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"Enhancing Respiratory Assessment and Monitoring to Extend Survival in Amyotrophic Lateral Sclerosis."

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Abstract

Respiratory failure is the first cause of death in amyotrophic lateral sclerosis (ALS) and currently ventilatory support is the only available treatment for it. Respiratory monitoring is essential to start non-invasive ventilation (NIV) timely, enhancing quality of life and extending survival in ALS. Different pulmonary function tests (PFTs) are available for respiratory function monitoring, each one with specific strengths and limits. However, there is no consensus about the correct type of monitoring, so as the cut-off values that should be used to indicate NIV prescription.

Based on this, we aimed at evaluating the sensitivity of different widely used PFTs, such as forced vital capacity (FVC), slow vital capacity (SVC), sniff nasal inspiratory pressure (SNIP) and peal cough flow (PCF), in monitoring respiratory function. For this aim we performed a longitudinal observational multi-centre study that assessed patterns of decline of different PFTs during disease course, based on various clinical and demographic characteristics, with specific focus on sex and type of onset. We demonstrated how pattern of decline is different between sexes and types of onset, with PCF and SNIP having a faster decline in females with bulbar onset, thus being particularly indicated for respiratory monitoring in this category of patients.

We then explored the role of other less used tools in ALS, such as maximal voluntary ventilation (MVV) and serum chloride levels, as potential markers of respiratory failure and survival in ALS. For this aim, we studied the correlations of MVV and serum chloride levels with other respiratory measures and clinical variables in a large retrospective cohort of ALS patients included in the PARALS registry. We showed how both MVV and serum chloride levels at diagnosis can detect early respiratory impairment in ALS, being good predictors of survival and time to NIV initiation. Particularly, a reduction of MVV score at diagnosis could be an earlier marker of respiratory failure in ALS in comparison with FVC, being associated with a reduced endurance and increased fatigability.

Additionally, since ALS is a clinical heterogeneous disease, based on both respiratory and clinical features, we aimed at identifying prognostic clusters of patients with different survivals and benefits from NIV. By performing a consensus cluster analysis, we identified five distinct clusters of patients, that could be useful in both clinical practice to stratify risk guiding decision making processes, and in clinical trials to identify prognostic categories of patients with different expected benefits from treatment.

Finally, in the last part of our project we studied the prevalence of ventilatory support usage in the last years, including both NIV and tracheostomy, in a large retrospective study including ALS patients from PARALS registry, diagnosed from 2008 to 2015. We confirmed a positive trend in ventilatory support usage, in line with current guidelines indications. We also

identified the clinical and demographic determinants influencing NIV prescription and survival after NIV initiation, helping clinicians in the decision of ventilatory support prescription, based on the expected benefit from it.

Summary

Declaration of original authorship

I Maria Claudia Torrieri confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

The work I am presenting in my PhD thesis is a project that took shape thanks to an extensive net of collaborations that led to a complex and fascinating work. I collaborated with the clinical research group 'CRESLA' at University of Torino, for data collection, study concept and design, and statistical analyses of all research presented in the study, with particular help from Prof. Andrea Calvo, Prof. Adriano Chiò and Dr. Umberto Manera. I also collaborated with REVEALS Group (including other than CRESLA at University of Torino, five European ALS centers (Beaumont, Hospital, Dublin, Ireland; King's College Hospital, London, UK; University Medical Center, Utrecht, The Netherlands; Sheffield Teaching Hospitals, Sheffield and University Hospitals Leuven, Belgium) in collecting data and performing clinical follow-up visits in ALS patients for REVEALS study.

Part of the data presented and discussed in this PhD thesis has already been published in peer-reviewed journals. Below I report the references for the already published articles:

1) for REVEALS study results: Rooney J, Murray D, Meldrum D, Al-Chalabi A, Bunte T, Chiwera T, Choudhury M, Chio A, Fenton L, Fortune J, Maidment L, Manera U, McDermott CJ, Meyjes M, Tattersall R, Torrieri MC, Van Damme P, Vanderlinden E, Wood C, van den Berg LH, Hardiman O. REVEALS-a longitudinal cohort study of multifaceted respiratory assessment in ALS. Amyotroph Lateral Scler Frontotemporal Degener. 2024 Nov;25(7-8):661-671. doi: 10.1080/21678421.2024.2359556.

2) for MVV results: Manera U, Torrieri MC, Moglia C, Canosa A, Vasta R, Palumbo F, Matteoni E, Cabras S, Grassano M, Bombaci A, Mattei A, Bellocchia M, Tabbia G, Ribolla F, Chiò A, Calvo A. Calculated Maximal Volume Ventilation (cMVV) as a Marker of Early Respiratory Failure in Amyotrophic Lateral Sclerosis (ALS). Brain Sci. 2024 Feb 3;14(2):157. doi: 10.3390/brainsci14020157

3) for serum chloride levels results: Manera U, Grassano M, Matteoni E, Bombaci A, Vasta R, Palumbo F, Torrieri MC, Cugnasco P, Moglia C, Canosa A, Chiò A, Calvo A. Serum chloride as a respiratory failure marker in amyotrophic lateral sclerosis. Front Aging Neurosci. 2023 May 24;15:1188827. doi: 10.3389/fnagi.2023.1188827.

4) for ventilation support data prevalence: Chiò A, Moglia C, Canosa A, Manera U, Vasta R, Grassano M, Palumbo F, Torrieri MC, Solero L, Mattei A, Ribolla F, Launaro N, De Marchi F, Mazzini L, Mora G, Calvo A. Respiratory support in a population-based ALS cohort: demographic,

timing and survival determinants. J Neurol Neurosurg Psychiatry. 2022 Jul 11;93(9):1024–6. doi: 10.1136/jnnp-2021-327968.

All the data not mentioned in this declaration were obtained by my personal work.

1. Introduction

1.1 Epidemiology, clinical phenotypes and etiology of Amyotrophic Lateral Sclerosis (ALS)

ALS, the most common form of motor neuron disease (MND), is a fatal neurodegenerative disease characterized by the premature death of both upper and lower motor neurons (respectively UMNs and LMNs), leading to general skeletal muscles weakness. Death is usually due to respiratory failure secondary to respiratory muscles function impairment and occurs typically within 3-5 years from onset [Brown RH, 2017].

ALS is a rare disease, showing a mean incidence of 2.8/100,000 and a mean prevalence of 5.4/100,000 in Europe [Chiò A, 2013]. Men are slightly more frequently affected than women, with a male-to-female incidence rate ratio of 1.4 [Logroscino G, 2010]. The mean age at disease onset is around 60 years [Chiò A, 2013].

ALS diagnosis is based on both clinical and instrumental evaluation, according to the revised El Escorial criteria [Brooks BR, 2000]. Clinically ALS may present with three distinct types of onset. Spinal onset is the most frequent one, affecting about 65-70% of cases, and being characterized by an initial muscular weakness in either upper or lower limbs [Chiò A, 2011]. Bulbar onset, characterized by weakness in the bulbar district, causing dysphagia, dysarthria, or tongue wasting, appears in about 30-35% of cases [Chiò A, 2011]. In contrast with spinal onset, bulbar onset is more frequent in females than males, has a higher correlation with cognitive/behavioral impairment and shows a worse prognosis (median survival 2.0 years for bulbar onset vs 2.6 years for spinal onset) [Chiò A, 2011]. Finally, respiratory onset affects usually less than 1% of ALS patients and is associated with a primary impairment of respiratory function and poor prognosis (median survival 1.4 years), [Chiò A, 2011]. However, regardless of clinical presentation, patients develop progressive muscle weakness and wasting, that spread with disease course at different progression rates, leading to a gradual impairment of respiratory muscles, and thus to death.

ALS is a clinically complex and heterogeneous disease, with various degrees of involvement of UMNs and LMNs, different body regions affected at onset, different degrees of involvement of other systems, especially cognition and behavior, and different progression rates among clinical phenotypes [Ravits JM, 2009]. Clinical heterogeneity of ALS also reflects the complex genetic architecture of the disease. Indeed, in ALS known and unknown susceptibility genes interact with environmental factors to modulate disease risk [Al-Chalabi A, 2013]. About 10% of ALS is classified as familial (fALS), whereas the remaining 90% of cases are considered sporadic (sALS) [Rowland LP, 2001]. Genetic mutations may now explain 70-80% of fALS [Ghasemi M, 2018]. However, in 3-16% of cases of sALS, a monogenic etiology can be recognized [Perrone B, 2020], so genetic

testing for the major genes associated with the disease (C9orf72, SOD1, TARDBP and FUS) is recommended in all ALS patients, regardless of familial history [Roggenbuck J, 2023].

The search for disease-modifying therapies has long been hampered by a poor understanding of disease mechanisms, partly due to the complex clinical and genetic heterogeneity of the disease. Only two drugs, riluzole [Bensimon G, 1994; Bellingham MC, 2011] and edaravone [Abe K, 2014; Writing Group on Behalf of Edaravone, 2017; Yoshino H, 2006], have been approved for many years by the US FDA for ALS treatment with limited benefits on survival, both targeting non-specific factors, such as excitotoxicity or oxidative stress. Recent advances in our understanding of ALS pathophysiology, including the discovery of many of its genetic underpinnings, have enabled the development of increasingly genetic targeted therapies, with today's clinical trials holding growing promise for efficacious treatments.

1.2 Monitoring disease progression in ALS

Despite the huge efforts and progresses made to find targeted therapies for ALS, the way to discover efficient treatments for all the clinical, genetic, and pathological shades of MND is still long and currently monitoring patients regularly to promptly identify signs of disease progression or bulbar/respiratory impairment remains a mainstay in the treatment of ALS. Indeed, when dysphagia occurs along the disease, it is of utmost importance to indicate food consistency changes initially, and later, the recurse to gastrostomy for nutrition and hydration. Similarly, when respiratory failure occurs, ventilatory support, consisting of either non-invasive ventilation (NIV) or invasive mechanical ventilation (IMV), i.e. tracheostomy, remains the only available treatment to increase survival in ALS patients. Consequently, it is clear how a multidisciplinary approach, involving many different specialists, such as neurologists, pulmonologists, dietitians, is fundamental for a ASL care to reduce hospital admissions, extend survival and improve quality of life [EFNS Task Force on Diagnosis and Management of Amyotrophic Lateral Sclerosis, 2012].

During neurological follow-up visits, that are usually set every 2-3 months, clinical evaluation and administration of the revised ALS Functional Rating Scale (ALSFRS-r) scale are recommended to monitor disease progression [EFNS Task Force on Diagnosis and Management of Amyotrophic Lateral Sclerosis, 2012]. The ALSFRS-r is a validated rating scale used to monitor the progression of disability in patients with ALS and is constituted of 12 items, scoring from 4 (normal) to 0 (complete dependance), assessing bulbar, upper and lower limbs, and respiratory functions [Cederbaum JM, 1999]. The maximum possible score is 48, representing no functional impairment, while a score of 0 indicating complete loss of function [Cederbaum JM, 1999]. The ALSFRS-r shows strong internal consistency and validity and has demonstrated a good inter-rater and intra-rater reliability [Cedarbaum JM, 1999; Kimura F, 2006; Rooney J, 2017]. Anyway, the sum of items to a

total score shows weak sensitivity for disease progression monitoring. Additionally, ALSFRS-r score does not change linearly over time, reaching a ceiling effect in the more advanced stages of disease, and is complicated by heterogeneity [Mandrioli J, 2015].

Another approach to monitor disease progression in ALS is resorting to a staging system. Among staging systems, the most widely studied are the Milano-Torino (MiToS) functional staging and the King's clinical staging systems [Chiò A, 2015; Roche JC, 2012]. The MiToS system identifies six stages, from 0 (normal function) to 5 (death), based on functional ability as assessed by the ALSFRS-r scale [Chiò A, 2015]. On the other side, the King's system is not based on the ALSFRS-r scale but can be easily estimated from it with high concordance. It uses five stages, from 1 (symptom onset) to 5 (death), being based on disease burden as measured by clinical involvement of one or more regions, and significant feeding or respiratory failure [Roche JC, 2012]. While King's staging system can well differentiate early to mid-disease, the MiToS staging better classifies late disease stages, being two complementary staging systems [Fang T, 2017].

The use of validated scales and staging systems, even with the described limits, remains an important instrument to quickly frame disease disability in ALS patients and to monitor disease progression in clinical practice and during clinical trials.

1.3 Pathophysiology of respiratory failure in ALS

1.3.1 Sleep-disordered breathing (SDB)

Progressive degeneration of motor neurons results in diaphragmatic and accessory respiratory muscles impairment, leading to the gradual appearance of signs and symptoms of respiratory failure. Sleep-disordered breathing (SDB) is one of the earliest signs of respiratory impairment, preceding day-time respiratory symptoms in most cases, and is secondary to both respiratory muscles weakness and loss of central ventilatory drive [Quaranta VN, 2017].

Nocturnal hypoventilation (NH) is the most common form of SDB in ALS, affecting about 70% of patients. NH is defined by the presence of increased carbon dioxide levels ($pCO2 > 45$ mmHg) and decreased oxygen levels in the blood during sleep, especially during the REM phase, when respiratory muscle tone physiologically decreases [Quaranta VN, 2017].

The second most common form of SDB in ALS is obstructive sleep apnea (OSA), being characterized by repetitive episodes of complete or partial upper airway obstruction (apneas and hypopneas) in the presence of respiratory effort, associated with blood desaturation [Lo Coco D, 2011]. Prevalence rate of OSA in ALS patients vary greatly among studies, with a median value of 45% [Boentert M, 2018]. Interestingly, severe bulbar impairment has been associated with a reduced risk of OSA, probably

due to tongue atrophy that leads to an increased upper airway patency [Boentert M, 2019]. Moreover, OSA seems to be more common in the early stage of the disease than during the later phases, probably due to the increasing inspiratory muscle weakness, that reduces the inspiratory pressure above the closing pressure of the upper airway [Prell T, 2016].

Both NH and OSA are associated with significant reduction in pulmonary function and reduced survival in ALS [Prell T, 2016]. However, early detection of SDB may be challenging, since its symptoms can be absent at all or may be subtle and insidious, being related mostly to morning headache, daytime fatigue and cognitive difficulties. Commonly, only when day-time respiratory symptoms appear, patients undergo sleep studies or arterial blood gas analysis (ABG), leading to an underestimated or delayed diagnosis of SDB.

SDB may be diagnosed using different instrumental tests. Nocturnal pulse oximetry is a widely used, low-cost, simple, and non-invasive tool for screening SDB in ALS. This method assesses the oxygen levels during sleep, and it is a valid instrument for both SDB diagnosis and monitoring after NIV initiation. However, nocturnal oximetry does not directly assess pCO2 levels, and this affects its sensitivity in detecting NH, where oxygen saturation may appear normal even in presence of hypercapnia [Boentert M, 2019].

NH can also be defined by peak transcutaneous CO2 (PtcCO2) levels, that allows a non-invasive measurement of the pCO2 in the blood by using skin-surface sensors for CO2. Usually, PtCO2 >50 mmHg or a PtcCO2 increase of >10 mmHg from the awake baseline value is suggestive of NH [Engel M, 2021]. However, PtcCO2 readings can be influenced by technical factors, such as skin temperature and perfusion, and calibration of sensors.

Capnography is another simple, non-invasive and reliable tool for SDB screening, allowing an indirect monitoring of ventilatory status by measuring the end-tidal carbon dioxide (ETCO2) levels in the expired air, that highly correlate with pCO2 [Magnan A, 1993]. ETCO2 levels greater than 50 mmHg are usually considered suggestive of NH. On the other side, its accuracy can be affected by technical issues, such as equipment calibration, sensor placement, and leaks in the ventilation circuit [Magnan A, 1993].

In all cases nocturnal oximetry, PtcCO2 and capnography do not provide comprehensive data on other important aspects of sleep architecture and respiratory function, so they are usually used as a screening method for selecting patients for more accurate tests, such as polysomnography (PSG). PSG is a multi-parameter test, that provides comprehensive data about sleep quality and respiratory function during sleep. Particularly, it measures brain activity via electroencephalogram, eye movements via electro-oculogram, muscle activity via electromyogram, heart rate and rhythm via electrocardiogram, breathing patterns and blood oxygen levels via pulse oximetry [Rundo JV, 2019]. PSG remains the gold standard in diagnosing SDB, also giving information about the specific type

of apneas (obstructive vs. central), but it is considered a second-level approach, mostly due to its higher cost and to the fact that it is often conducted in specialized sleep centers rather than at patients' home [Natsky AN, 2021]. Moreover, the presence of sensors and monitoring equipment may affect patient comfort and sleep quality, potentially impacting the quality of data. Finally, technical issues related to equipment malfunction can reduce PSG accuracy [Natsky AN, 2021].

Being SDB associated with early respiratory impairment and reduced survival in ALS, it is of utmost importance to detect it early in the course of the disease to start NIV timely. Indeed, NIV has been demonstrated to correct SDB, enhancing quality of life and increasing survival in ALS [Lyall RA, 2001; Engel M, 2021]. Specifically, NH patients may be started with nocturnal bilevel positive airway pressure ventilation, while for OSA continuous positive airway pressure is the preferred approach for ventilation. However, despite the evidence of a significant benefit of NIV usage in ALS patients with SDB, there is no international consensus about the correct management of SDB in ALS. Indeed, only European and American guidelines indicate the presence of significant SDB as a criterion for NIV initiation [EFNS Task Force on Diagnosis and Management of Amyotrophic Lateral Sclerosis, 2012; Miller RG, 2009], while NICE guidelines do not consider it for NIV initiation [NICE, 2010].

1.3.2 Daytime respiratory symptoms

Respiratory failure in most cases develops slowly, leading to hypercapnia that appears first at night, being associated with SDB, and later, during the day as well. As described, NH usually does not cause any daytime symptoms, or sometimes may be related to mild daytime fatigue, cognitive impairment, and depression. In later stages, when respiratory muscles weakness worsens, other more recognizable symptoms may appear, such as orthopnea and dyspnea, initially for moderate and later for mild efforts or even at rest.

However, respiratory symptoms, as measured by the ASLFRS-r respiratory sub-items, can vary significantly among ALS patients, showing only a mild correlation with real pulmonary function [Manera U and Torrieri MC, 2020; Cederbaum JM, 1999]. Indeed, some patients may suffer severe respiratory failure without noticeable symptoms. The subjective nature of respiratory symptoms in combination with the evidence of their delayed onset during disease course makes it difficult to assess respiratory function based only on subjective reports. Moreover, respiratory symptoms may be the consequence of other medical conditions, potentially leading to misinterpretation of their significance in relation to respiratory failure. Finally, respiratory symptoms, such as orthopnea and dyspnea, may be dependent on position or activity, so patients might not report them unless they are in specific situations.

The ability of the ALSFRS-r respiratory domain to predict residual respiratory function is still a matter of discussion. The ALSFRS-r respiratory sub-items, either considered individually or globally, show only a weak-moderate association with FVC [Manera U and Torrieri MC, 2020; Pinto S, 2017] and their change during disease course are poorly correlated with SVC change [Pinto S, 2017]. The lack of a strong association has been interpreted as the result of deconditioning or increased effort and fatigability during activity in ALS patients, leading to sensations of breathlessness, even with normal FVC [Cederbaum JM, 1999] or, on the contrary, as the consequence of reduced mobility due to muscle weakness, that could decrease the symptoms of dyspnea in individuals with severe FVC reduction [Pinto S, 2015].

1.4 Management of respiratory failure in ALS: NIV and IMV

Respiratory failure, secondary to diaphragmatic weakness, so as pulmonary complications (i.e. pneumonia), due to aspiration and reduced bronchial secretions clearance capacity, are the main cause of death in ALS.

To reduce incidence of pulmonary infections in patients having difficulties in effectively clearing bronchial secretions, it is possible to resort to mechanical cough-assisting devices (i.e. in–exsufflator). These devices are able to simulate a natural cough via a facemask by delivering positive pressure to inflate the lungs (insufflation), followed by a rapid shift to negative pressure (exsufflation) [Nicolini A, 2022].

NIV and, less frequently IMV, are the only available treatments to alleviate respiratory symptoms, to slow respiratory deterioration and extend survival [Kleopa KA, 1999; Miller RG, 2009]. NIV consists of a ventilatory support through the upper airways without using invasive artificial airways like endotracheal tubes or tracheostomy [Dorst J, 2019]. It is performed using a breathing device with a ventilation mask, that can be used at home, being well accepted by patients and carers in most cases [Dorst J, 2019]. ALS patients usually require NIV at night initially and with disease progression its usage may be extended during daytime. The aim of NIV is to alleviate hypercapnic symptoms, improve quality of life and increase survival. NIV optimization, including both quality and adherence to ventilation, is fundamental to extend survival in ALS. Indeed, the survival rate at 1-year reduces from 75% to 43% if there is no evidence of good correction of hypoxia on NIV [Gonzalez-Bermejo J, 2013]. Moreover, patients tolerating NIV for at least 4 hours a day show a slower decline of respiratory function and increased survival post-NIV initiation in comparison with patients tolerating it for less than 4 hours a day [Kleopa KA, 1999].

Despite the important therapeutic role of NIV in ALS, currently there is not an international consensus about the correct timing for NIV prescription. This refers particularly to the cut-off value of pulmonary tests results used for NIV indication.

European guidelines indicate that NIV should be initiated in ALS patients with respiratory symptoms/signs (at least one among dyspnea, tachypnea, orthopnea, disturbed sleep due to nocturnal desaturation/arousals, morning headache, use of auxiliary respiratory muscles at rest, paradoxical respiration, daytime fatigue, excessive daytime sleepiness) and in presence of abnormal pulmonary function tests (at least one among: forced vital capacity, FVC, <80% of predicted value; sniff nasal pressure, SNIP, <40 cmH2O; maximal inspiratory pressure, MIP <60 cmH2O; significant nocturnal desaturation on overnight oximetry; morning blood gas pCO2 >45 mmHg) [EFNS Task Force on Diagnosis and Management of Amyotrophic Lateral Sclerosis, 2012].

On the other side, NICE guidelines recommend NIV adaptation if: FVC or VC <50% of predicted value; SNIP or MIP <40 cmH2O; FVC or VC <80% of predicted value plus any symptoms or signs of respiratory impairment, particularly orthopnea; SNIP or MIP <65 cmH2O for men or 55 cmH2O for women plus any symptoms or signs of respiratory impairment, particularly orthopnea; repeated regular tests show a rate of decrease of sniff nasale inspiratory pressure (SNIP) or MIP of more than 10 cmH2O per 3 months [NICE, 2019].

Finally, American guidelines suggest NIV initiation when one among the following symptoms/signs are present: orthopnea or SNIP <40cmH2O or MIP <60cmH2O or abnormal nocturnal oximetry or FVC < 50% [Miller RG, 2009].

However, recent evidence show that earlier NIV initiation (FVC > 80%) is associated with a slower decline of pulmonary function, leading to a longer free-tracheostomy survival [Jacobs TL, 2016; Vitacca M, 2018].

Another aspect that is not fully clarified for NIV is the correct choice of ventilation masks for ALS patients. Ventilation masks include nasal, full-face and total face masks. Nasal masks, covering only the nose, even if generally better tolerated, can be problematic for bulbar patients, since air escapes due to incomplete closure of the mouth, especially at night during sleep. Full-face masks cover both nose and mouth, overcoming this limit, but are not ideal for patients' communication. Moreover, both nasal and full-face masks can lead to facial pressure ulcers, which may limit their application, and necessitate the use of total face masks. Total face masks encompass the whole face, being more effective in preventing leaks, but may be uncomfortable and restrict vision.

NIV usage may be limited and problematic in some categories of patients, especially patients with cognitive/behavioral and/or bulbar impairment. Patients with cognitive/behavioral dysfunction have reduced compliance to NIV with adaptation being often time consuming and in more severe cases not possible at all. On the other side, bulbar impairment is often associated with hypersalivation that

hinders mask ventilation, reducing compliance to NIV [Peysson S, 2008]. Moreover, contrasting evidence about the benefits of NIV usage in patients with severe bulbar impairment limit its indication in these cases [Bourke SC, 2006; Vrijsen B, 2015]. NIV usage has progressively increased from 16-20% of ALS patients with respiratory impairment in 2004 to 51% in 2008, but this percentage is still not enough, and NIV is still underutilized in ALS patients [Chiò A, 2012; Gordon PH, 2012]. However, data regarding NIV usage prevalence in the last 15 years are missing and knowing if the positive trend recorded during the first decade of the new Millenium is confirmed is important to understand if ALS patients' treatment remains aligned with guidelines recommendations.

Even IMV is able to significantly prolong survival in ALS, but there is no evidence of improvement in quality of life. Indeed, IMV allows patients to reach advanced stages of disease up to locked-in syndrome, so most patients reject it considering that condition unacceptable. Moreover, the availability and cultural acceptability of IMV varies greatly between different cultures, having emotional and social impacts on patients and caregivers. Additionally, it is costly and associated with a higher risk of infections [EFNS Task Force on Diagnosis and Management of Amyotrophic Lateral Sclerosis, 2012]. Prevalence data about IMV usage are scarce, however about 30% of ALS patients underwent tracheostomy from 2001 and 2010 in a single ALS center in Italy [Spataro R, 2012]. In 46% of cases, procedures are performed unfortunately as emergency interventions due to acute respiratory failure [Heritier Barras AC, 2013]. The possibility of recurring to IMV in case of severe respiratory failure should be discussed on time with ALS patients to decide advance directives to avoid emergency IMV [EFNS Task Force on Diagnosis and Management of Amyotrophic Lateral Sclerosis, 2012]. It is fundamental that patients are informed in detail about the benefits and the consequences of IMV, particularly underlying that IMV can treat respiratory failure, but disease will proceed in other districts. This information should be given when patients are conscious, and the respiratory situation is still stable for having time to take a thoughtful decision about their end-life directives. Currently, there is no consensus about the exact timing when IMV should be considered, and it is usually regarded as a possibility when NIV is not sufficient anymore for ventilatory function or is not tolerated [Dorst J, 2019]. IMV could be taken into consideration earlier in case of severe bulbar impairment with consequent difficulty in secretions management [Dorst J, 2019].

Despite the importance of respiratory support in ALS, studies systematically assessing the factors influencing ventilatory support prescription and the determinants of survival after NIV/IMV are still missing. This information could be useful for clinicians to better guide choices in respiratory management of ALS patients and to predict the expected benefit from ventilatory support.

1.5 Respiratory monitoring in ALS

It is fundamental to monitor pulmonary function to detect early respiratory muscles weakness for starting NIV timely. The clinical presentation of early respiratory impairment in ALS can vary widely among patients, therefore being difficult to recognize. Clinical practice guidelines recommend routine monitoring of respiratory symptoms in ALS patients to promptly detect respiratory impairment, however a great part of ALS patients do not complain of respiratory symptoms, due to compensation processes made during chronic respiratory failure. In the last decades, different methodologies and approaches have been used to assess pulmonary function in ALS with the aim of increasing sensitivity in detecting early respiratory impairment and monitoring pulmonary function decline, but all of them show some limits. In this paragraph, we are going to review the available methods for monitoring respiratory function in ALS.

1) ALSFRS-r respiratory sub-score

Regular monitoring for the appearance of respiratory symptoms is recommended to detect changes in pulmonary function in ALS. This aspect is explored by the ALSFRS-r respiratory sub-score, that investigates for the presence of dyspnea and orthopnea. However, ALSFRS-r respiratory sub-score only weakly correlates with FVC and other respiratory tests [Manera U and Torrieri MC, 2020; Cederbaum JM, 1999], since about 30% of ALS patients do not report dyspnea/orthopnea despite having FVC <75% as predicted [Miller RG, 2009]. Additionally, ALSFRS-r respiratory sub-items slope does not seem to be prognostic in ALS [Rooney J, 2017].

The absence of respiratory symptoms also in patients showing moderate/severe respiratory impairment may depend on the fact that during chronic respiratory failure there is time for the organism to adapt to the new condition and to implement some compensatory mechanism, such as blood bicarbonate increase, to avoid respiratory acidosis. This could lead to a delayed onset of respiratory symptoms during the course of the disease. Moreover, respiratory symptoms are subjective and may also be caused by other medical conditions, not directly related to respiratory failure. The lack of a strong association between ALSFRS-r respiratory domain has also been interpreted as the result of deconditioning or increased effort and fatigability during activity in ALS patients, leading to sensations of breathlessness, even with normal FVC [Cederbaum JM, 1999] or, on the contrary, as the consequence of reduced mobility due to muscle weakness, that could decrease the symptoms of dyspnea in individuals with severe FVC reduction [Pinto S, 2015].

Regardless of the subjectivity of respiratory symptoms and the weak association with real pulmonary function, it is recommended to monitor their appearance during disease course, to address ALS patients to more specific pulmonary instrumental tests. Moreover, current guidelines consider the presence of respiratory symptoms for NIV indication together with other PTFs scores [EFNS, 2012; NICE, 2019; Miller RG, 2009].

2) Forced vital capacity (FVC) and slow vital capacity (SVC)

FVC is the most commonly used respiratory measurement in ALS to assess pulmonary function at baseline, monitor disease progression and indicate ventilation [Czaplinski A, 2006]. FVC is the volume of air that can be exhaled forcefully after a maximal inhalation, and its value is commonly expressed as a percentage of the predicted value, based on patient's age and height (FVC%). It may be performed upright or supine. Even if in clinical practice upright FVC is more commonly used than supine FVC, since it is easier to perform, supine FVC seems to correlate more strongly with diaphragm weakness, being more sensitive than upright FVC for detecting respiratory muscle weakness. Consequently, American guidelines suggest performing supine FVC in addition to upright FVC [Miller RG, 2009].

FVC has a good predictive power of survival in ALS, indeed it has been observed that patients with FVC <75% at baseline show a median survival of 2.91 years, compared with 4.08 years for patients with higher values of FVC at baseline [Czaplinski A, 2006].

Due to its prognostic value, FVC assessment should be part of the initial assessment to diagnose ALS to establish the baseline respiratory function, together with blood oxygen saturation evaluation at rest [NICE, 2019]. FVC is also used to monitor disease progression and to indicate NIV. Particularly, European guidelines recommend NIV initiation with FVC values <80% in presence of respiratory symptoms/signs [EFNS Task Force on Diagnosis and Management of Amyotrophic Lateral Sclerosis, 2012]. In addition to it, American and NICE guidelines suggest NIV adaptation also in case of FVC <50% regardless of respiratory symptoms [Miller RG, 2009; NICE, 2019].

Despite the central role of FVC measurement for the assessment of pulmonary function in ALS, FVC does not show a good sensitivity in detecting NH, since in patients not reporting respiratory symptoms and having a FVC >75%, NH is observed in nearly half cases [Prell T, 2016]. So, it has been suggested to perform a screening for SDB also in patients with normal FVC values and not complaining of respiratory symptoms for an earlier detection of NH [Prell T, 2016].

SVC, even if less used than FVC for ALS pulmonary monitoring, is also a sensitive marker of respiratory function and is highly predictive of survival in ALS [Pinto S, 2019]. Unlike FVC, SVC assesses the maximum volume of air that can be exhaled slowly after a full inhalation. SVC and FVC strongly correlate with each other, and their decline rate is similar in ALS (i.e. 2%/month), also in patients with high grade of spasticity or with bulbar impairment [Pinto S, 2017]. Moreover, FVC and SVC strongly correlate with other respiratory tests, such as MIP and MEP, and moderately correlate with clinical scores, although these correlations are weaker in patients with bulbar impairment, that could have some difficulties in FVC/SVC performing due to facial weakness and poor coordination in the bulbar district [Pinto S., 2017]. However, studies have shown that SVC may provide a more accurate estimate of respiratory function in ALS patients with bulbar onset, being SVC slightly easier to perform in comparison with FVC in patients with bulbar impairment [Huang X, 2022]. Another population for which FVC and SVC assessment may be problematic corresponds to patients with

cognitive impairment, who are not able to properly collaborate, leading to inaccurate measures [Kim SM, 2007]. Finally, both FVC and SVC seem to be less useful in the advanced stages of disease, particularly after NIV initiation, probably due to the floor effect existing for both SVC and FVC [Pinto S, 2019].

3) MIP

MIP allows an accurate evaluation of inspiratory muscles strength, corresponding to the maximum pressure generated during a maximal inspiratory effort against a closed system, typically measured at residual volume. For its assessment the patient is asked to inhale maximally through a mouthpiece with their nose occluded. It is a low-cost, easy and rapid to perform test with well-established reference values (normal value >60 cmH_2O for females and >80 cmH_2O for males) [Caruso P, 2015].

It has been shown that MIP could be a more sensitive marker of early respiratory impairment than FVC in ALS, since patients with MIP values <60 may show normal FVC values [Jackson CE, 2001]. This could be explained by the fact that for FVC assessment maximal respiratory strength is not required to fully inflate and deflate, so MIP could be an earlier marker of respiratory impairment. Moreover, MIP is a sensitive prognostic marker in ALS, correlating with survival and a patient with normal MIP has a chance higher than 90% of tracheostomy-free survival at one year [Schimdt EP, 2006].

As for FVC, MIP sensitivity could be lower in patients with advanced bulbar disease, since facial weakness is associated with leaks around the mouthpiece [Schimdt EP, 2006]. Moreover, MIP assessment could be troublesome in patients with cognitive impairment, for whom, performing the test from residual volume can increase the complexity of the task [Mendoza M, 2007].

MIP is also used for NIV indication. As for other respiratory measures, different guidelines use distinct cut-off values for MIP to indicate NIV. Particularly, European guidelines recommend NIV initiation in ALS patients with respiratory symptoms/signs in presence of a MIP value <60 cmH2O [EFNS Task Force on Diagnosis and Management of Amyotrophic Lateral Sclerosis, 2012]. NICE guidelines recommend NIV adaptation if MIP values is <40 cmH2O, or <65 cmH20 for men and 55 cmH20 for women in presence of any symptom or sign of respiratory impairment, or if repeated regular tests show a rate of decrease of MIP of more than 10 cmH2O per 3 months [NICE, 2019]. Finally, American guidelines suggest NIV initiation when MIP is <60cmH2O regardless respiratory symptoms [Miller RG, 2009].

4) SNIP

SNIP is an easy test for assessing inspiratory muscular strength in ALS, corresponding to the pressure generated at the contralateral nostril during maximal inspiration from functional residual capacity, measured by a cannula connected to a plug occluding one nostril [Pinto S, 2018]. Normal values are

considered >60 cmH20 for females and >70 cmH20 for males [Caruso P, 2015]. It is more reliable than MIP in patients with facial weakness, due to the absence of leaks around the mouthpiece in bulbar patients that invalidate the scores for MIP [Pinto S, 2018]. SNIP shows similar reproducibility to MIP [Maillard JO, 1998] and it measures inspiratory muscle fatigue probably with higher sensitivity than MIP in ALS [Lyall RA, 2001]. However, SNIP and MIP should be considered complementary measures, since they reflect distinct ventilatory mechanics: for SNIP the generated effort is rapid and explosive, while for MIP it is more prolonged.

SNIP can detect NH, especially in spinal patients [Crescimanno G, 2021] and can predict survival in ALS [Morgan RK, 2005], but its decline rate is lower than FVC [Pinto S, 2009]. SNIP may be used for monitoring ALS pulmonary function, being particularly indicated in bulbar patients with weak lips, even if in cases of severe bulbar impairment, sensitivity of SNIP may be low, as for FVC [EFNS, 2012]. It has been shown that SNIP shows a curvilinear correlation with FVC, probably being more sensitive in the early phases of the disease [Barnes N, 2014].

Based on NICE guidelines, the initial assessment of MND patients useful to establish the person's baseline respiratory function could include SNIP as a valid alternative to FVC [NICE, 2019].

SNIP is also recommended by different guidelines for NIV indication: particularly, European guidelines indicate that NIV should be initiated in ALS patients with SNIP <40 cmH2O in presence of respiratory symptoms/signs [EFNS Task Force on Diagnosis and Management of Amyotrophic Lateral Sclerosis, 2012]. On the other side, NICE guidelines recommend NIV adaptation if SNIP is <40 cmH2O even without respiratory symptoms or if SNIP is <65 cmH2O for men or 55 cmH2O for women plus any symptoms or signs of respiratory impairment, particularly orthopnea [NICE, 2010]. Moreover, NICE guidelines also take into consideration for NIV indication a rate of decrease of SNIP of more than 10 cmH2O per 3 months at repeated regular tests [NICE, 2019]. Finally, American guidelines suggest NIV initiation when SNIP is <40cmH2O regardless of respiratory symptoms [Miller RG, 2009].

However, the high variability of techniques used for SNIP assessment is a major issue and evidence still lacks about the sensitivity of different technical approaches for respiratory function evaluation in ALS, with poor agreement especially about the number of trials that should be performed. This issue should be addressed, particularly for standardization in clinical trials.

5) Peak cough expiratory flow (PCF)

PCF is the most widely used measure of cough effectiveness. It is assessed by performing a maximal inspiration, followed by a cough as forcefully as possible, with the lips sealed around the tube [Tilanus TBM, 2017]. In healthy subjects, PCF is >500 L/min for all ages. In ALS PCF values correlate with survival, and patients with a mean PCF >337 L/min have a greater chance of survival

at 18 months in comparison with those with lower PCF values [Heffernan C, 2006]. Moreover, it has been shown that PCF has a great predictive value for NIV indication [Tilanus TBM, 2017].

PCF assessment may help decision about cough assistance in ALS patients. In this context, guidelines do not indicate a cut-off value for cough augmentation indication. Particularly, they generically suggest offering as the first-line treatment cough augmentation techniques, such as manual assisted cough, to ALS patients with ineffective cough, and to consider a mechanical cough assist device if assisted breath stacking is not effective, or during a respiratory tract infection [NICE, 2019]. However, in clinical practice usually a cut-off value of 270 L/min is considered for cough augmentation [Sancho J, 2017].

For bulbar patients who may not generate effective PCF levels due to collapse of the upper airways during exsufflation [Sancho J, 2004], or for patients with ineffective cough despite unassisted breath stacking, guidelines suggest considering assisted breath stacking (i.e., using a lung volume recruitment bag) [NICE, 2019].

6) **Maximal expiratory pressure (MEP)**

MEP is a pulmonary test for assessing expiratory function, allowing to evaluate patient's ability to cough and clear airway secretions. MEP corresponds to a maximal expiration through the mouth after a full inspiration using a mouthpiece. In healthy subjects MEP normal values are considered >120 cmH2O for females and >150 cmH2O for males [Caruso P, 2015], while for ALS the cut-off value commonly used for normality corresponds to 70 cmH2O. Its values moderately correlate with tests for inspiratory function evaluation, such as MIP and SNIP [Park KH, 2016]. Evidence shows that MEP highly correlates with survival in ALS with patients showing MEP values >70 cmH20 having a chance higher than 90% of tracheostomy-free survival at 1 year [Schimdt EP, 2006]. However, current guidelines for ALS respiratory management do not mention its use in clinical practice. As for other pulmonary function tests, MEP shows a reduced specificity for respiratory function assessment in patients with bulbar impairment, since facial weakness does not allow a proper adherence to the mouthpiece [Baumann F, 2010].

7) Arterial blood gas analysis (ABG)

Very few studies investigated the role of ABG in ALS to detect early respiratory failure and monitoring pulmonary function in ALS. Consequently, in our retrospective study [Manera U and Torrieri MC, 2020] we examined the usefulness of ABG and FVC in a large cohort of ALS patients, followed in the Turin ALS Center, diagnosed between 2000 and 2015, to identify the best cut-off values for FVC and ABG parameters (carbon dioxide, pCO2; carbonate, HCO3-; standard base excess, SBE) to predict NIV initiation and survival. We found out that a significant reduction in FVC was recorded when pCO2 exceeded 42 mmHg, that is a value usually considered as within normal

range. So, a significant diaphragm weakness is already present when ABG values are considered as normal. Moreover, we found out that about 25% of patients showed an isolated increase of HCO3- at ABG (>26 mmol/L), a well-known marker of NH, being associated with significant FVC decline and reduced survival. In our study we confirmed that NH is often asymptomatic and respiratory symptoms become more frequent only when both HCO3- and pCO2 are increased at ABG. The importance of ABG in respiratory monitoring in ALS has been confirmed by Alarcan H et al., that found out a significant correlation between HCO_3^-, pO_2, pCO_2 and SBE with FVC both in patients with spinal and bulbar onset [Alarcan H, 2023]. However, they describe HCO_3^- and SBE being associated with survival only in spinal forms of ALS [Alarcan H, 2023].

Only European guidelines mention the use of ABG for NIV indication, in particular only morning pCO2 >45 mmHg is considered for NIV adaptation [EFNS, 2012]. However, our study revealed that the most sensitive and specific cut-off values for starting NIV within 3 months were 42 mmHg for pCO2, 26 mmol/L for HCO3- and 2 mmol/L for SBE. So, taken together, our data suggest that pCO2 cut-off value currently used by guidelines for NIV indication could lead to a delayed ventilation support in ALS, and that other ABG parameters, such as SBE and HCO3-, could be used for this purpose, due to their high sensitivity in detecting NH and thus early respiratory impairment.

ABG, despite being a minimally invasive test, is a rapid, easy to perform and non-volitional test that shows high sensitivity for NH and early respiratory impairment and has a good predictive power for NIV initiation and survival. Moreover, since it does not require patients' collaboration, it can be an objective measure of respiratory function in patients with bulbar or cognitive impairment. However, further studies in independent cohorts, especially with prospective design, are needed to confirm and validate our results.

8) **SDB monitoring (PtcCO2, nocturnal oximetry, capnography, PSG)**

As previously described, SDB, including NH and OSA, is associated with early respiratory impairment and reduced survival in ALS. Different techniques are used to detect SDB.

Nocturnal pulse oximetry, based on assessing oxygen levels during sleep, is a widely used, low-cost and non-invasive tool for screening SDB in ALS. However, not directly assessing pCO2 levels, nocturnal oximetry has reduced sensitivity in detecting NH, since hypercapnia may be present at normal oxygen saturation levels [Boentert M, 2019].

PtcCO2 and capnography instead measure transcutaneous pCO2 levels or pCO2 levels in the exhaled air respectively [Engel M, 2021, Magnan A, 1993], highly correlating with real blood pCO2. However, both techniques, despite being simple and non-invasive tools, can be influenced by technical factors, such as skin temperature and perfusion for PtcCO2, leaks in the ventilation circuit for capnography, and calibration of sensors for both.

Usually nocturnal oximetry, PtcCO2 and capnography are considered as screening tools for SDB. To obtain comprehensive data on other important aspects of sleep architecture and respiratory function, such as airflow obstruction or apneic events, PSG is commonly used. PSG is the gold standard for SDB diagnosis and is a multi-parameter test measuring brain activity via electroencephalogram, eye movements via electro-oculogram, muscle activity via electromyogram, heart rate and rhythm via electrocardiogram, breathing patterns and blood oxygen levels, via pulse oximetry [Rundo JV, 2019]. Due to its higher cost and more complex organization, PSG is considered a second-level approach.

Detecting SDB is of utmost importance in ALS, since NIV has been demonstrated to correct SDB, enhancing quality of life and increasing survival [Lyall RA, 2001; Engel M, 2021]. However, currently there is no international consensus about the correct management of SDB in ALS. Indeed, only European and American guidelines indicate the presence of significant SDB as a criterion for NIV initiation [EFNS Task Force on Diagnosis and Management of Amyotrophic Lateral Sclerosis, 2012; Miller RG, 2009], while NICE guidelines do not consider SDB for NIV initiation [NICE, 2010]. It has been suggested that tests for SDB screening should be performed in all ALS patients not complaining of respiratory symptoms, with FVC values >50% and not able to perform spirometry (i.e. patients with bulbar/cognitive impairment). In these cases, the evidence of SDB should induce clinicians to initiate NIV to increase survival [Prell T, 2016].

9) Other less common respiratory measurements

Maximal voluntary ventilation (MVV) is a poorly studied measure in ALS, and it assesses respiratory muscle strength and endurance. For its evaluation the subject is required to breathe as rapidly and deeply as possible over 12 seconds, then the result is multiplied by 5 to estimate the value over 1 minute, and it is expressed in L/min [de Carvalho M, 2022]. MVV only moderately correlates with other respiratory tests, such as FVC, SVC, MIP and MEP, but strongly correlates with PEF and clinical parameters (ALSFRS-r score) [de Carvalho M, 2022], being an independent predictive factor for survival in ALS [Zhang QJ, 2022]. MVV seems to detect changes over a longer period in comparison with FVC, and its assessment is not influenced by the UMN vs LMN involvement [de Carvalho M, 2022]. However, as for other respiratory tests, it can be influenced by facial weakness in bulbar patients. MVV assesses endurance, corresponding to another aspect of respiratory function, that is not normally evaluated during routine pulmonary tests. Currently only a few clinical trials used MVV to assess pulmonary function in ALS [Berto MC, 2007; Sathyaprabha TN, 2010; Heiman-Patteson TD, 2021], and its role in diagnosing early respiratory impairment and monitoring pulmonary function has to be deepened.

Peak expiratory flow (PEF) measures the highest flow rate achieved during expiration, assessed using a handheld device namely peak flow meter. PEF has the advantage of being an easy-to-perform and cheap test and can be self-monitored at home. This could lead to a regular monitoring of PEF at home, facilitating timely interventions based on changes in respiratory status. PEF correlates with

FVC and disease severity. Some evidence shows that PEF is also a predictive factor for survival in ALS [Zhang QJ, 2022].

Phrenic nerve conduction study (PNCS) allows the measurement of the amplitude of the diaphragm motor evoked response by transcutaneous phrenic nerve stimulation, that is an important marker of hypoventilation [Pinto S, 2009b] and survival [Pinto S, 2012] in ALS, and whose rate of decline correlate with FVC and SNIP change over time. PNCS is usually a well-tolerated, easy to perform, reliable and non-volitional test, that could be particularly useful in patients with weak facial muscles or cognitive impairment, that are unable to cooperate with conventional respiratory tests [Torrieri MC, 2020].

1.6 Type and frequency of respiratory monitoring in ALS

Different approaches are recommended by different guidelines for the type and the frequency of respiratory monitoring in ALS.

The EFNS guidelines recommend using FVC regularly before and after NIV adaptation, except for bulbar patients for whom SNIP is highly suggested [EFNS, 2012]. The NICE guidelines recommend performing respiratory function tests (including oxygen saturation, FVC, SNIP/MIP) every 2–3 months and ABG analysis only in case of desaturation. On the other side American guidelines suggest monitoring respiratory symptoms and pulmonary function testing (VC, MIP, SNIP, or PCF), at least every three months [Miller RG, 2009].

After NIV initiation, the AAN guidelines mention the importance of ABG monitoring, but only pCO2 values are considered for assessing NIV adaptation, compliance and efficiency [Miller RG, 2009]. The German National Guideline for Treating Chronic Respiratory Failure guidelines indicate to monitor pCO2 at least 1–2 times per year after NIV starting, as the gold standard to monitor ventilatory therapy adherence, even if values of daytime pCO2 cannot rule out NH, particularly in neuromuscular patients [Windisch W, 2018].

2. Aims

Respiratory failure, secondary to diaphragm weakness, is the main cause of death in ALS and currently ventilatory support is the only available treatment, being able to enhance quality of life and prolong survival. Consequently, monitoring respiratory function in ALS is of utmost importance to detect early respiratory impairment to start NIV timely and to assess adherence and efficiency of ventilatory support after NIV initiation. Many different pulmonary function tests (PFTs) are available

for respiratory function monitoring in ALS, each one with different sensitivity for pulmonary function assessment in different categories of patients. Evidence regarding the role of PFTs in ALS derives mostly from retrospective data, since longitudinal studies addressing this issue are scarce and with small cohorts included, thus limiting the generalizability of results. Hence, there is no international consensus about the correct approach to monitor respiratory function in ALS, since different guidelines recommend different types of PFTs to be used, so as different timing for patients monitoring. Additionally, guidelines do not agree about the cut-off values to be used to define respiratory impairment and to indicate NIV, leading to different timing of ventilatory support starting among patients. The lack of standardization in respiratory function monitoring reflects in the lack of standardization of inclusion criteria regarding respiratory function for ALS patients in RCTs, leading to selection bias and compromised external validity, with results that may not be applicable to real-world settings.

On these bases, the main aims of our project were:

1) Finding sensitive, specific and reliable markers to detect early respiratory impairment, to start NIV timely and thus to increase survival in ALS. Since there is no consensus about the correct monitoring and management of respiratory failure, our major aim is to examine the sensitivity of different PFTs in assessing respiratory function in ALS, to evaluate the relationships among different PFTs, and to examine and compare the strengths and the limits of different PFTs. In this respect, we will examine the impact of possible confounders in respiratory measurements (i.e. spinal vs bulbar impairment, cognitive impairment) to define ALS patients' demographic and clinical characteristics that could influence respiratory assessment. The obtained information could be used to define a respiratory function evaluation and monitoring that could be more standardized among centers on one side, and more tailored to the single patient, based on clinical features, on the other side. Standardizing respiratory monitoring in ALS is crucial in clinical practice to ensure that respiratory function is measured consistently across different clinical settings, and at the same time, individualizing respiratory function assessment based on clinical features, such as bulbar impairment, is important to obtain informative data about the real pulmonary function in ALS patients.

For this aim, we used different strategies that passed through:

2) Assessing longitudinal change in respiratory measures in ALS to study the pattern of decline of different PFTs, identifying the most sensitive tools for monitoring respiratory function in ALS patients, based on clinical features, such as sex and type of onset. Most evidence about respiratory function assessment in ALS derives from retrospective studies, that are more susceptible to selection and information biases and to quality of data issues, since they rely on existing records or participant recall about past symptoms, leading to inaccuracies in data

interpretation. Longitudinal studies about respiratory measures in ALS are scarce and included only small cohorts. We aim at assessing the pattern of decline of different PFTs in ALS patients, depending on clinical and demographic features, in a longitudinal study, to overcome the limitations of retrospective studies, enabling repeated measures within individuals, and facilitating the assessment of multiple variables over time. This could lead to more reliable and valid results that could be exported to real-world populations.

3) Exploring the potential of less used tools in ALS (i.e. MVV and blood serum chloride levels) to detect early respiratory impairment and to predict NIV starting and survival in ALS. Poor evidence is available about the potential role of MVV in pulmonary function assessment in ALS. We think that its usage in clinical practice could give additional information about respiratory function in ALS, since MVV measures endurance. An increased fatigability and a reduced endurance are among the first symptoms complained by many ALS patients, and other PFTs commonly used to monitor respiratory function, such as FVC or MIP, do not give insights about fatigability, being a static picture of respiratory muscles strength. Based on this, we wondered if MVV could be a marker of early respiratory impairment in ALS patients, being able to describe diaphragm fatigability in early stages of disease, when other PFTs are still normal. Similarly, we aimed at exploring the potential role of serum chloride levels in ALS, that may provide valuable and objective information about electrolyte balance and acid-base status, since chloride imbalance can be linked to respiratory acidosis or alkalosis. Our interest in serum chloride levels derived especially from the non-invasive nature of its assessment, being obtained from a simple venous blood sample during routine laboratory tests, differently from ABG that requires arterial puncture.

Additionally, we aimed at:

4) Defining respiratory subgroups for both prognostication and stratification of participants in both clinical practice and randomized clinical trials (RCTs). The identification of prognostic clusters of patients, based on both clinical features and respiratory scores in the most widely used PFTs, could be highly beneficial in clinical practice, especially in a clinical heterogeneous disease like ALS, helping to guide clinician decision-making processes, i.e. understanding which subgroups may respond favorably or not to a treatment can help personalized medicine strategies, improving patient outcomes. On the other side, defining clinical and prognostic clusters of patients based on respiratory data may be extremely helpful in RCTs to identify subgroups with different responses to treatment, and thus populations that may benefit most from specific interventions. This is crucial for validating the generalizability and robustness of trial results. Moreover, standardizing inclusion criteria and respiratory function monitoring in RCTs is essential for comparing results and drawing meaningful conclusions about disease progression and treatment efficacy in ALS patients.

5) Assessing demographic characteristics, timing and survival determinants for respiratory support in ALS. Fortunately, NIV usage has more than doubled from early years of 2000, reaching a rate of about 50% of ALS patients with respiratory impairment to be ventilated in 2008 [Chiò A, 2012; Gordon PH, 2012]. However, being ventilatory support the only efficacious treatment for respiratory impairment in ALS, this percentage is still not enough. Data regarding NIV usage prevalence in the last 15 years is missing and our aim was to examine if a positive trend of NIV usage has been confirmed in this period. Additionally, we aimed at analyzing which demographic and clinical characteristics could influence NIV prescription and adaptation. These data are of utmost importance to increase and optimize NIV usage, leading to an enhanced quality of life and survival in ALS. Similarly, since prevalence data about IMV positioning are scarce, we aimed at investigating the prevalence data of IMV and clinical and demographic factors that influenced IMV placement. Finally, we examined the benefit on survival obtained by NIV/IMV usage in different categories of patients, to help clinicians in managing respiratory failure in ALS patients by predicting the expected benefit from ventilatory support.

3. Material and methods

3.1 Assessing longitudinal change in respiratory measures in ALS to study the pattern of decline of different PFTs, identifying the most sensitive tools for monitoring respiratory function in ALS

To assess sensitivity of different PFTs in monitoring respiratory impairment and to study their longitudinal change in ALS, the REVEALS study (Registry of Endpoints and Validated Experiences in ALS) was conducted in six European ALS centers, including ours (A.O.U. Città della Salute e della Scienza, Turin, Italy) and other 5 specialized centers (Beaumont, Hospital, Dublin, Ireland; King's College Hospital, London, UK; University Medical Center, Utrecht, The Netherlands; Sheffield Teaching Hospitals, Sheffield and University Hospitals Leuven, Belgium). REVEALS study was a longitudinal, observational study that recruited ALS patients with the aim of assessing sensitivity of different PFTs in evaluating respiratory function in ALS in a real-world setting. Inclusion criteria for participants were: 1) having a confirmed diagnosis of spinal or bulbar onset ALS; 2) ALS King's stage 2 or 3 at recruitment (involvement of 2 or 3 clinical regions at recruitment); 3) having the ability to provide informed consent; 4) having the ability to complete PFTs (defined as ability to generate consistent scores, i.e. two valid scores within 10% in SVC and FVC); 5) having the ability to correspond remotely, either independently or with the assistance of a

caregiver. Oppositely, exclusion criteria were: 1) NIV usage at the time of recruitment; 2) having another concomitant active respiratory condition, such as chronic obstructive pulmonary disease (COPD), bronchiectasis, lung cancer, etc.

Pulmonary function was assessed at recruitment and every 3 months; however, reassessment at irregular intervals was considered as acceptable in line with the "real world" design of the study.

To ensure intra-rater and inter-rater reliability in PFTs performing, all assessors completed training in PFTs procedures during an investigator meeting before study initiation. Moreover, all centers received regular site visits, that included data collection observation, ensuring consistency of the protocol across the sites.

At baseline and during each follow-up visit, clinical and demographic variables were collected, including age, gender, height, weight, site of ALS onset, date of onset, date of diagnosis, King's stage, ALSFRS-r score and its respiratory sub-score, history of chest infections, smoking history, the presence of respiratory symptoms and of a regular productive cough.

At each visit four different PFTs were performed in the same order, recording the maximum score for analysis. The first measure to be performed was SNIP, measured in cmH2O, assessed using a MicroRPM respiratory pressure meter (MicroRPM, VIASYS Healthcare, Hochberg, Germany) at an un-occluded nostril, according to the ENCALS respiratory measurement standard operating procedures [ENCALS, 2018]. Particularly, for its performing it is important to ensure that the patient is seated comfortably, with their back straight and feet flat on the floor, and to choose appropriately sized nasal probe. After a complete exhalation, the patient is asked to perform a forcefully short and sharp sniff through their nose, while keeping their mouth closed. At least 10 measurements were completed, and the maximum score was recorded. For SNIP assessment, trials were performed for both nostrils, and any nasal congestion, deviation or surgical history was noted.

PCF, measured in L/min, was the second PFT to be performed, being assessed using Wright's peak flow meter with a facemask. At least 6 trials were completed, and the maximum score was recorded. For PCF assessment, the patient was asked to cough as strongly as possible into the facemask, that was held firmly over their nose and mouth by the assessor.

Finally, FVC and SVC were performed using a MicroLab spirometer (CareFusion Germany 234 GmbH. Leibnizstrasse 7, D-97204 Hoechberg, Germany) according to ENCALS standard operating procedures [ENCALS, 2018]. Specifically, for both FVC and SVC the patient is seated comfortably with their back straight and feet flat on the floor and the patient is asked to breathe normally for a few seconds through the facemask. To perform FVC the patient is asked to perform a deep inhalation to fill their lungs completely, and then to exhalate as forced and rapidly as possible through the facemask until no air remains in their lungs. On the other side, to perform SVC, after a deep inhalation, the patient is asked to slowly exhalate through the facemask until their lungs are empty.

Since bulbar patients show some difficulties in using a mouthpiece for FVC/SVC recording, due to facial weakness that leads to an inadequate seal on the mouthpiece, causing leaks and thus untruthful results, a facemask was used for all patients. A measure was considered as valid if the spirometer noted that American Thoracic Society/European Respiratory Society (ATS/ERS) criteria [Graham BL, 2019] had been met and the maximum score was recorded in liters and, consequently, as % of the predicted value, based on the Global Lung Function Initiative (GLI) reference equations [Quanjer PH, 2012].

Subjects who did not complete measurements of all respiratory outcomes were excluded from the analysis (<5%). Descriptive statistics of all patients and stratified by study site was performed.

Firstly, the correlation between outcomes measures (FVC, SVC, SNIP, PCF, ALSFRS-r respiratory sub-score) across all individuals and all time points was calculated for the entire cohort and also stratified by spinal and bulbar onset. For this aim, a Bayesian multiple outcomes random effects model was developed to study the correlations of multiple longitudinal respiratory outcomes measured concomitantly in the same subjects. To account for individual variability, random intercepts per individual were included for each outcome. Fixed effects incorporated in the model were: time from baseline (corresponding to enrollment date); site of onset (spinal or bulbar) and gender coded as a single four level variable ("Male spinal onset", "Male bulbar onset", "Female spinal onset", and "Female bulbar onset") in interaction with time from baseline, gender, study site, King's stage, history of respiratory tract infections, smoking history, presence of respiratory symptoms or regular productive cough. This allowed us to explore different rates of decline depending on sex and site of onset. Weakly informative Bayesian priors were selected, and prior predictive checks were conducted according to Gabry et al. [Gabry JS, 2019]. The model was run for a total of 3,500 iterations, with model convergence and fit assessed through the bulk effective sample size, tail effective sample size, and posterior predictive checks. This model was subsequently extended to include random slopes. A third model was then fit, which further extended the previous model by incorporating the correlation of group effects between outcomes, thus modeling correlations both between and within individuals. These three models were compared using K-fold cross-validation with twenty folds. For each fold, the model excluded data from a randomly selected 5% of all longitudinal measurements. Posterior samples were drawn from the model, and the fitted correlation between outcomes stratified by site of onset was calculated.

R statistical software 3.6.2 with additional packages [Yoshida K, 2019; Wickham H, 2019; Pedersen T, 2019; Bengsston H, 2020] was used for data preparation and descriptive analysis, while R packages brms [Bürkner P-C, 2020], tidybayes [Kay M, 2020], bayesplot [Gabry J, 2020], and Stan software version 2.21.0 were used to fit and assess Bayesian models.

Analysis code is available on Github: https://github.com/jpkrooney/REVEALS_interim_analysis, and archived on Zenodo: http://doi.org/10.5281/zenodo.4549667.

Using this Bayesian model, in our interim analysis we have previously shown that all the outcome measures (FVC, SVC, SNIP, and PCF) declined over time and particularly, SNIP and PCF turned out to have a higher decline in bulbar onset patients in comparison with spinal onset patients [Murray D, 2021]. Moreover, we found out that FVC and SVC were strongly correlated in ALS patients as expected, while SNIP only moderately correlated with FVC and SVC. These data, taken together, confirmed that SNIP may be a sensitive tool to examine pulmonary function especially in patients with bulbar impairment, and it may be considered an early marker of respiratory impairment in ALS, examining a different aspect of respiratory function, associated with inspiratory muscles fatigue [Murray D, 2021].

In this PhD thesis we are going to discuss the final validated results derived from the Bayesian model used for REVEALS study, re-examining the relationships between the respiratory measures and reporting their decline during disease course with particular attention to possible confounders, such as sex and types of onset (spinal vs bulbar). In addition to the interim analysis, also some clinical outcomes, such as ALSFRS-r respiratory sub-score, have been included in the analysis to evaluate their correlation with respiratory outcomes and their decline over time. Finally, we will examine the impact of respiratory symptoms and history at baseline on the outcome measures.

3.2 Exploring the potential of less used tools in ALS (i.e. MVV and blood serum chloride levels) to detect early respiratory impairment and to predict NIV starting and survival in ALS

3.2.1 MVV

All patients diagnosed with ALS in Turin ALS Centre from 1995 to 2015 and included in the Piemonte and Valle D'Aosta ALS register (PARALS) were screened [Chiò A, 2017]. To study the predictive power of MVV at diagnosis on survival and time to NIV, only patients who underwent spirometry within 4 months from diagnosis were included, and each spirometry was associated with the nearest ALSFRS-r score recorded during disease course. Additionally, to study the relationship between ABG and MVV, we collected data from the first ABG and spirometry performed simultaneously during the respiratory monitoring, using another dataset previously published [Manera U and Torrieri MC, 2020], that also reported the nearest ALSFRS-r score recorded. From ALSFRS-r score we were able to derive information about respiratory symptoms (by using ALSFRS-r items 10 and 11, referring to the presence of dyspnea and orthopnea, respectively) and bulbar dysfunction (by using ALSFRS-r items 1,2 and 3, referring to the presence of speech difficulties, saliva management and the presence of dysphagia, respectively). Bulbar involvement was considered as present when the sum score of items 1, 2, and 3 was <12. Exclusion criteria were: 1) presence of

uncompensated acidosis/alkalosis at ABG (pH <7.35 or >7.45, respectively); 2) concomitant presence of severe pulmonary, metabolic, or kidney disease. More specifically, pulmonary disease was considered as severe if 1) severe ongoing asthma/COPD was documented by the persistent use of bronchodilator/corticosteroid drugs or if 2) lung tumor (both historical and ongoing) was present or in presence of 3) a documented both historical and ongoing primary lung disease (pulmonary fibrosis, sarcoidosis, etc.,) or of 4) an active pulmonary infectious disease (namely pneumonia).

Clinical and demographic data were collected for each patient (age at onset, sex, site of onset, date of diagnosis, death/tracheostomy status, date of ABG/spirometry, and date of ALSFRS-r scale). Progression rate at diagnosis (Δ ALSFRS-r) was expressed in points/month and calculated as the ratio between the difference 48−ALSFRS-r score at diagnosis, and the time interval between onset and diagnosis (in months). Survival was computed from diagnosis to the date of death/IMV, or to December 31st, 2018, corresponding to the censoring date.

To derive MVV from a routine spirometry, different equations are available in literature for its calculation for both healthy adults and patients with pulmonary diseases [Campbell SC, 1982; Keith W, 1979; Ferris BG, 1978]. Among these, we selected the below equation for the so called calculated MVV(40), cMVV(40), derived from a measure that is routinely performed during spirometry, namely the forced expiratory ventilation in the first second (FEV1). The formula used for cMVV(40) calculation is: FEV1 (L) $*$ 40. Other methods were discarded either to simplify the interpretation of the results, or because they had been designed for specific populations. To validate cMVV performance, we applied the method, proposed by Dillard et al. [Dillard TA, 1993], that uses the peak inspiratory flow (PIF) to adjust cMVV calculation according to the following formula: cMVV(Dillard) = 30.77 $*$ FEV1 (L) + 5.94 $*$ PIF (L/s) − 4.77.

Differences in discrete and continuous variables were computed using the $\chi 2$ test and the Student's t test, or the Kruskal-Wallis and Mann-Whitney U tests, depending on the parametric/non parametric distribution of data, respectively. Correlations between cMVV(40), FVC, and other clinical variables were assessed using the non-parametric two-tailed Spearman's rank correlation test.

Since cMVV(40) derives from the absolute value of FEV1 (expressed in liters, not accounting for anthropometric measures), we decided to include in our analysis also FEV1%, as well as the FEV1/FVC ratio (modified Tiffeneau-Pinelli index), to better explore the correlation between cMVV(40) and obstruction signs.

Then, we evaluated the effect of cMVV(40), cMVV(Dillard), and FEV1% on overall survival and time to NIV starting. For time-to-event analysis, we computed Kaplan-Meier curves using log-rank tests and Cox proportional hazard models, which were adjusted for several well-known prognostic factors in ALS. Consequently, we stratified time-to-event analyses according both to the most widely used value of normality for FVC (≥80%) and to quartiles for cMVV(40). This sub-analysis was useful

to find a cut-off value for cMVV(40) associated with an increased risk for NIV starting or for death/IMV.

In this sense, to better define the best cut-off value for cMVV(40), ROC curves were computed evaluating the sensitivity and specificity of each cut-off for survival at 3, 6, and 12 months, and NIV starting within 6 months from spirometry. The Youden Index (computed as sensitivity + specificity − 1) was then calculated.

For all analyses a p value < 0.05 was considered significant.

Data were analyzed using IBM SPSS Statistics for Windows, Version 28.0.1.0 (IBM Corp. Released 2021).

3.2.2 Blood serum chloride

We wondered if serum chloride levels at diagnosis could be a prognostic biomarker for survival and time to NIV adaptation in ALS. Indeed, venous serum chloride correlates with HCO3- blood levels, being a marker of respiratory acidosis, due to an increase of pCO2 levels in a context of initial respiratory failure. More precisely, to compensate respiratory acidosis the renal proximal tubule increases the secretion of hydrogen ions, resulting in higher retention of sodium bicarbonate and lower retention of sodium chloride. So, in presence of a compensation of respiratory acidosis reduced serum chloride levels are detected.

Serum chloride correlates with respiratory failure in other chronic pulmonary diseases [Naal T, 2018], however poor evidence is known about its potential role as prognostic biomarker in ALS. Previously Stambler et al. and Hadjikoutis et al. showed how serum chloride correlates with respiratory symptoms appearance and survival in ALS, using data from ALS clinical trials and from a prospective study, respectively [Stambler N, 1998; Hadjikoutis S, 2001]. Despite being a non-invasive, easy to perform and low-cost tool, serum chloride is not usually checked in ALS patients and is not even considered by international guidelines for respiratory monitoring.

Based on this evidence, we decided to study the potential role of serum chloride at diagnosis as a prognostic marker in ALS, using a retrospective center-based cohort of ALS patients. All ALS patients diagnosed in Turin ALS Centre from 2007 to 2019 and included in the PARALS registry, having serum chloride assessment within 3 months from diagnosis were included. We excluded patients with significant renal comorbidities or other medical conditions that could influence serum chloride levels. For each patient clinical and demographic data were collected, including age, sex, date of ALS onset, site of onset, date of diagnosis, ALSFRS-r score at diagnosis, weight at diagnosis, weight before disease onset, FVC% at diagnosis, serum chloride (mmol/L), serum sodium (mmol/L),

serum potassium (mmol/L), white blood cell count (WBC, 10^9/L), neutrophils count (10^9/L), lymphocyte count (10^9/L), date of NIV start, and date of death/IMV. Disease progression rate at the time of diagnosis (ΔALSFRS-r) was expressed in points/month and calculated using the following formula: (48 – ALSFRS-r score at diagnosis)/(time from onset to diagnosis in months). Finally, we calculated the neutrophil-to-lymphocyte ratio (NLR) as a marker of inflammatory status [Leone MA, 2022].

For statistical analysis differences between discrete and continuous variables of interest were analyzed using the χ2-test and Mann–Whitney *U*-test, as appropriate. Correlation analysis among serum chloride, clinical data and other respiratory outcomes, was performed using Pearson's correlation coefficient. Time-to-event analysis was modeled performing backward stepwise Cox regression (unadjusted and adjusted) and Kaplan–Meier curves adjusted for significant prognostic factors for ALS were then computed with the log-rank test to predict survival and NIV starting. For time-to-event analysis the censoring date corresponded to December 31st, 2021.

A *p*-value <0.05 was considered to be statistically significant.

Data were analyzed using IBM SPSS Statistics for Windows, version 28.0.1.0 (Armonk, NY: IBM Corp.).

3.3 Defining respiratory subgroups for both prognostication and stratification of participants in clinical practice and RCTs

Identifying respiratory clusters of ALS patients based on clinical features, PFTs, and ABG parameters, could improve respiratory management and prognostic stratification for both clinical practice and RCTs.

For this aim, we selected for the analysis all ALS patients included the PARALS registry [Chiò A, 2017] and diagnosed from 2000 to 2015, collecting a dataset including the first ABG performed concomitantly with a spirometry during disease course. We excluded all patients with severe pulmonary, metabolic and kidney diseases. Demographic and clinical information (sex, age at onset, site of onset, date of diagnosis, death/tracheostomy, date of ABG/spirometry, date of ALSFRS-r scale, cognitive evaluation) were noted for all patients. Regarding ALSFRS-r score, we selected for the analysis the nearest to the date of ABG/spirometry. Disease progression rate was computed as previously shown. For a better evaluation of functional impairment regardless respiratory symptoms, we also computed the total ALSFRS-r score excluding the respiratory domain (ALSFRS-r^{36} with a maximal score of 36) and the ΔALSFRS^{36}, (36-ALSFRS-r^{36} score at time of ABG/spirometry)/time interval between disease onset and ABG in months.

In a subgroup of patients, cognitive classification was evaluated based on the revised diagnostic criteria for ALS-FTD [Strong MJ, 2017]. Cognitive dysfunction was identified if a diagnosis of cognitive impairment (ALSci), behavioural impairment (ALSbi), cognitive-behavioural impairment (ALScbi), or frontotemporal dementia (ALS-FTD) was present.

In our study, all ABGs were performed in the morning through radial arterial puncture by a trained nurse or pulmonologist and immediately analyzed using a blood gas analyzer, routinely calibrated.

We considered NIV-treated all the patients who were prescribed NIV and used it for at least one day (intention-to-treat analysis). NIV was always prescribed by an expert pulmonologist, following the most updated clinical practice guidelines, as appropriate (EFNS, 2012; NICE, 2018; Miller RG, 2009].

Clustering analysis was performed in Python language, version 3.10.9. Model variables selected for cluster analysis were divided into two groups 1) clinical variables, including presence and severity of respiratory symptoms (ALSFRS-r items 10 and 11 sum score), disease progression rate ($\Delta ALSFRS^{36}$) and presence of severe bulbar involvement; 2) instrumental variables, including FVC%, pCO2 and HCO3- arterial blood levels. The clinical variables were chosen for their importance as markers of respiratory failure (respiratory symptoms and progression rate in other body regions) or as possible confounders of FVC values (severe bulbar impairment). We included severe bulbar involvement and respiratory symptoms as a binary variable (present/absent). Patients with at least one among ALSFRS-r items 1 (speech), 2 (salivation) or 3 (swallowing) scoring ≤ 2, were classified as having a severe bulbar involvement. We considered respiratory symptoms as present if ALSFRS-r items 10 (dyspnea) and/or 11 (orthopnea) scored <4. Before clustering, the aforementioned features underwent a min-max renormalization, thus being rescaled to the interval 0-1. Then, consensus K-means clustering was applied using the Python library pykmeans; specifically, it is an unsupervised ensemble clustering that performs the K-Means algorithm multiple times, where each K-Means is trained on a subset of the data (random subset) and a subset of the features (random subspace). The predicted cluster memberships are combined through a consensus (or co-association) matrix, determining the number of times each pair of samples was clustered together over all clustering iterations. This matrix can be interpreted as a similarity matrix and can be used to resolve the final consensus clustering [Monti S, 2003]. Consequently, the resulting cluster groups are expected to be more robust than those obtained by a single run of the K-means algorithm. The fraction of patients in each run of the algorithm was fixed to 0.8, while the number of repetitions was set to 200. The optimal number (K) of clusters was determined by considering both silhouette scores and consensus matrix analyses. Specifically, the silhouette score measures how similar a sample is to its own cluster with respect to other clusters, with its values ranging from -1 to 1, while the consensus matrix provides a visual representation of the robustness of the obtained clusters, showing the frequency of clustering two samples into the same group. Generally, we aimed at

identifying clusters yielding a silhouette score greater than 0.3, considered as a valid threshold for reliable clustering. T-distributed stochastic neighbor embedding (t-SNE) was employed for visualization purposes.

After having identified robust respiratory clusters of patients, differences of discrete and continuous variables among clusters were analyzed using the χ^2, the Kruskal-Wallis test and the Dunn test. A backward stepwise Cox regression was used to evaluate if the chosen cluster solution was able to predict survival, considering sex and age as confounders, while Kaplan-Meier curves with log-rank test were then performed to compare survival distributions between clusters. We also evaluated time-to-event analyses to compare the effect of NIV use in patients stratified by the respiratory clusters identified. A p-value <0.05 was considered significant.

The statistical analyses were performed in IBM SPSS Statistics for Windows environment, Version 26.0. IBM Corp. Released 2019 and GraphPad Prism version 9.3.0 for Windows, GraphPad Software, San Diego, California USA.

3.4 Assessing demographic characteristics, timing and survival determinants for respiratory support in ALS

All ALS patients diagnosed between 2008 and 2015 included in the PARALS register [Chiò A, 2017] were included. Demographic and clinical information, including age, sex, date of diagnosis, site of onset, ALSFRS-r, date of NIV/IMV, were collected. In 64.2% of patients cognitive tests were performed at diagnosis, allowing a classification of the patients into five categories according to the consensus criteria for ALS-FTD [Strong MJ, 2017]. Disease progression rate at diagnosis (Δ ALSFRS-r) was calculated using the following formula: (48 - ALSFRS-r score at diagnosis)/(months from onset to diagnosis).

Binary logistic regression analysis (backward) was used to identify the determinants of NIV, IMV and NIV to IMV transition.

Overall survival was computed from disease onset to death/tracheostomy or to the censoring date. Survival after NIV was computed to death/IMV or to the censoring date, so as survival after IMV was computed to death or to censoring date. Censoring date corresponded to December 31st, 2019. Survival analyses were performed using the Kaplan-Meier method and compared with the log-rank test. Multivariable analysis for survival was performed with the Cox proportional hazards model (stepwise backward) with a retention criterion of $p<0.1$.

A p level <0.05 was considered significant.

Statistical analyses were carried out using the SPSS 26.0 statistical package (SPSS, Chicago, IL, USA).

4. Results

4.1 Assessing longitudinal change in respiratory measures in ALS to study the pattern of decline of different PFTs, identifying the most sensitive tools for monitoring respiratory function in ALS

A total of 280 patients were recruited for the REVEALS study. In **Table 1** we summarize the baseline characteristics of participants, stratified by participant centers. The median follow-up time was about 12 months. In total, there were 974 in-clinic visits, of which 14.2% were missing at least one respiratory assessment (SNIP 9.2%, SVC 12.5%, FVC 11.8%, PCF 9.4%).

Initially, we studied the correlations among different respiratory outcomes within individuals (**Table 2**). As expected, considering all patients, FVC and SVC turned out to be highly correlated (R=0.95), while PCF had a moderate correlation with FVC (R=0.77) and SVC (R=0.77). SNIP only moderately correlated with FVC (R=0.55), SVC (R=0.56) and PCF (R=0.60). On the other side, the ALSFRS-r respiratory sub-score was poorly correlated with all respiratory outcomes, confirming perplexities about its sensitivity and specificity for respiratory function in ALS. When we stratified the patients based on site of onset, we noted that the correlation between PCF and SNIP was higher in the bulbar group (R=0.76) vs spinal group (R=0.55), suggesting that these two measures could be easier tools to monitor respiratory function in ALS patients with facial weakness and bulbar impairment in comparison with FVC/SVC. Particularly, despite remaining low, the correlation between the ALSFRS-r respiratory sub-score and respiratory measures was slightly higher in spinal than in bulbar patients. In our opinion, this could be explained by the difficulty in saliva management in bulbar patients, that could lead to a subjective sensation of choking, and thus to patients' reporting of respiratory symptoms, also in presence of a normal respiratory function.

	Total Cohort	Dublin	London	Leuven	Sheffield	Turin	Utrecht	p*
n	280	63	22	59	22	56	58	
Female n (%)	93 (33.2)	22 (34.9)	9 (40.9)	21 (35.6)	8 (36.4)	17 (30.4)	16 (27.6)	
Male n (%)	187 (66.8)	41 (65.1)	13 (59.1)	38 (64.4)	14 (63.6)	39 (69.6)	42 (72.4)	0.845
Spinal-onset n (%)	227 (81.1)	53 (84.1)	16 (72.7)	49 (83.1)	21 (95.5)	43 (76.8)	45 (77.6)	
Bulbar-onset n (%)	53 (18.9)	10 (15.9)	6 (27.3)	10 (16.9)	1 (4.5)	13 (23.2)	13 (22.4)	0.316
Kings 2 n (%)	167 (59.6)	50 (79.4)	15 (68.2)	24 (40.7)	13 (59.1)	37 (66.1)	28 (48.3)	
Kings 3 n (%)	113 (40.4)	13 (20.6)	7 (31.8)	35 (59.3)	9 (40.9)	19 (33.9)	30 (51.7)	
Height in cm (mean, SD)	172.10 (10.05)	170.55 (8.99)	170.56 (8.58)	170.19 (9.71)	172.82 (12.58)	169.71 (8.57)	178.24 (10.07)	**<0.001**
Weight in kg (mean, SD)	74.67 (14.05)	76.28 (13.20)	75.00 (15.06)	73.72 (14.51)	76.15 (17.49)	67.01 (11.47)	81.00 (11.77)	**<0.001**
Age at Diagnosis (mean, SD)	61.85 (11.85)	63.26 (12.10)	60.43 (13.28)	61.61 (11.99)	56.49 (12.69)	64.37 (10.85)	60.70 (11.05)	0.115
Median diagnostic delay (months, SD)	9.99 [6.67, 16.33]	9.28 [5.89, 16.13]	ND	9.26 [6.46, 12.80]	ND	11.99 [6.97, 18.25]	10.87 [6.80, 16.33]	0.404
Median time from onset to enrolment (months, SD)	19.42 [11.65, 35.12]	17.71 [9.58, 28.48]	ND	26.28 [12.07, 55.72]	ND	21.49 [14.92, 35.43]	17.26 [11.40, 26.60]	0.056
Median in-clinic follow-up time (months, IQR)	8.0 [2.3,14.1]	5.8 [2.1,11.6]	0.00 [0.0,0.6]	11.9 [5.3,23.9]	4.1 [0.0, 7.4]	8.9 [2.7,13.4]	12.3 [3.4, 5.9]	**<0.001**
Median remote follow-up time (months, IQR)	12.0 [4.9,18.1]	15.9 [10.5,25.0]	4.7 [3.8,10.5]	15.6 [8.6, 26.6]	9.1 [2.7, 11.9]	9.7 [4.5,15.7]	13.9 [5.3, 16.5]	**<0.001**
Median number of assessments per individual [IQR]	3.00 [2.0, 5.0]	3.00 [2.0, 4.0]	1.00 [1.0, 2.0]	4.00 [3.0, 7.0]	2.00 [1.0, 2.8]	4.00 [2.0, 4.0]	4.00 [2.0, 6.0]	**<0.001**

Table 1. Baseline characteristics of the REVEALS study stratified by recruitment site.

*p-value calculated using Fisher's exact test for categorical variables, Student's t-test for normally distributed continuous variables, and the Kruskal–Wallis Rank Sum Test for non-normal continuous variables.

All patients (n=280)					
	FVC	**SVC**	**PCF**	**SNIP**	**ALSFRS-r resp**
FVC	1.0	0.95	0.77	0.55	0.31
SVC	0.95	1.0	0.77	0.56	0.32
PCF	0.77	0.77	1.0	0.60	0.30
SNIP	0.55	0.56	0.60	1.0	0.36
ALSFRS-r resp	0.31	0.32	0.30	0.36	1.0
Spinal onset only (n=227)					
	FVC	**SVC**	**PCF**	**SNIP**	**ALSFRS-r resp**
FVC	1.0	0.95	0.78	0.53	0.33
SVC	0.95	1.0	0.78	0.54	0.35
PCF	0.78	0.78	1.0	0.55	0.33
SNIP	0.53	0.54	0.55	1.0	0.40
ALSFRS-R resp	0.33	0.35	0.33	0.40	1.0
Bulbar onset only (n=53)					
	FVC	**SVC**	**PCF**	**SNIP**	**ALSFRS-r resp**
FVC	1.0	0.95	0.71	0.60	0.22
SVC	0.95	1.0	0.72	0.60	0.21
PCF	0.71	0.72	1.0	0.76	0.18
SNIP	0.60	0.60	0.76	1.0	0.17
ALSFRS-r resp	0.22	0.21	0.18	0.17	1.0

Table 2. Crude correlations of respiratory outcomes in all patients and stratified by site of onset.

Consequently, we examined the decline in the respiratory outcomes (FVC, SVC, SNIP and PCF), stratified by sex and site of onset. As shown in **Figure 1**, females reached lower values in all respiratory measures. Particularly, the decline of all respiratory outcomes during time was steeper in females with bulbar onset, in comparison with both males and females with spinal onset. Moreover, females with bulbar onset showed lower values in all respiratory outcomes, compared to females with spinal onset. On the contrary, in males, FVC and SVC showed similar values and decline similarly regardless site of onset, while SNIP and PCF showed lower values and higher decline in the bulbar group. In **Table 3** we show the modelled intercepts and slopes for the four respiratory outcome measures estimated by our Bayesian model.

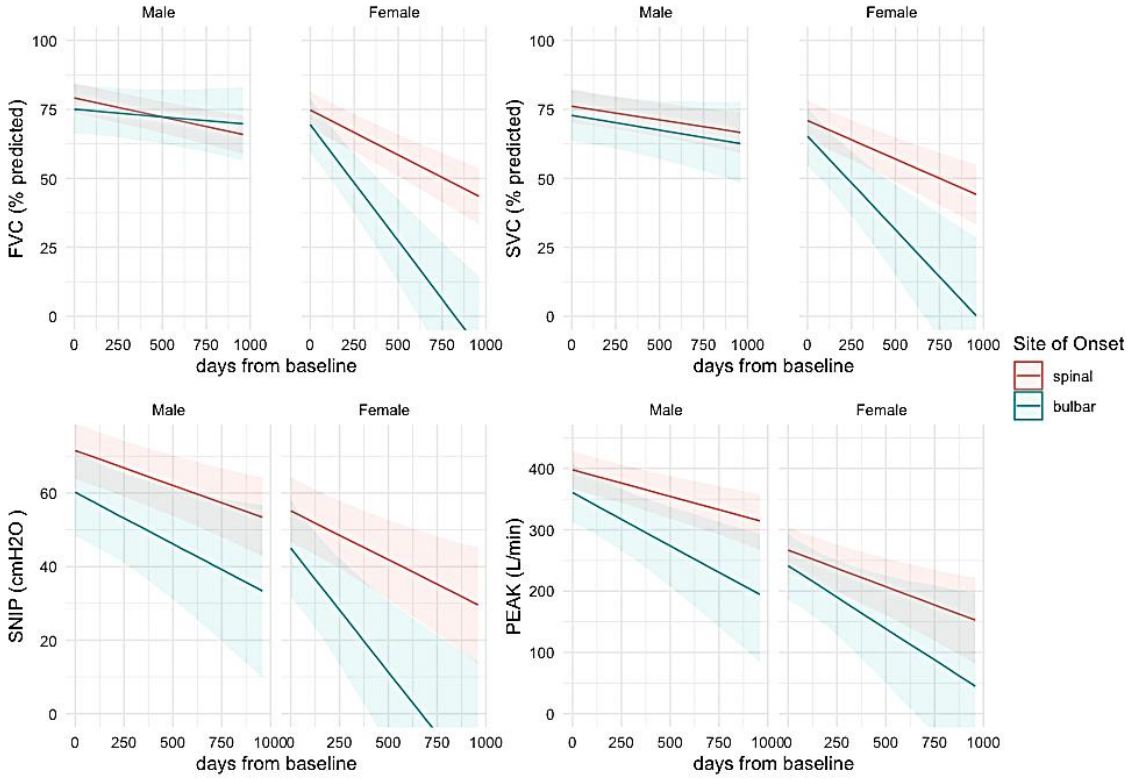

Figure 1. Conditional effect of site of onset and sex sampled from the multi-outcome Bayesian model for the 4 outcome measures (FVC%, SVC%, SNIP and PCF).

		Intercept		Slope (unit decline per month)	
		Estimate	**CI (Q2.5, Q97.5)**	**Estimate**	**CI (Q2.5, Q97.5)**
Males					
FVC %pred	**Spinal**	79.1	(73.6, 84.6)	-0.42	(-0.58, -0.27)
	Bulbar	75.0	(61.3, 88.9)	-0.17	(-0.72, 0.39)
SVC % pred	**Spinal**	76.1	(70.2, 82.0)	-0.30	(-0.46, -0.13)
	Bulbar	72.8	(58.1, 87.8)	-0.32	(-0.92, 0.29)
SNIP (cmH_2O)	**Spinal**	71.5	(63.9, 78.9)	-0.57	(-0.84, -0.30)
	Bulbar	60.1	(41.3, 78.8)	-0.85	(-1.88, 0.16)
PCF (L/min)	**Spinal**	397.9	(366.1, 428.2)	-2.67	(-3.88, -1.50)
	Bulbar	360.6	(282.2, 436.8)	-5.31	(-9.96, -0.94)
Females					
FVC %pred	**Spinal**	74.7	(63.0, 86.2)	-0.99	(-1.47, -0.53)
	Bulbar	69.4	(54.7, 83.8)	-2.56	(-3.53, -1.57)
SVC % pred	**Spinal**	71.0	(58.4, 83.3)	-0.85	(-1.35, -0.34)
	Bulbar	65.2	(49.5, 80.4)	-2.06	(-3.11, -0.99)
SNIP (cmH2O)	**Spinal**	55.2	(39.7, 70.6)	-0.82	(-1.60, -0.03)
	Bulbar	44.9	(25.3, 64.1)	-2.05	(-3.41, -0.67)
PCF (L/min)	**Spinal**	267.1	(201.7, 330.7)	-3.64	(-7.12, -0.15)
	Bulbar	240.9	(158.9, 318.6)	-6.23	(-12.26, -0.04)

Table 3. Slopes and intercepts of respiratory measurements after Bayesian modelling of the 4 outcomes.

When we included in the model also the ALSFRS-r respiratory sub-score, we observed that it is not able to distinguish type of onset in females, contrarily to the other respiratory measures (**Figure 2** and **Table 4**).

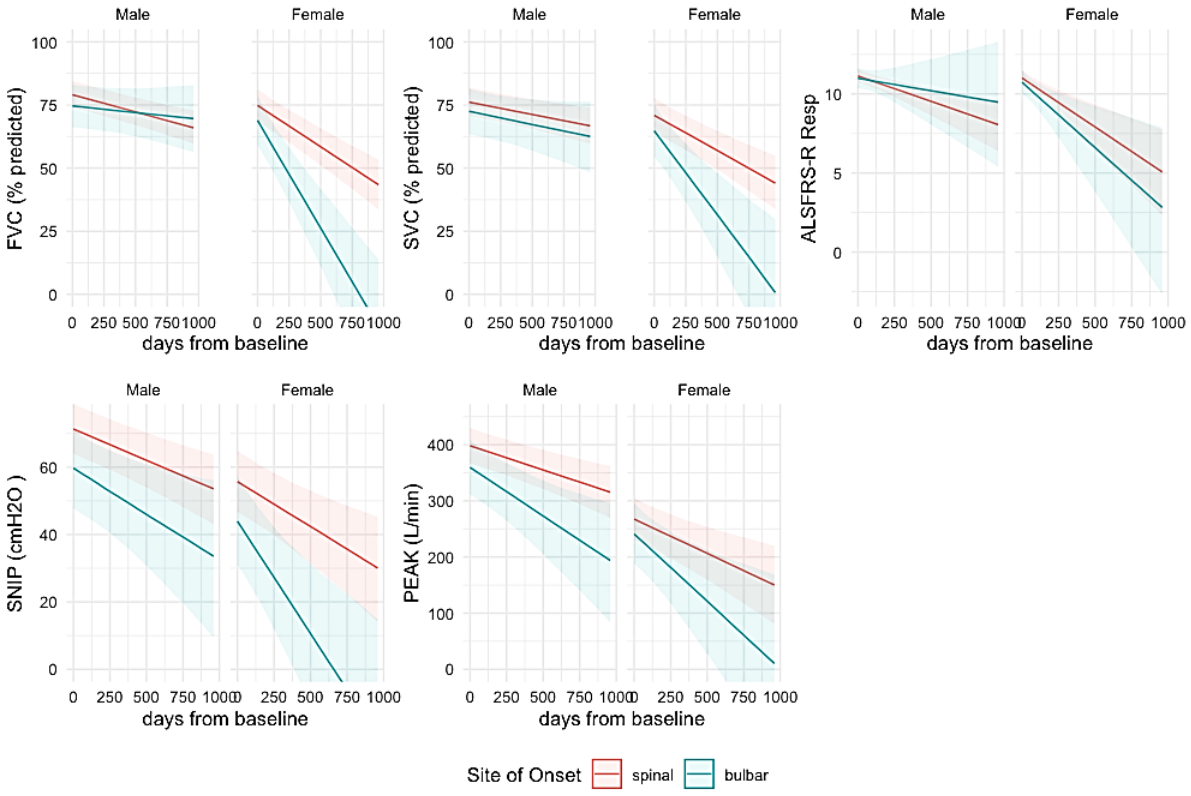

Figure 2. Conditional effect of site of onset and sex sampled from the multi-outcome Bayesian model extended to include the ALSFRS-r respiratory sub-score as an additional outcome.

		Intercept		Slope (unit decline per month)	
		Estimate	CrI (Q2.5, Q97.5)	Estimate	CrI (Q2.5, Q97.5)
Males					
FVC %pred	**Spinal**	79.1	(74.0, 84.5)	-0.41	(-0.57, -0.26)
	Bulbar	74.6	(61.1, 88.1)	-0.16	(-0.72, 0.38)
SVC % pred	**Spinal**	76.2	(70.7, 81.2)	-0.30	(-0.46, -0.13)
	Bulbar	72.4	(57.8, 86.7)	-0.32	(-0.91, 0.27)
SNIP (cmH_2O)	**Spinal**	71.4	(64.0, 78.8)	-0.57	(-0.83, -0.31)
	Bulbar	59.6	(40.5, 78.0)	-0.83	(-1.84, 0.16)
PCF (L/min)	**Spinal**	398.2	(367.0, 429.4)	-2.62	(-3.85, -1.47)
	Bulbar	359.6	(280.5, 437.7)	-5.31	(-10.08, -0.82)
ALSFRS-r respiratory sub-score	**Spinal**	11.1	(10.8, 11.6)	-0.10	(-0.15, -0.05)
	Bulbar	11.0	(10.0, 12.0)	-0.05	(-0.25, 0.14)
Females					
FVC %pred	**Spinal**	74.8	(63.4, 86.2)	-1.00	(-1.46, -0.53)
	Bulbar	68.9	(54.5, 83.7)	-2.59	(-3.61, -1.58)
SVC % pred	**Spinal**	70.8	(58.9, 83.0)	-0.85	(-1.34, -0.34)
	Bulbar	64.9	(49.5, 80.4)	-2.03	(-3.11, -0.93)
SNIP (cmH2O)	**Spinal**	55.8	(40.2, 71.9)	-0.82	(-1.59, -0.04)
	Bulbar	44.1	(24.2, 64.3)	-2.05	(-3.43, -0.67)
PCF (L/min)	**Spinal**	267.3	(202.8, 332.8)	-3.72	(-7.22, -0.23)
	Bulbar	241.0	(159.3, 323.9)	-7.32	(-13.81, -0.99)
ALSFRS-R respiratory sub-score	**Spinal**	11.0	(10.2, 11.9)	-0.19	(-0.35, -0.04)
	Bulbar	10.7	(9.7, 11.8)	-0.25	(-0.49, -0.02)

Table 4. Slopes and intercepts of respiratory measurements, including the ALSFRS-r respiratory sub-score after Bayesian modelling of the 5 outcomes.

Finally, by using the same model, we investigated the potential role of explanatory variables, shown in **Table 5**. As expected, King's stage 3 at baseline was associated with lower respiratory measures during disease course in comparison with King's stage 2, confirming a good prognostic power of King's staging system. A history of smoking or of respiratory tract infections was not associated with differences in respiratory metrics. The presence of respiratory symptoms at baseline was associated with reduced respiratory outcomes, with dyspnea at rest having the most negative estimates among all the respiratory symptoms. Finally, having a regular productive cough at baseline was associated with reduced PCF scores, confirming the sensitivity of PCF in detecting cough difficulties in clinical practice.

Variable/ Strata (N)	FVC%		SVC %		SNIP (cmH2O)		PCF (L/min)		ALSFRS-r resp subscore	
	Estim.	CrI: 2.5-97.5	Estim.	CrI: 2.5-97.5	Estim.	CrI: 2.5-97.5	Estim.	CrI: 2.5-97.5	Estim.	CrI: 2.5-97.5
Kings stage 3 (vs stage 2) at baseline	-9.1	-14.3, -3.86	-9.4	-15.0, -3.7	-8.7	-16.1, -1.5	-44.3	-73.6, -15.0	-0.7	-1.1, -0.3
Ever vs never smoked at baseline	-1.9	-6.9, 3.2	-2.5	-7.9, 3.0	3.3	-3.6, 10.1	-24.4	-52.1, 3.1	0.1	-0.3, 0.4
Occasional chest infections	0.8	-5.7, 7.4	1.5	-5.4, 8.5	3.5	-5.3, 12.2	-11.7	-47.8, 26.1	0.2	-0.3, 0.7
Moderate/Frequent chest infections	-1.0	-15.7, 14.0	-2.4	-18.1, 14.0	-4.1	-24.0, 15.5	12.9	-69.8, 97.2	-0.2	-1.3, 0.9
Orthopnea	-9.3	-16.8, -1.8	-10.4	-18.4, -2.3	-18.6	-28.8, -8.7	-43.4	-85.3, -0.7	-2.2	-2.7, -1.7
Dyspnea at rest	-18.3	-30.5, -6.4	-22.6	-35.5, -9.9	-18.1	-34.5, -1.8	-69.6	-138.1, -0.8	-2.6	-3.5, -1.8
Dyspnea when active	-13.3	-19.1, -7.5	-15.7	-21.7, -9.7	-15.6	-23.5, 7.6	-44.4	-77.1, -10.7	-1.6	-2.0, -1.2
Regular productive cough	-5.0	-10.7, 1.0	-4.5	-10.7, 2.00	-6.5	-14.5, 1.7	-40.8	-75.0, -6.4	-0.5	-0.9, 0.0

Table 5. Regression coefficients for investigational explanatory variables.

4.2 Exploring the potential of less used tools in ALS (i.e. MVV and blood serum chloride levels) to detect early respiratory impairment and to predict NIV starting and survival in ALS

4.2.1 MVV

To assess the potential role of MVV in detecting early respiratory impairment and predict NIV starting and survival in ALS, we included 1287 ALS patients who underwent spirometry at diagnosis. Descriptive statistics for the whole cohort and for cohorts with PIF or ABG are summarized in **Table 6**. The PIF cohort (N = 576) did not show any significant difference when compared to the whole cohort. Oppositely, the ABG cohort (N=484), for which we collected a second spirometry concomitant to ABG, turned out to have a higher functional impairment, lower values of FVC%, FEV1% and cMVV(40). This was expected since ALS patients usually perform ABG later in the course of disease, when spirometry values are reduced or when they are symptomatic for respiratory symptoms.

	Whole cohort (N=1287)	Cohort with PIF (N=567)	Cohort with ABG (N=484)	Whole vs PIF cohort	Whole vs ABG cohort
	Median (IQR)	**Median (IQR)**	**Median (IQR)**	***p* ***	***p* ***
Age at onset (years)	66.2 (58.9–72.7)	65.9 (57.9–72.5)	66.1 (58.8–73.5)	0.520	0.588
Age at PFT performance (years)	67.4 (60.0–73.8)	67.2 (59.5–73.6)	67.6 (60.1–73.5)	0.487	0.941
Onset-PFT interval (months)	11.0 (6.0–18.0)	11.0 (7.0–18.0)	13.5 (8.6–22.2)	0.893	**<0.001**
ALSFRS-r total score	42.0 (37.0–45.0)	42.0 (37.0–45.0)	40.0 (34.0–44.0)	0.968	**<0.001**
ΔALSFRS-r	0.50 (0.29–1.00)	0.50 (0.30–0.90)	0.55 (0.33–1.00)	0.774	0.137
FVC%	85.9 (66.9–101.2)	88.7 (70.6–101.9)	75.6 (58.5–92.0)	0.069	**<0.001**
FEV1%	86.4 (68.1–102.7)	89.6 (71.5–102.7)	77.2 (58.4–95.5)	0.106	**<0.001**
FEV1/FVC ratio	81.8 (75.9–87.7)	81.1 (75.6–86.2)	-	0.054	-
cMVV(40)	81.6 (59.6–104.8)	84.0 (63.3–109.4)	72.2 (53.7–94.9)	0.084	**<0.001**
PEF (L/s) (N = 1115)	4.3 (3.0–5.7)	4.3 (3.1–5.7)	-	0.984	-
PIF (L/s)	-	2.6 (1.7–3.6)	-	-	-
cMVV(Dillard)	-	85.7 (64.1–110.1)	-	-	-
pH	-	-	7.43 (7.41–7.44)	-	-
pO_2 (mmHg)	-	-	81.0 (73.8–87.0)	-	-
pCO_2 (mmHg)	-	-	40.0 (34.0–44.0)	-	-
BE (mEq/L)	-	-	1.38 (-0.20-3.12)	-	-
HCO_3^- (mmol/L)	-	-	25.5 (23.7–27.3)		-
	N (%)	**N (%)**	**N (%)**	***p* §**	***p* §**
Sex					
Female	587 (45.6)	253 (43.9)	217 (44.8)	0.532	0.792
Male	700 (54.4)	323 (56.1)	267 (55.2)		
Site of onset					
Bulbar onset	427 (33.2)	184 (31.9)	162 (33.5)	0.872	0.912
Spinal onset	849 (66.0)	387 (67.2)	316 (65.3)		
Respiratory onset	11 (0.9)	5 (0.9)	6 (1.2)		
Total	1287 (100.0)	576 (100.0)	484 (100.0)		

Table 6. Descriptive statistics for MVV cohort.

* Mann-Whitney U test; § Chi-square test; all significant results ($p < 0.05$) are written in bold.

Firstly, we studied the correlations among cMVV(40) and different respiratory outcomes and clinical variables at diagnosis (**Table 7**). We found out that cMVV(40) moderately correlates in order of correlation strength with FVC% and FEV1% and ALSFRS-r total score, being a good marker of respiratory function and functional impairment in ALS. However, cMVV(40) shows a weak-moderate positive correlation with ALSFRS bulbar score, that could be due either to a more advanced disease in patients with bulbar impairment, or to a reduced ability of patients with facial weakness in

performing spirometry. Moreover, cMVV(40) weakly correlates with disease progression rate and ALSFRS-r respiratory items, confirming that ALSFRS-r respiratory domain reflects subjective reports that are not specific for respiratory failure. No significant correlation was found between cMVV(40) and FEV1/FVC ratio, demonstrating that cMVV(40) is not influenced by pulmonary obstructive signs.

In the PIF cohort cMVV(Dillard) and cMVV(40) were highly correlated. As expected, being cMVV(Dillard) a measure derived from PIF, PIF turned out to be strongly correlated with cMVV(Dillard), but a moderate correlation was also found with cMVV(40). Similarly, PEF was highly correlated with both cMVV(40) and cMVV(Dillard).

When we evaluated the correlation of cMVV(40) with ABG parameters, we found out a moderate correlation of cMVV(40) with HCO3-, SBE, and pO2, and only a weak correlation with pCO2. This information collimates with our previous finding that HCO3- and SBE are an earlier marker of respiratory failure in ALS than pCO2 [Manera U and Torrieri MC, 2020].

	Whole cohort (N=1287)	**Cohort with PIF (N=567)**	**Cohort with ABG (N=484)**	
cMVV(40)	**R**	**R**	**R**	***p***
Age at onset (years)	-0.426	-	-	**<0.001**
Age at PFT (years)	-0.429	-	-	**<0.001**
Onset-PFT interval (months)	-0.072	-	-	**<0.001**
ALSFRS-r total score	0.319	-	-	**<0.001**
ALSFRS-r bulbar score	0.356	-	-	**<0.001**
ALSFRS-r item 10	0.214	-	-	**<0.001**
ALSFRS-r item 11	0.176	-	-	**<0.001**
ALSFRS-r item 12	0.116	-	-	**<0.001**
ΔALSFRS-r	-0.209	-	-	**<0.001**
FVC%	0.626	-	-	**<0.001**
FEV1%	0.669	-	-	**<0.001**
FEV1/FVC ratio	0.033	-	-	0.237
PEF (L/s) (N = 1115)	0.823	-	-	
PIF (L/s)	-	0.693	-	**<0.001**
cMVV(Dillard)	-	0.983	-	**<0.001**
pO_2 (mmHg)	-	-	0.350	**<0.001**
pCO_2 (mmHg)	-	-	-0.278	**<0.001**
SBE (mEq/L)	-	-	-0.318	**<0.001**
HCO_3^- (mmol/L)	-	-	-0.323	**<0.001**
cMVV(Dillard)	**R**	**R**	**R**	***p***
FVC%	-	0.584	-	**<0.001**
FEV1%	-	0.611	-	**<0.001**
PIF	-	0.807	-	**<0.001**
PEF	-	0.851	-	**<0.001**
FEV1/FVC ratio	-	0.028	-	0.504
Total	1287 (100.0)	576 (100.0)	484 (100.0)	

Table 7. Correlations of cMVV(40) and cMVV(Dillard) with other respiratory measures and clinical variables.

After having investigated the correlations among cMVV and other respiratory measures and clinical variables, we aimed at exploring the influence of cMVV on time to NIV and survival. For this aim, we applied different Cox proportional hazard models (**Table 8**).

Cox proportional hazard models adjusted for sex, age, progression rate, and site of onset confirmed that cMVV(40) and cMVV(Dillard) values were positively associated with both survival and time to NIV, in univariate and multivariate analysis. To exclude a possible influence of bulbar involvement in PFT performance, we stratified the analysis for bulbar involvement, but it did not significantly change the results, meaning that cMVV is not strictly dependent on bulbar impairment. However, when we adjusted Cox models also for FVC%, cMVV(40) lost its significant association with survival. To better understand the interplay between these two measures, we stratified the analysis according to the FVC% limit of normality (80%); we found out that in patients with normal FVC (≥80), cMVV(40) kept to be significantly associated with survival. This data could be explained by the fact that FVC is a measure strongly associated with respiratory function, so when FVC is reduced under normal limits, cMVV is less indicative of survival in comparison with FVC; however, when FVC is still normal, cMVV seems to be an early marker of respiratory failure in ALS, suggesting its use in the initial screening and in the early phases of ALS patients to stratify risk in patients.

Based on the Cox proportional hazard models, we then performed Kaplan-Meier curves to predict survival and time to NIV initiation in ALS patients based on both FVC and cMVV values (**Figure 3a-b**). For this aim we divided patients according to the cut-off of 80% for both FVC% and cMVV(40) for two reasons: 1) to simplify the memorization of the cut-off in clinical practice; 2) the median cMVV(40) value of our entire cohort was about 80%. Interestingly, Kaplan-Meier analysis confirmed that there is a subgroup of patients, showing normal FVC values, but reduced cMVV values, that have an already significant reduction in both time to NIV initiation and survival in comparison with patients with both FVC and cMVV normal values. Again, cMVV could be an earlier marker of respiratory failure in ALS, to be used to stratify patients in the initial phases of the disease and to detect the appearance of respiratory failure timely.

Univariate Analysis - Overall Survival

	HR (95% CI)	*p*		HR (95% CI)	*p*
Age at PFT (continuous)	1.022 (1.016–1.028)	**<0.001**	**FVC%**	0.983 (0.980–0.985)	**<0.001**
Sex	1.132 (1.010–1.269)	**0.033**	**FEV1%**	0.985 (0.982–0.987)	**<0.001**
Site of onset (B/S)	1.308 (1.166–1.467)	**<0.001**	**cMVV(40) (continuous)**	0.990 (0.988–0.992)	**<0.001**
ΔALSFRS	1.231 (1.176–1.289)	**<0.001**	**FEV1/FVC ratio**	1.006 (1.000–1.012)	0.067
Bulbar involvement	0.699 (0.618–0.791)	**<0.001**	**PIF (continuous, N = 576)**	0.832 (0.777–0.891)	**<0.001**
			cMVV(Dillard) (continuous, N = 576)	0.991 (0.988–0.994)	**<0.001**
cMVV(40) (median adj)			**cMVV(40) (quartiles adj)**		
<80	1		**<60**	1	
≥80	0.595 (0.531–0.667)	**<0.001**	**60–80**	0.611 (0.520–0.717)	**<0.001**
			80–105	0.489 (0.417–0.573)	**<0.001**
			≥105	0.428 (0.364–0.502)	**<0.001**
cMVV(Dillard) (median adj)			**cMVV(Dillard) (quartiles adj)**		
<85	1		**<65**	1	
≥85	0.677 (0.572–0.802)	**<0.001**	**65–85**	0.611 (0.520–0.717)	**<0.001**
<80	1		**85–105**	0.489 (0.417–0.573)	**<0.001**
≥80	0.617 (0.520–0.731)	**<0.001**	**≥105**	0.428 (0.364–0.502)	**<0.001**

Multivariate analysis - Overall survival - cMVV(40)

	Adjustments		HR (95% CI)	*p*
	Age at onset (cont), Sex, Site of onset (B/S), ΔALSFRS (N = 1287)	**cMVV(40) (continuous)**	0.988 (0.986–0.990)	**<0.001**
		cMVV(40) (median adj)		
		<80	1	
		≥80	0.598 (0.517–0.691)	**<0.001**
		cMVV(40) (quartiles adj)		
		<60	1	
		60–80	0.633 (0.531–0.755)	**<0.001**
		80–105	0.490 (0.409–0.587)	**<0.001**
		≥105	0.404 (0.326–0.500)	**<0.001**
	Age at onset (cont), Sex, Site of onset (B/S), Δ ALSFRS, bulbar involvement (N = 1083)	**cMVV(40) (continuous)**	0.989 (0.986–0.991)	**<0.001**

Stratified analysis - Overall survival - cMVV(40)

FVC ≥ 80	Age at onset (cont), Sex, Site of onset (B/S), ΔALSFRS (N = 1287)	**cMVV(40) (median adj)**		
		<80	1	
		≥80	0.785 (0.630–0.979)	**0.032**
FVC < 80	Age at onset (cont), Sex, Site of onset (B/S), ΔALSFRS (N = 1287)	**cMVV(40) (median adj)**		
		<80	1	
		≥80	0.931 (0.718–1.206)	0.586

Multivariate analysis - time to NIV - cMVV(40)

	Age at onset (cont), Sex, Site of onset (B/S), ΔALSFRS (N = 1287)	**cMVV(40) (continuous)**	0.983 (0.980–0.987)	**<0.001**
		cMVV(40) (median adj)		
		<80	1	
		≥80	0.525 (0.417–0.660)	**<0.001**
		cMVV(40) (quartiles adj)		
		<60	1	
		60–80	0.626 (0.472–0.830)	**<0.001**
		80–105	0.482 (0.360–0.645)	**<0.001**
		≥105	0.278 (0.200–0.388)	**<0.001**

Table 8. Cox proportional hazard models (univariate and multivariate analysis). Hazard ratios are shown for different respiratory measures, including FVC, cMVV(40), cMVV(Dillard), PIF, FEV1 and FEV1/FVC ratio.

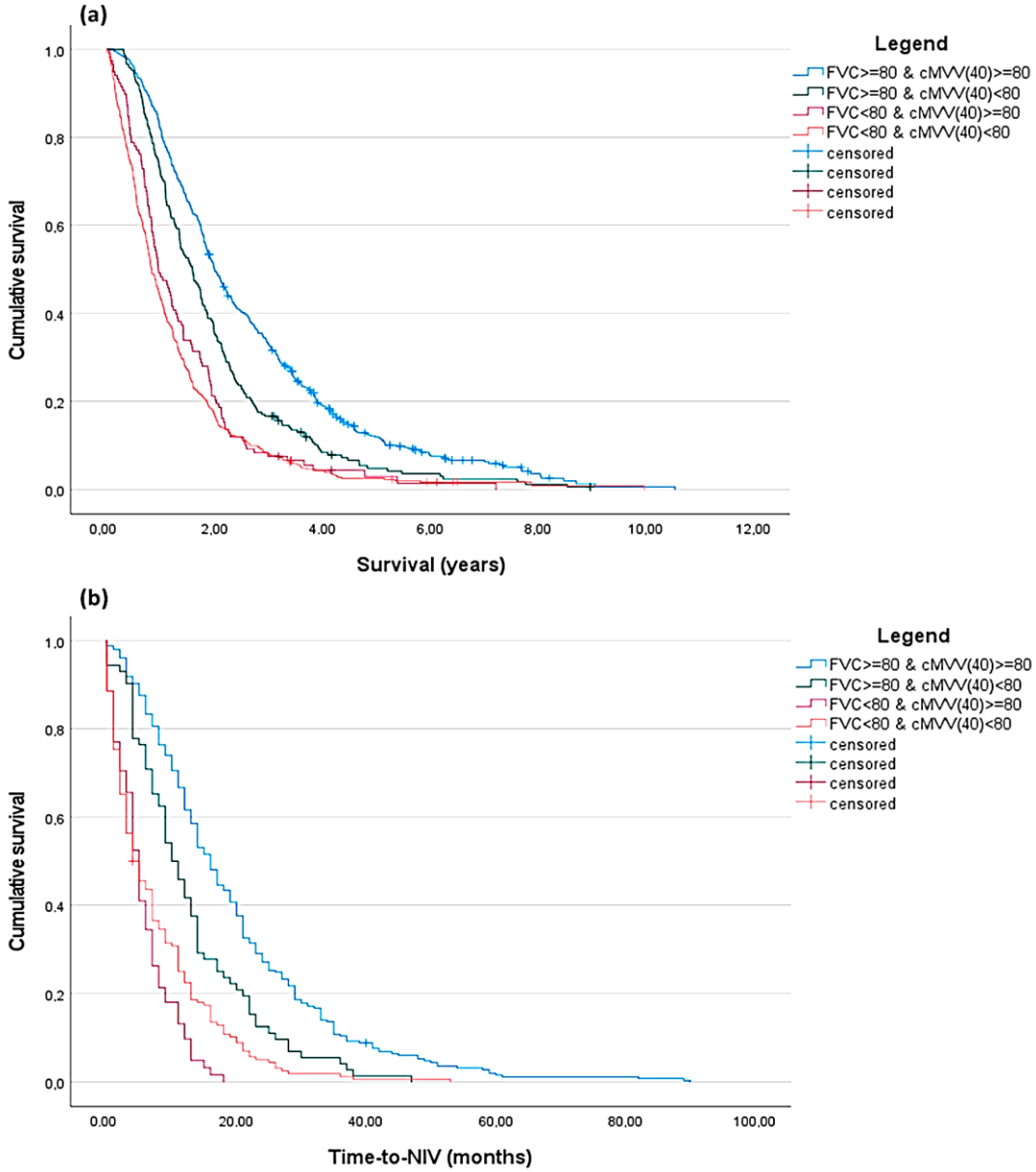

Figure 3. Kaplan-Meier curves for overall survival and time to NIV. Patients were subdivided into four categories according to the combination of FVC% and cMVV(40) values, using for both a cut-off of 80. **(a)** Overall survival (years) from PFT performance. The result of the pairwise log-rank test p was <0.001 for all combinations, except for the FVC% < 80 groups (p = 0.064); **(b)** Time-to-NIV (months) from PFT performance. The result of the pairwise log-rank test p was <0.001 for all combinations, except for the FVC% < 80 groups (p = 0.079).

Finally, to evaluate the sensitivity and specificity of cMVV(40) in predicting survival and NIV initiation we performed ROC analysis. Particularly, we considered survival at 3, 6, and 12 months and NIV start at 6 months (**Figure 4**). The AUC value was near to 0.7 for all of the curves, meaning a good accuracy of cMVV(40) in predicting survival and time to NIV initiation. Finally, Youden index values for survival at 1 year (YI 77.8) and for NIV initiation at 6 months (YI 82.6) confirmed

that the cut-off value of 80% for cMVV(40) can be considered valid to stratify patients with and without respiratory impairment.

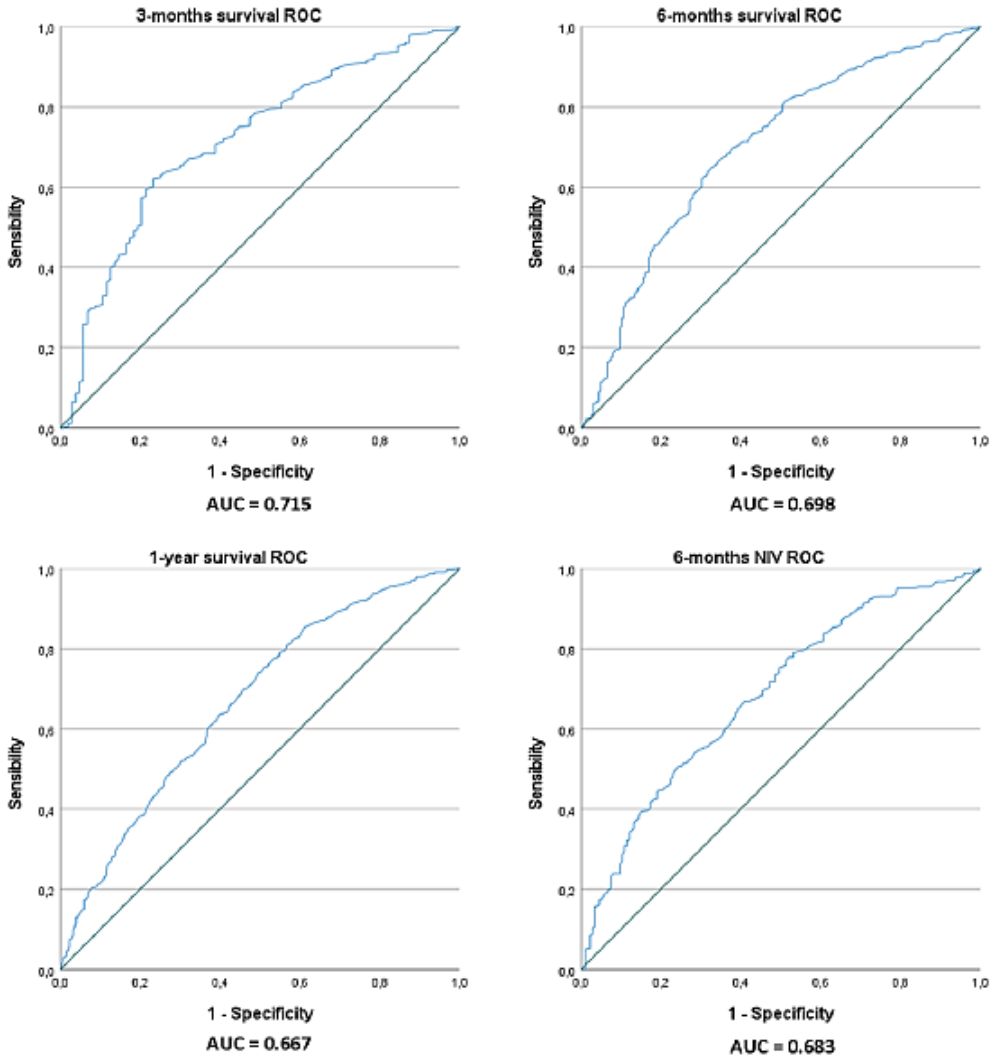

Figure 4. ROC curves for cMVV(40) for survival at 3, 6, and 12 months and NIV start at 6 months. Youden index value for survival at 1 year resulted to be of 77.8 and for NIV at 6 months of 82.6, confirming that values of cMVV(40) around the adjusted median can be considered as valid cut-off for ALS population.

4.2.2 Blood serum chloride

To assess the potential role of blood serum chloride levels at diagnosis as a marker of respiratory function, we collected data from 760 ALS patients. Descriptive statistics of the cohort are summarized in **Table 9**.

Median serum chloride values at diagnosis were 103.0 mmol/L (IQR 101.0–105.0 mmol/L), without showing any significant between genders (p = 0.936). Bulbar and spinal onset patients did not differ in serum chloride levels (p = 0.329), while, as expected, respiratory onset patients showed significantly lower serum chloride levels in comparison with other types of onset (98 mmol/L, IQR 94–105 vs. 103 mmol/L, IQR 101–105 mmol/L, p = 0.024).

	Median (IQR)
Age at onset (years)	67.6 (60.1-74.5)
Onset-diagnosis interval (months)	8.0 (5.0-13.0)
ALSFRS-r total score	43.0 (39.0-45.0)
ΔALSFRS-r at diagnosis (points/month)	0.600 (0.285-1.200)
Serum chloride (mmol/L)	103.0 (101.0-105.0)
Serum sodium (mmol/L)	142.0 (140.0-143.0)
Serum potassium (mmol/L)	4.2 (3.9-4.4)
Creatinine (mg/dL)	0.74 (0.61-0.89)
FVC (%) at diagnosis	92.0 (73.0-104.0)
Weight loss (kg/months)	0.27 (0.00-1.00)
Survival (months)	22.4 (11.9-43.0)
	n (%)
Sex	
Male	416 (54.7)
Female	344 (45.3)
Site of onset	
Bulbar onset	257 (33.8)
Upper limbs onset	212 (27.9)
Lower limbs onset	280 (36.8)
Respiratory onset	11 (1.4)
Total	760 (100.0)

Table 9. Descriptive statistics of ALS patients' cohort with blood serum chloride values at diagnosis (N=760).

As for MVV, we first studied the crude correlations between serum chloride levels and other variables, as shown in **Table 10**, where we included only the significant correlations found. Particularly, serum chloride had a moderate negative correlation with inflammatory markers, especially neutrophils and NLR, confirming the hypothesis of an inflammatory etiopathogenetic

component in ALS, that could worsen with disease spreading. As expected, depending on the renal compensatory mechanism for respiratory acidosis, serum sodium was moderately correlated to serum chloride. However, serum chloride levels showed only a weak correlation with FVC% values, general functional impairment (ALSFRS-r score) and ALSFRS-r respiratory items.

	R	p
WBC (10^9/L)	-0.276	<0.001
Neutrophils (10^9/L)	-0.328	<0.001
Lymphocyte (10^9/L)	0.117	0.001
Neutrophil-to-lymphocyte ratio (NLR)	-0.310	<0.001
Serum sodium (mmol/L)	0.531	<0.001
Serum potassium (mmol/L)	0.088	<0.001
ALSFRS-R item 10	0.209	<0.001
ALSFRS-R item 11	0.206	<0.001
ALSFRS-R total score	0.147	<0.001
Weight loss (kg/months)	-0.101	0.009
FVC (%)	0.151	<0.001
Age at diagnosis (years)	-0.152	<0.001

Table 10. Correlations between serum chloride levels at diagnosis and other clinical features and blood biomarkers. Only significant correlations have been reported (Pearson's correlation coefficient, $p < 0.05$).

Cox proportional hazard models confirmed in both univariate and multivariate analysis the significant influence of serum chloride levels at diagnosis on survival and time to NIV initiation with reduced levels being associated with a reduced survival and time to NIV initiation (**Table 11**). Particularly, when serum chloride levels were above >103.0 mmol/L there was a protective effect for both survival (HR 0.840, IQR 0.713–0.988, $p = 0.036$) and time to NIV (HR 0.668, IQR 0.461–0.968, $p = 0.033$). As expected, being serum chloride a non-volitional test, multivariate analysis stratified for site of onset revealed its predictive role for NIV starting in either spinal (HR 0.918, IQR 0.875–0.963, $p < 0.001$) or bulbar onset patients (HR 0.850, IQR 0.766–0.942, $p = 0.002$). Due to the limited number of patients with respiratory onset included in the analysis ($n = 11$), the time-to-event analysis for serum chloride resulted in non-significance in this subgroup of patients.

Overall survival	**HR**	**p**
Serum chloride Cox unadjusted (continuous)	0.962 (0.946-0.979)	**<0.001**
Serum chloride Cox unadjusted (median)	0.795 (0.682-0.926)	**0.003**
Serum chloride Cox unadjusted (quartiles)	0.902 (0.844-0.965)	**0.003**
Serum chloride Cox unadjusted (quartiles categories)	1	
	0.896 (0.732-1.097)	0.289
	0.759 (0.615-0.937)	**0,010**
	0.755 (0.612-0.932)	**0.009**
Serum chloride Cox adjusted* (continuous)	0.975 (0.957-0.993)	**0.007**
Serum chloride Cox adjusted* (median)	0.840 (0.713-0.988)	**0.036**
Time to NIV		
Serum chloride Cox adjusted* (continuous)	0.951 (0.924-0.979)	**<0.001**
Serum chloride Cox adjusted* (median)	0.668 (0.461-0.968)	**0.033**

Table 11. Time-to-event analysis using Cox proportional hazard models. The median serum chloride value was 103.0 mmol/L (IQR 101.0–105.0).

*Adjusted for sex, age at diagnosis, site of onset (B/S), weight loss (kg/months), ΔALSFRS-R (point loss per month), serum sodium (mmol/L), and serum potassium (mmol/L). Significant values are written in bold (p <0.05).

When we performed Kaplan–Maier curves (**Figure 5a-b**) we confirmed that patients with serum chloride levels <103.0 mmol/L at diagnosis had a reduced survival (p = 0.003, log-rank for pairwise comparison) and time NIV indication (p = 0.010, log-rank for pairwise comparison).

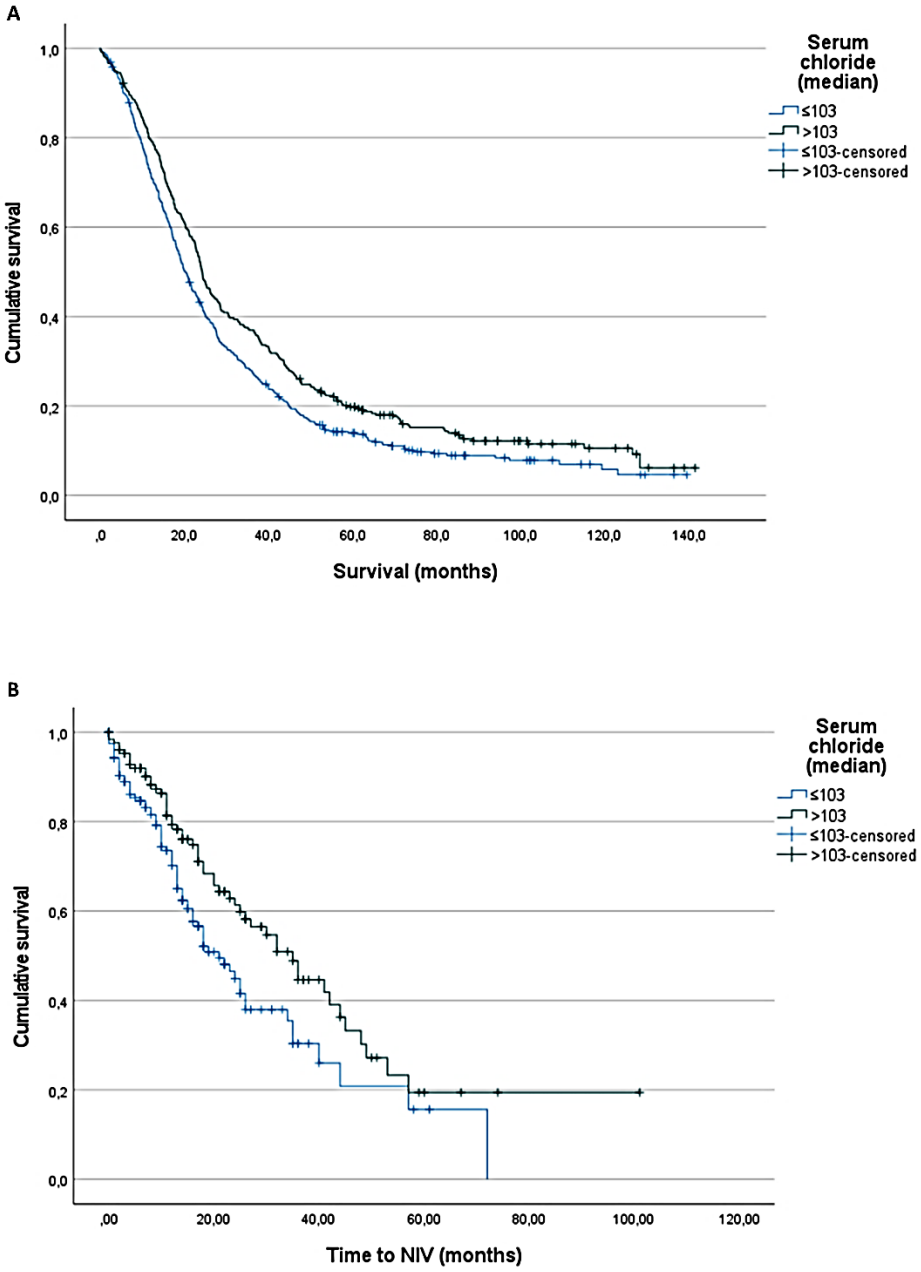

Figure 5. (a) Kaplan–Meier analysis on overall survival, comparing patients based on serum chloride values (median value 103.0 mmol/L). Log-rank test $p = 0.003$. **(b)** Kaplan–Meier analysis on time to NIV start, based on serum chloride values (median value 103.0 mmol/L). Log-rank test $p = 0.010$.

4.3 Defining respiratory subgroups for both clinical prognostication and stratification of participants in RCTs

A total of 550 patients were included for cluster analysis to define respiratory prognostic subgroups of ALS patients. Clinical and demographic data of all patients are shown in **Table 12**.

All patients (n=550)	
	Median (IQR)
Age at onset	67.2 (59.9-72.9)
ALSFRS-r total score	40.0 (34.0-44.0)
ALSFRS-r[36]	28.0 (23.0-32.0)
ΔALSFRS	0.60 (0.33-1.08)
ΔALSFRS[36]	0.57 (0.31-1.00)
FVC%	79.5 (60.8-94.3)
pH	7.43 (7.41-7.44)
pCO2	39.5.0 (37.0-42.0)
pO2	81.2 (74.1-87.0)
HCO3-	25.1 (23.6-26.9)
Disease duration at PFTs (years)	1.1 (0.7-1.9)
	N (%)
Sex	
Female	251 (45.6)
Male	299 (54.4)
Type of onset	
Bulbar onset	193 (35.1)
Spinal onset	353 (64.2)
Respiratory onset	4 (0.7)
Respiratory symptoms (present)	134 (24.4)
Dyspnea only	124 (22.5)
Orthopnea only	89 (16.2)
Both dyspnea and orthopnea	79 (14.4)
Severe bulbar involvement (present)	123 (22.4)
NIV	
Yes	262 (47.6)
No	259 (47.1)
Unknown	29 (5.3)
Tracheostomy	
Yes	82 (14.9)
No	456 (82.9)
Unknown	12 (2.2)
Cognitive status (n=211)	
Normal cognition	110 (20.0)
ASLci, ALSbi, ALScbi	65 (11.9)
FTD	36 (6.5)
Not available	339 (61.6)

Table 12. Descriptive statistics for ALS patients' cohort used to find respiratory clusters.

We then proceeded to cluster analysis, as previously described in the Methods section. We identified five unique patients' clusters, with their optimal number (K) determined by considering both silhouette score (0.51) and consensus matrix, displayed in **Figure 6**.

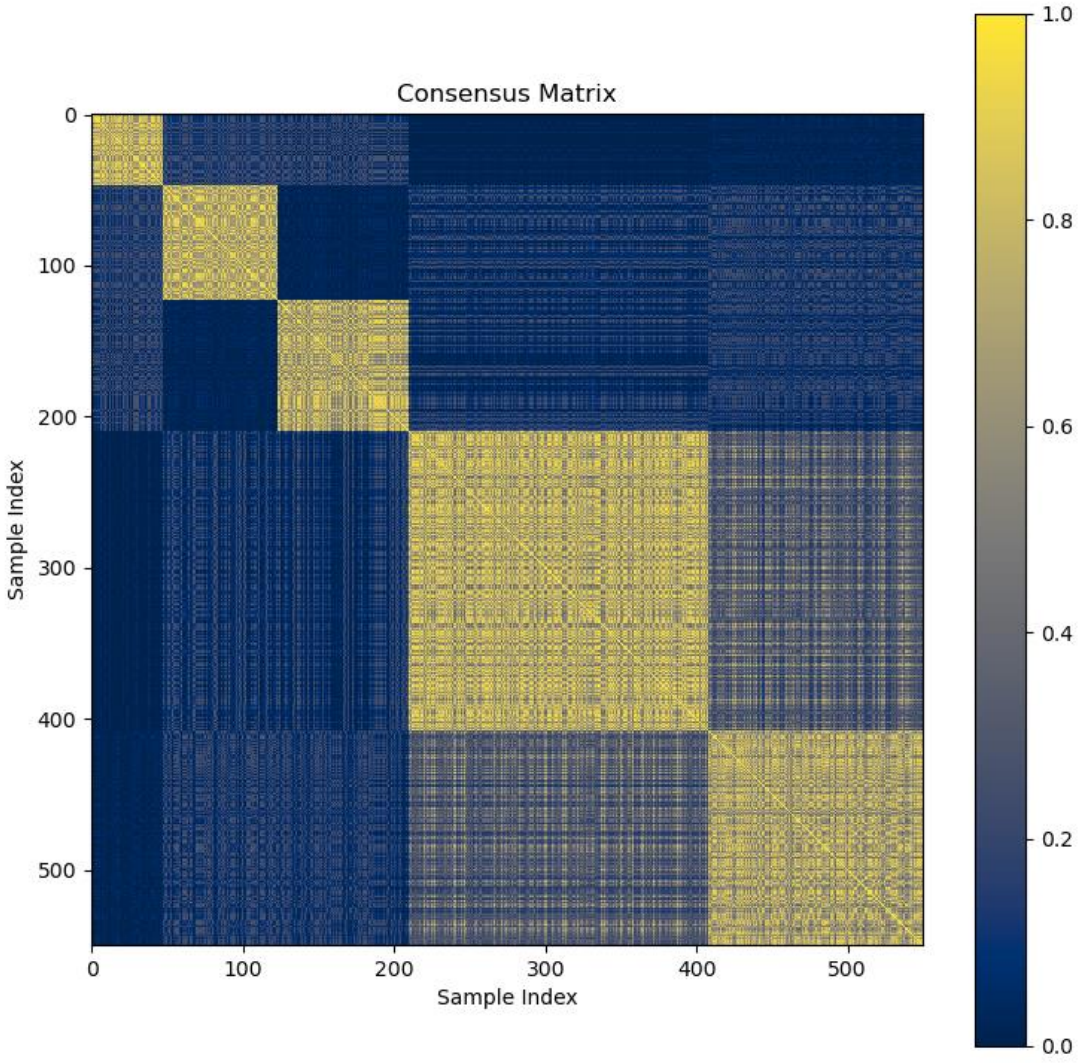

Figure 6. Consensus matrix for cluster stability assessment. The consensus matrix is constructed based on pairwise clustering co-occurrence of patients among different iterations of the clustering algorithm. The color yellow represents perfect consensus, the color blue represents no consensus, while the gradient between them, different degrees of consensus. The presence of five distinct yellow blocks suggests the presence of five different clusters of patients.

An additional validation of the solidity of patients' division into five different clusters was sought in t-SNE plots, shown in **Figure 7.** Based on the subsequent analysis of clinical and respiratory features of each cluster, we named them as follows:

1) normal ventilation cluster (NV);
2) asymptomatic hypoventilation cluster (AH);
3) severe bulbar cluster (SB);
4) symptomatic hypoventilation cluster (SH);
5) fast-progressing cluster (FP).

Significant differences in the distribution of certain variables were found to be statistically significant ($p < 0.05$) across the five clusters, while other variables showed differences only within specific cluster subsets. Demographic and clinical data of each cluster are shown in **Table 13** and in **Figure 8**.

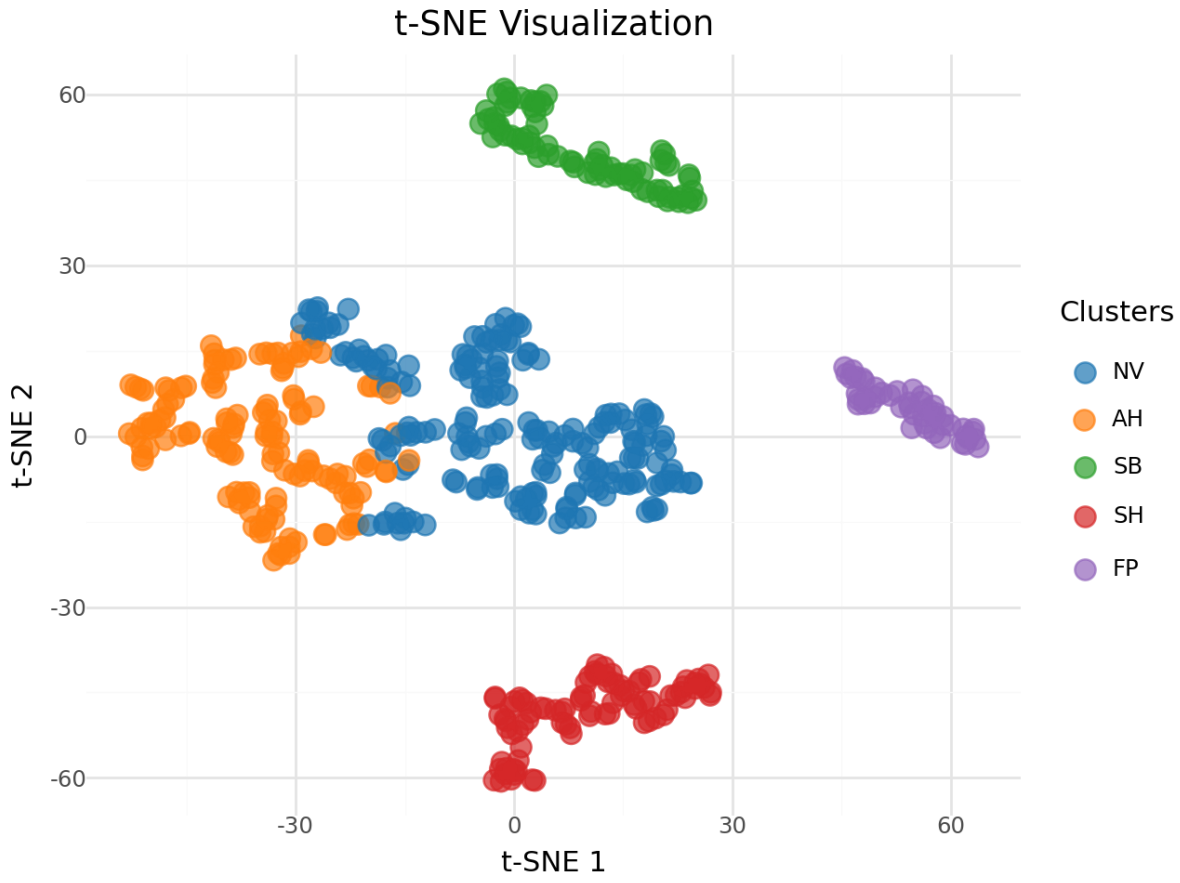

Figure 7. t-SNE plot. t-SNE plot visualizing the clustering of different data points into five distinct groups: NV, normal ventilation cluster; AH, asymptomatic hypoventilation cluster; SB, severe bulbar cluster; SH, symptomatic hypoventilation cluster; FP, fast-progressing cluster.

	Cluster 1 NV patients (n=198)	Cluster 2 AH patients (n=142)	Cluster 3 SB patients (n=76)	Cluster 4 SH patients (n=87)	Cluster 5 FP patients (n=47)	
	Median (IQR)					p*
Age at PFTs	64.6 (57.5-72.1)	67.8 (61.0-72.3)	70.4 (64.3-74.7)	68.0 (58.7-76.6)	66.1 (62.2-72.2)	**0.004**
ALSFRS-r total score	43.0 (39.0-45.0)	41.0 (37.0-45.0)	39.0 (33.0-43.0)	37.0 (32.0-40.0)	29.0 (22.0-36.0)	**<0.001**
ALSFRS-r^{36}	31.0 (27.0-33.0)	29.0 (25.0-33.0)	27.0 (21.0-31.0)	26.0 (21.0-30.0)	19.0 (13.0-25.0)	**<0.001**
ΔALSFRS	0.50 (0.25-1.00)	0.50 (0.30-1.00)	0.58 (0.40-0.94)	0.72 (0.50-1.33)	1.25 (0.78-2.00)	**<0.001**
ΔALSFRS^{36}	0.50 (0.25-1.00)	0.50 (0.30-1.00)	0.58 (0.40-0.94)	0.62 (0.30-1.00)	1.13 (0.75-1.77)	**<0.001**
ALSFRS-r bulb domain	12.0 (10.0-12.0)	11.0 (10.0-12.0)	7.5 (6.0-8.0)	11.0 (10.0-12.0)	7.0 (5.0-8.0)	**<0.001**
ALSFRS-r resp domain	12.0 (12.0-12.0)	12.0 (12.0-12.0)	12.0 (12.0-12.0)	10.0 (9.0-11.0)	10.0 (9.0-11.0)	**<0.001**
FVC%	96.9 (88.9-108.8)	67.4 (57.3-75.5)	70.9 (49.9-86.9)	54.5 (42.9-77.1)	50.9 (38.2-63.7)	**<0.001**
pCO2	38.0 (36.0-40.0)	41.0 (38.0-43.6)	40.0 (38.0-42.0)	40.5 (38.0-45.1	41.3 (37.0-46.0)	**<0.001**
HCO3-	24.2 (23.0-25.4)	26.2 (24.6-27.6)	25.2 (24.0-26.9)	26.1 (24.2-28.0)	26.3 (24.0-30.1)	**<0.001**
pO2	84.2 (79.0-92.0)	78.0 (71.3-84.0)	84.0 (78.0-87.1)	76.8 (71.0-83.3)	77.0 (70.4-84.9)	**<0.001**
Disease duration at PFT (years)	1.0 (0.6-1.8)	1.1 (0.7-1.6)	1.6 (0.9-2.0)	1.2 (0.8-2.3)	1.2 (0.8-1.7)	**0.006**
	n (%)					p§
Sex						**0.025**
Female	90 (45.5)	61 (43.0)	44 (57.9)	30 (34.5)	26 (55.3)	
Site of onset						**<0.001**
Bulbar onset	48 (24.2)	42 (29.6)	61 (80.3)	12 (13.8)	30 (63.8)	
Spinal onset	150 (75.8)	100 (70.4)	15 (19.7)	71 (81.6)	17 (36.2)	
Respiratory onset	0 (0.0)	0 (0.0)	0 (0.0)	4 (4.6)	0 (0.0)	
Respiratory symptoms	0 (0.0)	0 (0.0)	0 (0.0)	87 (100.0)	47 (100.0)	**<0.001**
Severe bulbar inv.	0 (0.0)	0 (0.0)	76 (100.0)	0 (0.0)	47 (100.0)	**<0.001**
NIV						**<0.001**
Yes	87 (43.9)	78 (54.9)	29 (38.2)	49 (56.3)	19 (40.4)	
No	92 (46.5)	63 (44.4)	44 (57.9)	32 (36.8)	28 (59.6)	
Unknown	19 (9.6)	1 (0.7)	3 (3.9)	6 (6.9)	0 (0.0)	
Tracheostomy						0.365
Yes	29 (14.6)	20 (14.1)	7 (9.2)	18 (20.7)	8 (17.0)	
No	162 (81.8)	121 (85.2)	67 (88.2)	67 (77.0)	39 (83.0)	
Unknown	7 (3.5)	1 (0.7)	2 (2.6)	2 (2.3)	0 (0.0)	
Cognitive status						0.305
Missing data	121 (61.1)	97 (68.3)	48 (63.2)	44 (50.6)	29 (61.7)	
Normal cognition	46 (23.2)	24 (16.9)	11 (14.5)	19 (21.8)	10 (21.3)	
Impaired cognition	31 (15.7)	21 (14.8)	17 (22.4)	24 (27.6)	8 (17.0)	

Table 13. Descriptive statistics of ALS patients' cohort, divided by respiratory clusters. NV, normal ventilation cluster; AH, asymptomatic hypoventilation cluster, AH; SB, severe bulbar cluster; SH, symptomatic hypoventilation cluster; FP, fast progressing cluster; IQR, interquartile ranges; PFTs, Pulmonary function tests.

*Kruskal-Wallis test, § chi-square test, significant differences ($p<0.05$) are written in bold.

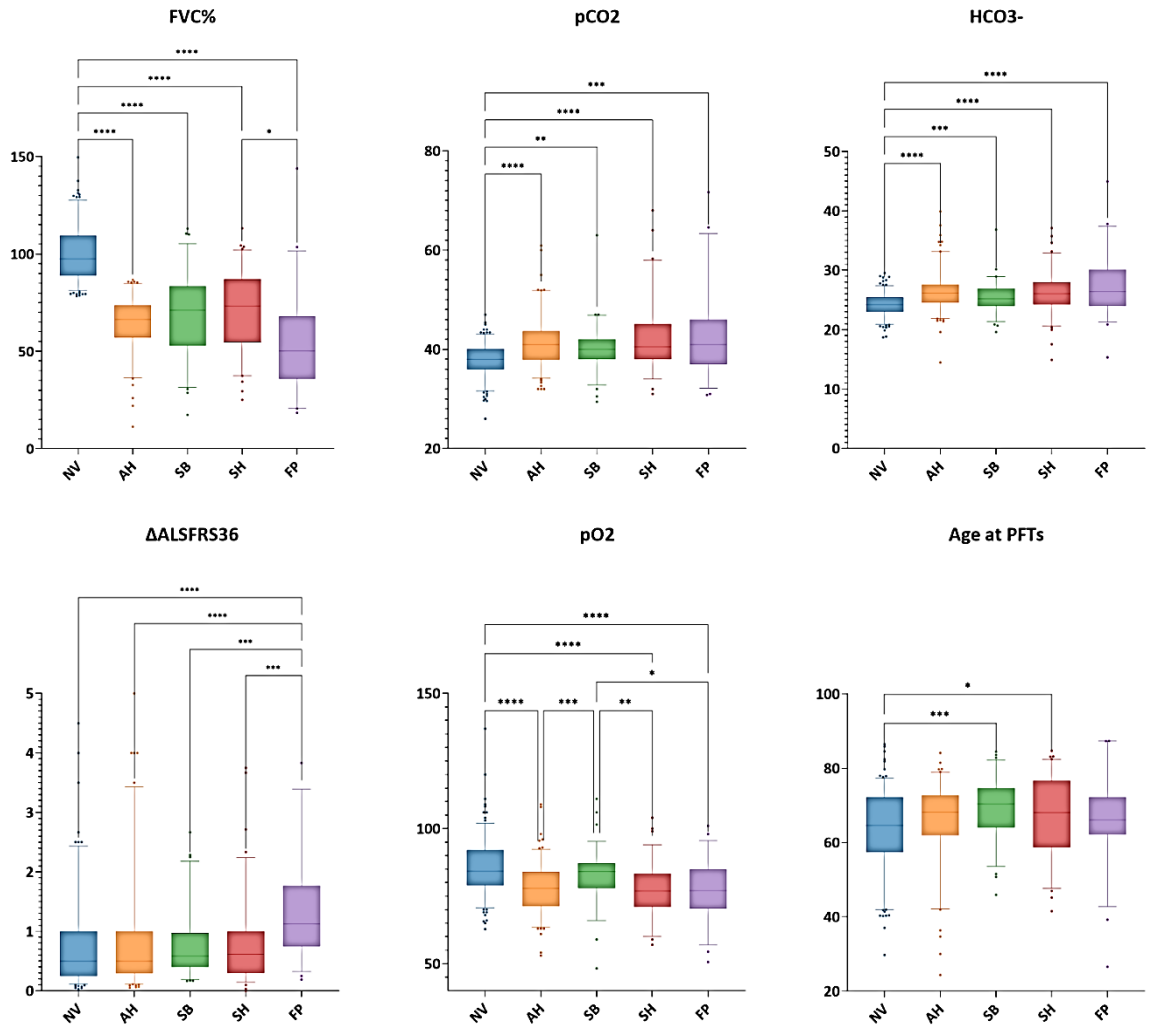

Figure 8. Box-plot representation of respiratory clusters main features.

FVC%, forced vital capacity; pCO2, partial pressure of carbon dioxide; HCO3- blood carbonate; ΔALSFRS36, progression rate, excluding ALSFRS-R respiratory items; pO2, partial pressure of oxygen; PFTs, pulmonary function tests; NV, normal ventilation cluster; AH, asymptomatic hypoventilation cluster; SB, severe bulbar cluster; SH, symptomatic hypoventilation cluster; FP, fast-progressing cluster.

Dunn's multiple comparison test: * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

The 5 clusters can be summarized as follows:

1. **Cluster 1 (NV, N=198)**: patients without evidence of respiratory failure. All patients did not report any respiratory symptoms and resulted to have significantly higher FVC values and lower pCO2 and HCO3- values when compared to all other groups.

2. **Cluster 2 (AH, N=142)**: patients with asymptomatic hypoventilation. These patients did not differ significantly from the NV cluster regarding ALSFRS-r total score, disease duration, ΔALSFRS36 and did not report respiratory symptoms or severe bulbar dysfunction, but all the respiratory measures resulted to be significantly altered when compared to NV cluster.

3. **Cluster 3 (SB, N=76)**: patients with severe bulbar impairment. This cluster included patients with severe bulbar dysfunction as prominent feature: the majority of them (80.3%) had a bulbar onset and for this reason were also prevalently women and older than the other clusters. Despite showing FVC reduction at spirometry, they interestingly showed higher pO2 values than AH, SH and FP.

4. **Cluster 4 (SH, N=87)**: patients with symptomatic hypoventilation. The patients in cluster 4 were indistinguishable from patients with asymptomatic hypoventilation (cluster 2) when considering FVC and ABGs values. Additionally, disease duration at the time of the first respiratory evaluation was similar in the two groups, while the ALSFRS-r total score and ΔALSFRS36 indicated a slightly greater functional impairment and faster progression rate in symptomatic patients, though the differences did not reach the threshold for significance.

5. **Cluster 5 (FP, N=47)**: patients with fast progression. With a median ΔALSFRS of 1.25 (0.78-2.00) points loss per month and a median ΔALSFRS36 of 1.13 (0.75-1.77) points loss per month, this cluster included patients with a rapidly progressing disease. All these patients showed respiratory and bulbar symptoms, very low ALSFRS-r total score and widespread regional involvement.

No significant differences in cognitive status were observed across the clusters. The use of NIV was less frequent in the SB and FP groups (p=0.039), whereas the rate of tracheostomy placement did not differ among the groups.

To analyze the effects on survival determined by the identified respiratory clusters, we performed Cox regression, that confirmed our clustering as an important covariate to predict survival ($p<0.001$). Considering NV cluster as reference, all other clusters showed higher HRs (AH 2.43, 95%CI 1.93-3.06; SB 1.85, 95%CI 1.41-2.45; SH 1.65, 95%CI 1.26-2.15; FP 3.36, 95%CI 2.41-4.70).

Kaplan-Meier curves analysis shown in **Figure 8** confirmed that NV cluster patients showed the highest survival from inclusion (24.4 months, 95% CI 21.3-27.6, log rank test p<0.001 vs all clusters) while FP cluster resulted in the shorter (8.7 months, 95% CI 5.1-12.3, log rank test p<0.05 vs all clusters). SH patients (14.5 months, 95% CI 10.3-18.6) differentiated in survival from AH patients (11.1 months, 95% CI 8.4-13.6, log rank test p=0.016) but not from SB patients log rank test (11.5 months, 95% CI 9.4-13.5, log rank test p=0.511).

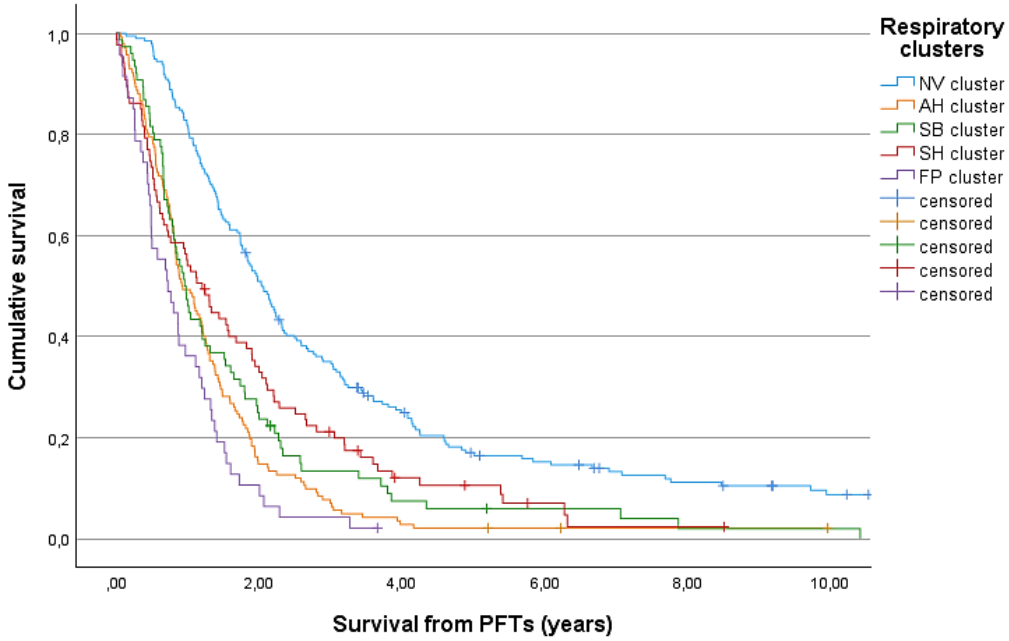

Figure 8. Kaplan-Meyer curves showing survival depending on identified clusters.

NV cluster initiated NIV significantly later than other groups, with no timing differences observed among the other groups (**Table 14**).

In our cohort, NIV significantly extended survival by approximately 6 months, with patients adapted to NIV showing a median survival of 17.4 months (95% CI: 15.6-19.2) compared to 11.4 months (95% CI: 9.9-12.9) for those not adapted (log-rank test, p<0.001).

The benefit of NIV varied by cluster (**Table 14**). SH patients showed the greatest survival advantage with a median difference of 13.5 months (Mann-Whitney U test p<0.001; Cox proportional hazards model: HR 0.546, 95% CI 0.380-0.785, p=0.001). AH patients also demonstrated a significant survival benefit, with a median difference of 6.1 months (Mann-Whitney U test p<0.001; Cox proportional hazards model: HR 0.542, 95% CI 0.343-0.856, p=0.009). While FP patients appeared to benefit from NIV adaptation (14.8 months vs 6.0 months, Mann-Whitney U test p=0.020), the

limited sample size rendered the Cox model results non-significant. No survival differences related to NIV use were observed in NV and SB patients at the time of PFT classification.

	PFT/NIV interval	**Survival from PFT (months)**				**Cox proportional hazard model adjusted for age and sex**	
	Median (IQR)	**NIV N [%] Median (IQR)**	**No NIV N [%] Median (IQR)**	**d**	**p***	**HR (95%CI)**	**p**
NV cluster	13.3 (7.6-21.5)	87 [48.6] 22.1 (15.3-38.1)	92 [51.6] 23.7 (11.6-38.7)	-1.7	0.601	0.979 (0.720-1.333)	0.895
AH cluster	4.2 (1.7-9.8)	78 [55.3] 15.5 (9.1-23.2)	63 [44.7] 9.4 (4.6-14.7)	6.1	**<0.001**	0.546 (0.380-0.785)	**0.001**
SB cluster	6.0 (3.4-12.7)	29 [39.7] 14.1 (8.6-23.6)	44 [60.3] 10.8 (6.8-20.4)	3.3	0.243	0.877 (0.541-1.419)	0.592
SH cluster	3.3 (0.8-11.5)	49 [60.5] 18.7 (8.6-30.1)	32 [39.5] 5.2 (1.9-12.5)	13.5	**<0.001**	0.542 (0.343-0.856)	**0.009**
FP cluster	2.4 (1.2-4.5)	19 [40.4] 14.8 (5.9-19.2)	28 [59.6] 6.0 (3.2-11.9)	8.8	**0.020**	0.549 (0.300-1.002)	0.051

Table 14. Survival according to NIV use in patients stratified in respiratory clusters.

PFT, pulmonary function test; NV: normal ventilation cluster; AH: asymptomatic hypoventilation cluster; SB: severe bulbar cluster; SH: symptomatic hypoventilation cluster; FP: fast progressing cluster.

*Mann-Whitney U test

4.4 Assessing demographic characteristics, timing and survival determinants for respiratory support in ALS

In the final part of our project, we aimed at studying the demographic characteristics, the timing and the survival determinants for respiratory support in ALS, to better guide choices in respiratory management of ALS patients and to predict the expected benefit from ventilatory support. For this

aim we collected information from 1159 patients that were diagnosed with ALS from 2008 to 2015, included in the PARALS registry (median age at onset of 68.4 years (IQR 60.3–74.7); 540 females (46.6%); 395 (34.1%) bulbar onset). The characteristics of patients according to the different respiratory supports are reported in **Table 15**. Patients adapted to NIV were the 33.7% of the whole cohort, 7.6% of patients underwent NIV followed by IMV, and 7.0% of patients underwent IMV without prior NIV initiation. On the other side, 620 patients (53.5%) did not undergo ventilation at all.

	Overall (n=1159)	**NIV (n=391)**	**NIV+IMV (n=88)**	**IMV (n=81)**	**No vent (n=599)**	**p**
Median age at onset, years (IQR)	68.4 (60.3-74.7)	66.8 (59.7-73.6)	62.3 (54.1-67.3)	68.3 (61.5-73.7)	69.9 (63.2-76.3)	**<0.001**
Median diagnostic delay, months (IQR)	9.0 (5.1-14.0)	9.0 (5.1-13.9)	8.1 (4.9-14.0)	9.0 (5.1-13.0)	8.9 (5.1-14.0)	0.57
Sex (female, %)	540 (46.6%)	174 (44.5%)	33 (37.5%)	33 (40.7%)	300 (50.1%)	0.054
Site of onset (bulbar, %)	395 (34.1%)	110 (28.1%)	32 (36.4%)	36 (44.4%)	217 (36.2%)	0.009
Education (≥11 years)	240 (20.7%)	80 (20.5%)	22 (25.0%)	16 (19.8%)	122 (20.4%)	0.78
Marital status (Married vs. Single/Widow-widower) §	857 (75.0%)	308 (79.6%)	67 (76.1%)	64 (80.0%)	418 (71.1%)	0.016
Median FVC% at diagnosis (IQR) #	89 (68-103)	87 (66-102)	87 (74-100)	81 (53-99)	90 (70-104)	0.017
Median monthly ALSFRS-R decay at diagnosis (IQR) °	0.66 (0.31-1.35)	0.59 (0.33-1.14)	0.59 (0.25-1.44)	0.79 (0.39-1.56)	0.68 (0.30-1.41)	0.076
ALS multidisciplinary clinic (yes)	1000 (86.3%)	366 (93.3%)	80 (90.9%)	67 (82.7%)	487 (81.3%)	**<0.001**
Cognitive status (FTD vs non-FTD) *	144 (19.4%)	37 (13.4%)	4 (6.0%)	13 (24.1%)	90 (26.0%)	**<0.001**

Table 15. Demographic characteristics of ALS patients included, according to ventilatory choice.

§ Available for 1143 patients (98.6%); # available for 1027 patients (88.6%); ° available for 1137 patients (98.1%); * available for 744 patients (64.2%).

The median survival time after NIV initiation was 1.00 year (IQR 0.51–2.34). Prognostic factors associated with a longer survival after NIV initiation are reported in **Table 16**, where two different models for multivariate analysis are shown: the first model not including the FVC% before NIV

initiation, and the second one including it (pre-NIV spirometry values were available for 308 patients).

In both models age at NIV remains an important factor influencing survival after NIV, with younger patients having an enhanced survival after NIV adaptation. When not considering FVC, disease progression rate and bulbar involvement at time of NIV initiation seem to influence survival, however when FVC was included in the model, they lost their significance with FVC being the strongest determinant of NIV outcome. Particularly, when FVC was included in the model, the grade of functional impairment of upper limbs was significantly associated with survival after NIV. In our opinion, this could be explained by the prion-like mechanism, that remains one of the etiopathogenetic hypothesis for the disease: indeed, lower motor neurons for upper limbs innervation, especially for the proximal muscles, are located near to the motor neurons for diaphragm innervation in the cervical medulla. So, a more severe upper limbs weakness could be associated with a more rapid worsening of respiratory function due to the spreading of disease with a prion-like mechanism [Gromicho M, 2020]. Finally, other variables, such as lower limbs functional impairment, the presence of respiratory symptoms, site of onset (bulbar/spinal), marital status, King's stage at time of NIV did not influence significantly survival after NIV (all variables included in the model are listed in Table 16 legend).

Model A				Model B			
Factor	**Level**	**HR (95% CI)**	**P value**	**Factor**	**Level**	**HR (95% CI)**	**P value**
Age at NIV	≥80	1	**0.017**	**Age at NIV**	≥80	1	**0.002**
	70-79	1.27 (0.85-1.90)			70-79	1.76 (1.04-2.86)	
	60-69	1.38 (0.96-2.05)			60-69	1.89 (1.16-3.09)	
	50-59	1.84 (1.23-2.73)			50-59	2.39 (1.44-3.97)	
	20-49	2.49 (1.47-4.22)			20-49	3.67 (1.78-7.59)	
ALSFRS-r decline from onset to NIV (points/months)	≥0.74	1	**0.0001**	**FVC% before NIV**	≥60	1	**0.0001**
	<0.74	1.76 (1.33-2.33)			<60	1.92 (1.43-2.66)	
ALSFRS-r bulbar sub-score at time of NIV	0-3	1	**0.003**	**ALSFRS-r upper limbs sub-score at time of NIV**	0-3	1	**0.026**
	4-7	1.56 (1.13-2.15)			4-7	1.28 (0.86-1.77)	
	8-11	1.71 (1.20-2.43)			8	1.83 (1.10-3.02)	
	12	1.89 (1.22-2.91)					

Table 16. Factors related to improved survival after NIV. Model A excluded FVC% (n=456); model B included FVC (n=296). Model A did not include FVC%, model B included FVC%.

The other variables included in the model were: ALSFRS-r lower limbs score (items 8 + 9); ALSFRS-r respiratory score (items 10 + 11); site of onset (bulbar/spinal); PEG performed before NIV (yes vs. no); time from diagnosis to NIV (≥1 year vs. <1 year); BMI mean monthly decline before NIV; education (≥11 years vs. <11 years); marital status (married vs. single, divorced, widow/widower); family history of ALS and/or FTD (yes/no); King's stage at time of NIV; MiToS stage at time of NIV.

Eighty-eight (18.4%) of the 479 patients who were initially adapted to NIV subsequently underwent IMV. In 74 cases (84.1%), IMV was performed when the dependence on NIV exceeded 20 hours/day, and in the remaining 14 for intervening acute events (infective or aspiration pneumonia).

A total of 81 patients with ALS (7.0%) underwent directly IMV. In these cases, the events leading to IMV were acute respiratory infections (31, 38.3%), aspiration pneumonia (23, 28.4%) and sudden respiratory failure (27, 33.3%). Factors related to an improved survival after IMV are reported in **Table 17**. Particularly, a younger age, a lower disease progression rate, the previous use of NIV and the married status were associated with a better survival after IMV. In **Figure 9** we show the Kaplan-Meyer curves comparing survival after NIV initiation depending on FVC% values before NIV (**Figure 9a**), comparing survival after IMV depending on previous NIV adaptation (**Figure 9b**) and comparing survival from onset depending on the choice of ventilatory support (**Figure 9c).** As expected, survival after NIV adaptation strongly depended on FVC values before adaptation (**Figure 9a**). Patients undergoing IMV who had been initially adapted to NIV showed a longer survival after IMV compared with those who had not (3.00 years (IQR 0.70–8.54) vs 1.58 years (IQR 0.59–3.66), p=0.014) (**Figure 9b**). Finally, we confirmed a positive impact on survival from ventilatory support (NIV/IMV) (**Figure 9c**).

Factor	Level	HR (95% c.i.)	P value
Age at IMV	≥80	1	0.0001
	70-79	1.80 (1.07-3.03)	
	60-69	2.30 (1.39-3.78)	
	50-59	3.98 (2.34-6.78)	
	20-49	4.66 (2.10-10.34)	
Previous NIV	No	1	0.009
	Yes	1.48 (1.10-1.98)	
ALSFRS-r decline from onset to IMV (points/months)	≥0.91	1	0.015
	<0.91	1.41 (1.07-1.86)	
Marital status	Single, divorced, widow/er	1	0.024
	Married	1.45 (1.05-2.00)	

Table 17. Factors related to improved survival in ALS after IMV.

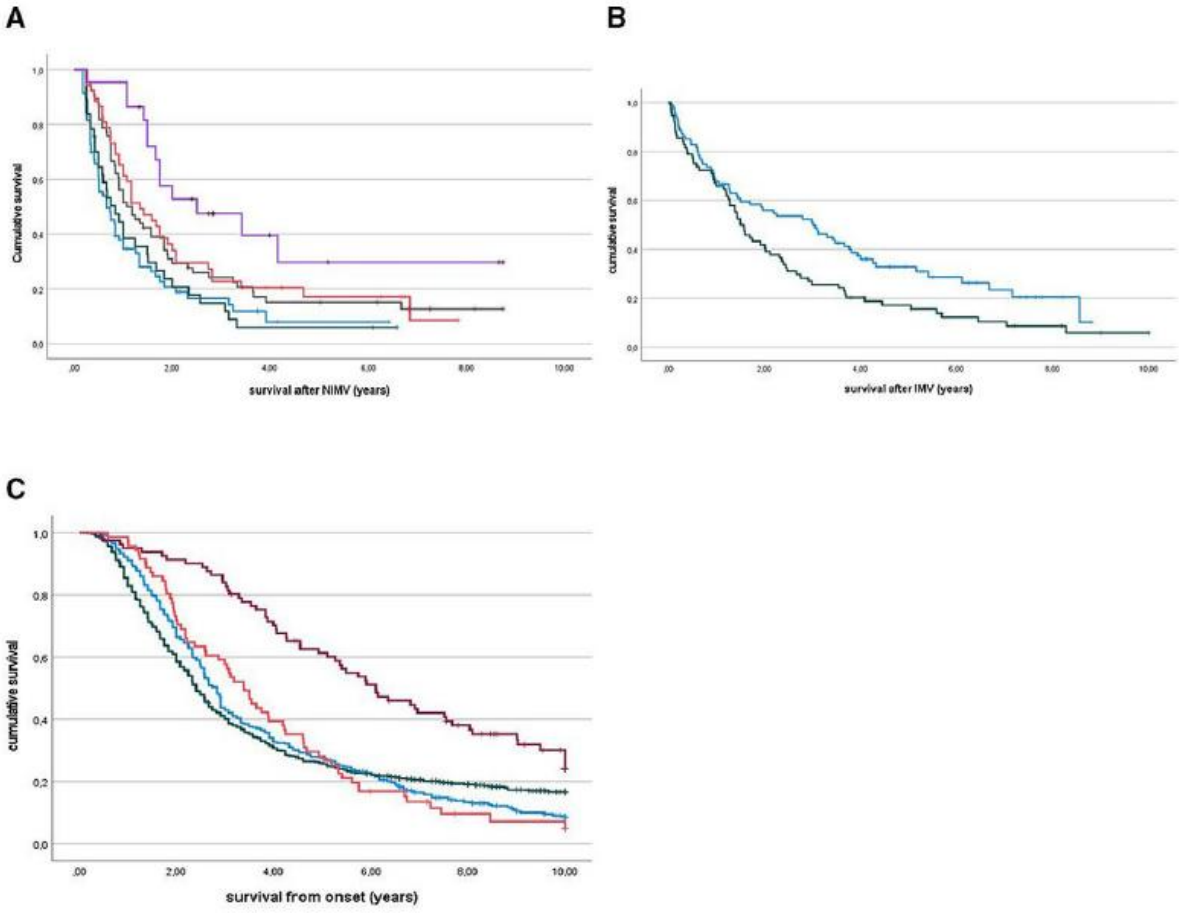

Figure 9. (A) Survival after NIV according to FVC% performed before NIV (n=308). FVC% <50% (blue) 85 cases, median survival time 0.66 year (IQR 0.33–1.66); FVC% 50–59 (green) 48 cases, median survival time 0.84 (IQR 0.43–1.84); FVC% 60–69 (black) 85 cases, median survival time 1.08 (IQR 0.74–2.75), FVC% 70–79 (red) 65 cases, median survival time 1.34 (IQR 0.75–2.82), FVC% ≥80 (violet) 25 cases, median survival time 2.51 (1.49– 5.00) (p<0.0001). **(B)** Survival after IMV in patients who did (blue) and did not (green) undergo NIV before performing IMV (p<0.014). **(C)** Survival from ALS onset. NIV+IMV (violet), median survival time 6.09 (IQR 3.89->10); IMV alone (red) median survival time 3.40 (IQR 1.95–5.41) (p<0.0001); NIV (blue) median survival time 2.84 (IQR 1.75–5.25); non-respiratory support (green) median survival time 2.41 (IQR 1.33–5.18).

5. Discussion

This PhD thesis had the main aim of increasing knowledge about respiratory markers in ALS, to facilitate and standardize respiratory monitoring in clinical practice, ensuring that respiratory function is measured consistently across different disease phenotypes and clinical settings. At the same time, we explored the main determinants affecting sensitivity and specificity of different respiratory markers, to individualize respiratory function assessment based on clinical characteristics, obtaining information about the real pulmonary function in ALS patients. Indeed, current different guidelines do not agree about the correct pulmonary management in ALS, regarding

both the type of monitoring and the cut-offs that should be used to indicate ventilatory support [EFNS, 2012; NICE, 2019; Miller RG, 2009].

5.1 Pattern of decline of respiratory measures varies depending on sex and site of onset. SNIP and PCF could be useful in monitoring bulbar patients

In our attempt of putting some order in respiratory function monitoring in ALS, we firstly assessed how some of the most widely used respiratory measures (FVC, SVC, SNIP and PCF) correlated among each other and changed during disease course, in a longitudinal observational multicenter study, named REVEALS. The necessity of resorting to a longitudinal study relied on the need to overcome the limitations of retrospective studies, from which most evidence about respiratory function assessment in ALS derives. Indeed, retrospective studies are more susceptible to selection and information biases and to quality of data issues, since they rely on existing records or participant recall about past symptoms, leading to inaccuracies in data interpretation. On the other hand, longitudinal studies overcome these limits and allow us to obtain more reliable and valid results, that could be exported to real-world populations.

In REVEALS a large cohort of more than 250 ALS patients were recruited and followed-up for a median time of one year. The decision to recruit patients in the middle stages of ALS (King's stage 2 and 3) was related to the need of selecting patients who were likely to experience respiratory decline during study duration, while being well enough to attend for assessment during follow-up.

The novelty of this study is that we focused the analysis on the different patterns of decline of respiratory measures, based on sex and site of onset, to discriminate how these two variables affect respiratory monitoring, and to identify more suitable tools to assess pulmonary function in ALS depending on them. The first evidence obtained from this stratification is that females patients showed the lowest scores for all respiratory measures, with bulbar females having the worst values among the groups, confirming a worse prognosis of bulbar onset disease, that is typically more common in females.

When we examined the crude correlations among different respiratory measures, we confirmed a strong correlation between FVC and SVC, as already known in literature [Pinto S, 2017]. Moreover, FVC and SVC showed similar pattern of decline in all patients, regardless site of onset and sex. This data aligns with previous findings [Pinto S, 2017], even if some studies recommend using SVC in patients with bulbar involvement to provide a more accurate estimate of respiratory function, being SVC easier to perform in this category of patients [Huang X, 2022]. Moreover, despite bulbar patients had slightly lower values for both FVC/SVC in comparison with spinal patients, we showed how type of onset did not influence FVC/SVC performance, since both spinal and bulbar patients revealed

similar pattern of decline during disease course. This could be explained by our choice of using facemasks for FVC/SVC assessment instead of the most commonly used mouthpieces, overcoming the limits deriving from leaks around mouthpieces in bulbar patients, secondary to lips weakness. Based on our results, we suggest using FVC and SVC interchangeably in clinical practice, regardless of site of onset, having the foresight to use facemasks, especially for bulbar patients. The slope of decline that we found for FVC% is in line with the one found in a previous study (−2.1%/month) [Pinto S, 2017]. On the other side, the SVC% slope was reported previously by three studies [Pinto S, 2017; Tattersall R, 2020; Andrews JA, 2018], two of which showing faster decline than our fastest sub-group (-2.2%/month in Pinto S, 2017; and -2.7%/month in Andrews JA, 2018).

On the other side, PCF and SNIP moderately correlated with both FVC and SVC, confirming previous reports [Zhang QJ, 2022; Park KH, 2016], so as PCF and SNIP moderately correlated with each other. At our knowledge, no previous study analyzed the correlation between PCF and SNIP. The moderate correlation among these respiratory measures is explained by the fact that all these measures are prognostic variables in ALS, despite examining different aspects of respiratory function. Indeed, while FVC and SVC assess the function of both inspiratory and expiratory muscles, PCF explores the strength of expiratory muscles involved in generating a forceful cough, including abdominal and intercostal muscles, while SNIP examines the strength of inspiratory muscles only. In our cohort PCF and SNIP showed lower values and a faster decline in patients with bulbar onset, regardless of sex. This trend is particularly evident in females with bulbar onset, that showed the lowest values and the steepest pattern of decline among all groups. Moreover, the correlation between PCF and SNIP was higher in bulbar patients when compared to spinal patients, suggesting that PCF and SNIP could be useful in monitoring respiratory function, especially in patients with facial weakness and bulbar impairment in comparison with FVC/SVC, in line with European guidelines recommendations [EFNS, 2012]. Regarding the slope of decline, for PCF one previous study found a decline of −5.77 L/min/month, that falls between our slowest and fastest subgroups [Rafiq MK, 2015], while Tattersall et al. observed a faster decline at −10.4 L/min/month, probably depending on the inclusion of patients with more advanced disease [Tattersall R, 2020]. On the other side, the slope of SNIP was only reported in two studies [-1.6 cmH2O/month for Tattersall R, 2020; - 3.00 cmH2O/months for Enache I, 2017], one of which showed a faster decline than our fastest sub-group [Enache I, 2017].

The ALSFRS-r respiratory sub-score was poorly correlated with other respiratory measures, confirming previous evidence and existing perplexities about its sensitivity and specificity for assessing respiratory function in ALS [Manera U and Torrieri MC, 2020; Cederbaum JM, 1999]. Moreover, ALSFRS-r respiratory sub-score was not able to distinguish patients with bulbar and spinal onset, as the other respiratory outcomes. Respiratory symptoms are a subjective report that could be absent also in patients with moderate/severe respiratory impairment, due to the

compensatory mechanism implemented by the organism during chronic respiratory failure, such as blood bicarbonate increase to avoid respiratory acidosis. Moreover, respiratory symptoms could be not specific of respiratory failure, since they could be caused also by other medical conditions. Finally, the reduced mobility due to muscle weakness in ALS patients could hide the appearance of respiratory symptoms even in individuals with severe pulmonary function reduction [Pinto S, 2015]. In our cohort the correlation between respiratory symptoms and other respiratory measures turned to be slightly higher in spinal than in bulbar patients. This could be explained by the fact that bulbar patients may have a difficulty in oral secretions management, leading to a subjective sensation of air hunger's feelings, even with a normal respiratory function. The slope of the ALSFRS-r respiratory sub-score was previously reported only by one study [Pinto S, 2017] and its value (-0.15 points/month) falls within the range spanned by our slowest and fastest groups.

Among all respiratory symptoms, the complaining of dyspnea at rest turned out to be more associated with survival than other symptoms, such as orthopnea and dyspnea when active. Moreover, a regular productive cough at baseline was associated with reduced PCF scores, so interrogating the patient about its presence could be useful to identify a red flag during disease course to address patients to respiratory function assessment. However, regardless of the subjectivity of respiratory symptoms and the weak association with real pulmonary function, monitoring their appearance during disease course remains a simple and fundamental tool to address ALS patients to more specific pulmonary instrumental tests, in line with current guidelines [EFNS, 2012; NICE, 2019; Miller RG, 2009].

5.2 Clusters of patients with distinct clinical and respiratory features and different survival and benefit from ventilatory support could be useful to stratify risk in ALS patients and guide decision making process in clinical practice

After having studied the pattern of decline of some important respiratory measures during disease course in ALS, we aimed at identifying prognostic clusters of patients, based on both respiratory and clinical features, that could be useful to stratify risk in patients, simplifying and guiding the respiratory monitoring and management in clinical practice. Indeed, respiratory failure presentation in ALS patients is heterogeneous and, in some patients, may be insidious, making it difficult to identify timely. Clustering analysis recently helped to differentiate subgroups based on clinical features, especially when analyzing longitudinal data [M Amaral D, 2024; Faghri F, 2022] and we thought that its use for identifying respiratory clusters would be useful to stratify risk and guide therapeutic decisions, such as the recourse to ventilatory support. Additionally, the definition of prognostic clusters of patients could inform RCTs, allowing to identify subgroups with different responses to treatment, and thus populations that may benefit most from specific interventions. In a

heterogeneous disease like ALS, this information could be crucial for validating the generalizability and robustness of trial results.

Specifically, in our study, consensus clustering was employed to identify distinct clusters of ALS patients based on clinical characteristics, FVC, and ABG results. The five respiratory clusters identified comprised patients without any clinical or instrumental signs of respiratory dysfunction (normal ventilation cluster, NV), patients with asymptomatic hypoventilation (asymptomatic hypoventilation cluster, AH), patients experiencing symptomatic hypoventilation (symptomatic hypoventilation cluster, SH), patients with significant bulbar impairment (severe bulbar cluster, SB), and patients with rapid disease progression and overt respiratory failure (fast progressing cluster, FP). This clustering analysis effectively revealed clinically interpretable groups, each characterized by unique survival outcomes and varying degrees of benefit from NIV use.

Even in this case, we confirmed the poor correlation between respiratory symptoms identified by the ALSFRS-r respiratory sub-score and the respiratory measures. Indeed, about 25% of patients were classified in the asymptomatic hypoventilation (AH) group, that despite not complaining of respiratory symptoms, already showed altered respiratory measures in comparison with patients with normal respiratory status (NV). Particularly, they had lower FVC% values (<70%), increased levels of HCO3- (>26 mmol/L) and reduced levels of pO2 (78 mmHg) at ABG, meaning the appearance of nocturnal hypoventilation, the earliest marker of respiratory failure in ALS. Notably, these patients, despite maintaining relatively preserved functional abilities and demographic and clinical features similar to NV patients, including same disease duration at time of PFTs, had a reduced survival and a shorter time to NIV adaptation in comparison with NV patients. This finding highlights the importance of performing spirometry, or even ABG or nocturnal saturimetry, at diagnosis and during regular follow-up, since respiratory symptoms may lack even in presence of initial respiratory failure.

Another unexpected finding was that patients with AH had a shorter survival compared to those in the symptomatic hypoventilation (SH) cluster, despite the latter group having a lower ALSFRS-r total score and FVC. This evidence could be explained by the fact that SH patients showed the highest benefit from NIV in comparison with all the other clusters (more than 18 months), probably because patients with respiratory symptoms tend to adapt more easily and to adhere more strictly to NIV than asymptomatic patients.

The cluster of patients characterized by severe bulbar involvement (SB), despite having FVC% values around 70% and ABG values altered in comparison with NV patients although remaining in the normal range, did not complain of respiratory symptoms. ABG is a more sensitive tool for respiratory failure in ALS, being a non-volitional test, that could be particularly useful in bulbar patients in comparison with FVC [Manera U and Torrieri MC, 2020], especially when for FVC performance, a mouthpiece is used instead of a facemask, as commonly happening in clinical settings. However, this cluster of patients, that had a higher frequency of bulbar onset disease,

showed a reduced survival that was similar to SH patients, confirming a poor prognosis in patients with bulbar onset. For this cluster of patients NIV was prescribed with lower frequency (less than 40%), confirming previous evidence, due to a more complicated adaptation to ventilatory support secondary to difficulty in oral secretion management and higher frequency of cognitive dysfunction [Sancho J, 2018], even though in our analysis we did not find an increased frequency of cognitive impairment for this cluster, probably depending on the limited number of patients with an available cognitive evaluation. However, our survival analysis revealed that patients with severe bulbar involvement did not have any benefit from NIV prescription, confirming perplexities about its use for this category of patients.

The last cluster identified corresponded to fast progressing patients (FP), that showed the highest disease progression rate (1.25 points/month) and the worst prognosis among groups. Clinically they often complained of respiratory symptoms, showing markedly reduced FVC values and signs of nocturnal hypoventilation at ABG with still normal pCO2 values, but reduced in comparison with NV patients.

With our cluster analysis, we confirmed that the emergence of respiratory symptoms in ALS patients could depend on a combination of several factors, including FVC values, pCO2, general impairment and disease progression rate, each one playing a different role in different clusters of patients, thus explaining the heterogeneity of respiratory failure presentation.

An important information that we gave with our study is the time to NIV indication for each cluster of patients, with FP showing the shortest and NV the longest time to NIV prescription among clusters. This data could allow to stratify risk in ALS patients, based on clinical and respiratory features, being particularly useful in clinical practice to plan respiratory monitoring and start NIV timely, thus increasing quality of life and survival.

Finally, we found out that NIV benefit differs among patients. Indeed, when considering all patients NIV significantly extended survival by approximately 6 months, while when we stratified survival analysis by the identified clusters, we found out that SH patients showed the greatest survival advantage (almost 14 months of benefit), followed by FP patients (almost 9 months) and AH patients (6 months). As mentioned for SB patients we did not find any significant benefit on survival by starting NIV, confirming previous evidence [Sancho J, 2018]. Knowing the expected benefit from ventilatory support, based on clinical and respiratory features of ALS patients, is of utmost importance for guiding clinical choices, including NIV prescription, to ameliorate patients' outcome.

5.3 MVV and blood serum chloride are markers of early respiratory failure in ALS

After having examined the presence of prognostic clusters of patients, based on the most widely used respiratory markers, particularly ABG and FVC, we aimed at exploring the potential of less used tools for respiratory function monitoring in ALS. Indeed, both ABG and FVC, despite having a good prognostic value and a good sensitivity in pulmonary function assessment, show some limitations. Particularly ABG has a higher sensitivity in diagnosing NH, in comparison with FVC [Manera U and Torrieri MC, 2020], and its role is particularly useful not only for NIV indication, but also for monitoring NIV adherence after NIV initiation [NICE, 2019; Miller RG, 2009; Windisch W, 2018]. Moreover, it is an easy to perform and quick tool to assess respiratory function and it has the advantage of being a non-volitional test, particularly useful in patients with bulbar impairment or cognitive dysfunction, that could show some difficulties in performing PFTs. However, ABG is also considered a minimally invasive exam, that could cause discomfort to patients, limiting its use in some clinical contexts. On the other side, FVC is the main respiratory marker in ALS, that is strongly associated with survival, being widely used to assess respiratory function at diagnosis, during follow-up, for NIV indication and after NIV adaptation [EFNS, 2012; NICE, 2019; Miller RG, 2009]. However, as mentioned, its sensitivity has been questioned in patients with bulbar impairment or cognitive dysfunction, since the lack of coordination or cooperation may compromise the validity of the obtained score, so that European guidelines recommend using SNIP for bulbar patients [EFNS, 2012].

To overcome these limits, we aimed at exploring the potential role of other less used markers for assessing respiratory function, evaluating their sensitivity for early detection of respiratory failure and their prognostic value in ALS.

Firstly, we examined the role of MVV at diagnosis in a retrospective study including a large single-center cohort of ALS patients. MVV is a poorly studied measure in ALS, being able to assess respiratory muscle strength and endurance. Currently only a few clinical trials used MVV to assess pulmonary function in ALS [Berto MC, 2007; Sathyaprabha TN, 2010; Heiman-Patteson TD, 2021], and its role in diagnosing early respiratory impairment and monitoring pulmonary function is not clear.

For all patients of our cohort we calculated MVV from FEV1 at diagnosis, obtaining the so called cMVV, while for a subset of patients for which PIF was available, we obtained the so called cMVV(Dillard), demonstrating that the two measures highly correlate and thus, they can be used interchangeably for assessing respiratory function.

We showed how cMVV(40) moderately correlate with FVC% and FEV1% and ALSFRS-r total score, being a good marker of respiratory function and of functional impairment in ALS, confirming previous findings [de Carvalho M, 2022]. The moderate correlation between cMVV(40) and FVC/FEV1 could be explained by the fact that cMVV examines a different aspect of respiratory function. Particularly, cMVV assesses not only the strength of respiratory muscles, but also the

endurance, that is not normally explored with routine spirometry. Fatigability is one of the earliest symptoms in ALS when muscles weakness appears and, based on this, MVV could be an earlier marker of respiratory failure detection.

When we evaluated the correlation of cMVV(40) with ABG parameters, we found out a moderate correlation of cMVV(40) with HCO3-, SBE, and pO2, and only a weak correlation with pCO2, showing how cMVV(40) could be a tool to screen for NH, and confirming the importance of both HCO3- and SBE as earlier and more sensitive markers of respiratory failure in ALS in comparison with pCO2 [Manera U and Torrieri MC, 2020].

However, in our cohort cMVV(40) showed a negative correlation with bulbar impairment, so as at higher grades of bulbar impairment corresponded cMVV(40) lower values. This could be due either to a more advanced disease in patients with bulbar impairment, reflecting a higher chance of having respiratory failure, or to a reduced ability of patients with facial weakness in performing spirometry, and thus MVV, from which it is derived. The fact of MVV potentially being influenced by bulbar impairment is not novel and has already been reported by de Carvalho et al. [De Carvalho M, 2022]. We think that this limit could be overcome by using facemasks instead of mouthpieces, as suggested by our findings during REVEALS study. However, the influence of bulbar impairment on MVV score seems to be little, since we demonstrated how MVV is an independent predictive factor of survival in ALS, regardless of bulbar involvement.

Additionally, as for other respiratory tests, cMVV(40) showed only weak correlation with disease ALSFRS-r respiratory items, confirming that ALSFRS-r respiratory domain reflects subjective reports that are not specific for respiratory failure. Finally, both cMVV(40) and cMVV(Dillard) values are not influenced by pulmonary obstructive signs at spirometry (FEV1/FVC ratio), potentially being able to be used also in ALS patients with pulmonary obstructive diseases, such as COPD.

In our analysis cMVV(40) showed good sensitivity and specificity in predicting survival and time to NIV initiation, as shown by ROC curves, and we found out that the cut-off value of 80% for cMVV(40) could be used as the limit for normality in clinical practice to stratify patients with and without respiratory impairment with good accuracy.

As mentioned, our study confirmed previous findings about the role of MVV as an independent predictive factor for survival in ALS [Zhang QJ, 2022]. Indeed, we showed how both cMVV(40) and cMVV(Dillard) were associated with survival and time to NIV initiation, regardless of other important prognostic factors in ALS, such as sex, age, progression rate, site of onset and bulbar involvement. Notably, we found out that cMVV(40) could be particularly useful for detecting early respiratory failure in patients with normal FVC values. Indeed, when multivariate analysis was stratified for patients with normal/not normal FVC values, cMVV maintained its prognostic role only

in patients with normal FVC values. This result suggests that when FVC, that is strongly associated with pulmonary function and survival in ALS, is already reduced, overcomes and hides cMVV prognostic role; on the other side, when FVC is still normal, cMVV values reduction under 80% is associated with a significant reduction in survival and time to NIV, being an earlier marker of respiratory failure in ALS. This evidence suggests using MVV as a marker of early respiratory failure in ALS, to be performed at diagnosis and in the initial phases of disease, to stratify risk in patients.

As for MVV, we examined the potential prognostic role of serum chloride levels at diagnosis in ALS. We thought that serum chloride could be useful to detect early respiratory impairment, since its values reduce as a renal compensatory mechanism in a context of respiratory acidosis. More precisely, to compensate respiratory acidosis the renal proximal tubule increases the secretion of hydrogen ions, resulting in higher retention of sodium bicarbonate and lower retention of sodium chloride. Its role as biomarker has already been studied in other chronic pulmonary diseases [Naal T, 2018], however poor is known about its potential function in ALS. Particularly, Stambler et al. and Hadjikoutis et al. showed how serum chloride correlates with respiratory symptoms appearance and survival in ALS, using data from ALS clinical trials and from a small cohort perspective study, respectively [Stambler N, 1998; Hadjikoutis S, 2001]. Despite being a non-invasive, easy to perform and low-cost tool, serum chloride is not usually checked in ALS patients and is not even considered by international guidelines for respiratory monitoring.

Based on this evidence, we assessed the potential role of blood serum chloride levels at diagnosis as a marker of respiratory function in a retrospective center-based study with a large cohort of ALS patients.

As expected, serum chloride levels at diagnosis were not influenced by demographic features. Bulbar and spinal onset patients had similar levels of serum chloride, while respiratory onset patients showed a reduction in its levels, being associated with respiratory impairment and reduced survival.

Again, ALSFRS-r respiratory items only weakly correlated with serum chloride levels. Notably, also FVC% values did not show a good correlation with serum chloride. This could be due to the non-linear reduction in time of serum chloride, in comparison with FVC. Indeed, the longitudinal ACTS study showed that serum chloride levels decline slowly in the initial phases of disease, and then drop sharply at 4–5 months before death [Stambler N, 1998].

In our cohort serum chloride levels at diagnosis showed a prognostic independent role in predicting survival and time to NIV initiation in ALS, with reduced levels being associated with a worse prognosis, despite site of onset. Particularly, the cut-off value of 103.0 mmol/L can help at diagnosis to stratify patients in different prognostic categories. This is unexpected, since normal range is usually considered between 96-106 mmol/L [Walker HK, 1990]. Based on our results, we suggest addressing patients with serum chloride <103 mmol/L at diagnosis to a prompt and stricter

respiratory monitoring for checking signs of initial respiratory failure on time. The importance of serum chloride levels as prognostic factor had been described by Qureshi et al., that using a random effects model multivariate analysis, observed that vital capacity decline was faster and survival was shorter in participants who had low blood chloride levels [Qureshi M, 2008]. Moreover, serum chloride at the baseline affected the rate of progression of the vital capacity, with a decrease by 0.62 in %VC slope for each serum chloride unit decreased [Qureshi M, 2008]. This evidence was confirmed by our study, in which we found out that lower chloride levels were associated with faster ALSFRS-r rate of decline.

A particular aspect that we found is that lower serum chloride levels were associated with an increase of blood inflammatory markers, especially neutrophils and NLR, confirming the hypothesis of an inflammatory etiopathogenetic component in ALS, that could worsen with disease spreading [McCombe PA, 2011]. Moreover, the association of serum chloride with systemic inflammation could highlight the role of serum chloride as a marker of NH, since several studies found a significant association between SDBs and some inflammatory indexes, such as the NLR [Uygur F, 2016; Bozkuş F, 2018].

5.4 Ventilatory support usage remained stable in the last years and is able to enhance survival in ALS, depending on clinical features and respiratory measures

In the final part of this PhD thesis, we described the demographic characteristics, the timing and the survival determinants for respiratory support in ALS, including both NIV and IMV, to better guide choices in respiratory management of ALS patients and to predict the expected benefit from ventilatory support.

Initially we examined the data about prevalence of NIV in a retrospective study involving ALS patients diagnosed from 2008 to 2015 included in the PARALS registry. Indeed, prevalence data of NIV usage in the last 15 years are missing and the last available information dates back to 2008, when a rate of about 20% of ALS patients with respiratory impairment had been ventilated with NIV, with about half patients receiving ventilatory support, considering both NIV/IMV [Chiò A, 2012; Gordon PH, 2015]. In our cohort from 2008 to 2015 about 33% of patients were adapted to NIV, confirming a positive trend in NIV usage in the last years. About 50% of ALS patients underwent ventilatory support in our study, including patients receiving NIV, and patients who underwent primary IMV (without prior NIV adaptation, 7% of ALS patients) and IMV following NIV (about 7% of ALS patients). Overall, the proportion of patients undergoing ventilatory support remained stable in the last years, comparable to the one of early years of 2000 [Chiò A, 2012]. Prevalence data of IMV in our cohort was slightly superior to that found in a large German cohort from 2007 to 2019

(9.5% of patients, including both primary IMV in 7.2% of patients and IMV with preceding NIV in 2.3% of patients) [Spittel S, 2021]. In our cohort, about 18% of patients using NIV underwent IMV, being generally younger and with a lower frequency of cognitive dysfunction in comparison with patients who did not undergo IMV after NIV and with patients who received primary IMV. Additionally, the transition from NIV to IMV was significantly more frequent in patients followed by multidisciplinary clinics, being a choice almost invariably planned in advance by the patients with clinicians and caregivers. Indeed, only in about 15% of cases IMV was performed for intervening acute events, such as infective or aspiration pneumonia. On the other side, in case of primary IMV indications for its positioning were emergency situations, such as acute respiratory infections (38.3%), aspiration pneumonia (28.4%) and sudden respiratory failure (33.3%).

We confirmed a benefit from ventilatory support initiation, confirming its use as the gold standard for respiratory failure treatment in ALS. The median survival time after NIV initiation was 1 year in our cohort, highlighting an increased benefit of NIV on survival in comparison with previous years, when the increase in survival was about 9 months [Chiò A, 2012]. This could be due to the increasing expertise of NIV usage and management by tertiary centers, that leads to an increased adherence and thus efficacy of ventilatory support.

As expected, the strongest independent prognostic factor influencing survival after NIV was FVC values before NIV adaptation. However, other significant variables enhancing survival after NIV were younger age, lower disease progression rate, lower bulbar involvement and lower functional impairment of upper limbs. While it seems easy to explain how a higher bulbar involvement could be associated with reduced survival after NIV, mainly linked to a more difficult management of oral secretions with an increased risk of aspiration pneumonia, explaining how upper limbs (and not lower limbs) involvement is associated with survival could be more challenging. To explain this association, we call into question the prion-like mechanism, a well-known etiopathogenetic hypothesis for ALS disease: indeed, lower motor neurons for upper limbs innervation, especially for the proximal muscles, are located near to the motor neurons for diaphragm innervation in the cervical medulla. So, a more severe upper limbs weakness could be associated with a more rapid worsening of respiratory function due to the spreading of disease with a prion-like mechanism [Gromicho M, 2020].

A younger age and a lower disease progression rate were associated with an improved survival also after IMV, so as a previous use of NIV (3.00 years for IMV following NIV vs 1.58 years for primary NIV). Particularly the married status did not influence survival after NIV but turned out to be associated with survival after IMV. Indeed, married patients showed a higher survival when compared to single/divorced/widow patients, being probably linked to the importance of a caregiver in tracheostomy management.

6. Limitations

Despite being a comprehensive study about respiratory markers in ALS and their use in respiratory monitoring and management, our analysis shows some limits.

First, in our longitudinal study, so as in the retrospective analysis for MVV and serum chloride, and in cluster analysis, we did not include other important respiratory measures, such as MIP, MEP and nocturnal oximetry, to make a proper comparison. The reasons why we did not include these measures are different, depending on different studies. Particularly, for REVEALS study, we decided to include only the longitudinal assessment of the most widely used respiratory markers in ALS, to simplify the study design and the interpretation of the results. For MVV and serum chloride analysis, instead, it depended on the retrospective nature of the data, that did not include respiratory measures other than those described. However, this aspect could be deepened in future longitudinal studies.

Second, when we studied the demographic and clinical determinants of ventilatory support in ALS, we could not include cognitive impairment in the multivariable models, since FTD patients were less likely to undergo NIV, hindering the possibility to unbiasedly assess the effect of cognitive impairment on survival. Additionally, most patients undergoing ventilatory support attended a multidisciplinary clinic, limiting the possibility to evaluate the effect of multidisciplinary care on mechanical ventilation outcome.

7. Conclusions

Our study investigated the complex issue of respiratory function monitoring and management in ALS.

In a longitudinal study we showed how the most widely used respiratory measures correlate with each other and decline over time. We demonstrated how pattern of decline varies, depending not only on the type of respiratory measure, but also on sex and type of onset. Particularly, female patients show lower values in all respiratory measures and female patients with bulbar onset have the faster decline in respiratory scores, confirming poor prognosis for this subgroup of patients. SNIP and PCF turned out to be particularly useful in female patients with bulbar onset. We showed how FVC and SVC strongly correlate and can be used interchangeably in clinical practice. Additionally, they are not influenced by bulbar involvement if facemasks are used in place of mouthpieces. We confirmed that ALSFRS-r respiratory sub-score accuracy in predicting respiratory failure in ALS in low, even though regular monitoring of respiratory symptoms appearance remains recommended in clinical practice.

Our cluster analysis identified prognostic clusters of ALS patients, based on both respiratory and clinical features, being useful to stratify risk in clinical practice, guiding the timing of respiratory monitoring and helping in clinical choices. We showed how specific clusters of patients had a higher benefit from NIV prescription, such as symptomatic and fast progressing patients, while others did not have any significant effect on survival, such as patients with severe bulbar involvement.

We showed how less used tools could be helpful for monitoring respiratory function in ALS. Particularly, both MVV and serum chloride levels at diagnosis turned out to have a prognostic function in ALS, being able to predict time to NIV initiation and survival. Specifically, MVV is able to detect respiratory failure in ALS earlier than FVC and should be assessed at diagnosis to stratify risk in patients and guide respiratory monitoring.

Finally, we described an increased use of NIV in ALS patients in the last years, confirming the positive trend of the early years of 2000. We confirmed the significant benefit on survival deriving from both NIV/IMV, strengthening ventilatory support use as the gold standard of respiratory failure treatment in ALS. Factors influencing survival after NIV/IMV were described and FVC turned out to be the strongest independent prognostic factor, while only bulbar involvement at time of ventilatory support initiation and not bulbar onset influenced survival after NIV. Knowing the factors affecting survival after ventilatory support and the expected benefit from it is particularly useful to guide the choice of starting ventilatory support in clinical practice.

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