UNIVERSITA' VITA-SALUTE SAN RAFFAELE

CORSO DI DOTTORATO DI RICERCA INTERNAZIONALE IN MEDICINA MOLECOLARE

CURRICULUM IN NEUROSCIENZE E NEUROLOGIA SPERIMENTALE

BRAIN FUNCTIONAL CONNECTIVITY AND COGNITIVE DYSFUNCTION IN PATIENTS WITH MOTOR NEURON DISORDERS

DoS: Dr. Elisa Canu
Second Supervisor: Prof. Sharon Abrahams

Tesi di DOTTORATO di RICERCA di Veronica Castelnovo
Matr. 013832
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DECLARATION

This thesis has been composed by myself and has not been used in any previous application for a degree. Throughout the text I use both 'I' and 'We' interchangeably.

All the results presented here were obtained by myself, except for:

1) Patient recruitment (Chapters 2-5) has been performed by Dr. Riva and collaborators, Unit of Neurology, San Raffaele Scientific Institute, Vita-Salute San Raffaele University, Milan, Italy, and Professor Silani and collaborators, Department of Pathophysiology and Transplantation, "Dino Ferrari" Center, Università degli Studi di Milano, Milano, Italy.

2) MRI acquisition (Chapters 3-5) has been performed in collaboration with Prof. Falini and collaborators, Department of Neuroradiology, San Raffaele Scientific Institute, Vita-Salute San Raffaele University, Milan, Italy.

3) Statistical analysis using generalized linear model for longitudinal data (Chapter 2) were performed by Dr. A. Fontana, Unit of Biostatistics, Fondazione IRCCS Casa Sollievo della Sofferenza, San Giovanni Rotondo, Foggia, Italy.

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ABSTRACT

Motor neuron diseases (MND) are heterogeneous neurodegenerative disorders in which the possible presence of cognitive and/or behavioural symptoms is a universally known neuropsychological feature. Detecting even subtle cognitive changes in MND, monitoring them over time and identifying new markers both in terms of cognition and of neuroimaging is critical due to their considerable clinical impact. This thesis examines the presence of early markers of cognitive and behavioural alterations, of disease progression and brain correlates in MND patients, using specific neuropsychological evaluations and advanced magnetic resonance imaging (MRI) techniques. The results from this thesis demonstrated that computer-based neuropsychological batteries, rather than the standard ones', are able to identify subtle cognitive changes in amyotrophic lateral sclerosis (ALS), unique to this condition. These findings highlight the need of specific, accurate and well-tolerated tools for monitoring cognitive deficits in MND. Assessing the resting-state functional connectivity (RS-FC) in ALS patients over 6 months, we observed an increased functional connectivity over time within the frontostriatal and the frontoparietal networks. RS-FC was related to patients' frontal-executive dysfunction, and we identified the middle frontal gyrus as a potential core region in the framework of a frontoparietal functional disconnection, which is typical of frontotemporal lobar degeneration (FTLD). Finally, in a sample of cognitively/behaviourally unimpaired patients with ALS, we investigated the emotional processing and we observed that a lower performance in recognizing disgust was related with a reduced volume of the left pallidum and with a lower performance on the Edinburgh Cognitive and Behavioral ALS Screen, suggesting that altered disgust recognition could be an early marker of cognitive decline in ALS patients. In pure motor patients we also demonstrated an altered RS-FC between pallidum and the rest of the brain and, specifically, that a reduced left pallidum-temporo-striatal RS-FC may have a role in the lower ability of ALS patients in recognizing disgust. Taken together, our studies provide novel cognitive and imaging markers for detecting early signs of disease progression and for monitoring MND over time. Future studies are needed to verify whether the information of cognitive and MRI alterations may be able to classify new ALS patients with a FTLD-like progression at the single subject level.

TABLE OF CONTENTS

ACRONYMS AND ABBREVIATIONS

ALS=Amyotrophic Lateral Sclerosis

ALS-FTD=ALS with Frontotemporal Dementia

BvFTD=Behavioral Variant of Frontotemporal Dementia

CSF=Cerebrospinal Fluid

C9orf72=Chromosome 9 Open Reading Frame 72

ECAS= Edinburgh Cognitive and Behavioural ALS Screen

fMRI=Functional Magnetic Resonance Imaging

FA=Fractional Anisotropy

FTLD=Frontotemporal Lobar Degeneration

FUS= Fused in Sarcoma

GM=Grey Matter

LMN=Lower Motor Neuron

MAPT=Microtubule Associated Protein Tau

MD=Mean Diffusivity

MMSE=Mini Mental State Examination

MND=Motor Neuron Disease

MRI=Magnetic Resonance Imaging

PLS=Primary Lateral Sclerosis

PMA=Progressive Muscular Atrophy

ROI=Region Of Interest

RS-FC=Resting-State Functional Connectivity

SBM=Surface Based Morphometry

SOD1=Superoxide Dismutase 1 Gene

TAP=Test of Attentional Performance

TDP-43=TAR DNA-Binding Protein 43

UMN=Upper Motor Neuron

VBM=Voxel-Based Morphometry

WM=White Matter

Chapter 1 – General introduction

1.1 Brief overview on MND

1.1.1 Definition

Motor neuron diseases (MND) are progressive and heterogeneous neurodegenerative disorders characterised by the degeneration of upper motor neurons (projecting from the cortex to the brainstem and the spinal cord) in the precentral gyrus of the frontal lobe and/or lower motor neurons (projecting from the brainstem or spinal cord to muscle) in the ventral horn of the spinal cord (Foster & Salajegheh, 2019, Hardiman, Al-Chalabi et al., 2017).

The neurodegeneration of the upper motor neurons and/or lower motor neurons determines different clinical phenotypes: amyotrophic lateral sclerosis (ALS), primary lateral sclerosis (PLS) and progressive muscular atrophy (PMA) (Norris, Shepherd et al., 1993).

1.1.2 Clinical subtypes of MND and diagnostic criteria

ALS is the most common type of motor neuron disease, a fatal and heterogeneous neurodegenerative disorder of the motor system and its wider connections in which both upper and lower motor neurons are affected. The initial symptoms of ALS can vary between patients: the 80% of patients present with limb (or spinal)-onset and manifest limb muscle weakness, which leads to difficulties, for instance, in writing, turning keys in locks, raising the arm over the head. The 20% of patients presents with a bulbar onset and their initial symptoms are difficulty in speech (dysarthria), and difficulty in swallowing (dysphagia) (Foster & Salajegheh, 2019, Hardiman et al., 2017). The diagnosis of ALS is currently made according to El Escorial revised criteria (Brooks, Miller et al., 2000). ALS disease duration is rapid and can range from 2 to 5 years from onset, the death is usually due to respiratory failure (van Es, Hardiman et al., 2017).

According to the El Escorial revised criteria (Brooks et al., 2000), the diagnosis of ALS

requires:

A. the presence of:

1. evidence of lower motor neuron (LMN) degeneration by clinical, electrophysiological or neuropathologic examination;
2. evidence of upper motor neuron (UMN) degeneration by clinical examination, and
3. progressive spread of symptoms or signs within a region or to other regions, as determined by history or examination,

together with

B. the absence of:

1. electrophysiological or pathological evidence of other disease processes that might explain the signs of LMN and/or UMN degeneration, and
2. neuroimaging evidence of other disease processes that might explain the observed clinical and electrophysiological signs.

PLS is a slowly progressive and selective neurodegenerative disorder affecting mainly the central motor system (Turner, Barohn et al., 2020). The initial symptom is a progressive muscle stiffness which leads to an insidious loss of mobility, together with the development of corticobulbar dysfunction causing dysarthria, dysphagia and weakness of tongue. Compared to ALS, in PLS the presence of significant progressive lower motor neuron (LMN) involvement is not inevitable, but it usually manifests several years from the initial clinical UMN syndrome (D'Amico, Pasmantier et al., 2013). For this reason, the criteria for a definite diagnosis of PLS require the absence of significant active LMN degeneration for at least 3 years from symptom onset (Pringle, Hudson et al., 1992). PLS is typically slowly progressive and the mean symptom duration can range from 7.2 to 14.5 years (Statland, Barohn et al., 2015).

PMA is a rare neurodegenerative disorder affecting mainly lower motor neurons, including anterior horn cells and brainstem motor nuclei. From a clinical point of view, patients complain progressive weakness, muscle atrophy, fasciculations, and reduced or absent tendon reflexes. Weakness and atrophy initially start in distal limb muscles asymmetrically. PMA differs from ALS by the absence of clinical evidence of UMN dysfunction (Liewluck & Saperstein, 2015). The criteria for a definite diagnosis of PMA

include a minimum duration of symptoms of 4 years (van den Berg-Vos, Visser et al., 2003). The disease progression in PMA can vary from slow (decades) to very rapid (months to a year) and the median disease duration is 12 months longer than in patients with ALS (Kim, Liu et al., 2009).

1.1.3 Epidemiology

The incidence of ALS in Europe ranges from 2 to 3 cases per 100.000 individuals (Chio, Logroscino et al., 2013, Hardiman et al., 2017). Incidence is lower in east Asia (0.8 cases per 100,000 individuals) and south Asia (0.7 cases per 100.000 individuals). In areas where different ancestral populations live (such as North America), the incidence of ALS in indigenous populations is low (0.63 cases per 100.000 individuals) (Gordon, Mehal et al., 2013), while in more homogeneous populations (such as in Ireland, Scotland and the Faroe Islands) is high (2.6 cases per 100.000 individuals) (Joensen, 2012, Logroscino, Traynor et al., 2010). Disease onset occurs on average between 40 and 70 years of age, despite it can occur also in younger patients. Heterogeneity has been observed also in the survival: in Europe is about 24 months, while in central Asia is 48 months (Marin, Logroscino et al., 2016). Regarding the onset site, in Europe, spinal-onset has been observed more in man, while women have a higher probability to have a bulbar-onset. In southern Europe bulbar-onset is higher than in Asia (Logroscino et al., 2010).

PLS has a lower incidence compared to ALS and it accounts approximately for 1% to 4% of all patients with MND (Statland et al., 2015). The mean age at symptom onset is 50 years and a male predominance has been observed (range 2–4:1) (Turner et al., 2020).

PMA represents about 2.5% to 11% of MND patients (de Carvalho, Scotto et al., 2007, Kim et al., 2009). Its incidence is about 0.02 per 100.000. PMA seems to be more common in men and the age of onset is older than in patients with ALS (63.4 ± 11.7 years) (de Carvalho et al., 2007, Kim et al., 2009).

1.1.4 Extramotor features

Despite the principal symptoms of ALS are linked to motor impairment, in ALS cognitive and/or behavioural disturbances are observed in about 50% of patients, and about 13% of

patients presents with a concomitant behavioural variant frontotemporal dementia (bvFTD) (Hardiman et al., 2017, Pender, Pinto-Grau et al., 2020).

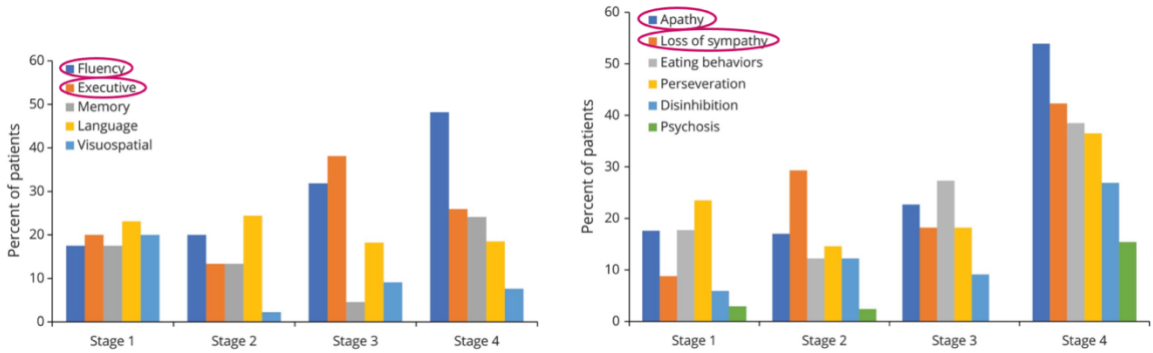

Figure 1. *Extramotor symptoms in ALS [from Crockford et al., Neurology 2018].*

Cognitive deficits in ALS patients can occur in multiple domains, but the most frequently affected abilities are the executive functions (Figure 1). Verbal fluency is a sensitive early marker of executive functions difficulties in ALS (Goldstein & Abrahams, 2013, Phukan, Pender et al., 2007). Other cognitive deficits are observed in language, social cognition, cognitive flexibility, abstract reasoning, working memory, and inhibitory control (Strong, Abrahams et al., 2017).

Language deficits were initially thought to be due to executive functions impairment. However, recent evidences shows the presence of specific language difficulties (Ash, Olm et al., 2015, Consonni, Catricala et al., 2016, Taylor, Brown et al., 2013) in word retrieval, spelling and grammatic and syntactic processing (Taylor et al., 2013, Tsermentseli, Leigh et al., 2015).

Memory impairments have been reported inconsistently in ALS, and more in the immediate than in the delayed recall (Massman, Sims et al., 1996, Rakowicz & Hodges, 1998).

Deficits in social cognition, such as emotion recognition and processing, are frequent in ALS (Burke, Pinto-Grau et al., 2016), although often under-recognized in a clinical setting. However, they can impact on relationships with caregivers, as the patient may appear selfish and thoughtless (Pender et al., 2020).

Concerning behavioural changes, the most common symptom is apathy, which occurs in up to 60% of patients (Crockford, Newton et al., 2018). Other behavioural symptoms that ALS patients can manifest overlap with those described by Rascovsky for

the behavioural variant of FTD, namely, disinhibition, loss of sympathy or empathy, perseverative behaviours and dietary changes. These features are often associated with a deficit of social cognition (Rascovsky, Hodges et al., 2011, Strong et al., 2017).

More incongruous and few data have been reported in patients with other MND. A study in patients with PLS observed an impaired neuropsychological profile in about 20% of patients, characterized by deficits in executive functions, social cognition (mainly in the affective theory of mind), fluency, and memory domains (de Vries, Spreij et al., 2019b). Other studies reported percentages and involved cognitive domains similar to ALS (Agarwal, Highton-Williamson et al., 2018, de Vries, Rustemeijer et al., 2019a, Le Forestier, Maisonobe et al., 2001). In PMA, some studies observed a neuropsychological profile similar to ALS (de Vries et al., 2019a), others a less severe picture (Raaphorst, de Visser et al., 2011, Raaphorst, van Tol et al., 2014).

1.1.5 Revised Strong's criteria

The considerable clinical impact of cognitive and behavioural impairments in these patients brought to the publication of the revised consensus criteria for the diagnosis of frontotemporal impairments in ALS (Strong et al., 2017), which provide a classification based on deficits in specific cognitive domains.

According to these criteria, patients are classified as cognitively unimpaired, cognitively impaired, behaviourally impaired, cognitively and behaviourally impaired (without dementia) or as having frontotemporal dementia (ALS-FTD).

Specifically, patients are considered to have a cognitive impairment if there is evidence of executive dysfunction or language impairment. Executive dysfunction is defined by the presence of impaired verbal fluency (where verbal fluency must take into account motor impairment) or of impairment of two non-overlapping tests assessing executive functions (including social cognition) or if there is a language impairment, or a combination of executive and language dysfunction.

Behavioural impairment is diagnosed when a patient presents apathy or/and two or more behavioural features drawn from the criteria for the behavioural variant of FTD (Rascovsky et al., 2011).

A diagnosis of ALS-FTD is made when there is a progressive worsening of the behavioural and cognitive symptoms and if the patient presents with at least three of the

bvFTD symptoms, if there is evidence of loss of insight or psychotic symptoms or if language impairments meet criteria of a primary progressive aphasia (Strong et al., 2017).

1.1.6 Genetics

Although most cases are sporadic, genetic factors play important roles in the pathogenesis of ALS. About the 10% of patients with ALS have a positive family history of ALS and present a Mendelian pattern of inheritance (Zou, Zhou et al., 2017).

At present, the most common mutated genes in ALS, which explain up to 70% of all cases of familial ALS, are: C9orf72, SOD1 (encoding superoxide dismutase), TARDBP (encoding TAR DNA-binding protein 43, TDP-43) and FUS (encoding RNA-binding protein FUS) (Chio, Battistini et al., 2014, Zou et al., 2017). Familial ALS is generally inherited in an autosomal dominant pattern and rarely as an autosomal recessive or X-linked trait (Renton, Chio et al., 2014).

C9orf72 is the most frequently genetic cause of familial ALS and familial FTD. It explains about 40% of familial ALS and 7% of sporadic ALS (Majounie, Renton et al., 2012) and about 25% of familial FTD (Baumer, Talbot et al., 2014). Before the identification of the pathogenic repeat expansion in C9orf72, only 20–30% of the familial ALS cases could be ascribed to mutations in the SOD1, and TARDBP and FUS (van Blitterswijk, DeJesus-Hernandez et al., 2012). The C9ORF72 repeat expansions are related with different phenotypes: ALS, PMA, PLS, ALS-FTD, and pure FTD (Boylan, 2015). Bulbar onset and cognitive deficits are more common, and mean survival seems to be lower than in patients with TARDBP or SOD1 mutations (Sabatelli, Conte et al., 2013).

SOD1 is the first identified causative gene of ALS. The majority of patients with SOD1 mutations develops a rapidly progressive ALS, but the phenotype (age of onset and severity of the disease) depends on the variants implicated; for instance, patients with p.A4V, p.H43R, p.L84V, p.G85R, p.N86S, or p.G93A mutations present with a more severe form of ALS, shorter disease duration (less than 3 years), while individuals with p.G93C, p.D90A, or p.H46R mutations have longer disease duration (Yamashita & Ando, 2015). Cognitive impairment in patients with SOD1 mutation is uncommon and bulbar onset is less frequent than in other mutations (Sabatelli et al., 2013).

Patients with TARDBP mutations generally present a typical ALS phenotype, absence of overt dementia and variable disease progression and duration (Kirby, Goodall et al., 2010). Other phenotypes related with TARDBP mutations are FTD (Gitcho, Bigio et al., 2009), ALS-FTD (Benajiba, Le Ber et al., 2009), ALS with extrapyramidal signs (Origone, Caponnetto et al., 2010), FTD with parkinsonism (Quadri, Cossu et al., 2011) and PD (Rayaprolu, Fujioka et al., 2013).

FUS gene mutations are the second most common cause of ALS. The phenotypes associated include adult-onset ALS, juvenile ALS, ALS-FTD, and, more infrequently, pure FTD (Deng, Gao et al., 2014). The majority of patients with FUS mutations show a classical ALS phenotype with no cognitive deficits and the clinical course is variable. ALS patients with FUS mutations have an earlier age at onset, more frequent bulbar onset, and a more rapid progression than those with SOD1 mutations (Yan, Deng et al., 2010).

According to a recent meta-analysis on 111 studies reported, the overall pooled mutation frequencies of these four main ALS-related genes were 47.7% in familial ALS and 5.2% in sporadic ALS and that the frequency of these mutations differed in European and Asian patients. More in detail, in European populations the most common mutations were in C9orf72 (familial ALS 33.7%, sporadic ALS 5.1%), followed by SOD1 (familial ALS 14.8%, sporadic ALS 1.2%), TARDBP (familial ALS 4.2%, sporadic ALS 0.8%) and FUS mutations (familial ALS 2.8%, sporadic ALS 0.3%), while in Asian populations the most common mutations were in SOD1, followed by FUS, C9orf72 and TARDBP (Zou et al., 2017).

Since 2014, seven new genes associated with ALS have been identified by genome-wide association studies, whole genome studies, or exome sequencing technologies: MATR3, CHCHD10, TBK1, TUBA4A, NEK1, C21orf2, and CCNF. Each genes code for proteins associated with one or more molecular pathways involved in ALS. Frequency data about these novel genes are still scarce (Chia, Chio et al., 2018) (Figure 2).

ALS shares mutations in multiple genes with FTD, namely, C9orf72 repeat expansion, TBK1, VCP, and TARDBP mutations (Nguyen, Van Broeckhoven et al., 2018). For this reason, genetic testing is usually done in patients who have a family history of MND or FTD, or in patients who are young at disease onset. In asymptomatic

adult relatives, the identification of mutation carriers before the disease becomes clinically overt.

Although more than 30 genes yield a major risk of ALS, recent findings suggest a possible role of oligogenic inheritance (in which a phenotypic trait is determined by more than one gene) and of genetic pleiotropy (in which a single gene has different phenotypic manifestations) (Hardiman et al., 2017).

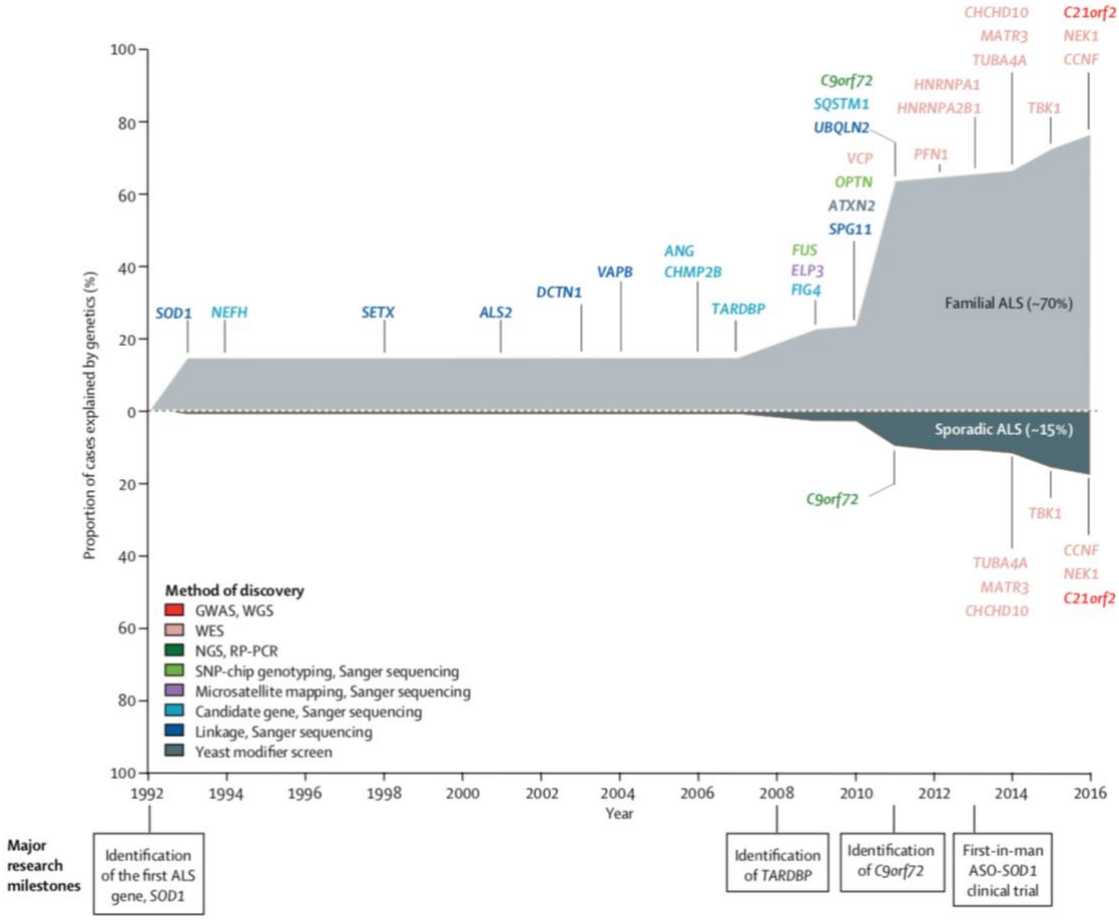

Figure 2. *Genes involved in ALS discovered until 2016 [from Chia et al., The Lancet Neurology 2018]*

1.1.7 Pathological biomarkers

The main pathophysiological mechanisms underlying ALS are still not well known. The neuropathological hallmark of ALS is the aggregation and accumulation of ubiquitinated inclusions in motor neurons. In the majority of ALS subtypes, the main constituent of these inclusions is TDP-43 (Kabashi, Valdmanis et al., 2008, Van Deerlin, Leverenz et al., 2008). About 97% of ALS patients with familial ALS presents characteristics of a TDP-43 proteinopathy, with TDP-43 depletion in the nucleus and with

the cytoplasmic aggregates in residual motor neurons (Figure 3a) (Hardiman et al., 2017). Four sub-types of TDP-43 pathology have been identified, which differ in morphology and distribution, and are associated with different phenotypes: type A is characterized by round intracytoplasmic TDP-43 inclusions and short neurites principally in the upper cortical layers; type B display round TDP-43 inclusions throughout the cortex; type C has long dystrophic neurites; and the rare type D shows intranuclear inclusions.

TDP-43 inclusion pathology is present also in about 40–50% of frontotemporal dementia (Burrell, Halliday et al., 2016), but the pattern of TDP-43 deposition in the brain differs from that of MND. Brettschneider et al., proposed a staging system in ALS where stage 1 affects motor systems; stage 2 posterior frontal and other brainstem regions; stage 3 more widespread pathology in prefrontal, sensory, and striatal brain regions; and stage 4 the temporal lobe, including hippocampus. According to this system, the spreading between regions takes place through corticofugal connections (Brettschneider, Del Tredici et al., 2013)

Other forms of protein aggregates which might be present are neurofilamentous hyaline conglomerate inclusions (Figure 3b) and the accumulation of misfolded SOD1 in ALS patients with SOD1 mutation and sequestosome 1-positive, TDP-43-negative inclusions, which are caused by dipeptide repeat proteins and could be find outside the motor system in ALS patients with C9orf72 mutations (Figure 3c) (Hardiman et al., 2017).

Despite the hallmark of ALS are protein aggregates, the high molecular-weight complexes that anticipate the generation of the aggregates (Marino, Papa et al., 2015, Ross & Poirier, 2005), could be the toxic species. The diffusion of higher molecular-weight protein complexes could arbitrate cell-to-cell propagation of disease, relating ALS progression to a prion-like mechanism, as for Synucleinopathies and Tauopathies (Aguzzi & Rajendran, 2009, Polymenidou & Cleveland, 2011).

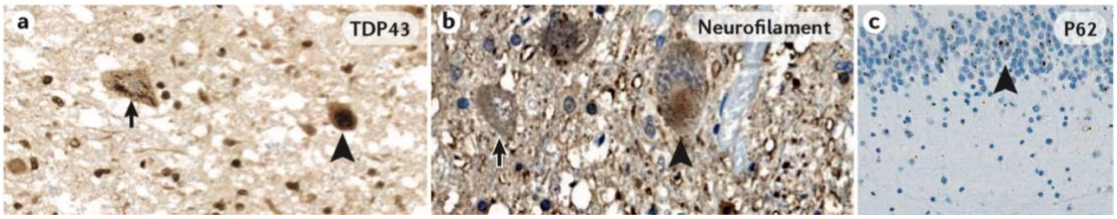

Figure 3. *Pathological markers in ALS [from Hardiman et al., The Lancet 2017]*

1.1.8 FTLD spectrum

In light of the clinical and pathological overlapping features, ALS and FTD are two ends of a disease spectrum. Up to 50% of ALS patients have executive dysfunctions and 15% of FTD patients develop ALS (Lee & Huang, 2017).

The belonging of ALS to a frontotemporal lobar (FTLD) degeneration spectrum is also sustained by the observations that patients with FTD–ALS and ALS present similar genetic mutations, especially those involving the non-coding GGGGCC hexanucleotide expansion of the C9ORF72 gene and two RNA/DNA binding protein: TDP-43 and FUS (Lee, Lee et al., 2011, Ling, Polymenidou et al., 2013).

In the majority of cases, FTD–ALS and ALS patients carrying mutations in the same gene (in particular C9ORF72, TARDBP or FUS) manifest similar neuropathological features with neurons including different protein aggregates of TDP-43 or FUS proteins. For this reason, the neuropathology of these patients can be classified according to the nature of the proteinopathy into FTLD-tau, FTLD-TDP, FTLD-FUS, ALS-TDP, ALS-FUS or ALS-SOD1 (Figure 4) (Lee & Huang, 2017).

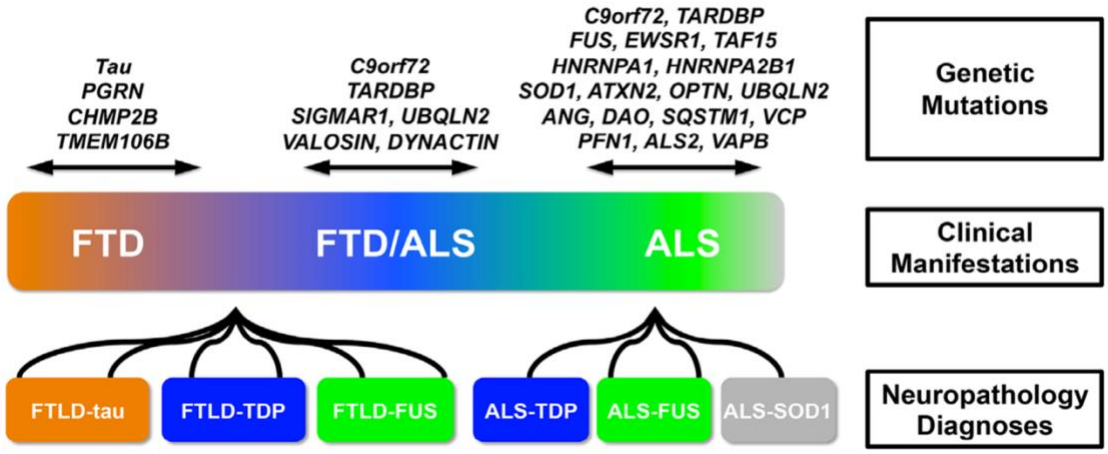

Figure 4. *Genetics, phenotype and pathology of FTLD spectrum [from Lee and Huang, Brain research 2017].*

1.2 MRI in MND:

Brain MRI has been shown to be a powerful tool to better understand the pathophysiological mechanisms underlying ALS and to detect and monitoring in vivo structural and functional brain abnormalities within the central nervous system of patients with MND.

1.2.1 Structural MRI

1.2.1.1 Gray matter

Many studies performed voxel-based morphometry (VBM) on high-resolution 3D T1-weighted MRI scans to assess grey matter (GM) volume in patients with ALS. VBM is a reliable and reproducible method which enables to detect subtle volume alterations through a voxel-by-voxel comparison of the regional attenuation of brain GM intensity between groups. The earliest VBM studies reported different outcomes: some studies showed focal atrophy in motor and premotor regions (Agosta, Pagani et al., 2007, Turner, Hammers et al., 2007), others observed a widespread frontotemporal atrophy with no involvement of the motor cortex (Mezzapesa, Ceccarelli et al., 2007), others reported no significant atrophy (Abrahams, Goldstein et al., 2005). This variability could be due to differences in image processing pipelines and statistical methods used. A meta-analysis on 29 VBM studies including more than 600 ALS patients, reported significant GM volume loss in the right precentral gyrus and in bilateral inferior frontal cortex (Shen, Cui et al., 2016).

Atrophy patterns differs in each specific clinical presentation. ALS patients with bulbar and limb onset differ in the GM alterations within the primary motor cortex, in line with their disease severity (Bede, Bokde et al., 2013). Patients with a concomitant FTD present a more severe pattern of atrophy which spreads to frontotemporal regions and to the caudate nucleus (Lillo, Mioshi et al., 2012, Masuda, Senda et al., 2016). GM loss in widespread cortical and subcortical regions has been found also in patients with mild cognitive/ behavioural deficits (Alruwaili, Pannek et al., 2018). A longitudinal study on patients with sporadic ALS showed a significant GM decline over a two-years follow-up in motor and non-motor frontotemporal cortex (Menke, Proudfoot et al., 2018). A

shape analysis of the sub-cortical structures showed overlapping patterns of atrophy of the thalamus, caudate, and pallidum bilaterally, and for the right putamen, hippocampus and amygdala, linked to the clinical and cognitive worsening (Figure 5) (Menke et al., 2018).

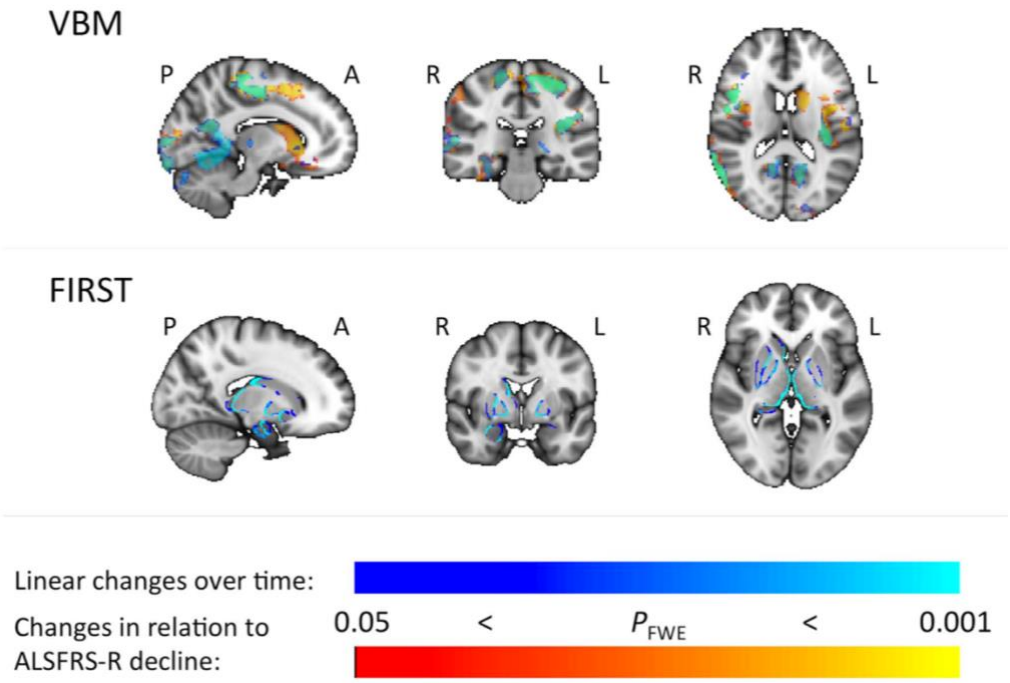

Figure 5. *Pattern of progressive grey matter loss over two years (blue), overlayed (semi-transparent) with changes regarding ALSFRS-R worsening (orange). Overlapping regions are displayed in green [from Menke et al., NeuroImage: Clinical 2018].*

Another method to assess brain cortical alterations is surface-based morphometry (SBM), which estimates cortical thickness on T1-weighted images. Studies performing SBM reported a significant reduction of cortical thickness in primary motor and extra-motor regions of ALS patients (Agosta, Valsasina et al., 2012, Verstraete, van den Heuvel et al., 2010). Extra motor involvement was greater in ALS cases with cognitive or behavioural impairments (Agosta, Ferraro et al., 2016, Schuster, Kasper et al., 2014).
As for VBM, also SBM is able to detect focal cortical alterations specific of each clinical presentation (Schuster, Kasper et al., 2013). Thinning of the motor cortex is typical of upper motor neuron degeneration, and it is not found in patients with predominant LMN involvement and ALS-mimic syndromes (Kwan, Meoded et al., 2012, Walhout, Westeneng et al., 2015). Significant correlations were reported between the thinning of

primary motor cortex over one year and the progression of disease severity (Spinelli, Agosta et al., 2016). A VBM study reported cerebellar atrophy mainly in the inferior lobules and vermis in ALS patients with motor symptoms and in the superior lobules, Crus I and II in patients with cognitive and behavioural disturbances (Tan, Devenney et al., 2014). More recent works used VBM or SBM to study subcortical structures in ALS and observed an involvement of the caudate, nucleus accumbens (Bede, Elamin et al., 2013), and thalamus (Menke, Korner et al., 2014). Basal ganglia volume loss has also been reported to correlate with a worse prognosis (Westeneng, Verstraete et al., 2015) and with cognitive deficits and apathy in ALS patients (Machts, Loewe et al., 2015). A recent study in ALS patients without cognitive and behavioural impairments observed regional shape contractions of the bilateral pallidum, right putamen, and right accumbens compared to healthy controls, suggesting early involvement of basal ganglia in ALS disease course (Tae, Sung et al., 2020).

1.2.1.2 White matter

White matter (WM) tract changes have been assessed in patients with ALS using diffusion tensor (DT) MRI, a method which is sensitive to the random thermal movement of water molecules in neural tissues. Evaluating the magnitude and directionality of water diffusion, it allows to have data on the microstructural integrity of brain structures. The DT technique assesses the absolute magnitude of water diffusion, which is quantified by mean diffusivity (MD), and fractional anisotropy (FA), which represents the degree of anisotropy of the diffusion. WM presents an anisotropic diffusion of water molecules attributable to the linear structure of tracts; the water molecules spread freely along the axons but the myelin hampers their direction perpendicularly to the axonal main direction. FA and MD values are markers of damage to the microarchitectural structure of neuronal projection (Agosta, Spinelli et al., 2018).

In ALS patients, a reduced FA and increased MD in the corticospinal tracts (CST) and in the middle and posterior parts of the corpus callosum have been observed (Muller, Turner et al., 2016). Microstructural alterations in non-motor frontotemporal tracts has been also reported, in particular in patients with cognitive or behavioural symptoms (Agosta et al., 2016, Lillo et al., 2012, Spinelli et al., 2016, Trojsi, Corbo et al., 2013).

Patients with PLS present great DT MRI alterations in both motor and extra-motor regions, associated to the severity of cognitive impairment (Agosta, Galantucci et al., 2014b). In patients with predominant LMN involvement opposite findings have been reported pointing toward a least diffuse WM damage (Prudlo, Bissbort et al., 2012, Rosenbohm, Muller et al., 2016, Spinelli et al., 2016).

Some longitudinal studies on alterations in the motor tracts of MND patients showed a significant change of CST damage after 6 to 8 months (Keil, Prell et al., 2012, van der Graaff, Sage et al., 2011), others did not observed this progression (Kassubek, Muller et al., 2018, Kwan et al., 2012). A 6-months longitudinal study in ALS patients reported a progressive involvement of non-motor frontotemporal and cerebellar WM regions (Keil et al., 2012).

Another longitudinal study used DT MRI to categorize ALS patients on the basis of the involvement of WM damaged at each neuropathological stage, reflecting the clinical progression (Kassubek et al., 2018). DT MRI could be a valuable biomarker of disease progression in ALS. Additional works are needed to deeply assess the topographical pattern of pathological change and its association with cognition and prognosis (Agosta et al., 2018).

1.2.2 Functional MRI

Functional MRI (fMRI) consists in the assessment of fluctuations in blood flow and blood- oxygen-level dependent contrast that result from the neuronal activation, using the intrinsic paramagnetic properties of the blood. Brain regions which activate at the same time during resting conditions define the "resting-state" (RS) functional networks. Studying the connectivity changes among RS networks allows to understand the functional reorganization of brain in various neurodegenerative diseases. This is of particular importance for ALS patients in which, because of their motor impairments, the use of task-based fMRI cannot be always possible (Agosta et al., 2018).

In ALS patients some studies reported reduced functional connectivity of the sensorimotor network (Fekete, Zach et al., 2013, Mohammadi, Kollewe et al., 2009, Trojsi, Esposito et al., 2015), others observed increased functional connectivity (Agosta, Canu et al., 2014a, Douaud, Filippini et al., 2011), or patterns of both reduced and increased functional connectivity (Zhou, Xu et al., 2014). Functional connectivity

changes were also found in brain networks associated with cognitive functions and behaviour such as default mode and frontoparietal networks (Agosta, Canu et al., 2013, Luo, Chen et al., 2012).

On the basis of RS fMRI results, it has been suggested that an increased functional connectivity could be present in earlier stages of ALS as a compensatory mechanism, with a following reduction as the pathological burden increases. Coherently, functional connectivity was reported to be higher in ALS patients with a less severe CST microstructural damage (Agosta, Valsasina et al., 2011), it was associated with a lower disease progression rate, reduced disease duration, and retained motor function (Agosta et al., 2011).

Interestingly, a recent 2-year longitudinal study on 13 patients with ALS and 3 with PLS observed a reduced RS functional connectivity within the sensorimotor and thalamic networks, in line with the clinical decline (Menke et al., 2018). The progressive increased functional connectivity in non-motor networks observed in the same study (Figure 6), namely the left frontoparietal and the temporal RS networks (Menke et al., 2018), is coherent with a "disconnection" hypothesis due to the loss of compensation.

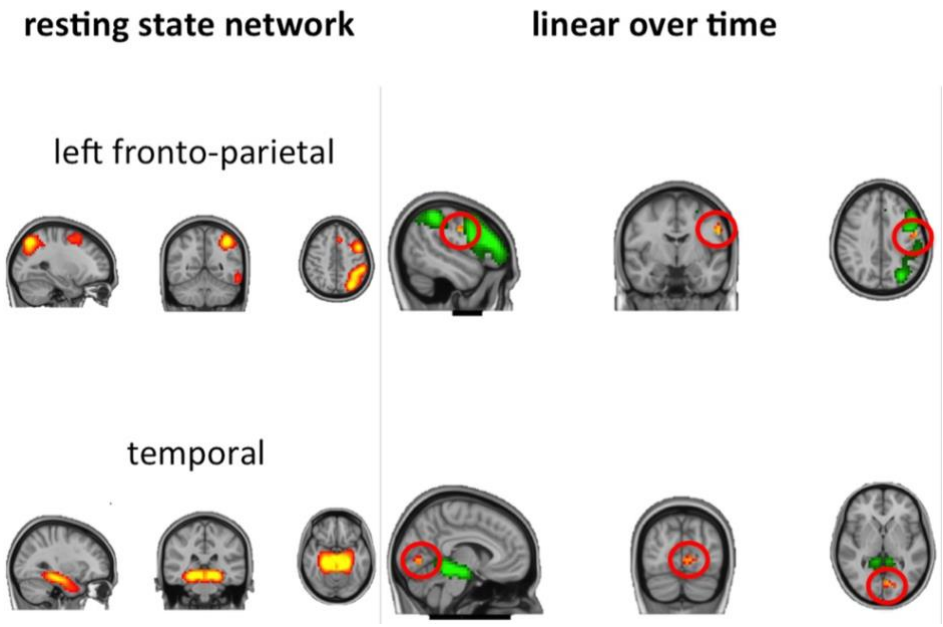

Figure 6. *Progressive increases in resting state functional connectivity within the left fronto-parietal and the temporal resting state networks (red-yellow colours in the graph on the left and green colours in the graph on the right). Significant results are shown in red-yellow and circled [from Menke et al., NeuroImage: Clinical 2018].*

Despite this, other studies reported increased functional connectivity in ALS-typical regions in association with faster disease progression (Douaud et al., 2011) and higher clinical and executive functions deficits (Agosta et al., 2014a). For this reason, another explanation of the increased functional connectivity could be the loss of local inhibitory circuitry within the primary motor and frontal cortex, which is suggested also by magnetic resonance spectroscopy (Foerster, Callaghan et al., 2012) and ^{11}C-flumazenil positron emission tomography studies (Lloyd, Richardson et al., 2000).

1.3 Cognitive and behavioural profiles of MND

1.3.1 Neuropsychological assessment and longitudinal monitoring

Identifying and monitoring over time the early cognitive difficulties in MND is of fundamental due to their great clinical relevance: patients who show cognitive impairment at the initial stages present a faster disease progression, and the presence of executive deficits has been linked with a shorter survival (Elamin, Bede et al., 2015). The presence of dysexecutive symptoms, including impairments in social cognition, in ALS requires major attention, since it can also influence the ability of the patient in taking decisions, the adherence to treatment, and the potential of taking advantage from non-pharmacological treatments (Caga, Hsieh et al., 2019).

The studies that have so far assessed cognitive deficits progression in MND reported heterogeneous findings. Those assessing cognitive impairment of ALS detected no or only mild cognitive changes over time, in particular within the executive function and, less frequently, language domains (Beeldman, Govaarts et al., 2020, Bock, Duong et al., 2017, Chio, Moglia et al., 2019, Elamin, Bede et al., 2013, Kilani, Micallef et al., 2004). The only study evaluating cognition changes over time in PLS focused on executive function assessment and observed a cognitive stability over time (Proudfoot, Menke et al., 2015). In PMA no studies have so far investigated cognitive and behavioural decline over time.

Some longitudinal studies support the hypothesis of a relative stability of cognition in patients who are unimpaired at first visit, and a progression of the

cognitive/behavioural deficits in patients already compromised (Bock et al., 2017, Elamin et al., 2013, Kasper, Zydatiss et al., 2016, Kilani et al., 2004, Poletti, Solca et al., 2018, Schreiber, Gaigalat et al., 2005). Other studies report that cognitive and behavioural deficits are more frequent and severe in later stages of the disease (Crockford et al., 2018). A recent longitudinal study demonstrated that some patients who are cognitively unimpaired at first visit could develop cognitive difficulties during the course of the disease, while those with cognitive deficits experienced cognitive and motor function worsening, and shorter survival (Bersano, Sarnelli et al., 2020).

The concern of the heterogeneity of neuropsychological findings in MND could be due to different reasons. One of the most important is the high patient attrition rate in longitudinal studies, due to the rapid rates of motor functional decline or bulbar function or death. The second possible explanation is the use of the different test administration approaches and whether the tests used consider the patient motor impairment (due, for instance, to difficulties in speaking caused by dysarthria).

Up to date, the neuropsychological batteries which have been used to assess and monitor cognition in MND are standard paper and pencil batteries, specific screening tools such as the Edinburgh Cognitive and Behavioral ALS Screen (ECAS) (Poletti et al., 2018), and computer-based batteries. Standard batteries are the most commonly used, but they can be proper and sensitive to initial cognitive deficits in MND only if they take into account MND motor deficits (Abrahams, Newton et al., 2014). For instance, a cross-sectional study which used both standard and computerized-based (the ALS Computerized Frontal Battery; ALS-CFB) batteries showed that the ALS-CFB was able to detect frontal impairments, compared to a standard neuropsychological battery which reported negative findings (Gillingham, Yunusova et al., 2017). Some computer-based batteries used in the longitudinal cognitive assessment in MND patients which have been demonstrated to be able to identify even subtle cognitive alterations over time in ALS were the Test of Attentional Performance (TAP) (Zimmerman, Eslinger et al., 2007), the ALS-CFB (Gillingham et al., 2017), and the Computerized Sentence Completion Test (Abrahams, Leigh et al., 2005).

1.4 Thesis aims

In the context of the existing need of detecting even subtle cognitive changes in ALS, PLS and PMA, to monitor them over time and to identify new markers both in terms of cognition and neuroimaging, the experimental chapters of this thesis had the following broad aims:

- To monitor the progression of cognitive and behavioural deficits in patients with ALS, PLS and PMA using two different neuropsychological batteries: a comprehensive standard neuropsychological assessment and a computer-based battery.
- To investigate the progression of resting-state functional connectivity (RS-FC) changes in patients with ALS and to define the relationship between ALS cognitive alterations and brain FC changes over time.
- To identify which emotions are altered in ALS and to investigate the relationship between emotion recognition and the integrity of basal ganglia, hippocampus and amygdala.
- To study the RS-FC of the pallidum compared to healthy controls, and the relationship between pallidal RS-FC changes and disgust recognition.

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Chapter 2 - Progression of cognitive and behavioural disturbances in motor neuron diseases assessed using standard and computer-based batteries

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RESEARCH ARTICLE

Progression of cognitive and behavioral disturbances in motor neuron diseases assessed using standard and computer-based batteries

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Abstract

Objective: Detecting and monitoring cognitive and behavioral deficits in motor neuron diseases (MND) is critical due to their considerable clinical impact. In this scenario, computer-based batteries may play an important role. In this study, we investigated the progression of cognitive and behavioral deficits in MND patients using both standard and computer-based neuropsychological batteries.

Methods: This is a retrospective study on 74 MND patients (52 amyotrophic lateral sclerosis [ALS], 12 primary lateral sclerosis [PLS], and 10 progressive muscular atrophy [PMA]) who were followed up for 12 months and underwent up to three cognitive/behavioral assessments, 6 months apart, including standard and/or computerized based (the Test of Attentional Performance [TAP]) batteries. Behavioral/cognitive changes were investigated over time using generalized linear model for longitudinal data accounting for time and revised-ALS Functional Rating Scale.

Results: Over 12 months, ALS patients showed a global cognitive decline (Mini Mental State Examination) at the standard battery and reduced performance in the alertness, sustained and divided attention, go/nogo, cross-modal and incompatibility TAP tasks. Most of these findings remained significant when ALSFRS-R changes over time were included as covariate in the analyses. ALS patients did not show significant behavioral abnormalities over time. No cognitive and behavioral changes were found in PLS and PMA cases.

Conclusions: Computer-based neuropsychological evaluations are able to identify subtle cognitive changes in ALS, unique to this condition. This study highlights the need of specific, accurate and well-tolerated tools for the monitoring of cognitive deficits in MND.

Keywords: *Motor neuron disease, amyotrophic lateral sclerosis, cognitive decline, computer-based neuropsychological evaluation, test of attentional performance*

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2.1 Introduction

In amyotrophic lateral sclerosis (ALS), cognitive and behavioural disturbances are observed in about 50% of patients (van Es, Hardiman et al., 2017). Detecting and monitoring even subtle cognitive deficits in ALS is critical due to their considerable clinical impact: patients who present with cognitive impairment at the early stage show a faster progression of motor impairment (Elamin, Bede et al., 2013), and the occurrence of executive dysfunction during the disease course has been associated with a reduced survival (Elamin, Bede et al., 2015). Furthermore, the presence of dysexecutive symptoms in ALS impacts on patient and caregiver psychological well-being, adherence to treatment, decision-making, and ability to benefit from non-pharmacological interventions (Caga, Hsieh et al., 2019). However, the studies that have so far investigated cognitive deficit progression of ALS detected no or only mild cognitive changes (Beeldman, Govaarts et al., 2020, Bock, Duong et al., 2017, Chio, Moglia et al., 2019, Elamin et al., 2013, Kilani, Micallef et al., 2004).

More discordant and poorer data have been reported in patients with other motor neuron diseases (MND). Some studies in primary lateral sclerosis (PLS) observed cognitive deficits in about 20% of patients (de Vries, Spreij et al., 2019b), others reported percentages similar to ALS (Agarwal, Highton-Williamson et al., 2018, de Vries, Rustemeijer et al., 2019a, Le Forestier, Maisonobe et al., 2001). The only study assessing the progression of cognitive dysfunctions in PLS reported negative findings (Proudfoot, Menke et al., 2015). In progressive muscular atrophy (PMA), some studies observed a neuropsychological profile similar to ALS (de Vries et al., 2019a, de Vries et al., 2019b), others a less severe picture (Raaphorst, de Visser et al., 2011, Raaphorst, van Tol et al., 2014). No studies have so far investigated PMA cognitive and behavioural decline over time.

The heterogeneity of neuropsychological findings in MND may have several explanations. One of the most important is patient attrition rate in longitudinal studies. The second possible reason is the effect of the different test administration modalities and whether it takes into account the patient physical impairment. Up to date, different neuropsychological batteries have been used to monitor the cognitive profile in MND: standard (‘paper and pencil’), specific screening (i.e., Edinburgh Cognitive and

Behavioural ALS Screen [ECAS] (Poletti, Solca et al., 2018)), and computer-based batteries. Although standard batteries are the most used tools and can be adequate and sensitive to cognitive decline in MND if corrections for motor impairment are applied, they are not specifically designed for MND and are likely not appropriate to detect and monitor cognition in patients with motor disturbances (Abrahams, Newton et al., 2014). In fact, so far, the majority of longitudinal studies using standard batteries reported no cognitive decline over 6 (Kasper, Zydatiss et al., 2016, Robinson, Lacey et al., 2006, Strong, Grace et al., 1999, Taylor, Brown et al., 2013), 12 (Kilani et al., 2004, Woolley, Goetz et al., 2018) or 24 months (Gillingham, Yunusova et al., 2017), whereas others showed a mild cognitive worsening over time (Abrahams, Leigh et al., 2005, Beeldman et al., 2020, Elamin et al., 2013, Floeter, Traynor et al., 2017, Schreiber, Gaigalat et al., 2005). The need of a neuropsychological battery able to overcome these limitations has been at least partially satisfied by the ECAS (Abrahams et al., 2014). However, all longitudinal studies using ECAS for monitoring the cognitive profile of ALS patients did not reveal a significant cognitive worsening throughout 6 (Xu, Alruwaili et al., 2017), 12 (Burkhardt, Neuwirth et al., 2017, Trojsi, Di Nardo et al., 2020), or 24 months (Poletti et al., 2018), even in samples where 20-40% of patients was cognitively impaired at baseline (Burkhardt et al., 2017, Poletti et al., 2018, Xu et al., 2017). This could be due to the fact that, as a screening tool, ECAS may have limited sensitivity in detecting the progression of cognitive decline when present.

In this scenario, computer-based batteries may play a crucial role for different reasons: use of a response box that limits the impact of motor impairment on cognitive performance; assessment of a large number of outcomes within a short time; precision of measurements in terms of both accuracy and reaction times; reduced inter-rater variability; and good test-retest reliability (Gillingham et al., 2017, Kemp, Hatch et al., 2009, Yunusova, Ansari et al., 2019). Specifically, a cross sectional study comparing standard and computerized-based (ALS Computerised Frontal Battery [ALS-CFB]) batteries observed that the ALS-CFB reported a profile of extramotor frontal dysfunction in patients, which standard neuropsychological screening tests were not able to show (Gillingham et al., 2017). Other longitudinal studies have monitored cognitive dysfunction in MND patients through computer-based batteries employing the Test of Attentional Performance (TAP) (Zimmermann, 1992), the ALS-CFB (Gillingham et al.,

2017), and the Computerised Sentence Completion Test (Abrahams et al., 2005). Although these batteries were able to detect even subtle longitudinal cognitive changes in ALS, they focused only on few domains (language only (Abrahams et al., 2005) or few executive TAP subtests (Schreiber et al., 2005)) or did not consider mood as possible influencing factor for cognitive changes (Gillingham et al., 2017).

The aim of the present study was to investigate the progression of cognitive and behavioural deficits in a population of MND patients (ALS, PLS and PMA) using (when available) two different neuropsychological batteries: a comprehensive standard neuropsychological assessment investigating the main cognitive domains and behaviour, and a computer-based battery, the TAP, which allows the investigation of the whole spectrum of frontal involvement in MND accounting for verbal and/or physical impairment.

2.2 Materials and methods

2.2.1 Sample selection

The study was performed retrospectively in a sample of MND patients recruited between 2009 and 2017 at two centres in Milan, Italy (IRCCS San Raffaele Scientific Institute and IRCCS Istituto Auxologico Italiano). We initially selected patients who had undergone at least two visits (6 months apart) within 12 months, each including a clinical evaluation and a standard neuropsychological assessment (minimum dataset at follow up: Mini Mental State Examination [MMSE], Fluency tests and indices, Beck Depression Inventory [BDI]). Then, ALS, PLS and PMA groups were age- and education matched. Finally, the subgroup of MND patients who underwent also a computer-based battery (TAP) was identified. See Figure 1 for a schematic representation of the sample selection. Additional inclusion criteria were: native Italian-speaking; no significant respiratory failure; no significant medical illnesses or substance abuse that could interfere with cognitive functioning; no any major systemic, psychiatric, or (other) neurological illnesses; and no (other) causes of focal or diffuse brain damage, including cerebrovascular disease at conventional MRI scans. All patients were in treatment with Riluzole at study entry. Disease severity was assessed using the ALS Functional Rating Scale-revised (ALSFRS-R) (Cedarbaum, Stambler et al., 1999).

Local ethical standards committee (clinical trial center: https://research.hsr.it/en/clinical-trial-center/index.html) on human experimentation of IRCCS San Raffaele Scientific Institute approved the study protocols (protocol IDs: RF-2010-2313220 and RF-2011-02351193) and all participants provided written informed consent.

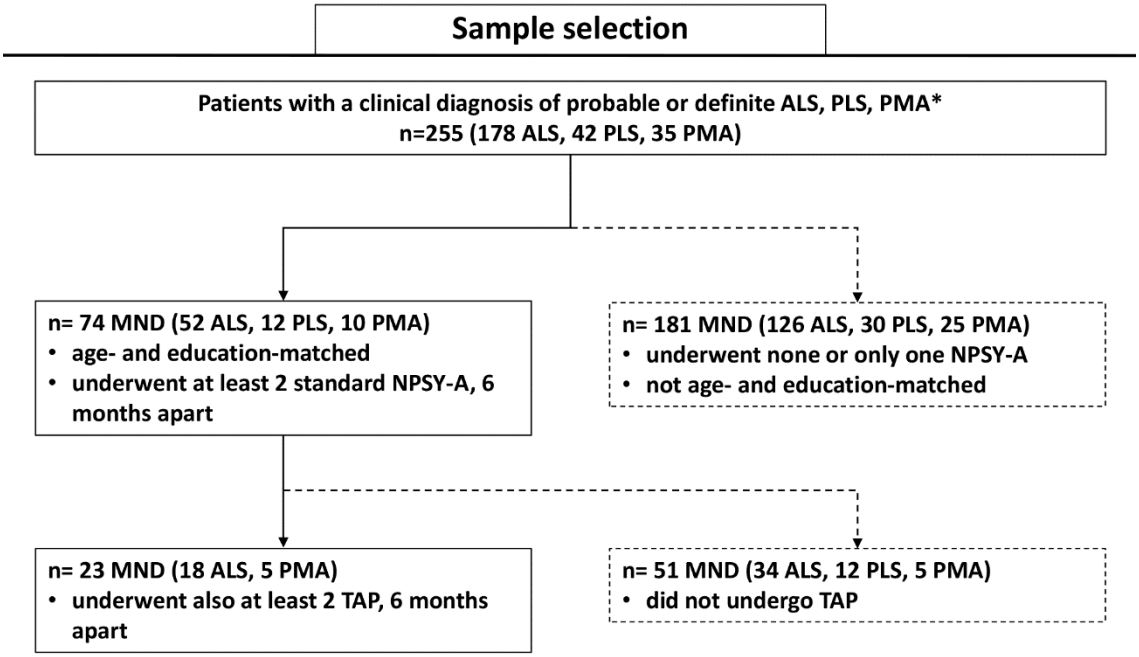

Figure 1. *Schematic representation of the sample selection process. Abbreviations: ALS=Amyotrophic Lateral Sclerosis; MND=Motor Neuron Diseases; NPSY-A=neuropsychological assessment; PLS=Primary Lateral Sclerosis; PMA=Primary Motor Atrophy; TAP=Test of Attentional Performance. *According to the current diagnostic criteria (Brooks et al. 2000; Pringle et al. 1992; van den Berg-Vos et al. 2003).*

2.2.2 Cognitive and behavioural assessment

Neuropsychological assessments were performed at each visit by experienced neuropsychologists unaware of the purpose of the present study and consisted of a standard neuropsychological assessment in all sample (see supplementary material for details) and a computer-based battery in a subsample of ALS and PMA cases. Neuropsychological data were collected at both centres after a common training provided by a senior neuropsychologist. The presence of cognitive and/or behavioural impairment

was defined according to Strong's criteria (Strong, Abrahams et al., 2017) (see supplementary material).

2.2.3 Computer-based assessment

The computer-based assessment consisted of the administration of six subtests of the TAP (see Figure 2 and supplementary material) assessing attention and executive functions (Zimmermann, 1992). The administration of TAP strictly followed standardized procedures and was conducted through an automated computerized system. In order to account for patients' verbal and/or physical impairment, patients performed each task with a response-box consisting of a single facilitator press-button for all sub-tests (with the only exception of the Incompatibility sub-test which required two press-buttons), not requiring either patient fine movements or strength. In order to reduce the number of comparisons, TAP variables with observed ceiling effects in patients were not considered (see S-Table 1).

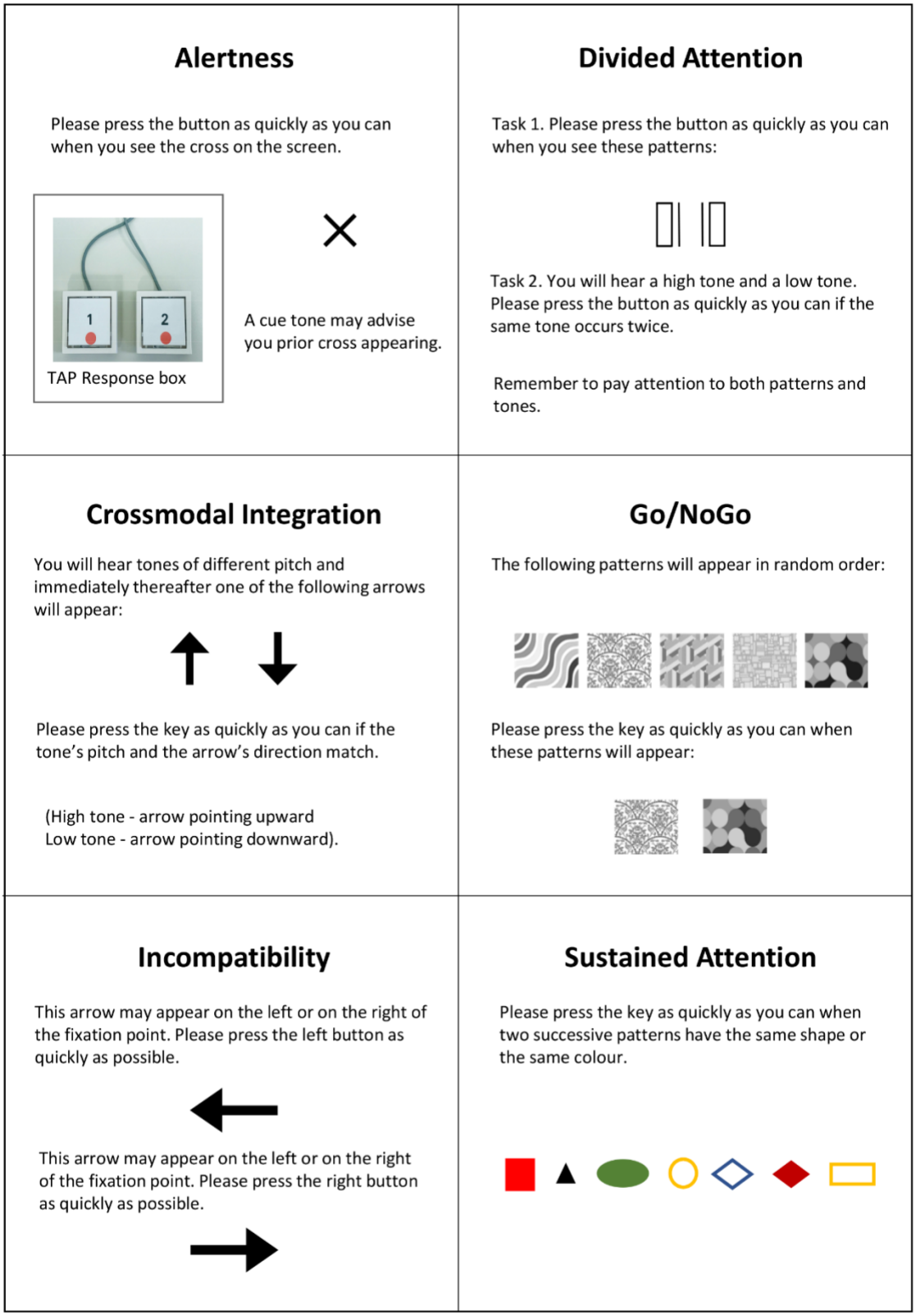

Figure 2. *The six subtests of the Test of Attentional Performance (TAP). For illustrative purposes, stimuli represent an adaptation of the original version.*

2.2.4 Statistical Analysis

To compare sociodemographic and clinical features between groups at baseline, Kruskal-Wallis one-way ANOVA models followed by post-hoc pairwise comparisons (using U test of Mann-Whitney and Bonferroni-correction for multiple comparisons) and Fisher's exact test were performed for continuous and categorical variables, respectively. Two-sided p-values < 0.05 denote statistical significance (SPSS, version 24.0; IBM Corp., Armonk, NY, USA).

Changes of cognitive and behavioural performances within patient groups over time were assessed by generalized linear model for longitudinal data, assuming Poisson and Normal distributions for score and continuous outcomes, respectively. Means (along with 95% confidence interval) were estimated for each outcome at baseline, at 6 and 12 months from models, which included time as categorical variable and baseline ALSFRS-R as further covariate. The overall comparison among patient groups at baseline was assessed, for each outcome, by means of statistical contrasts derived from each model (joint test). Moreover, a test for linear trend across the estimated means was performed, within each patient group, including the time as continuous variable as well as baseline ALSFRS-R into the ANOVAs. Only for significant findings in ALS, we re-run the analyses including ALSFRS-R changes over time as covariate. We finally investigated a potential relationship between changes of patients' mood (BDI, since all patients consistently underwent this scale) and the observed significant changes of cognition (MMSE and TAP scores reported in Table 4) using partial correlations accounted for baseline ALSFRS-R (see S-Table 2 for more details about partial correlation analyses). Two-sided $p < 0.05$ denote statistical significance. All analyses were performed using SAS Software, Release 9.4 (SAS Institute, Cary, NC, USA).

2.2.5 Data availability statement

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

2.3 Results

74 patients (52 ALS, 12 PLS, 10 PMA) were included (Figure 1). A subgroup of 23 patients (18 ALS and 5 PMA), who underwent also the TAP battery, was identified. At study entry, compared to the original sample (255 patients), the study groups had lower disease severity (p=0.01).

PLS patients were more frequently women and had greater disease severity and longer disease duration than ALS and PMA cases (Table 1). Five patients were *C9orf72* mutation carriers (4 ALS, 1 PMA). ALS and PMA subgroups included in the TAP analysis did not differ for any sociodemographic and clinical feature (Table 2). At baseline, 19% of ALS, 25% of PLS, and 14% of PMA patients from the main sample, and 21% of ALS and 14% of PMA from the TAP were classified as cognitively and/or behaviourally impaired according to Strong's criteria (Strong et al., 2017). No difference was observed at baseline between patient groups in terms of cognition, behaviour and mood (Tables 3 and 4).

Table 1. Sociodemographic and clinical features of patient sample at baseline.

	ALS	PLS	PMA	Overall p	p ALS vs PLS	p ALS vs PMA	p PLS vs PMA
N	52	12	10	-	-	-	-
Age [years]	62.54 ± 10.14	61.59 ± 9.59	58.77 ± 7.60	0.28	1.00	0.89	0.43
Sex, women	19 (37)	10 (83)	3 (30)	0.01	0.003	0.69	0.01
Education [years]	11.15 ± 4.25	9.17 ± 3.93	11.40 ± 4.14	0.28	0.12	0.89	0.20
ALSFRS-R, 0-48	39.85 ± 4.71	35.83 ± 6.24	41.80 ± 4.24	0.01	0.01	0.21	0.01
Disease duration [months]	23.78 ± 30.25	80.42 ± 36.76	29.78 ± 19.44	<0.001	<0.001	0.05	0.004
Disease progression rate	0.63 ± 0.60	0.21 ± 0.20	0.23 ± 0.15	<0.001	0.001	0.01	0.39
Site of onset (limb/ bulbar/limb+bulbar)	40/10/2	11/1/0	10/0/0	0.51	0.63	0.37	0.35

Values denote mean ± standard deviation or number (percentage). P values refer to Kruskal-Wallis one-way ANOVA models followed by post-hoc pairwise comparisons (using U test of Mann-Withney and Bonferroni-correction for multiple comparisons) and to Fisher's exact test for continuous and categorical variables, respectively. Abbreviations: ALS=Amyotrophic Lateral Sclerosis; PLS=Primary Lateral Sclerosis; PMA=Primary Motor Atrophy; ALSFRS-R=ALS Functional Rating Scale revised. Disease Progression Rate has been obtained as following: (48–ALSFRS-R score)/time between symptom onset and first visit.

Table 2. Baseline sociodemographic and clinical features of the subgroups of patients who performed the TAP.

	ALS	PMA	P
N	18	5	-
Age at baseline [years]	61.82 ± 11.39	59.51 ± 10.41	0.60
Sex, women	6 (33%)	1 (20%)	0.57
Education [years]	11.72 ± 4.23	11.80 ± 4.96	0.94
ALSFRS-R [0-48]	41.22 ±4.48	43.20 ± 3.42	0.39
Disease duration [months]	19.41 ± 15.31	25.60 ± 4.56	0.04
Disease progression rate	0.43 ± 0.30	0.18 ± 0.10	0.046
Site of onset (limb/ bulbar/limb+bulbar)	15/2/1	5/0/0	0.62

Values denotes mean ± standard deviations or numbers (percentages). P-values refer to Kruskal Wallis one-way ANOVA and Fisher's exact test for continuous and categorical variables, respectively. Abbreviations: ALS=Amyotrophic Lateral Sclerosis; PMA=Primary Motor Atrophy; ALSFRS-R=ALS Functional Rating Scale revised. Disease Progression Rate has been obtained as following: (48–ALSFRS-R score)/time between symptom onset and first visit.

Over time, the standard neuropsychological battery identified a global cognitive decline (MMSE) in ALS patients; however, this finding was not observed when the analysis accounted for ALSFRS-R changes (Table 3). No other significant changes were detected using the standard battery either in other cognitive domains or in mood or behaviour. No longitudinal changes were observed for the available standard tests in the TAP subsample (Table 4). The computer-based battery revealed a significantly worse performance of ALS patients after 12 months, in terms of both accuracy and reaction times, in the alertness, sustained and divided attention, go/nogo, crossmodal and incompatibility tasks. Most of these findings remained significant when ALSFRS-R changes over time were included as covariate in the analyses (Table 4, Figure 3). MMSE and TAP changes in ALS patients were not associated with mood modifications over time ($p>0.05$; see S-Table 2 for partial correlation results).

PLS and PMA patients did not show changes over 12 months at the standard neuropsychological tests (Table 3). Using TAP, PMA patients showed improvement over time in alertness (without cue) and go/nogo (Table 4; Figure 3).

Table 3. Cognitive performance at the standard neuropsychological battery at baseline, 6 months and 12 months.

Outcome	At baseline (0m)			At 6 months (6m)			At 12 months (12m)			Baseline comparison (p-value)	Test for linear trend (p-values)		
	ALS	PLS	PMA	ALS	PLS	PMA	ALS	PLS	PMA	ALS vs PLS vs PMA	Within ALS	Within PLS	Within PMA
Global cognition													
MMSE [%corrected/performed items]	96 (73 – 100)	96 (83 – 100)	97 (93 – 100)	96 (73 – 100)	96 (70 – 100)	98 (90 – 100)	88 (60 – 100)	100 (100 – 100)	96 (87 – 100)	0.98	0.01	0.60	0.72
Memory													
RAVLT, delayed recall	8.59 (7.63-9.67)	8.37 (6.98-10.05)	9.03 (6.41-12.74)	9.21 (8.18-10.36)	8.96 (7.51-10.68)	7.57 (4.82-11.9)	9.27 (7.63-11.27)	10.1 (7.34-13.9)	12.95 (9.05-18.53)	0.93	0.44	0.39	0.20
Digit span forward	5.42 (5.04-5.84)	5.33 (4.73-6.01)	6.68 (5.45-8.2)	5.43 (5.04-5.86)	5.33 (4.73-6.01)	5.68 (4.55-7.09)	5.76 (5.1-6.5)	6.28 (5.09-7.75)	6.2 (4.74-8.1)	0.14	0.48	0.30	0.51
Executive functions													
WCST, global score	55.3 (35.92-85.12)	39.76 (16.99-93.05)	-	46.43 (28.39-75.95)	36.22 (14.88-88.19)	-	51.77 (20.56-130.35)	-	-	0.48	0.74	0.88	-
WCST, perseverations	17.74 (11.54-27.28)	34.11 (14.19-82.02)	-	17.39 (10.97-27.57)	26.43 (9.75-71.61)	-	8.31 (2.34-29.52)	-	-	0.16	0.36	0.70	-
Digit span, backward	4.13 (3.74-4.56)	4.19 (3.58-4.91)	5.35 (4.09-6.99)	4.09 (3.7-4.53)	3.84 (3.25-4.53)	4.34 (3.23-5.85)	4.94 (4.2-5.81)	5.63 (4.34-7.3)	5.65 (4.06-7.86)	0.20	0.16	0.26	0.94
Weigl's Test	11.39 (10.44-12.42)	12.53 (10.6-14.81)	12.07 (9.54-15.26)	12.65 (11.6-13.79)	12.26 (10.36-14.51)	15.03 (11.62-19.45)	12.25 (9.94-15.11)	13.83 (11.09-17.26)	13.73 (10.4-18.13)	0.58	0.11	0.54	0.41
Raven's matrices	28.49 (26.6-30.51)	27.04 (23.88-30.62)	31.36 (25.83-38.08)	27.89 (25.96-29.96)	28.46 (25.21-32.13)	32.36 (26.73-39.18)	31.03 (27.87-34.55)	33.2 (27.5-40.08)	29.91 (23.37-38.29)	0.44	0.33	0.09	0.80
Cognitive Estimation Task	13.96 (12.65-15.42)	15.9 (13.68-18.48)	10.57 (7.06-15.83)	12.24 (10.94-13.7)	15.7 (13.49-18.26)	14.1 (9.94-20.00)	14.78 (12.47-17.51)	10.71 (7.72-14.85)	14.25 (8.82-23.04)	0.12	0.87	0.09	0.31
Fluency													

Phonemic verbal fluency test	31.97 (28.92-35.35)	25.7 (20.22-32.67)	31.1 (24.41-39.62)	30.96 (27.78-34.51)	28.52 (22.71-35.82)	28.87 (22.45-37.11)	28.94 (24.68-33.92)	31.87 (21.54-47.15)	29.2 (21.28-40.05)	0.43	0.30	0.33	0.72
Phonemic fluency index*	7.29 (5.05-9.54)	8.43 (4.08-12.79)	5.51 (0.45-10.57)	7.8 (5.34-10.25)	4.54 (-0.07-9.16)	6.26 (1.02-11.51)	7.85 (4.67-11.04)	4.84 (-7.45-17.13)	6.01 (0.28-11.74)	0.70	0.66	0.18	0.84
Semantic verbal fluency test	40.77 (37.74-44.03)	37.65 (31.7-44.73)	38.46 (31.87-46.4)	40.31 (37.13-43.76)	33.69 (28.09-40.42)	40.55 (33.77-48.68)	38.91 (34.55-43.81)	43.87 (32.83-58.61)	34.29 (26.69-44.06)	0.79	0.54	0.71	0.55
Semantic fluency index*	5.03 (3.28-6.79)	5.79 (2.5-9.09)	4.93 (0.95-8.91)	4.55 (2.73-6.37)	6.87 (3.43-10.31)	4.54 (0.5-8.58)	5.4 (3.27-7.53)	4.73 (-4.24-13.7)	5.39 (1.18-9.61)	0.92	0.95	0.70	0.84
Language													
BADA [nouns]	28.81 (28.33-29.3)	29.08 (28.18-30.01)	29.67 (28.16-31.26)	28.79 (28.29-29.3)	29.3 (28.4-30.24)	29.67 (28.16-31.26)	28.79 (28.29-29.3)	29.99 (28.48-31.59)	29.03 (27.2-30.98)	0.53	0.52	0.41	0.54
BADA [actions]	26.55 (25.94-27.17)	26.44 (25.26-27.67)	27.00 (25.11-29.03)	26.8 (26.16-27.44)	26.81 (25.62-28.05)	27.34 (25.43-29.38)	26.62 (25.57-27.71)	27.64 (25.72-29.69)	27.08 (24.76-29.61)	0.89	0.54	0.52	0.79
Mood and behaviour													
HDRS	5.21 (3.82-7.11)	6.17 (4.04-9.44)	3.82 (1.1-13.25)	4.09 (2.81-5.94)	5.17 (3.26-8.2)	5.25 (1.82-15.18)	9.21 (5.36-15.82)	2.31 (0.48-11.14)	4.47 (0.77-26.04)	0.69	0.39	0.22	0.82
BDI	8.44 (5.66-12.59)	-	6.23 (3.39-11.47)	8.88 (5.78-13.64)	-	5.83 (2.93-11.58)	7.93 (4.66-13.5)	-	7.10 (3.54-14.25)	0.41	0.68	-	0.72
ALS-FTD-Q	14.11 (9.82-20.28)	8.01 (3.04-21.1)	9.77 (1.39-68.69)	16.26 (11.44-23.13)	10.97 (4.79-25.11)	8.79 (1.13-68.63)	19.6 (11.34-33.87)	10.94 (3.02-39.59)	15.63 (3.33-73.38)	0.52	0.26	0.57	0.56
FBI	2.83 (1.63-4.93)	2.9 (1.3-6.48)	-	3.65 (2.16-6.15)	3.42 (1.63-7.19)	-	5.93 (2.32-15.2)	2.45 (0.47-12.91)	-	0.96	0.20	0.99	-

*Values denote means (along with 95% confidence interval) estimated from a repeated-measure ANOVA model, which included time as categorical variable and baseline ALS Functional Rating Scale Revised (ALSFRS-R) as further covariate. The overall comparison among patient groups at baseline was assessed by means of statistical contrasts derived from each model (joint test). To assess the presence of a linear trend across the estimated means within each patient group, a repeated-measure ANOVA model, which included time as continuous variable (and baseline ALSFRS-R), was performed. *=Verbal fluency indices were obtained as following: time for generation condition - time for control condition (reading or writing generated words)/total number of items generated. Abbreviations: ALS=Amyotrophic Lateral Sclerosis; ALS-FTD-Q=ALS-Frontotemporal lobar degeneration Questionnaire; BADA=battery for the analysis of aphasic deficits; BDI=Beck Depression Inventory; FBI=Frontal Behavioral Inventory; HC=Healthy Controls; HDRS=Hamilton Depression Rating Scale; MMSE=Mini Mental State Examination; PLS=Primary Lateral Sclerosis; PMA=Progressive Muscular Atrophy; RAVLT=Rey Auditory Verbal Learning Test; WCST=Wisconsin Card Sorting Test.*

Table 4. Test of Attentional Performance (TAP) at baseline, 6 and 12 months. Standard neuropsychological test scores of the same sample are also shown.

Outcome	Value	At baseline(0m)		At 6 months (6m)		At 12 months (12m)		Baseline comparison (p-value)	Test for linear trend (p-values)	
		ALS	PMA	ALS	PMA	ALS	PMA	ALS vs. PMA at 0m	Within ALS	Within PMA
Alertness, no cue	RT	381.88 (319.29-456.74)	458.52 (332.9-631.52)	441.24 (352.69-552.03)	334.39 (202.94-550.97)	469.14 (382.72-575.06)	366.96 (248.58-541.73)	0.32	0.03#	0.11
	MDN	370.14 (308.15-444.59)	450.34 (324.99-624.05)	428.12 (340.98-537.53)	327.60 197.33-543.85)	455.52 (374.74-553.73)	374.07 (258.33-541.64)	0.30	0.03#	0.16
	T-MDN	34.26 (30.23-38.81)	27.59 (21.3-35.74)	48.69 (24.01-98.76)	37.43 (8.71-160.8)	29.29 (25.33-33.87)	32.02 (25.43-40.33)	0.13	0.03#	0.04
	SD	71.41 (51.52-99)	115.35 (69.22-192.21)	75.52 (55.43-102.87)	68.87 (36.6-129.6)	71.53 (26.02-196.58)	69.95 (9.27-528.14)	0.07	0.88	0.05
	T-SD	42.17 (37.45-47.49)	35.76 (28.02-45.65)	41.99 (36.86-47.83)	47.03 (37.22-59.42)	39.11 (28.93-52.86)	38.38 (23.62-62.35)	0.17	0.79	0.15
Alertness, with cue	RT	354.24 (306.51-409.41)	429.20 (332.23-554.45)	406.64 (329.57-501.73)	334.40 (214.25-521.95)	420.76 (352.15-502.73)	360.14 (264.74-489.9)	0.19	0.02	0.10
	MDN	345.55 (297.43-401.46)	427.53 (328.75-555.98)	396.34 (322.32-487.38)	331.78 (214.88-512.27)	413.54 (344.91-495.84)	355.45 (258.62-488.54)	0.16	0.02#	0.09
	T-MDN	35.05 (31.05-39.56)	27.46 (21.27-35.47)	47.76 (25.83-88.31)	35.84 (9.87-130.12)	32.25 (25.91-40.12)	30.75 (21.68-43.6)	0.22	0.52	0.28
	SD	56.94 (42.47-76.33)	79.12 (48.36-129.43)	63.58 (44.73-90.38)	70.25 (36.17-136.47)	57.71 (41.48-80.3)	56.5 (32.14-99.33)	0.16	0.82	0.13
	T-SD	46.36 (42.06-51.09)	39.8 (32.68-48.47)	46.47 (40.55-53.26)	43.92 (33.87-56.94)	46.33 (39.91-53.78)	45.17 (35.71-57.14)	0.24	0.98	0.17
Sustained attention	T-MDN	42.78 (38.21-47.9)	51.76 (41.82-64.07)	103.98 (32.58-331.91)	51.76 (2.6-1029.5)	43.92 (14.61-132.02)	46.44 (2.26-953.67)	0.76	0.81	0.84
	T-DS	41.11 (27.47-61.53)	45.43 (20.5-100.69)	57.95 (40.6-82.71)	48.41 (22.39-104.67)	41.32 (17.32-98.61)	40.53 (14.1-116.56)	0.64	0.48	0.91

	Omissions	16.64 (12.89-21.49)	20.61 (12.9-32.91)	19.57 (14.61-26.22)	17.00 (8.98-32.18)	23.67 (18.66-30.02)	19.65 (12.52-30.86)	0.41	0.02	0.79
Go/NoGo	RT	680.33 (623.12-742.8)	608.89 (516.8-717.4)	721.31 (658.59-790)	591.21 (491.29-711.46)	736.98 (664.53-817.33)	609.85 (517.94-718.07)	0.23	0.02[#]	0.97
	MDN	660.51 (605.9-720.04)	607.19 (518.53-711.02)	699.61 (640.77-763.86)	565.59 (474.64-673.98)	724.83 (657.09-799.56)	554.00 (468.15-655.59)	0.34	0.01[#]	0.045
	T-MDN	40.24 (33.32-48.61)	45.12 (33.57-60.65)	34.31 (27.89-42.2)	51.02 (38.39-67.8)	34.94 (28.12-43.4)	53.52 (40.94-69.96)	0.50	0.04[#]	0.91
	SD	115.87 (88.55-151.61)	90.27 (52.99-153.79)	125.93 (99.66-159.13)	97.79 (59.45-160.85)	115.66 (74.14-180.44)	81.92 (37.88-177.16)	0.74	0.94	0.73
	T-SD	40.29 (34.24-47.42)	49.68 (38.68-63.81)	37.46 (31.82-44.1)	44.83 (34.03-59.06)	43.51 (32.11-58.97)	48.57 (33.37-70.7)	0.77	0.97	0.73
	Errors	1.66 (0.1-28.58)	0.19 (0-292463.44)	10.31 (3.28-32.36)	0.61 (0-4423.66)	11.9 (1.72-82.55)	0.38 (0-210876.71)	0.91	0.26	0.94
Incompatibility	RT	668.26 (579.35-770.81)	568.65 (434.69-743.89)	715.79 (612.11-837.04)	565.18 (416.5-766.93)	757.58 (664.98-863.08)	606.72 (488.8-753.09)	0.27	0.046	0.57
	MDN	646.92 (555.72-753.07)	560.81 (422.7-744.03)	694.60 (601.5-802.12)	552.88 418.07-731.14)	740.79 (660.81-830.45	572.04 (470.89-694.92)	0.36	0.03[#]	0.97
	T-MDN	38.03 (32.14-45)	44.92 (34.26-58.89)	34.08 (28.18-41.2)	45.67 (34.31-60.8)	31.51 (26.71-37.17)	40.88 (32.91-50.77)	0.28	0.01[#]	0.82
	SD	132.83 (104.42-168.96)	120.69 (77.91-186.95)	145.56 (113.9-186.02)	117.96 (73.58-189.12)	141.16 (110.61-180.14)	128.08 (88.69-184.96)	0.79	0.82	0.63
	T-SD	49.45 (45.37-53.91)	49.33 (42.45-57.32)	46.43 (42.15-51.14)	51.34 (44.05-59.84)	47.38 (44.38-50.59)	46.63 (42.9-50.67)	0.83	0.51	0.51
	Errors	1.88 (1.13-3.11)	3.94 (2.12-7.32)	1.41 (0.88-2.26)	3.41 (2.08-5.59)	1.5 (0.16-14.34)	4.9 (0.98-24.46)	0.94	0.55	0.72
	T-Errors	59.34 (53.63-65.67)	50.37 (41.65-60.91)	60.47 (56.73-64.47)	50.86 (45.79-56.49)	64.1 (41.81-98.27)	47.27 (27.57-81.03)	0.96	0.72	0.88
Divided attention	RT	572.64 (536.37-611.37)	552.08 (485.07-628.35)	590.5 (525.75-663.23)	495.86 (389.03-632.04)	635.02 (558.24-722.36)	539.33 (443.57-655.77)	0.19	0.09	0.63
	MDN	563.48 (525.78-603.89)	545.36 (475.99-624.84)	556.37 (509.55-607.49)	483.81 (403.9-579.54)	632.96 (570.36-702.43)	544.52 (458.2-647.1)	0.17	0.06	0.73

	T-MDN	44.44 (38.21-51.68)	46.82 (35.16-62.34)	57.38 (36.02-91.41)	57.62 (23.81-139.45)	34.87 (28.63-42.48)	46.43 (34.75-62.03)	0.74	0.01	0.95
	SD	90.41 (73.27-111.57)	100.94 (68.39-148.99)	137.04 (74.18-253.18)	86.58 (18.66-401.7)	77.28 (40.57-147.22)	79.56 (12.49-506.81)	0.95	0.31	0.53
	T-SD	46.48 (41.18-52.46)	42 (33.06-53.36)	45.03 (39.77-50.98)	47.59 (37.91-59.75)	50.21 (44.66-56.46)	47.09 (39.79-55.74)	0.99	0.19	0.38
Crossmodal	RT	516.3 (476.4-559.54)	495.01 (427.79-572.8)	549.13 (492.26-612.58)	433.12 (343.95-545.4)	674.63 (481.65-944.93)	487.5 (274.07-867.16)	0.07	0.07	0.27
	MDN	491.57 (450.39-536.51)	451.49 (384.27-530.48)	513.13 (459.04-573.6)	393.4 (310.79-497.97)	625.79 (419.93-932.55)	525.75 (273.34-1011.24)	0.17	0.27	0.33
	T-MDN	44.55 (35.34-56.17)	41.85 (30.46-57.49)	42.55 (33.57-53.94)	46.87 (34.68-63.34)	31.35 (19.96-49.24)	36.27 (23.03-57.14)	0.46	0.32	0.92
	SD	114.76 (94.32-139.63)	132.76 (95.78-184.03)	137.82 (96.92-195.98)	112.68 (50.36-252.12)	123.12 (45.15-335.71)	40.08 (3.15-509.37)	0.15	0.48	0.20
	T-SD	45.3 (36.59-56.09)	45.77 (34.68-60.4)	43.5 (34.98-54.09)	58.61 (45.81-74.98)	53.95 (29.45-98.86)	62.66 (39.51-99.38)	0.35	0.87	0.14
	T-Errors	43.70 (38.96-49.01)	44.78 (38.58-51.99)	50.91 (45.77-56.63)	45.11 (38.88-52.35)	54.64 (41.6-71.78)	48.39 (37.65-62.21)	0.76	0.01	0.67
MMSE	-	0.96 (0.93-0.99)	0.95 (0.93-0.98)	0.97 (0.93-1.00)	0.96 (0.89-1.00)	0.90 (0.83-0.98)	0.96 (0.86-1.00))	0.16	0.28	0.48
Phonemic fluency test	-	31.67 (25.05-38.29)	29.40 (23.15-35.65)	32.79 (23.44-42.13)	28.60 (20.48-36.72)	26.67 (20.50-32.83)	28.67 (22.93-34.40)	0.60	0.90	0.83
Phonemic fluency index*	-	6.57 (4.60-8.55)	5.60 (4.21-7.00)	9.16 (0.43-17.90)	6.13 (3.53-8.73)	8.66 (4.27-13.05)	5.63 (4.52-6.74)	0.88	0.52	0.69
Semantic fluency test	-	42.78 (36.21-49.35)	36.20 (28.09-44.31)	42.79 (34.77-50.80)	39.60 (28.97-50.23)	37.33 (29.07-45.60)	32.00 (8.30-55.70)	0.17	0.62	0.07
Semantic fluency index*	-	4.71 (2.7-6.68)	4.78 (3.64-5.93)	4.77 (1.73-7.81)	4.32 (2.42-6.23)	6.38 (1.22-11.52)	5.82 (-0.24-11.87)	0.12	0.33	0.24
BDI	-	7.92 (4.34-11.50)	8.00 (1.61-14.39)	8.82 (4.76-12.88)	6.40 (2.82-9.98)	9.56 (4.80-14.31)	9.67 (-1.53-20.87)	0.84	0.98	0.053

Values denote means (along with 95% confidence interval) estimated from a repeated-measure ANOVA model, which included time as categorical variable and baseline ALS Functional Rating Scale Revised (ALSFRS-R) as further covariate. # reflects findings that remained significant when ALSFRS-

*R changes over time were included as covariate in the analyses. The overall comparison among patient groups at baseline was assessed by means of statistical contrasts derived from each model (joint test). To assess the presence of a linear trend across the estimated means within each patient group, a repeated-measure ANOVA model, which included time as continuous variable (and baseline ALSFRS-R), was performed. T is a TAP standardized value with a mean of 50 and a standard deviation of 10. Higher T values mean better performance. *Verbal fluency indices were obtained as follows: time for generation condition - time for control condition (reading or writing generated words)/total number of items generated. Abbreviations: ALS=Amyotrophic Lateral Sclerosis; BDI=Beck Depression Inventory; MDN=median; MMSE=Mini Mental State Examination; PMA=Primary Motor Atrophy; RT=mean reaction time; SD= standard deviation; T-Errors=T value of errors; T-MDN=T value of the median; T-SD= T value of the standard deviation.*

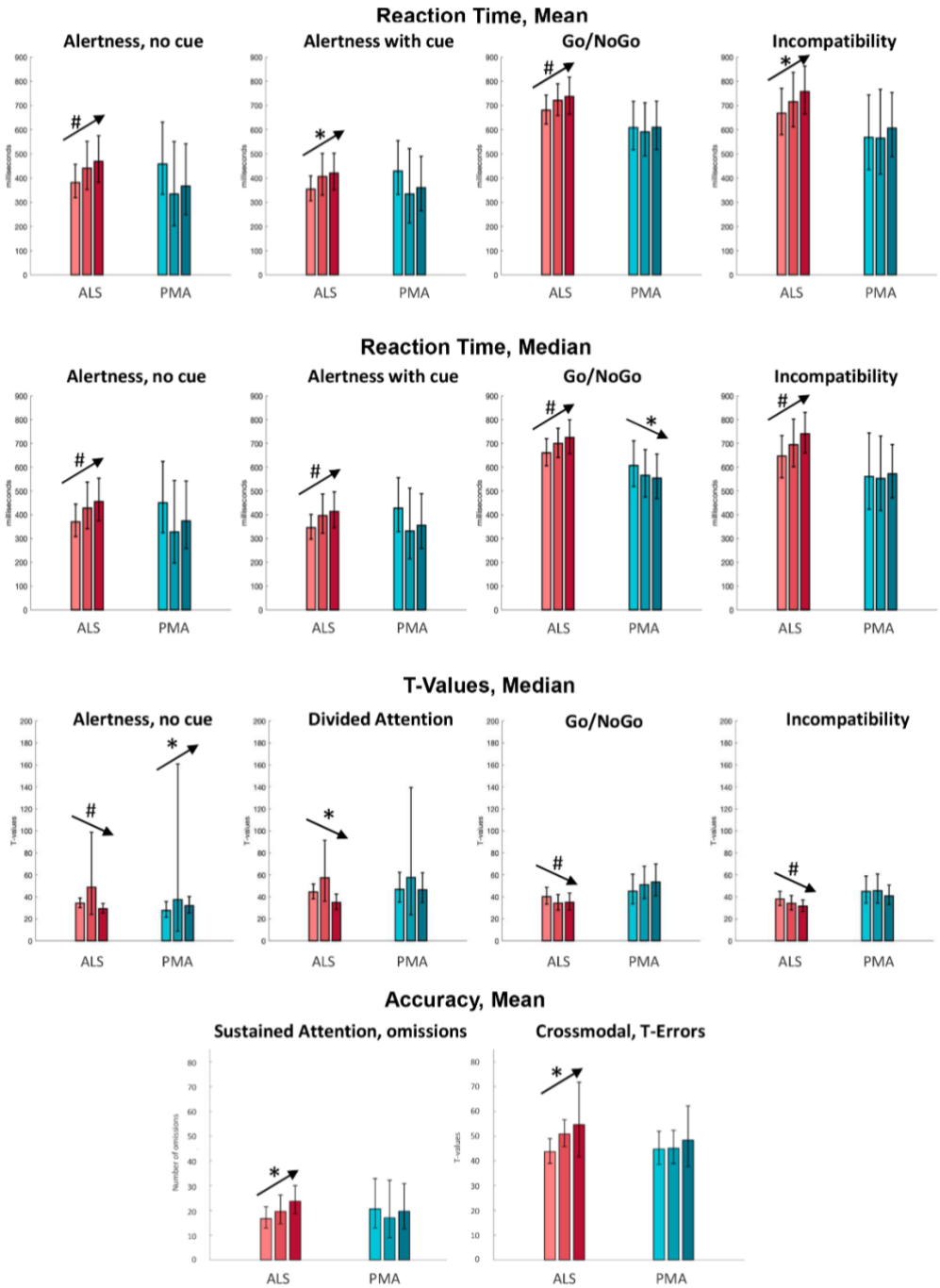

Figure 3. *Performance at the Test of Attentional Performance (TAP) subtests in ALS and PMA patients at baseline, month 6 and month 12. Histograms denote means or medians (along with 95% confidence interval) estimated from a repeated-measure ANOVA model, which included time as categorical variable and baseline ALS Functional Rating Scale Revised (ALSFRS-R) as further covariate. Only variables with significant changes over time were shown and are reported with the asterisk (*). In addition, the symbol # indicates findings that remained significant in ALS when ALSFRS-R changes over time were included as covariate in the analyses. T is a TAP standardized value with a mean of 50 and a standard deviation of 10. Higher T values mean better performance. Abbreviations: ALS=Amyotrophic Lateral Sclerosis; MDN=median; PMA=Primary Motor Atrophy; RT=mean reaction time; T-MDN=T value of the median; T-Errors=T value of errors.*

2.4 Discussion

In this study, we investigated the progression of cognitive and behavioural changes within a time window of 12 months in MND patients using standard and/or computer-based neuropsychological batteries. Cognitive changes over time were observed in ALS patients, involving attentive and executive functions, and were detected by the computer-based approach. Our small samples of PLS and PMA patients did not show any change over time.

Cognitive and behavioural findings in patients with MND are highly dependent on test administration modality. In addition, it is important to consider whether tests are specific and sensitive for MND, and whether they take into account the patient's verbal and/or physical impairment.

Considering the clinical evolution of the disease, a neuropsychological investigation that does not consider all these aspects is likely to fail in detecting subtle cognitive deficits and their progression over time. In this study, precautions were adopted for the standard neuropsychological tests that are most sensitive to motor impairment. We used a weighted score for global cognition (MMSE; i.e., the percentage of corrected items on the total administered items) and fluency indices, which account for reading/writing speed (Abrahams, Leigh et al., 2000). Furthermore, during FBI administration, we avoided to include items questioning on logopenia/aphasia and alien hand/apraxia since they cannot clearly distinguish behavioural symptoms from physical disability. Finally, all analyses were corrected for ALSFRS-R. Nevertheless, using this battery, we did not observe performance differences within any of the MND groups after 12 months, except for MMSE changes in ALS. However, this finding was not significant when the analysis accounted for ALSFR-R changes in ALS patients over time.

Concerning the other standard tests, the negative findings are in line with other studies which used a standard neuropsychological approach (Kasper et al., 2016, Kilani et al., 2004, Robinson et al., 2006, Strong et al., 1999, Woolley et al., 2018). Using this type of cognitive assessment, two studies (Bock et al., 2017, Elamin et al., 2013) observed a cognitive decline in ALS patients who were already cognitively impaired at baseline. In our ALS sample, only 6% of patients were classified as cognitively impaired at study entry and low percentage of cases were also detected in previous studies showing negative findings (Kilani et al., 2004, Robinson et al., 2006, Strong et al., 1999). Thus, we can

hypothesize that a standard approach is useful for detecting the cognitive progression in patients that already present with cognitive impairment at study entry, but it could be not as efficient in detecting a cognitive decline in cases with a subclinical cognitive profile.

Furthermore, standard batteries may have the additional limitation of being not specific for MND because they have not been designed for this clinical population. On the other hand, studies using screening tools specifically designed for ALS, as the ECAS or the Amyotrophic Lateral Sclerosis Cognitive-Behavioral Screen, did not identify cognitive longitudinal changes even with long follow-up periods (Abrahams et al., 2014, Bock et al., 2017). The lack of findings in these cases may depend on the nature of the 'screening' batteries, which may have the limitation of not assessing all possible dysfunctions (Bock et al., 2017).

For both standard and screening batteries, in general, we must acknowledge that the majority of tests adopted have a smaller range of possible responses compared to the typical outcomes obtained with computerized batteries, so they may have less chance to detect subtle changes.

On this purpose, computer-based batteries, might allow for a deeper investigation of MND-typical altered cognitive functions and accommodate for motor disability. Using TAP, we observed that ALS patients experienced a significant worsening of performance, in terms of both speed and accuracy, in specific subtests assessing attention and dual tasks (alertness, sustained and divided attention) and proper executive tasks (inhibitory control, multimodal sensory integration and parallel processing of divergent information). All these functions are subtended by the frontal cortices and their main connections with the parietal and ventro-striatal networks (Di Martino, Scheres et al., 2008). These findings are consistent with the significant decline found in the same subtest by Schreiber et al. (Schreiber et al., 2005), which focused only on speed performance during this test and divided attention. Interestingly, in this study patients were also administered a standard paper and pencil battery and worsened significantly in the naming-time score of the colour word interference test and in fluency (Schreiber et al., 2005), a performance that might have been affected by motor impairment. A more recent study also administered both the standard and the ALS-CFB to ALS patients (Gillingham et al., 2017) and reported that patients worsened exclusively in the Simple Reaction Time task of the ALS-

CFB, once more demonstrating the ability of computerised batteries to detect subtle cognitive deficits compared to standard neuropsychological tests.

We are aware that increased reaction times in ALS patients after 12 months, mainly in specific domains such as alertness, may be attributed to several factors other than a frontal pathological involvement, such as hypoxia due to respiratory failure, progressive physical impairment, and abnormal mood. However, we paid particular attention in reducing the impact of all these factors: we excluded patients with significant respiratory failure; the simple TAP key press response minimizes motor demands; cognitive analyses were adjusted for ALSFRS-R; we verified the potential relationship between patient mood and TAP changes.

With regard to the two other MND variants, our results should be considered with caution, considering the small sample. The absence of cognitive changes over 12 months at the standard battery is in line with the only previous study which assessed the progression of cognitive dysfunctions in PLS (Proudfoot et al., 2015). Unfortunately, since we did not collect performances at the computer-based battery for the PLS group, we cannot exclude that cognitive changes were present but not detected by the standard battery. Regarding PMA, this was the first longitudinal study monitoring the cognitive profile of these patients. After 12 months, we observed no significant cognitive worsening either at the standard battery, or at the computerised battery. In this group, we rather observed a significant improvement in alertness and go/nogo, suggesting a maintaining of these domains, as well as a (still) possible role of familiarity processes while approaching the same tests one year later. A recent cross-sectional study in PMA patients with a mean disease duration of 60 months reported frequencies of cognitive impairment equal to ALS and PMA (de Vries et al., 2019a). In our study, all PMA patients had shorter disease duration and were cognitively normal at study entry. Thus, we can hypothesize that PMA patients may manifest cognitive deficits only later in the course of the disease.

Some limitations of our study should be noted. This is a retrospective study. A major limitation of this longitudinal study is the relatively high attrition rate: only 33/74 patients of the main sample and 9/23 patients of the subsample performing the TAP underwent the last 12-month visit, although this was expected given the aggressive course of MND. In addition, the samples of patients with PLS and PMA are small and this could explain negative findings. Furthermore, with such as small groups, we were unable to

perform comparisons between groups over time. Computer-based battery was not used in PLS patients and we did not collect ECAS. Finally, although the cognitive/behavioural classification of our patients was out of the main purpose of the study, the two language tests adopted were fewer compared to those assessing executive functions and both tests analysed the same language sub-function (i.e., confrontation naming). Despite these shortcomings, this study revealed a unique progressive cognitive decline in ALS patients, involving the domains of attention and executive functions. The longitudinal changes of cognitive abilities shown by ALS patients well reflect the link of the disease to the frontotemporal lobar degeneration spectrum. The computer-based approach, more than the standard and comprehensive neuropsychological battery, was able to identify even subtle cognitive changes, specific to ALS condition, and should be tested in future prospective studies.

Disclosure statement

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2.6 Appendix

2.6.1 Standard neuropsychological assessment

The following cognitive functions were evaluated: global cognitive functioning with the MMSE (Folstein, Folstein et al., 1975) (as ratio between number of correct items/maximum number of administered items), long and short term verbal memory with the Rey Auditory Verbal Learning Test (Carlesimo, Caltagirone et al., 1996) and the digit span forward (Orsini, Grossi et al., 1987), respectively; executive functions with the digit span backward (Monaco, Costa et al., 2013), the Cognitive Estimation Task (Della Sala, MacPherson et al., 2003), the Weigl's Sorting test (Spinnler, 1987), the Wisconsin Card Sorting Test (Laiacona, Inzaghi et al., 2000), and the Raven's coloured progressive matrices (Basso, Capitani et al., 1987); fluency with the phonemic and semantic fluency tests (Novelli G, 1986) and the relative fluency indices (Abrahams, Leigh et al., 2000); language with the naming sub-tests of the Italian battery for the assessment of aphasic disorders (Miceli, 1994); mood with the Hamilton Depression Rating Scale (HDRS) (Hamilton, 1960) and the Beck Depression Inventory (Beck, Ward et al., 1961); and the presence of behavioural disturbances with the Amyotrophic Lateral Sclerosis-Frontotemporal Dementia-Questionnaire (Raaphorst, Beeldman et al., 2012) and the Frontal Behavioural Inventory (FBI) (Alberici, Geroldi et al., 2007) administered to patients' caregivers.

The standard neuropsychological battery was also used to define the potential presence of cognitive and/or behavioural impairment according to Strong's criteria (Strong, Abrahams et al., 2017) for each group at the study entry. Specifically, cognitive impairment was defined by the presence of executive dysfunctions (i.e., impaired letter fluency or impairment on two other non-overlapping measures of executive functions which may include social cognition) or language dysfunctions, or a combination of the two. Behavioural impairment was defined by the presence of apathy or at least two non-overlapping behavioural disturbances. Each cognitive domain required for the cognitive/behavioural classification was represented by at least two tests (S-Table 1).

2.6.2 Computer-based assessment

The Test of Attentional Performance (TAP) subtests were the following: 1) alertness, which estimates patients' reaction time to visual stimuli preceded or not by warning acoustic signals; 2) divided attention, a dual task which requires to elaborate visual and acoustic stimuli in parallel; 3) crossmodal integration, which values the capacity to integrate multimodal sensory stimuli; 4) go/nogo, which investigates the ability to suppress the response to irrelevant stimuli; 5) incompatibility, which investigates the ability to process in parallel divergent stimulus information; 6) sustained attention, which requires mentally demanding activity for a sustained period of time.

S-Table 1. Outcome measures of the standard neuropsychological battery and the Test of Attentional performance.

Test	**Reference**	**Cognitive function**	**Correction for physical impairment**	**Outcome: speed**	**Outcome: accuracy**	**Cognitive/ behavioral classification according to Strong et al., 2017**
MMSE [%corrected/performed items]	Folstein et al., 1975	Global cognition	N of correct items/maximum N of administered items	/	Adjusted N corrected responses, Total score	
RAVLT, delayed recall	Carlesimo et al., 1996	Memory, verbal long-term free recall	/	/	N of remembered words	
Digit span forward	Orsini et al., 1987	Memory, verbal brief-term free recall	/	/	N of digits recalled in the right order	
WCST	Laiacona et al., 2000	Executive functions, problem solving	/	/	Global score, perseverations	X
Digit span, backward	Monaco et al., 2013	Executive functions, verbal working memory	/	/	N of digits recalled in the reverse order	X
Weigl's Test	Spinler et al., 1987	Executive functions, abstract sorting	/	/	N of sorted categories	
Raven's matrices	Basso et al., 1987	Executive functions, abstract reasoning	/	/	N corrected responses, Total score	X

Cognitive Estimation Task	Della Sala et al., 2003	Executive functions, cognitive estimation	/	/	N corrected responses, Total score; N of bizarre responses.	X
Phonemic fluency index	Novelli et al., 1986	Fluency	Time for generation condition - time for control condition (reading/writing generated words)/total number of items generated	/	Index obtained for produced words in 60 seconds (or written words in 120 seconds)	X
Semantic fluency index	Novelli et al., 1986	Fluency		/		X
BADA [nouns]	Miceli et al., 1994	Language, confrontation naming of visual stimuli	/	/	N of correctly named stimuli, Total score	X
BADA [actions]	Miceli et al., 1994	Language, confrontation naming of actions	/	/	N of correctly named stimuli, Total score	X
HDRS	Hamilton, 1960	Mood	/	/	Total score	
BDI	Beck et al., 1961	Mood	/	/	Total score	
ALS-FTD-Q	Raaphorst et al., 2012	Behaviour	/	/	Total score (Item subscores were used for the behavioral classification)	X
FBI	Alberici et al., 2007	Behaviour	Items on logopenia and alien hand/apraxia were not considered	/	Total score (Item subscores were used for the behavioral classification)	X
Alertness, no cue	Zimmermann et al., 1992	Condition of general wakefulness that enables a person to respond quickly and appropriately to any given demand	/	Mean, Median, T-MND, SD, T-SD		
Alertness with cue			/	Mean, Median, T-MND, SD, T-SD		
Sustained attention		Continue maintenance of attention	/	T-MND, T-SD	Omissions	

		in tasks with different levels of cognitive demands				
Go/NoGo		Ability to perform an appropriate reaction and to simultaneously inhibit an inappropriate behavioural response	/	Mean, Median, T-MND, SD, T-SD	Errors	
Incompatibility		The ability to face a conflict situation in which divergent stimulus information has to be processed in parallel	/	Mean, Median, T-MND, SD, T-SD	Errors, T-Errors	
Divided attention		The capacity to pay attention to several things at once	/	Mean, Median, T-MND, SD, T-SD		
Crossmodal integration		The capacity to integrate multimodal sensory stimuli	/	Mean, Median, T-MND, SD, T-SD	T-Errors	

Abbreviations: ALS=Amyotrophic Lateral Sclerosis; MDN=median; SD=standard deviation; T-Errors=T value of errors; T-MDN=T value of the median; T-SD=T value of the standard deviation.

S-Table 2. Partial correlations between mood (BDI) and cognition changes over time in ALS patients accounting for ALSFRS-R at baseline.

Significant outcomes	Value	R	Significance
MMSE	-	0.293	0.271
Alertness, no cue	RT	-0.500	0.118
	MDN	-0.547	0.081
	T-MDN	0.152	0.655
Alertness, with cue	RT	-0.528	0.095
	MDN	-0.497	0.120
Sustained attention	Errors	0.341	0.409
Go/Nogo	RT	0.024	0.959
	MDN	0.322	0.482
	T-MDN	-0.352	0.438
Incompatibility	RT	-0.140	0.765
	MDN	-0.233	0.615
	T-MDN	0.172	0.712
Divided attention	T-MND	-0.168	0.621
Crossmodal	T-Errors	0.655	0.546

Abbreviations: ALS=Amyotrophic Lateral Sclerosis; MDN=median; RT=mean reaction time; T-MDN=T value of the median; T-Errors=T value of errors. To assess changes of mood (BDI) and cognition over time, delta scores for each significant outcome were obtained by subtracting baseline scores from follow-up scores.

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Chapter 3 - Progression of brain functional connectivity and frontal cognitive dysfunction in ALS

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Progression of brain functional connectivity and frontal cognitive dysfunction in ALS

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ABSTRACT

Objective: To investigate the progression of resting-state functional connectivity (rs-FC) changes in patients with amyotrophic lateral sclerosis (ALS) and their relationship with frontal cognitive alterations.
Methods: This is a multicentre, observational and longitudinal study. At baseline and after six months, 25 ALS patients underwent 3D T1-weighted MRI, resting-state functional MRI (rs-fMRI), and the computerized Test of Attentional Performance (TAP). Using independent component analysis, rs-FC changes of brain networks involving connections to frontal lobes and their relationship with baseline cognitive scores and cognitive changes over time were assessed. With a seed-based approach, rs-FC longitudinal changes of the middle frontal gyrus (MFG) were also explored.
Results: After six months, ALS patients showed an increased rs-FC of the left anterior cingulate, left middle frontal gyrus (MFG) and left superior frontal gyrus within the frontostriatal network, and of the left MFG, left supramarginal gyrus and right angular gyrus within the left frontoparietal network. Within the frontostriatal network, a worse baseline performance at TAP divided attention task was associated with an increased rs-FC over time in the left MFG and a worse baseline performance at the category fluency index was related with increased rs-FC over time in the left frontal superior gyrus. After six months, the seed-based rs-FC analysis of the MFG with the whole brain showed decreased rs-FC of the right MFG with frontoparietal regions in patients compared to controls.
Conclusions: Rs-FC changes in ALS patients progressed over time within the frontostriatal and the frontoparietal networks and are related to frontal-executive dysfunction. The MFG seems a potential core region in the framework of a frontoparietal functional breakdown, which is typical of frontotemporal lobar degeneration. These findings offer new potential markers for monitoring extra-motor progression in ALS.

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3.1 Introduction

Amyotrophic lateral sclerosis (ALS) is the most common type of motor neuron disease, a fatal and heterogeneous neurodegenerative disorder characterized by progressive damage to upper and lower motor neurons (Swinnen & Robberecht, 2014). At present, its multisystem nature and clinical, pathological, and genetic overlap with frontotemporal lobar degeneration (FTLD) are firmly established (Proudfoot, Bede et al., 2018). Cognitive and behavioural disturbances in ALS have been observed in about 50% of patients (van Es, Hardiman et al., 2017), with executive functions, verbal fluency, and apathy as the most frequently affected domains (Goldstein & Abrahams, 2013). Being increasingly recognized, these non-motor features in ALS have been shown to hold significant prognostic implications (Agosta, Spinelli et al., 2019).

In recent years, several neuroimaging techniques, such as magnetic resonance imaging (MRI), have proved to be useful in the search of biomarkers of ALS *in vivo*. Resting-state functional connectivity (rs-FC) is offering a unique contribution for understanding the integrity of brain networks related to motor, cognition and behavior in ALS (Smith, Fox et al., 2009). Cross-sectional rs-functional MRI (rs-fMRI) studies reported rs-FC alterations within motor and extra-motor networks in ALS patients relative to healthy controls (Agosta, Canu et al., 2013, Mohammadi, Kollewe et al., 2009).

Longitudinal studies are critical for understanding, monitoring and timing the progression of neurodegenerative diseases. In ALS, these studies are particularly relevant in order to identify novel biomarkers of motor and cognitive decline and to better define the patient's prognosis. Up to date, only few studies have investigated rs-FC changes over time in ALS (Menke, Proudfoot et al., 2018, Schulthess, Gorges et al., 2016, Shen, Xu et al., 2018, Trojsi, Di Nardo et al., 2020). Most of them have used network-based approaches and have commonly showed increased rs-FC within the sensorimotor network after six months of follow-up. On the other hand, although findings in extra-motor networks are heterogeneous, all studies evidenced altered rs-FC of the frontal regions (Menke et al., 2018, Schulthess et al., 2016, Shen et al., 2018, Trojsi et al., 2020). At present, no studies have yet investigated the relationship between the progressive rs-FC changes involving the connections to the frontal lobes and ALS cognitive alterations over time. Specifically, it is not clear whether and how the progression of brain frontal

alterations are related to the cognitive dysfunction in ALS, and therefore whether they reflect a sign of disease progression or a mechanism of compensation.

It is common knowledge that cognitive assessment in ALS patients requires a correction for motor speed. This adjustment has been excellently achieved by the Edinburgh Cognitive and Behavioural ALS Screen (ECAS) (Abrahams, Newton et al., 2014), a brief neuropsychological battery for detecting cognitive and behavioural disturbances in ALS patients. However, as a screening tool, ECAS may have the limitation of not identifying subtle cognitive deficits in ALS patients. On this purpose, other approaches, such as computer-based batteries, might allow for a deeper investigation of ALS-typical altered cognitive functions and, at the same time, accommodate for motor disability.

In this study, we collected and analyzed clinical, cognitive computer-based, and fronto-connected network (those networks which hold functional connections that reach the frontal lobes, such as frontostriatal and frontoparietal networks) rs-fMRI data in ALS patients at baseline and after six months. We also assessed the relationship of longitudinal rs-FC alterations with baseline and longitudinal performance of patients at a neuropsychological computer-based battery (the Test of Attentional Performance-TAP) (Zimmermann & Fimm, 1992), which investigates the whole spectrum of frontal involvement in ALS accounting for motor impairment. We hypothesized that rs-FC changes could provide an extra-motor brain marker for the study of ALS disease progression.

3.2 Material and methods

3.2.1 Participants

From a large sample of 255 MND cases, twenty-five patients with a clinical diagnosis of probable or definite ALS according to the revised El Escorial criteria (Brooks, Miller et al., 2000) with no significant respiratory failure were selected from those attending two centres in Milan, Italy (IRCCS San Raffaele Scientific Institute and IRCCS Istituto Auxologico Italiano). We selected only patients with two visits (at baseline and six months), each including a clinical evaluation, an MRI scan and a computer-based battery assessing attention and executive functions. A standard

neuropsychological assessment covering all cognitive domains was also obtained at study entry. Thirty-nine healthy controls, age-, sex-, and education-matched with patients, were recruited among non-consanguineous relatives and by word of mouth based on the following criteria: normal neurological exam, mini mental state examination (MMSE) score ≥28, and no family history of neurodegenerative diseases. All healthy controls underwent the baseline visit, which included the standard neuropsychological assessment and an MRI scan. Ten healthy subjects performed also the same MRI protocol at six months. All participants were excluded if they had significant medical illnesses or substance abuse that could interfere with cognitive functioning; any (other) major systemic, psychiatric, or neurological illnesses; and (other) causes of focal or diffuse brain damage, including cerebrovascular disease at conventional MRI scans. No participants were excluded for motion-related artifacts in the MR images. Local ethical standards committee on human experimentation approved the study protocol and all participants provided written informed consent.

3.2.2 Clinical assessment

Disease severity in patients was assessed at baseline and after six months using the ALS Functional Rating Scale-revised (ALSFRS-R, with a maximum score of 48) (Cedarbaum, Stambler et al., 1999). The rate of disease progression was defined according to the following formula: (48–ALSFRS-R score)/time between symptom onset and first visit.

3.2.3 Cognitive and behavioral assessment

At the two centers, neuropsychological assessments were performed by experienced neuropsychologists, who were trained by the same senior neuropsychologist and were unaware of the MRI results. Cognitive evaluation consisted in the administration of: a comprehensive standard neuropsychological battery (to patients and controls at study entry) in order to define the potential presence of cognitive and/or behavioural impairment according to Strong's criteria (Strong, Abrahams et al., 2017), and a computer-based battery (to patients at baseline and after six months) as cognitive outcome to monitor the cognitive progression over time.

In the standard battery, the following cognitive functions were evaluated: global cognitive functioning with the mini mental state examination (MMSE) (Folstein, Folstein et al., 1975); long and short term verbal memory with the Rey Auditory Verbal Learning Test (Carlesimo, Caltagirone et al., 1996) and the digit span forward (Orsini, Grossi et al., 1987), respectively; executive functions with the digit span backward (Monaco, Costa et al., 2013), the Cognitive Estimation Task (CET) (Della Sala, MacPherson et al., 2003), the Weigl's Sorting test (Spinnler & Tognoni, 1987), the Wisconsin Card Sorting Test (Laiacona, Inzaghi et al., 2000) and the Raven's colored progressive matrices (CPM) (Basso, Capitani et al., 1987); fluency with the phonemic and semantic fluency tests (Novelli, 1986) and the relative fluency indices (controlling for individual motor disabilities) (Abrahams, Leigh et al., 2000); language with the Italian battery for the assessment of aphasic disorders (Miceli, Laudanna et al., 1994); mood with the Beck Depression Inventory (BDI) (Beck, Ward et al., 1961); and the presence of behavioral disturbances with the Amyotrophic Lateral Sclerosis-Frontotemporal Dementia-Questionnaire (ALS-FTD-Q) (Raaphorst, Beeldman et al., 2012) administered to patients' caregivers. Healthy controls underwent the entire assessment except for the CET and the Weigl's Sorting test, which were used in patients to deeply investigate executive functions.

The computer-based assessment consisted in the administration of six subtests of the TAP (alertness, divided attention, crossmodal integration, go/nogo, incompatibility and sustained attention) (Zimmermann & Fimm, 1992). The TAP battery accounts for patients' verbal and/or physical impairment and allows the investigation of cognitive abilities, such as attention and executive functions, requiring the involvement of the frontal regions typically affected in ALS. The TAP administration followed strictly standardized procedures. In order to account for patients' verbal and/or physical impairment, patients performed each task with a response-box consisting in a single facilitator press-button for all sub-tests (with the only exception of the Incompatibility sub-test which required two press-buttons), not either requiring patient fine movements nor strength.

3.2.4 MRI acquisition

Using a 3.0 T scanner (Intera, Philips Medical Systems, Best, the Netherlands), the following brain MRI sequences were obtained from all participants at the same center (San Raffaele Scientific Institute): T2-weighted spin echo (repetition time [TR]=3500 ms; echo time [TE]=85 ms; echo train length=15; flip angle=90 [degrees]; 22 contiguous, 5-mm-thick, axial slices; matrix size=512 x 512; field of view [FOV]=230 x 184 mm^2); fluid-attenuated inversion recovery (TR=11 s; TE=120 ms; flip angle=90 [degrees]; 22 contiguous, 5-mm-thick, axial slices; matrix size=512 x 512; FOV=230 mm^2); 3D T1-weighted fast field echo (FFE) (TR=25 ms; TE=4.6 ms; flip angle=30 [degrees]; 220 contiguous axial slices with voxel size=0.89 x 0.89 x 0.8 mm, matrix size=256 x 256, FOV=230 x 182 mm^2); and T2*-weighted single-shot echo planar imaging sequence for rs-fMRI (TR=3000 ms; TE=35 ms; flip angle=90; FOV=240 x 240 mm^2; matrix size=128 x 128; slice thickness=4 mm; 200 sets of 30 contiguous axial slices; acquisition time=10 minutes). Before starting the rs-fMRI scanning, the technician talked with the participants through their earphones instructing them to remain motionless, to keep their eyes closed, not to fall asleep, and not to think about anything in particular. At the end of the rs-fMRI acquisition, the technician talked again with the participants asking whether they remained awake during the sequence.

3.2.5 MRI analysis

MRI analysis was performed at the Neuroimaging Research Unit, Scientific Institute San Raffaele, Milan, Italy.

3.2.5.1 Resting-state fMRI preprocessing

Rs-fMRI data processing was carried out using the FMRIB software library (FSLv5.0) as described previously (Canu, Agosta et al., 2020). The first four volumes of the rs-fMRI data were removed to reach complete magnet signal stabilization. The following FSL-standard preprocessing pipeline was applied: (1) motion correction using MCFLIRT; (2) high-pass temporal filtering (lower frequency: 0.01 Hz); (3) spatial smoothing (Gaussian Kernel of FWHM 6 mm); (4) single-session independent component analysis-based automatic removal of motion artifacts (ICA_AROMA)

(Pruim, Mennes et al., 2015) in order to identify those independent components (ICs) representing motion-related artifacts.

3.2.5.2 Network-based functional connectivity: Independent component analysis (for a brief overview of the method see Figure 1).

Rs-fMRI data set ('clean' from motion-related ICs) were co-registered to the participant's 3D T1-weighted TFE image using affine boundary-based registration as implemented in FLIRT (Greve & Fischl, 2009) and subsequently transformed to the Montreal Neurological Institute (MNI) 152 standard space with 4 mm isotropic resolution using non-linear registration through FNIRT (Andersson, Jenkinson et al., 2007). Pre-processed rs-fMRI data for each subject were temporally concatenated across participants to create a single 4D data set. This rs-fMRI data set was then decomposed into Ics with a free estimate of the number of components using MELODIC (Multivariate Exploratory Linear Optimized Decomposition into Independent Components) (Beckmann, DeLuca et al., 2005). Among group-IC spatial maps (Figure A.1), Ics of interest (frontostriatal, left and right frontoparietal networks) were selected by visual inspection of neuroimaging experts based on previous literature (Smith et al., 2009). In order to identify the subject-specific temporal dynamics and spatial maps associated with each group IC, a dual regression analysis was applied (Filippini, MacIntosh et al., 2009). Finally, spatial maps of all participants were collected into single 4D files for each original IC.

To assess rs-FC changes in ALS patients, delta rs-FC maps for each IC (network) were obtained by subtracting baseline subject-specific spatial maps (in MNI standard space) from follow-up maps.

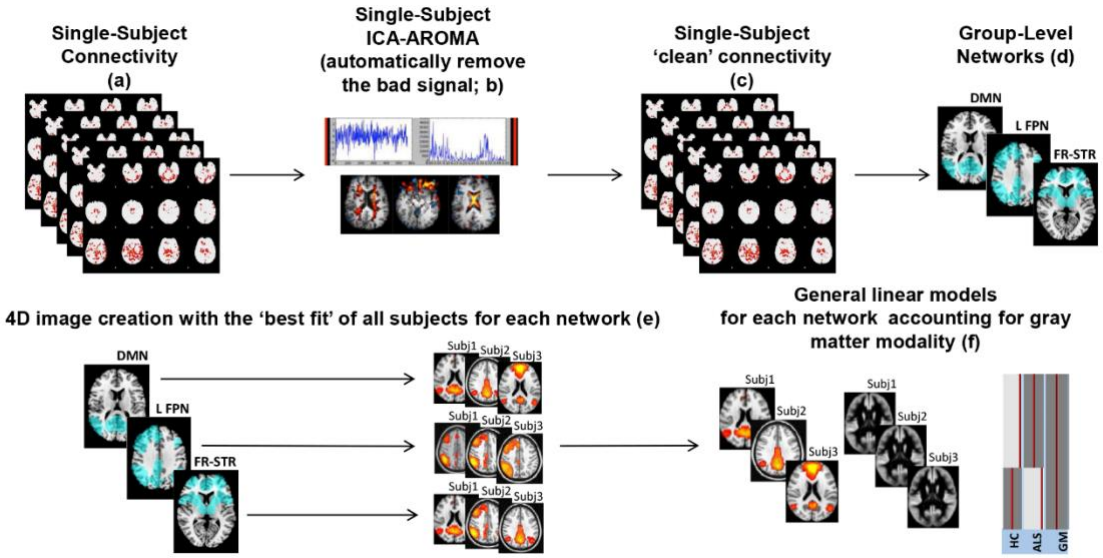

Figure 1. Schematic representation of the procedure for the network-based independent component (IC) analysis. *In each single-subject connectivity map (a), independent component (IC) analysis-based automatic removal of motion artifacts (ICA_AROMA) was applied (b). Pre-processed 'clean' resting state functional MRI data (c) were temporally concatenated across participants to create 4D group-level IC networks (d). A dual-regression procedure was performed and spatial maps of all participants were collected into single 4D files for each original IC (e). Finally, functional connectivity was investigated within each IC according to a specific general linear model. Here we provided the illustrative example of analysis at baseline (f): functional connectivity was compared between ALS patients and controls within each IC using a general linear model which includes the group as independent factor and accounts for voxel-based grey matter density. Abbreviations: ALS=amyotrophic lateral sclerosis; HC= healthy controls; GM=grey matter.*

3.2.5.3 Post-hoc seed-based resting-state functional connectivity (for a brief overview of the method see Figure 2).

Based on findings observed from the independent component analyses (i.e., an increased rs-FC of the MFG was present in ALS patients after six months within the frontostriatal and the left frontoparietal networks, and was related with the patients cognitive dysfunction at baseline; see Results for details), we performed a seed-based analysis, as previously described (Canu et al., 2020). Two regions of interest were selected: left and right MFG. These regions were defined in the MNI space using the automated anatomical labelling atlas (AAL) in WFU PickAtlas (toolbox of SPM12), moved to each subject native T1-weighted space trough non-linear and affine registrations, and visually inspected in the individual brains by neuroimaging expert

researchers. Seed-based rs-FC was then performed using a 2-step regression analysis as implemented in the FMRIB software library (FSLv5). First, time series of white matter (WM), cerebrospinal fluid, and whole brain volumes in rs-fMRI native space were extracted from the preprocessed and denoised data and their effects were regressed out using the FMRI Expert Analysis Tool. ROI mean time-series were then calculated. The output of this step is represented by subject-level maps of all positively and negatively predicted voxels for each regressor. Subject-level maps were registered to the MNI standard template to enter the statistical analysis.

For the longitudinal analysis in ALS patients, delta rs-FC maps for each seed of interest were obtained by subtracting baseline subject-level maps from follow-up maps.

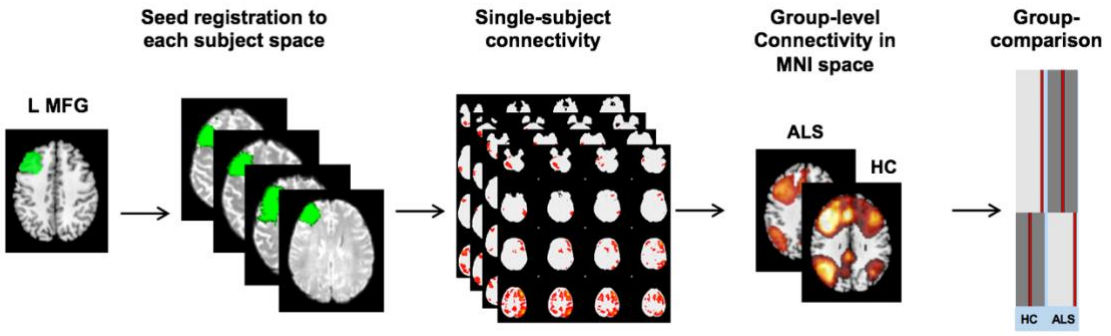

Figure 2. Schematic representation of the procedure for the seed-based resting state functional connectivity. *Each seed of interest (in the figure an example is provided for the left middle frontal gyrus) was defined in MNI space and moved to each subject native space. From each seed, mean time-series were extracted and subject-level maps of all positively and negatively predicted voxels for each regressor were obtained. Subject-level maps were finally registered to the MNI standard template and were ready for the statistical analysis. Here we provided the illustrative example of analysis at baseline: seed-based functional connectivity was investigated in each patient group versus the matched group of controls using a general linear model which includes the group as independent factor. Abbreviations: ALS=amyotrophic lateral sclerosis; HC= healthy controls; L=left; MFG=middle frontal gyrus.*

3.2.6 Statistical analysis

3.2.6.1 Demographic, clinical and cognitive data

To compare the baseline demographic characteristics and cognitive performance between groups at baseline, T-test models and Pearson's $\chi 2$ test were performed for

continuous and categorical variables, respectively. The statistical analyses were performed with SPSS software (version 24.0; IBM Corp., Armonk, NY, USA).

For the longitudinal analysis of cognitive (TAP) scores in ALS patients, we used general linear models (GLM) for repeated-measures in SPSS accounting for changes in ALSFRS-R and time between scans. Analyses were thresholded at $p<0.05$ adjusted for multiple comparisons using Bonferroni's correction. We finally investigated a potential relationship between significant TAP changes over time and patients' mood at baseline assessed with the Beck Depression Inventory (BDI) using partial correlations accounted for ALSFRS-R changes and time between scans.

3.2.6.2 Network-based functional connectivity: Independent component analysis

We performed**:** a) between-group (patients *vs* controls) rs-FC comparisons within each rs-network of interest at baseline; b) in ALS patients (within-group), analysis of each rs-network changes between baseline and follow-up; and c) in patients, correlations between rs-FC changes within each network of interest and measures of TAP, letter and category verbal fluency indices, and CPM (baseline and changes over time). All analyses were carried out using GLMs and nonparametric permutation tests (5000 permutations), and were restricted within the spatial rs-network of interest using binary masks obtained by thresholding the corresponding Z map image ($Z>2.3$). A family-wise error (FEW) correction for multiple comparisons was performed, implementing the threshold-free cluster enhancement using a significance threshold of $p<0.05$. The between-group comparisons at baseline a) were performed with GLMs including 4D spatial maps of all participants as dependent variable accounting for voxel-based gray matter (GM) density. The within-group analyses b) in ALS patients were performed with GLMs including patients' delta rs-FC maps as dependent variables and delta GM density maps, ALSFRS-R changes, and time between scans as nuisance variables. Finally, for correlations c), GLMs included delta rs-FC maps of patients masked for significant (in b) findings as dependent variable, TAP scores and standard neuropsychological scores (fluency indices and CPM) at baseline and TAP and standard score changes over time as covariates of interest, and delta GM density maps, ALSFRS-R changes, and time between scans as nuisance variables. In order to exclude the possibility that our results could be driven by

those patients with behavioral impairment (N=3) and with *C9orf72* mutation (N=2), all analyses were performed also by excluding those patients.

3.2.6.3 Post-hoc seed-based resting-state functional connectivity

Seed-based rs-FC was compared between patients (at baseline and at follow-up) and healthy controls using GLM, which included rs-FC maps as dependent variables. In ALS patients, changes over time were assessed applying GLM using delta rs-FC maps as dependent variables accounting for ALSFRS-R changes and time between scans. Corrections for multiple comparisons were carried out at a cluster level using Gaussian random field theory, $z > 2.3$; cluster significance: $p < 0.05$, corrected for multiple comparisons.

In order to confirm our results at follow-up, seed-based analyses were repeated comparing ALS patients at month six with a sub-sample of 10 age-matched healthy controls who performed the same MRI protocol both at baseline and follow-up (see Table B.3 for sociodemographic features of ALS patients and healthy controls at baseline).

3.3 Results

At baseline, ALS patients and controls did not significantly differ in age, sex and education (Table 1). Two patients were *C9orf72* mutation carriers. According to Strong's criteria (Strong et al., 2017), three patients were classified as behaviourally impaired and none held cognitive abnormalities. The standard neuropsychological battery did not reveal differences between groups, except for subtle mood alterations (evident at the BDI) in ALS patients compared to controls (Table 1). However, no patient showed clinical depression according to BDI neither to clinical interview. After six months, patients showed a clinical progression of the disease (ALSFRS-R at six months: 37.79 ± 6.63; $p<0.01$), and the computer-based battery identified significant cognitive changes in the alertness (with and without cue) TAP subtests (Table 2). No significant associations were observed between patients' mood at baseline and TAP significant changes over time ($p>0.05$).

Table 1. Sociodemographic, clinical and neuropsychological features of ALS patients and healthy controls at baseline.

	ALS	**HC**	**P-value**
N	25	39	
Sex, women	6 (25%)	19 (47.5%)	0.07
Age at MRI [years]	61.56 ± 10.79	64.17 ± 7.44	0.29
Education [years]	10.96 ± 3.92	12.28 ± 4.15	0.21
Disease duration [months]	17.56 ± 14.06	-	-
ALSFRS-R baseline, 0-48	42.36 ± 4.02	-	-
Disease Progression Rate	0.47 ± 0.40	-	-
Site of onset (limb/bulbar/limb+bulbar)	21/3/1	-	-
Visit time interval [months]	5.1 ± 2.1	-	-
Global cognition			
MMSE	28.94 ± 1.14	29.33 ± 0.92	0.21
Memory			
Digit span, forward	6.20 ± 1.48	6.10 ± 0.98	0.85
RAVLT, immediate	47.00 ± 11.13	45.55 ± 10.05	0.75
RAVLT, delayed	8.50 ± 2.43	9.03 ± 3.32	0.71
Spatial span, forward	-	4.83 ± 1.01	-
Executive function			
CPM	32.50 ± 2.88	31.11 ± 3.19	0.34
Digit span, backward	5.00 ± 1.23	4.40 ± 1.16	0.30
CET	13.00 ± 4.42	-	-
Weigl's test	10.83 ± 2.71	-	-
WCST, perseverative responses	5.50 ± 1.73	11.75 ± 7.27	0.13
Language			
BADA (nouns)	30.00 ± 0.00	29.31 ± 1.45	0.08
BADA (actions)	27.50 ± 0.83	27.25 ± 1.77	0.75
Fluency			
Phonemic fluency	33.05 ± 12.63	38.21 ± 8.54	0.11
Phonemic fluency, Index	6.28 ± 3.54	4.82 ± 2.27	0.14
Semantic fluency	42.48 ±10.39	44.03 ± 8.66	0.57
Semantic fluency, Index	3.89 ± 1.09	3.93 ± 1.08	0.85
Mood & Behavior			
BDI	10.32 ± 6.78	4.35 ± 3.64	0.002*
ALS-FTD-Q	22.00 ± 10.74	-	-

*Values denote mean ± standard deviations or numbers (percentages). Neuropsychological values are reported as raw scores. P-values refer to T-test models and Pearson's χ^2 test. *=significant differences between groups at p<0.05. Abbreviations: ALS=amyotrophic lateral sclerosis; ALS-FTD-Q=amyotrophic lateral sclerosis-frontotemporal dementia-questionnaire; ALSFRS-R=ALS Functional Rating Scale Revised; BADA=battery for the analysis of aphasic deficits; BDI=Beck Depression Inventory; CET=cognitive estimation test; CPM=coloured progressive matrices; HC=healthy controls; MMSE=mini-mental state examination; MRI=Magnetic Resonance Imaging. RAVLT=Rey auditory verbal learning test; WCST=Wisconsin card sorting test. Disease Progression Rate has been obtained as following: (48–ALSFRS-R score)/time between symptom onset and first visit. Fluency indices have been obtained as following: time for generation condition – time for control condition (reading or writing generated words)/total number of items generated.*

Table 2. Cognitive performance of ALS patients at the Test of Attentional Performance (TAP), fluency tests and coloured progressive matrices at baseline and at six months of follow-up.

Outcome	**Value**	**Baseline**	**Month 6**	**P-value**
Alertness, no cue	RT	344.00 ± 131.86	383.96 ± 210.29	0.01*
Alertness, no cue	Omissions	0.00 ± 0.00	0.20 ± 1.00	0.13
Alertness, no cue	T-MDN	40.17 ± 11.54	37.13 ± 10.76	0.02*
Alertness with cue	RT	312.32 ± 98.98	349.48 ± 166.42	0.01*
Alertness, with cue	Omissions	0.00 ± 0.00	0.04 ± 0.20	0.35
Alertness with cue	T-MDN	41.71 ± 11.99	39.13 ± 11.53	0.06
Sustained attention	RT	715.64 ± 120.89	718.55 ± 141.13	0.84
Sustained attention	Errors	11.27 ± 9.89	12.32 ± 16.98	0.64
Sustained attention	T-MDN	41.92 ± 7.84	41.50 ± 8.70	0.79
Go/NoGo	RT	643.35 ± 103.20	665.00 ± 115.74	0.15
Go/NoGo	Errors	2.22 ± 3.94	1.39 ± 2.46	0.17
Go/NoGo	T-MDN	45.09 ±13.14	42.68 ± 15.19	0.16
Incompatibility	RT	633.72 ± 157.42	634.94 ± 181.54	0.96
Incompatibility	Errors	5.00 ± 7.66	3.55 ± 5.38	0.19
Incompatibility	T-MDN	42.44 ± 11.80	41.83 ±12.93	0.76
Divided attention	RT	562.40 ± 87.18	568.52 ± 127.67	0.74
Divided attention	Omissions	0.56 ± 1.12	0.32 ± 0.85	0.46
Divided attention	T-MDN	47.24 ±14.47	48.24 ±13.50	0.67
Crossmodal	RT	518.73 ± 119.30	514.59 ± 135.36	0.87
Crossmodal	Errors	2.14 ± 4.04	1.18 ± 2.06	0.21
Crossmodal	T-MDN	44.50 ± 7.07	46.50 ± 10.41	0.52
Letter fluency, Index	Adjusted score	6.28 ± 3.54	8.43 ± 10.69	0.40

Category fluency, Index	Adjusted score	3.89 ± 1.09	4.37 ± 2.01	0.32
CPM	Correct items	32.50 ± 2.88	31.29 ± 4.15	0.05

*Values denote means (reaction times or accuracy) and T-value of the median (T-MDN) ± standard deviations. RT (reaction times) have been reported in terms of seconds. Comparisons among the two time points were assessed using general linear models for repeated-measures, corrected for changes at the ALS Functional Rating Scale Revised (ALSFRS-R) and time between scans, adjusting for multiple comparisons using Bonferroni's correction. *=significant differences between groups at p<0.05. T is a standardized value with a mean of 50 and a standard deviation of 10. Higher T values mean better performance. Abbreviations: CPM=coloured progressive matrices. Fluency indices have been obtained as follows: time for generation condition - time for control condition (reading or writing generated words)/total number of items generated.*

3.3.1 Network-based functional connectivity: Independent component analysis.

At baseline, we did not observe differences between groups. Over six months, ALS patients showed increased rs-FC in the bilateral supplementary motor area, and left dorsal anterior cingulate cortex (ACC), MFG, superior frontal and superior medial frontal gyri within the frontostriatal network; and in the left MFG and supramarginal gyrus and right angular gyrus within the left frontoparietal network (Figure 3; Table B.1).

In ALS patients, within the frontostriatal network, we observed that: a worse performance at the TAP divided attention subtest (the T normative value of the median) was related with increased rs-FC over time in the left MFG (MNI coordinates, x=-34, y=38; z=32; Z=0.959; p<0.001; Pearson correlation coefficient, R=-0.691), and a worse performance at the category fluency (index scores) at baseline was related with increased rs-FC over time in the left frontal superior gyrus (MNI coordinates, x=-18, y=30; z=44; Z=0.999; p<0.001; R=0.822) (Figure 4). No other significant correlations were observed.

The analysis performed excluding 5 ALS patients with behavioral imapirment and *C9orf72* mutation confirmed the findings of the entire sample (Figures A.2 and A.3).

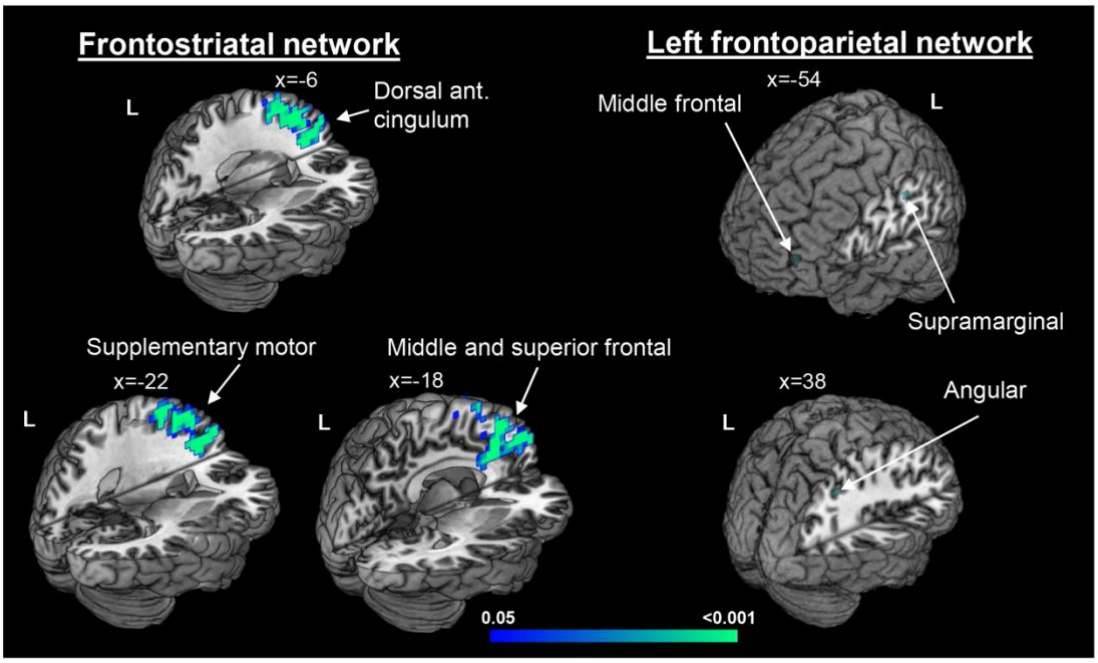

Figure 3. Independent component analysis. *Increased resting-state functional connectivity in ALS patients after six months accounting for changes at the ALS Functional Rating Scale Revised (ALSFRS-R), time between scans and voxel-based grey matter density. Results are overlaid on the Montreal Neurological Institute (MNI) standard brain in neurological convention and displayed at p<0.05 Family-wise error corrected for multiple comparisons. X values denote x-MNI coordinates. Colour bar represents p values.*

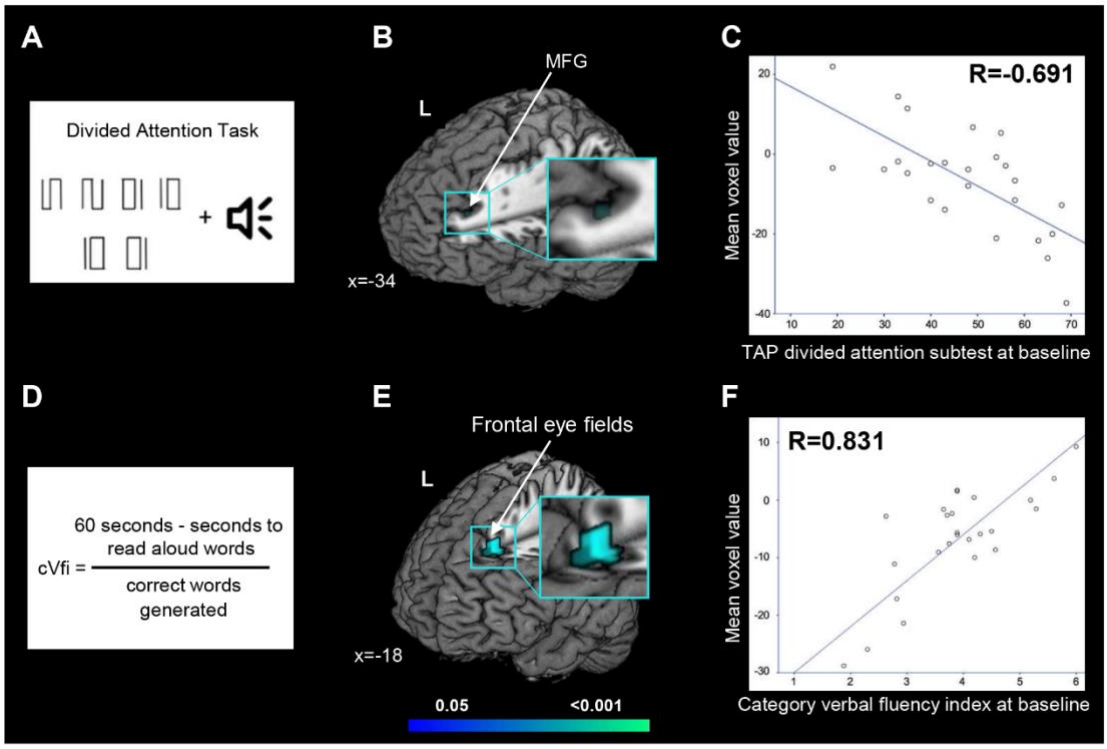

Figure 4. Cognitive-fMRI correlations. *In ALS patients, worse performance at baseline TAP divided attention subtest (A) related with increased functional connectivity over time in the left middle frontal gyrus, MFG within the frontostriatal network (B) ($p<0.001$; R=-0.691 [C]), and worse performance at baseline category verbal fluency index, cVfi (D) related with increased functional connectivity over time in the frontal superior gyrus within the frontostriatal network (E) ($p<0.001$; R=0.831 [F]). Results are overlaid on the Montreal Neurological Institute (MNI) standard brain and displayed at $p<0.05$ Family-wise error corrected for multiple comparisons. Delta gray matter density maps, changes at the ALS Functional Rating Scale Revised (ALSFRS-R) and time between scans were included as nuisance variables. X values denotes x-MNI coordinates. Colour bar represents p values.*

3.3.2 Post-hoc seed-based resting-state functional connectivity

At baseline, compared to controls, ALS patients showed increased rs-FC between the left MFG and the right supramarginal gyrus, primary somatosensory and primary motor cortices; and between the right MFG and the bilateral primary visual and primary somatosensory cortices, bilateral dorsal posterior cingulate cortex and lingual gyrus, left parahippocampal gyrus, left precunes, right supramarginal gyrus, right primary motor cortex, right ventral ACC, right fusiform and inferior occipital gyri (Figure 5A, Table B.2). At follow-up, we observed that compared to controls, ALS patients presented reduced rs-FC between the right MFG and the bilateral angular gyrus, left supramarginal

gyrus, and left inferior frontal gyrus (pars opercularis and triangularis) (Figure 5B, Table B.2). No rs-FC changes over time within ALS patients survived the correction for multiple comparisons. The results of the analysis in which we compared the 25 ALS patients with a sub-sample of 10 controls at the six month of follow-up confirmed a pattern of significant, although less extended, decreased rs-FC between the right and left MFG and anterior (mainly frontal) and posterior brain regions (Figure A.4).

The analysis performed excluding 5 ALS patients with behavioral imapirment and *C9orf72* mutation confirmed the findings of the entire sample (Figures A.2 and A.3).

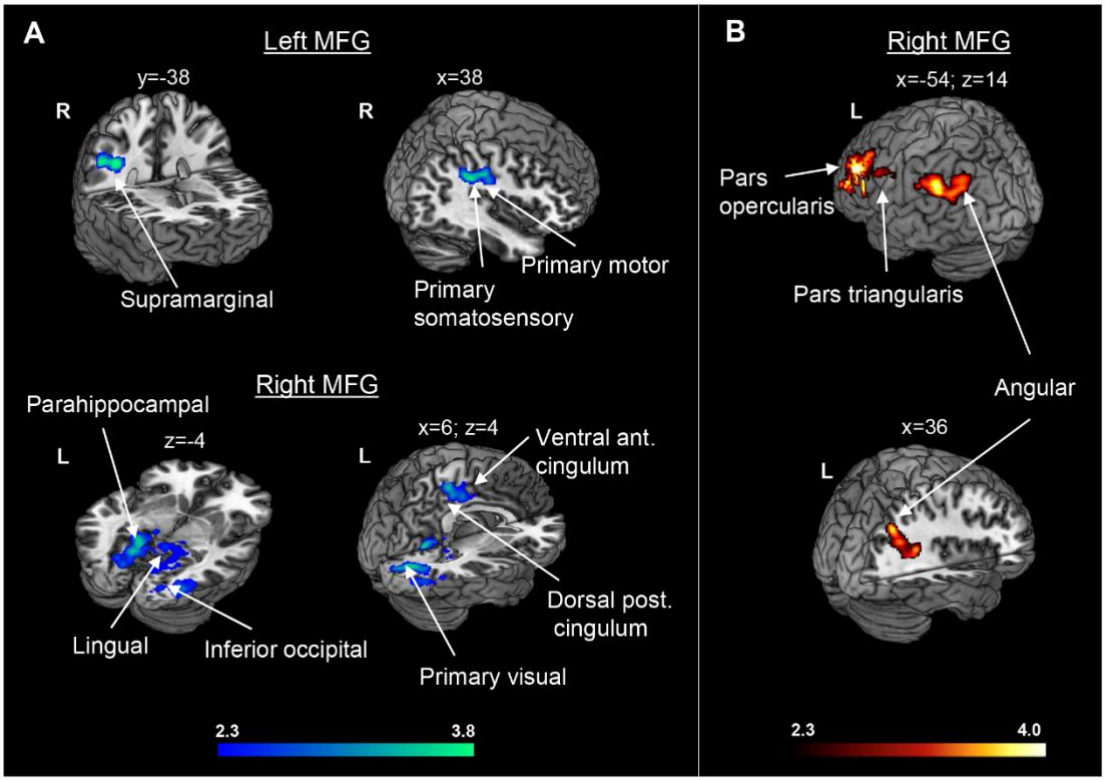

Figure 5. Seed-based functional connectivity analysis. *Regions where ALS patients showed increased (cold colors) or reduced (warm colors) functional connectivity with the left and right middle frontal gyrus (MFG) compared to healthy controls at baseline (A) and at follow-up (6 months) (B), accounting for changes at the ALS Functional Rating Scale Revised (ALSFRS-R) and time between scans. Results are overlaid on the Montreal Neurological Institute (MNI) standard brain and displayed at p<0.05 Family-wise error corrected for multiple comparisons at a cluster level. X, y and z values denotes MNI coordinates. Colour bar represents Z values.*

3.4 Discussion

In this study, three major findings emerge: 1) rs-FC alterations of the frontostriatal and frontoparietal networks progressed in ALS patients over six months; 2) such alterations are related to patients' frontal-executive low performance at baseline; 3) pivotal 'frontal-executive' regions, such as the MFG, are subject to a progressive frontoparietal breakdown.

Over time, in ALS patients, we observed increased rs-FC in middle and superior frontal gyri and ACC within the frontostriatal and left frontoparietal networks, and a progressive reduced rs-FC between the MFG and other frontal and parietal regions. Previous studies assessing longitudinal rs-FC changes in ALS demonstrated a mixed picture of decreased and increased rs-FC within motor and non-motor cortical networks (Menke et al., 2018, Schulthess et al., 2016, Shen et al., 2018, Trojsi et al., 2020), together with a loss of integrity of WM connections (Menke et al., 2018) and progressive disability (Menke et al., 2018, Schulthess et al., 2016). The 'direction' of rs-FC alterations seems to be dependent on the period of observation, with longer follow-up having higher chance of detecting decreased connectivity (Menke et al., 2018, Trojsi et al., 2020).
Additionally, the hypothesis of a cerebral disconnection in ALS when increasing FC is present comes from several imaging studies which combined structural and functional connectivity using standard and connectome approaches (Agosta, Spinelli et al., 2018). In line with neuropathological studies, these works demonstrated that local FC increased even when structural connectivity with distant brain regions of a network is disrupted (Basaia, Agosta et al., 2020). Although the observations of increased FC in established ALS might reflect a compensatory plasticity (Mohammadi et al., 2009), a loss of local inhibitory neuronal circuits, which then overlaps with the established concept of cortical excitability, is becoming the most consistent hypothesis (Douaud, Filippini et al., 2011). In our cohort of ALS, the correlations that we observed between increased FC over time and low cognitive performances at baseline corroborate the disconnection hypothesis.

The expanding involvement of frontal and parietal networks in the progression of the disease is in keeping with pathological studies (Braak, Brettschneider et al., 2013, Brettschneider, Del Tredici et al., 2013) and supports the notion of ALS as part of the FTLD spectrum (Burrell, Halliday et al., 2016). Specifically, the finding of altered rs-FC in the ACC during the disease course well reflects a recent neuropathological study on

TDP-43 which reported that agranular/disgranular regions of the ACC became involved in advanced pathological stages of sporadic ALS (Braak & Del Tredici, 2018). Pathological changes in this region have been identified also in cases of frontotemporal dementia (Santillo, Nilsson et al., 2013, Seeley, 2008). Beside neuropathology, our data are also in line with a recent connectome study (Meier, van der Burgh et al., 2020), which described a similar spatiotemporal progression of WM damage in ALS by simulating the disease propagation from the motor cortex using a network-based analysis.

In this study, we observed that the MFG plays a central role in the complex pattern of rs-FC changes in ALS patients. Specifically, we observed a progressive increased rs-FC of this region within both the frontostriatal and the left frontoparietal network, its association with a low performance at the TAP divided attention (dual task) subtest at baseline, and a continuous de-coupling between the MFG and a set of frontoparietal regions over time. MFG is known to have functional connections with the lateral and medial parietal structures (i.e., supramarginal gyrus, angular gyrus and posterior cingulate cortex), medial frontal cortices (i.e., ventral ACC) (Japee, Holiday et al., 2015), and striatum (Di Martino, Scheres et al., 2008). In healthy individuals, all these regions subtend attentional and executive functions, and in particular working memory, with MFG being one of the regions with high network centrality (in terms of WM connectivity) and frontostriatal network having the unique role of not only maintaining but also updating working memory information (as represented by our dual task) (Ekman, Fiebach et al., 2016). The MFG has been shown to be involved in ALS in previous studies using functional imaging (Abrahams, Goldstein et al., 1996; Abrahams, Goldstein et al., 2004; Trojsi, Di Nardo et al., 2017) and other imaging modalities (Alruwaili, Pannek et al., 2018, Verstraete, Veldink et al., 2012). A study used a word-fluency paradigm (which requires high-order executive functions including working memory) during positron emission tomography and task-based fMRI (Abrahams et al., 1996). Authors found reduced metabolism and activation, respectively, in both cognitively impaired and unimpaired ALS patients compared to controls in a cluster including the MFG and dorsolateral prefrontal cortices (Abrahams et al., 1996). Furthermore, MFG rs-FC was found altered within the salience and the left frontoparietal networks in ALS patients who exhibited affective and cognitive theory of mind (a complex frontal function) deficits after six months and was observed to be related with patients' disease duration (Trojsi et

al., 2017). A previous study (Pettit, Bastin et al., 2013) also observed that abnormal dual-task performance in ALS patients correlated with low fractional anisotropy in the WM underneath the MFG, confirming the important role of this region in executive dysfunctions typical of ALS patients.

The computer-test battery (TAP) (Zimmermann & Fimm, 1992) revealed the progression of patients' frontal dysfunctions, despite most of our patients had normal cognition according to Strong's criteria (Strong et al., 2017) at study entry, suggesting that this is a valid tool to monitor even subtle frontal changes in cognitively unimpaired patients. It would be important to compare the performance of TAP in measuring cognitive changes over time with that of alternative versions of the ECAS, whenever available in Italian. We observed increased reaction times in patients' alertness after six months. Alertness alterations in ALS patients may be attributed to several factors other than a frontal pathological involvement, such as hypoxia due to respiratory failure, progressive physical impairment and abnormal mood. However, we paid particular attention in reducing the impact of all these factors. Specifically, we excluded patients with significant respiratory failure; the TAP accounts for motor impairment and cognitive analyses were adjusted for ALSFRS-R changes; and we verified and excluded the potential relationship between patients' mood and alertness.

Some limitations of our study should be noted. The sample of patients is relatively small and this could have led to negative findings, mainly in the correlation analysis. Second, we did not report longitudinal data for healthy controls, for both imaging and cognitive features. However, the sub-analysis that we performed with 10 healthy controls who underwent the MRI scan also after six months, confirmed our findings. Finally, the cognitive data of the present study have been collected before the publication of the Italian version of the ECAS. Thus, we cannot compare data obtained by using this ALS specific battery with those observed with the TAP. Despite these shortcomings, this manuscript has several strengths. The ALS sample has been cognitively well defined using standard and computer-based batteries. Finally, we used both network- and seed-based rs-FC, thus revealing comprehensive ALS extra-motor brain alterations.

3.5 Conclusions

In conclusion, this study investigated the relationship between cognitive and rs-fMRI longitudinal changes in ALS. In ALS, the increased connectivity in frontal regions in relation with lower frontal-executive performance at study entry suggests that it is likely not a mechanism of compensation but rather a sign of disease progression, as previously observed in patients with FTLD (Farb, Grady et al., 2013). This is also revealed by the reduced rs-FC between MFG and antero-posterior regions at follow-up that could reflect the typical FTLD functional disconnection (Reyes, Ortega-Merchan et al., 2018). The combined information of cognitive and fMRI alterations (and their relationship) can be useful in ALS to predict the disease progression beyond the motor network, even in patients with not frank cognitive impairment. We believe that these findings offer new potential markers for monitoring the ALS evolution. Further studies are needed to verify whether the information of cognitive and fMRI alterations may be able to classify new ALS patients with a FTLD-like progression at the single subject level.

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Declaration of competing interest

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3.7 Appendix

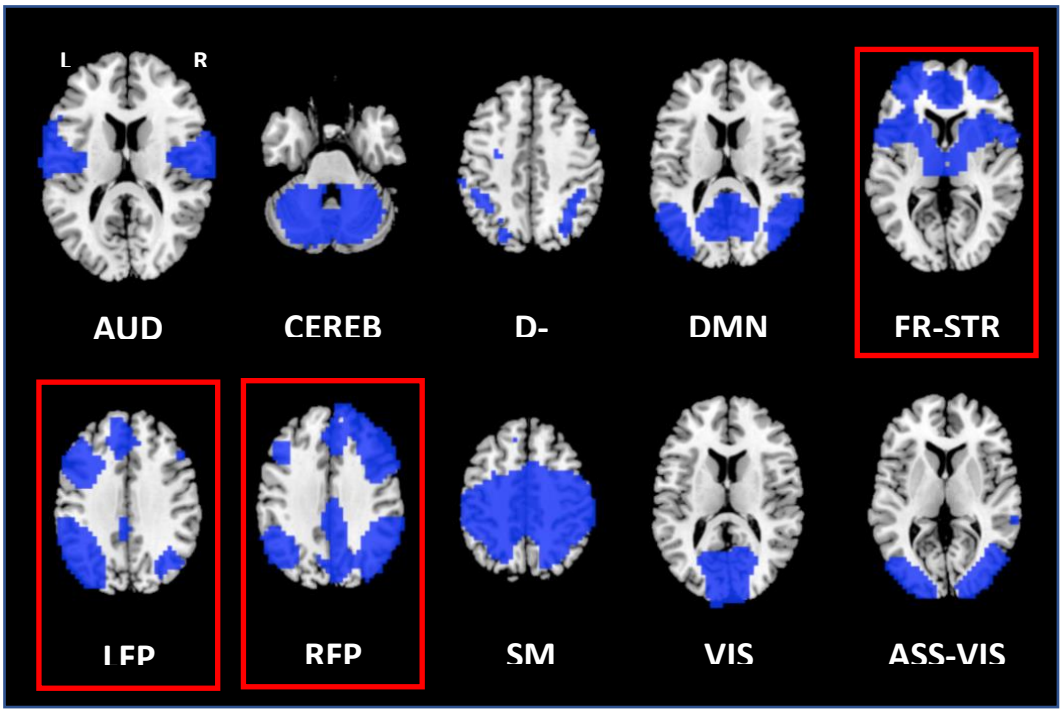

Figure A.1. *Independent components (IC) of interest identified by ICA. Red rectangles highlights the fronto-connected networks of interest. IC binary masks are overlaid on the 3D Montreal Neurological Institute template in neurological convention (right is right). Abbreviations: AUD=auditory; CEREB=cerebellar; D-ATT=dorsal attention; DMN=default mode; FR-STR=fronto-striatal; L=left; LFP=left frontoparietal; RFP=right frontoparietal; SM=sensorimotor; VIS=primary visual; ASS-VIS=associative visual.*

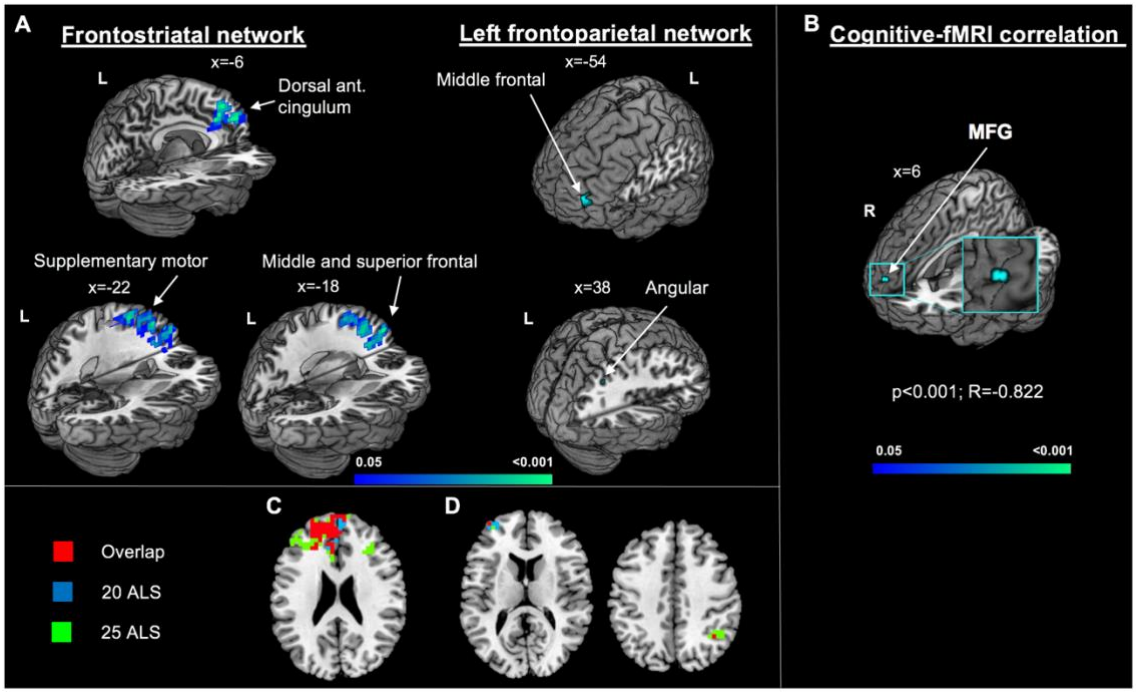

Figure A.2. *Independent component analysis rerun excluding 2 patients with C9orf72 mutation and 3 patients with behavioral impairment. Increased resting-state functional connectivity in ALS patients after six months accounting for changes at the ALS Functional Rating Scale Revised (ALSFRS-R), time between scans and grey matter density (A). Worse performance at baseline TAP divided attention subtest related with increased functional connectivity over time in the most rostral part of middle frontal gyrus, MFG within the frontostriatal network in ALS patients. Results are overlaid on the Montreal Neurological Institute (MNI) standard brain and displayed at p<0.05 Family-wise error corrected for multiple comparisons. X values denotes x-MNI coordinates. Color bar represents p values (B). Overlay between the significance maps of the frontostriatal (C) and left frontoparietal (D) networks.*

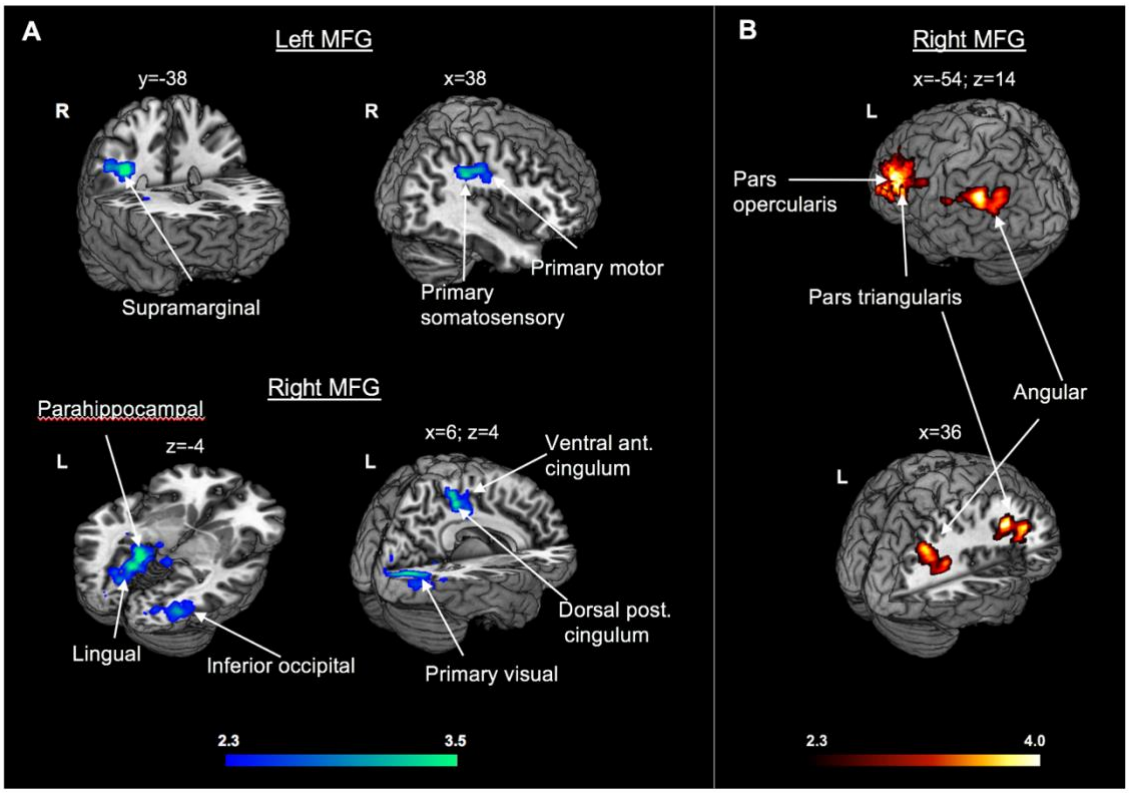

Figure A.3. *Seed-based functional connectivity analysis rerun excluding 2 patients with C9orf72 mutation and 3 patients with behavioral impairment. Regions where ALS patients showed enhanced (cold colors) or reduced (warm colors) functional connectivity with the left and right middle frontal gyrus (MFG) compared to healthy controls at baseline (A) and at follow-up (6 month) (B). Results are overlaid on the Montreal Neurological Institute (MNI) standard brain and displayed at p<0.05 Family-wise error corrected for multiple comparisons at a cluster level. X,y,z values denotes MNI coordinates. Color bar represents Z values.*

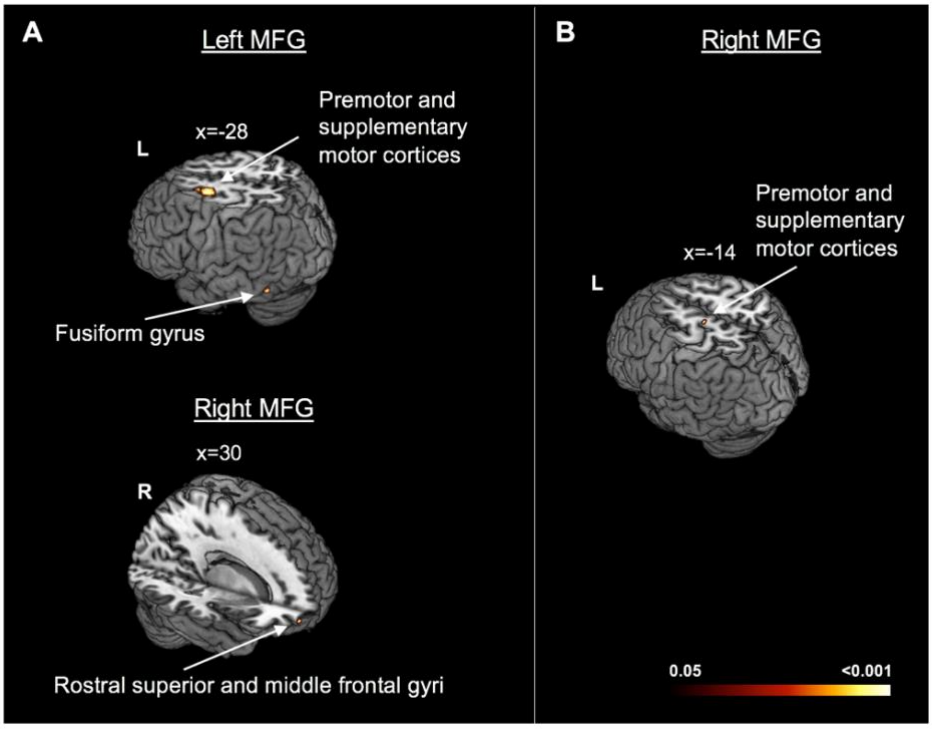

Figure A.4. *Seed-based functional connectivity analysis rerun comparing the 25 ALS patients with 10 HC who performed the same MRI scan both at baseline and follow-up. (A) Regions where ALS patients showed reduced functional connectivity with the left and right middle frontal gyrus (MFG) compared to healthy controls at follow-up (month 6). (B) Regions where ALS patients showed reduced functional connectivity with the left and right middle frontal gyrus (MFG) compared to healthy controls over time. Results are overlaid on the Montreal Neurological Institute (MNI) standard brain and displayed at p<0.05 Family-wise error corrected for multiple comparisons. X values denotes x-MNI coordinates. Color bar represents p values.*

Table B.1. Sociodemographic, clinical and neuropsychological features of ALS patients and healthy controls at baseline and follow-up.

	ALS baseline	HC baseline	P-value
N	25	10	-
Sex, women	6 (25%)	7 (70%)	0.01
Age at MRI at baseline [years]	61.56 ± 10.79	58.10 ± 6.67	0.35
Age at 6 month MRI [years]	61.92 ± 10.88	60.40 ± 7.22	0.68
Education [years]	10.96 ± 3.92	12.60 ± 3.37	0.25

Values denote mean ± standard deviations or numbers (percentages). P-values refer to T-test models and Pearson's χ^2 test. Abbreviations: ALS=amyotrophic lateral sclerosis; HC=healthy controls.

Table B.2. Independent component analysis. Significant differences of resting state functional connectivity in ALS patients over six months.

	Region	N of voxels	p-value	x	y	z
Follow-up (6 months): ALS						
Frontostriatal network						
Increased rs-FC	L dorsal anterior cingulate	688	<0.001	-6	34	28
	L superior medial frontal		0.001	-10	42	40
	L superior frontal		0.002	-18	58	28
	L middle frontal		0.01	-34	38	32
	L supplementary motor		0.003	-22	14	60
	R supplementary motor	2	0.03	26	18	60
Left frontoparietal network						
Increased rs-FC	R angular	8	0.03	38	-54	44
	L middle frontal	5	0.04	-38	58	12
	L supramarginal	2	0.02	-54	-26	40

Coordinates (x, y, z) are in Montreal Neurological Institute (MNI) space. Results are shown at p<0.05 FWE corrected for multiple comparisons, accounting for grey matter density. Longitudinal analyses were further corrected for changes at the ALS Functional Rating Scale Revised (ALSFRS-R) and time interval between scans. Abbreviations: ALS=amyotrophic lateral sclerosis; HC=healthy controls: rs-FC=resting state-functional connectivity.

Table B.3. Seed-based functional connectivity analysis. Significant differences of middle frontal gyrus functional connectivity between ALS patients and healthy controls at baseline and after six months.

	Region	N of voxels	Z	x	y	z
Baseline: ALS *vs* HC						
Left MFG						
ALS>HC	R supramarginal gyrus	773	3.66	48	-38	38
	R primary somatosensory		3.60	38	-22	36
	R primary motor		3.09	38	-18	30
Right MFG						
ALS>HC	L parahippocampal	1922	4.23	-17	-40	-4
	R lingual gyrus		3.89	16	-40	-2
	L lingual gyrus		3.71	-14	-54	-6
	L primary visual		3.32	-12	-90	10
	R primary somatosensory	983	3.68	58	-18	38
	R supramarginal		3.57	42	-36	38
	R primary motor		3.03	36	-28	52
	R primary visual	854	3.93	22	-80	4
	R inferior occipital		3.14	40	-74	-4
	R fusiform		2.84	40	-62	-8
	L precuneus	674	3.61	-12	-40	60
	R dorsal posterior cingulate		3.56	6	-28	46
	R ventral anterior cingulate		3.21	6	-16	40
	L primary somatosensory		2.96	-24	-34	58
	L dorsal posterior cingulate		2.95	-10	-22	40
Follow-up (6 months): ALS *vs* HC						
Right MFG						
ALS <HC	L angular	820	4.10	-52	-42	28
	L supramarginal		2.97	-65	-36	32
	R angular	724	3.55	36	-66	38
	L inferior frontal (pars opercularis)	696	4.10	-56	26	26
	L inferior frontal (pars triangularis)		4.07	-56	26	22
	L inferior frontal (pars triangularis)		3.65	-58	22	16

Coordinates (x, y, z) are in Montreal Neurological Institute (MNI) space. Results are shown at p<0.05 corrected for multiple comparisons at a cluster level. Abbreviations: ALS=amyotrophic lateral sclerosis; HC=healthy controls; MFG=middle frontal gyrus.

Chapter 4 - Impaired recognition of disgust in amyotrophic lateral sclerosis is related to basal ganglia involvement

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Impaired recognition of disgust in amyotrophic lateral sclerosis is related to basal ganglia involvement

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ABSTRACT

In the present study we investigated emotion recognition in pure motor amyotrophic lateral sclerosis (ALS) patients and its relationship with the integrity of basal ganglia, hippocampus and amygdala. Twenty ALS patients without either cognitive or behavioural impairment, and 52 matched healthy controls performed a neuropsychological assessment including the Comprehensive Affect Testing System (CATS) investigating emotion recognition. All participants underwent also a 3T brain MRI. Volumes of basal ganglia, hippocampus and amygdala bilaterally were measured using FIRST in FSL. Sociodemographic, cognitive and MRI data were compared between groups. In ALS patients, correlations between CATS significant findings, brain volumes, cognition, mood and behaviour were explored. ALS patients showed altered performances at the CATS total score and, among the investigated emotions, patients were significantly less able to recognize disgust compared with controls. No brain volumetric differences were observed between groups. In ALS patients, a lower performance in disgust recognition was related with a reduced volume of the left pallidum and a lower performance on the Edinburgh Cognitive and Behavioural ALS Screen. Cognitively/behaviourally unimpaired ALS patients showed impaired disgust recognition, which was associated with pallidum volume. The association with cognitive alterations may suggest impaired disgust recognition as an early marker of cognitive decline.

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4.1 Introduction

Alterations of socioemotional behaviour are important early features of frontotemporal lobar degeneration (FTLD), particularly of the behavioural variant of frontotemporal dementia (FTD) and the semantic variant of primary progressive aphasia (Werner, Roberts et al., 2007). Social cognition deficits have been reported also in amyotrophic lateral sclerosis (ALS) patients as alterations in theory of mind and emotion processing (Girardi, MacPherson et al., 2011). The first studies on emotion processing in ALS are dated back to 2005 (Lule, Kurt et al., 2005, Papps, Abrahams et al., 2005), and up to date several investigations reported emotion perception impairment in ALS (Aho-Ozhan, Keller et al., 2016, Andrews, Staios et al., 2017, Crespi, Cerami et al., 2014, Crespi, Cerami et al., 2016, Girardi et al., 2011, Lule et al., 2005, Oh, Oh et al., 2016, Palmieri, Naccarato et al., 2010, Zimmerman, Eslinger et al., 2007), such as emotion recognition (both facial and prosodic), emotion attribution, and reduced psychophysiological excitability to emotional stimuli (Benbrika, Desgranges et al., 2019). According to a recent meta-analysis, the most frequent alteration in ALS patients is in facial recognition for disgust and surprise (Bora, 2017), followed by inability in recognizing anger (Andrews et al., 2017, Crespi et al., 2014, Girardi et al., 2011, Oh et al., 2016, Savage, Lillo et al., 2014, Zimmerman et al., 2007) and sadness (Aho-Ozhan et al., 2016, Andrews et al., 2017, Oh et al., 2016, Zimmerman et al., 2007). On the other hand, recognition of fear and happiness seems to be relatively spared (Aho-Ozhan et al., 2016, Girardi et al., 2011). Although emotion processing deficits in ALS have been observed both in patients with and without cognitive impairment (Andrews et al., 2017, Crespi et al., 2014, Crespi et al., 2016, Girardi et al., 2011), whether they appear before and/or regardless of cognitive and behavioural abnormalities is still not clear.

Previous MRI studies on emotion processing in ALS reported associations with the neurodegeneration of cortical and limbic brain structures. Specifically, in ALS patients an increased emotion attribution ability was related to greater gray matter (GM) density of the right fronto-insular and anterior cingulate cortices (Cerami, Dodich et al., 2014). Furthermore, two diffusion tensor imaging studies (Crespi et al., 2014, Crespi et al., 2016) observed a relationship between emotion recognition difficulties in ALS patients and microstructural changes in ventral associative bundles connecting occipital, temporo-limbic and orbitofrontal regions of the right hemisphere. Finally, task-based

functional MRI (fMRI) studies including paradigms which present emotional words, Ekman's faces or pictures of people in social situations (Aho-Ozhan et al., 2016, Lule, Diekmann et al., 2007, Palmieri et al., 2010), reported higher activation in the bilateral inferior frontal gyrus and right supramarginal area, and reduced activation in the bilateral hippocampus and extrastriate visual areas in ALS patients compared with healthy controls.

In ALS, basal ganglia damage appears in the stages 3 and 4 of TDP-43 pathology (Brettschneider, Del Tredici et al., 2013) and was confirmed by several MRI studies (Bede, Bokde et al., 2013, Bede, Elamin et al., 2013, Verstraete, Veldink et al., 2014, Westeneng, Verstraete et al., 2015). Structural and task-based functional MRI investigations (Abidi, de Marco et al., 2020, Feron, Couillandre et al., 2018) highlighted a role of basal ganglia in mediating extra-pyramidal motor manifestations and gait impairment in ALS. Recent works found correlations between cognitive deficits, apathy and basal ganglia damage (Machts, Loewe et al., 2015) or frontostriatal circuits (Castelnovo, Canu et al., 2020) in ALS patients (Castelnovo et al., 2020, Machts et al., 2015). Regional shape contractions of the bilateral pallidum, right putamen, and right accumbens were recently observed in cognitively/behaviourally unimpaired ALS patients compared to controls, suggesting that basal ganglia are involved early in the disease course (Tae, Sung et al., 2020). Due to the growing attention on basal ganglia, we decided to focus the present work on these brain regions.

In a sample of early diagnosed ALS patients without cognitive and behavioural deficits, the aim of our study was twofold: 1) to identify which emotions are altered in ALS compared to healthy controls; and 2) to investigate the relationship between emotion recognition and the integrity of basal ganglia, hippocampus and amygdala. For these purposes, we used a specific battery, the Comprehensive Affect Testing System (CATS), which investigates several subcomponents of the emotion processing.

4.2 Materials and Methods

4.2.1 Participants

From a sample of 72 MND cases prospectively recruited at IRCCS San Raffaele Scientific Institute in Milan, Italy, from 2017 to 2019, we selected 20 sporadic ALS

patients (Brooks, Miller et al., 2000) with no significant respiratory failure. We included only patients with available clinical assessment, neuropsychological assessment including an evaluation of emotion processing (see details below) and brain 3T MRI scan, with no cognitive and/or behavioural impairment according to Strong's criteria (Strong, Abrahams et al., 2017), and no mood alterations. All patients were screened for known pathogenic mutations, including *C9orf72*. Fifty-two healthy controls, age-, sex-, and education-matched with patients were recruited among non-consanguineous relatives and by word of mouth based on the following criteria: normal neurological exam, mini mental state examination (MMSE) score ≥28, and no family history for neurodegenerative diseases. Healthy controls underwent neuropsychological assessment and brain MRI scan. All participants were excluded if they had significant medical illnesses or substance abuse that could interfere with cognitive functioning; any (other) major systemic, psychiatric, or neurological illnesses; and (other) causes of focal or diffuse brain damage, including cerebrovascular disease at conventional MRI scans. No participants were excluded for motion-related artifacts in the MR images.

4.2.1.1 Standard Protocol Approvals, Registrations, and Patient Consents

Local ethical standards committee on human experimentation approved the study protocol and all participants provided written informed consent (Ethical committee numbers: GR-2013-02357415 and StG-2016_714388_NeuroTRACK).

4.2.2 Clinical assessment

Disease severity in patients was assessed using the ALS Functional Rating Scale-revised (ALSFRS-R, with a maximum score of 48) (Cedarbaum, Stambler et al., 1999). The rate of disease progression was defined according to the following formula: (48–ALSFRS-R score)/time between symptom onset and first visit.

4.2.3 Genetic analysis

Blood samples were collected from all patients and healthy controls. The presence of GGGGCC hexanucleotide expansion in the first intron of *C9orf72* was assessed using a repeat-primed polymerase chain reaction (PCR) assay (Renton, Majounie et al., 2011).

A cut-off of ≥30 repeats combined with a typical saw-tooth pattern was considered pathological. In addition, the coding sequences and intron/exon boundaries of *GRN*, *MAPT*, *TARDBP*, *SOD1*, *FUS*, *TBK1*, *TREM2*, *OPTN* and *VCP* genes were amplified by PCR using optimized protocols, looking for known pathogenic mutations (Pozzi, Valenza et al., 2017).

4.2.4 Cognitive and behavioural assessment

Neuropsychological assessments were performed by experienced neuropsychologists, unaware of the MRI results. Cognitive evaluation consisted in the administration of a comprehensive neuropsychological battery in order to define performance differences in patients and controls, and the potential presence of cognitive and/or behavioural impairment according to Strong's criteria in ALS patients (Strong et al., 2017).

In the neuropsychological battery, the following cognitive functions were administered to patients as previously described (Castelnovo, Canu et al., 2021): global cognitive functioning with the MMSE and the Edinburgh Cognitive and Behavioural ALS Screen (ECAS); long and short term verbal memory with the Rey Auditory Verbal Learning Test and the digit span forward, respectively; executive functions with the digit span backward, the Cognitive Estimation Task (CET), the Modified Card Sorting Test; fluency with the phonemic and semantic fluency tests and the relative fluency indices (controlling for individual motor disabilities); language with the confrontation naming subtests of Italian battery for the assessment of aphasic disorders (BADA); emotion processing with the CATS; mood with the Hamilton Depression Rating Scale (HDRS); and the presence of behavioural disturbances with the Amyotrophic Lateral Sclerosis-Frontotemporal Dementia-Questionnaire (ALS-FTD-Q) administered to patients' caregivers. Healthy controls underwent the entire assessment except for the ECAS, CET and BADA subtests; moreover, in healthy controls the Beck Depression Inventory (BDI) was used to assess mood.

The CATS is a battery which investigates different aspects of the emotion processing by using the Ekman's faces expressing the six basic emotions (disgust, surprise, happiness, anger, fear and sadness) (Froming, Levy et al., 2006). From this battery, we selected and administered six subtests: identity discrimination (which is the

control condition that investigates the correct discrimination of faces with no emotion involvement), affect discrimination, affect naming, affect matching, affect selection, affect confrontation) (Figure 1).

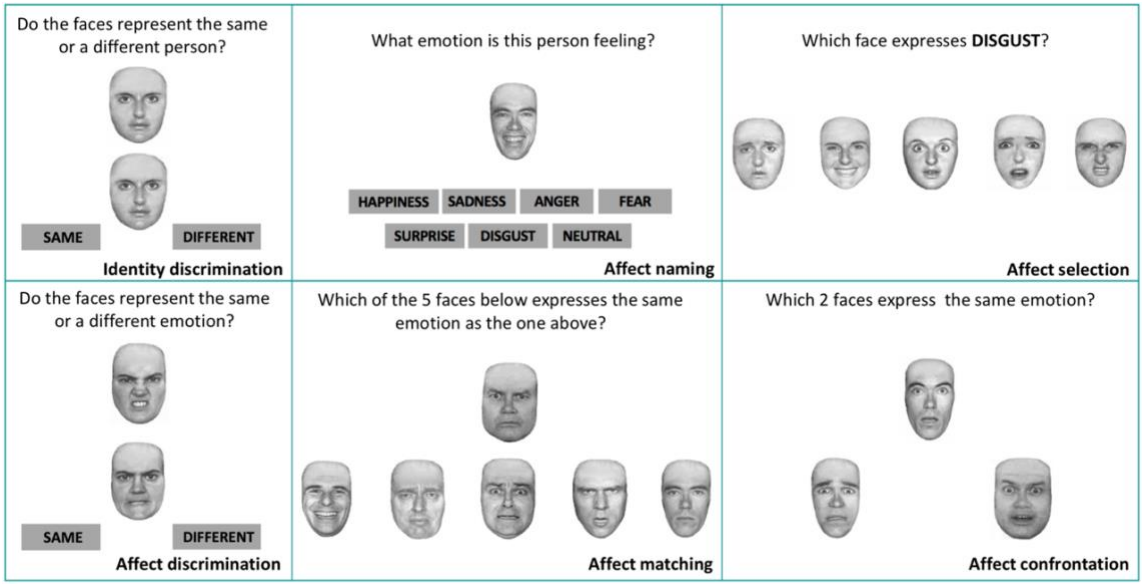

Figure 1. *The six subtests of the Comprehensive Affect Testing System (CATS). For illustrative purposes, the stimuli represent an adaptation of the original version.*

Based on the number of correct answers, we obtained a total score and specific scores for each CATS subdomain. Finally, composite scores were calculated by summing up the correct answers for each of the six basic emotions.

4.2.5 MRI acquisition

Using a 3.0 T scanner (Ingenia CX, Philips), the following brain MRI sequences were obtained from all participants: 3D T1-weighted (TFE) (TR=7 ms; TE=3.2 ms; flip angle=9 [degrees]; 204 contiguous sagittal slices with voxel size=1 x 1 x 1 mm, matrix size=256 x 240, FOV=256x240 mm^2); 3D FLAIR (TR=4800 ms; TE=267 ms; TI=1650 ms; ETL=167; NEX=2; 192 contiguous sagittal slices with voxel size=0.89 x 0.89 x 1 mm, matrix size=256 x 256, FOV=256x256 mm^2); 3D T2 (TR=2500 ms; TE=330 ms; ETL=117; NEX=1; 192 contiguous sagittal slices with voxel size=0.89 x 0.89 x 1 mm, matrix size=256 x 258, FOV=256x256 mm^2).

4.2.6 MRI analysis

MRI analysis was performed at the Neuroimaging Research Unit, IRCCS Scientific Institute San Raffaele, Milan, Italy. The potential presence of white matter hyperintensities was evaluated on 3D FLAIR and 3D T2-weighted images. FMRIB's Integrated Registration and Segmentation Tool (FIRST) in FSL (http://www.fmrib.ox.ac.uk/fsl/first/index.html) was applied to TFE images of each subject and used to automatically segment GM regions, i.e., caudate, pallidum, putamen, thalamus and nucleus accumbens, amygdala and hippocampus, bilaterally. Mean GM volumes were calculated and multiplied by the normalization factor derived from SIENAx to correct for subject head size (http://www. fmrib.ox.ac.uk/fsl/sienax/index.html).

4.2.7 Statistical analysis

To compare the sociodemographic characteristics, cognitive performances and MRI data between groups, we used Kruskal-Wallis one-way ANOVA models followed by post-hoc pairwise comparisons (using U test of Mann–Whitney and Bonferroni-correction for multiple comparisons) and Fisher's exact test for continuous and categorical variables, respectively.

In ALS patients and controls, CATS significant findings were correlated with the volumes of basal ganglia, hippocampus and amygdala, patients' performances at the ECAS, patients' mood (HDRS) and behaviour (ECAS behaviour score and ALS-FTD-Q) using Pearson's correlation analysis. The statistical analyses were performed with using SPSS software (version 24.0; IBM Corp., Armonk, NY, USA). All analyses were performed with using the same models also excluding those patients with *C9orf72* mutation.

4.3 Results

ALS patients and healthy controls were similar in age, sex and education (Table 1). Two patients were *C9orf72* mutation carriers. According to Strong's criteria (Strong et al., 2017), none of the patients were classified as cognitive and/or behavioural impaired. No patients nor controls showed depression according to self-administered

questionnaires (HDRS or BDI) and/or clinical interview. At the time of the visit, 25% of patients were on stable SSRI-type antidepressants and 5% on benzodiazepines. The neuropsychological assessment reveals that patients performed similarly to controls in all considered cognitive domains (Table 1).

Table 1. Sociodemographic, clinical and cognitive features of the sample.

	Healthy controls	**ALS patients**	**F value**	**Effect size**	**P value**
N	52	20	-	-	-
Sex [women]	33 (63%)	11 (55%)	0.435	0.006	0.35
Age at MRI [years]	60.36±8.49	57.98±11.35	0.819	0.012	0.37
Education [years]	12.40±3.79	12.55±4.99	0.124	0.002	0.73
Handedness [right]	50 (96%)	20 (100%)			
Disease duration [months]	-	25.64±18.18	-	-	-
ALSFRS-R, 0-48	-	39.00±7.53	-	-	-
Disease progression rate	-	0.53±0.47	-	-	-
Site of onset, limb	-	86%	-	-	-
Global cognition					
MMSE	29.36±0.80	28.63±1.80	1.946	0.29	0.16
ECAS,* Language	-	24.65±3.94	-	-	-
ECAS,* Fluency	-	19.65±2.76	-	-	-
ECAS,* Executive Functions	-	38.65±3.46	-	-	-
ECAS,* ALS-specific functions	-	82.65±8.29	-	-	-
ECAS,* Memory	-	18.47±2.92	-	-	-
ECAS,* Visuospatial	-	11.53±0.62	-	-	-
ECAS,* ALS non-specific functions	-	30.00±3.04	-	-	-
ECAS,* total score	-	111.82±10.06	-	-	-
Memory					
Digit span, forward	5.96±1.07	5.56±1.34	1.886	0.028	0.17
Digit span, backward	4.82±1.20	4.56±0.98	0.461	0.007	0.50
RAVLT, immediate	49.62±7.08	45.95±9.02	2.521	0.037	0.11
RAVLT, delayed	10.92±2.42	9.95±2.06	2.945	0.043	0.09
Executive function					
CET	-	13.36±3.48	-	-	-
MCST, perseverative responses	3.52±3.63	4.71±6.49	0.133	0.002	0.72

Language					
BADA (nouns)	-	28.86±1.22	-	-	-
BADA (actions)	-	27.71±1.50	-	-	-
Fluency					
Phonemic fluency, Index	5.10±1.84	4.22±1.56	3.474	0.062	0.06
Semantic fluency, Index	3.81±1.58	4.12±1.48	0.434	0.008	0.51
Mood & Behaviour					
BDI	7.04±4.81	-	-	-	-
HDRS	-	4.68±2.69	-	-	-
ECAS,* behaviour score	-	0.19±0.40	-	-	-
ALS-FTD-Q	-	8.10±13.11	-	-	-

*Values denote mean ± standard deviations or numbers (frequency). P values refer to Kruskal-Wallis one-way ANOVA models followed by post-hoc pairwise comparisons (using U test of Mann–Whitney and Bonferroni-correction for multiple comparisons) and Fisher's exact test for continuous and categorical variables, respectively. *ALS-FTD-Q was administered to 18 patients. *ECAS was administered to 17 patients. Abbreviations: ALS=Amyotrophic Lateral Sclerosis; ALSFRS-R=ALS Functional Rating Scale Revised; ALS-FTD-Q=Amyotrophic Lateral Sclerosis-Frontotemporal Dementia-Questionnaire; BADA= Italian battery for the assessment of aphasic disorders; BDI=Beck Depression Inventory; CET= Cognitive Estimation Task; ECAS=Edinburgh cognitive and behavioural ALS screen; HDRS=Hamilton depression rating scale; MCST= Modified Card Sorting Test; MMSE=Mini Mental State Examination; MRI=Magnetic Resonance Imaging; RAVLT= Rey Auditory Verbal Learning Test.*

At the CATS, patients performed worse than controls in the total score, affect selection and affect matching subtests (Table 2). When the emotion target was disgust, patients provided less correct answers than controls (Table 2).

Table 2. Performances of healthy controls and ALS patients at the Comprehensive Affect Testing System.

	Healthy controls	**ALS patients**	**F value**	**Effect size**	**P value**
CATS Total score	56.79±4.77	53.05±5.08	8.766	0.123	**0.003**
CATS Identity Discrimination	11.81±0.40	11.45±1.05	1.522	0.021	0.22
CATS Affect Discrimination	11.59±0.98	10.95±0.95	2.654	0.037	0.10
CATS Affect Naming	4.63±0.97	4.50±1.15	0.247	0.003	0.62
CATS Affect Selection	5.52±0.67	5.05±0.95	4.166	0.059	**0.04**
CATS Affect Matching	9.31±1.92	8.05±1.40	7.950	0.112	**0.005**
CATS Affect Confrontation	14.23±3.04	13.05±3.35	2.617	0.037	0.11
Disgust, correct answers (0-9)	5.85±1.50	4.75±1.83	6.060	0.085	**0.01**

Surprise, correct answers (0-8)	6.56±1.34	6.35±0.93	1.458	0.021	0.23
Happiness, correct answers (0-9)	8.65±0.52	8.45±0.69	1.309	0.018	0.25
Anger, correct answers (0-10)	5.69±2.11	4.80±1.61	3.053	0.043	0.08
Fear, correct answers (0-8)	5.15±1.29	5.45±1.28	0.564	0.008	0.45
Sadness, correct answers (0-9)	6.90±1.54	6.10±1.89	2.622	0.037	0.11

Values denote mean ± standard deviations. P values refer to Kruskal-Wallis one-way ANOVA models followed by post-hoc pairwise comparisons (using U test of Mann–Whitney and Bonferroni-correction for multiple comparisons). BOLD p values denote significant differences between groups at p< 0.05. Abbreviations: ALS=Amyotrophic Lateral Sclerosis; CATS=Comprehensive Affect Testing System.

These latter differences remained significant when the analyses were re-run without the two *C9orf72* patients (p=0.04). For the other target emotions, the two groups showed similar scores. ALS patients and controls did not differ in any of the investigated GM volumes (Table 3).

Table 3. Gray matter volumes in healthy controls and ALS patients.

	Healthy controls	**ALS patients**	**F value**	**Effect size**	**P value**
L caudate	4427.33±388.76	4534.65±500.52	0.710	0.010	0.40
L pallidum	2391.91±253.82	2450.66±280.97	0.913	0.013	0.34
L putamen	6489.57±595.81	6410.40±556.56	0.031	0.000	0.86
L accumbens	694.62±129.06	649.88±126.93	1.089	0.015	0.30
L thalamus	10509.67±819.14	10168.84±523.05	2.201	0.031	0.14
L hippocampus	5228.55±665.85	5069.76±378.92	2.164	0.030	0.14
L amygdala	1793.19±243.30	1731.25±223.50	1.089	0.000	0.30
R caudate	4690.06±421.20	4792.63±542.87	0.266	0.004	0.61
R pallidum	2420.09±274.77	2444.17±291.22	0.162	0.002	0.69
R putamen	6262.95±562.95	6290.94±526.22	0.003	0.000	0.96
R accumbens	530.60±129.39	498.75±121.47	0.710	0.010	0.40
R thalamus	10206.60±835.17	9767.73±543.74	3.416	0.048	0.07
R hippocampus	5309.90±691.76	5094.08±368.83	3.098	0.044	0.08
R amygdala	1692.05±294.43	1707.46±262.09	0.013	0.000	0.91

Values (mm^3) denote mean ± standard deviations. P values refer to Kruskal-Wallis one-way ANOVA models followed by post-hoc pairwise comparisons (using U test of Mann–Whitney and Bonferroni-correction for multiple comparisons). Abbreviations: ALS=Amyotrophic Lateral Sclerosis.

In ALS patients, a positive relationship was observed between the disgust correct recognition at the CATS (number of correct answers when disgust was the emotion target) and the volume of the left pallidum (Figure 2). When this analysis was re-run without the two *C9orf72* patients, a trend toward statistical significance was observed (r=0.459; p=0.056). Furthermore, in ALS patients, a positive relationship was found between disgust correct recognition at the CATS and patients' performance at the ECAS ALS-specific and executive functions scores (ECAS was administered to 17 patients; Figure 2).

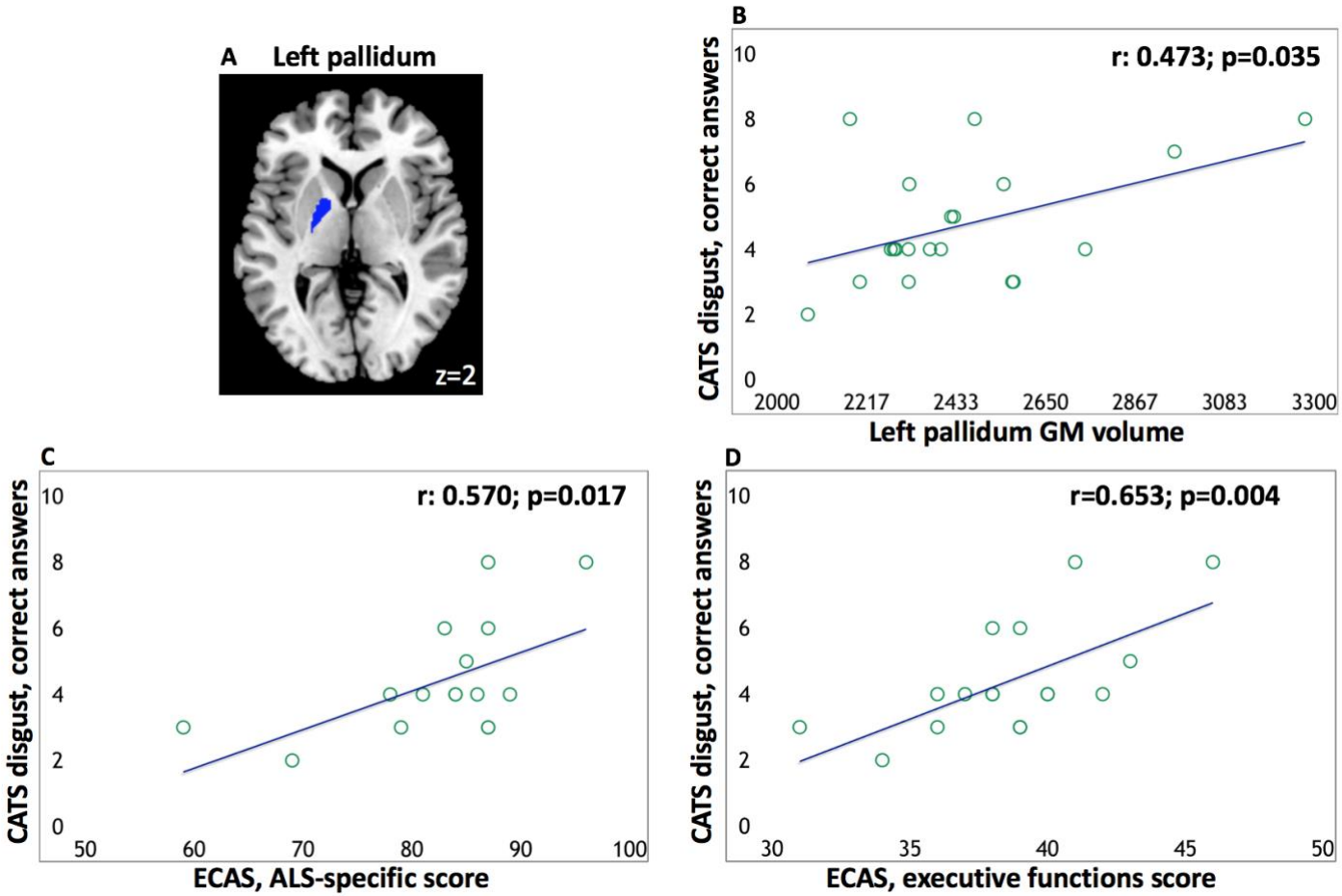

Figure 2. *Region of interest (left pallidum) overlaid on the Montreal Neurological Institute template (A); relationship between disgust recognition and volume of the left pallidum (B) and between disgust recognition and ECAS ALS-specific and executive functions score (C and D, respectively). R values are Pearson's correlation coefficients. ECAS scores were available for 17 patients only. Abbreviations: CATS=Comprehensive Affect Testing System; GM=grey matter; ECAS=Edinburgh cognitive and behavioural ALS screen.*

When these analysis were re-run without the two *C9orf72* patients, a significant correlation was still observed (r=0.625; p=0.013 and r=0.659; p=0.01, respectively). In patients, no relationships were observed between CATS disgust correct recognition and degree of depression and behavioural disturbances assessed with the ECAS and ALS-FTD-Q (ALS-FTD-Q was administered to 18 patients). In controls, no relationship was observed between disgust correct recognition at the CATS and the volumes of the pallidum.

4.4 Discussion

In a sample of cognitively and behaviourally unimpaired patients with ALS, we observed an altered ability to correctly recognize disgust and a potential role of the basal ganglia, specifically of the left pallidum, in the altered processing of this emotion in ALS.

The finding of disgust recognition difficulties in ALS patients is in line with previous studies, which investigated the emotional processing in these patients, mainly during the processing of facial expressions from the Ekman's series (Aho-Ozhan et al., 2016, Andrews et al., 2017, Crespi et al., 2014, Girardi et al., 2011, Oh et al., 2016, Zimmerman et al., 2007). What should be underlined about the impairment in recognising emotions, and specifically disgust in ALS, are the clinical implications on everyday life for patients and caregivers. Disgust is an adaptative emotion, which together with fear, has got implications for human survival since the origin of our species (Castillo-Huitron, Naranjo et al., 2020). It is considered as a "moral" emotion, since it helps us to react toward something representing a risk like situation, act and people, by producing strong internal signals of avoidance (Eckart, Sturm et al., 2012, Rozin, Haidt et al., 2009). Given that everyday life often requires us to identify the emotions of several faces, these difficulties, even if subtle, could have a negative impact on social interactions, and also important moral implications. Awareness of difficulties in emotion processing becomes crucial in life-limiting and physically debilitating diseases as ALS, where keeping social relationships is fundamental to safeguard good quality of life (Swinnen & Robberecht, 2014). Since emotional and social deficits may lead to poorer quality of life and increased carer burden (Caga, Hsieh et al., 2019), the study of social cognition in ALS has been focus of increasing attention over the past few years. Changes in emotional processing in ALS have been described in an early report by Lulè and colleagues (Lule et al., 2005),

who demonstrated that patients judged socio-emotional stimuli as more positive than controls and exhibited reduced subjective arousal. Caregivers of ALS patients meeting also criteria for FTD described a significant reduction of their empathic concern and perspective taking abilities after disease onset (Arshad, Paplikar et al., 2020). From the current literature, we know that impairment in empathy, emotion recognition, fluency and executive functions are present also in non-demented ALS, sharing similarities with behavioral changes and cognitive decline in bvFTD (Beeldman, Raaphorst et al., 2018). Thus, studies examining management of bvFTD may be relevant to ALS, though caution should be used in extrapolating their conclusions.

Disgust recognition alterations have been previously reported in a sample of bulbar ALS patients in whom the half presented dementia (Zimmerman et al., 2007), in a sample of non-demented sporadic ALS of which only 14/22 were pure motor (Crespi et al., 2014) and in a sample of Korean ALS patients who presented different levels of cognitive dysfunction (Oh et al., 2016). The absence of impairment in cognition, behaviour and in the recognition of other emotions in our sample suggests that disgust could be the first emotion that ALS patients misrecognize in the course of the disease. The relationship we observed between the altered disgust recognition and the performance at the ECAS ALS-specific score and ECAS executive functions score, may suggest impaired disgust recognition as an early marker of cognitive decline. Therefore, from a clinical point of view, it would be important to assess regularly cognition, including social cognition and, in particular, emotional processing, also in those patients without manifest cognitive deficits.

There is an ongoing debate about whether impaired emotion recognition in neurodegenerative disorders, such as ALS, is linked to a more general cognitive decline or if it is independent of other cognitive processes. Specifically, some researchers suggested a causative role of executive dysfunctions in the social cognition impairment in ALS patients (Bora, 2017, Savage et al., 2014, Watermeyer, Brown et al., 2015), others reported social cognitive impairment, in absence of frontal lobe dysfunctions (Consonni, Catricala et al., 2016, Girardi et al., 2011, Lule et al., 2005). On the other hand, a relationship was not observed with the degree of depression or behavioural disturbances. Larger longitudinal studies are needed to confirm the specificity of disgust recognition deficit in ALS and its predictive value.

In our study, we observed an association between disgust recognition ability and the pallidum volume in ALS patients, while such a finding was not present in controls. Thus, this relationship was unique for patients only and could reflect an initial vulnerability of this brain region in cognitively and behavioural unimpaired ALS with starting emotional processing failure. Several studies define the anteroventral insula as a point of convergence for disgust, which has reciprocal connections with several other brain regions including the somatomotor orofacial area and the pallidum, both involved in disgust sensitivity (Calder, Beaver et al., 2007). According to a meta-analysis of 106 positron emission tomography and fMRI studies on human emotions, the most consistently activated regions for facial expression of disgust are insula/operculum and pallidum (Murphy, Nimmo-Smith et al., 2003). More specifically, several studies define the ventral pallidum as an "hedonic hot spot," which supports the sensory experience of disgust, controlling negative motivation to produce avoidance behaviour (Calder et al., 2007, Ho & Berridge, 2014, Holtmann, Bruchmann et al., 2020).

Interestingly, disgust misrecognition in our patients was related with the left pallidum volume. One prominent set of functional neuroimaging studies supported a left rather than a right activation of insular cortex to disgust stimuli (Royet, Plailly et al., 2003, Small, Gregory et al., 2003, Sprengelmeyer, Rausch et al., 1998, Wicker, Keysers et al., 2003). This was also observed in a study where authors applied a direct electrical stimulation of the insula in awake surgery in patients with a left hemisphere tumor, which demonstrated a selective decrease of disgust recognition, compared to other emotional facial expressions (Papagno, Pisoni et al., 2016). Furthermore, a recent study reported that left-lesioned patients in the insular cortex-basal ganglia complex presented significantly lower disgust composite scores compared to right-lesioned patients, or healthy controls, and suggested a primarily left-hemispheric basis of disgust (Holtmann et al., 2020). Authors attributed this lateralized functional difference to a potential psychobiological mechanism. They suggested that, since the left insular cortex supports the modulation of the parasympathetic tone, and since the activation of the parasympathetic nervous system is essential in initiating disgust responses, the lesions to the left insular cortex could alter the integration of information necessary to successfully process disgust (Guo, Sturm et al., 2016, Holtmann et al., 2020). Finally, an effect of handedness on hemispheric specialization for disgust processing cannot be ruled out.

However, this has not been demonstrated yet since all studies on disgust, including the present, involved mainly right-handers.

One of the major limitations of our study is the relatively small sample size, which could have led to negative findings. Second, it is a cross-sectional study, thus the evolving trajectory of emotion recognition deficits in these patients as well as their ability to predict ALS prognosis should be further investigated. Third, no volumetric differences between ALS patients and controls were observed. However, the unique association between pallidum volume and disgust recognition in our group of patients suggests a potential role of this subcortical region in the early alterations of emotional processing in ALS. Finally, we could not identify which emotions (among fear, anger, surprise, happiness and sadness) were more frequently confused with disgust, since the CATS has not been implemented with this purpose.

4.5 Conclusions

In conclusion, this is the first study which demonstrates an altered ability to correctly recognize disgust and a potential role of the left pallidum in the altered processing of this emotion in a sample of cognitively and behaviourally unimpaired ALS patients. Although the specific involvement of disgust (and not of other emotions) in pure motor ALS needs to be confirmed, these findings, together with the relationship between the altered disgust recognition with worse ECAS performance, suggest that disgust could be one of the first emotion that ALS patients fail to recognize in the course of the cognitive decline.

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Declaration of Competing Interest

The authors declare the following financial interests/personal re- lationships which may be considered as potential competing interests: V. Castelnovo, M.A. Magno, S. Basaia, N. Riva, B. Poletti report no dis- closures. E. Canu has received research supports from the Italian Ministry of Health. V. Silani received compensation for consulting services

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Chapter 5 - Pallidal functional connectivity changes are associated with disgust recognition in pure motor amyotrophic lateral sclerosis

Part of the following data have been published in Castelnovo et al., *Neuroimage Clin.* 2021; 32:102803.

5.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal and heterogeneous neurodegenerative disease of the motor system and its wider connections in which the possible presence of cognitive and/or behavioural symptoms is a universally known neuropsychological feature (McKenna, Corcia et al., 2021). In the past few years, a growing literature focused on the study of social cognition in ALS, from emotional processing to others' intention attribution. Emotional and social deficits in ALS have a great clinical impact, since they may influence the quality of life of patients and increase caregiver burden (Caga, Hsieh et al., 2019). Several studies on emotion perception impairment reported that ALS patients have a diminished psychophysiological arousal to emotional stimuli and have difficulties in recognizing and attributing emotions (Andrews, Staios et al., 2017, Crespi, Cerami et al., 2014, Girardi, MacPherson et al., 2011, Oh, Oh et al., 2016, Savage, Lillo et al., 2014, Zimmerman, Eslinger et al., 2007) judging socio-emotional stimuli as more positive than healthy controls (Lule, Kurt et al., 2005). In particular, the emotions that ALS patients, with and without cognitive impairment, mostly fail to recognise are disgust and surprise (Bora, 2017), followed by fear (Aho-Ozhan, Keller et al., 2016), anger (Oh et al., 2016, Zimmerman et al., 2007), and sadness (Martins, Prado et al., 2019).

ALS's failure in both emotion recognition and attribution has been mainly ascribed to the degeneration of fronto-striatal, temporal and occipital brain regions and their WM connections. In ALS populations in which nearly the 30% of patients were cognitively/behaviourally impaired, one voxel-based morphometry study found positive correlations between emotion attribution scores and gray matter density of right fronto-insular and anterior cingulate cortices (Cerami, Dodich et al., 2014), and two diffusion tensor imaging studies (Crespi et al., 2014, Crespi, Cerami et al., 2016) reported an association between emotion recognition difficulties and microstructural changes in ventral associative WM bundles connecting occipital, temporo-limbic and orbito-frontal

areas of the right hemisphere. In a recent study in which the 40% of ALS patients were cognitively impaired, positive correlations were observed between emotion recognition and the cortical thickness of the left caudal middle frontal, bilateral precentral, right middle and inferior temporal, lateral occipital and inferior parietal regions (Benbrika, Desgranges et al., 2019). Using a task-based functional magnetic resonance imaging (fMRI) paradigm with emotional pictures as stimuli, it emerged that ALS patients without major cognitive deficits, compared to controls, showed decreased activation of extra-striate visual areas and increased activation of the right supramarginal gyrus (Lule, Diekmann et al., 2007). Authors stated the reduced activity of extra-striate visual regions could be an indicator of lower subjective excitement at neural and behavioral level in ALS patients, while the increased response of supramarginal gyrus may be interpreted as a compensatory recruitment of somatosensory regions (Lule et al., 2007). More interestingly, some authors observed a decreased functional connectivity in the default mode, fronto-parietal, and salience networks at baseline, and the occurrence of affective theory of mind deficits after 6 months in cognitively unimpaired ALS patients (Trojsi, Di Nardo et al., 2017). Alterations of resting-state fMRI have been considered a possible biomarker of social cognition impairment in ALS patients (Trojsi et al., 2017).

In pure-motor ALS patients, we recently observed that an altered processing of disgust was related with smaller volume of the left pallidum (Castelnovo, Canu et al., 2021a) in absence of significant structural differences between groups in terms of volume. For this reason, in the present study, we aimed to investigate the resting-state functional connectivity (RS-FC) of the pallidum with the rest of the brain in ALS patients compared to healthy controls, in order to explore the presence of early functional connectivity alterations which could anticipate structural ones. We further investigated to which pallidal RS-FC changes were related the difficulties in recognising disgust.

5.2 Material and methods

5.2.1 Participants

From a sample of 87 patients with motor neuron disease, we selected 26 cognitively and behaviourally unimpaired sporadic patients with a clinical diagnosis of probable or definite ALS (Brooks, Miller et al., 2000) and without significant respiratory

failure, from those attending IRCCS San Raffaele Scientific Institute and IRCCS Istituto Auxologico Italiano. We included only patients who underwent the Comprehensive Affect Testing System (CATS), a comprehensive standard neuropsychological assessment, an MRI scan and a clinical evaluation. Fifty-two healthy controls, age-, sex-, and education-matched with patients, were recruited among non-consanguineous relatives. Inclusion criteria for healthy controls were: a normal neurological exam, a mini mental state examination (MMSE) score ≥28, and no familiarity with neurodegenerative disorders. Healthy controls underwent the same standard neuropsychological assessment and an MRI scan of ALS patients. Exclusion criteria for all participants were the following: significant medical illnesses or substance abuse which could interfere with cognitive functioning; any (other) major systemic, psychiatric, or neurological illnesses; and (other) causes of focal or diffuse brain damage, including cerebrovascular disease at conventional MRI scans. No participants were excluded for motion-related artifacts in the MR images. The 92% of participants described in the present study overlap with a recently published sample (Castelnovo et al., 2021a). The study protocol was approved by local ethical standards committee on human experimentation and all participants provided written informed consent.

5.2.2 Clinical assessment

Disease severity was scored by the revised ALS Functional Rating Scale (ALSFRS-R ALSFRS-R, with a maximum score of 48) (Cedarbaum, Stambler et al., 1999). Disease duration was calculated from symptom onset to MRI date in months. The disease progression rate was calculated with to the following formula: (48–ALSFRS-R score)/disease duration.

5.2.3 Cognitive and behavioral assessment

Cognitive evaluation were performed by experienced neuropsychologists, who were unaware of the MRI results, and consisted in the administration of a comprehensive standard neuropsychological battery, which was necessary to detect the presence of cognitive and/or behavioural deficits according to Strong's revised criteria (Strong, Abrahams et al., 2017). In the neuropsychological battery, the following cognitive

functions were administered to patients as previously described (Castelnovo, Canu et al., 2021b): global cognitive functioning with the MMSE and the Edinburgh Cognitive and Behavioural ALS Screen (ECAS); long and short term verbal memory with the Rey Auditory Verbal Learning Test and the digit span forward, respectively; executive functions with the digit span backward, the Cognitive Estimation Task (CET), the Modified Card Sorting Test; fluency with the phonemic and semantic fluency tests and the relative fluency indices (controlling for individual motor disabilities); language with the confrontation naming subtests of Italian battery for the assessment of aphasic disorders (BADA); emotion processing with the CATS; mood with the Hamilton Depression Rating Scale (HDRS); and the presence of behavioral disturbances with the Amyotrophic Lateral Sclerosis-Frontotemporal Dementia-Questionnaire (ALS-FTD-Q) administered to patients' caregivers. Healthy controls underwent the entire assessment except for the ECAS, CET and BADA subtests; moreover, in healthy controls the Beck Depression Inventory (BDI) was used to assess mood.

CATS is a battery measuring different aspects of the emotion processing by using the famous Ekman's faces expressing the six basic emotions (disgust, surprise, happiness, anger, fear and sadness) (Froming, Levy et al., 2006). From this battery, we selected and administered six subtests: identity discrimination (which is the control condition that investigates the correct discrimination of faces with no emotion involvement), affect discrimination, affect naming, affect matching, affect selection, affect confrontation). See Figure 1 of chapter 4 for an overview of the administered CATS subtests.
Summing up the number of correct answers at the CATS subtests, we calculated a total score for each of the six basic emotions.

5.2.4 MRI acquisition

Using a 3.0 T scanner (Ingenia CX, Philips), the following brain MRI sequences were obtained from all participants: 3D T1-weighted (TFE) (TR=7 ms; TE=3.2 ms; flip angle=9 [degrees]; 204 contiguous sagittal slices with voxel size=1 x 1 x 1 mm, matrix size=256 x 240, FOV=256x240 mm^2); 3D FLAIR (TR=4800 ms; TE=267 ms; TI=1650 ms; ETL=167; NEX=2; 192 contiguous sagittal slices with voxel size=0.89 x 0.89 x 1 mm, matrix size=256 x 256, FOV=256x256 mm^2); 3D T2 (TR=2500 ms; TE=330 ms; ETL=117; NEX=1; 192 contiguous sagittal slices with voxel size=0.89 x 0.89 x 1 mm,

matrix size=256 x 258, FOV=256x256 mm^2); and T2* weighted (GE-EPI) sequence for RS-fMRI (TR=1567 ms; TE=35 ms; flip angle=70; MB=2; SENSE=2; FOV=240x240; pixel size=2.5x2.5 mm; slice thickness=3 mm; 320 sets of 48 contiguous axial slices; acquisition time=8' and 32''). Before starting the RS-fMRI scanning, the technician talked with the participants through their earphones instructing them to remain motionless, to keep their eyes closed, not to fall asleep, and not to think about anything in particular.

5.2.5 MRI analysis

MRI analysis was performed at the Neuroimaging Research Unit, IRCCS Scientific Institute San Raffaele, Milan, Italy. The potential presence of WM hyperintensities was evaluated on 3D FLAIR and 3D T2-weighted images.

5.2.5.1. Resting-state fMRI preprocessing

RS-fMRI data processing was performed using the FMRIB software library (FSLv5.0) as outlined formerly (Canu, Agosta et al., 2020). The first four volumes of the RS-fMRI data were removed to reach complete magnet signal stabilization. The following FSL-standard preprocessing pipeline was implemented: (1) motion correction using MCFLIRT; (2) high-pass temporal filtering (lower frequency: 0.01 Hz); (3) spatial smoothing (Gaussian Kernel of FWHM 6 mm); (4) single-session independent component analysis-based automatic removal of motion artifacts (ICA_AROMA)(Pruim, Mennes et al., 2015) in order to identify those independent components (ICs) representing motion-related artifacts.

5.2.5.2. Seed-based resting-state functional connectivity (for a brief overview of the method see Figure 2 of chapter 3).

Based on findings observed from the results of the study presented in chapter 4, we performed a seed-based analysis, as previously described (Canu et al., 2020, Castelnovo, Canu et al., 2020). Left and right pallidum were selected as regions of interest and were defined in the MNI space using the automated anatomical labelling atlas (AAL) in WFU PickAtlas (toolbox of SPM12), moved to each subject native T1-weighted space

trough non-linear and affine registrations, and visually inspected in the individual brains by neuroimaging expert researchers. Seed-based RS-FC was then performed using a 2-step regression analysis as implemented in the FMRIB software library (FSLv5). First, time series of WM, cerebrospinal fluid, and whole brain volumes in RS-fMRI native space were extracted from the preprocessed and denoised data and their effects were regressed out using the FMRI Expert Analysis Tool. ROI mean time-series were then calculated. The output of this step is represented by subject-level maps of all positively and negatively predicted voxels for each regressor. Subject-level maps were registered to the MNI standard template to enter the statistical analysis.

5.2.6 Statistical analysis

5.2.6.1 Demographic, clinical and cognitive data.

Demographic characteristics and cognitive performance between groups, were compared using Kruskal-Wallis one-way ANOVA models (using U test of Mann–Whitney) and Fisher's exact test, for continuous and categorical variables, respectively.

5.2.6.2 Seed-based resting-state functional connectivity.

Seed-based RS-FC was compared between patients and healthy controls using GLM, which included RS-FC maps as dependent variables. Corrections for multiple comparisons were carried out at a cluster level using Gaussian random field theory, $z > 2.3$; cluster significance: $p < 0.05$, corrected for multiple comparisons.

For the correlation analysis between significant RS-FC changes and cognitive scores, we firstly extracted the mean RS-FC value for each subject from each significant cluster emerging from the seed-based RS-FC analysis. In all subjects, CATS disgust score was correlated with mean RS-FC values of the significant clusters using Pearson's correlation analysis. Analyses were thresholded at $p<0.05$ adjusted for multiple comparisons using Bonferroni's correction. We finally investigated a potential relationship between significant CATS disgust score and patients' mood as assessed with the HDRS (Hamilton, 1960). The statistical analyses were performed with SPSS software (version 24.0; IBM Corp., Armonk, NY, USA).

5.3. Results

ALS patients and controls did not differ in age, sex and education (Table 1). All patients were cognitively and behaviourally normal according to Strong's criteria (Strong et al., 2017). The standard neuropsychological battery did not reveal differences between groups (Table 1), except for better performances of ALS patients compared to controls in the perseverative responses of MCST and in the phonemic fluency index. No patient showed clinical depression according to HDRS neither to clinical interview.

At the CATS, compared to healthy controls, patients obtained significantly lower scores in the total score, affect selection and affect matching subtests and they were significantly less able to recognize disgust (Table 2). No significant associations were observed between patients' mood and CATS disgust score.

Table 1. Sociodemographic, clinical and neuropsychological features of ALS patients and healthy controls.

	Healthy controls	**ALS patients**	**P value**
N	52	26	-
Sex [women]	33 (63%)	12 (46%)	0.16
Age at MRI [years]	60.36±8.49	56.90±12.30	0.25
Education [years]	12.40±3.79	13.27±4.68	0.54
Disease duration [months]	-	27.91±21.06	-
ALSFRS-R, 0-48	-	38.16±7.04	-
Disease progression rate	-	0.51±0.48	-
Site of onset, limb	-	87%	-
Global cognition			
MMSE	29.36±0.80	29.17±1.37	0.95
ECAS,* Language	-	25.34±2.06	-
ECAS,* Fluency	-	20.08±2.15	-
ECAS,* Executive Functions	-	38.80±5.00	-
ECAS,* ALS-specific functions	-	84.72±6.47	-
ECAS,* Memory	-	18.92±3.45	-
ECAS,* Visuospatial	-	11.68±0.56	-
ECAS,* ALS non-specific functions	-	30.40±3.45	-
ECAS,* total score	-	115.64±9.65	-

Memory			
Digit span, forward	5.96±1.07	6.13±1.39	0.56
Digit span, backward	4.82±1.20	5.27±1.83	0.38
RAVLT, immediate	49.62±7.08	49.84±11.04	0.88
RAVLT, delayed	10.92±2.42	10.12±2.73	0.18
Executive function			
CET	-	12.24±3.83	-
MCST, perseverative responses	3.52±3.63	1.91±2.74	0.03*
Language			
BADA (nouns)	-	28.75±0.96	-
BADA (actions)	-	27.50±1.00	-
Fluency			
Phonemic fluency, Index	5.10±1.84	4.35±2.47	0.01*
Semantic fluency, Index	3.81±1.58	3.84±1.44	0.73
Mood & Behaviour			
BDI	7.04±4.81	-	-
HDRS	-	6.87±4.38	-
ECAS,* behaviour score	-	0.50±0.70	-
ALS-FTD-Q	-	5.63±7.46	-

*Values denote mean ± standard deviations or numbers (percentages). Neuropsychological values are reported as raw scores. P-values refer to Kruskal-Wallis one-way ANOVA models (using U test of Mann–Whitney) and Fisher's exact test, for continuous and categorical variables, respectively. *=significant differences between groups at p<0.05. Abbreviations: ALS=amyotrophic lateral sclerosis; ALS-FTD-Q=amyotrophic lateral sclerosis-frontotemporal dementia-questionnaire; ALSFRS-R=ALS Functional Rating Scale Revised; BADA=battery for the analysis of aphasic deficits; CET=cognitive estimation test; CPM=coloured progressive matrices; HC=healthy controls; HDRS; Hamilton Depression Rating Scale; MMSE=mini-mental state examination; MRI=Magnetic Resonance Imaging. RAVLT=Rey auditory verbal learning test; WCST=Wisconsin card sorting test. Disease Progression Rate has been obtained as following: (48–ALSFRS-R score)/time between symptom onset and first visit. Fluency indices have been obtained as following: time for generation condition - time for control condition (reading or writing generated words)/total number of items generated.*

Table 2. Cognitive performance of ALS patients at the Comprehensive Affect Testing System (CATS).

	HC	ALS	P-value
CATS Total score	56.79±4.77	53.92±5.75	0.02*
CATS Identity Discrimination	11.81±0.40	11.46±0.99	0.16
CATS Affect Discrimination	11.29±0.98	10.92±0.98	0.07
CATS Affect Naming	4.63±0.97	4.15±1.19	0.07
CATS Affect Selection	5.52±0.67	5.08±0.89	0.03*
CATS Affect Matching	9.31±1.92	8.46±1.53	0.03*
CATS Affect Confrontation	14.23±3.04	13.85±3.33	0.52
Disgust, correct answers (0-9)	5.85±1.59	5.04±1.80	0.048*
Surprise, correct answers (0-8)	6.56±1.34	6.46±1.21	0.54
Happiness, correct answers (0-9)	8.65±0.52	8.62±0.57	0.83
Anger, correct answers (0-10)	5.69±2.11	4.77±1.58	0.06
Fear, correct answers (0-8)	5.15±1.29	5.35±1.26	0.57
Sadness, correct answers (0-9)	6.90±1.54	6.58±1.60	0.38

*Values denote mean ± standard deviations. P values refer to Kruskal-Wallis one-way ANOVA models (using U test of Mann–Whitney). *=significant differences between groups at p<0.05. Abbreviations: ALS=Amyotrophic Lateral Sclerosis; CATS=Comprehensive Affect Testing System.*

5.3.1 Seed-based resting-state functional connectivity

For each group, Figure 1 reports the RS-FC mean connectivity between left and right pallidum and the rest of the brain.

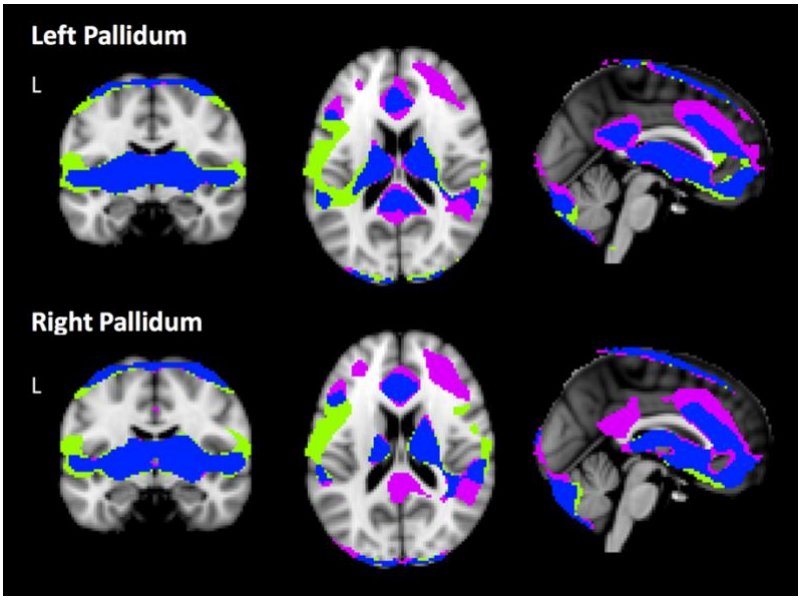

Figure 1. Seed-based functional connectivity analysis. *RS-FC mean connectivity between left and right pallidum and the rest of the brain for healthy controls (pink colour), ALS patients (green colour) and their overlap (blue colour).*

In ALS patients compared to controls, the seed-based analysis showed reduced RS-FC between bilateral pallidum and bilateral middle and superior frontal gyri and right middle cingulate gyri, and increased RS-FC between bilateral pallidum and bilateral postcentral gyrus and Rolandic operculum, left superior temporal and right supramarginal gyrus (Figures 2 and 3). Furthermore, decreased RS-FC was observed between left pallidum and right superior frontal, left middle and inferior temporal gyri and bilateral caudate, and between right pallidum and left anterior cingulate gyrus (Figure 2). RS-FC was increased between left pallidum and right superior temporal, left middle frontal and precentral and left supramarginal gyri, and between right pallidum and left lingual, supramarginal and fusiform gyri (Figure 3).

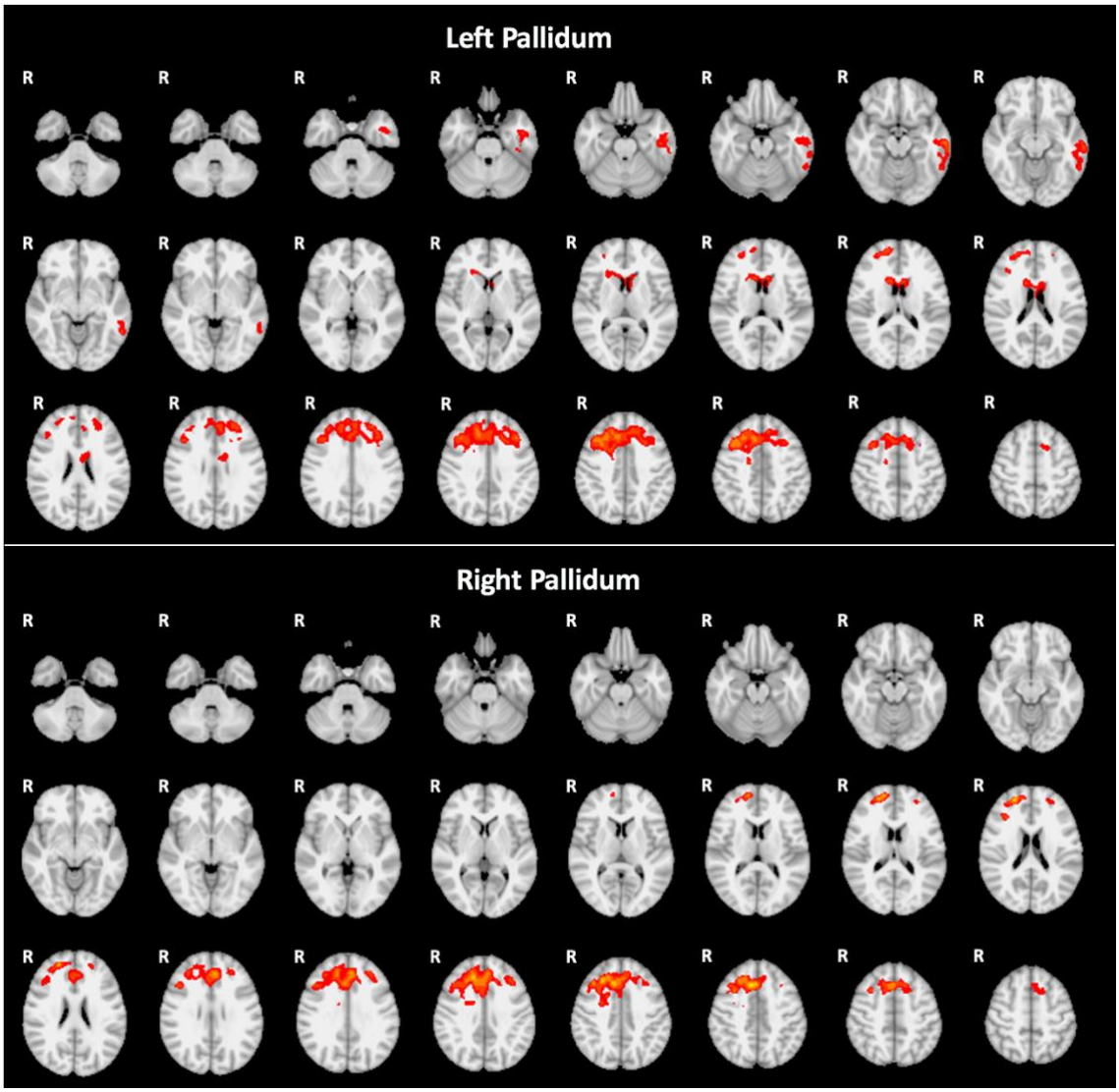

Figure 2. Seed-based functional connectivity analysis. *Regions where ALS patients showed decreased resting state functional connectivity with left pallidum and right pallidum compared to healthy controls.*

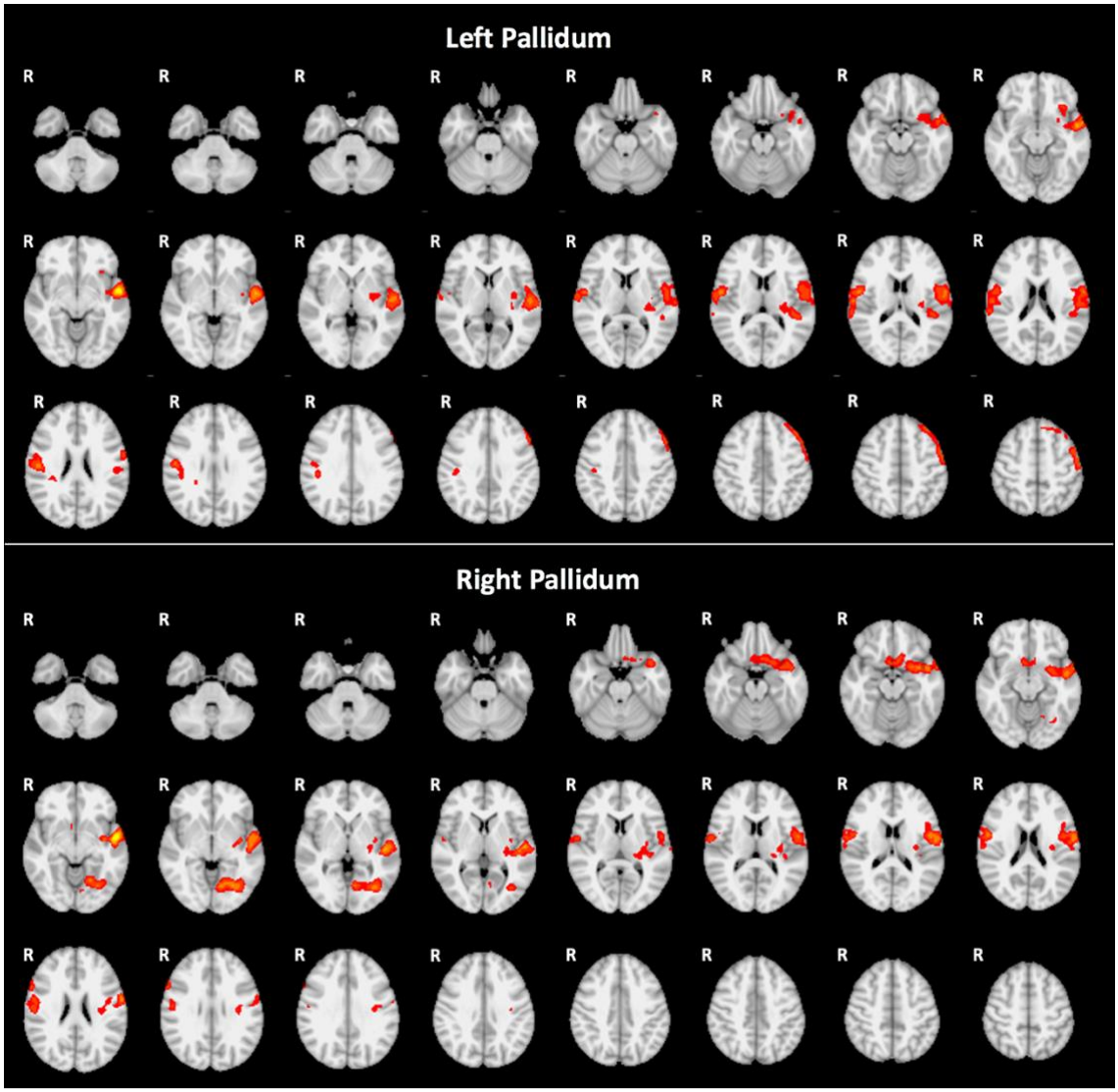

Figure 3. Seed-based functional connectivity analysis. *Regions where ALS patients showed increased resting state functional connectivity with left pallidum and right pallidum compared to healthy controls.*

In ALS patients and healthy controls, a lower performance in recognizing disgust was related with a reduced RS-FC between the left pallidum and the left middle and inferior temporal gyrus (r=0.282; p=0.048, corrected for multiple comparisons; Figure 4).

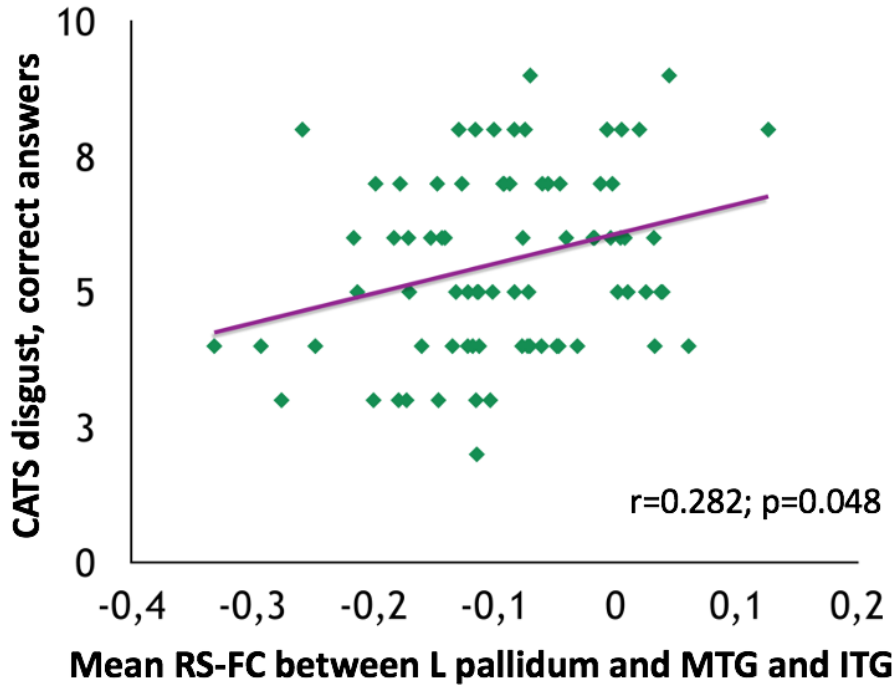

Figure 4. Cognitive-fMRI positive relationship. *In ALS patients and healthy controls, worse performance in recognizing disgust was related with decreased resting state functional connectivity of the left pallidum with left middle and inferior temporal gyrus (r=0.282; p=0.048, corrected for multiple comparisons).*

5.4 Discussion

In a sample of cognitively unimpaired ALS patients, we confirmed our previous findings of initial difficulties in emotion recognition limited to the facial identification of disgust. In the previous work we observed an association with pallidum volume although no significant structural differences were found between ALS and healthy controls (Castelnovo et al., 2021a). Here, on the basis of our initial hypothesis, we demonstrated an altered RS-FC of the bilateral pallidum both in terms of increased and decreased RS-FC in ALS patients compared to healthy controls. Furthermore, in ALS patients and healthy controls, we observed that a reduced RS-FC of the left pallidum with the left

middle and inferior temporal gyri was associated with a lower ability in recognizing disgust. Taken together, our results suggest that emotional processing in ALS is associated with pallidum functioning and that resting state functional changes of this region and related circuits may precede structural alterations.

In ALS patients compared to controls, we observed a reduced RS-FC between pallidum, medial frontal and temporal cortices, and caudate. These brain regions were observed to activate together with pallidum, insula, other basal ganglia, and fusiform gyrus during the processing of disgusted faces in healthy subjects (Calder, Keane et al., 2000, Fusar-Poli, Placentino et al., 2009, Phillips, Young et al., 1998). This is an important circuit, named cortico-basal ganglia-thalamo-cortical loop (Pessoa, 2017), involved in emotional processing, especially in disgust, in which the cortex is directly connected with striatum, which projects to pallidum which, in turn, communicates with the thalamus projecting back to the cortical regions and closing the loop (Pessoa, 2017). In this circuit, cortical regions, such as the medial frontal cortex, participate in the conscious experience of emotion, inhibition of excessive emotion, and control emotional states to make important decisions (Fusar-Poli et al., 2009). The temporal cortex and the fusiform gyrus have a role in higher-order visual information processing (Wicker, Keysers et al., 2003), since they activate during the processing of human emotional faces (Fusar-Poli et al., 2009). In young adults, fMRI studies suggested that temporal regions (Gallagher and Frith, 2003; Völlm et al., 2006) have a role in social and emotional processes, including the recognition of facial expressions and the theory of mind (Olson et al., 2007).

Cortical regions project to basal ganglia, which, beyond motor function, have a role in limbic function, such as motivation and reward valuation, and contribute to the evaluation of the affective valence and creation of emotional states. In particular, the striatum is a key station for the reward processing and the dorsal regions (putamen, caudate) contribute to goal-directed behavior such as the choosing and initiation of an action, inhibitory control, cognitive reappraisal, through the integration of sensorimotor, cognitive, and motivational data (Jahanshahi, Obeso et al., 2015). Pallidum is the main target of striatal outflow and its ventral part belongs to the ventro-striatopallidal system. It plays a role in the regulation of emotion, specifically in the sensory perception of disgust and in initiating a movement in reaction to an emotional stimuli to produce an

avoidance behavior (Calder, Beaver et al., 2007, Ho & Berridge, 2014, Holtmann, Bruchmann et al., 2020, Singh-Bains, Waldvogel et al., 2016, Smith, Fox et al., 2009). Therefore, in our patients, a reduced RS-FC between fronto-temporo-striatal regions and the pallidum, could lead to an altered processing of the stimulus, performed by cortical regions and, consequently, to an altered ability to recognize disgust.

We further observed that a reduced RS-FC between the left pallidum and the left middle and inferior temporal gyri was associated with lower performances in recognizing disgust in ALS. This association confirms once again the left lateralization of the disgust circuit, as proposed by a prominent set of neuroimaging studies (Guo, Sturm et al., 2016, Holtmann et al., 2020). These works withstand a primary left activation of insular cortex to disgust stimuli (Royet, Plailly et al., 2003, Small, Gregory et al., 2003, Sprengelmeyer, Rausch et al., 1998, Wicker et al., 2003) and observed that left-lesioned patients in the insular cortex-basal ganglia complex performed significantly worse in disgust recognition compared to right-lesioned patients or healthy controls (Holtmann et al., 2020).

The brain regions which played a role in this observed association, the middle and inferior temporal gyri, are involved in multiple high-cognitive functions including social cognition and, specifically, emotion regulation (Lin, Young et al., 2020, Xu, Lyu et al., 2019). In fact, extensive connections are present between the inferior temporal gyrus and limbic regions such as the ventral striatum through the inferior longitudinal fasciculus (Lin et al., 2020). A resting state study investigating functional connectivity from different subregions of the middle temporal gyrus in healthy controls, observed that the most anterior ones participated in social cognition processing (Xu et al., 2019).

On the other hand, in ALS patients compared to controls, we observed an increased RS-FC of the Rolandic operculum (i.e., part of the insular cortex), postcentral gyrus and supramarginal gyrus with bilateral pallidum. These brain regions were observed to activate during emotion recognition fMRI-tasks in pure motor ALS (Lule et al., 2007, Lule et al., 2005, Yoshimura, Kawamura et al., 2005). According to some authors (Lule et al., 2007, Yoshimura et al., 2005) the activation of these somatosensory regions in ALS could reflect a compensatory mechanism: they would create internal somatosensory representations of facial and emotional cues in order to remedy the initial emotional difficulties. In line with these findings, the increased connectivity of these regions with bilateral pallidum in our sample could be in part explained by compensatory

attempts. On the other hand, the increased RS-FC of brain regions directly involved in the circuit of disgust, such as the insular cortex, could reflect a pathophysiological consequence of the disconnection between the pallidum and frontal and temporal regions.

Our study has some limitations, mainly related to the relatively small sample size and the cross-sectional nature of the design, which did not allow us to investigate the evolving trajectory of emotion recognition deficits in these patients. Furthermore, it was not possible to detect which emotions were more frequently confused with disgust, because the CATS has not been realised with this aim.

In conclusion, in a sample of cognitively unimpaired ALS patients, we confirmed our previous findings of early difficulties in recognizing disgust and we demonstrated an altered RS-FC between pallidum and the rest of the brain corroborating the role of left pallidum in the altered processing of disgust. Specifically, the reduced left pallidum-frontotemporal-striatal RS-FC may play a role in the lower ability of patients in recognizing disgust and could be linked to future frontotemporal dementia-like alterations in ALS. These findings offer new potential markers for monitoring extra-motor clinical progression in ALS.

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Chapter 6 - General discussion

In this thesis we explored the presence of early markers of cognitive and behavioural alterations and their progression in MND patients, using novel neuropsychological evaluations and advanced MRI techniques.

In **chapter 2**, we investigated the progression of cognitive and behavioural changes over 12 months in MND patients using standard and/or computer-based neuropsychological assessments (Castelnovo, Canu et al., 2021b). In this work, we demonstrated that the progressive cognitive changes in ALS patients, which mainly involved attentive and executive function domains, were identified by the computer-based battery rather than by the standard neuropsychological tests. This study underlines an important point in the current neuropsychological literature. In fact, cognitive and behavioural findings in MND patients are affected by the administration modality, and, for this reason, it is important to consider whether the tests are specific and sensitive for MND, and whether they consider the patient verbal and/or physical difficulties. While using the standard battery, we payed particular attention to the tests which are most sensitive to motor impairment: we used fluency indices, which account for reading/writing speed (Abrahams, Leigh et al., 2000); we calculated a weighted score for the MMSE; FBI items questioning on logopenia/aphasia and alien hand/apraxia were not administered to caregivers, due to their potential difficulties in discriminating between behavioural symptoms and physical disability; and, finally, all analyses were corrected for the disease severity (ALSFRS-R). Nevertheless, using the standard assessment, compared to baseline, no neuropsychological changes were detected after 12 months in each of the three MND groups, except for MMSE changes in ALS. These negative findings are in line with other studies which used standard neuropsychological batteries (Kasper, Zydatiss et al., 2016, Kilani, Micallef et al., 2004, Robinson, Lacey et al., 2006, Strong, Grace et al., 1999, Woolley, Goetz et al., 2018) and which had low percentages of patients with cognitive impairment (Kilani et al., 2004, Robinson et al., 2006, Strong et al., 1999). Using this type of cognitive assessment, only two works (Bock, Duong et al., 2017, Elamin, Bede et al., 2013) observed a cognitive worsening in ALS patients who already presented cognitive deficits at baseline. It could be hypothesized that a standard

approach is able to identify cognitive worsening in patients who already manifest a cognitive impairment at the first visit, but not in those with initial subclinical deficits.

Using a computer-based battery, the TAP, which investigates attention and executive functions, accounting for motor impairment, we observed a significant worsening of the performance in ALS patients, in terms of both speed and accuracy. This cognitive worsening in patients was evident in subtests assessing attention and dual tasks, and in proper executive tests, all functions subtended by the frontal regions and their main connections with the parietal and ventro-striatal networks (Di Martino, Scheres et al., 2008). The progressive decline of the performance of ALS patients in the TAP alertness subtest is in line with the significant worsening found in the same subtest after 12 months by Schreiber et al. (Schreiber, Gaigalat et al., 2005). A more recent study using both the standard battery and the ALS-CFB (a computerized battery) to assess cognition in ALS patients (Gillingham, Yunusova et al., 2017), reported a worsening only in the Simple Reaction Time task of the ALS-CFB, demonstrating again that computerized batteries can detect subtle cognitive deficits compared to standard assessments.

Concerning PLS and PMA patients, due to the small sample size, our findings should be considered with caution. Coherently with the only previous study assessing cognitive progression in PLS (Proudfoot, Menke et al., 2015), we observed no cognitive changes over 12 months at the standard battery in PLS patients. Regarding PMA, this was the first longitudinal study which monitored the cognitive profile of these patients over time. After 12 months, we observed no significant cognitive worsening neither at the standard battery, nor at the TAP. However, since in a cross-sectional work on PMA patients with a longer mean disease duration, frequencies of cognitive impairment equal to ALS have been reported (de Vries, Rustemeijer et al., 2019), we can hypothesize that PMA patients may manifest cognitive deficits later in the course of the disease.

In **chapter 3** we aimed to clarify whether and how the progression of the observed cognitive dysfunctions in ALS was related to frontal brain alterations (Castelnovo, Canu et al., 2020). We investigated RS-FC changes in patients with ALS over 6 months of observation and their relationship with ALS frontal cognitive alterations over time evaluated with the TAP. Over time, ALS patients, showed increased RS-FC in middle and superior frontal gyri and anterior cingulate cortex within the frontostriatal and left

frontoparietal networks, and a decreased RS-FC of the right middle frontal gyrus with frontoparietal regions. The involvement of frontal and parietal networks in the course of the disease is in line with pathological studies (Braak, Brettschneider et al., 2013, Brettschneider, Del Tredici et al., 2013) and with the definition of ALS as part of the FTLD spectrum (Burrell, Halliday et al., 2016). In line with the present work, a connectome study, which simulated the disease spreading from the motor cortex performing a network-based analysis, delineated a progression of WM damage in ALS similar to our findings (Meier, van der Burgh et al., 2020). Previous studies investigating the progression of RS-FC changes in ALS reported both reduced and increased RS-FC within motor and extra-motor networks (Menke, Proudfoot et al., 2018, Schulthess, Gorges et al., 2016, Shen, Xu et al., 2018, Trojsi, Di Nardo et al., 2020). The 'direction' of RS-FC changes appear to rely on the observation period, since longer follow-up have been associated with a decreased RS-FC (Menke et al., 2018, Trojsi et al., 2020).

In this study, a central role of the middle frontal gyrus in the pattern of RS-FC alterations in ALS patients emerged. In particular, we observed that a progressive increased RS-FC of the middle frontal gyrus within both the frontostriatal and the left frontoparietal networks, was associated with a reduced ability of patients at the TAP divided attention (dual task) subtest at the first visit. In healthy individuals, middle frontal gyrus contributes to attention, executive functions and working memory, while frontostriatal network contributes to maintain and update information in working memory (as represented by our dual task) (Ekman, Fiebach et al., 2016). Previous studies reported an alteration of middle frontal gyrus in ALS using fMRI (Abrahams, Goldstein et al., 1996, Abrahams, Goldstein et al., 2004, Trojsi, Di Nardo et al., 2017) and other imaging techniques (Alruwaili, Pannek et al., 2018, Verstraete, Veldink et al., 2012). A previous study (Pettit, Bastin et al., 2013) reported that worse dual-task performance in ALS patients was associated with low fractional anisotropy in the WM underneath the middle frontal gyrus, corroborating the contribute of middle frontal gyrus in the executive dysfunctions observed in ALS patients. The higher connectivity in frontal regions in association with reduced frontal-executive abilities at baseline that we observed in ALS patients, can be an index of disease progression, as found in patients with FTLD (Farb, Grady et al., 2013). Furthermore, the seed-based RS-FC analysis of the middle frontal gyrus demonstrated a reduced RS-FC between this region and antero-posterior regions at

follow-up, which could show the typical FTLD functional disconnection (Reyes, Ortega-Merchan et al., 2018). Combining both cognitive and fMRI data in ALS can be a valuable method to monitor the disease progression in extra-motor regions, even in patients without manifest cognitive deficits.

Beyond frontal alterations, several studies reported basal ganglia involvement in ALS. In **chapter 4** we focused our attention on emotion recognition, which has a great clinical impact in ALS patients, and on its association with basal ganglia, hippocampus and amygdala (Castelnovo, Canu et al., 2021a).

Deficits in social cognition, such as empathy and emotion recognition, are features also of non-demented ALS patients, who present behavioural and cognitive mild alterations similar to those of bvFTD patients (Beeldman, Raaphorst et al., 2018). In a group of ALS patients without cognitive and behavioural impairment, we observed initial difficulties in the ability to recognise disgust, in agreement with previous literature (Aho-Ozhan, Keller et al., 2016, Andrews, Staios et al., 2017, Crespi, Cerami et al., 2014, Girardi, MacPherson et al., 2011, Oh, Oh et al., 2016, Zimmerman, Eslinger et al., 2007). In this work, we hypothesized that disgust could be one of the first emotions that ALS patients have difficulties to recognise. Furthermore, the finding of an association between the disgust recognition score and the ECAS ALS-specific score and ECAS executive functions score, could indicate that the deficits in recognising disgust are an early feature of neuropsychological worsening in ALS. This highlights the importance of including emotional processing evaluation in the regular cognition monitoring, also in pure-motor ALS patients.

In our study, we also found an association between the recognition of facial expression of disgust and the volume of the left pallidum, exclusively in the ALS patient group. From a meta-analysis on 106 positron emission tomography and fMRI studies on human emotions emerged that insula/operculum and pallidum were the main activated regions for facial expression of disgust (Murphy, Nimmo-Smith et al., 2003). In particular, the ventral part of the pallidum seems to have a role in the sensory experience of disgust, controlling negative motivation to produce avoidance behaviour (Calder, Beaver et al., 2007, Ho & Berridge, 2014, Holtmann, Bruchmann et al., 2020). The left lateralization of this circuit in response to disgusting stimuli is supported by several

functional neuroimaging studies (Royet, Plailly et al., 2003, Small, Gregory et al., 2003, Sprengelmeyer, Rausch et al., 1998, Wicker, Keysers et al., 2003) and studies on patients with lesions in the insular cortex-basal ganglia complex (Holtmann et al., 2020). Some authors suggest that since the left insula contributes to the modulation of the parasympathetic tone, whose activation is fundamental in starting responses to disgust stimuli, lesions to the left insula and linked brain regions, including pallidum, could influence the integration of information required to correctly process disgust (Guo, Sturm et al., 2016, Holtmann et al., 2020).

This study has demonstrated a potential role of the left pallidum in the altered processing of disgust in a sample of pure-motor ALS patients, which together with the association between worse disgust recognition ability and worse ECAS scores, indicates that disgust could be one of the first emotions that ALS patients have difficulties to recognise in their cognitive worsening.

In the study presented in chapter 4 we observed that an altered processing of disgust was related with smaller volume of the left pallidum (Castelnovo et al., 2021a) in absence of significant structural volumetric differences between patients and controls. In the study described in **chapter 5**, we investigated the resting-state functional connectivity (RS-FC) of the pallidum with the rest of the brain in patients with ALS compared to healthy controls. We hypothesized that early functional connectivity alterations could precede structural changes. As expected, ALS patients showed an altered RS-FC of the bilateral pallidum in specific brain circuits compared to healthy controls. Furthermore, in ALS patients and healthy controls, a reduced RS-FC of the left pallidum with the left middle and inferior temporal gyri was related with a reduced ability in recognizing disgust.

More in detail, ALS patients showed a reduced RS-FC between pallidum and medial frontal and temporal cortices, and caudate, which were reported to activate with pallidum, insula, other basal ganglia, and fusiform gyrus when stimuli of disgusted faces were presented (Calder, Keane et al., 2000, Fusar-Poli, Placentino et al., 2009, Phillips, Young et al., 1998). These regions belong to the cortico-basal ganglia-thalamo-cortical loop (Pessoa, 2017), which contributes to emotional processing, especially of disgust (Pessoa, 2017). In this circuit, cortical areas, as the medial frontal cortex, support the

conscious experience of emotion and the regulation of emotional states (Fusar-Poli et al., 2009). Also the temporal cortex and the fusiform gyrus activate when a facial expression is presented (Fusar-Poli et al., 2009) and they are involved in higher-order visual information processing (Wicker et al., 2003).

Cortical regions project to basal ganglia, which contribute to the evaluation of the affective valence, generation of emotional states and motivation. In particular, the striatum has a role in the reward processing and contributes to goal-directed behaviour. Striatum then projects to pallidum, whose ventral part has been shown to mediate the sensory perception of disgust, and the initiation of an avoidance behaviour (Calder et al., 2007, Ho & Berridge, 2014, Holtmann et al., 2020, Singh-Bains, Waldvogel et al., 2016, Smith, Fox et al., 2009). For this reason, the reduced RS-FC between fronto-temporo-striatal regions and the pallidum observed in our ALS patients could lead to an altered processing of this emotion.

The reduced RS-FC between the left pallidum and the left middle and inferior temporal gyri in relation with lower performances of our ALS patients and healthy controls in recognizing disgust further confirms the left lateralization of the disgust circuit, as discussed in chapter 4 (Guo et al., 2016, Holtmann et al., 2020). Furthermore, the middle and inferior temporal gyri are involved in multiple high-cognitive functions including social cognition and, specifically, emotion regulation.

On the other hand, the increased RS-FC of the Rolandic operculum, which is part of the insular cortex, postcentral gyrus and supramarginal gyrus with bilateral pallidum in ALS patients compared to controls, may have two possible explanations. Firstly, as suggested by previous fMRI studies in response to emotional stimuli, the increased connectivity of these somatosensory regions could reflect a compensatory mechanism: these regions would contribute to internally create somatosensory representations of facial and emotional stimuli in order to remedy the initial emotional difficulties (Lule, Diekmann et al., 2007, Yoshimura, Kawamura et al., 2005). Secondly, the increased RS-FC of brain regions, which are directly involved in the circuit of disgust, such as the insular cortex, could reflect a pathophysiological consequence of the disconnection between the pallidum and frontal and temporal regions.

In the study described in this chapter we therefore confirmed our previous findings of early difficulties in recognizing disgust and we demonstrated that the reduced left

pallidum-frontotemporal-striatal RS-FC may contribute to the lower ability of patients in recognizing disgust and could be linked to future frontotemporal dementia-like alterations in ALS.

This thesis has provided important insights regarding the cognitive and RS-fMRI longitudinal changes in MND. Concerning cognition, the cognitive worsening in executive functions and social cognition domains that we observed in ALS patients well reflect the belonging of ALS to the frontotemporal lobar degeneration spectrum. In addition, our findings highlight the need of specific, accurate and well-tolerated tools for monitoring the cognitive worsening in MND. Our longitudinal imaging analysis corroborates again the link of ALS with the FTLD spectrum. Over time, we have shown a progression of RS-FC changes within frontostriatal and frontoparietal networks, as well as the relationship between these changes and patients' frontal-executive dysfunction. Furthermore, in pure motor ALS, the seed-based RS-FC analysis of pallidum demonstrated the presence of early alterations of the left pallidum-frontotemporal-striatal functional connectivity, which are linked to patients' emotional dysfunction and precede structural brain damage. Thus, RS-FC could be considered as a valuable and sensitive biomarker for detecting and monitoring in vivo functional brain changes within the central nervous system even in MND patients with not frank cognitive impairment.

Longitudinal neuropsychological and neuroimaging investigations in neurodegenerative disorders as MND are challenging. However, as we discussed in the chapters of this thesis, not only identifying, but also monitoring over time the early brain alterations and cognitive difficulties in MND is fundamental due to their great clinical relevance in terms of prognosis. Future studies are needed to verify if the information of cognitive and MRI alterations may be able to identify individual ALS patients at onset who are more likely to present an extra-motor progression during the disease course.

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