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DIPLÔME D'ÉTAT DE DOCTEUR EN MÉDECINE

Qualification en NEUROLOGIE

DETECTION OF TDP-43 PATHOLOGY IN MINOR SALIVARY GLANDS OF PATIENTS WITH AMYOTROPHIC LATERAL SCLEROSIS

Détection de la pathologie TDP-43 dans les glandes
salivaires accessoires de patients atteints de Sclérose
Latérale Amyotrophique

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Liste des abréviations (français)

BGSA	Biopsie des Glandes Salivaires Accessoires
SLA	Sclérose Latérale Amyotrophique
SNC	Système nerveux central

Liste des abréviations (anglais)

AD	Alzheimer's disease
ALS	Amyotrophic Lateral Sclerosis
ALSFRS-R	ALS Functional Rating Scale – Revisited
CNS	Central Nervous System
HRP	Horse Radish Peroxidase
MAG	Myeline Associated Glycoprotein
MRI	Magnetic Resonance Imaging
MSG	Minor Salivary Gland
PD	Parkinson's Disease
pTDP-43	Hyperphosphorylated TDP-43 Protein
TDP-43	Transactive Response DNA-binding Protein 43

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INTRODUCTION

Ce travail de thèse, encadré par le Pr Philippe CODRON, porte sur la recherche de marqueurs pathologiques de Sclérose Latérale Amyotrophique (SLA) dans les biopsies de glandes salivaires accessoires (BGSA).

1. Sclérose latérale amyotrophique, présentation et enjeux

1.1. La sclérose latérale amyotrophique

La sclérose latérale amyotrophique (SLA), également connue sous le nom de maladie de Charcot en France, est une maladie neurodégénérative caractérisée par une perte progressive des motoneurones centraux et périphériques conduisant à une paralysie progressive et généralisée. La survie médiane est de 2 à 4 ans, principalement liée à la progression de l'atteinte respiratoire (1). Avec plus de 7 000 patients touchés en France, la SLA est la troisième maladie neurodégénérative de l'adulte (2). En France, l'incidence est de 2,5 nouveaux cas / 100 000 habitants / an, avec un âge médian au diagnostic de 70 ans (3). Dans la grande majorité des cas, la SLA est dite sporadique, sans étiologie identifiée à ce jour mais avec une très probable participation multigénique et environnementale (exposome). Dans 10% des cas, la SLA est génétique, liée à la présence d'une mutation sur l'un des gènes pouvant être responsable de la maladie (4). Il n'existe pas à ce jour de traitement curatif de la SLA, la seule molécule possédant une AMM en France, le RILUZOLE, a démontré un effet protecteur permettant une prolongation de la survie de plusieurs mois (5). Le développement récent de thérapies géniques permettant de stabiliser la maladie dans certaines formes génétiques de SLA laisse toutefois entrevoir des perspectives encourageantes, tant dans les situations familiales que sporadiques (6).

1.2. Des difficultés diagnostiques

Le diagnostic de SLA peut être difficile ; en l'absence de test diagnostique, il reste principalement clinique et électrophysiologique, basé sur l'association de signes d'atteinte des motoneurones centraux et périphériques aux niveaux bulbaire, cervical, thoracique et lombaire. Le diagnostic de SLA est rendu particulièrement complexe par l'importante diversité des phénotypes observés dans la SLA (7, ANNEXE 1), pouvant conduire à un retard diagnostique avec un délai médian de 10 à 16 mois entre les premiers symptômes et l'établissement du diagnostic (8). Dans ce contexte, élucider les voies pathologiques impliquées dans la maladie afin d'identifier un biomarqueur qui puisse aider au diagnostic constitue un enjeu majeur en recherche.

1.3. Une physiopathologie complexe et encore imparfaitement comprise

En effet, en dehors des formes génétiques de la maladie, l'étiologie de la SLA reste inconnue, et sa physiopathologie mal comprise (1). Toutefois, malgré la diversité des phénotypes cliniques, presque tous les patients, indépendamment de leur présentation, partagent un profil neuropathologique post-mortem similaire, suggérant des voies physiopathologiques communes. La protéine TDP-43, dont l'implication dans la SLA a été identifiée en 2006, se trouve en effet chez les patients hyperphosphorylée (pTDP-43) et agrégée dans le cytoplasme des neurones (9,10) (Figure 1). La protéine pathologique pTDP-43 diffuse au sein du neurone via des mécanismes de type prion-like, et est également transférée passivement et activement aux cellules environnantes tels que les neurones ou les cellules gliales, contribuant à la progression de la pathologie dans le système nerveux central (SNC) des patients (11,12).

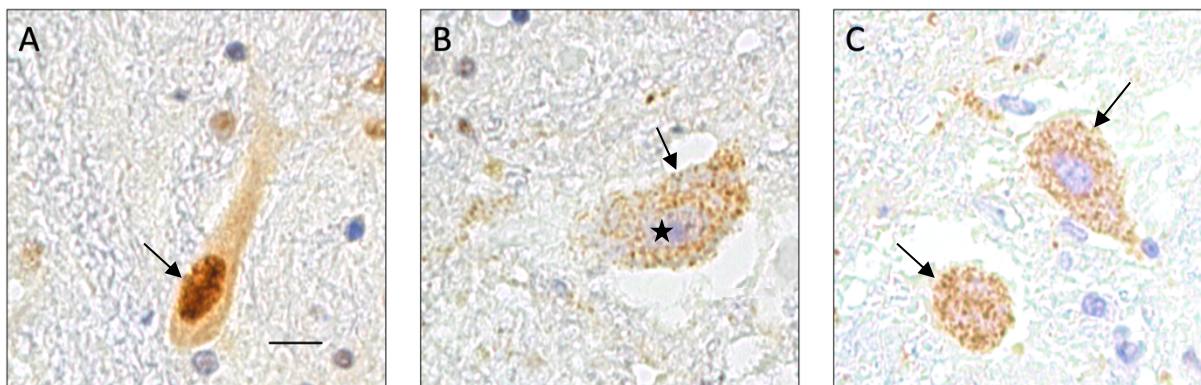


Figure 1 : (A-B) Immunohistochimie dirigée contre TDP-43, étude post-mortem de neurones moteurs de moelle épinière : (A) Marquage physiologique majoritairement nucléaire chez un sujet contrôle non atteint de SLA (flèche). (B) Translocation nucléocytoplasmique et agrégats cytoplasmiques pathologiques observés chez un patient atteint de SLA (flèche), disparition de la protéine au niveau du noyau (étoile). (C) Immunohistochimie dirigée contre pTDP-43, étude post-mortem de neurones moteurs de moelle épinière de patient atteint de SLA révélant la présence d'agrégats cytoplasmiques pathologiques (flèche). Grossissement x40, barre d'échelle 100 μ m. Images M.GARNIER, laboratoire de neuropathologie CHU Angers.

Plusieurs travaux récents décrivent également la présence de ce marqueur pathologique en dehors du SNC, en particulier au niveau musculaire (13–16) mais également dans d'autres tissus tels que la muqueuse digestive ou encore la rétine (17,18). Ainsi, la possibilité de détecter des signes moléculaires de cette pathologie dans les tissus périphériques permet l'hypothèse d'un diagnostic anatomopathologique par biopsie chez les patients.

2. Objectif du projet

Dans cette étude, nous avons cherché à déterminer si la pathologie TDP-43 peut être détectée dans la biopsie de glandes salivaires accessoires de patients atteints de SLA. Le choix de ce tissu se justifie par sa facilité d'accès par simple biopsie,

ainsi que par les données de la littérature démontrant la présence d'anomalies dans les glandes salivaires accessoires chez des patients atteints d'autres maladies neurodégénératives notamment la maladie de Parkinson (19–21). Nous avons étudié de façon rétrospective des échantillons tissulaires résiduels de BGSA prélevées chez des patients atteints de SLA dans le cadre de leur exploration diagnostique initiale. Ces échantillons ont été comparés à des BGSA de patients suivis dans le service de neurologie du CHU pour des pathologies non neurodégénératives (principalement des maladies inflammatoires du système nerveux central ou périphérique). Ce projet a été rendu possible grâce à la collaboration entre le centre de référence pour la Sclérose Latérale Amyotrophique du service de neurologie du CHU d'Angers et les laboratoires d'anatomopathologie et de neuropathologie du CHU d'Angers.

3. Présentation des centres et laboratoires

Le centre SLA d'Angers a été créé en 1985 par le Pr Jean EMILE. Il devient centre de référence en 2023 sous la responsabilité du Pr Julien CASSEREAU. Il couvre le Maine et Loire, la Mayenne, la Sarthe ainsi que la Loire Atlantique et la Vendée en collaboration avec le centre SLA de Nantes. Sur une période de 20 ans (2003-2023) plus de 1300 patients ont été diagnostiqués et suivis au centre SLA d'Angers (ANNEXE II, Chevet et al.). La file active est d'environ 225 patients par an, suivis sur le plan médical par le Pr CASSEREAU, le Dr SCHERER-GAGOU et le Pr CODRON, avec une prise en charge pluridisciplinaire organisée en hôpital de jour par deux infirmières de coordination associant ergothérapeutes, psychologue, diététicienne, assistante sociale mais également consultations de pneumologie, ORL, gastro-entérologie, rééducation, soins palliatifs.

Le laboratoire de neuropathologie et neurobiologie, fondé en 1986 par le Pr Frédéric DUBAS, et dirigé par le Dr Franck LETOURNEL est consacré au diagnostic biologique et histologique des maladies neurologiques par l'analyse de prélèvements biologiques, cutanés, nerveux, musculaires et cérébraux. Une part importante de l'activité du service est dédiée à la recherche en neurosciences à partir de l'analyse des collections de liquides biologiques et de tissus, et de la banque de cerveaux du laboratoire comptant plus de 500 cerveaux et moelles épinières de patients provenant de Maine-et-Loire, Loire-Atlantique, Mayenne, Sarthe et Indre-et-Loire, dont plus de soixante-dix prélèvements de patients atteints de SLA. Toutes les informations cliniques et histologiques des patients autopsiés sont répertoriées dans une base de données nommée Brain BASE développée en 2020 dans le cadre du travail de thèse du Dr Isabelle JOURNE (22).

4. Résultats du travail de thèse

Les résultats de ce projet sont présentés dans le manuscrit ci-après.

Vous trouverez en annexe un article publié en 2024 portant sur la stratégie diagnostique en cas de suspicion de SLA (ANNEXE 1), ainsi qu'un manuscrit présentant les résultats d'une étude portant sur l'épidémiologie de la SLA dans les pays de la Loire, menée en 2023-2024, en cours de finalisation (ANNEXE 2).

Detection of tdp-43 pathology in the minor salivary glands of patients with amyotrophic lateral sclerosis

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Detection of TDP-43 Pathology in the Minor Salivary Glands of Patients with Amyotrophic Lateral Sclerosis

Abstract: Emerging evidence suggests the presence of TDP-43 pathology in peripheral tissues of patients with Amyotrophic Lateral Sclerosis (ALS), providing avenues to better understand the disease and identify biomarkers. Here, we investigated pathological TDP-43 in minor salivary glands (MSG) from 10 patients with ALS and 20 controls. Only one patient showed pTDP-43 immunoreactivity in a few cells. No significant difference in staining surface was found between groups using digital quantification. These results suggest that pTDP-43 rarely reach salivary glands, limiting its diagnostic value. Further studies are needed to clarify the temporal and spatial patterns of peripheral pTDP-43 diffusion in ALS.

Keywords: Amyotrophic lateral sclerosis, TDP-43, minor salivary glands, biopsy, peripheral pathology.

Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder that affects motor neurons in the cortex, brainstem, and spinal cord.¹ The pathological hallmark of the disease is the aggregation of hyperphosphorylated TDP-43 protein (pTDP-43) in the cytoplasm of neurons and glial cells.^{2,3} TDP-43 pathology spreads through the central nervous system (CNS) via prion-like mechanisms, thereby contributing to disease progression. Interestingly, TDP-43 pathology has also recently been identified in peripheral tissues of patients with ALS, similar to what has been observed for proteins involved in other neurodegenerative disorders, such as tau and alpha-synuclein.^{4,5} Indeed, pTDP-43 immunoreactivity has been reported in skeletal and cardiac muscle, skin, retina, and digestive tract of patients with ALS, sometimes prior to symptom onset.⁶⁻¹¹ These results not only improve our understanding of ALS pathophysiology, but also provide an opportunity to identify a disease biomarker since ALS diagnosis currently relies on clinical evaluation and electrophysiology. However, the results from these studies are not consistent enough to establish a reliable biomarker. Additionally, some of the analyzed tissues are inaccessible or too invasive to sample *in vivo*, limiting their applicability in routine.

In this regard, the minor salivary gland (MSG) is a promising tissue for investigating TDP-43 pathology since it can be easily obtained from patients via minimally invasive biopsy. Furthermore, autonomic nervous system impairment has been reported in ALS,¹² including early subclinical salivary gland dysfunction and pTDP-43 pathology in peripheral autonomic gland fibers.^{7,13} Lastly, tau and alpha-synuclein pathology has been identified in these glands in patients with Alzheimer's disease (AD) and Parkinson's disease (PD), suggesting that they could be peripheral sites of pathological CNS protein accumulation.¹⁴⁻¹⁹ In this pilot study, we aimed to determine whether TDP-43 pathology can be detected in the MSG of ALS patients.

Methods

Subjects

Minor salivary gland samples were collected from biopsies performed as part of routine care in the Neurology Department at the University Hospital of Angers between 2015 and 2025. Patients who had an MSG biopsy and were diagnosed according to the Gold Coast criteria²⁰ were included as ALS patients. Subjects who had an MSG biopsy in the Neurology Department during the same period and were not diagnosed with a neurodegenerative disorder were included as controls. ALS patients and control subjects were excluded if they had a disorder affecting the salivary glands (e.g., sialadenitis, abscess, or neoplasm).

Biopsy procedure

All MSG biopsies were performed using lidocaine 2% as a local anesthetic on the inner side of the lower lip. A horizontal incision ranging from 0.5 to 1 cm in length was made along the long axis of the lower lip mucosa, just lateral to the midline, while stretching the lip. This allowed for the removal of at least two MSGs with a scalpel. The MSGs were then fixed in 10% neutral-buffered formalin. Hemostasis was achieved by applying pressure with a compress. No antibiotics or pain medications were needed.

Immunohistochemistry

Formalin-fixed tissue samples embedded in paraffin were sectioned at room temperature into 4 µm thick sections using a Leica RM125 microtome (Leica Biosystems, Wetzlar, Germany). A minimum of 3 sections were collected for each sample and immunostained using a fully

automated Leica BOND-III stainer (Leica Biosystems, Wetzlar, Germany). Washes, blocking, antigen retrieval, incubation with primary antibody against TDP-43 (Proteintech, Cat# 18280-1-AP, 1:6000), or phospho-TDP-43 Ser409/410 (Proteintech, Cat# 22309-1-AP, 1:6000), enzymatic detection with horse radish peroxidase (HRP) and hematoxylin counterstain were automatically performed following manufacturer's instructions.

Microscopy evaluation

Sections were imaged using a Zeiss Axioscop40 microscope equipped with an Axiocam MRC5 camera (Carl Zeiss, Oberkochen, Germany) and scanned using an Aperio CS2 whole slide scanner (Leica Microsystems, Wetzlar, Germany). The evaluation was conducted blindly by 3 trained pathologists.

Surface quantification

For each subject, three representative images of glandular tissue immunostained against pTDP-43 without major staining artifacts were acquired at a similar magnification. Each image was analyzed using QuPath software²¹ to separate the hematoxylin signal from the HRP signal. Then, the area of pTDP-43 immunostaining relative to the field area was measured using an automatic detection threshold relative to the nuclear signal. The final surface area value for each subject was the mean of the values calculated from the three images.

Statistical analysis

Statistical analyses were conducted using PRISM software version 8.0 for Windows (GraphPad, La Jolla, CA). Comparisons of means were performed using the Mann-Whitney test, and comparisons of proportions were performed using the Chi-square test. Differences were considered significant at $p < 0.05$.

Ethical approval

The use of human biological samples in this study has been given ethical approval by the regional ethics committee West II (biological resource center national identifier BB-0033-00038, declaration number 2024-169). The study protocol has been declared to the French

commission for information technology and civil liberties (declaration number ar24-0081v0). The General Data Protection Regulation (GDPR) was observed in accordance with European legislation. Data were collected anonymously, and a correspondence list was kept under the responsibility of the principal investigator.

Results

Study population

Ten MSG samples from patients with ALS and 20 samples from control subjects were immunostained for TDP-43 and pTDP-43 and analyzed. There were no significant differences between the two groups regarding age (mean age of 60.2 ± 16.2 years in the ALS group vs. 62.6 ± 14.6 years in the control group, $p = 0.71$) or sex ratio (6 men out of 10 subjects in the ALS group vs. 11 men out of 20 subjects in the control group, $p = 0.79$). No biopsy-related complications were recorded. Clinical findings are reported in the Table 1 (ALS patients) and Table S1 (control subjects).

TABLE 1. Demographic and clinical characteristics of patients with ALS at the time of biopsy.

Patient	Gender	Age	Disease duration (months)	Site of onset	Disease Progression	ALSFRS-R	Spinal impairment	Bulbar impairment	Respiratory impairment	Cognitive impairment	<i>SOD1</i> mutation	<i>C9ORF72</i> mutation
1	M	55	4	Spinal	Fast	43/48	+	-	-	-	-	-
2	F	41	11	Spinal	Slow	42/48	+	+	-	-	-	-
3	M	61	12	Spinal	Fast	38/48	+	+	+	-	-	-
4	M	67	9	Spinal	Moderate	43/48	+	+	-	-	-	-
5	M	85	4	Bulbar	Fast	26/48	-	+	-	-	-	-
6	F	56	6	Spinal	Fast	36/48	+	+	-	-	-	+
7	F	36	5	Spinal	Fast	37/48	+	-	-	-	-	-
8	M	86	20	Spinal	Slow	47/48	+	-	-	+	-	-
9	F	62	37	Spinal	Moderate	38/48	+	-	-	-	-	-
10	M	53	36	Spinal	Slow	48/48	+	-	-	-	-	-

Abbreviations: ALSFRS-R = ALS Functional Rating Scale-Revised. Disease Progression: Slow: <0.47 ALSFRS-R points per month; Moderate: <0.47 and <1.1 ALSFRS-R points per month; Fast: >1.1 ALSFRS-R points per month.

TDP-43 pathology in MSG

We first studied samples from patients and controls immune-stained for unphosphorylated TDP-43 to detect ALS pathological hallmarks, in particular nucleo-cytosolic translocation of the protein, cytosolic aggregates, and nuclear aggregates. Numerous fibroblasts and glandular cells with no TDP-43 nuclear staining were identified in both the ALS and control groups in

similar proportions (Fig 1A). However, no pathological cytosolic or nuclear TDP-43 aggregation was observed in any of the samples.

pTDP-43 immunoreactivity in MSG

We then looked for pTDP-43 (pSer409/410) immunoreactivity in the MSG of ALS patients and control to detect the pathological fraction of TDP-43. In one patient (ALS₃), we observed immunoreactivity with no aggregates in the cytosol of a few glandular cells and fibroblasts (Fig 1B, Fig S1). Artefacts and non-specific extracellular immunoreactivity were observed in the MSG lumen of all patients with ALS and controls (Fig S1). An automated quantification of the HRP signal surface was performed using Qupath software²¹ for each subject (Fig 1C). No significant difference in the % area of pTDP-43 immunoreactivity was observed between the two groups (Fig 1D), although the mean for the ALS group tended to be higher than that for the control group ($p = 0.096$). The ALS₃ patient had the highest value in the ALS group.

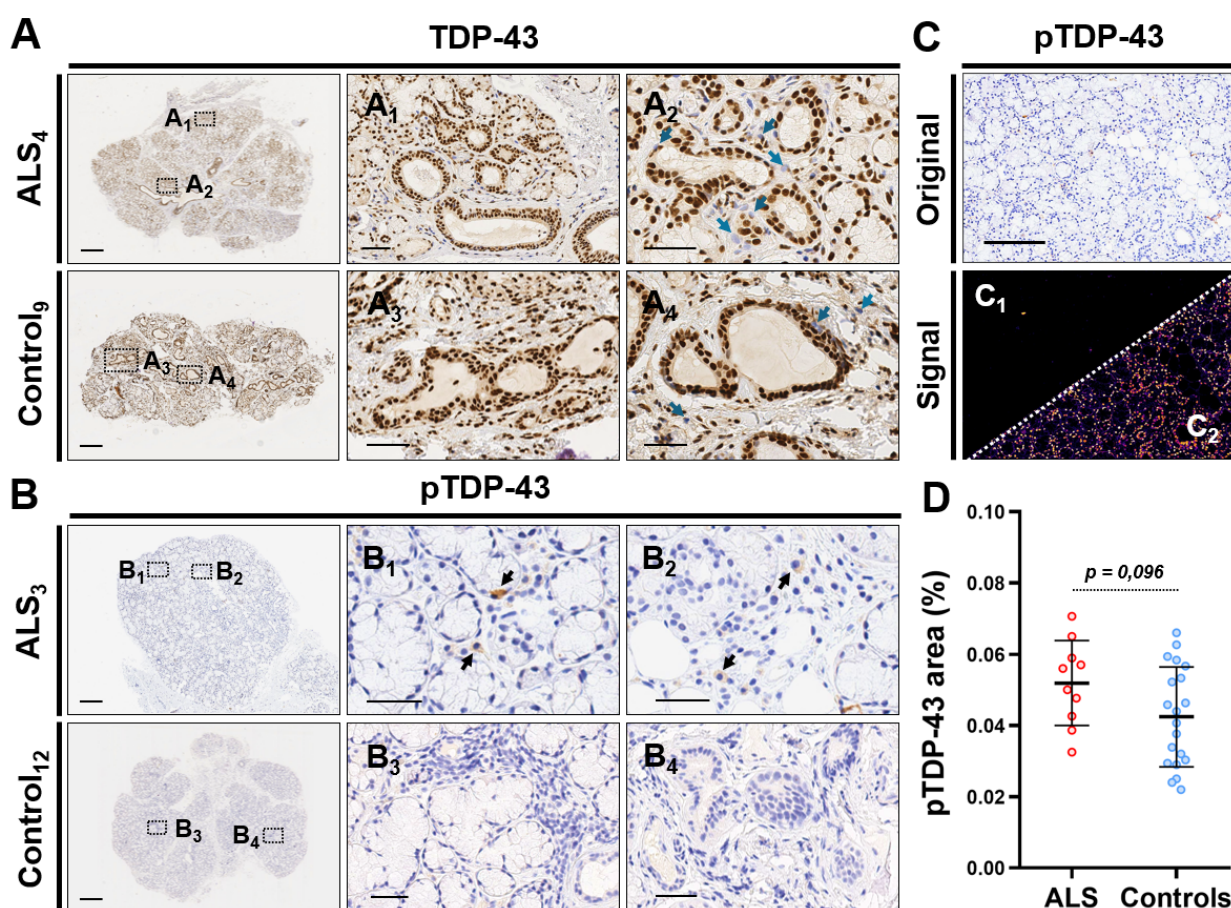


FIGURE 1. Detection of TDP-43 pathology in the minor salivary glands (MSGs) of patients with ALS. (A) Representative images of MSG sections from an ALS patient and a control subject immunostained for TDP-43. The absence of nuclear immunoreactivity is indicated by blue arrows. Scale bars: 350 μ m

(broadfield) and 50 μm (insets). (B) Representative images of MSG sections from an ALS patient and a control subject immunostained for phospho-TDP-43 Ser409/410 (pTDP-43). Cytosolic immunoreactivity is indicated by arrowheads. Scale bars: 350 μm (broadfield) and 50 μm (insets). (C) Automated quantification of pTDP-43 immunoreactivity on MSG sections using Qupath software²¹ to separate the horse radish peroxidase (HRP) signal (C1) from the hematoxylin signal (C2). Scale bar: 100 μm . (D) Mean percent area of pTDP-43 immunoreactivity on MSG sections for each ALS patient and control subject. Error bars indicate the mean with standard deviation.

Discussion

In this study, we investigated the presence of pathological TDP-43 in the minor salivary glands of patients with ALS compared to control subjects. Our findings suggest that while pTDP-43 immunoreactivity can occasionally be detected in the MSG of patients with ALS, the low detection rate precludes its use as a reliable diagnostic biomarker.

In previous studies, pTDP-43 pathology has been identified in several peripheral tissues, with varying sensitivity depending on the anatomical site and methodology. Peripheral immunoreactivity has mainly been studied in skeletal muscle, with positivity rates ranging from 30% to 100%, depending on the series.^{6,8,11} In the skin and retina, the reported immunoreactivity rates are 33% and 90%, respectively.^{7,10} Although studies have been conducted on other peripheral sites, such as the lymph nodes, colon, and gallbladder, the small number of cases makes it difficult to estimate the prevalence of immunoreactivity among patients with ALS.⁹ In our study, pTDP-43 immunoreactivity was observed in only one out of ten patients (10%). Interestingly, the patient exhibited rapid progression and was the only one with diffuse spinal, bulbar, and respiratory involvement. This suggests that the diffusion of peripheral TDP-43 pathology in ALS may vary not only according to tissue type, but also according to the stage and clinical profile of patients, which could explain the differences observed between series. Variability in staining protocols and tissue processing between studies could also impact the sensitivity of pathological detection. Furthermore, the biopsy procedure only allows for a limited examination of the tissue sample and may therefore fail to reveal an existing pathology that would have been detected at other sampling sites. This is particularly true for MSG, where only two to three glands are analyzed out of the hundreds in the labial mucosa. Further *ante-* and *postmortem* investigations of patients with advanced disease and various clinical profiles using standardized, wide-ranging detection protocols, may help clarify whether and when pTDP-43 accumulates in MSG in ALS.

The presence of pathological CNS proteins in salivary glands has also been studied in other neurodegenerative diseases. Several studies have been conducted in PD due to the known clinical and pathological impairment of autonomic innervation in the disease.²² The detection of phosphorylated alpha-synuclein in the MSG of PD patients has shown elevated but heterogeneous results, with immunoreactivity rates ranging from 15% to 70%.¹⁴⁻¹⁸ By contrast, a post-mortem study analyzing the presence of phosphorylated tau protein in the submandibular salivary glands of patients with AD reported an immunoreactivity rate of only 6%, which is closer to our results.¹⁹ The higher rates observed in PD compared to ALS and AD may be due to the more severe clinical and pathological autonomic impairment associated with the disease. Overall, these heterogeneous findings suggest that the diffusion profiles of tau, alpha-synuclein and TDP-43 differ in the salivary glands of patients with neurodegenerative disorders. This hypothesis can be extended to all peripheral tissues for these proteins, which highlights the importance of defining diffusion for each disease.

In conclusion, this study furthers our understanding of peripheral TDP-43 pathology in ALS. Our findings suggest that although pTDP-43 immunoreactivity can be detected in the MSG of patients with ALS, this pathological approach is currently unusable for clinical applications due to its low detection rate. In recent years, our knowledge of the presence of pathological CNS proteins in peripheral tissues has improved, but our understanding of the systemic distribution of pTDP-43 pathology in ALS remains limited. Future research should aim to clarify peripheral tissue vulnerability across endophenotypes and disease stages using standardized detection protocols in order to provide an integrated picture of the spatial and temporal diffusion of TDP-43. This approach is critical to better understanding disease progression and considering diagnostic avenues.

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Author Contributions

MG, JG, AR, FL and PC conceived and designed the study. MG, JC, CS, and PC recruited participants. MG, JG, AR, FL and PC analyzed data. MG and PC drafted the manuscript and figures. All authors revised and approved the final manuscript.

Data Availability

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

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Supplementary Material

Table S1. Demographic and clinical characteristics of control subjects at the time of biopsy.

Subject Number	Gender	Age at biopsy	Disease
1	M	65	Anti-MAG polyneuropathy
2	M	71	Chronic Inflammatory Demyelinating Polyneuropathy
3	M	50	Axonal polyneuropathy
4	F	68	Axonal polyneuropathy
5	F	57	Axonal polyneuropathy
6	M	88	Axonal polyneuropathy
7	F	59	White matter MRI hypersignal
8	M	59	Bilateral cervical plexopathy
9	M	77	Axonal polyneuropathy
10	F	47	Axonal polyneuropathy
11	F	49	CNS inflammation
12	F	36	CNS inflammation
13	M	54	Lewis-Sumner syndrome
14	F	39	CNS inflammation
15	F	75	Axonal polyneuropathy
16	M	83	Axonal polyneuropathy
17	F	70	Pachymeningitis
18	M	53	CNS inflammation
19	M	70	Axonal polyneuropathy
20	M	82	Axonal polyneuropathy

Abbreviations: CNS: central nervous system; MAG: myelin associated glycoprotein; MRI: magnetic resonance imaging.

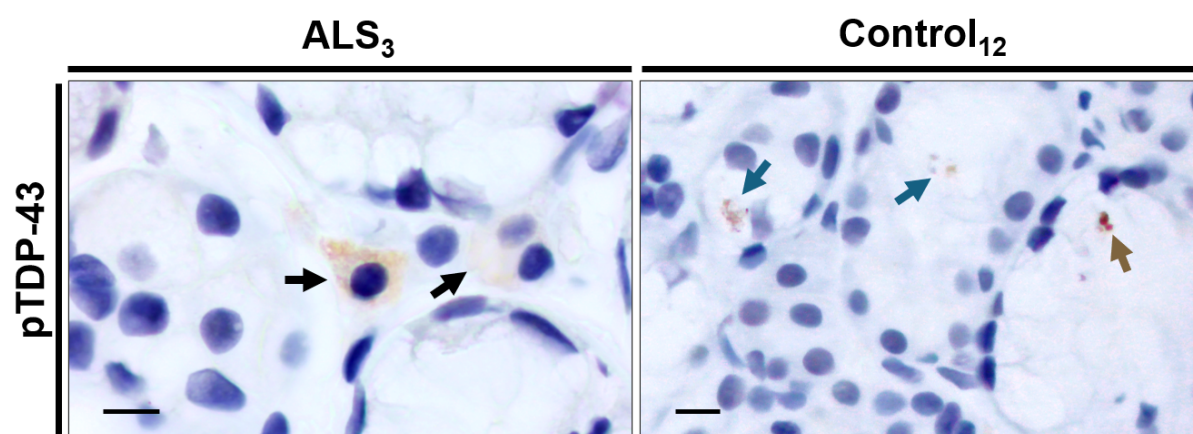


Figure S1. Representative images of minor salivary glands (MSG) sections from patients ALS₃ and a control subject immunostained for phospho-TDP-43 Ser409/410 (pTDP-43). Immunoreactivity in the cytosol of a few glandular cells was observed in the MSG of patient ALS₃ (black arrows). Artifacts (blue arrows) and non-specific extracellular immunoreactivity (brown arrow) were observed in the MSG lumen of all patients with ALS and controls. Scale bars: 10 μ m.

CONCLUSION ET PERSPECTIVES

Cette étude approfondit notre compréhension de la pathologie périphérique de TDP-43 dans la SLA. Nos résultats suggèrent que l'immunoréactivité pTDP-43 est rarement détectable dans les glandes salivaires accessoires de patients atteints de SLA. Toutefois ce marqueur pathologique ne peut être considérée en pratique en clinique compte tenu de sa très faible prévalence chez les patients.

Bien que nos connaissances sur la présence de protéines pathologiques du SNC dans les tissus périphériques se soient développées ces dernières années, notre compréhension de la distribution systémique de la pathologie de pTDP-43 dans la SLA reste limitée. Les recherches futures doivent donc viser à fournir une image intégrée de la diffusion spatiale et temporelle de la pathologie TDP-43 dans les tissus périphériques que ce soit en ante ou post-mortem chez les patients atteints de SLA, mais également chez les patients atteints d'autres tardopathies (telles que la dégénérescence lobaire fronto-temporale-TDP et le LATE).

Une investigation post-mortem de la distribution périphérique de la protéinopathie TDP-43 pourrait être portée par notre laboratoire, par le biais de prélèvements systématiques de multiples sites superficiels lors de l'autopsie (muscles, peau, glandes salivaires accessoires et glande sous-mandibulaire,).

Afin de mieux caractériser la cinétique de progression de la pathologie, nous pourrions également réaliser une étude ante-mortem comportant la réalisation systématique de biopsies de réalisation aisées (BGSA, biopsie de peau...) chez les patients à différents stades évolutifs de la maladie afin d'évaluer la dynamique de diffusion de la protéinopathies.

Il pourrait enfin être envisageable de conduire d'autres études sur le modèle de celle de Pattle et al., 2023, visant à collecter des échantillons tissulaires résiduels de biopsies

ou de résections d'organes effectuées dans le cadre du parcours de soins des patients atteints de SLA (que ce soit avant le diagnostic ou au cours du suivi) afin d'y rechercher des figures pathologiques caractéristiques.

Nous travaillons actuellement sur l'écriture d'un article de synthèse portant sur la pathologie TDP-43 périphérique dans la SLA afin de faire un état des lieux des connaissances actuelles et les perspectives de ce domaine de recherche.

Pour conclure, cette étude complète les données de la littérature existantes et enrichit nos connaissances sur la pathologie périphérique TDP-43. De futures recherches sont à poursuivre afin de mieux apprécier la dynamique de diffusion de cette pathologie.

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ANNEXE 1

From suspicion to diagnosis: exploration strategy for suspected amyotrophic lateral sclerosis

From suspicion to diagnosis: exploration strategy for suspected amyotrophic lateral sclerosis

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ABSTRACT

The diagnosis of amyotrophic lateral sclerosis (ALS) is based on evidence of upper and lower motor neuron degeneration in the bulbar, cervical, thoracic, and lumbar regions in a patient with progressive motor weakness, in the absence of differential diagnosis. Despite these well-defined criteria, ALS can be difficult to diagnose, given the wide variety of clinical phenotypes. Indeed, the central or peripheral location of the disease varies with a spectrum ranging from predominantly central to exclusively peripheral, symptoms can be extensive or limited to the limbs, bulbar area or respiratory muscles, and the duration of the disease may range from a few months to several decades. In the absence of a specific test, the diagnostic strategy relies on clinical, electrophysiological, biological and radiological investigations to confirm the disease and exclude ALS mimics. The main challenge is to establish a diagnosis based on robust clinical and paraclinical evidence without delaying treatment initiation by increasing the number of additional tests. This approach requires a thorough knowledge of the phenotypes of ALS and its main differential diagnoses.

KEY MESSAGES

- The diagnosis of amyotrophic lateral sclerosis (ALS) is based on progressive degeneration of upper and lower motor neurons.
- ALS can be difficult to diagnose due to the wide range of clinical phenotypes (central/peripheral location, symptom distribution, disease duration).
- A thorough diagnostic strategy including clinical, electrophysiological, biological and radiological investigations is essential to confirm ALS and exclude differential diagnoses.

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Introduction

Amyotrophic lateral sclerosis (ALS), also known as Charcot's disease, is a neurodegenerative disease affecting the upper motor neurons (UMN) in the cerebral cortex and the lower motor neurons (LMN) in the brainstem and the spinal cord. In Europe, the incidence of the disease is estimated at 2.5/100,000 (4 new cases per day) [1,2]. In 90% of cases, ALS is sporadic, with no known cause to date. Cumulative lifetime environmental exposures, known as the exposome, and genetic susceptibility are increasingly recognized as playing a role in the development and progression of ALS. Air pollution, neurotoxic pesticides, head and spinal injuries, electric shock, smoking, military service and physical activity are considered potential risk factors for ALS [3,4]. In 10% of cases, the disease is caused by the transmission of a pathogenic gene mutation. The average age of onset of ALS is around 65 years [2,5]. Patients affected by the disease develop a progressively worsening diffuse motor

impairment leading to muscle weakness in the upper and lower limbs, and/or difficulty speaking and swallowing, and/or restrictive respiratory failure. Median survival is approximately 3 years from symptom onset [1,2]. The only drug treatment available in France to slow the progression of the disease is riluzole, which reduces the 1-year mortality rate by one third and prolongs life without a tracheostomy [6,7]. A multidisciplinary functional approach is also essential (non-invasive ventilation, enteral nutrition, physiotherapy, speech therapy, psychological support, analgesia), to improve survival and quality of life for patients [8].

Diagnostic challenges and issues

ALS may be difficult to diagnose, given the wide variety of clinical phenotypes and the fear of missing a curable differential diagnosis. The main challenge is therefore to establish a diagnosis based on robust clinical and paraclinical evidence, without delaying the

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diagnosis announcement and the initiation of treatment by increasing the number of sometimes expensive additional tests. The diagnosis of ALS is based on the combination of evidence of central and peripheral motor neuron damage, progressive involvement of the bulbar, cervical, thoracic, and lumbar areas, and the absence of a differential diagnosis to explain the symptoms. These elements are included in the Gold Coast diagnostic criteria published in 2019 [9] (Table 1), which progressively replace the revised El Escorial [10] and Awaji-shima [11] criteria. These new criteria are less restrictive and replace the degrees of certainty (possible, probable, definite ALS) with a positive or negative diagnosis, which is clearer for both patients and physicians. The first 3 steps of the diagnosis should be clinical, electrophysiological, and paraclinical, with the aim of both supporting the diagnosis of ALS and ruling out pathologies – especially curable ones – that may mimic the disease. This approach therefore requires a thorough knowledge of the phenotypes of ALS and its main differential diagnoses.

Table 1. Gold Coast diagnostic criteria for ALS [9].

Progressive motor impairment documented by history or repeated clinical assessment, preceded by normal motor function, AND
Presence of upper and lower motor neuron dysfunction in at least 1 body region ^a (in the same body region if only one is involved) OR lower motor neuron dysfunction in at least 2 body regions, AND
Investigations excluding other disease processes

^aBulbar, cervical, thoracic, and lumbosacral.

Clinical phenotypes of ALS

There is considerable clinical variability among ALS patients [12,13] (Figure 1), although they share a common neuropathological profile with neuronal degeneration and hyperphosphorylated TDP-43 inclusions in neurons and glial cells [14,15]. This clinical heterogeneity has several aspects. First, the central or peripheral location of the disease may vary, with a spectrum ranging from exclusively central disease, known as Primary Lateral Sclerosis (PLS), to purely peripheral forms, known as Progressive Muscular Atrophy (PMA). Furthermore, the topography of the muscle weakness may vary from patient to another and, at least at the onset of the disease, the symptoms may be limited to the limbs, the bulbar level, or even the respiratory level only. The oculomotor and sphincter systems are however usually spared. Nearly half of patients have cognitive or psychobehavioural impairment, and 10–15% of cases fulfill criteria for frontotemporal lobar degeneration (FTLD) [16]. In addition, 30% to 60% of patients may present with hypermetabolic syndrome, defined as an increased resting energy consumption leading to weight loss and accelerated decline in motor function and survival [17,18]. Finally, the duration of the disease varies from patient to patient, ranging from a few months to several decades [19].

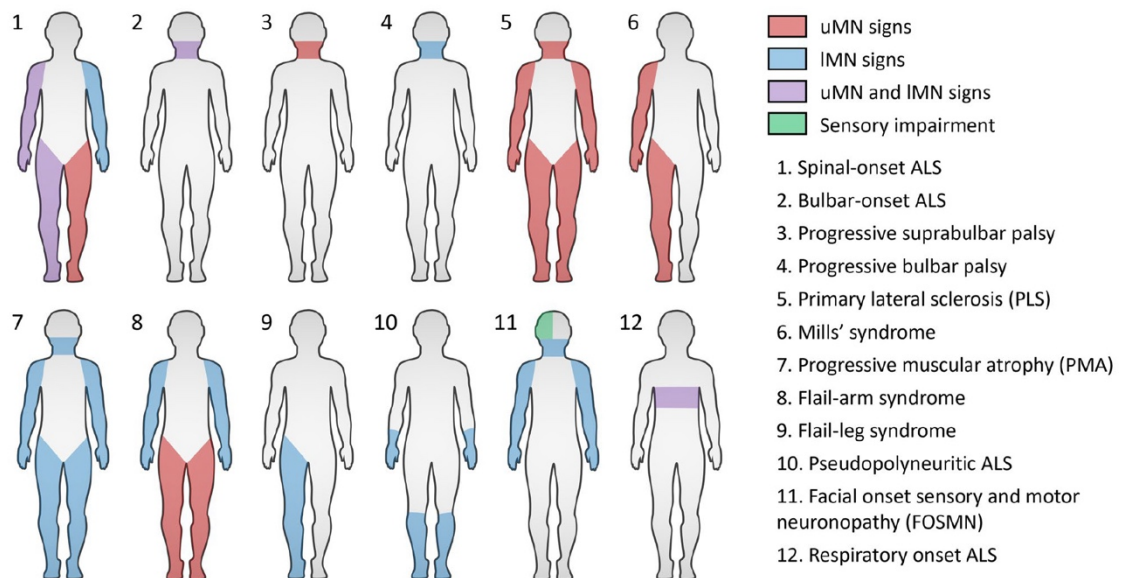


Figure 1. Clinical phenotypes of ALS (initial presentation). ALS: amyotrophic lateral sclerosis, LMN: lower motor neuron, UMN: upper motor neuron.

Table 2. Main differential diagnoses of ALS.**Central nervous system lesions**

- Brain lesion
- Degenerative cervical myelopathy
- Other lesions of the spinal cord with radicular involvement
- Hirayama's Disease

Autoimmune diseases of the peripheral nervous system

- Multifocal motor neuropathy
- Chronic inflammatory demyelinating polyneuropathy

Infectious diseases

- HIV infection, West Nile virus, Lyme disease, Syphilis
- Post-polio syndrome

Metabolic and toxic diseases

- Dysthyroidism
- Hyperparathyroidism
- Vitamin B12 deficiency
- Lead poisoning

Genetic diseases

- Hereditary spastic paraplegia
- Adrenomyeloneuropathy
- Spinal muscular atrophy (type III or type IV)
- Hereditary neuropathy with motor expression
- X-linked spinal and bulbar muscular atrophy (Kennedy's disease)
- GM2 gangliosidosis
- Alexander disease
- Brown-Vialetto-Van-Laere disease

Cancer-related disorders

- Paraneoplastic syndrome
- Postirradiation myeloradiculopathy

Muscular pathologies and neuromuscular junction disorders

- Myopathy
- Inclusion body myositis
- Autoimmune myasthenia gravis

Peripheral nerve hyperexcitability disorder

- Cramp fasciculation syndrome

Table 3. Clinical signs of upper motor neuron (UMN) and lower motor neuron (LMN) impairment that should be evaluated during the clinical examination of a patient suspected of having ALS.**UMN impairment in the limbs and trunk**

- Diffused and polykinetic osteotendinous reflexes
- Preserved osteotendinous reflexes in a region with LMN impairment
- Hoffman sign, Babinski sign
- Clavicle reflex
- Pyramidal hypertonia (spasticity)
- Ankle clonus, patellar clonus

LMN impairment in the limbs and trunk

- Muscle weakness
- Cramps
- Muscle atrophy
- Fasciculations
- Osteotendinous reflexes reduced or absent

UMN impairment in the bulbar region

- Dysphonia, Dysarthria, Dysphagia
- Jaw clonus
- Buccofacial apraxia
- Hyperactive or repeating jaw jerk reflex
- Spasmodic laughing and crying

LMN impairment in the bulbar region

- Dysphonia, Dysarthria, Dysphagia
- Tongue fasciculations
- Tongue atrophy
- Salivary stasis
- Velar hypotonia

Respiratory impairment

- Dyspnea, tachypnea, orthopnea
- Use of the accessory respiratory muscles
- Clinical signs of hypercapnia (headache, asterixis, hypercrinia)

Main differential diagnoses

The European Federation of Neurological Associations (EFNA) listed 77 differential diagnoses for ALS [20]. The main are grouped according to their mechanism of pathogenicity in Table 2.

These pathologies partly guide the investigations to be performed when a patient presents with symptoms suggestive of the disease, with three main steps: clinical, electrophysiological and paraclinical (biological and radiological) investigations.

Clinical examination

The clinical examination is the first step in the diagnostic work-up. Motor, bulbar, and respiratory signs must be examined in detail, each time trying to identify elements associated with UMN and LMN involvement (Table 3). It is also important to look for non-motor signs, which may be part of the disease (dysexecutive syndrome, behavioral impairment, weight loss) or in opposition to it (sensory loss, oculomotor paralysis, vesicosphincter signs). Lastly, particular importance should be attached to establish a family tree that spans at least three generations and includes all antecedents in the family, looking specifically for relatives with motor neuron disease, cognitive disorders, or psychiatric

symptoms, and family tree censorships. The risk factors for ALS mentioned above should also be investigated.

Electrophysiology exploration

Electroneuromyography (ENMG) is the key test for the detection of LMN involvement and the specification of its topography [21]. In this disease, there is usually no change in either motor or sensory nerve conduction. The amplitude of motor potentials may be reduced, reflecting axonal loss, and pseudo-blocks may be observed in case of rapid motor neuron loss. Detection records and accurately defines LMN damage. Abnormal resting activity (fasciculation potentials, fibrillation potentials, positive slow waves) and neurogenic recruitment (motor unit potentials of increased duration and/or amplitude) indicate active and chronic denervation. A region is considered affected if denervation is recorded in two muscles innervated by different roots and nerves for the cervical and lumbar regions, and in one muscle for the bulbar and thoracic regions [9]. These criteria therefore require a systematic exploration of all four anatomical regions, without limiting the examination to clinically affected areas. A standardized protocol for the electrophysiological examination of a patient suspected of having ALS was proposed in 2022 by Lenglet and Camdessanché [21] (Table 4).

Table 4. Gold Coast criteria for lower motor neuron (LMN) impairment on electroneuromyogram (EMNG) [9] and proposed protocol for investigating a patient suspected of having ALS [21].

Gold Coast criteria for LMN dysfunction on EMNG

- Evidence of chronic neurogenic change, defined by large motor unit potentials of increased duration and/or increased amplitude (with polyphasia), and motor unit instability regarded as supportive but not obligatory evidence, AND
- Evidence of ongoing denervation, including fibrillation potentials or positive sharp waves, or fasciculation potentials.
- In TWO limb muscles innervated by different roots and nerves, or ONE bulbar muscle, or ONE thoracic muscle

ENMG exploration protocol proposed by Lenglet and Camdessanché

Nerve conduction studies (sensory and muscle action potentials)

- Median, ulnar (to Erb's point if necessary), fibular and tibial nerves, in addition to nerves in the affected areas.
- First *interosseus dorsalis* and *abductor digiti minimi* used to study ulnar nerve conduction. Myography

Lower limbs: *tibialis anterior* and *vastus lateralis*

- Upper limbs: *interosseus dorsalis* and *deltoidus* or *biceps brachii*
- Thoracic region: *rectus abdominis* or *paraspinalis dorsalis*
- Bulbar region : *sternocleidomastoideus*, *genioglossus*, *trapezius superior*
- And additional muscles depending on the clinical presentation
- Repetitive nerve stimulation
- In the case of proximal upper limb deficits, isolated bulbar involvement or in the absence denervation: radial nerve/*anconeus*, spinal nerve/*trapezius superior*, V-XII nerves/submental triangle on both sides.

Table 5. Paraclinical tests recommended by the EFNS for patients with suspected ALS [20].

Biology

- Ionogram, urea, creatinine
- Fasting blood glucose, AST, ALT, LDH, albumin
- Calcium, Thyroid-stimulating hormone, free T₄, free T₃ hormone assays
- Haematological screen
- Serum protein electrophoresis, serum immunoelectrophoresis
- C-reactive protein
- Vitamin B₁₂ and folate
- Creatine kinase
- Serologies (Borrelia, viruses including HIV, syphilis)^a

Radiology

- Chest X-ray
- Brain and spinal cord MRI

^aIf exposed or risk of infection.

Paraclinical tests

Systematic prescription of a standardized biological examination (Table 5) and a frontal chest X-ray is recommended by the EFNA to screen for the main differential diagnoses of ALS [20]. Brain and spinal MRI should also be organized to rule out lesions in the central nervous system. In patients with ALS, it is sometimes possible to observe a T2 hypersignal of the cortico-spinal tract, from the semi-oval centers to the brainstem, as well as a cortical atrophy predominant in the frontal regions.

Diagnostic strategy

Several situations can be identified at the end of this initial clinical, electrical, biological, and radiological examination (Figure 2).

ALS mimic identified

In this case, clinical and paraclinical investigations should then be directed to the pathology causing the patient's symptoms. Occasionally, MRI may reveal spinal cord injury with radicular involvement, especially in degenerative cervical myelopathy. This can complicate the diagnosis in a patient with purely motor symptoms. In this case, signs of LMN damage more diffuse than expected from the lesion and/or bulbar signs may point to ALS.

Gold Coast criteria fulfilled, UMN and LMN signs without atypia

The diagnosis of ALS can be announced to the patient. If the disease is in its early stages, or if two pathologies are evoked, a new EMNG at 3 or 6 months - depending on the rate of worsening - will help to be more conclusive.

Isolated UMN signs

If brain and spinal MRI exclude central nervous system lesion, the diagnosis of PLS or Mills syndrome can be made. Repeated clinical and eventually electrical evaluations must be performed to ensure that there is no delayed LMN impairment. In a young patient presenting with very progressive weakness predominantly in the lower limbs, hereditary spastic paraplegia or adrenoleukodystrophy may be investigated.

Isolated LMN signs

The absence of UMN damage may be confirmed by normal motor evoked potentials. In this case, the search for undetected blocks may motivate the repetition of EMNG and the use of high-voltage radicular stimulation techniques or triple stimulation in the hypothesis of multifocal motor neuropathy (MMN) or chronic dysimmune neuropathy [21]. In addition, anti-ganglioside antibodies testing, CSF analysis, plexus MRI, and trial treatment with intravenous immunoglobulins may be considered. In a young man presenting with asymmetric progressive pseudo-ulnar motor signs, a cervical MRI with dynamic flexion sequences can be performed to look for a compression of the anterior spinal cord with the posterior part of the vertebral bodies. This may be a sign of Hirayama's disease. Finally, in the case of a young subject with very progressive motor symptoms and chronic signs on EMNG (giant motor unit potentials, electroclinical dissociation), four hypotheses can be investigated: spinal muscular atrophy (type III or type IV), hereditary distal motor neuropathy (*BSCL2*, *HSPB1*, *HSPB8* and *GARS* genes in particular), Charcot-Marie-Tooth disease (CMT2) with motor

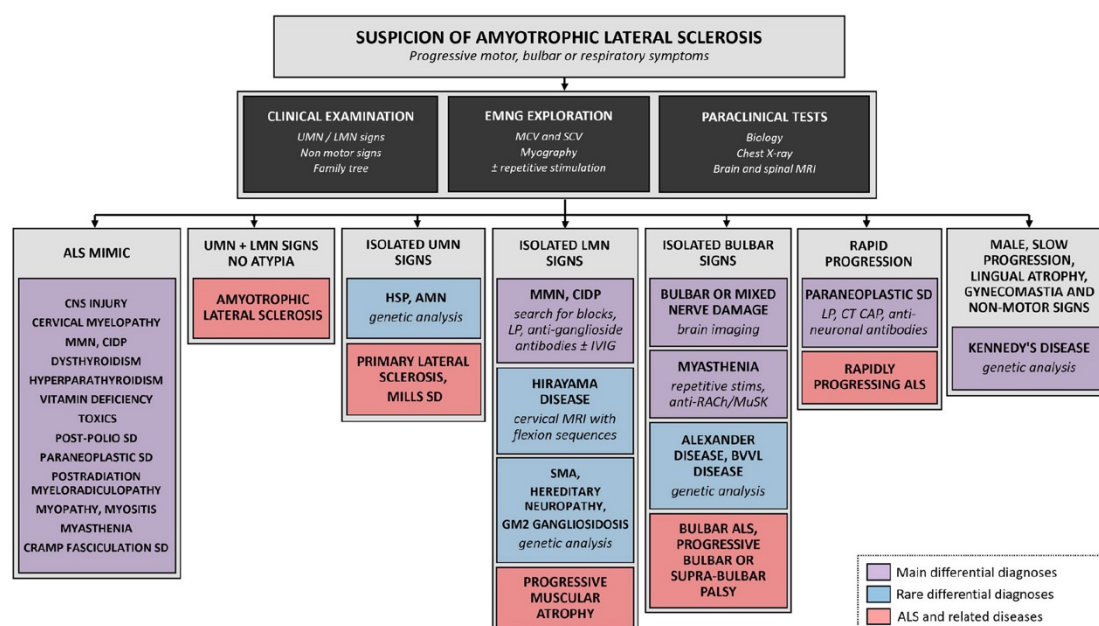


Figure 2. Diagnostic strategy for a patient with suspected ALS. AMN: adrenomyeloneuropathy, CIDP: chronic inflammatory demyelinating polyradiculoneuropathy, CNS: Central nervous system, CT CAP: CT scan chest abdomen pelvis, EMNG: electromyoneurography, HSP: hereditary spastic paraplegia, LMN: lower motor neuron, LP: lumbar puncture, MCV: motor nerve conduction velocity, MMN: multifocal motor neuropathy, SCV: sensory nerve conduction velocity, SD: syndrome, SMA: spinal muscular atrophy, UMN: upper motor neuron.

predominance (*MFN2* and *MT-ATP6* genes in particular) and GM2 gangliosidosis. In the absence of elements suggestive of these conditions, and if two regions are affected, the diagnosis of PMA can be made according to the Gold Coast criteria.

Isolated bulbar signs

Special attention must be paid to the course of the mixed nerves on radiologic studies in the search for a lesion. Similarly, in the presence of atypical or fluctuating symptoms, repetitive nerve stimulation and anti-RACH and anti-MuSK antibodies testing will help to rule out autoimmune myasthenia, which can sometimes mimic bulbar ALS. It should be noted that decrements are sometimes observed in ALS, so their presence does not formally exclude the disease. Less frequently, Alexander disease (hereditary leukodystrophy caused by mutation of the *GFAP* gene) may be suspected if there is cervical cord atrophy on MRI, and Brown-Vialetto-Van-Laere disease in patients under 30 with hearing loss (mutation of the *SLC52A2/A3* genes). In the absence of further evidence, the diagnosis of bulbar ALS with UMN and/or LMN impairment can be made.

Signs restricted to the respiratory function

This is a rare situation usually seen in patients admitted to a pulmonary department or intensive care unit for acute or subacute alveolar hypoventilation. The search for more diffuse motor neuron damage is essential in this situation. The diagnosis is based on the absence of other respiratory causes and the clinical course, in particular the development of motor or bulbar signs.

Rapid progression and associated non-motor signs

The rapid progression of symptoms, the presence of non-motor neurological signs (sensory, cerebellar, limbic), a generalized impairment, and paraclinical abnormalities (blood count, monoclonal gammopathy, pulmonary lesion) should prompt further investigations in search of a paraneoplastic origin that may lead to UMN and/or LMN impairment [22]. The most associated cancers are small cell lung cancer, breast adenocarcinoma, and hematologic malignancies (especially lymphoma). Investigations may be completed by a CSF analysis, thoraco-abdominal-pelvic CT scan or preferentially positron emission tomography, and anti-neuronal antibody testing.

Slow progression predominantly in the lower limbs, lingual atrophy and non-motor signs in a male

In a male, the presence of a marked lingual atrophy, peribuccal myokimia, gynecomastia, endocrine disorders, postural tremor, sensory signs, and sensory neuropathy on ENMG should raise the suspicion of X-linked spinal and bulbar muscular atrophy (Kennedy's disease) due to the repetition of CAG triplets in exon-1 of the androgen receptor gene.

Frontotemporal spectrum disorder of ALS (ALS-FTSD)

As mentioned above, almost half of patients have cognitive dysfunction or behavioural changes, and the disease may begin with these symptoms. It is therefore essential to look for neuropsychological deficits in all ALS patients, especially those that are more specific to the disease: executive dysfunction (including deficits in social cognition), language impairment (dissociable from motor impairment) and behavioural changes (dissociable from pseudobulbar affect and depression, anxiety and sleep disturbance), in particular apathy. Reports from family members and carers are essential in this approach. The Edinburgh Cognitive and Behavioural ALS Screen (ECAS) [23] or the ALS Cognitive Behavioural Screen (ALS-CBS) [24] are rapid assessment batteries for frontotemporal dysfunction that are recommended for all patients. If impaired, further neuropsychological assessment with specific and targeted tests should be undertaken. Four cognitive-behavioural phenotypes are defined by the revised Strong criteria [16], all grouped under the term "frontotemporal spectrum disorder of ALS" (ALS-FTSD): 1) ALS with cognitive impairment (ALSci): patients with executive dysfunction (including social cognition), language dysfunction, or combination of both. 2) ALS with behavioural impairment (ALSbi): patients with apathy and/or two or more other behavioural symptoms (including disinhibition, loss of sympathy and empathy, perseverative, stereotyped or compulsive behaviour, hyperorality/dietary change, loss of insight and psychotic symptoms). 3) ALS with combined cognitive and behavioural impairment (ALS-cbi): patients fulfilling criteria for both ALSci and ALSbi, but not for FTD. 4) ALS with frontotemporal dementia (ALS-FTD): patients with ALS who also have frontotemporal dementia.

Genetics of ALS

More than 30 pathogenic genes have been identified as responsible for ALS. Four of these genes account for more than 60% of familial ALS and 10% of sporadic ALS: *C9orf72*, *SOD1*, *FUS* and *TARDBP* [25,26]. The recent

development of gene therapies led to systematic genetic analyses in France: any patient suffering from ALS, whether sporadic or familial, should benefit from a molecular biological study of the *C9orf72* and *SOD1* genes as soon as the disease is diagnosed [27]. Additional analysis (ALS Panel NGS) may be performed in cases of early onset (before age 40), slow or rapid progression, or if there is a suggestive family history. All samples should be sent to a reference laboratory. A dedicated consultation should then be organized to give the results to the patient in person.

Conclusion

The diagnosis of ALS must be based on robust clinical and paraclinical evidences. The identification of markers of the disease, such as neurofilament testing, diffusion tensor imaging techniques and omics approaches, is an active area of research. In the coming years, it should provide additional informations to help clinicians to diagnose the disease.

Authors contributions

MG and PC drafted the manuscript and the figures. JPC and JC revised the article for intellectual content. All authors approved the final manuscript.

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Data availability statement

The data are available from the corresponding author upon reasonable request.

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ANNEXE 2

Epidemiology of Amyotrophic Lateral Sclerosis in the Pays de la Loire, France: A 20-Year Study from a Centralized Diagnostic Center

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Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterized by the degeneration of motor neurons in the cortex, brainstem and anterior horn of the spinal cord [1]. ALS leads to the loss of voluntary muscle control, resulting in progressive, widespread paralysis. Death occurs within three to five years after symptoms onset, most often due to respiratory failure.

ALS is characterized by significant variability in personal history, motor and non-motor signs, and rate of progression [2]. This heterogeneity complicates not only diagnosis, but also pathophysiological understanding. Indeed, apart from the 5 to 10 % of individuals with a monogenic pathological mutation that causes the disease, the etiology of ALS remains unclear, likely caused by a combination of environmental risk factors and genetic susceptibility [3]. The significant variation in the incidence of ALS across the world and within geographical regions of countries supports this idea [4,5]. By defining the burden of disease, epidemiological studies can help identify risk factors and provide a basis for mechanistic, preventive, and therapeutic studies.

In France the incidence of ALS is estimated at 2.5 cases per 100,000 person-years [6-9]. However, the epidemiology of ALS in France is primarily based on the FRALim registry from the Limousin region, which is in the center of the country. The aim of this study was to provide additional epidemiological data by establishing the incidence and clinical features of ALS patients over a 20-year period in another French region in the west of France, the Pays de la Loire, which has a population of nearly 4 million.

Methods

Population

This study was performed in the French region of Pays de la Loire, which includes the departments of Loire-Atlantique (44), Maine-et-Loire (49), Mayenne (53), Sarthe (72) and Vendée (85), covering 32 082 km². The population was 3,247,675 in 2003, 3,902,115 in 2023 [10]. The ALS reference center of the University Hospital of Angers has been the centralized diagnostic center of the region since 1985. Patients with suspected ALS living in the Pays de la Loire are meant to be referred to the reference center of Angers for confirmation (based on revised El Escorial criteria) and treatment.

Data collection and management

Patients diagnosed with ALS at the ALS reference center in Angers between 2003 and 2023 were included in the study. ALS diagnosis was retained if the patient's medical records showed an explicit diagnosis by a neurologist from the ALS center. Data were collected retrospectively from medical records for these cases. Patients lacking sufficient data in their medical records for the study were excluded. The collected administrative and medical

data included the following: year of birth; year of death; sex; year of onset of first symptoms; year of ALS diagnosis; type of ALS (sporadic or genetic); initial presentation (spinal, bulbar or respiratory); occupation; and city and postal code of residence.

Statistical analysis

The statistical analysis was performed using PRISM software version 8.0 for Windows (GraphPad, La Jolla, CA). Qualitative variables were described in terms of frequency, and quantitative variables were analyzed using the mean, median, interquartile range, minimum, and maximum. Confidence intervals at the 95% level were calculated under the assumption of a Poisson distribution. The Kolmogorov-Smirnov and Shapiro-Wilk tests were used to examine the normality of continuous variables. Means were compared using a Student's t-test. Frequencies were compared using the Chi-squared test or Fisher's exact test if the sample size was less than five. All missing data were reported. Incidence rates were calculated using patients' years of ALS diagnosis and the Pays de la Loire's annual population estimates from the National Institute of Statistics and Economic Studies [10].

Epidemiological Maps

Choropleth maps in RStudio version 4.2.1 were created using the tidyverse, sf, readxl, dplyr, ggmap, leaflet, ggrepel, and cowplot libraries. We used the "Evolution and Structure of the Population in 2020" file from INSEE in GeoJSON format under the Open Database License (ODbL). The number of cases from 2003 to 2023, as well as the prevalence rate per 1,000 inhabitants, were calculated for each municipality using 2020 INSEE population structure data for individuals aged 15 to over 90 years. Technical-economic data on municipalities in the Pays de la Loire region were obtained from SIRSé [11]. Treatment frequency indices for 2021 were obtained from Solagro's Adonis pesticide map [12].

Results

Incidence

From 2003 to 2023, a total of 1,316 patients were diagnosed with ALS at the Angers Reference Center. The annual incidences ranged from 0.99 cases per 100,000 persons in 2003 to 2.68 cases per 100,000 persons in 2014 with no significant difference ($p = 0.62$) (Figure 1A). The crude incidence based on the number of new cases diagnosed over the 20-year period and the estimated average population size during the same timeframe was 1.88 cases per 100,000 person-years.

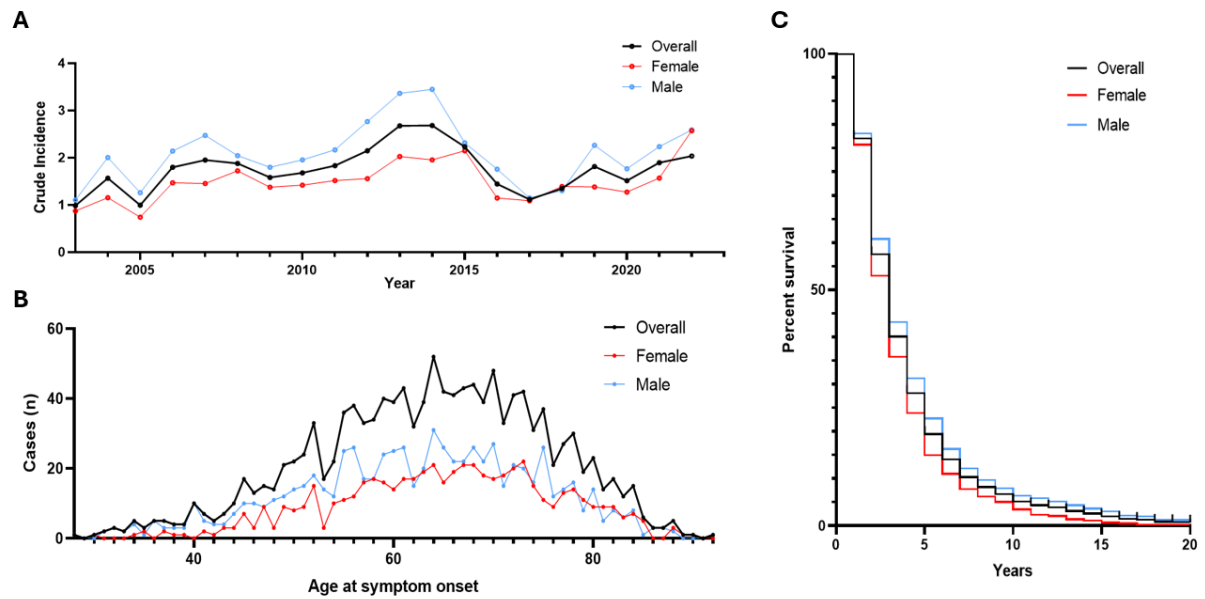


Figure 1. (A) Crude incidence of ALS (cases per 100,000 persons per year) from 2003 to 2023 (n= 1316). (B) Age at symptom onset (n= 1297). (C) Kaplan-Meier survival curves from date of diagnosis (n=1054).

Clinical characteristics

The patients' clinical characteristics are shown in Table 1. The mean age at symptom onset was 63.76 (95% CI: 63.13–64.39), with a normal distribution ($p = 0.23$) (Figure 1B). No increase in ALS diagnosis among younger individuals (<45 years) was observed between 2013 and 2023 compared to 2003 and 2013 (41 vs 42 patients, $p = 0.88$), and the mean age at symptom onset was similar during both periods (63.2 and 64.0, respectively). The mean disease duration, from symptom onset to death, was 3.73 years (95% CI: 3.54–3.92), ranging from less than 12 months to 19 years (Figure 1C). Sixty-one percent of patients died within three years of symptom onset, 33% died between four and nine years after symptom onset, and 6% died 10 years or more after symptom onset. Of the patients, 70.3% had spinal-onset ALS, 27.9% had bulbar-onset ALS, and 1.7% had respiratory-onset ALS. The mean age at symptom onset did not vary according to ALS type ($p = 0.29$).

More than half of the patients (58.7%) were male, resulting in a male-to-female ratio of 1.4:1. The mean age at symptom onset was significantly higher in women than in men (65.2 years, 95% CI 64.29–66.11 vs. 62.44 years, 95% CI 61.59–63.29, $p < 0.001$), as was the age at death (69.25 years, 95% CI 68.27–70.23 vs. 67.44 years, 95% CI 66.88–68.6, $p = 0.024$). The mean disease duration from the onset of symptoms to death was 4.21 years (95% CI: 3.90–4.52) in men versus 3.46 years (95% CI: 3.19–3.73) in women ($p < 0.001$). Regarding clinical forms, 76.87% of men presented with a spinal form, compared to 61.89% of women ($p = 0.016$). Meanwhile, 21.06% of men presented with a bulbar form, compared to 36.84% of women ($p < 0.001$).

Six percent of the patients had a genetic mutation that was retained to cause ALS. Significant differences were observed between patients with sporadic ALS and genetic ALS regarding the age at symptom onset (63.95 vs. 60.93, $p = 0.019$), age at diagnosis (65.50 vs. 62.49, $p = 0.017$), and age at death (68.45 vs. 64.70, $p = 0.008$).

Table 1. Clinical characteristics of ALS patients followed at the ALS Center of Angers between 2003 and 2023. Accessible data is specified in the *Cases* column.

	Cases	Male		Female	
Gender n (%)	1316	770 (58.5 %)		546 (41.5 %)	
	Cases	Median	Mean (95% CI)	IQR	Range (min-max)
Age at first symptoms (years)	1261	64	63.76 (63.13-64.39)	56.00-72.00	28.00-92.00
Age at diagnosis (years)	1282	66	65.37 (64.76-65.98)	58.00-66.00	31.00-93.00
Age at death (years)	1081	69	68.26 (67.61-68.91)	61.00-69.00	32.00-101.000
Time from 1st symptoms to diagnosis (years)	1252	1	1.50 (1.40-1.60)	1.00-2.00	0.00-22.00
Duration from 1st symptoms to death (years)	1029	3	3.73 (3.54-3.92)	2.00-5.00	0.00-28.00
Time from diagnosis to death (years)	1054	2	2.32 (2.17-2.47)	1.00-3.00	0.00-18.00
	Cases	Spinal n (%)	Bulbar n (%)	Respiratory n (%)	FTD n (%)
Initial presentation	1285	903 (70.3)	359 (27.9)	15 (1.7)	8 (0.6)

Abbreviations: CI: Confidence interval; FTD: Fronto-Temporal Dementia; IQR: Interquartile range (Q1-Q3).

Over-incidence areas and environmental risk factors

To identify potentially high-prevalence sites that could reflect the presence of environmental risk factors, over-incidence areas were defined based on 1) a crude incidence > 10 cases per 100,000 person-years with a minimum of 2 reported cases 2) place of residence of cases within an area of less than 5 km², and 3) an onset time range of less than 10 years between 2 cases. Four over-incidence areas were identified in the Pays de la Loire from 2003 to 2023 based on these criteria (Figure 2A). Juillé (1 on Figure 2A), a commune in the Nord-Sarthe region, had the highest crude incidence in the region, with 3 cases for 372 persons over the 20-year period (crude incidence 40.3 / 100 000 person-year). All three cases resided within a 100-meter radius. In Trans-sur-Erdre (2 on Figure 2A), the crude incidence was 17.9 / 100,000 person-years (3 cases for 837 inhabitants). The cases occurred in 2007, 2008, and 2010, and the patients lived within a two-kilometer radius. In Rouez (3 on Figure 2A), the crude incidence was 16.1 / 100,000 person-years (2 cases for 621 inhabitants). The two ALS cases lived within two kilometers of each other, with symptom onset in 2010 and 2014. Finally, in Le May-sur-Evre (4 on Figure 2A) the crude incidence was 11.2 / 100,000

person-years (7 cases for 3,110 inhabitants). Five sporadic cases of ALS (three spinal onset and two bulbar onset) were recorded there between 2010 and 2014; four of these cases occurred within a 300-meter radius.

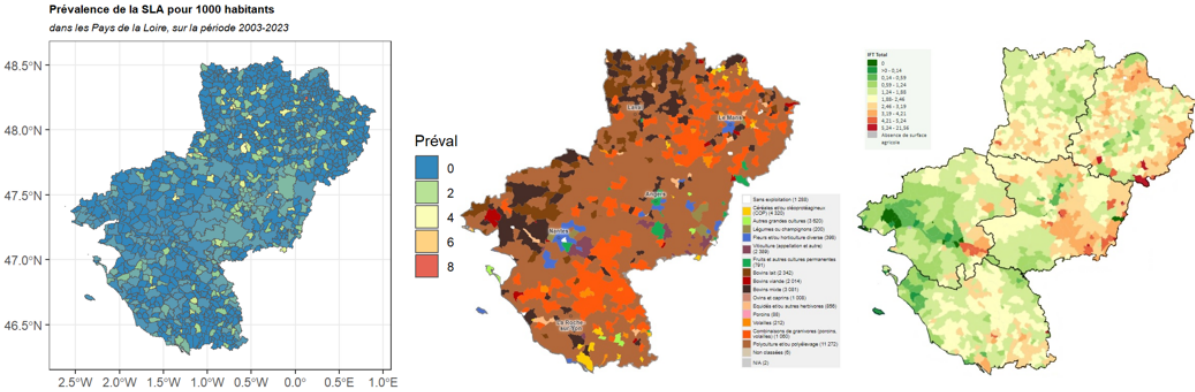


Figure 2. (A) Choropleth map of ALS prevalence per 1000 inhabitants per year over the period 2003-2023 in Pays de la Loire. (B) Agricultural practices in Pays de la Loire communes [11]. (C) Average treatment frequency index (TFI, herbicides and pesticides) by commune [12]. Black circles indicate identified over-incidence areas, black square indicate commune with professional over-representation.

After identifying these over-incidence areas, we investigated potential linked environmental risk factors. A general investigation with the mayors' offices of the communes did not reveal any known environmental issues, and the areas were not considered to be at nuclear risk, nor did they include any industrial establishments classified under the Seveso directive. We then examined the dominant farming practices [11], and the rate of pesticides in the ambient air in the region [12]. As with many others, all of the identified communes were involved in mixed farming and/or the cultivation of corn and soft wheat (Figure 2B). The declared phytosanitary treatment frequency indexes (TFI) were not particularly elevated, ranging from 1.14 to 2.54 (Figure 2C). The other way round, analyzing communes with specific farming practices and/or the highest levels of pesticides in the air did not reveal elevated crude incidences of ALS. Analysis of the air pollution concentration dataset (PM10, PM2.5, O3, and NO2) [13] also revealed no higher rates in the over-incidence areas. The overall and population-weighted annual rates were lower than those of the region's major cities. Finally, there was no over-representation of occupation among the cases that resided in the over-incidence areas.

Professional over-representation and work exposome

Information on occupation was collected for 874 patients: 81 (9.27%) were farmers, 33(3.78%) teachers, 37 (4.23%) factory workers, 25 (2.9%) truckdrivers, and 23 (2.6%) secretaries. To identify potential local occupational risk factors, professional over-representation was defined as follows: 1) more than 50% of cases having the same occupation and 2) cases residing in the same commune or within an area of less than 5 km². This approach allowed us to identify a professional over-representation in Longué-Jumelles/Saint-Philbert-du-Peuple (population 8,122, A on Figure 2A). Of the 7 cases diagnosed during the study period, 5 were farmers, 3 of whom worked in neighboring fields. The declared phytosanitary treatment frequency index (TFI) in this commune was not particularly elevated, 1.78 (A on Figure 2C). Due to this local over-representation, an occupational disease recognition program has been established for affected farmers in this commune, and an investigation is underway to define work exposome and identify contributing factors.

Discussion

In this study, we report the incidence and clinical features of ALS data from a centralized diagnostic center in a French region of 4 million inhabitants over a 20-year period. This is the first epidemiological study conducted in France outside of the Limousin region, providing additional national results.

In our study, the overall crude incidence of ALS was 1.88 cases per 100,000 person-years, which is lower than that observed in the French studies based on data from the FRALim registry, with an estimated incidence between 2.4 and 2.5 cases per 100,000 person-years [6-9]. The incidence is also lower than that observed in European studies, which have estimated the incidence to be around 2.1 cases per 100,000 person-years [14]. Although these variations may be due to distinct environmental and genetic factors within populations, differences in methodology (retrospective studies versus prospective register with correction by capture–recapture analysis) must also be considered. Furthermore, our lower incidence rate may also reflect under-reporting of ALS patients in the region, particularly in rural areas and among older patients. Evidence of this could be seen in the higher mean age of onset in the Limousin studies, at around 70 years, suggesting more effective coverage of older patients.

On a broader timescale, we have not observed an increase in ALS incidence in our region over the last two decades. This is consistent with the French Limousin study, which was conducted over a similar period [9]. Therefore, it appears that there have been no major contributing factors in the Loire region that could have led to an increase in incidence, such as environmental risk factors, an ageing population, changes in healthcare systems, or

greater awareness among the medical community and the general population. It should also be noted that the number of patients referred to the center was unaffected by the health restrictions implemented in France during the COVID-19 pandemic. Finally, we have not observed an increase in cases among young people (under 45 years old) over the last decade, and the average age at symptom onset has remained stable over the last 20 years.

Regarding the clinical profile of the ALS cases, the mean age of onset (63.7 years), mean disease duration (3.7 years) and rate of spinal onset (70.3%) are consistent with previous national and European studies [9,14]. The H:F ratio of 1.4:1 in our study and the more prevalent bulbar presentation in women and spinal presentation in men are also in line with recent French and European epidemiological studies [7,9,14]. These similarities with the literature suggest that our cohort is representative, although it is important to note again that the retrospective approach does not allow for the thoroughness of a study based on a registry.

In our study, six percent of patients had a mutation that was identified as causing ALS. This result should be interpreted with caution, given that the role of *SOD1* gene mutations in causing ALS has been known since 1991 [15,16], whereas the involvement of the other three main genes (*C9orf72*, *TARDBP* and *FUS*) was not established until 2008, 2009 and 2011, respectively [17-22]. Furthermore, in Angers, systematic screening of these four genes in all individuals with ALS has only been carried out since 2021 in accordance with national recommendations [23]. Previously, it was only proposed if the disease was familial. A recent prospective national study which aimed to establish the prevalence of *SOD1* and *C9orf72* variants among the French ALS population by systematically testing incident patients reported a prevalence of 9.2%, which is probably closer to the actual figure [24].

In epidemiology, the study of clusters is essential for identifying potential environmental risk factors. This approach has previously allowed two risk factors to be identified in France: exposure to BMAA toxins and consumption of the *Gyromitra gigas* fungus, which contains a neurotoxin called gyromitrin [25,26]. Our aim was to identify areas of over-incidence by defining criteria based on prevalence rates, geographical locations of cases and times of disease onset. Based on these criteria, we identified four municipalities with spatial and temporal over-incidence. Given the rural characteristics of the region and literature pointing to pesticides as a potential cause of ALS [27-29], we first analyzed agricultural exposure factors but did not observe any clear correlation. It is important to note that this global assessment over a long period of time does not assess the temporal dynamics of these factors or individual exposure over time. Similarly, the study of air pollution showed no correlation, although the studied maps concern background pollution and are therefore not representative of areas close to specific sources such as heavy traffic or industrial activity. Other potential lifestyle-associated risk factors, such as intense physical exercise, head injury, electric shock and military service [30], could not be evaluated due to the

retrospective nature of the study. Studying patients' occupations revealed an overrepresentation of farmers in one small commune, three of whom worked in neighboring fields. This observation led to the establishment of an occupational disease recognition program and the initiation of an investigation to identify local occupational risk factors.

This study identified epidemiological and occupational clusters that are currently being investigated and may help identify risk factors for the disease, highlighting the importance of conducting epidemiological studies on ALS. The main difficulty with this approach remains the fact that data relating to patients' lifestyles, residences and occupations is too extensive to be accurately collected, particularly in retrospective studies. Exposome analysis is therefore fundamentally limited by the large number of potential factors and the biases inherent in epidemiological studies such as memory bias, collection bias and confounding factors. Added to this complexity is the fact that the disease begins in the nervous system of patients years before symptom onset [31], making it challenging to identify risk factors from previous decades. The creation of prospective registries such as the one in Limoges should be encouraged to obtain the most reliable and complete analysis.

Conclusion

Here, we present 20 years of epidemiological data that contribute to the characterization of the French ALS population. Our data is relatively consistent with national and European studies, although the crude incidence rate is lower. We identified areas of over-incidence in specific geographical locations over short time periods, which may be linked to environmental factors that remain unknown. Our study of occupations identified a professional cluster with possible risk factors linked to agricultural activity, an administrative report has been drawn up, and a study of exposure factors is underway. Our results support the importance of conducting epidemiological studies through prospective and comprehensive registries to identify potential risk factors for the disease and contribute to our understanding of ALS and its prevention.

Acknowledgements

We would like to thank Ms. J. Muller for her help with this project.

Statement of Ethics

The project received authorization from the CNIL (Commission Nationale de l'Informatique et des Libertés, no. AR24-0021V0) on March 29, 2024, and from the Ethics Committee of

Angers (authorization no. 2024-101) on May 14, 2024. The General Data Protection Regulation (GDPR) was observed in accordance with European legislation. Data were collected anonymously, and a correspondence list was kept under the responsibility of the principal investigator.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Author Contributions

MLC, ML and PC conceived and designed the study. MF, JC, CS, and PC recruited participants. MLC, MG, ML and PC analyzed data. MLC, MG and PC drafted the manuscript and figures. All authors revised and approved the final manuscript.

Data Availability Statement

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

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Détection de la pathologie TDP-43 dans les glandes salivaires accessoires de patients atteints de Sclérose Latérale Amyotrophique

RÉSUMÉ

INTRODUCTION : De récentes données suggèrent la présence d'une pathologie TDP-43 dans les tissus périphériques des patients atteints de sclérose latérale amyotrophique (SLA), ouvrant ainsi la voie à une meilleure compréhension de la maladie et à l'identification de biomarqueurs.

SUJETS ET METHODES : Nous avons étudié la pathologie TDP-43 dans les glandes salivaires accessoires de 10 patients atteints de SLA et de 20 témoins.

RESULTATS : Seul un patient présentait une immunoréactivité pTDP-43 dans quelques cellules.

Aucune différence significative n'a été observée entre les groupes en termes de surface de coloration à l'aide d'une quantification numérique.

CONCLUSION : Ces résultats suggèrent que la pathologie TDP-43 peut être détectée au sein des glandes salivaires de patients atteints de SLA, La faible fréquence de l'atteinte tissulaire limite toutefois sa valeur diagnostique. Des études supplémentaires sont nécessaires pour clarifier les schémas temporels et spatiaux de la diffusion périphérique de pTDP-43 dans la SLA.

Mots-clés : Sclérose Latérale Amyotrophique, TDP-43, glandes salivaires accessoires, pathologie périphérique

Detection of tdp-43 pathology in minor salivary glands of patients with amyotrophic lateral sclerosis

ABSTRACT

INTRODUCTION: Emerging evidence suggests the presence of TDP-43 pathology in peripheral tissues of patients with Amyotrophic Lateral Sclerosis (ALS), providing avenues to better understand the disease and identify biomarkers.

SUBJECTS AND METHODS: Here, we investigated pathological TDP-43 in minor salivary glands (MSG) from 10 patients with ALS and 20 controls.

RESULTS: Only one patient showed pTDP-43 immunoreactivity in a few cells.

No significant difference in staining surface was found between groups using digital quantification.

CONCLUSION: These results suggest that TDP-43 pathology can be detected in the salivary glands of ALS patients. However, the low frequency of tissue involvement limits its diagnostic value. Further studies are needed to clarify the temporal and spatial patterns of peripheral pTDP-43 diffusion in ALS.

Keywords : Amyotrophic lateral sclerosis, TDP-43, minor salivary glands, biopsy, peripheral pathology.