.87,-0.58)	-0.55 (-0.81,-0.28)	---	0.538
	El Escorial ALS	-0.76 (-0.92,-0.60)	-0.68 (-0.97,-0.39)	---	0.562
	Education	-0.71 (-0.86,-0.55)	-0.62 (-0.90,-0.34)	---	0.563
	Diagnostic delay* + Age at onset	-0.71 (-0.82,-0.59)	-0.62 (-0.84,-0.41)	---	0.446
	Diagnostic delay* + El Escorial ALS	-0.74 (-0.86,-0.61)	-0.74 (-0.97,-0.51)	---	0.466
	Diagnostic delay* + Education	-0.69 (-0.81,-0.56)	-0.69 (-0.91,-0.46)	---	0.472
	Diagnostic delay* + Age at onset + El Escorial ALS	-0.75 (-0.86,-0.63)	-0.67 (-0.88,-0.45)	---	0.430
	Diagnostic delay* + Age at onset + El Escorial ALS + Education	-0.74 (-0.86,-0.63)	-0.67 (-0.89,-0.46)	---	0.428
Other types of tea (groups)	None (unadjusted)	-0.64 (-0.81,-0.47)	-0.78 (-1.02,-0.54)	---	0.351
1: 0 cups/day	Diagnostic delay*	-0.63 (-0.76,-0.49)	-0.81 (-1.00,-0.61)	---	0.251
2: 1-2 cups/day	Age at onset	-0.67 (-0.83,-0.51)	-0.72 (-0.94,-0.49)	---	0.317
	El Escorial ALS	-0.71 (-0.88,-0.54)	-0.80 (-1.04,-0.56)	---	0.344

Education	-0.65 (-0.82,-0.49)	-0.76 (-0.99,-0.52)	---	0.345
Diagnostic delay* + Age at onset	-0.65 (-0.78,-0.53)	-0.75 (-0.93,-0.57)	---	0.214
Diagnostic delay* + El Escorial ALS	-0.70 (-0.84,-0.56)	-0.82 (-1.01,-0.63)	---	0.234
Diagnostic delay* + Education	-0.64 (-0.77,-0.50)	-0.79 (-0.97,-0.60)	---	0.240
Diagnostic delay* + Age at onset +El Escorial ALS	-0.71 (-0.84,-0.59)	-0.76 (-0.94,-0.58)	---	0.199
Diagnostic delay* + Age at onset + El Escorial ALS + Education	-0.72 (-0.84,-0.59)	-0.75 (-0.93,-0.58)	---	0.197

Unadjusted and adjusted least square means from ANOVA models.

4.5 Cigarette Smoking

Paper II

Current smokers were 44 (18.3%), 187 (77.6%) were non-smokers, and 10 (4.1%) were former smokers. No patients started smoking after the ALS diagnosis. No difference was found for the status and modalities of smoking (Table 4.6). Table 4.7 shows unadjusted comparisons of clinical variables according to the intensity of smoking (cigarettes/day) categories. Former smokers were excluded from the analysis. Never smokers had a significantly higher age at ALS onset than current smokers, and a lower, although not statistically significant, ΔFS. All the other clinical factors (gender, BMI, FVC, El Escorial category), except the site of onset, were equally distributed across the categories. Pairwise associations between cigarettes/day, pack-years, duration of smoking, and log-transformed ΔFS (i.e., log-ΔFS) are reported in Figure 4.3. The log-ΔFS was not correlated with the duration of smoking ($r=0.13$, $p=0.406$), nor was it different between classes of cigarettes/day and pack-years. As expected, the number of pack-years was associated with the duration.

Table 4.6 Clinical and exposure variables overall and according to the tertiles of ΔFS.

Variable	Category	All (N=241)	I: Slow progression rate of disease (N=81)	II: Medium progression rate of disease (N=80)	III: Fast progression rate of disease (N=80)	p-value	SMD
Country - N(%)	Italy Moldova/Romania	206 (85.5) 35 (14.5)	71 (87.7) 10 (12.3)	67 (83.8) 13 (16.2)	68 (85.0) 12 (15.0)	0.762	0.074
Gender - N(%)	Males Females	145 (60.2) 96 (39.8)	53 (65.4) 28 (34.6)	44 (55.0) 36 (45.0)	48 (60.0) 32 (40.0)	0.401	0.143
Age at recruitment (years)	Mean±SD	62.4 ± 11.0	59.8 ± 12.3	63.6 ± 10.4	63.9 ± 9.8	0.032	0.241
Age at disease onset (years)	Mean±SD	59.9 ± 11.8	54.6 ± 12.9	62.0 ± 10.5	63.2 ± 9.8	<0.001	0.502
Diagnostic delay (years)	Median (range)	0.9 (0.1-15.8)	1.7 (1.0-2.8)	0.8 (0.5-1.1)	0.5 (0.3-0.8)	<0.001	0.820
Education (years)	Mean±SD	10.4 ± 4.4	11.1 ± 4.4	10.6 ± 4.3	9.5 ± 4.2	0.058	0.248
Site of onset - N(%)	Spinal Bulbar	187 (77.6) 54 (22.4)	71 (87.7) 10 (12.3)	53 (66.2) 27 (33.8)	63 (78.8) 17 (21.2)	0.005	0.349
Escorial ALS - N(%)	Definite Possible Probable Suspected	74 (30.7) 55 (22.8) 77 (32.0) 35 (14.5)	16 (19.8) 23 (28.4) 26 (32.1) 16 (19.8)	25 (31.2) 23 (28.7) 23 (28.7) 9 (11.2)	33 (41.2) 9 (11.2) 28 (35.0) 10 (12.5)	0.014	0.460
FVC - N(%)	<80% ≥80%	88 (43.8) 113 (56.2)	20 (29.0) 49 (71.0)	32 (47.1) 36 (52.9)	36 (56.2) 28 (43.8)	0.005	0.379
BMI - N(%)	<18.5 18.5-24.9 ≥25	15 (6.2) 121 (50.2) 105 (43.6)	5 (6.2) 42 (51.9) 34 (42.0)	4 (5.0) 40 (50.0) 36 (45.0)	6 (7.5) 39 (48.8) 35 (43.8)	0.967	0.083
Riluzole - N(%)	Yes No	129 (53.5) 112 (46.5)	41 (50.6) 40 (49.4)	47 (58.8) 33 (41.2)	41 (51.2) 39 (48.8)	0.517	0.109
Alcoholic drinking status - N(%)	Current drinker Former drinker	147 (61.0) 5 (2.1)	49 (60.5) 1 (1.2)	52 (65.0) 3 (3.8)	46 (57.5) 1 (1.2)	0.599#	0.173

	Non-drinker	89 (36.9)	31 (38.3)	25 (31.2)	33 (41.2)		
Smoking status - N(%)	Current smoker	44 (18.3)	12 (14.8)	12 (15.0)	20 (25.0)	0.326[#]	0.226
	Former smoker	10 (4.1)	3 (3.7)	5 (6.2)	2 (2.5)		
	Non-smoker	187 (77.6)	66 (81.5)	63 (78.8)	58 (72.5)		
Age at start smoking (years)	Mean±SD	17.0 ± 4.2	17.4 ± 4.0	18.1 ± 5.1	15.9 ± 3.5	0.252	0.353
Age at start drinking (years)	Mean±SD	19.7 ± 7.4	20.0 ± 6.7	18.4 ± 5.6	21.0 ± 9.4	0.192	0.240

SD: standard deviation; p-values from ANOVA models or Chi-Square (with continuity correction) statistics for continuous and categorical variables, respectively. [#]p-values from Fisher exact test. SMD: standardized mean difference (i.e. the average of all possible standardized mean differences). Tertiles of ΔFS distribution were ≤ 0.333 (I); $0.334 - 0.875$ (II); >0.875 (III).

Table 4.7 Clinical variables according to intensity of smoking during lifetime. Former smokers were excluded from the analysis.

Variable	Category	I: Non-smokers (N=187)	II: ≤14° cigarettes per day* (N=21)	III: >14° cigarettes per day* (N=23)	II vs. I (p-value)	III vs. I (p-value)	III vs. II (p-value)
Country - N(%)	Italy Moldova/Romania	157 (84.0) 30 (16.0)	21 (100.0) 0 (0.0)	19 (82.6) 4 (17.4)	0.049	0.772	0.109
Gender - N(%)	Males Females	103 (55.1) 84 (44.9)	16 (76.2) 5 (23.8)	18 (78.3) 5 (21.7)	0.102	0.043	1.000
BMI (Kg/m^2) - N(%)	<18.5 18.5-24.9 ≥25	11 (5.9) 94 (50.3) 82 (43.9)	2 (9.5) 8 (38.1) 11 (52.4)	2 (8.7) 13 (56.5) 8 (34.8)	0.426	0.596	0.506
Age at recruitment (years)	Mean±SD	63.9±10.8	55.5±12.1	58.3±8.6	0.001	0.017	0.396
Age at disease onset (years)	Mean±SD	61.3±11.8	54.0±12.4	56.6±8.1	0.006	0.067	0.457
Diagnostic delay (years)#	Median (range)	0.9 [0.1-9.3]	0.7 [0.1-4.0]	0.6 [0.1-4.1]	0.322	0.174	0.810
Education (years)	Mean±SD	10.5±4.5	10.8±4.3	10.0±3.3	0.778	0.593	0.544
Site of onset - N(%)	Spinal Bulbar	45 (24.1) 142 (75.9)	0 (0.0) 21 (100.0)	7 (30.4) 16 (69.6)	0.009	0.608	0.009
Escorial ALS - N(%)	Definite Possible Probable Suspected	61 (32.6) 45 (24.1) 57 (30.5) 24 (12.8)	6 (28.6) 5 (23.8) 6 (28.6) 4 (19.0)	4 (17.4) 4 (17.4) 11 (47.8) 4 (17.4)	0.862	0.244	0.590
FVC - N(%)	<80% ≥80%	69 (45.1) 84 (54.9)	9 (45.0) 11 (55.0)	7 (38.9) 11 (61.1)	1.000	0.803	0.752
ΔFS#	Median (range)	0.6 [0.0-5.3]	0.5 [0.0-2.4]	0.9 [0.1-2.7]	0.990	0.129	0.262

SD: standard deviation; p-values were reported from pairwise contrasts defined in ANOVA models or Fisher exact test from continuous and categorical variables, respectively; #log-transformed variable was used in the ANOVA model (because of skewed distribution); °Median cut-off; *The smoking intensity was computed as the weighted mean of the number of; cigarettes smoked per day at different age periods, with weights equal to the smoking duration within each age period.

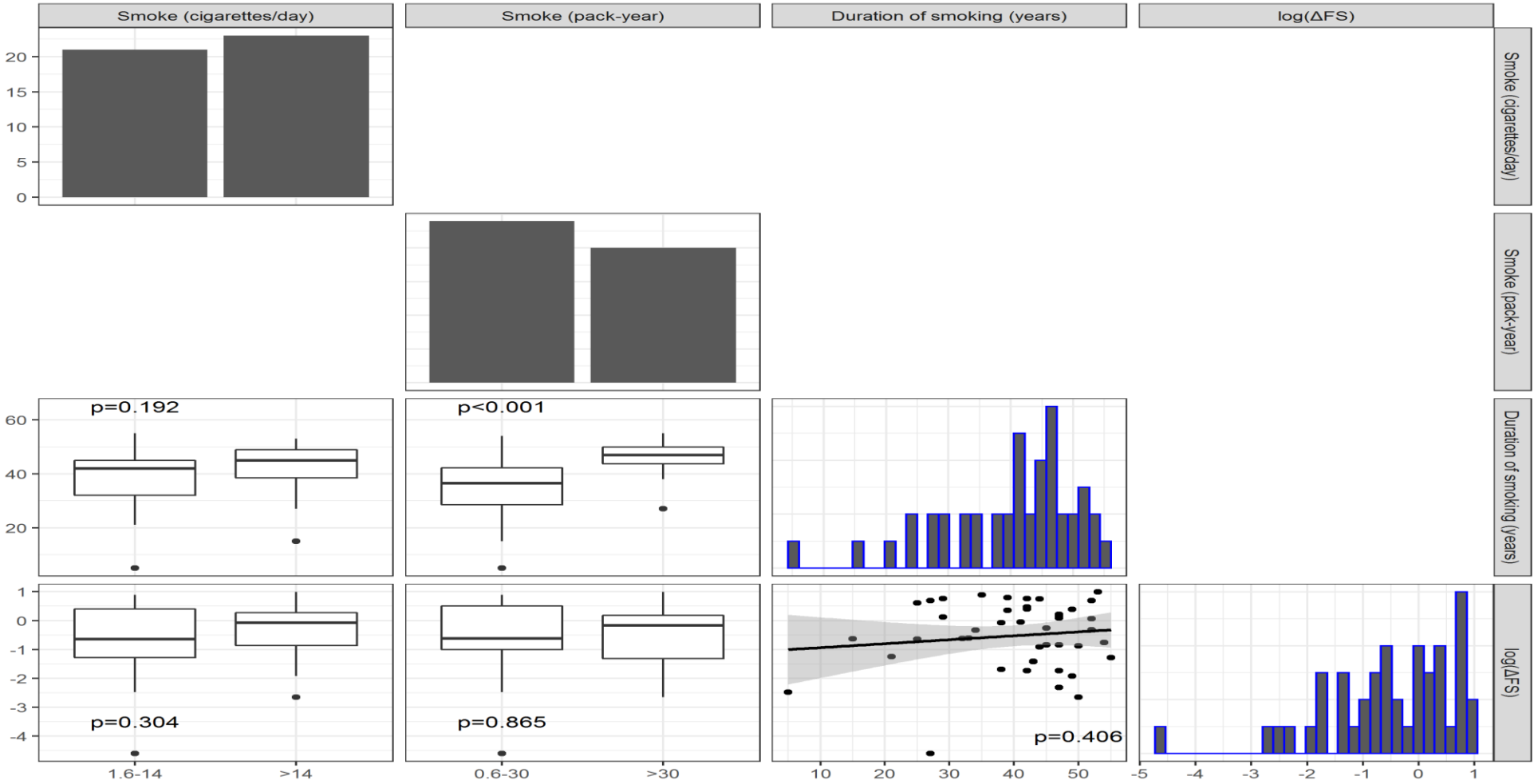

Figure 4.3 Plot matrix depicting pairwise associations between smoking load (i.e., cigarettes/day and pack-years), duration of smoking and log-transformed ΔFS (lower diagonal elements). Comparisons with smoking loads are reported as boxplots, whereas the correlation between log-transformed ΔFS and duration of smoking is reported as a scatterplot with fitted regression line. The distribution of each variable at issue is reported as bar chart or histograms in the diagonal. Only current smokers are considered for the present analysis.

4.6 Alcohol Drinking

Paper II

Current alcohol drinkers were 147 (61.0%), 5 patients (2.1%) were former-drinkers, and 89 (36.9%) non-drinkers. No patients started drinking alcohol after the ALS diagnosis. Table 4.8 shows unadjusted comparisons of clinical variables among non-drinkers and drinkers according to the intensity (drinks/day) categories. Former drinkers were excluded from the analysis. The disease rate of progression (median ΔFS score) was similar among all categories. All the clinical factors were equally distributed across the categories. Pairwise associations between drinks/day, drink-years, duration of alcohol consumption, and log-transformed ΔFS were assessed, and results are reported in Figure 4.4. The log-ΔFS was weakly (but statistically significant) correlated only with the duration of alcohol consumption ($r=0.18$, $p=0.028$), but not with the number of drinks/day or drink-years. As expected, the number of drink-years was associated with the duration.

Since a previous multicenter case-control study (Ovidio *et al.*, 2019) found an intriguing difference in the ALS risk between patients from Apulia Region (increased) and other areas (decreased or neutral), we analyzed separately the subset of patients from this Region (See Appendix E). However, no difference in the disease progression was found for exposure to alcoholic beverages, only wine, or smoking.

Table 4.8 Clinical variables according to intensity of alcohol intake during lifetime. Former drinkers were excluded from the analysis.

Variable	Category	I: Non-drinkers (N=89)	II: ≤1° drinks per day* (N=73)	III: >1° drinks per day* (N=74)	II vs. I (p-value)	III vs. I (p-value)	III vs. II (p-value)
Country - N(%)	Italy	75 (84.3)	57 (78.1)	70 (94.6)	0.319	0.045	0.004
	Moldova/Romania	14 (15.7)	16 (21.9)	4 (5.4)			
Gender - N(%)	Males	41 (46.1)	41 (56.2)	60 (81.1)	0.211	<0.001	0.001
	Females	48 (53.9)	32 (43.8)	14 (18.9)			
BMI (Kg/m^2) - N(%)	<18.5	6 (6.7)	7 (9.6)	1 (1.4)	0.719	0.237	0.062
	18.5-24.9	45 (50.6)	38 (52.1)	37 (50.0)			
	≥25	38 (42.7)	28 (38.4)	36 (48.6)			
Age at recruitment (years)	Mean±SD	62.7±11.1	59.2±11.5	65.3±9.7	0.044	0.120	0.001
Age at disease onset (years)	Mean±SD	60.1±12.2	56.8±12.3	62.9±10.1	0.071	0.121	0.001
Diagnostic delay (years)#	Median (range)	0.7 [0.1-9.3]	0.9 [0.1-7.5]	1.0 [0.1-15.8]	0.560	0.239	0.571
Education (years)	Mean±SD	10.4±4.5	11.0±4.3	9.9±4.4	0.342	0.454	0.104
Site of onset - N(%)	Spinal	63 (70.8)	55 (75.3)	65 (87.8)	0.596	0.012	0.058
	Bulbar	26 (29.2)	18 (24.7)	9 (12.2)			
Escorial ALS - N(%)	Definite	31 (34.8)	14 (19.2)	27 (36.5)	0.008	0.576	0.005
	Possible	16 (18.0)	19 (26.0)	19 (25.7)			
	Probable	25 (28.1)	34 (46.6)	16 (21.6)			
	Suspected	17 (19.1)	6 (8.2)	12 (16.2)			
FVC - N(%)	<80%	30 (41.1)	24 (43.6)	32 (46.4)	0.857	0.612	0.856
	≥80%	43 (58.9)	31 (56.4)	37 (53.6)			
ΔFS#	Median (range)	0.6 [0.0-5.3]	0.6 [0.0-4.3]	0.5 [0.1-4.8]	0.795	0.720	0.926

SD: standard deviation; p-values were reported from pairwise contrasts defined in ANOVA models or Fisher exact test from continuous and categorical variables, respectively; #log-transformed variable was used in the ANOVA model (because of skewed distribution); °Median cut-off; *The drinking intensity was computed as the weighted mean number of standard alcoholic units per day at different age periods with weights equal to the number of years spent drinking (i.e., drinking duration) within each age period for all type of beverages.

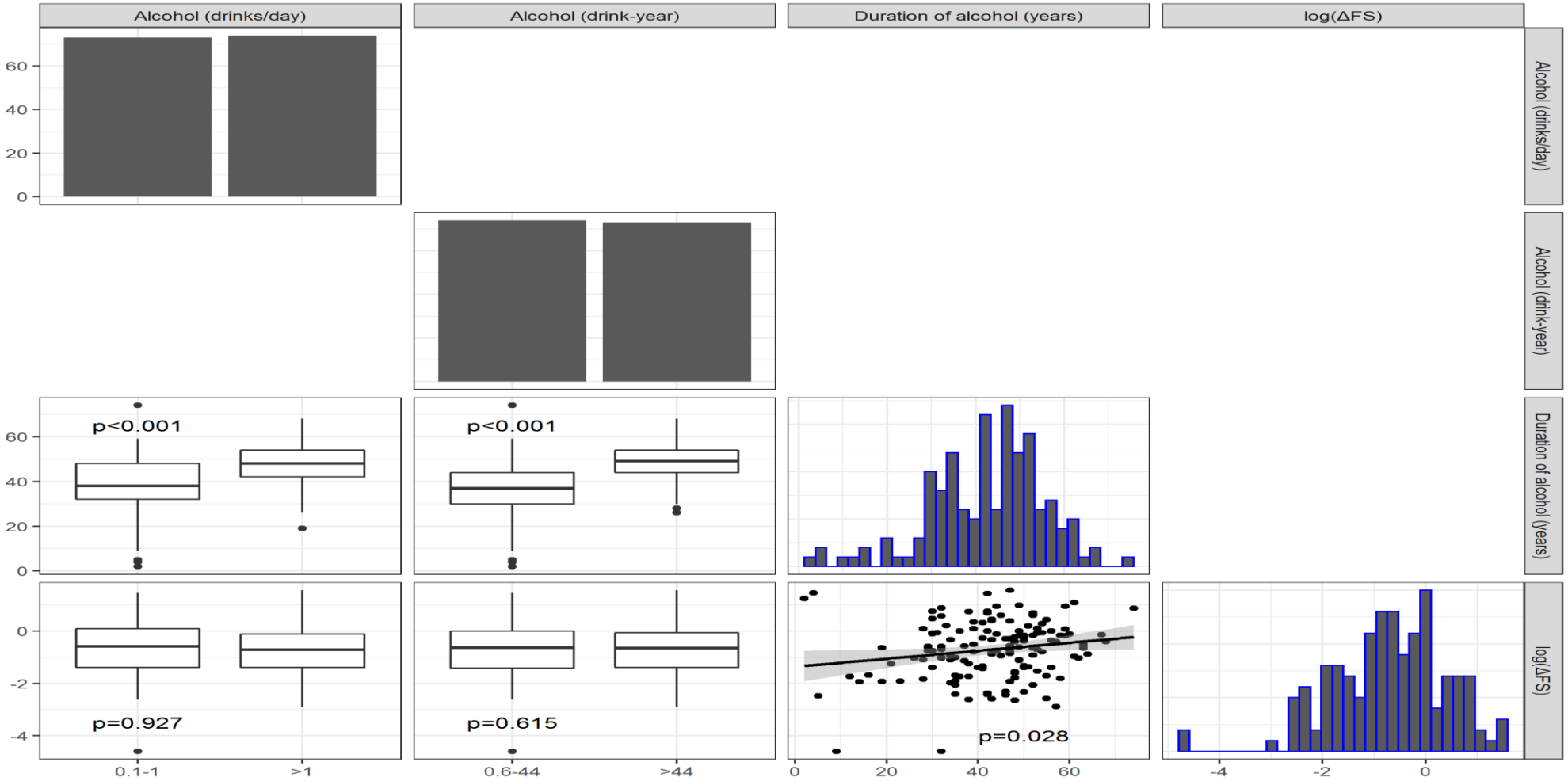

Figure 4.4 Plot matrices are depicting pairwise associations between alcohol loads (drinks/day and drink-year), duration of alcohol consumption and log-transformed ΔFS (lower diagonal elements). Comparisons are reported as boxplots whereas the association between log-transformed ΔFS and duration of alcohol consumption is reported as a scatterplot with fitted regression line. The distribution of each variable at issue is reported as bar chart or histogram in the diagonal. Only current drinkers are considered for the present analysis.

4.7 Predictors of ΔFS_Paper II

The VIMP provided by the conditional RF algorithm that we used to detect the variables most associated with ΔFS suggested that diagnostic delay, age at onset, El Escorial category, and education were the covariates that explained the largest amount of the log-ΔFS variance (Table 4.9). Specifically, the diagnostic delay was the strongest predictor, achieving the highest VIMP of 0.63, followed by age at onset and El Escorial classification, whereas drinking and smoking status were at the bottom of the list. The association between smoking and alcohol intensity (drinks/day) on the log ΔFS was eventually assessed both in a univariable and multivariable analysis, adjusting ANOVA models for four possible confounders (gender, age at onset, education, and diagnostic delay), both alone and in combination. Results are reported in Table 4.10: ΔFS least-square means (i.e., back-transformed on the original scale) did not significantly vary across smoking and alcohol consumption groups.

Table 4.9 Variable importance (VIMP) and relative variable importance (RVIMP) values from conditional Random Forest algorithm.

Variable	Conditional VIMP	Conditional RVIMP
Diagnostic delay	0.6302	100.0%
Age at onset	0.1680	26.7%
Escorial	0.0413	6.6%
Education	0.0278	4.4%
Site of onset	0.0072	1.1%
Alcohol (cups/year)	0.0043	0.7%
Alcohol (cups/day)	0.0043	0.7%
Smoke (cigarettes/day)	0.0016	0.3%
Country	0.0014	0.2%
Riluzole	0.0007	0.1%
Alcohol duration	0.0005	0.1%
Smoke (packs/year)	0.0002	0.0%
BMI	0.0000	0.0%
Smoking duration	0.0000	0.0%
Alcohol drinking status	0.0000	0.0%
Smoking status	0.0000	0.0%
Gender	0.0000	0.0%

Random Forest algorithm (100'000 trees) of each candidate clinical, demographical, pathological, treatment and smoking/alcohol consumption variables in explaining the variability of the ΔFS (log values). Variables are ranked from the most to the less important (rank). The VIMP of a specific variable is the sum of the decrease in prediction error values (of log-ΔFS) when a tree of the forest splits by that variable whereas RVIMP is the VIMP divided by the highest VIMP value so that values are bounded between 0 and 1 (or similarly between 0 and 100%).

Table 4.10 Association between smoke and alcohol consumption during lifetime on ΔFS.

		Estimated ΔFS means (95%CI)[#]			
Exposure (groups)	**Confounders**	**Group 1**	**Group 2**	**Group 3**	**p-value***
Smoke 1: 0 cigarettes/day 2: 1.5-14 cigarettes/day 3: >14 cigarettes/day	None	0.49 (0.42-0.57)	0.49 (0.31-0.78)	0.71 (0.45-1.10)	0.313
	Age at onset	0.47 (0.41-0.54)	0.61 (0.40-0.95)	0.80 (0.53-1.22)	0.255
	Gender	0.49 (0.42-0.58)	0.51 (0.32-0.81)	0.73 (0.47-1.16)	0.313
	Education	0.49 (0.42-0.57)	0.49 (0.31-0.78)	0.69 (0.44-1.07)	0.303
	Diagnostic delay (log)	0.51 (0.44-0.57)	0.44 (0.30-0.64)	0.60 (0.42-0.87)	0.174
	Age at onset + Gender	0.47 (0.41-0.55)	0.65 (0.42-1.01)	0.86 (0.56-1.31)	0.252
	Age at onset + education	0.47 (0.41-0.54)	0.61 (0.39-0.94)	0.79 (0.52-1.20)	0.255
Alcohol 1: 0 drinks/day 2: 0.1-1 drinks/day 3: >1 drinks/day	None	0.52 (0.41-0.65)	0.50 (0.39-0.64)	0.49 (0.38-0.63)	0.932
	Age at onset	0.52 (0.42-0.64)	0.56 (0.44-0.71)	0.44 (0.35-0.55)	0.921
	Gender	0.52 (0.41-0.65)	0.50 (0.39-0.64)	0.51 (0.39-0.66)	0.932
	Education	0.52 (0.41-0.65)	0.51 (0.40-0.66)	0.48 (0.37-0.61)	0.930
	Diagnostic delay (log)	0.49 (0.41-0.59)	0.50 (0.41-0.61)	0.52 (0.42-0.64)	0.899
	Age at onset + Gender	0.51 (0.42-0.64)	0.56 (0.44-0.71)	0.46 (0.36-0.58)	0.921
	Age at onset + education	0.52 (0.42-0.64)	0.56 (0.44-0.71)	0.44 (0.35-0.55)	0.921

Results from ANOVA models. Former consumers were excluded from the analysis.

* p-value from ANOVA model (Type 3 test); [#]log-transformed ΔFS values were used in the ANOVA models and their means were back-transformed on their original scales.

4.8 Paper III

Demographic and Clinical Data

We recruited 208 patients, 139 women, and 69 men, with a sex ratio of 2:1. The main demographic and clinical characteristics are presented in Table 4.11, categorized in the three tertiles of the MSSS distribution. Patients in the lower MSSS tertile were significantly younger at the onset, diagnosis, and recruitment and had a longer disease duration. Progressive forms were more represented in the worst MSSS tertile.

Table 4.11 Demographic and clinical variables overall and according to tertiles of MSSS distribution

								MSSS III vs. I	
Variable	**Category**	**All (N=208)**	**MSSS I (N=70)**	**MSSS II (N=69)**	**MSSS III (N=69)**	**p-value**	**SMD**	**OR (95%CI)**	**p-value (overall)**
Gender - N(%)	Males	69 (33.2)	22 (31.4)	22 (31.9)	25 (36.2)	0.803*	0.068	1.24 (0.61-2.51)	0.550
	Females	139 (66.8)	48 (68.6)	47 (68.1)	44 (63.8)			Ref	
Age at recruitment (years)	Mean±SD	42.8±11.2	40.7±10.2	41.4±11.4	46.3±11.3	0.005*	0.342	1.05 (1.02-1.09)	0.004
	<30	29 (13.9)	11 (15.7)	13 (18.8)	5 (7.2)	0.182*	0.410	Ref	0.113
	30-39	55 (26.4)	22 (31.4)	18 (26.1)	15 (21.7)			1.50 (0.43-5.21)	
	40-49	64 (30.8)	20 (28.6)	22 (31.9)	22 (31.9)			2.42 (0.72-8.18)	
	50-59	44 (21.2)	15 (21.4)	11 (15.9)	18 (26.1)			2.64 (0.75-9.31)	
	≥60	16 (7.7)	2 (2.9)	5 (7.2)	9 (13.0)			9.90 (1.54-63.68)	
Age at disease onset (years)	Mean±SD	32.0±9.7	29.2±9.1	32.2±8.6	34.5±10.7	0.005*	0.369	1.06 (1.02-1.10)	0.003
Age at diagnosis (years)	Mean±SD	35.0±10.4	32.8±10.2	34.1±9.5	37.6±11.1	0.028*	0.307	1.04 (1.01-1.08)	0.017
Education (years)	Mean±SD	12.1±3.7	12.7±3.4	12.3±3.7	11.3±3.9	0.056*	0.262	0.90 (0.82-0.99)	0.024
Disease duration (years)	Median [IQR]	9.4 [5.0-15.4]	11.0 [5.9-15.4]	7.7 [3.8-11.6]	10.0 [5.2-19.1]	0.037#	0.224	1.01 (0.96-1.05)	0.769
	<5	66 (31.7)	18 (25.7)	27 (39.1)	21 (30.4)	0.283*	0.252	Ref	0.801
	6-10	45 (21.6)	14 (20.0)	17 (24.6)	14 (20.3)			0.86 (0.32-2.27)	
	>10	97 (46.6)	38 (54.3)	25 (36.2)	34 (49.3)			0.77 (0.35-1.68)	
MS clinical form - N(%)	RR	181 (87.0)	67 (95.7)	67 (97.1)	47 (68.1)	<0.001#	0.625	Ref	0.111
	PP	13 (6.2)	3 (4.3)	1 (1.4)	9 (13.0)			4.28 (1.10-16.64)	
	SP	14 (6.7)	0 (0.0)	1 (1.4)	13 (18.8)			NE	
Coffee-drinking status - N(%)	Current drinker	176 (84.6)	60 (85.7)	55 (79.7)	61 (88.4)	0.390#	0.216	1.27 (0.47-3.44)	0.637
	Ex drinker	2 (1.0)	0 (0.0)	2 (2.9)	0 (0.0)			NE	
	Non-drinker	30 (14.4)	10 (14.3)	12 (17.4)	8 (11.6)			Ref	

Tea-drinking status - N(%)	Current drinker	104 (50.0)	40 (57.1)	31 (44.9)	33 (47.8)	0.390#	0.230	0.68 (0.35-1.34)	0.269
	Ex drinker	2 (1.0)	1 (1.4)	0 (0.0)	1 (1.4)			NE	
	Non-drinker	102 (49.0)	29 (41.4)	38 (55.1)	35 (50.7)			Ref	
Current drinkers of both coffee and tea - N(%)	Yes	91 (43.8)	33 (47.1)	27 (39.1)	31 (44.9)	0.617*	0.108	0.92 (0.47-1.78)	0.793
	No	117 (56.2)	37 (52.9)	42 (60.9)	38 (55.1)			Ref	
Coffee consumption (cups/day)	0 (non-drinkers)	30 (14.4)	10 (14.3)	12 (17.4)	8 (11.6)	0.785*	0.149	Ref	0.861
	1-3	141 (67.8)	48 (68.6)	43 (62.3)	50 (72.5)			1.30 (0.47-3.58)	
	4-8	37 (17.8)	12 (17.1)	14 (20.3)	11 (15.9)			1.14 (0.33-3.95)	
Coffee consumption (cups-year)	0 (non-drinkers)	30 (14.4)	10 (14.3)	12 (17.4)	8 (11.6)	0.850*	0.139	Ref	0.805
	1-52	92 (44.2)	33 (47.1)	28 (40.6)	31 (44.9)			1.17 (0.41-3.36)	
	53-294	86 (41.3)	27 (38.6)	29 (42.0)	30 (43.5)			1.39 (0.48-4.03)	
Tea consumption (cups/day)	0 (non-drinkers)	102 (49.0)	29 (41.4)	38 (55.1)	35 (50.7)	0.258*	0.183	Ref	0.272
	1-8	106 (51.0)	41 (58.6)	31 (44.9)	34 (49.3)			0.69 (0.35-1.34)	
Tea consumption (cups-year)	0 (non-drinkers)	102 (49.0)	29 (41.4)	38 (55.1)	35 (50.7)	0.241*	0.289	Ref	0.232
	1-53	53 (25.5)	19 (27.1)	13 (18.8)	21 (30.4)			0.49 (0.21-1.14)	
	54-126	53 (25.5)	22 (31.4)	18 (26.1)	13 (18.8)			0.92 (0.42-2.02)	

*p-values from ANOVA models or Chi-Square statistics for continuous and categorical variables, respectively; #p-values from Kruskal-Wallis test or Fisher exact test for continuous and categorical variables, respectively; §info available for 167 patients only; IQR: interquartile range (i.e., first-third quartiles); SMD: standardized mean difference (i.e. the average of all possible standardized mean differences). Tertiles of MSSS distribution were: ≤ 1.53 (I); 1.54 – 3.52 (II); >3.52 (III).

Coffee Consumption

Table 4.12 shows unadjusted comparisons between never and ever-drinkers for coffee (including 176 current and two former drinkers). Thirty-nine patients began to drink coffee after being diagnosed with MS. The mean age at the start of coffee drinking was 19.6 years (SD=7.3). Those consuming 4-8 cups of coffee per day (strong consumers) more frequently had a progressive form and were older, with a higher age at onset and a lower education level than never-drinkers. As expected, strong coffee consumers tend to smoke and drink alcohol more than people who never had drunk coffee. However, the severity of the disease, in terms of MSSS, was similar between people who drunk coffee or not.

Table 4.12 Clinical variables according to coffee consumption (cups/day)

					Comparisons (p-values)		
Variable	**Category**	**Non-drinkers (0 cups/day) (N=30)**	**1-3 cups/day* (N=141)**	**4-8 cups/day* (N=37)**	**1-3 vs. 0 cups/day**	**4-8 vs. 0 cups/day**	**4-8 vs. 1-3 cups/day**
Gender - N(%)	Males Females	10 (33.3) 20 (66.7)	41 (29.1) 100 (70.9)	18 (48.6) 19 (51.4)	0.664	0.225	0.031
Age at recruitment (years)	Mean±SD	36.5±9.9	43.2±11.2	46.5±10.4	0.002	<0.001	0.101
Age at onset (years)	Mean±SD	28.6±8.1	31.5±9.5	36.6±10.3	0.135	0.001	0.003
Education (years)	Mean±SD	13.3±3.9	12.1±3.7	11.4±3.4	0.103	0.034	0.300
MS clinical form - N(%)	RR PP SP	30 (100.0) 0 (0.0) 0 (0.0)	121 (85.8) 9 (6.4) 11 (7.8)	30 (81.1) 4 (10.8) 3 (8.1)	0.118	0.041	0.635
Alcoholic-drinking status - N(%)	Current drinker Ex drinker Non-drinker	17 (56.7) 1 (3.3) 12 (40.0)	108 (76.6) 12 (8.5) 21 (14.9)	28 (75.7) 4 (10.8) 5 (13.5)	0.010	0.041	0.899
Smoking habits - N(%)	Current smoker Ex smoker Non-smoker	8 (26.7) 4 (13.3) 18 (60.0)	34 (24.1) 37 (26.2) 70 (49.6)	19 (51.4) 8 (21.6) 10 (27.0)	0.334	0.027	0.005
MSSS°	Median (range)	2.4 [0.2-9.0]	2.6 [0.0-9.3]	2.1 [0.2-9.4]	0.367	0.526	0.890

SD: standard deviation; p-values were reported from ANOVA models or Fisher exact test from continuous and categorical variables, respectively; #log-transformed variable was used in the ANOVA model; °square root-transformed variable was used in the ANOVA model; *The mean number of cups per day was computed for each patient as the weighted mean of the number of cups drunk within each decade at different ages, with weights equal to the number of years spent drinking within each period, divided by 365.25; §The cup-year is the unit for measuring the amount a person has drunk over a long period of time. One cup-year is the equivalent of 365.25 cups of coffee, and it is calculated by multiplying the number of cups drunk per day by the number of years the person has drunk.

Tea Consumption

Table 4.13 shows unadjusted comparisons among never-drinkers and ever-drinkers for tea (including 104 current and two former drinkers). Twenty-four patients began to drink tea after being diagnosed with MS. The mean age at the start of tea drinking was 13.8 years (SD=8.9). The two groups were not different, except for a slightly higher education level and a lower percentage of smokers in tea drinkers. The MSSS was slight, although not significantly lower in tea drinkers.

Table 4.13 Clinical variables according to tea consumption (cups/day)

Variable	Category	Non-drinkers (0 cups/day) (N=102)	Drinkers (>0 cups/day) (N=106)	p-value
Gender - N(%)	Males	36 (35.3)	33 (31.1)	0.558
	Females	66 (64.7)	73 (68.9)	
Age at recruitment (years)	Mean±SD	43.8±11.4	41.8±10.9	0.206
Age at onset (years)	Mean±SD	32.7±9.4	31.3±10.0	0.283
Education (years)	Mean±SD	11.5±3.7	12.7±3.6	0.020
MS clinical form - N(%)	RR/CIS	90 (88.2)	91 (85.8)	0.907
	PP	6 (5.9)	7 (6.6)	
	SP	6 (5.9)	8 (7.5)	
Alcoholic-drinking status - N(%)	Current drinker	72 (70.6)	81 (76.4)	0.516
	Ex drinker	8 (7.8)	9 (8.5)	
	Non-drinker	22 (21.6)	16 (15.1)	
Smoking habits - N(%)	Current smoker	41 (40.2)	20 (18.9)	0.002
	Ex smoker	23 (22.5)	26 (24.5)	
	Non-smoker	38 (37.3)	60 (56.6)	
Tea type - N(%)	Black (normal)	---	82 (78.8)	---
	Green		6 (5.8)	
	Black+Green		16 (15.4)	
Tea cups-year[#, §]	Median (range)	---	53.0 [2.0-125.9]	---
MSSS[°]	Median (range)	2.8 [0.0-9.4]	2.1 [0.1-9.3]	0.108

SD: standard deviation; p-values were reported from two-sample t test or Fisher exact test from continuous and categorical variables, respectively; #log-transformed variable was used in performing two-sample t test; °square root-transformed variable was in performing two-sample t test; §The cup-year is the unit for measuring the amount a person has drunk over a long period of time. One cup-year is the equivalent of 365.25 cups of coffee and it is calculated by multiplying the number of cups drunk per day by the number of years the person has drunk.

Association between Coffee and Tea Consumption

The association between coffee/tea drinking intensity (drinks/day) on the MSSS was eventually assessed both in a univariable and multivariable analysis, adjusting for four possible confounders (age at onset, education, and alcoholic drinking status), alone or in combination. Results are reported in Table 4.14: MSSS means did not vary significantly across the exposure groups.

Table 4.14 Association between coffee and tea consumption on MSSS among patients who belong into extreme MSSS tertiles groups (III tertile vs. I tertile).

Confounders	Variable	Category	OR (95%CI)	p-value (overall)
None	Coffee consumption (cups/day)	0 (non-drinkers) 1-3 4-8	Ref 1.30 (0.47-3.58) 1.15 (0.33-3.95)	0.861
	Tea consumption (cups/day)	0 (non-drinkers) >0	Ref 0.69 (0.35-1.34)	0.272
Age at onset	Coffee consumption (cups/day)	0 (non-drinkers) 1-3 4-8	Ref 1.27 (0.45-3.61) 0.73 (0.20-2.75)	0.534
	Tea consumption (cups/day)	0 (non-drinkers) >0	Ref 0.73 (0.36-1.45)	0.367
Education	Coffee consumption (cups/day)	0 (non-drinkers) 1-3 4-8	Ref 1.18 (0.42-3.32) 0.88 (0.24-3.17)	0.808
	Tea consumption (cups/day)	0 (non-drinkers) >0	Ref 0.80 (0.40-1.59)	0.520
Age at onset + education	Coffee consumption (cups/day)	0 (non-drinkers) 1-3 4-8	Ref 1.15 (0.40-3.33) 0.60 (0.16-2.34)	0.452
	Tea consumption (cups/day)	0 (non-drinkers) >0	Ref 0.82 (0.40-1.67)	0.576
Smoking status	Coffee consumption (cups/day)	0 (non-drinkers) 1-3 4-8	Ref 1.24 (0.44-3.48) 0.88 (0.24-3.25)	0.7673
	Tea consumption (cups/day)	0 (non-drinkers) >0	Ref 0.71 (0.36-1.42)	0.330
Alcoholic-drinking status	Coffee consumption (cups/day)	0 (non-drinkers) 1-3 4-8	Ref 1.34 (0.47-3.79) 1.10 (0.31-3.91)	0.818
	Tea consumption (cups/day)	0 (non-drinkers) >0	Ref 0.66 (0.33-1.31)	0.232

Results from multivariable logistic regression models.

Finally, we evaluated the median cups/day in the HLA groups (Table 4.15; Table 4.16) for both beverages without finding any difference for both HLA-DRB1*15 and HLA-A*02.

Table 4.15 Median number of cups/day in coffee consumers stratified by HLA

		Coffee cups/day [Median; range]*	**p-value**
HLA-DR15	Neg	2.0 [1.8-6.0]	0.365
	Pos	2.0 [1.8-6.0]	
HLA-A02	Neg	2.0 [1.8-6.0]	0.421
	Pos	2.0 [1.8-6.0]	

*Among former and current consumers only

Table 4.16 Median number of cups/day in tea consumers stratified by HLA

		Tea cups/day [Median; range]*	**p-value**
HLA-DR15	Neg	2.0 [1.4-6.0]	0.868
	Pos	2.0 [0.2-6.0]	
HLA-A02	Neg	2.0 [0.2-6.0]	0.378
	Pos	2.0 [1.2-2.0]	

*Among former and current consumers only

Chapter 5: Discussion

5.1 Coffee and tea consumption impact on ALS progression

Paper I

In this study, it no correlation was found between coffee or tea consumption and disease progression. Log-ΔFS was only weakly correlated with the duration of coffee and other types of tea consumption, but not with the number of cups-year. Coffee and tea consumption have been studied in ALS for their possible role in the risk of developing the disease, but their possible role as predictors of the disease course once it has begun has not been evaluated so far. A pooled analysis based on over 1,000,000 individuals from five cohorts (Fondell *et al.*, 2015) did not show an association of caffeine and tea intake with the risk of dying from ALS. A pooled analysis of eight international prospective cohort studies, including 351,565 individuals (Petimar *et al.*, 2019), did not observe statistically significant associations between coffee, tea, or caffeine intake and ALS mortality risk. Only one study bucks these observations (Beghi *et al.*, 2011), showing that coffee intake was less frequent and prolonged among ALS patients than in different groups of sick or healthy controls. However, the odds for exposure among ALS patients decreased after excluding cases and controls who stopped consuming coffee after disease onset, and an exposure gradient was not detected. This study also found a small, although the significant protective effect of smoking, which is also bucking with most studies (Wang *et al.*, 2017), suggesting the possibility of bias. A case-control study conducted in almost the same population some

years later (Pupillo *et al.*, 2018) did not confirm these data but found a small risk reduction for tea.

To analyse the possible role of beverages on disease progression, we divided log-ΔFS into tertiles. Tertiles of ΔFS distribution are associated with survival (Riboli *et al.*, 2002; Labra *et al.*, 2016), indicating that this measure predicts different disease progressions. Slow progressors had a younger age at disease onset, more frequent spinal onset, better FVC%, and longer diagnostic delay, all positive predictive factors for ALS progression. Coffee and tea consuming status were equally distributed across progression categories. DeltaFS score, age at starting, and consumption duration were substantially similar for coffee, green tea, and other types of tea across consumption categories. All these findings are against a role for coffee or tea in influencing disease progression, in analogy with cohort studies indicating that coffee and tea intake are not risk factors for disease susceptibility. A few experimental studies do not help to understand the role of coffee in ALS. Chronic caffeine intake significantly reduced survival in superoxide dismutase1 G93A mice, an animal model of ALS (Potenza *et al.*, 2013), but in another study, coffee improved motor performance of male G93A mice (Seevaratnam *et al.*, 2009).

To analyse a possible interaction of coffee and tea consumption with other lifestyle factors and clinical variables, we firstly ranked variables using a variable importance measure, and eventually performed a multivariable model. None of the lifestyle variables analysis was ranked high. Clinical/demographic variables, such as diagnostic delay, age at onset, El Escorial category, and education, explained the largest amount of log-ΔFS variance. Adjusting for these four variables, the

multivariable analysis did not show any association between coffee and tea consumption and log ΔFS.

Study limitations are related to a possible recall bias, which seems improbable given that patients were unaware of the study hypothesis, and interviewers were blinded to clinical history and neurological status. We could not evaluate the influence of unmeasured variables, such as physical activity, trauma, or diet, but it is unlikely that these are confounders of coffee or tea consumption. Cross-sectional study prevents establishing a causal relation.

Although the findings should be interpreted with caution, this study has several strengths. Selection bias was minimized because patients were consecutively enrolled at five different Centres and included a large spectrum of disease severity. Previous cohort and case-control studies only assessed the baseline intake of coffee and tea, but not the personal history of consumption for every single patient. On the contrary, we studied the lifetime cumulative effect of both exposures using a cup-year measure in analogy to pack-year research on smoking.

This study does not support the hypothesis that coffee or tea intake is associated with a different ALS progression, contrarily to other neurodegenerative diseases. Although our findings seem rather strong, we cannot exclude a possible effect of coffee or tea on a subgroup of patients, for example, with positive family history. However, this could only be studied with a much larger sample of patients.

5.2 The Impact of Lifetime Alcohol and Cigarette Smoking Loads on Severity of ALS

Paper II

According to Al- Chalabi et al., ALS arises as to the final manifestation of a multistep process. However, the rapid progression of the pathological process after onset is an intriguing feature that remains unexplained. In this study, it was interesting to figure out the possible role of two exposures in accelerating disease progression, once it has started, and not in their role as risk/protective factors for the onset of ALS. For this reason, it was evaluated the smoking/drinking status at disease (clinical) onset, considering those who quitted smoking or drinking at least six months before onset as non-smokers/drinkers. To evaluate the possible impact of the two exposures at the earliest stage, we also included suspected ALS.

To analyze the possible role of smoking and alcohol exposures on disease progression, we divided the ΔFS into tertiles. Tertiles of the ΔFS distribution are associated with survival, thus indicating that this measure predicts different rates of disease progression. This was also true in our sample, where slow progressors had a younger age at disease onset, more frequent spinal onset, better FVC, and a longer diagnostic delay, all positive predictive factors for ALS progression.

It was determined no statistically significant association between alcohol drinking status and disease progression, measured with the ΔFS, or between age at disease onset or ΔFS and drinks per day. The log-ΔFS was only weakly correlated with the duration of alcohol consumption. On the other hand, the age of ALS onset was lower in current smokers than non-smokers, as already observed (Calvo *et al.*, 2016), pointing to a

possible effect of smoking in anticipating disease onset. Similarly, the ΔFS was slight, although not significantly, higher for smokers of >14 cigarettes/day. Indeed (Table 3), our sample achieved only 64% of statistical power to detect any significant difference of log-ΔFS means among smoking groups (exposure).

To analyze a possible interaction of smoking and alcohol consumption with other clinical variables, we firstly ranked variables using a variable importance measure and eventually performed a multivariable model. Clinical/demographic variables, such as diagnostic delay, age at onset, El Escorial category, and education, explained the largest amount of the log-ΔFS variance. In contrast, smoking and alcohol drinking retained only minor importance. Adjusting for these four variables, the multivariable analysis did not show any clear association between smoking or alcohol drinking and the log (ΔFS).

Taken together, these findings suggest a possible minor role for smoking, but not for alcohol drinking in worsening disease progression. Cohort studies have been performed only for smoking, with equivocal results: smoking was identified as an independent predictor of survival in both sexes in a population registry from North-western Italy (Calvo *et al.*, 2016), and in a US study, but only in women (Alonso *et al.*, 2010). In two other studies, smoking did not predict mortality (del Aguila *et al.*, 2003; Paillisse *et al.*, 2005).

This study has limitations intrinsic to its cross-sectional design that prevents to establish a causal relation; however, it is practical for testing hypotheses in rare disease and allows to prove associations with outcomes, if sufficiently strong, as for smoking and severity in multiple sclerosis (Ivashynka *et al.*, 2019). Also, we could not evaluate the possible confounding by unmeasured variables, such as physical activity, trauma,

or diet. On the other hand, our study does present some strengths. Selection bias was minimized because patients were consecutively enrolled and had a large spectrum of disease severity. A recall bias is unavoidable with this type of study, but patients were unaware of the study hypothesis, and interviewers were blinded to clinical history and neurological status. Collecting the personal history of consumption for every single patient, we were able to study the lifelong cumulative effect of both exposures and not only the amount of exposure at the time of the interview or immediately before.

5.3 Impact of Lifetime Coffee and Tea Loads on Multiple Sclerosis Severity

Paper III

To analyze the possible role of beverages on disease severity, we divided the MSSS distribution in tertiles. MSSS tertiles were associated with known risk factors for progression, such as female gender, age, and clinical form, but not with the status of coffee or tea consumer or the amount of cups/day or cups-year. The only study to which compare our results were in the opposite direction. D'Hooghe et al. (D'Hooghe M *et al.*, 2012) investigated coffee consumption in a sample of 1372 persons with definite MS, collected through a cross-sectional survey amongst MS persons registered by the Flemish MS society in Belgium. The hazard ratios for reaching EDSS 6 (requiring a cane or support to walk for a distance of 100 m) from the onset were 0.60 for daily consumers of coffee in RR-MS and 1.18 for progressive MS, compared with non-drinkers. However, this study suffers from possible biases due to the selection of

patients from MS patient association, low respondence rate, self-assessment of exposures and disability, and possible reverse causation.

Although the slight increase of risk for coffee drinkers in our study was not significant, we observed age of MS onset three years lower in drinkers of 1-3 cups of coffee per day and eight years lower in drinkers of 4-8 cups/day. Since age at starting drinking was at least one decade before the onset of MS, this finding could point to a possible effect of prolonged coffee intake in anticipating disease onset. Also, progressive forms were absent among non-consumers and slightly more frequent among high consumers than low-consumers. These findings could prospect a negative predictive role for coffee consumption in disease progression.

Furthermore, the simple distinction between exposed and non-exposed could be insufficient to evaluate the role of coffee consumption on disease progression and severity, and the dose might be important. For example, some of the conflicting results from susceptibility studies (Pekmezovic *et al.*, 2006; Massa *et al.*, 2013; Ponsonby *et al.*, 2013; Hedström *et al.*, 2016; Al Wutayd *et al.*, 2018) may be due to different preparation and dosages of coffee. We did not find any trend with the quantity of coffee drunk for both the intensity and cumulative exposures.

To analyze a possible interaction of coffee and tea consumption with other risk factors, we performed a multivariable model, using the categories of cups/day as a measure of consumption, adjusting for age at onset, education, and smoking. The multivariable analysis did not show any association between coffee and tea consumption (cups/day) and MSSS.

Regarding tea consumption, we found no correlation with disease severity, measured with the MSSS, age at onset, or clinical form. Compared to non-consumers, the ORs were 1.27 for coffee drinkers and 0.68 for tea drinkers. Although none of these figures was significant, it is noteworthy that they showed opposite direction; this finding is worthy of further evaluations with larger sample size. Since alcohol and smoking status were differently distributed in coffee and tea drinkers, we adjusted for these factors, but the results did not change significantly. Apart from caffeine, which is approximately half the amount contained in a single cup of coffee (D'Hooghe M *et al.*, 2012), tea contains high concentrations of polyphenols and other phytochemical compounds with anti-inflammatory and neuroprotective properties (de Mejia *et al.*, 2009). Although there is some experimental evidence that the severity of experimental allergic encephalomyelitis could be reduced by the green tea polyphenol epigallocatechin, one trial with this substance (Lovera *et al.*, 2015) was stopped because of hepatotoxicity.

Finally, since a strong interaction between high-risk HLA variants and heavy coffee intake has been found in rheumatoid arthritis (Pedersen *et al.*, 2007) and latent autoimmune diabetes in adults (Rasouli *et al.*, 2018), and this has never been explored in MS, we evaluated the possibility that the effect of exposure to coffee or tea may vary depending on the genetic characteristics of the individual. However, we did not observe any difference stratifying by HLA-DRB1*15 and HLA-A*02 for both coffee and tea.

The limitations of this study are mostly due to its cross-sectional design. As with any cross-sectional study, the outcomes of interest and exposures are carried out at the same point in time and do not indicate the sequence of events, whether exposure

determines the severity or vice versa. For this reason, it is not possible to infer causality. However, the lifestyle questionnaire we used made it possible to collect the entire exposure history retrospectively, and although recall biases were possible, the sequence of events was defined. Another limitation regards the impossibility to explore the effect of high doses of daily tea intake, which may be related to a more pronounced effect since the usual pattern of consumption in Italy does not exceed 1-2 cups/day. Studies in populations with higher consumption are warranted. Lastly, our study did not have enough power for subgroup analyses by sex and clinical types of MS, and we could not evaluate the influence of unmeasured confounders, such as BMI or vitamin D.

On the other hand, our study does present some strengths. Selection bias was minimized because patients were enrolled at a first-referral Center serving most patients of its catchment area, and recruitment was consecutive. Recall bias is unavoidable with this type of study, but patients were unaware of the study hypothesis, questionnaires were self-administered, and the helping interviewer was blinded to neurological status. On the contrary, using a cup-year measure in analogy to pack-year used in research on smoking, we were able to study the lifelong cumulative effect of both exposures and not only the amount of exposure at the time of the interview or immediately before.

Chapter 6: Conclusions

6.1 Coffee and tea consumption impact on ALS progression

Paper I

In this study was used a new approach to assess the role of potentially modifiable risk factors on the ALS progression - cumulative lifetime coffee and tea consumption load that were not previously studied at all. These values allow us to estimate the cumulative effect of coffee and tea consumption on disease course, even for low to moderate doses. Our study does not support the hypothesis that coffee or tea intake is associated with a different progression of ALS, contrary to other neurodegenerative diseases. Although our findings seem rather strong, we cannot exclude a possible effect of coffee or tea on a subgroup of patients, for example, with positive family history. However, this could only be studied with a much larger sample of patients.

6.2 The Impact of Lifetime Alcohol and Cigarette Smoking Loads on Severity of ALS

Paper II

This cross-sectional multicenter study does not support the hypothesis that alcohol drinking is associated with a different progression of ALS and suggests only a minor role for cigarette smoking, contrary to other neurodegenerative diseases (Ivashynka *et*

al., 2019; Belvisi *et al.*, 2020). The influence of potentially modifiable risk factors on ALS progression needs further investigation.

6.3 Impact of Lifetime Coffee and Tea Loads on Multiple Sclerosis Severity

Paper III

In conclusion, this study does not support the hypothesis that coffee or tea intake is associated with a different severity or progression of MS, contrarily to other neurodegenerative diseases (Belvisi *et al.*, 2020). However, we cannot exclude a possible effect of higher doses of coffee or tea or an effect on a subgroup of patients. Moreover, this could only be studied with a much larger sample of patients.

Appendices

Appendix A

QUESTIONARIO

_____

DATA INTERVISTA |__|__|____|

NUMERO IDENTIFICATIVO

Centro			Paziente		

INTERVISTATORE ……

L'INTERVISTA SVOLTA CON: ❑paziente ❑parente

Se parente, specificare (sono ammessi coniuge/convivente, genitori, figli, fratello/sorella):

……

I - Dati anagrafici

COGNOME ……

NOME ……

SESSO ❑Maschio ❑Femmina

CENTRO ……

ORA INIZIO ……. ORA FINE ……

Gentile Sig./Sig.ra. Le chiederò di rispondere ad alcune domande riguardanti le Sue abitudini di vita. Si tratta di un questionario da cui possiamo trarre informazioni utili anche in relazione alla malattia da cui Lei è colpito/a.

Leggere ciascuna domanda all'intervistato ed attendere la sua risposta: se l'intervistato mostra di non avere capito bene, o se richiesti, si può spiegare la domanda con esempi.
Evitare quanto più possibile le risposte "non so"; in tal caso insistere nella spiegazione.

II - Abitudini relative al fumo

1. Attualmente fuma sigarette?

❑Si, fumo sigarette → proseguire con la sezione**1a**
❑No, in passato ho fumato ma adesso non fumo più → proseguire con la sezione**1b**
❑No, non ho mai fumato. → proseguire con la sezione**1c**

1a. Domande per i fumatori

❖ ***In media quante sigarette fuma al giorno?***

❑ 1-3❑ 4-8❑ 9-13❑ 14-18❑ 19-23❑ 24-28❑ 29-33❑ 34 o più

❖ ***Abitualmente fuma sigarette:***

❑Con filtro ❑Senza filtro ❑Con e senza filtro

❖ ***Abitualmente aspira il fumo delle sigarette?***

❑ Sì, profondamente nei polmoni ❑ Sì, ma non profondamente ❑ No, non lo aspiro

❖ ***Quanti anni aveva quando ha iniziato a fumare?*** |___|

❖ ***Quante sigarette al giorno fumava di solito nelle età indicate sotto?***

	quando aveva circa 20 anni:	**quando aveva circa 30 anni:**	**quando aveva circa 40 anni:**	**quando aveva circa 50 anni:**	**quando aveva circa 60 anni:**	**quando aveva circa 70 anni:**
Con filtro o senza filtro	❑ con ❑ senza ❑ entrambe	❑ con ❑ senza ❑ entrambe	❑ con ❑ senza ❑ entrambe	❑ con ❑ senza ❑ entrambe	❑ con ❑ senza ❑ entrambe	❑ con ❑ senza ❑ entrambe
Numero di sigarette	❑ 0 ❑ 1-3 ❑ 4-8 ❑ 9-13 ❑ 14-18 ❑ 19-23 ❑ 24-28 ❑ 29-33 ❑ 34 o più	❑ 0 ❑ 1-3 ❑ 4-8 ❑ 9-13 ❑ 14-18 ❑ 19-23 ❑ 24-28 ❑ 29-33 ❑ 34 o più	❑ 0 ❑ 1-3 ❑ 4-8 ❑ 9-13 ❑ 14-18 ❑ 19-23 ❑ 24-28 ❑ 29-33 ❑ 34 o più	❑ 0 ❑ 1-3 ❑ 4-8 ❑ 9-13 ❑ 14-18 ❑ 19-23 ❑ 24-28 ❑ 29-33 ❑ 34 o più	❑ 0 ❑ 1-3 ❑ 4-8 ❑ 9-13 ❑ 14-18 ❑ 19-23 ❑ 24-28 ❑ 29-33 ❑ 34 o più	❑ 0 ❑ 1-3 ❑ 4-8 ❑ 9-13 ❑ 14-18 ❑ 19-23 ❑ 24-28 ❑ 29-33 ❑ 34 o più

❖ ***Ha mai smesso di fumare per almeno un mese?***

❑ Si ❑ No, mai

❖ ***Se sì, per quanto tempo in totale aveva smesso di fumare?***

❑ 1-4 mesi❑ 5-11 mesi❑ 1-2 anni❑ 3-4 anni❑ 5 anni o più

<u>1b. Domande per gli ex fumatori</u>

❖ ***A che età ha iniziato a fumare?*** |___|

❖ ***A che età ha smesso di fumare?*** |___|

❖ ***Quante sigarette al giorno fumava di solito nelle età indicate sotto?***

	quando aveva circa 20 anni:	**quando aveva circa 30 anni:**	**quando aveva circa 40 anni:**	**quando aveva circa 50 anni:**	**quando aveva circa 60 anni:**	**quando aveva circa 70 anni:**
Con filtro o senza filtro	❑ con ❑ senza ❑ entrambe	❑ con ❑ senza ❑ entrambe	❑ con ❑ senza ❑ entrambe	❑ con ❑ senza ❑ entrambe	❑ con ❑ senza ❑ entrambe	❑ con ❑ senza ❑ entrambe
Numero di sigarette	❑ 0 ❑ 1-3 ❑ 4-8 ❑ 9-13 ❑ 14-18 ❑ 19-23 ❑ 24-28 ❑ 29-33 ❑ 34 o più	❑ 0 ❑ 1-3 ❑ 4-8 ❑ 9-13 ❑ 14-18 ❑ 19-23 ❑ 24-28 ❑ 29-33 ❑ 34 o più	❑ 0 ❑ 1-3 ❑ 4-8 ❑ 9-13 ❑ 14-18 ❑ 19-23 ❑ 24-28 ❑ 29-33 ❑ 34 o più	❑ 0 ❑ 1-3 ❑ 4-8 ❑ 9-13 ❑ 14-18 ❑ 19-23 ❑ 24-28 ❑ 29-33 ❑ 34 o più	❑ 0 ❑ 1-3 ❑ 4-8 ❑ 9-13 ❑ 14-18 ❑ 19-23 ❑ 24-28 ❑ 29-33 ❑ 34 o più	❑ 0 ❑ 1-3 ❑ 4-8 ❑ 9-13 ❑ 14-18 ❑ 19-23 ❑ 24-28 ❑ 29-33 ❑ 34 o più

❖ ***Prima di smettere definitivamente, aveva mai interrotto di fumare per almeno un mese?***

❑ Si ❑ No, mai

❖ ***<u>Se sì,</u> per quanto tempo in totale aveva smesso di fumare?***

❑ 1-4 mesi ❑ 5-11 mesi ❑ 1-2 anni ❑ 3-4 anni ❑ 5 anni o più

1c. Domande per i non fumatori

❖ ***Ha mai provato a fumare?*** *(ad es. durante incontri con amici, a feste, a cene, o in particolari periodi della sua vita)*

❑ Si ❑ No

❖ ***Ha mai fumato anche solo occasionalmente?*** *(sigarette, sigari, pipe)*

❑ Si ❑ No

❖ ***Se sì, per quanti anni ha fumato anche solo occasionalmente?***

❑ 1 anno o meno ❑ 2-3 anni ❑ 4-6 ❑ 7-10 ❑ 11-20 ❑ 21 anni o più

2. Attualmente fuma altri prodotti?

❑ Si ❑ No

2a. Che tipo di prodotti? *(Specificare la frequenza giornaliera attuale)*

❑ Sigari (n° _____) ❑ Pipa (n° _____) ❑ Altro (n° _____)

III: Esposizione passiva a fumo di tabacco

1.Quando lei era bambino/a suo padre fumava? ❑Sì ❑No

2. Quando lei era bambino/a sua madre fumava? ❑Sì ❑ No

3. Durante la sua infanzia, trascorreva del tempo in locali in cui era presente fumo di tabacco?(ad es. in casa o in automobile)

❑ Non ricordo
❑ Molto raramente
❑ Occasionalmente (poche volte la settimana)
❑ Giornalmente o quasi ma per poche ore
❑ Giornalmente o quasi ma per parecchie ore

4. Il/la suo/a partner attualmente fuma?

❑Vivo da solo/a
❑ Sì, sigari/pipa
❑ Sì, sigarette
❑ No, da quando stiamo insieme non ha mai fumato → proseguire alla domanda 5
❑ No, ma in passato fumava→ proseguire alla domanda 5
❑ No, non ha mai fumato in vita sua→ proseguire alla domanda 5

4a. Se sì, quanti pacchetti di sigarette fuma al giorno?(si tenga presente il consumo abituale dell'ultimo anno)

❑ Mezzo pacchetto o meno
❑ Circa un pacchetto
❑ Uno e mezzo
❑ Due o più

4b.Fuma in sua presenza? ❑ Sì ❑ No

4c.Quante ore al giorno trascorre con lui/lei mentre fuma?

❑ Meno di 1 ora ❑ 1-2 ore ❑ 3-4 ❑ 5-6 ❑ 7 ore o più

5. Sul luogo di lavoro o nel tempo libero ci sono colleghi o amici che fumano?

❑ Sì ❑ No

5a. Se si, quante ore al giorno trascorre con loro mentre fumano?

❑ Meno di 1 ora ❑ 1-2 ore ❑ 3-4 ❑ 5-6 ❑ 7 ore o più

IV – Consumo attuale di bevande alcoliche

1. **In riferimento al consumo attuale di ciascuna delle seguenti bevande (vino, birra e superalcolici) classificare il paziente in una delle seguenti categorie:**

❑ Non bevitore (astemio oppure beve meno di 1 volta al mese)				
		Vino	**Birra**	**Superalcolici**
Bevitore	Età di inizio	|___|	|___|	|___|
Ex-bevitore	Età di inizio	|___|	|___|	|___|
	Età di cessazione	|___|	|___|	|___|

Se <u>non-bevitore</u>, proseguire con la sezione VI.

<u>Se bevitore</u> o <u>ex-bevitore</u>, continuare.

Le chiederò ora informazioni sul consumo di ciascuna bevanda alcoolica. <u>Da somministrare a bevitori (riferendo le domande ai 6 mesi precedenti) ed ex-bevitori(riferendo le domande ai 6 mesi precedenti l'astensione)</u>.

2.Lei beve/beveva vino bianco (compresi spumante o champagne**)?**

❑SI ❑ NO

2a. Quante volte la settimana? ❑<1 ❑ 1 ❑ 2-6 ❑ tutti i giorni

2b. Quante volte al giorno? ❑ 1 ❑ 2 ❑>2

2c. Quanti bicchieri per volta? ❑ 1 ❑ 2 ❑>2

3.Lei beve/beveva vino rosso?

❑SI ❑ NO

3a. Quante volte la settimana? ❑<1 ❑ 1 ❑ 2-6 ❑ tutti i giorni

3b. Quante volte al giorno? ❑ 1 ❑ 2 ❑>2

3c. Quanti bicchieri per volta? ❑ 1 ❑ 2 ❑>2

4.Lei beve/beveva birra?

❑SI ❑ NO

4a. Quante volte la settimana?	❑<1	❑ 1	❑ 2-6	❑ tutti i giorni
4b. Quante volte al giorno?	❑ 1	❑ 2	❑>2	
4c. Quante lattine (o equivalenti) *per volta?*	❑ 1	❑ 2	❑>2	

5.Lei beve/beveva aperitivi (Martini, Campari)**, amari o digestivi, vini da dessert** (Porto, Marsala)**?**

❑SI ❑ NO

5a. Quante volte la settimana?	❑<1	❑ 1	❑ 2-6	❑ tutti i giorni
5b. Quante volte al giorno?	❑ 1	❑ 2	❑>2	
5c. Quanti bicchieri per volta?	❑ 1	❑ 2	❑>2	

6. Lei beve/beveva superalcolici (grappa, whisky, vodka, cognac, brandy….)?

❑SI ❑ NO

5a. Quante volte la settimana?	❑<1	❑ 1	❑ 2-6	❑ tutti i giorni
5b. Quante volte al giorno?	❑ 1	❑ 2	❑>2	
5c. Quanti bicchieri per volta?	❑ 1	❑ 2	❑>2	

V – Consumo di bevande alcoliche nell'arco della vita

1.Vorremmo conoscere il suo consumo di bevande alcoliche in alcuni periodi della sua vita.

Numero di bicchieri/bicchierini

quando aveva circa 20 anni:	mai	Meno di 1 alla sett.	1-2 alla sett.	3-6 alla sett.	1 al dì	2 al dì	3-4 al dì	5-6 al dì	7 o più al dì	Solo d'estate
Vino	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Birra	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Liquori/superalcolici	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
quando aveva circa 30 anni:	mai	Meno di 1 alla sett.	1-2 alla sett.	3-6 alla sett.	1 al dì	2 al dì	3-4 al dì	5-6 al dì	7 o più al dì	Solo d'estate
Vino	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Birra	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Liquori/superalcolici	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
quando aveva circa 40 anni:	mai	Meno di 1 alla sett.	1-2 alla sett.	3-6 alla sett.	1 al dì	2 al dì	3-4 al dì	5-6 al dì	7 o più al dì	Solo d'estate
Vino	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Birra	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Liquori/superalcolici	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
quando aveva circa 50 anni:	mai	Meno di 1 alla sett.	1-2 alla sett.	3-6 alla sett.	1 al dì	2 al dì	3-4 al dì	5-6 al dì	7 o più al dì	Solo d'estate
Vino	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Birra	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Liquori/superalcolici	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
quando aveva circa 60 anni:	mai	Meno di 1 alla sett.	1-2 alla sett.	3-6 alla sett.	1 al dì	2 al dì	3-4 al dì	5-6 al dì	7 o più al dì	Solo d'estate
Vino	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Birra	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Liquori/superalcolici	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
quando aveva circa 70 anni:	mai	Meno di 1 alla sett.	1-2 alla sett.	3-6 alla sett.	1 al dì	2 al dì	3-4 al dì	5-6 al dì	7 o più al dì	Solo d'estate
Vino	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Birra	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑
Liquori/superalcolici	❑	❑	❑	❑	❑	❑	❑	❑	❑	❑

VI –ASSUNZIONE DI CAFFÈ

1. **In riferimento al <u>consumo attuale</u> di caffè classificare il paziente in una delle seguenti categorie:**

❑Non consumatore di caffè (Proseguire con le domande sul The)		
❑Beve solo caffè decaffeinato (Proseguire con le domande sul The)		
❑ Consumatore	Età di inizio|___|	Numero di tazzine al giorno|___|
❑ Ex-Consumatore	Età di inizio|___|	Numero di tazzine al giorno |___|
	Età di cessazione|___|	

2. In riferimento al <u>consumo passato</u>, quante tazzine al giorno beveva di solito nelle età indicate sotto? (Solo per consumatori ed ex-consumatori di caffè)

	Caffè (no decaffeinato)	Caffè decaffeinato
quando aveva circa 20 anni:	❑ 0❑ 1-3❑ 4-8	❑ 0❑ 1-3❑ 4-8
quando aveva circa 30 anni:	❑ 0❑ 1-3❑ 4-8	❑ 0❑ 1-3❑ 4-8
quando aveva circa 40 anni:	❑ 0❑ 1-3❑ 4-8	❑ 0❑ 1-3❑ 4-8
quando aveva circa 50 anni:	❑ 0❑ 1-3❑ 4-8	❑ 0❑ 1-3❑ 4-8
quando aveva circa 60 anni:	❑ 0❑ 1-3❑ 4-8	❑ 0❑ 1-3❑ 4-8
quando aveva circa 70 anni:	❑ 0❑ 1-3❑ 4-8	❑ 0❑ 1-3❑ 4-8

3.Lei beve caffè corretto?

❑SI ❑ NO

3a. Quante volte la settimana? ❑<1 ❑ 1 ❑ 3-6 ❑ tutti i giorni

3b. Quante volte al giorno? ❑ 1 ❑ 2 ❑>2

VII - ASSUNZIONE DI THE

1. **In riferimento al <u>consumo attuale</u> di the classificare il paziente in una delle seguenti categorie:**

❑Non consumatore di the (Proseguire con le domande sul The)		
❑ Consumatore	Età di inizio|___|	Numero di tazze al giorno |___|
❑ Ex-Consumatore	Età di inizio|___|	Numero di tazze al giorno |___|
	Età di cessazione|___|	

2. In riferimento al <u>consumo passato</u>, quante tazze al giorno beveva di solito nelle età indicate sotto? (Solo per consumatori ed ex-consumatori di the)

		The verde	Altri tipi di The
quando aveva circa	**20 anni:**	❑ 0 ❑ 1-3 ❑ 4-8	❑ 0 ❑ 1-3 ❑ 4-8
quando aveva circa	**30 anni:**	❑ 0 ❑ 1-3 ❑ 4-8	❑ 0 ❑ 1-3 ❑ 4-8
quando aveva circa	**40 anni:**	❑ 0 ❑ 1-3 ❑ 4-8	❑ 0 ❑ 1-3 ❑ 4-8
quando aveva circa	**50 anni:**	❑ 0 ❑ 1-3 ❑ 4-8	❑ 0 ❑ 1-3 ❑ 4-8
quando aveva circa	**60 anni:**	❑ 0 ❑ 1-3 ❑ 4-8	❑ 0 ❑ 1-3 ❑ 4-8
quando aveva circa	**70 anni:**	❑ 0 ❑ 1-3 ❑ 4-8	❑ 0 ❑ 1-3 ❑ 4-8

VIII - QUESTIONARIO ALIMENTARE

Riportare la frequenza di consumo dei seguenti alimenti:

	Porzione	**Primavera**	**Estate**	**Autunno**	**Inverno**
UVA	un grappolo (150 gr)	|__| volte al mese	|__| volte al mese	|__| volte al mese	|__| volte al mese
BANANE	Una banana	|__| volte al mese	|__| volte al mese	|__| volte al mese	|__| volte al mese
FRUTTI ROSSI (lamponi, mirtilli, more, melagrana)	una vaschetta (100 gr)	|__| volte al mese	|__| volte al mese	|__| volte al mese	|__| volte al mese
FRUTTA SECCA	(10 pistacchi,10 arachidi, 5 noci)	|__| volte al mese	|__| volte al mese	|__| volte al mese	|__| volte al mese
SUCCO DI FRUTTI ROSSI (lamponi, mirtilli, more, melagrana)	Un brick o un bicchiere	|__| vote al mese	|__| volte al mese	|__| volte al mese	|__| volte al mese
SUCCO DI UVA	Un brick o un bicchiere	|__| volte al mese	|__| volte al mese	|__| volte al mese	|__| volte al mese
FUNGHI	100 gr	|__| volte al mese	|__| volte al mese	|__| volte al mese	|__| volte al mese
CIOCCOLATO, CIOCCOLATINI, CIOCCOLATA CALDA, SNACKS A BASE DI CIOCCOLATO	30 g, 3 cioccolatini, 1 tazza	|__| volte al mese	|__| volte al mese	|__| volte al mese	|__| volte al mese
BIBITE GASSATE, APERITIVO ANALCOLICO (es. Coca-cola, Sprite, Red-bull…non acqua minerale)	1 bicchiere o 150 ml	|__| volte al mese	|__| volte al mese	|__| volte al mese	|__| volte al mese
DOLCI FARCITI CON CREME AL CIOCCOLATO (es. brioche, bomboloni, pasticcini, torte, merendine)	1 brioche, 1 fetta di torta, 3 pasticcini	|__| volte al mese	|__| volte al mese	|__| volte al mese	|__| volte al mese

Appendix B

ALS Functional Rating Scale Revised (ALS-FRS-R)

Total score |__|__|

Item 1: SPEECH
4 Normal speech process
3 Detectable speech disturbance
2 Intelligible with repeating
1 Speech combined with non-vocal communication
0 Loss of useful speech

Item 2: SALIVATION
4 Normal
3 Slight but definite excess of saliva in mouth; may have night-time drooling
2 Moderately excessive saliva; may have minimal drooling (during the day)
1 Marked excess of saliva with some drooling
0 Marked drooling; requires constant tissue or handkerchief

Item 3: SWALLOWING
4 Normal eating habits
3 Early eating problems – occasional choking
2 Dietary consistency changes
1 Needs supplement tube feeding
0 NPO (exclusively parenteral or enteral feeding)

Item 4: HANDWRITING
4 Normal
3 Slow or sloppy: all words are legible
2 Not all words are legible
1 Able to grip pen, but unable to write
0 Unable to grip pen

Item 5a: CUTTING FOOD AND HANDLING UTENSILS
Patients without gastrostomy Use 5b if >50% is through g-tube
4 Normal
3 Somewhat slow and clumsy, but no help needed
2 Can cut most foods (>50%), although slow and clumsy; some help needed
1 Food must be cut by someone, but can still feed slowly
0 Needs to be fed

Item 5b: CUTTING FOOD AND HANDLING UTENSILS
Patients with gastrostomy 5b option is used if the patient has a gastrostomy and only if it is the primary method (more than 50%) of eating .
4 Normal
3 Clumsy, but able to perform all manipulations independently
2 Some help needed with closures and fasteners
1 Provides minimal assistance to caregiver
0 Unable to perform any aspect of task

Item 6: DRESSING AND HYGIENE
4 Normal function
3 Independent and complete self-care with effort or decreased efficiency
2 Intermittent assistance or substitute methods
1 Needs attendant for self-care
0 Total dependence

Item 7: TURNING IN BED AND ADJUSTING BED CLOTHES
4 Normal function
3 Somewhat slow and clumsy, but no help needed
2 Can turn alone, or adjust sheets, but with great difficulty
1 Can initiate, but not turn or adjust sheets alone
0 Helpless

Item 8: WALKING
4 Normal
3 Early ambulation difficulties
2 Walks with assistance
1 Non-ambulatory functional movement
0 No purposeful leg movement

Item 9: CLIMBING STAIRS
4 Normal
3 Slow
2 Mild unsteadiness or fatigue
1 Needs assistance
0 Cannot do

Item 10: DYSPNEA
4 None
3 Occurs when walking
2 Occurs with one or more of the following: eating, bathing, dressing (ADL)
1 Occurs at rest: difficulty breathing when either sitting or lying
0 Significant difficulty: considering using mechanical respiratory support

Item 11: ORTHOPNEA
4 None
3 Some difficulty sleeping at night due to shortness of breath, does not routinely use more than two pillows
2 Needs extra pillows in order to sleep (more than two)
1 Can only sleep sitting up
0 Unable to sleep without mechanical assistance

Item 12: RESPIRATORY INSUFFICIENCY
4 None
3 Intermittent use of BiPAP
2 Continuous use of BiPAP during the night
1 Continuous use of BiPAP during day & night
0 Invasive mechanical ventilation by intubation or tracheostomy

Appendix C

Inter-Rater Agreement of a Romanian questionnaire designed to assess lifestyle habits in Amyotrophic Lateral Sclerosis

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Abstract

Several epidemiological studies have evaluated life-style habits as possible risk factors for Amyotrophic Lateral Sclerosis. We aimed to assess the inter-rater agreement of the Romanian version of an Italian questionnaire designed to ascertain the influence of exogenous pro-oxidative and antioxidative factors on the disease course. The Italian questionnaire was translated in Romanian and back-translated in Italian. The questionnaire is composed of three parts evaluating the smoking, the current consumption of alcoholic beverages and drinking history, and the consumption of antioxidant-rich beverages and foods. Forty patients admitted to our Institute in Chişinău, Republic of Moldova, were interviewed by two blinded raters, with a randomized sequence. Intraclass correlation coefficient and Cohen's kappa statistics were used to determine the level of agreement. We found an excellent agreement for broad indicators of consumption (yes/no/ex) for each type of alcoholic beverage, for smoking (yes/no/ex, age at onset and cessation, number of cigarettes, smoking load, and passive smoking), and for all dietary habits. On the contrary, agreement was only moderate for the total daily amount of alcohol, with high variability among the different beverages.

Key words: Lifestyle habits; questionnaire; Amyotrophic Lateral Sclerosis; Inter-rater Agreement

Introduction

Amyotrophic lateral Sclerosis (ALS) is a fatal, progressive neurodegenerative disease of upper and lower motor neurons with unknown etiology. Several epidemiological studies have evaluated life-style habits as possible risk factors for the disease, including smoking, consumption of alcoholic beverages, coffee, tea, and foods containing antioxidative substances.[1,2] We are currently doing a multicenter study in three countries (Italy, Republic of Moldova and Romania), to explore whether the above factors may influence ALS course and progression. Information on prognostic factors is collected through a questionnaire (available on request) divided in three parts: the first (smoking and drinking history) is part of the European Prospective Investigation into Cancer and Nutrition project (EPIC),[3] the second (current consumption of alcoholic beverages) was validated in Italy for a study on alcohol and epilepsy,[4] the third is an ad-hoc questionnaire collecting information about consumption of antioxidant-rich beverages and foods. The purpose of this study is to analyze the reliability of the Romanian version of the questionnaire (inter-rater agreement (IRA)).

Methods

The study was approved by the Ethical Committee of the Institute of Neurology and Neurosurgery in Chişinău, Republic of Moldova. The Italian questionnaire was translated in Romanian language by a Romanian mother language and back-translated in Italian by an Italian mother language. We assessed 40 patients admitted to our Institute during the year 2016 (22

women, 18 men, mean age 52 years, range 21-69). Written informed consent was obtained from all participants. Diagnoses of admission were headache, parkinsonism, myasthenia gravis, multiple sclerosis, seizure, benign paroxysmal positional vertigo, vegetative disturbances, radiculopathy, neurasthenia, and others. Each subject was interviewed in a dedicated room by two raters (VA and AR), who were previously trained in the use of the questionnaire. The sequence of interviews was randomized, and the randomization list was concealed. The two raters were not caring the interviewed patients and were blinded to the interviewee's diagnosis and to each other's responses. The two interviews were administered at least one day and no more than seven days apart; this was considered a sufficient time window for the subject being unable to remember his or her answers and not to change his or her smoking and alcohol consumption habits. The interviewers specified the start and end time of completion of the questionnaire.

Exposure assessment

All subjects were asked about their smoking status at recruitment (never, former, or current smoker). Ever smokers (former and current smokers) were asked about: the number of cigarettes per day smoked at recruitment and at ages 20, 30, 40, 50, and 60 years; age at smoking onset and cessation (for former smokers). For each age period, the mean number of cigarettes smoked per day was calculated based on the questionnaire information, and the number of years spent smoking was calculated considering age at onset, age at cessation, and age at recruitment. The total number of years spent smoking was computed summing the number of years in each period. An estimation of a cumulative lifetime smoking load was calculated for lifetime cigarettes per years smoked as the weighted sum of the mean number of cigarettes smoked per day at different ages, including recruitment, with weights equal to the number of years spent

smoking for each period. This measure of exposure was expressed in terms of packs of cigarettes, defining a pack as containing 20 cigarettes.

Current drinking: participants were asked about their drinking status at recruitment (never, former, or current drinker), and about the current consumption (for current drinkers) or the consumption during the six months before cessation (for former drinkers) of alcoholic beverages (red wine, white wine, beer, and spirits). For each type of beverage, subjects were asked to report the frequency of their drinks, in terms of number of standard alcoholic units per drink, number of drinks per day, and number of days per week. A standard alcoholic unit was assumed containing approximately 12 grams of pure ethanol. Using this information, the current intake of each type of alcoholic drink was computed, expressed in terms of grams of alcohol per day (g/day), and the intake for each beverage was summed to obtain the measure of current exposure to alcoholic beverage, expressed as the total amount of g/day.

To calculate an estimation of a cumulative lifetime alcohol load, drinkers (former and current) were asked about the amount of beverages consumed at ages 20, 30, 40, 50, and 60 years. For each age period, the mean number of drinking units per day of each type of beverage was calculated based on the questionnaire information, and the number of years spent drinking was calculated considering the questionnaire information, age at onset, age at cessation and age at recruitment. A cumulative lifetime exposure load for each beverage was calculated as the weighted sum of the mean number of drinking units per day at different ages, with weights equal to the number of years spent drinking for each period.

Antioxidants in food and beverages: participants were asked on the consumption of cups of coffee and green tea per day at recruitment and at ages 20, 30, 40, 50, and 60 years. A cumulative lifetime coffee and tea load was calculated as for alcohol and smoking. Participants were also asked on the consumption (yes/no) and the frequency of consumption (servings per

month) of a selected list of foods containing antioxidative substances, derived from Benzie & Choi[5].

Statistical analysis

Data missing for both raters were excluded from the analysis. The intraclass correlation coefficient (range 0-100%), was used to determine the level of agreement between the two raters for the continuous variables.[6] Inter-rater reliability (IRR) analyses based on the proportional agreement that could occur simply by chance, using Cohen's kappa statistics,[7] were used to determine the level of agreement between the two raters for the nominal variables. The strength of agreement (k-values) was interpreted as poor (<0.00), slight (0.00–0.20), fair (0.21–0.40), moderate (0.41–0.60), substantial (0.61–0.80), and almost perfect (0.81–1.00) according to Landis and Koch.[8] Analysis by Gamer et al.[9] were performed using the *IRR* package in R. A p-value of 0.05 was assumed to be the threshold for statistical significance.

Results

Consumption of alcoholic beverages (Table 1). The agreement for age at drinking onset was excellent. The agreement was also excellent for the broad indicators of current consumption (yes/no) for each type of alcoholic beverage. However, when analysing the current intake in grams/day, agreement varied depending on the type of alcoholic drink: it was much better for red wine than white wine, spirits or beer, and only moderate (0.57), considering the total daily consumption of alcohol. A worse agreement for beer than the other beverages was also obtained considering the cumulative lifetime load.

Table 1. Inter-rater agreement for questions related to drinking habits

Variable	Number of subjects *	ICC / Kappa **	95% CI / p-value
Wine – Yes/ No/Ex	40	0.877	<0.001
Beer – Yes/ No/Ex	40	0.908	<0.001
Spirits – Yes/ No/Ex	40	0.911	<0.001
Age at drinking onset of wine	30	0.997	0.994-0.999
Age at drinking onset of beer	22	0.912	0.803-0.962
Age at drinking onset of spirits	22	0.971	0.933-0.988
Current consumption (g/day):			
White wine	24	0.504	0.141-0.749
Red wine	26	0.710	0.456-0.858
Beer	20	0.242	0.000-0.609
Spirits	20	0.242	0.000-0.832
Current total alcohol consumption (g/day)	32	0.568	0.281-0.762
Cumulative lifetime wine load (Units)	30	0.595	0.308-0.783
Cumulative lifetime beer load (Units)	22	0.371	0.-0.678
Cumulative lifetime spirits load (Units)	22	0.801	0.584-0.912

* Number of pairs for whom both raters obtained a response to the question
** Agreement reported as Intraclass Correlation Coefficient (ICC) with 95% Confidence intervals for continuous variables or Kappa statistics with p-value for nominal variables

Smoking habits (Table 2). An almost perfect agreement between the two raters was obtained for all the variables concerning smoking: age at smoking onset and quitting, patterns of smoking, current number of cigarettes, pack load, and passive smoking, with the only exception of passive smoking in pubs.

Table 2. Inter-rater agreement for questions related to smoking habits

Variable	Number of subjects *	ICC / Kappa **	95% Confidence Intervals / p-value
Smoking - Yes / No	40	1.000	<0.001
Age at smoking onset	14	0.998	0.995-0.999
Age at smoking cessation	7	1.000	<0.001
Current number of packs	7	1.000	<0.001
Cumulative lifetime smoking load (packs)	14	0.974	0.924-0.992
Current number of packs	7	1.000	0.008
Cigarettes with filter - Yes / No	7	1.000	<0.001
Aspirate smoke - Yes / No	7	1.000	<0.001
Passive smoking:			
Father	40	1.000	<0.001
Mother	40	1.000	<0.001
In pubs	40	0.367	0.002
Partner	38	1.000	<0.001
Friends	40	0.935	<0.001

* Number of pairs for whom both raters obtained a response to the question
** Agreement reported as Intraclass Correlation Coefficient (ICC) with 95% Confidence intervals
for continuous variables or Kappa statistics with p-value for nominal variables

Antioxidative beverages and foods (Table 3). Agreement was excellent for current coffee and green tea consumption, and for cumulative lifetime tea load and moderate for cumulative coffee load. It was also excellent for yearly consumption of grapes, berries, nuts, berries juice, grapes juice, mushrooms, chocolate, carbonated soft drinks, and sweet creams, with kappa values ranging from 0.84 to 1.00.

Table 3. Inter-rater agreement for questions related to the consumption of antioxidant-rich beverages and food

Variable	Number of subjects*	ICC / Kappa**	95% CI / p-value
Coffee consumption - Yes / No	40	0.949	<0.001
Green tea consumption - Yes / No	40	1.000	<0.001
Total coffee consumption (years)	24	0.990	0.978-0.996
Cumulative lifetime coffee load (cups)	24	0.648	0.345-0.830
Total green tea consumption (years)	36	1.000	0.999-1.000
Cumulative lifetime green tea load (cups)	36	0.941	0.900-0.971
Dietary habits:			
Grapes - Yes / No	40	1.000	<0.001
Berries - Yes / No	40	1.000	<0.001
Nuts - Yes / No	40	0.844	<0.001
Berries juice - Yes / No	40	1.000	<0.001
Grapes juice - Yes / No	40	0.950	<0.001
Mushrooms - Yes / No	40	0.875	<0.001
Chocolate - Yes / No	40	1.000	<0.001
Carbonated soft drinks - Yes / No	40	0.942	<0.001
Sweet creams - Yes / No	40	1.000	<0.001
Consumption of (servings/month):			
Grapes	40	0.991	0.984-0.995
Berries	40	0.994	0.989-0.997
Nuts	40	0.998	0.997-0.999
Berry juice	40	0.993	0.986-0.996
Grape juice	40	0.998	0.996-0.999
Mushrooms	40	0.967	0.939-0.982
Chocolate	40	0.990	0.981-0.995
Carbonated soft drinks	39	0.998	0.995-0.999
Sweet creams	40	0.991	0.983-0.995

* Number of pairs for whom both raters obtained a response to the question

** Agreement reported as Intraclass Correlation Coefficient (ICC) with 95% Confidence intervals for continuous variables or Kappa statistics with p-value for nominal variables

Discussion

This study assessed the reproducibility of the Romanian version of a life-style questionnaire administered by different interviewers (IRA). The questionnaire covered smoking habits,

consumption of alcoholic beverages, and use of antioxidant- rich foods. This analysis was done as a preliminary study for a multicentre multinational cross-sectional study on life-style habits and progression of ALS.

The major findings of our study were: 1) an excellent agreement for broad indicators of consumption (yes/no/ex) for each type of alcoholic beverage, whereas agreement was only moderate for the total daily amount of alcohol, and highly variable among the different beverages for the current and past consumption; 2) an excellent agreement for smoking (yes/not) and all its characteristics (age at onset and cessation, number of cigarettes, smoking load, and passive smoking); 3) an excellent agreement for all dietary habits.

The knowledge of the IRA of a questionnaire is crucial in studies using multiple interviewers, as our ongoing study on life-style habits and ALS progression. Although we obtained excellent agreement in most items of the questionnaire, we must acknowledge a lower agreement regarding the calculation of the daily quantity of single beverages, especially beer, that is reflected also in the total daily amount of alcohol intake. The IRA of the Italian version of the same questionnaire was higher (>0.90) for the yes/no questions for all beverages, except white wine,[10] but showed a trend similar to this Romanian version, being lower for the total alcohol intake than for the single beverages. In conclusion, our questionnaire is a reliable instrument to measure life-style habits in our ALS multicenter study. The items related to alcohol use showed a lower reliability, but the finding of a similar trend in the Italian and Romanian version make us confident on its use in a multicentre multinational study, as well as in other studies in neurologic diseases. However, we must say that the results of this reliability study cannot be generalized, since the inter-rater agreements are unique to each individual study, depending on several factors including the context in which the study is being undertaken, the type of variables, and the expertise of interviewers.[11]

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Appendix D

STROBE Statement

Checklist of items of Paper II ”The Impact of Lifetime Alcohol and Cigarette Smoking Loads on Severity of Amyotrophic Lateral Sclerosis: a cross-sectional Study”

	Item No	Recommendation	Page No
Title and abstract	1	(*a*) Indicate the study’s design with a commonly used term in the title or the abstract	2
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	2
Introduction			
Background/ rationale	2	Explain the scientific background and rationale for the investigation being reported	4
Objectives	3	State specific objectives, including any prespecified hypotheses	4
Methods			
Study design	4	Present key elements of study design early in the paper	4
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	5
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	5
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	5-7

Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	5-7
Bias	9	Describe any efforts to address potential sources of bias	4-7
Study size	10	Explain how the study size was arrived at	13+suppl tab.3
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	5-7
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	8-9
		(*b*) Describe any methods used to examine subgroups and interactions	Suppl. Tab. 1-2
		(*c*) Explain how missing data were addressed	n/a
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	n/a
		(*e*) Describe any sensitivity analyses	n/a
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	9
		(b) Give reasons for non-participation at each stage	n/a
		(c) Consider use of a flow diagram	n/a

	Item No	Recommendation	Page No
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	9, tab. 1-3
		(b) Indicate number of participants with missing data for each variable of interest	n/a
Outcome data	15*	Report numbers of outcome events or summary measures	10-11
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Tab. 2-3
		(*b*) Report category boundaries when continuous variables were categorized	5
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	n/a
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Suppl tab.3
Discussion			
Key results	18	Summarise key results with reference to study objectives	12-14
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or	14

		imprecision. Discuss both direction and magnitude of any potential bias	
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	14
Generalisability	21	Discuss the generalisability (external validity) of the study results	n/a
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	18

*Give information separately for exposed and unexposed groups.

Appendix E

Supplementary Materials of Paper II

Supplemental Table 1. Alcohol and smoking status overall and according to the tertiles of ΔFS distribution among patients in Apulia region.

Variable	Category	All (N=57)	I: Slow progression rate of disease (N=21)	II: Medium progression rate of disease (N=13)	III: Fast progression rate of disease (N=23)	p-value	SMD
Alcoholic drinking status - N(%)	Current drinker	35 (61.4)	12 (57.1)	9 (69.2)	14 (60.9)	0.453#	0.385
	Former drinker	1 (1.8)	0 (0.0)	1 (7.7)	0 (0.0)		
	Non-drinker	21 (36.8)	9 (42.9)	3 (23.1)	9 (39.1)		
Wine drinking status - N(%)	Current drinker	33 (57.9)	12 (57.1)	8 (61.5)	13 (56.5)	0.194#	0.469
	Former drinker	2 (3.5)	0 (0.0)	2 (15.4)	0 (0.0)		
	Non-drinker	22 (38.6)	9 (42.9)	3 (23.1)	10 (43.5)		
Smoking habits - N(%)	Current smoker	10 (17.5)	4 (19.0)	4 (30.8)	2 (8.7)	0.241#	0.524
	Former smoker	2 (3.5)	0 (0.0)	1 (7.7)	1 (4.3)		
	Non-smoker	45 (78.9)	17 (81.0)	8 (61.5)	20 (87.0)		

Patients represent a subgroup of all 241 ALS patients, with residency in Apulia. Tertiles of ΔFS distribution were ≤ 0.333 (I); $0.334 - 0.875$(II); >0.875 (III).

Supplemental Table 2. ΔFS distribution according to alcohol load (during lifetime) in Apulia ALS patients. Former drinkers were excluded from the analysis.

Variable	Statistic	All (N=56)	I: Non-drinkers (N=21)	II: ≤1° drinks per day* (N=14)	III: >1° drinks per day* (N=21)	II vs. I (p-value)	III vs. I (p-value)	III vs. II (p-value)
ΔFS#	Median (range)	0.68 [0.00-5.33]	0.64 [0.02-5.33]	0.65 [0.00-4.33]	0.72 [0.08-4.20]	0.921	0.781	0.881

Patients represent a subgroup of all 241 ALS patients, with residency in Apulia. SD: standard deviation; p-values were reported from pairwise contrasts defined in ANOVA models; #log-transformed variable was used in the ANOVA model (because of skewed distribution); °Median cut-off; *The drinking intensity was computed as the weighted mean number of standard alcoholic units per day at different age periods with weights equal to the number of years spent drinking (i.e. drinking duration) within each age period for all type of beverages.

Supplemental Table 3. Details for power calculation to detect a statistically significant ($p<0.05$) difference of log-ΔFS means among smoke groups (i.e. non-smokers vs. light vs. heavy smokers) using a one-way ANOVA model. Former smokers are not considered in the present analysis.

		log-ΔFS	
Smoke groups	**N**	**Mean**	**SD**
Non-smokers	187	-0.714	1.067
≤14 cigarettes per day	21	-0.717	1.344
>14 cigarettes per day	23	-0.349	0.991
Overall	231	-0.678	1.088
$\mathbf{SDm} = \sqrt{\frac{[(-0.714)-(-0.678)]^2}{3} + \frac{[(-0.717)-(-0.678)]^2}{3} + \frac{[(-0.349)-(-0.678)]^2}{3}} \approx 0.19$			
Given the groups sample size of 187, 21 and 23 subjects and under the assumption that the log-ΔFS's SD of 1.1 was the same within each group, this sample achieved 80% of statistical power (i.e. 1- type II error) to detect a SDm of 0.23 as statistically significant, using a one-way ANOVA model, having fixed a type I error of 5%. Because the observed SDm was lower than the expected, we found that the actual statistical power was 64%.			

N: number of subjects; SD: standard deviation of log-ΔFS; SDm: standard deviation of log-ΔFS means

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