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Systemische Faktoren bei der amyotrophen Lateralsklerose – Blut-Liquor-Schranke und Metabolismus

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Abkürzungsverzeichnis

AD	Alzheimer Demenz
ALS	Amyotrophe Lateralsklerose
ALS-FRS	ALS-Functional Rating Scale
ApoA1	Apolipoprotein A1
ApoB	Apolipoprotein B
ANCOVA	Kovarianzanalyse (Analysis of Covariance)
ANOVA	Varianzanalyse (Analysis of Variance)
BMI	Körpermasseindex (body mass index)
C9ORF72	Chromosom-9-Open-Reading-Frame-72-Gen
COPD	Chronisch obstruktiver Lungenerkrankung (chronic obstructive pulmonary disease)
CSF	Cerebrospinalflüssigkeit (cerebrospinal fluid)
CXCL13	C-X-C-motif chemokine ligand 13
DM	Diabetes mellitus
DZNE	Deutsches Zentrum für Neurodegenerative Erkrankungen
fALS	Familiäre ALS
FP	Fazialisparese
FUS	Fused in sarcoma
GWAS	Genome-wide association study
HDL	High density lipoprotein
HR	Hazard ratio
HSD	Honestly significant difference
HSP	Hereditäre spastische Spinalparalysen
HTML	Hypertext markup language
ID	Identifikationsnummer
IG	Immunglobulin
IQR	Interquartilsabstand (interquartile range)
IV	Instrumentalvariablen
KI	Konfidenzintervall
LDL	Low Density Lipoprotein
LMN	Unteres Motoneuron (lower motor neuron)
LP	Lumbalpunktion
MCI	Mild Cognitive Impairment
MR	Mendelsche Randomisierung
NfL	Neurofilament leichte Kette (Neurofilament light chain)
OKBs	Oligoklonale IgG Banden
OR	Odds ratio
pNfH	Neurofilament phosphorylierte schwere Kette (phosphorylated neurofilament heavy chain)
pTDP43	phosphorylated transactive response DNA-binding protein 43
PUFAs	Mehrfach ungesättigte Fettsäuren (Polyunsaturated Fatty Acids)
QAlb	Liquor-Serum-Albumin-Quotient
RKU	Rehabilitationskliniken Ulm

sALS	Sporadische ALS
SD	Standardabweichung (Standard Deviation)
SMA	Spinale Muskelatrophie
SNP	Single nucleotide polymorphism
SOD1	Superoxide Dismutase 1
TARDBP	TARDNA Binding Protein
TH	Spannungskopfschmerzen (Tension headache)
UMN	Oberes Motoneuron (upper motor neuron)
VZV	Varizella Zoster Virus
XML	Extensible markup language

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1. Einleitung

1.1 Amyotrophe Lateralsklerose

Das klinische Bild

Die amyotrophe Lateralsklerose (ALS) ist eine neurodegenerative Erkrankung des Erwachsenenalters, welche chronisch progressiv verläuft und das erste und zweite Motoneuron betrifft. Klinisch zeigt sich durch die Schädigung des ersten Motoneurons ein erhöhter Muskeltonus; durch die des zweiten Motoneurons Paresen und Muskelatrophien. Zusätzlich wird zwischen spinalem und bulbärem Krankheitsbeginn unterschieden. Die irreversiblen Schädigungen breiten sich rapide in allen Muskelgruppen des Körpers aus, was nach durchschnittlich drei bis sechs Jahren meist aufgrund einer Betroffenheit der Atemmuskulatur letal endet. Das durchschnittliche Erkrankungsalter der ALS liegt in Deutschland bei 61 Jahren (Dorst et al., 2019). Die Prävalenz liegt bei 4.1 - 8.4, die Inzidenz bei 2.1 – 3.8 pro 100.000 Personen in Europa (E. Longinetti & Fang, 2019). In 5-10% der Fälle ist die ALS familiär (fALS), genetisch bedingt, beim Rest der Patienten tritt sie sporadisch (sALS) auf (Kiernan et al., 2011). Die Progression der ALS und der damit einhergehende Verlust der Mobilität, der Sprache und bei fortgeschrittener Krankheit der Fähigkeit selbständig zu atmen sind weiterhin eine enorme psychische Belastung sowohl für Patienten als auch Angehörige, da eine Heilung bisher nicht existent ist (Lulé et al., 2019).

Pathogenese, Diagnostik und Therapie

Die ALS wurde 1874 von Jean Martin Charcot erstmals klinisch als auch anatomisch („anatomo-clinical method“) beschrieben. Charcot beschrieb die Erkrankung vorwiegend nach dem Tod der Patienten (Rowland, 2001). Heiko Braak autopsierte 76 Gehirne von ALS Patienten, wodurch 2013 eine neuropathologische Stadieneinteilung, welche auf dem Verteilungsmuster des zellschädigenden Proteins „phosphorylated transactive response DNA-binding protein 43“ (pTDP-43) basiert, veröffentlicht werden konnte (Brettschneider et al., 2013).

Stadium 1: Geringstes Vorkommen von pTDP-43 im agranulären motorischen Kortex, α-Motoneuronen des spinalen Cortex und bulbären Motorneuronen der Hirnerven V, VII, X-XII

Stadium 2: Stadium 1 + Betroffenheit des präfrontalen Neocortex, Formatio reticularis des Hirnstamms, präcerebellärer Zellkerne und des roten Nukleus im Mittelhin

Stadium 3: Ausbreitung von Läsionen des präfrontalen Neocortex (Gyrus rectus und Orbital Gyri), Striatum und post-zentralen Cortex.

Stadium 4: Höchste Ausbreitung von pTDP-43 zusätzlich zu den Regionen der Stadien 1-3 in anteromedialen Anteilen des Temporallappens

Damit wurde nachgewiesen, dass es sich bei der ALS um eine Multisystemerkrankung handelt. Außerdem wurde es basierend auf der Stadieneinteilung möglich, ALS Patienten mithilfe bildgebender Verfahren hinsichtlich ihres Erkrankungsstadiums zu diagnostizieren. Die grundsätzliche Diagnosestellung erfolgt jedoch basierend auf dem klinischen Bild anhand der El Escorial Kriterien (Brooks et al., 2000). Im Gegensatz zu Parkinson oder Alzheimer ist durch die Stadieneinteilung und die El Escorial Kriterien bei der ALS keine präklinische Phase definiert (Oeckl et al., 2019). Das Gen, welches pTDP-43 kodiert ist TARDBP (Gendron et al., 2013), welches neben C9ORF72, SOD1 und FUS eines der am

häufigsten mutierten Gene der ALS darstellt, die bis jetzt bekannt sind (siehe Abbildung 1) (Zou et al., 2017).

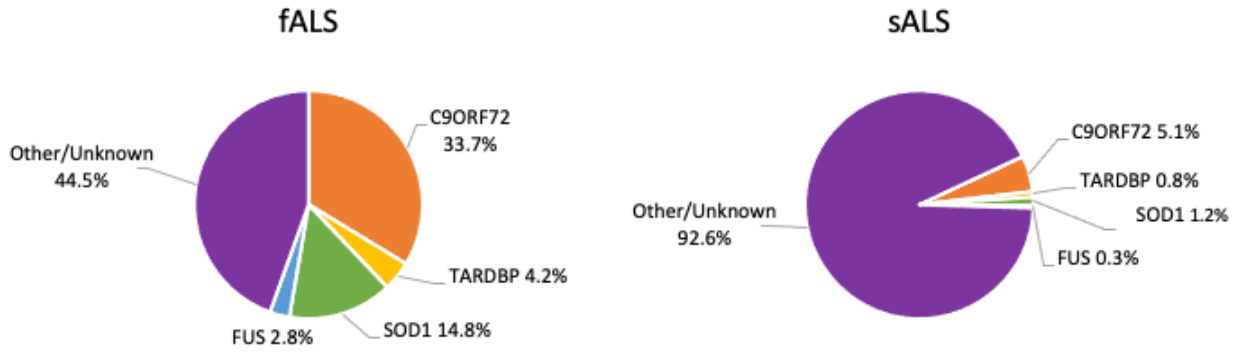

Abbildung 1: Verteilung von Amyotrophe Lateralsklerose (ALS) Mutationen in Europa (in Anlehnung an (Zou et al., 2017), mit freundlicher Genehmigung von BMJ Publishing Group Ltd.); Chromosom-9-Open-Reding-Frame-72-Gen (C9ORF72), TARDNA Binding Protein (TARDBP), Superoxide Dismutase 1 (SOD1), Fused in sarcoma (FUS)

Neben genetischen Mutationen wurden noch keine eindeutigen Risikofaktoren, welche das Risiko ALS zu entwickeln begünstigen, identifiziert. Es ist davon auszugehen, dass Menschen mit einem besseren Fitnesslevel und kardiovaskulären Profil ein erhöhtes Risiko haben an ALS zu erkranken (Visser et al., 2017). Bei ALS-Patienten zeigt sich außerdem häufig ein Hypermetabolismus (Funalot et al., 2009), Hyperlipidämie und Gewichtsverlust. Diese Faktoren wirken sich umso stärker sie ausgeprägt sind meist negativ auf die Prognose aus und verkürzen die Überlebenszeit der ALS Patienten (Luc Dupuis et al., 2011). Des Weiteren wurde vor allem bei fALS-Patienten ein Zusammenhang des Körpermasseindexes (BMI) mit Atrophien des Hypothalamus entdeckt, sogar bei präklinischen ALS Patienten. Außerdem konnte ein geringeres Hypothalamus-Volumen, das durch diese Atrophien hervorgerufen wurde mit einem früheren Ausbruch der ALS in Verbindung gebracht werden (Gorges et al., 2017). Ein weiterer Hinweis auf eine bereits ausgebrochene ALS ist die Neuroinflammation, welche mit dem klinischen Bild einhergeht (Oeckl et al., 2019), und die Aktivierung von Mikroglia und Astrozyten einschließt (Evans et al., 2013). Nachweisbar sind diese Entzündungszeichen im Liquor. In Tiermodellen (Mäusen mit SOD1-Mutation) konnte ebenfalls eine Inflammation mit Beteiligung von Mikroglia und Astrozyten im symptomatischen Stadium der ALS gefunden werden (McCombe & Henderson, 2011). Bei Anwendung entzündungshemmender Medikamente zeigte sich hier ein verbessertes Überleben. Beim Menschen könnte diese Behandlungsstrategie auch in Betracht gezogen werden, da hier wie auch in Tiermodellen die erhöhte Aktivierung von Mikroglia mit der erhöhten Schädigung der Motorneurone einherzugehen scheint (Turner et al., 2004).
Innerhalb der Pathogenese der ALS wurde auch immer wieder die Möglichkeit eines Autoimmunmechanismus diskutiert (Drachman & Kuncl, 1989). Eine schwedische Studie fand heraus, dass bei ALS Patienten das Risiko um 47 % erhöht war vor dem Ausbruch der ALS eine Autoimmunerkrankung diagnostiziert zu bekommen. Es ist jedoch oft nicht klar, ob die ALS mit den Autoimmunerkrankungen zusammenhängt oder diese vorab fälschlicherweise als Fehldiagnose gestellt wurde. Bei Autoimmunerkrankungen wie Diabetes mellitus Typ 1 und Multipler Sklerose wird jedoch weiterhin ein Zusammenhang mit der ALS vermutet (Cui et al., 2021).

Derzeit gibt es noch keine kurative Therapie der ALS. Vorwiegend wird die ALS bisher mit Riluzol, einem Glutamatfreisetzungsblocker behandelt, welcher das Leben der Patienten ursprünglich um durchschnittlich drei Monate verlängerte (Bensimon et al., 1994). In neueren Studien geht man bei der Behandlung mit Riluzol mittlerweile von einer durchschnittlichen Verlängerung der Überlebenszeit zwischen sechs und 19 Monaten aus (Andrews et al., 2020). Bei ALS Patienten mit SOD1 Mutation scheint das Medikament Tofersen vielversprechend zu sein, da es zu einer Abnahme der NfL (Neurofilament leichte Kette) führt (Meyer et al., 2023). Hinsichtlich der Wirkung und Nebenwirkungen bedarf es hier aber weiterer Langzeitbeobachtungen und klinischer Studien.

1.2 Fragestellung der Arbeit

Ziel dieser Arbeit ist es modifizierende Faktoren der ALS im Liquor und Blut von Patienten zu identifizieren. Als ersten Parameter möchte ich den Liquor-Serum-Albumin-Quotient (QAlb) untersuchen, da dieser eine Aussage über die Funktion und Permeabilität der Blut-Liquor-Schranke ermöglicht. Diese wird neben dem Alter und dem BMI möglicherweise auch durch neurologische Erkrankungen wie die ALS beeinflusst oder ist dafür verantwortlich, dass Moleküle das Gehirn erreichen, vor denen es durch eine intakte Blut-Liquor-Schranke geschützt ist (Brettschneider et al., 2005). Der zweite Faktor, der innerhalb dieser Dissertation, im Liquor von ALS Patienten (> 1000) untersucht wird, sind Marker der Inflammation; dies schließt die Untersuchung von oligoklonalen Banden mit ein. Im Blut hingegen soll der Frage nach klinischen und präklinischen metabolischen Veränderungen nachgegangen werden. Hierzu kam das statistische Verfahren der Mendelschen Randomisierung zum Einsatz. Für die präklinischen Untersuchungen des Lipid- und Glukosemetabolismus stand ein weltweit einzigartiges Kollektiv von 80 präklinischen Genträgern und ihren Geschwistern zur Verfügung.

2. Allgemeine Methodik

2.1 Extraktion der Liquordaten

Die aufgezeichneten Liquordaten der Biobank der Rehabilitationskliniken Ulm (RKU) lagen erst ab 2019 teilweise digitalisiert vor. Davor erfolgte die Dokumentation der Liquorparameter ausschließlich in Papierform. Durch das Nephelometer wurden jedoch unstrukturierte bis semi-strukturierte Daten der Messungen in Form von 2.021 Text-Dateien (.txt Format) erzeugt. Diese Text-Dateien reichten von November 2011 bis Dezember 2021. Als unstrukturierte Daten bezeichnet man Daten, die in einer nicht normalisierten Datenstruktur außerhalb einer relationalen Datenbank meist im Textformat vorliegen. In dieser Form sind die Daten für wissenschaftliche Analysen unbrauchbar (Boulton & Hammersley, 2006). Als semi-strukturierte Daten kann man wiederum zum Beispiel XML- oder HTML-Dateien bezeichnen, welche anhand von vordefinierten Tags innerhalb des Texts eine gewisse Struktur aufweisen (Abiteboul, 1997), jedoch in dieser Form ohne weitere Modifizierung auch nicht in Datenbanken eingepflegt werden und für Analysen genutzt werden können.

Die Daten des Nephelometers wiesen teilweise eine Struktur auf, die aber nicht vordefiniert war, somit sind diese Daten zwischen unstrukturierten und semi-strukturierten Daten einzuordnen. In der Distribution Anaconda 3 (Version 2020.02) wurde die Programmiersprache Python (Version 3.7.6) innerhalb eines Jupyter Notebooks (Version 6.03) angewendet, um die Liquordaten in eine nutzbare Form zu bringen.

Für die Textdateien habe ich pro Jahr ein individuell zugeschnittenes Python-Programm zur Extrahierung der Daten geschrieben, da sich a) die Struktur der Dokumente jedes Jahr geringfügig änderte; b) Ausnahmefälle in den Daten so einfacher identifizierbar waren und angepasst bzw. korrigiert werden konnten (zum Beispiel an einer Stelle im Text, an dem das LP-Datum erwartet war stand ein Vorname); c) von 2012 bis 2016 ein Nephelometer mit der Software Protis® und in darauffolgenden Jahren mit Medeora® eingesetzt wurde wodurch sich auch die Textdateien änderten und d) unterschiedliche Geräte zur Bestimmung unterschiedlicher Werte verwendet wurden (zum Beispiel BN-Prospec für Albumin und Immunglobuline, Olympus AU für Protein und Laktat).

```
H|\^&|||Protis|||||||P|DB 100102|20120401165205|
P|1||||Mustermann^Max^^||19660508|M|^||||^^^^|||||^|||||\||Su^Stroke Unit|||||^|||||
C|1|I|CHECK^||
C|2|I|CSFChart3^ [  ]  Schrankendysfunktion||
C|3|I|CSFChart4^ [  ]  Erh^hte Zellzahl||
C|4|I|OLIGO^Identische oligoklonale Banden in CSF und Serum.||
C|5|I|CSFChart5^ [x]  Entz¸ndlicher Prozess im ZNS||
C|6|I|CSFChart6^ [x]  Spez. AK-Synthese im ZNS||
C|7|I|CSFChart1^ [  ]  Normaler Liquorbefund||
C|8|I|CSFChart2^ [  ]  Normaler Liquor-Proteinbefund||
C|9|I|CSF^__||
O|1|max08056620120401C||^^^0107^1601^ALBliq|R|||||N|||20120329120704|C^|^^^^||1|1|||
20120401165205|||F||||||
R|1|^^^0107^1601^ALBliq|130|mg/L|0-350|||F|||20120401165205|20120401165205|10648|
L|1|N|
```

Abbildung 2: Beispiel für rohe Textdaten erstellt durch Protis® (2012)

Für alle Python Programme wurden die Bibliotheken Pandas (Version 1.1.5), NumPy (Version 1.18.1), re (Version 2.2.1) und datetime verwendet. Nach dem Einlesen einer Textdatei in Python wurde diese in Einträge unterteilt und in ein Array eingeschlossen. Ein Eintrag begann hierbei immer mit „H| \^&|||" (siehe Abbildung 2) und konnte somit eindeutig identifiziert werden. Anschließend habe ich die Textdateien optisch inspiziert und

herausgefunden, welche relevanten Parameter aus den Liquordaten exportiert werden konnten, dies waren folgende:

- Proben-Identifikationsnummer (ID)
- Datum der Lumbalpunktion (LP)
- Nachname
- Vorname
- Geburtsdatum
- Geschlecht

Extrahierte Messparameter, individuell bei jedem Patienten vorhanden/nicht vorhanden, je nachdem was gemessen wurde:

- Lymphozyten (n=2.845)
- Plasmazellen (n=604)
- Monozyten (n=2.819)
- Leukozyten (n=18.337)
- Erythrozyten (n=13.656)
- Granulozyten (n=302)
- Gesamteiweiß (n=23.490)
- Laktat (n=22.345)
- IgG Oligoklonale Banden (OKB; n=17.726)
- IgG (n=24.196; n=24.276), IgA (n=23.979; n=24.024) und IgM (n=23.927; n=21.569) in Serum und Liquor
- Albumin in Serum und Liquor (n=24.184; n=24.246)
- CXCL13 in Serum und Liquor (n=1.463; n=1.820)
- Glukose (n=1.668)
- Tau Protein (n=4.269)
- ß-Amyloid 1-42 (n=4.269)
- Röteln Antikörper (im IgG; n=4.106)
- Masern Antikörper (im IgG; n=4.110)
- Herpes Simplex Antikörper (im IgG; n=3.398)
- Epstein-Barr Antikörper (im IgG; n=681)
- Borrelien Antikörper (im IgG & IgM; n=1.446 & n=102)
- Frühsommer-Meningoenzephalitis Antikörper (im IgG & IgM; n=921 & n=94)
- Varizella Zoster Antikörper (im IgG; n=4.782)
- Neurofilament phosphorylierte schwere Kette (pNfH; n =1.224)
- Ferritin in Serum und Liquor (n=494; n=473)
- ß-2 Mikroglobulin in Serum und Liquor (n=381; n=377)

Ab dem Einsatz von Medeora® konnten aus dem aktuellen Nephelometer (seit 2017) die Messdaten auch aus dem Gerät in eine Excel Datei exportiert werden (siehe Abbildung 3). Dadurch konnten von diesem Zeitpunkt an die exportierten Daten aus den Textdateien mit den Exceldaten validiert und zusammengeführt (da in den Exceldateien z.B. OKBs nicht enthalten waren) werden.

PNR	Nachname	Vorname	geb.	G.	Auftrag	Anforderung	Einsender	Analyse	Gerät	Ergebniseingabe	Ergebnis
1234567891	Mustermann	Max	08.05.1966	M	46523165	19.08.2017 15:27	Ambulanz Neurologie	Albumin Serum	BN-Prospec	19.08.2017 15:51	42.5
1234567891	Mustermann	Max	08.05.1966	M	46523165	19.08.2017 15:27	Ambulanz Neurologie	Albumin im Liquor	BN-Prospec	19.08.2017 15:44	130
1234567891	Mustermann	Max	08.05.1966	M	46523165	19.08.2017 15:27	Ambulanz Neurologie	IgA Liquor	BN-Prospec	19.08.2017 15:37	0.5
1234567891	Mustermann	Max	08.05.1966	M	46523165	19.08.2017 15:27	Ambulanz Neurologie	IgA Serum	BN-Prospec	19.08.2017 15:30	1.3
1234567891	Mustermann	Max	08.05.1966	M	46523165	19.08.2017 15:27	Ambulanz Neurologie	IgG Liquor	BN-Prospec	19.08.2017 15:23	12
1234567891	Mustermann	Max	08.05.1966	M	46523165	19.08.2017 15:27	Ambulanz Neurologie	IgG Serum	BN-Prospec	19.08.2017 15:16	8.7
1234567891	Mustermann	Max	08.05.1966	M	46523165	19.08.2017 15:27	Ambulanz Neurologie	IgM Serum	BN-Prospec	19.08.2017 15:09	1.4
1234567891	Mustermann	Max	08.05.1966	M	46523165	19.08.2017 15:27	Ambulanz Neurologie	Laktat im Liquor	Olympus AU480	19.08.2017 15:02	1.68

Abbildung 3: Beispiel für exportierte Exceldatei direkt aus dem Nephelometer (erst ab 2017 möglich)

Diese Daten wurden anschließend innerhalb der Entwicklungsumgebung RStudio (Version

1.2.5033, R Version 4.0.3) bereinigt und auf Plausibilität geprüft. Insgesamt konnte ich mit diesen Verfahren die Daten von 31.053 Liquoruntersuchungen von 24.546 Patienten digitalisieren, strukturieren und somit für Analysen nutzbar machen. In den Daten ließen sich unter anderem 1203 ALS Patienten mit ihren zugehörigen Liquordaten finden. Diese habe ich mit den folgenden klinischen Parametern aus dem ALS Schwaben Register ergänzen können:

- Datum der Erstmanifestation
- Visitendatum
- ALS-Functional Rating Scale (ALS-FRS) bei Diagnose und jeder Vorstellung
- BMI bei Diagnose und jeder Vorstellung
- Todesdatum (falls vorhanden)
- Datum an dem die Diagnose gestellt wurde
- Lokalisation der Erstmanifestation (bulbär, spinal)

Daraus wurden Parameter errechnet, wie das Alter bei Erstmanifestation und die Progressionsrate, welche falls der ALS-FRS bei mehreren Besuchen dokumentiert wurde wie folgt berechnet wurde: (Erster ALS-FRS – letzter ALS-FRS)/ Monate zwischen erster und letzter ALS-FRS Dokumentation; falls jedoch nur der ALS-FRS bei Diagnose vorlag wurde die Progressionsrate ersatzweise folgendermaßen definiert: (48-ALS-FRS)/Monate zwischen Erstmanifestation und LP Datum (Halbgebauer et al., 2022; Simon et al., 2014).

2.2 Mendelsche Randomisierung

In einer in diese kumulative Dissertation eingebundenen Publikation („Lipids and amyotrophic lateral sclerosis: A two sample Mendelian randomization study“) wird die Methode der Mendelschen Randomisierung (MR) verwendet. Diese soll mithilfe von genetischen Markern bei nicht-experimentellen Studien die Beziehung zwischen veränderlichen Expositionen auf das Risiko eine Erkrankung zu entwickeln abbilden (Burgess & Thompson, 2015). In Abbildung 4 ist das Prinzip der MR mit dem Beispiel der ALS dargestellt.

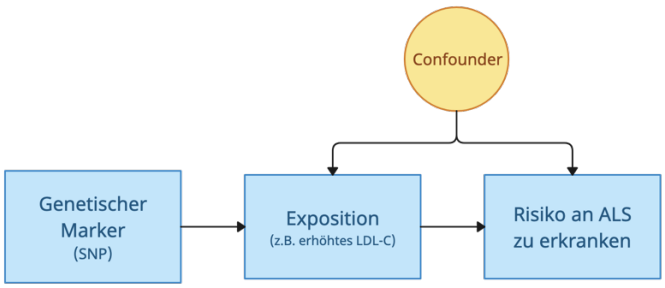

Abbildung 4: Prinzip der Mendelschen Randomisierung (in Anlehnung an (Burgess & Thompson, 2015), Seite 28, mit freundlicher Genehmigung von INFORMA UK LIMITED)

Die MR hat drei Grundannahmen für die genetischen Marker, welche in dieser Methode auch als Instrumentalvariablen (IV) bezeichnet werden und meist Single Nucleotide Polymorphisms (SNPs) sind.

I. Die IV ist mit der Exposition assoziiert
II. Die IV steht in keinem Zusammenhang mit einer Störvariable (Confounder), welche sich auf die Beziehung zwischen Exposition und dem Risiko, in unserem Fall an ALS, zu erkranken

III. Die IV beeinflusst nicht das Risiko der Erkrankung, außer über die Verbindung zur Exposition

Die Randomisierung erfolgt bei der MR anhand der genetischen Marker (siehe Abbildung 5), welche direkt mit einer Exposition assoziiert sind. Wenn nachgewiesen wird, dass der genetische Marker mit einer Erkrankung assoziiert ist wird der Zusammenhang zwischen dieser Erkrankung und der Exposition als kausal eingeschätzt („causal estimation“).

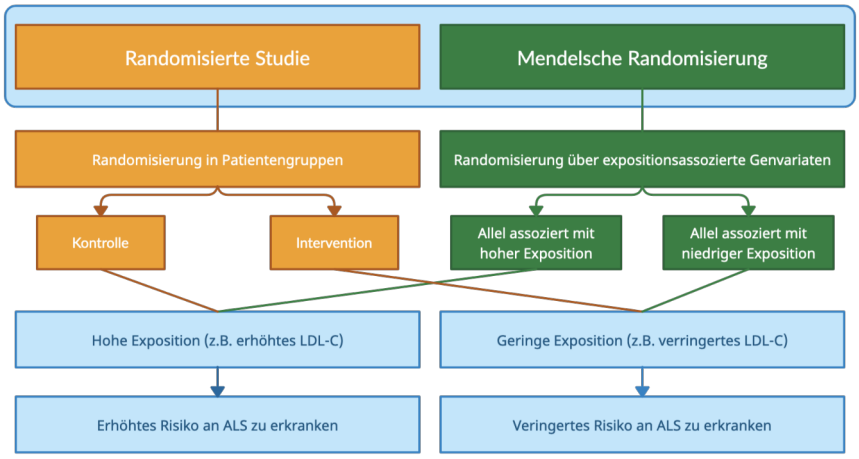

Abbildung 5: Vergleich einer randomisierten kontrollierten Studie und der Mendelschen Randomisierung (in Anlehnung an (Burgess & Thompson, 2015), Seite 17, mit freundlicher Genehmigung von INFORMA UK LIMITED)

3. Ergebnisse

Zum Überblick und Abgrenzung der einzelnen Publikationen, welche in die vorliegende kumulative Dissertation eingebundenen wurden, und deren Ergebnisse dient Tabelle 1.

Tabelle 1: Überblick zu eingebundenen Publikationen der kumulativen Dissertation

Publikation	Studienkohorte	Zielgrößen	Ergebnisse
Blood-CSF barrier integrity in amyotrophic lateral sclerosis	ALS Patienten aus dem ALS Schwaben Register (n=1059) Kontrollen: Patienten mit Alzheimer (n=336), Spannungskopfschmerz (n=105), Fazialisparese (n=36)	Liquor-Serum-Albumin-Quotient (QAlb), Überleben	Blut-Liquor-Schranke bei der ALS intakt; keine Altersabhängigkeit des QAlb bei der ALS; kein Hinweis eines Zusammenhangs der Funktion der Blut-Liquor-Schranke mit dem Überleben in der Gesamtpopulation; Längeres Überleben bei männlichen ALS Patienten mit niedrigerem QAlb (beeinflusst durch DM und BMI); Kürzeres Überleben bei männlichen Patienten mit erhöhtem QAlb (beeinflusst durch DM und BMI); kein Zusammenhang von Überleben und QAlb bei weiblichen ALS Patienten
CSF oligoclonal IgG bands are not associated with ALS progression and prognosis	ALS Patienten aus dem ALS Schwaben Register (n=457) Kontrollen: Patienten mit Alzheimer (n=506), Mild Cognitive Impairment (MCI) (n=91), Spannungskopfschmerz (n=152), Fazialisparese (n=94)	Oligoklonale IgG Banden (OKBs)	Kein Hinweis auf Inflammation bei der ALS, OKBs sind ein Hinweis auf eine infektiöse oder entzündliche Komorbidität
Lipids and Amyotrophic Lateral Sclerosis: A two-sample Mendelian Randomization Study	Genome-wide association study (GWAS) Daten: Gesamtcholesterin (n=188.578), High Density Liporotein (HDL) Cholesterin (n=403.943), Low Density Lipoprotein (LDL) Cholesterin (n=440.546), Apolipoprotein A1 (ApoA1) (n=391.193),	Lipide (Gesamtcholesterin, HDL, LDL, ApoA1, ApoB)	Zusammenhang zwischen präklinisch erhöhten Lipiden und dem Risiko an ALS zu erkranken; vermittelnde Rolle von LDL-Cholesterin in der Verbindung zwischen Polyunsaturated Fatty Acids (PUFAs) und ALS

Fortsetzung der Tabelle auf Seite 9

Fortsetzung der Tabelle von Seite 8

	Apolipoprotein B (ApoB) (n=439.214), ALS Fälle (n=12.577) und Kontrollen (n=23.475)		
Mutation-specific metabolic profiles in presymptomatic amyotrophic lateral sclerosis	Präsymptomatische ALS Genträger (n=60) Kontrollen: Nicht-Genträger aus den gleichen Familien (n=70)	Metabolisches Profil	Kein Unterschied zwischen allen ALS Genträgern und Kontrollen; niedrigeres kardiovaskuläres Risikoprofil bei präsymptomatischen C9ORF72 ALS Genträgern (niedrigerer BMI, systolischer Blutdruck, nüchterner Glukosewert im Serum, höheres HDL) im Vergleich zu Kontrollen; höheres kardiovaskuläres Risikoprofil bei präsymptomatischen SOD1 ALS Genträgern (höherer BMI, Nüchtern - glukose im Serum, niedrigeres HDL) im Vergleich zu Kontrollen

3.1 Publikation I: Blood-CSF-Barrier integrity in amyotrophic lateral sclerosis

Klose V., Jesse S., Lewerenz J., Kassubek J., Dorst J., Rosenbohm A., Nagel G., Wernecke D., Roselli F., Tumani H, Ludolph A.C. (2024). Blood-CSF barrier integrity in amyotrophic lateral sclerosis. Brain. 2024 May 14: awae144. doi: 10.1093/brain/awae144. PMID: 38743595. (Klose et al., 2024)

Fragestellung

In der ersten Publikation, die in diese Dissertation miteinfließt, wurde untersucht, ob es signifikante Unterschiede bei der Blut-Liquor-Schranke zwischen ALS Patienten und anderen Patientenkohorten (Kontrollkohorten) mit Alzheimer Demenz (AD = Alzheimer Disease), Spannungskopfschmerz (TH = Tension Headache) und Fazialisparese (FP) gibt. Außerdem sollte anhand der vorliegenden Daten ermittelt werden, ob ein Zusammenhang zwischen der Überlebensdauer von ALS Patienten und der Blut-Liquor-Schranke existiert.

Methodik

Für diese Studie wurden die extrahierten Liquordaten verwendet, die zuvor in 2 Allgemeine Methodik erwähnt wurden. Da der Liquor-Serum-Albumin-Quotient (QAlb), welcher sich aus dem Albumin im Serum und dem im Liquor ergibt, eine Beurteilung über die Permeabilität der Blut-Liquor-Schranke ermöglicht (Hansotto Reiber, 2003), wurde dieser instrumentalisiert um Unterschiede zwischen den Krankheitskohorten zu identifizieren. Um sicher zu gehen, die Blut-Liquor-Schranke durch QAlb adäquat beurteilen zu können, habe ich mir zusätzlich auch die Synthese der Immunglobuline IgG, IgA und IgM in Form ihrer Serum-Liquor-Quotienten (QIgG, QIgA und QIgM) angesehen. Patienten ohne gemessenes Albumin im Serum oder Liquor, sodass kein QAlb ermittelbar war, oder Patienten mit einem QAlb Wert über 20×10^{-3} wurden von unserer Studie exkludiert. Um entzündliche Komorbiditäten auszuschließen wurden Patienten mit einer Leukozytenzahl > 10 nicht mit in die Analysen einbezogen, ebenso wurden Patienten unter 16 Jahren ausgeschlossen. Bei 40 Patienten war die Enddiagnose „spinale Muskelatrophie“ (SMA), bei 20 „hereditäre spastische Spinalparalyse“ (HSP), daher wurden sie für diese Studie auch nicht berücksichtigt. Somit standen für die Analysen 1059 ALS Patienten mit ihren Liquorparametern zur Verfügung. Eine Störung der Blut-Liquor-Schranke ist laut Reiber altersabhängig definiert, so liegt der Normalbereich des QAlb $< (4 + \text{Alter in Jahren}/15) \times 10^{-3}$ (H. Reiber et al., 2001). Aufgrund der Abhängigkeit des QAlb vom Alter wurden die Kontrollkohorten der Altersstruktur der ALS Kohorte angeglichen, um sie vergleichbar zu machen, daher ergaben sich für meine Analysen 336 AD, 105 TH und 36 FP Patienten als Kontrollen. Die Studie wurde in Übereinstimmung mit der Deklaration von Helsinki durchgeführt und von der Ethikkommission der Universität Ulm genehmigt (Genehmigungsnummer 20/10). Die Vergleiche von demografischen Daten und Liquorparametern zwischen den Kohorten wurden mit dem Pearson's χ^2-Test für kategoriale bzw. qualitative Variablen und einer einfaktoriellen ANOVA (Analysis of Variance) für quantitative bzw. kontinuierliche Variablen durchgeführt. Eine zweifaktorielle ANOVA wurde angewendet um den Unterschied des QAlb zwischen den Geschlechtern in den Kohorten zu evaluieren. Um Unterschiede zwischen dem Verlauf des QAlb Werts mit dem Alter zwischen den Krankheitskohorten zu ermitteln wurde ein lineares Regressionsmodell

und anschließend eine ANCOVA (Analysis of Covariance) angewendet. Mithilfe der paarweisen Pearson-Korrelation konnten wir eine Korrelationsmatrix erstellen, durch welche ermittelbar war, welche Variablen signifikant miteinander zusammenhängen. Das Überleben der ALS Patienten wurde mit einem univariaten Cox-Proportional-Hazard-Ratio-Modell (HR) untersucht, welches noch durch die multivariante Version dieses Modells validiert wurde, um die Einflüsse von möglichen Störfaktoren zu berücksichtigen. Außerdem wurden Untergruppen von ALS Patienten, basierend auf ihrem QAlb Wert innerhalb eines Kaplan-Meier Plots dargestellt, und mit dem Log-Rank-Test verglichen. Die Unterschiede in allen multivariaten Cox-Modellen wurden durch ANOVA verglichen. Bei allen Analysen wurde $\alpha = 0{,}05$ als Signifikanzniveau angesehen. Alle Analysen wurden mit der Programmiersprache R (Version 4.0.3) innerhalb der Entwicklungsumgebung RStudio (Version 1.2.5033) durchgeführt.

Ergebnisse

Die Werte von QAlb ebenso wie QIgG, QIgA und QIgM waren bei ALS Patienten nicht signifikant verschieden von den neurologischen Kontrollkohorten (ANOVA: QAlb p = 0,26; QIgG p = 0,35; QIgA p = 0,17; QIgM p = 0,46). Auch der Anteil, der über dem von Reiber definierten Normalbereich für QAlb (H. Reiber et al., 2001) und damit für eine Störung der Blut-Liquor-Schranke spricht, war in den Populationen vergleichbar (ALS: 27%, TH: 22%, FP: 30%, AD: 28%; p = 0,16). Ich konnte durch die zweifaktorielle ANOVA einen signifikanten Einfluss des Geschlechts auf den QAlb Wert feststellen (p < 0,001; QAlb Mittelwert bei ALS männlich: 7,56 [Standardabweichung (SD) = 3,01]; weiblich: 6,11 [SD = 2,46]), welcher jedoch nicht von der Diagnose abhängig war (p = 0,24; QAlb Mittelwert aller Kontrollkohorten männlich: 7,50 [SD = 3,17]; weiblich: 6,17 [SD = 2,78]). Der altersabhängige Verlauf von QAlb, welchen Reiber beschrieben hatte (Hansotto Reiber, 1980), konnte zwar in den Kontrollkohorten beobachtet werden (TH: R = 0,36, p = 0,03; FP: R = 0,19, p = 0,09; AD: R = 0,16, p < 0,001), jedoch nicht in der ALS Kohorte (R = 0,01, p = 0,65), hier blieb der QAlb Wert mit dem Alter konstant und stieg nicht signifikant an. Ein signifikanter Unterschied zwischen den Steigungen der QAlb-Alter-Beziehung konnte jedoch nicht nachgewiesen werden (ANCOVA, p = 0,26).

Innerhalb der ALS Kohorte korrelierte ich den QAlb mit den klinischen Parametern (Alter bei LP, Alter bei Erstmanifestation, Geschlecht, BMI beim ersten Visit, Lokalisation der Erstmanifestation (bulbär, spinal), ALS-FRS bei Diagnose und Progressionsrate); diese waren bei 860 der ursprünglich 1059 Patienten vollständig verfügbar. Hier fand ich abermals die signifikante Korrelation des QAlb mit dem Geschlecht (p < 0,001), aber auch mit dem BMI (p < 0,001). Alle anderen einbezogenen klinischen Variablen zeigten keinen signifikanten Zusammenhang mit dem QAlb. Abgesehen vom QAlb, korrelierte das Alter der Erstmanifestation mit der Lokalisation dieser (p < 0,001), dem Geschlecht (p = 0,02) und der Progressionsrate (p < 0,001). Außerdem korrelierte der BMI positiv mit dem ALS-FRS bei Diagnose (p < 0,001). Wenn hier nach Geschlecht unterschieden wurde kam man zu ähnlichen Ergebnissen mit der Ausnahme, dass der BMI bei Frauen eine lineare Korrelation mit deren QAlb zeigte (p < 0,001).

Bei 327 der untersuchten 1059 ALS Patienten war ein Todesdatum bekannt. Um die Überlebenszeit bei den Patienten ohne bekanntes Todesdatum einzuschätzen, wurde in die Analysen miteinbezogen, dass die betroffenen Patienten bis zum Datum ihrer letzten

Vorstellung noch gelebt haben. In der ALS Gesamtpopulation konnte ein geringer Einfluss des QAlb auf das Überleben ermittelt werden (HR = 1,07; p < 0,001), welcher geschlechtsspezifisch bei Männern etwas mehr ausgeprägt und im Gegensatz zu dem Effekt bei Frauen auch signifikant war (Männer: HR = 1,10; p < 0,001; Frauen: HR = 1,03; p = 0,39). Somit zeigte sich der Effekt eines kürzeren Überlebens bei Männern mit einem erhöhten QAlb. Ebenso unter Einbeziehung möglicher Störfaktoren wie BMI, Geschlecht, Alter bei LP, Alter bei Erstmanifestation und deren Lokalisation zeigte sich weiterhin ein ähnlicher Effekt des QAlb auf das Überleben (HR 1,07 – 1,10), abermals ausgeprägter bei männlichen Patienten.

Aufgrund der möglichen nicht linearen Beziehung zwischen QAlb und den benannten Störfaktoren, habe ich eine weitere unabhängige Analyse durchgeführt um die Auswirkungen von QAlb auf das Überleben zu untersuchen. Hierzu wurden die QAlb Werte mit dem z-Score normalisiert und die Patienten in je 3 Kategorien unterteilt (z < -1 = niedriger QAlb; -1 < z < 1 = mittlerer QAlb; z > 1 = hoher QAlb), dies geschah sowohl für die Gesamtpopulation als auch für die männliche (n = 650) und weibliche (n = 409) ALS Subkohorte (Tabelle 2).

Tabelle 2: Liquor-Serum-Albumin-Quotient (QAlb) Gruppen bei ALS Patienten

ALS Patienten	Niedriger QAlb	Mittlerer QAlb	Hoher QAlb
Gesamtpopulation (n=1059)	< 4,35	[4,96; 9,69]	> 9,69
Männer (n=650)	< 4,79	[4,79; 10,32]	> 10,32
Frauen (n=409)	< 3,87	[3,87; 8,40]	> 8,40

Der BMI war in den drei Gruppen der Gesamtpopulation (p = 0,004), wie auch innerhalb der männlichen Subkohorte (p = 0,04) signifikant unterschiedlich, jedoch nicht bei Frauen (p = 0,06). Dies lässt darauf schließen, dass der BMI vorwiegend bei Männern als Störfaktor bei der Beziehung zwischen dem Überleben und QAlb agiert. Jedoch ist diese Beziehung invers da ein höherer BMI, welcher unseren Daten und vorrangegangenen Studien nach protektiv für das Überleben ist (Dorst et al., 2019), in der höheren QAlb Kategorie gefunden wurde, welche das kürzeste Überleben unserer Gruppen hatte (Kaplan-Meier mittlere Überlebenszeit: 19,8 Monate). Dies war signifikant unterschiedlich von der mittleren Kategorie (Kaplan-Meier mittlere Überlebenszeit: 26,2 Monate; p = 0,02). Welche wiederum auch ein signifikant kürzeres Überleben im Vergleich zur Gruppe mit niedrigem QAlb (Kaplan-Meyer mittlere Überlebenszeit: 31,8 Monate; p = 0,04) zeigte. Ein signifikanter Effekt zwischen den Gruppen hinsichtlich des Überlebens wurde geschlechterspezifisch abermalig nur in der männlichen Subkohorte (männlich: p < 0.001; weiblich: p = 0,45) gefunden. Interessanterweise war der Überlebensvorteil der niedrigen QAlb Kategorie ein Jahr nach LP statistisch noch nicht signifikant (Gesamtpopulation: p = 0,07; männliche Subkohorte: p = 0,06; weibliche Subkohorte: p = 0,4), wurde aber 2 Jahre nach der Entnahme stark signifikant (Gesamtpopulation: p = 0,003; männliche Subkohorte: p < 0,001; weibliche Subkohorte: p = 0,3) und blieb auch nach 3, 4 und 5 Jahren signifikant (mehr Jahre waren aufgrund der Patientenanzahl nicht verwertbar), jedoch blieb dieser Effekt in allen Jahren der Erkrankung bei den Frauen aus. Als wir das Alter der Erstmanifestation und den BMI als Störfaktoren miteinbezogen, ging der Effekt des niedrigen QAlb für das längere Überleben in der Gesamtpopulation und bei Männern

verloren, aber der Effekt des hohen QAlb blieb signifikant für ein kürzeres Überleben (HR = 1,86; p = 0,001).
Wir führten eine weitere Analyse nur anhand der ALS Patienten durch, zu denen wir ein Sterbedatum vorliegen hatten (n = 327), um mögliche Fehlinterpretationen hinsichtlich des geschätzten Überlebens bei Patienten ohne Sterbedatum auszuschließen. Die QAlb Gruppen wiesen hier kein signifikant unterschiedliches Überleben auf (p = 0,16), wobei der Trend zum längeren Überleben bei niedrigeren QAlb (17,4 Monate, 95% Konfidenzintervall (KI) [11,3; 21,0]) und kürzeren Überleben bei mittleren (15,6 Monate, 95% KI [12,98; 17,8]) und hohen QAlb (13,7 Monate 95% KI [9,76; 15,4]) auffiel. Der Effekt war bei der männlichen Kohorte wieder statistisch signifikant (n = 192; p = 0,03), bei der weiblichen jedoch nicht (n = 135; p = 0,71).
Um sicherzustellen, dass der Effekt von QAlb auf das Überleben vorrangig bei männlichen Patienten nicht durch weitere Störfaktoren bedingt ist, haben wir die bekannten Komorbiditäten der Patienten in eine weitere Analyse miteinbezogen. Wir konnten keinen signifikanten Zusammenhang zwischen QAlb und entzündlichen Erkrankungen, Asthma, arteriellem Bluthochdruck, kardiovaskulären Erkrankungen und Chronisch obstruktiver Lungenerkrankung (COPD) finden, weder bei den weiblichen noch bei den männlichen ALS Patienten. Diabetes Mellitus (DM) wies jedoch innerhalb der QAlb Gruppen eine signifikant verschiedene Prävalenz auf (p = 0,001). Bei Männern mit hohem QAlb trat DM deutlich häufiger aus als in den anderen QAlb Gruppen (p = 0,008), was den Effekt des kürzeren Überlebens dieser Gruppe zumindest teilweise erklären könnte. Bei den Frauen konnte kein Unterschied von DM innerhalb QAlb Gruppen gefunden werden (p = 0,35). Die Einbeziehung von DM als Confounder in das multivariate Cox-Modell änderte nichts an der statistischen Signifikanz von QAlb. Unter Ausschluss der Patienten mit DM führten wir erneut die eine univariate und multivariate Cox-Analyse basierend auf den QAlb Gruppen durch. Hier war auffällig, dass nur die männliche Gruppe mit den niedrigen QAlb Werten signifikant mit einer besseren Prognose assoziiert war (HR = 0,64, p = 0,05), und hoher QAlb mit der schlechteren Prognose korrelierte (HR = 1,77, p = 0,003). Im multivariaten Modell, in dem die möglichen Confounder miteinbezogen wurden, blieb der Effekt des hohen QAlb bei männlichen ALS Patienten (Gesamtpopulation: HR = 1,30, p = 0,11; Männer: HR = 1.78, p = 0,007). Wurden nur die Patienten mit vorliegendem Todesdatum berücksichtigt und die ALS Patienten mit DM ausgeschlossen ergab sich in der univariaten Analyse eine signifikant bessere Prognose für die niedrige QAlb Gruppe bei Männern (HR = 0,55, p = 0,005). Unter Berücksichtigung der Confounder in dieser Analyse bestand diese Signifikanz bei der männlichen Kohorte weiterhin (niedriger QAlb bei Gesamtpopulation: HR = 1,01, p = 0,95; Männer: HR = 0,61, p = 0,03; Frauen: HR = 1,28, p = 0,38).

Diskussion

In dieser Arbeit konnten wir zeigen, dass eine Korrelation zwischen dem Überleben von ALS Patienten und dem QAlb und somit der Blut-Liquor-Schranke besteht. Ein niedrigerer QAlb und somit eine weniger durchlässige Blut-Liquor-Schranke konnte mit einem längeren Überleben der männlichen Patienten assoziiert werden. Das reduzierte Überleben bei erhöhtem QAlb und damit erhöhter Permeabilität der Blut-Liquor-Schranke ist vermutlich zumindest teilweise auf Störfaktoren wie Diabetes und BMI zurückzuführen. Diese Ergebnisse stützen die Hypothese, dass ein gewisses Maß an Störung der Blut-Liquor-

Schranke durch metabolische, toxische oder reaktive Mechanismen bedingt ist und ein Biomarker für eine schlechtere Prognose bei ALS ist.
Der Geschlechterunterschied, welcher in unserer Studie bezüglich der QAlb Werte und dem Überleben gefunden wurde stimmt mit einer französischen Studie, welche 307 Patienten untersuchte von denen 30% einen abnormalen QAlb hatten, überein. Auch hier wurde ein signifikanter Zusammenhang zwischen dem Überleben bei männlichen ALS Patienten (hoher QAlb = kürzeres Überleben, niedriger QAlb = längeres Überleben) gefunden, jedoch nicht bei weiblichen. Störfaktoren wie BMI und DM, die in unserer Studie relevant für die Ergebnisse waren konnten jedoch – wohl aufgrund der geringeren Fallzahl – nicht miteinbezogen werden (Alarcan et al., 2023). Verde et al. (Verde et al., 2023) berichtete auch von 10% abnormalen QAlb Werten bei 328 ALS Patienten und wie auch von uns beobachtet von einer konstanten Beziehung von QAlb zum Alter, entgegen dem altersbedingten Anstieg von QAlb von dem laut Reiber (Hansotto Reiber, 1980) bei anderen Populationen ausgegangen wird. Diesen bekannten Effekt konnten wir auch in unseren Kontrollpopulationen beobachten. Zusätzlich wurde in unserer Studie auch die Überlebenszeit der Patienten mit dem QAlb korreliert. Li et al. (Li et al., 2022) berichtete von einer Korrelation erhöhter QAlb Werte und einer schlechteren Prognose bei 113 ALS und 12 ALS/FTD Patienten. Welche nach Berücksichtigung von Störfaktoren (Alter, Geschlecht, Dauer der Erkrankung, Lokalisation der Erstmanifestation, vorwiegende Betroffenheit von unterem oder oberem Motoneuron [LMN; UMN], ALS-FRS) bestehen blieb, wobei hier BMI und DM nicht einbezogen wurden. Auch weitere frühere Studien lieferten bereits Hinweise, dass ein erhöhter QAlb mit einem schnelleren Fortschreiten und ein niedriger QAlb mit längerem Überleben bei der ALS zusammenhängen könnte (Apostolski et al., 1991; Leonardi et al., 1984; Mirian et al., 2022; Norris et al., 1993; Winkler et al., 2013).
Unsere Ergebnisse decken sich mit diesen vorangegangenen Studien und zeigen, dass ein niedrigerer QAlb-Wert und somit eine weniger permeable Blut-Liquor-Schranke mit einem längeren Überleben assoziiert ist. Der Marker ist jedoch nicht mit dem Alter bei Krankheitsbeginn, dem ALSFRS und der Progressionsrate in Verbindung zu setzen. Dank unseres großen Datensatzes mit über 1000 Patienten und der Einbeziehung zusätzlicher Variablen konnten wir außerdem die Störfaktoren wie den BMI und DM identifizieren, welche in vorrangegangenen Studien gar nicht oder nicht vollständig berücksichtigt wurden. Durch die Berücksichtigung dieser war es uns möglich, den scheinbar bestehenden Effekt zwischen kürzerem Überleben und erhöhtem QAlb zumindest teilweise zu erklären und mit Adjustierung an diese fast zu neutralisieren. Der geschlechtsspezifische Unterschied, des niedrigen QAlb und längerem Überleben bei Männern, welcher auch von Alarcan et al. (Alarcan et al., 2023) entdeckt wurde könnte unter anderem einem anderen Risikoprofil basierend auf dem BMI liegen (Gallo et al., 2013) oder an metabolischen Faktoren wie dem unterschiedlichen Fettstoffwechsel (Lindauer et al., 2013). Die Evaluation dieser Ergebnisse könnte in nachfolgenden Studien unter Einschluss von weiteren Störfaktoren erfolgen, über die wir für unsere Studie nicht verfügten.

Originalpublikation

Referenz:
Klose V., Jesse S., Lewerenz J., Kassubek J., Dorst J., Rosenbohm A., Nagel G., Wernecke D., Roselli F., Tumani H, Ludolph A.C. (2024). Blood-CSF barrier integrity in amyotrophic lateral sclerosis. Brain. 2024 May 14: awae144. doi: 10.1093/brain/awae144. PMID: 38743595. (Klose et al., 2024)

Darstellung des Eigenanteils:
Bei dieser Publikation hatte ich die Idee die Liquordaten von 2012 – 2021 zu digitalisieren, strukturieren und zu bereinigen. Dies habe durch die Programmierung in Python (Version 3.7.6) umgesetzt. Aus diesen Daten habe ich die benötigten Kontrollkohorten, wie auch die ALS Kohorte extrahiert. Anschließend habe ich die Liquorparameter mit den klinischen Daten der ALS Patienten korreliert. Durch Anregungen von Prof. Albert Ludolph, Prof. Hayrettin Tumani und Prof. Francesco Roselli kam die Untersuchung der Blut-Liquor-Schranke bei diesem Patientenkollektiv zustande. Ich habe dazu die Datenanalyse wie auch die Auswertung in R (Version 4.0.3) durchgeführt. Die Tabellen und Abbildungen, welche in der Publikation enthalten sind wurden ebenfalls von mir umgesetzt. Die erste schriftliche Ausarbeitung des Manuskripts habe ich gemeinsam mit Prof. Francesco Roselli erstellt. Zusätzliche wichtige Empfehlungen vor allem hinsichtlich der Interpretation der Ergebnisse kamen von Prof. Hayrettin Tumani und Prof. Albert Ludolph. Diese habe ich, wie auch die Kommentare der Reviewer in das Manuskript eingearbeitet.

Copyrightvermerk:
Die Verwendung der Publikation „Blood-CSF barrier integrity in amyotrophic lateral sclerosis“ (Klose et al., 2024) im Rahmen dieser Dissertation wurde von Oxford University Press genehmigt.

BRAIN
ORIGINAL ARTICLE

Blood–CSF barrier integrity in amyotrophic lateral sclerosis

Veronika Klose,[1,2] Sarah Jesse,[1,2] Jan Lewerenz,[1] Jan Kassubek,[1,2] Johannes Dorst,[1] Angela Rosenbohm,[1] Gabriele Nagel,[3] Deborah Wernecke,[3] ALS Registry Swabia Study Group Francesco Roselli,[1,2,†] Hayrettin Tumani[1,†] and Albert C. Ludolph[1,2,†]

[†]These authors contributed equally to this work.

The integrity of the blood–CSF barrier plays a major role in inflammation, but also in shielding the CNS from external and systemic—potentially toxic—factors. Here we report results of measurements of the albumin quotient—which is thought to mirror the integrity of the blood-CSF barrier—in 1059 patients with amyotrophic lateral sclerosis. The results were compared with groups of patients suffering from Alzheimer's disease, facial palsy and tension headache. The albumin quotient, an accepted measure of the blood-CSF barrier integrity, was not significantly different from control populations. In addition, we found that the albumin quotient correlated with survival of the patients; this effect was mainly driven by male patients and influenced by age, body mass index and diabetes mellitus.
We conclude that the blood-CSF barrier is intact in this large cohort of patients with amyotrophic lateral sclerosis and that the albumin quotient correlates with survival. Whether this is important for the pathogenesis of the disease, requires mechanistic studies.

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Introduction

Amyotrophic lateral sclerosis (ALS) has been classically conceptualized as a progressive neurodegenerative process affecting spinal motor neurons and corticospinal neurons in motor cortex.[1] However, there is experimental evidence for the involvement of inflammatory and vascular processes in the pathogenic cascade[2-4]; their potential contribution in determining the progression and survival of ALS patients is the background of this study.

Vascular alterations in ALS hypothesized to be related to metabolic, toxic or reactive mechanisms, have been identified in post-mortem samples: degeneration of endothelial cells, deposits of

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extravasated albumin and toxic haemoglobin degradation products, together with decrease in pericyte number and activation of perivascular fibroblasts have been found in patients' spinal cord specimens.[4-9] Further vascular abnormalities in the form of loss of endothelial tight-junction proteins and disruption of the continuity of CD31+ vascular contour have been discovered in the choroid plexus.[10] Likewise, signs of altered microvascular integrity, such as loss of endothelial tight junctions and retraction of vascular astrocytic end-feet, were observed in murine models carrying mutations in the SOD1, TDP-43, FUS, TBK1 or C9ORF72 genes.[3,11-14]

The integrity of the blood–brain barrier (BBB) and blood–CSF barrier (BCSFB) is affected by multiple factors positively or negatively correlated with the risk and prognosis of ALS. Diabetes mellitus is recognized to delay onset of ALS,[15-18] but is also a disruptor of the BCSFB.[19-21] Toxic chemicals, such as pesticides, are associated with increased ALS risk by epidemiological evidence[22,23] and may gain access to the brain and spinal cord upon BBB/BCSFB disruption; likewise, iron toxicity and haemoglobin degradation products may accumulate in the spinal cord with neurotoxic consequences in the case of increased BBB/BCSFB permeability.[24,25] Furthermore, complement factors and inflammatory mediators may penetrate the CNS upon disruption of the BBB/BCSFB and contribute to the neurotoxic environment.[26,27] Thus, the evaluation of BCSFB integrity may determine not only the contribution of comorbidities to the pathogenic cascade in ALS but also the vulnerability of the CNS to toxic and reactive insults.

The CSF/serum ratio for albumin (QAlb) has been employed as a biomarker of BCSFB function[28] in metabolic, toxic and degenerative neurological conditions, and it is easily determined and widely available. However, to date, only a few studies[29-32] have explored the alterations of QAlb in ALS patients, often with inconsistent results, possibly due to small cohort sizes, retrospective design and limited clinical characterization of patients. Therefore, two main questions remain to be addressed: (i) is QAlb detecting alterations of BSCFB function in ALS patients? and (ii) is QAlb related to clinical features and prognosis?

Here we addressed these issues in a large (>1000 patients) prospective cohort of ALS patients [and three control cohorts diagnosed with facial palsy (FP), tension-type headache (TTH) or Alzheimer's disease, altogether >400 patients], subjected to CSF examination, standardized clinical examination and extensive follow-up. We demonstrate that, although ALS patients do not display QAlb higher than control cohorts, lower QAlb values relate to better prognosis.

Materials and methods

Patient cohorts and clinical assessment

The present study was carried out in agreement with the Declaration of Helsinki and was approved by the Ethical Committee of Ulm University (license no. 20/10).

Four cohorts of patients were considered in the present work, including patients diagnosed with TTH, peripheral FP, Alzheimer's disease (AD) and ALS. All CSF samples and clinical data of these patients were obtained at the Department of Neurology of Ulm University between April 2012 and December 2021.

Patients with TTH were diagnosed according to International Classification of Headache Disorders-3rd edition (ICHD-3; https://ichd-3.org/) after clinical assessment, neuroimaging evaluation and measurements of blood and CSF parameters. From an original cohort of 289 patients, 22 were excluded because of CSF leucocyte counts >10/µl. Patients with AD were diagnosed according to current criteria.[33,34] In addition to history and third-party history, clinical neurological examination and laboratory for exclusion diagnostics, we also included neuropsychological testing and CSF determination of dementia markers total Tau, pTau181, $A\beta_{1\text{-}40}$, $A\beta_{1\text{-}42}$ and $A\beta_{1\text{-}42}/A\beta_{1\text{-}40}$.

To have a comparable demographic distribution in the control and in the ALS cohorts, control subjects were randomly adjusted to the age structure of the ALS cohort (~1% <30 years, ~12% 40–50 years, ~83% 50–80 years, ~4% >80 years), whereby the balance between female and male patients was also adapted (Supplementary Fig. 1). Therefore, we ended up with 105 patients in the TTH cohort, 36 patients with FP and 336 patients in the AD cohort.

All CSF samples and clinical data were examined from patients at the Department of Neurology of Ulm University between April 2012 and December 2021. In this interval, 1203 ALS patients were prospectively evaluated; diagnosis of definite or probable ALS was established according to the revised El Escorial criteria.[35] Inclusion criteria included (i) diagnosis of suspected, possible, probable and definite ALS; and (ii) age of onset >16 years. Exclusion criteria were (i) erythrocyte count >1000/µl or leucocyte count >10/µl in CSF (to avoid samples with possible blood contamination or with coincidental inflammatory/infectious conditions); (ii) final diagnosis of spinal muscular atrophy (SMA) or hereditary spastic paraparesis (HSP); (iii) QAlb > 20 or QAlb missing; and (iv) age of onset > 90 years. The final cohort included 1059 ALS patients (Fig. 1).

At the first visit, patients underwent a full neurological examination [including the determination of the body mass index (BMI) and of the revised ALS Functional Rating Scale (ALSFRS)[36]], blood and CSF as well as neuroimaging examination and the documentation of comorbidities. The BMI was classified according to the World Health Organization (WHO) guidelines: underweight (<18.5 kg/m^2), healthy weight (18.5–24.9 kg/m^2) and overweight (> 24.9 kg/m^2). Clinical and ALSFRS assessments were performed at follow-up visits. The calculation of the progression rate based on the revised ALSFRS was performed as follows: (48 – ALSFRS)/months between disease onset (defined as occurrence of first paresis) and sampling,[37,38] if more than one ALSFRS was available in one patient (ALSFRS-first – ALSFRS-last)/months between the date of ALSFRS-first and ALSFRS-last. Information relative to the disease onset and the available death certificates were obtained from the ALS Swabia Register.[39]

Lumbar puncture and CSF analysis

CSF was obtained and analysed as previously reported according to standard procedures of the laboratory.[38,40] Briefly, CSF samples were collected in polypropylene tubes and were analysed within 1 h of sampling. Leucocyte count was performed microscopically in a Fuchs–Rosenthal chamber, as previously reported.[40,41] CSF samples with contamination of erythrocytes were excluded to avoid false positive values of CSF albumin. Total protein, albumin, IgG, IgA and IgM were measured by standard immunochemical nephelometry in CSF and serum (Dade–Behring nephelometer analyser) using a polyclonal antibody in the case of albumin and IgG as well as a latex particle-amplified antibody reaction for IgA. The inter- and intra-assay variabilities were <10% for the method-dependent absolute levels as well as for the method-independent CSF/serum quotients of albumin, IgG and IgA. CSF lactate was determined by a lactate oxidase reaction (Greiner GmbH). The limit for QAlb as a measure for the upper reference limit was determined according to the age-adapted linear formula $QAlb^* = [(4 + \text{age in years})/15] \times 10^{-3}$.[28]

Statistical analysis

Statistical analyses were performed using the R software (version 4.0.3) on RStudio (version 1.2.5033). Pearson's chi square test (for

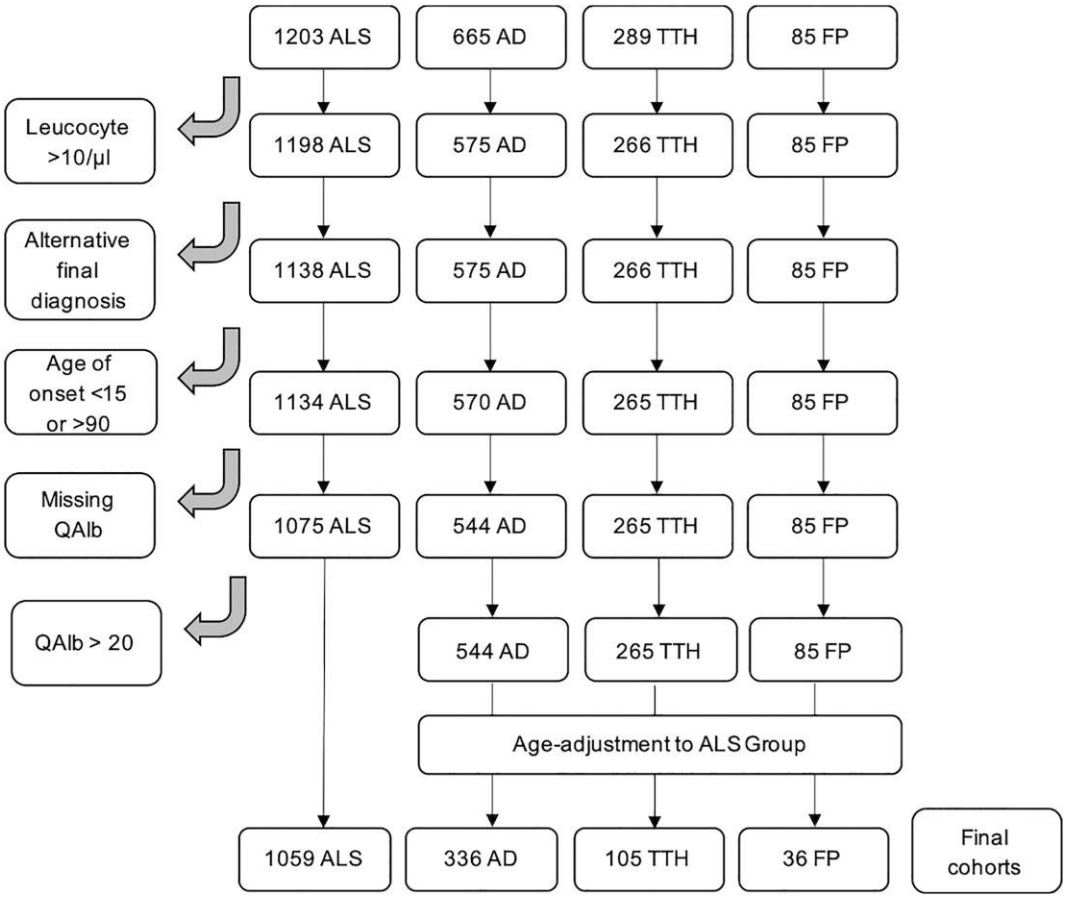

Figure 1 Algorithm of the application of exclusion criteria to the four clinical cohorts under study. AD = Alzheimer's disease; ALS = amyotrophic lateral sclerosis; FP = peripheral facial palsy; QAlb = albumin quotient; TTH = tension-type headache.

categorical variables) or one-way ANOVA with a *post hoc* pairwise *t*-test (for continuous variables) was applied to compare the socio-demographic, clinical and CSF parameters among the age-adjusted disease cohorts (ALS, AD, TH and FP); two-way ANOVA to evaluate the sex effect on QAlb in the four disease cohorts. Linear regression model and ANCOVA were used to compare QAlb/age slopes across the four cohorts. The cross-correlation matrices were obtained by pair-wise Pearson's correlation. The effect of the clinico-demographic and CSF parameters on survival time for the ALS patients was investigated using a univariate Cox proportional hazards ratio model and then a multivariate Cox model to account for possible clinical confounders. A step-wise multivariate Cox model was used due to the variability of the confounding variables. Survival of ALS patient subgroups was compared using the Kaplan-Meier method with the log-rank test. The differences in all multivariate Cox models were compared by ANOVA. In all of the analyses we considered $\alpha = 0.05$ as the level of significance. The ALS subgroups were defined by QAlb normalized z-scores (≤ -1, between -1 and $+1$, $\geq +1$), to handle outliers and the spread of the data with a score relative to the study population.

Results

BCSFB indices are comparable in ALS patients and neurological controls

We explored if ALS patients ($n = 1059$; 38.6% female, 61.4% male) displayed significant differences in QAlb, QIgG, QIgA or QIgM compared to three age-matched (Supplementary Fig. 1) neurological control populations: TTH ($n = 105$), FP ($n = 36$) and AD ($n = 336$). The demographic, clinical and CSF features of the four groups are reported in Table 1. Values of QAlb, QIgG, QIgA and QIgM were comparable across groups (one-way ANOVA; $P = 0.70$, $P = 0.35$, $P = 0.17$ and $P = 0.46$, respectively). Using the upper normal reference limit calculated according to the Reiber's age-adapted linear formula (refer to the 'Materials and methods' section), we determined that in the ALS cohort ~27% of subjects had abnormal QAlb, a fraction comparable to the other groups (~22%, 30% and 28% in TTH, FP and AD, respectively; $P = 0.16$, Table 1). The fraction of subjects with abnormal QIgG was very small and comparable across the four groups (Table 1).

When male and female patients subgroups were compared across the four cohorts, two-way ANOVA identified a significant effect of sex ($P < 0.001$) but not of diagnosis ($P = 0.24$) and no interaction sex:diagnosis ($P = 0.61$; Supplementary Fig. 2). We further evaluated the progressive age-related changes in QAlb[42,43] occurring in the four cohorts. In contrast to the positive correlation between QAlb and age in TTH and FP ($R = 0.36$, $P = 0.03$; $R = 0.19$, $P = 0.09$) and AD ($R = 0.16$, $P < 0.001$) surprisingly no such correlation existed in the ALS cohort ($R = 0.01$, $P = 0.65$). There was no significant difference between the slopes of the age/QAlb (Supplementary Fig. 3) in the four groups (ANCOVA, $P = 0.26$).

Thus, ALS patients do not display a significantly different QAlb, QIgG or QIgA at population level (Table 1), nor as a function of age or sex when compared to age-matched neurological controls. Notably, QAlb does not increase with age in ALS patients.

Table 1 Demographic and CSF features of the four cohorts

	ALS cohort ($n = 1059$)	Alzheimer's disease ($n = 336$)	Tension headache ($n = 105$)	Facial palsy ($n = 36$)	P-value
Sex female/male, *n* (%)	409/650 (38.62%/61.38%)	140/196 (41.67%/58.33%)	44/61 (41.9%/58.1%)	16/20 (44.44%/55.56%)	0.63[b]
Age, mean (SD)	62.61 (11.5)	63.44 (9.9)	63.01 (12.96)	60.98 (13.66)	0.51[a]
Serum albumin (g/l), mean (SD)	42.41 (4.35)	42.47 (4.4)	42.62 (4.58)	43.24 (3.88)	0.72[a]
CSF albumin (g/l), mean (SD)	296.65 (126.01)	286.63 (123.1)	292.67 (130.88)	331.67 (150.52)	0.20[a]
QAlb, mean (SD) $\times 10^{-3}$	7.00 (2.89)	6.83 (3.02)	6.85 (2.86)	7.82 (3.85)	0.26[a]
Percentage of abnormal QAlb according to age-adapted linear formula by Reiber	27.38%	21.62%	30.21%	27.78%	0.16[b]
QIgG, mean (SD) $\times 10^{-3}$	3.34 (1.44)	3.41 (1.8)	3.58 (2.08)	3.64 (1.83)	0.35[a]
Percentage of abnormal QIgG according to hyperbolic formula by Reiber	0.67%	1.86%	2.11%	0%	0.21[b]
QIgA, mean (SD)	1.85 (1.11)	1.85 (1.01)	2.09 (1.89)	2.1 (1.43)	0.17[a]
Percentage of abnormal QIgA according to hyperbolic formula by Reiber	0.48%	0.47%	1.05%	0%	0.86[b]
QIgM, mean (SD) $\times 10^{-3}$	0.43 (0.47)	0.4 (0.34)	0.49 (0.91)	0.47 (0.53)	0.46[a]
Percentage of abnormal QIgM according to hyperbolic formula by to Reiber	0.72%	0.53%	0%	0%	0.81[b]
Leucocyte cell count, mean (SD)/µl	0.98 (1.27)	0.52 (0.5)	1.53 (1.75)	1.39 (1.29)	**1.523×10^{-13}**[a]

The four patient cohorts under study (ALS, Alzheimer's disease, tension headache and facial palsy) do not differ for clinico-demographic parameters and BCSFB indexes; a significant difference (bold) is detected in the leucocyte count, with Alzheimer's disease patients showing a significantly lower count than the others. ALS = amyotrophic lateral sclerosis; SD = standard deviation.
[a]One-way ANOVA with Tukey *post hoc* multiple comparisons.
[b]χ^2 test.

Table 2 Cross-correlation matrix of clinico-demographic parameters and BCSFB permeability indices for ALS patients (overall, $n = 860$)

	Age	Age-onset	Sex: male	BMI	Site onset: spinal	ALSFRS	Progression rate	QAlb
Age	–	**0.97**[b]	**−0.07**[a]	−0.06	**−0.20**[c]	**−0.12**[b]	0.04	−0.02
Age-onset	–	–	**−0.08**[a]	−0.06	**−0.22**[c]	**−0.11**[b]	**0.09**[b]	−0.02
Sex:male	–	–	–	0.10	**0.16**[c]	**0.10**[b]	−0.04	**0.26**[b]
BMI	–	–	–	–	**0.11**[b]	**0.09**[b]	−0.06	**0.13**[b]
Site onset: spinal	–	–	–	–	–	0.05	**−0.09**[b]	0.03
ALSFRS	–	–	–	–	–	–	**−0.22**[c]	−0.01
Progression rate	–	–	–	–	–	–	–	0.03
QAlb	–	–	–	–	–	–	–	–

Albumin quotient (QAlb) is significantly correlated with sex and body mass index (BMI) but not with onset site, ALS Functional Rating Scale (ALSFRS) severity at sampling or with progression rate. Pearson's correlation with pairwise deletion. All significant values are in bold. ALS = amyotrophic lateral sclerosis.
Correlation coefficient 1 (or −1) = strong correlation; 0 = no correlation.
Significance:
[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.

BCSFB integrity does not correlate with disease severity or progression rate in ALS

We then explored the correlation between QAlb and clinical indices of progression as well as with clinico-demographic parameters including BMI, topographical site of disease onset, ALS-FRS at sampling, and progression rate. A sub-cohort of 860 ALS patients, for which the full dataset was available, was subject to the investigation (Table 2). Notably, QAlb significantly correlated with BMI ($P < 0.001$) and with sex ($P < 0.001$) but showed no correlation with age at lumbar puncture ($P = 0.54$), age of onset ($P = 0.66$), site of onset (spinal, bulbar) ($P = 0.37$), ALSFRS at diagnosis ($P = 0.85$) and progression rate ($P = 0.27$) (Table 2). Confirming previous observations,[44-46] age of onset correlated with site of onset ($R = -0.15$, $P < 0.001$), with sex ($R = -0.08$, $P = 0.02$) and with progression rate ($R = 0.10$, $P < 0.001$); ALSFRS at diagnosis positively correlated with BMI ($R = 0.10$, $P < 0.001$).

The separated analysis of female ($n = 338$; Supplementary Table 1) and male ($n = 522$; Supplementary Table 1) ALS patient subgroups revealed similar findings (no correlation with age of onset, onset site, ALSFRS at diagnosis or progression rate) with one exception: QAlb displayed a significant linear correlation with BMI in

Table 3 Clinico-demographic characteristics of ALS patient subgroups divided according to the QAlb z-score and sex

Overall	QAlb $z \leq -1$ ($n = 169$)	QAlb $-1 < z < +1$ ($n = 725$)	QAlb $z \geq +1$ ($n = 165$)	*P*-value
Sex female/male, *n* (%)	95/74 (56.2% 43.8%)	276/449 (38.1% 61.9%)	38/127 (23.0% 77.0%)	$\mathbf{3.272 \times 10^{-9}}$
Age, mean (SD)	61.95 (12.55)	62.88 (11.15)	62.14 (11.89)	0.55[a]
Age of onset, mean (SD)	60.12 (12.93)	61.07 (11.67)	60.90 (12.00)	0.65[a]
BMI, mean (SD)	24.00 (4.12)	24.96 (4.11)	25.57 (3.82)	0.0036[a]
Progression rate, mean (SD)	0.87 (0.99)	0.90 (1.04)	0.93 (0.88)	0.88[a]
Onset site, *n* (bulbar, spinal, na)	39, 122, 8	171, 533, 21	35, 121, 9	0.47[b]
Female	**QAlb $z \leq -1$ ($n = 67$)**	**QAlb $-1 < z < +1$ ($n = 282$)**	**QAlb $z \geq +1$ ($n = 60$)**	***P*-value**
Age, mean (SD)	63.32 (12.22)	63.32 (10.59)	63.63 (12.49)	0.96[a]
Age of onset, mean (SD)	61.79 (12.89)	61.77 (10.95)	62.12 (12.44)	0.98[a]
BMI, mean (SD)	23.47 (4.23)	24.54 (4.53)	25.52 (4.91)	0.06[a]
Progression rate, mean (SD)	0.85 (0.86)	0.98 (1.02)	0.86 (0.90)	0.52[a]
Onset site, *n* (bulbar, spinal, na)	20, 45, 2	88, 186, 8	23, 37, 0	0.59[b]
Male	**QAlb $z \leq -1$ ($n = 99$)**	**QAlb $-1 < z < +1$ ($n = 447$)**	**QAlb $z \geq +1$ ($n = 104$)**	***P*-value**
Age, mean (SD)	60.79 (12.55)	62.17 (11.65)	63.06 (11.06)	0.38[a]
Age of onset, mean (SD)	59.14 (12.68)	60.24 (12.24)	61.72 (11.27)	0.42[a]
BMI, mean (SD)	24.22 (3.83)	25.35 (3.74)	25.20 (3.55)	**0.04**[a]
Progression rate, mean (SD)	0.76 (0.88)	0.90 (1.10)	0.94 (0.86)	0.43[a]
Onset site, *n* (bulbar, spinal, na)	14, 81, 4	80, 350, 17	20, 77, 7	0.57[b]

The three albumin quotient (QAlb) subgroups were comparable in age, age of onset, site of onset and progression rate (overall population as well as male and female subgroups). Males were over-represented in the high QAlb group, and body mass index (BMI) was significantly higher in the high QAlb group (in males but not in females). Overall: z-score −1 = QAlb 4.35; z-score +1 = QAlb 9.69. Female: z-score −1 = QAlb 3.87; z-score +1 = QAlb 8.40. Male: z-score −1 = QAlb 4.79; z-score +1 = QAlb 10.32. ALS = amyotrophic lateral sclerosis; SD = standard deviation; na = not applicable.

[a]One-way ANOVA.

[b]χ^2 test.

females but not in males (Supplementary Table 1). Thus, QAlb does not predict age of onset or progression rate. This effect will be considered in further covariate analysis.

QAlb at sampling predicts survival of ALS patients

We next explored if QAlb could predict the long-term survival of ALS patients. We considered the whole ALS patient cohort ($n = 1059$), using the time from diagnosis to last hospital visit as proxy of survival. The univariate proportional Cox model (Supplementary Table 2) revealed a significant effect of QAlb on survival in the overall cohort [hazard ratio (HR) = 1.07, $P < 0.001$] with patients with higher QAlb having a shorter survival, which was significant in males (HR = 1.10, $P < 0.001$) but only showed a trend in females (HR = 1.03, $P = 0.39$). Among the clinico-demographic parameters, BMI showed an inverse effect in the overall cohort (HR = 0.96, $P = 0.024$) but was not significant in male or female subgroups (trend in males, $P = 0.094$); increased age at sampling, age of onset and bulbar site of onset (not in male patients) also negatively affected survival in the overall cohort and in the sex-divided subgroups (Supplementary Table 2). Also, increased BMI is statistically related to an increased QAlb (if groups with increased and normal QAlb are compared) (data not shown). If males and females are stratified by BMI categories, in all groups QAlb was related to BMI; consistently the BMI was higher in males than females. On the other hand, sex as a variable influenced the quantitative effect of BMI on the albumin quotient (significant positive correlation only in females; Supplementary Table 1A). The comparisons such as overweight versus healthy weight, underweight versus overweight, and underweight versus overweight in males, as well as in females, revealed no discrepancies in QAlb values among subgroups when stratified by sex (Supplementary Table 1B).

We employed a stepwise multivariate proportional Cox model to account for the possible confounding role of BMI, sex, age at sampling, age of onset and site of onset in the effect of QAlb on survival. The stepwise addition of one covariate per step (Supplementary Table 3) demonstrated that in the overall population, QAlb was correlated with survival with a similar HR (range 1.07–1.10) in bivariate, three-variate and multivariate models; the effect was more pronounced in male patients.

Because of the possible non-linear relationship between QAlb, BMI and demographic variables (and the different correlation between QAlb and BMI in male and females), linear models may not completely compensate for the effect of confounders. We performed an independent analysis to investigate the effect of QAlb on survival. Because the QAlb data were not normally distributed (Shapiro-Wilk normality test $P < 0.001$), we used logarithmic transformation, which led to a normal distribution of the data (Shapiro-Wilk normality test: $P = 0.3431$). After log-transformation and z-normalization, we divided the overall population in three groups according to their z-scores (low QAlb: $z < -1$, high QAlb: $z > 1$, intermediate QAlb: $-1 < z < 1$). We divided male and female subgroups according to their own internal z-scores (Table 3). For the overall cohort, the three groups corresponded to a lower group with z-score ≤ -1 with QAlb ≤ 4.35 (male: 4.35, female: 3.87) an upper group with z-score ≥ 1 with QAlb ≥ 9.69 (male: 10.32, female: 8.40) and an intermediate group with a z-score in between −1 and 1 with QAlb z-score between 4.35 and 9.69 [male: (4.35; 10.32), female: (3.87; 8.40)].

The three QAlb groups (overall or sex-divided; Table 3) were closely matched in terms of clinical and demographic variables with two notable exceptions: males were over-represented in the high QAlb group of the overall population (as expected) and—most importantly—BMI was significantly different across the three groups ($P = 0.0036$), but this effect was only slightly statistically significant for males ($P = 0.04$) and not for females ($P = 0.06$). Thus, in female patients, BMI does not differ across QAlb subgroups, whereas in males BMI is higher in patients in the ≥1 z-score group. QAlb,

QIgG, QIgA and QIgM were different in the three groups, as expected (Supplementary Table 4).

Importantly, in the survival analysis, the ≤−1 z-score group (low QAlb) displayed a significantly longer median survival than the z-score between −1 and 1 group (Kaplan-Meyer: 31.8 months versus 26.2 months, $P = 0.06$) whereas the ≥1 z-score group (high QAlb) displayed a shorter survival than the intermediate group (high QAlb: 19.8 months versus 26.2 months, $P = 0.02$; Fig. 2A). A significant effect of QAlb groups was observed in males (Fig. 2B; $n = 650$, $P < 0.001$) but not significantly in females (Fig. 2C; $n = 409$, $P = 0.22$). We performed an exploratory analysis with five QAlb categories ($z < -1.5$, $-1.5 < z < -1.0$, $-1.0 < z < 1.0$, $1.0 < z < 1.5$, $z > 1.5$; Supplementary Fig. 4), which demonstrated a strong effect of QAlb subgroup on survival in males ($P = 0.0004$; Supplementary Fig. 4A), with high z-score patients displaying substantially shorter survivals, but not in females ($P = 0.35$; Supplementary Fig. 4B).

The survival difference of the QAlb categories was not statistically significant 1 year after the CSF sampling (overall population, $P = 0.07$; male patients, $P = 0.06$) but became strongly significant 2 years after sampling ($P = 0.003$; Supplementary Table 5) and remained significant after 3, 4 and 5 years (Supplementary Table 5; test beyond 5 years was limited by the small number of surviving patients). In female patients, no significant difference in the survival probability was observed among the QAlb categories over time.

We performed a stepwise multivariate proportional Cox model to verify whether the difference in survival observed in the three QAlb groups was not due to confounders (Supplementary Table 6). In the univariate model, QAlb groups showed a significant effect on survival (overall $P = 0.004$, low QAlb $P = 0.07$ and high QAlb $P = 0.02$); the effect was more pronounced in males ($n = 650$; low QAlb $P = 0.11$; high QAlb $P = 0.001$) than in females ($n = 409$; low QAlb $P = 0.10$; high QAlb $P = 0.86$). When BMI and age of onset were added, the effect of low QAlb in males was lost and only the high QAlb remained significant (HR = 1.86, $P = 0.001$). Thus, the multivariate analysis supports an effect of QAlb group on survival, but it identifies confounding effects from BMI and age of onset, in particular in males.

As overall survival based on last hospital visit (mean observation period: 13.08 months from ALS onset to last visit or death) could add biases to the analysis due to the uncertainty of the time passed between the last visit and the death of the patients, we considered a sub-cohort of patients ($n = 327$; Supplementary Table 7 and Supplementary Fig. 4) for whom death certificates were available in the ALS Swabia Register.[39] The death certificate cohort (mean observation period: 17.32 months from ALS onset to death) was significantly different from the overall cohort (patients displayed on average faster progression and shorter overall survival in the death certificate subgroup; clinico-demographic features of this sub-cohort and the comparison with the overall cohort are reported in Supplementary Table 7). In the death certificate cohort we again converted the QAlb values into z-scores (or male and female patients independently) and we subgrouped patients according to their z-score [as the baseline populations are different, z-score threshold differed when considering the whole ($n = 1059$) cohort or the death certificates cohort ($n = 327$)].

In the death certificate cohort, only trend toward different survival in QAlb groups was detected, ($P = 0.16$) with $z < -1$ [low QAlb: 17.4 months (11.3; 21.0)] displaying a longer survival than $-1 < z < 1$ [intermediate QAlb: 15.6 months (12.98; 17.8)] and $z > 1$ [high QAlb: 13.7 months (9.76; 17.8), Supplementary Fig. 5A]; the effect was statistically significant in male patients ($n = 192$; $P = 0.027$;

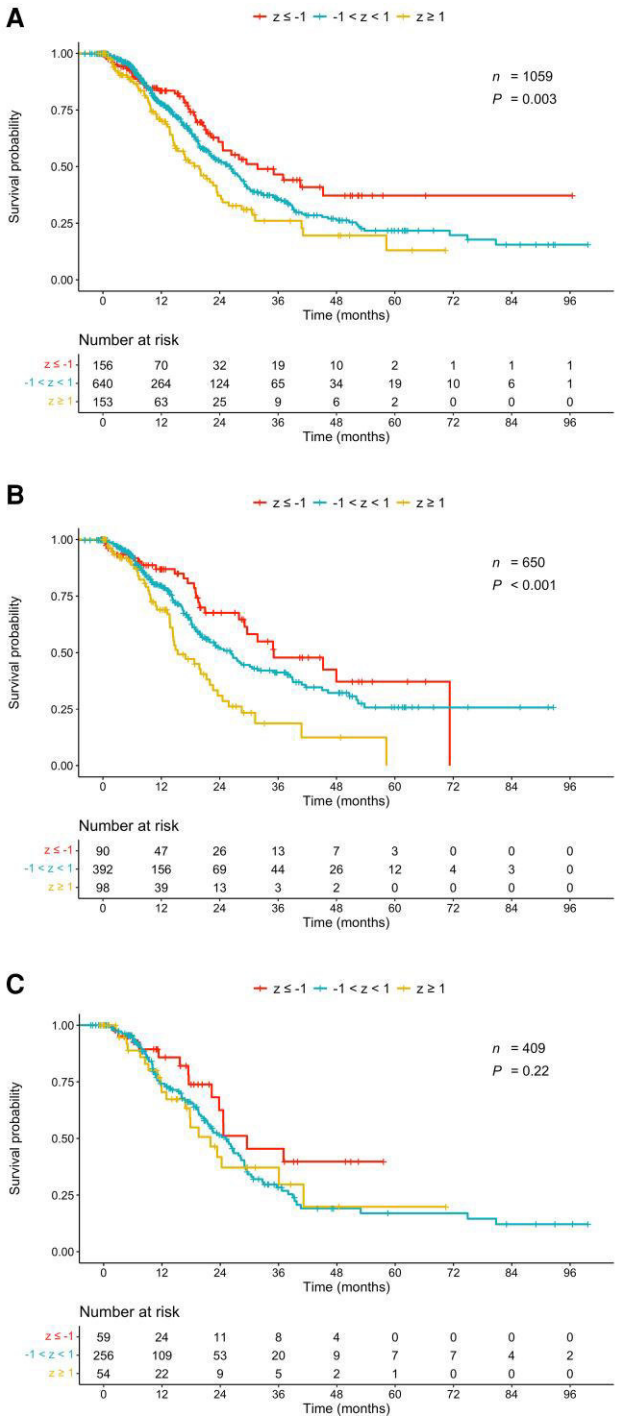

Figure 2 Kaplan-Meyer analysis of the effect of QAlb categories on the survival of ALS patients. (**A**) Overall cohort, (**B**) male and (**C**) female patients. The low albumin quotient (QAlb) subgroup display a longer median survival than the intermediate group [Kaplan-Meyer: 31.8 months (24.6; 45.2) versus 26.2 months (22.1; 28.9), $P = 0.06$] whereas the high QAlb group displays a significantly worse survival than the intermediate group [high QAlb: 19.8 months (14.9; 23.6) versus 26.2 months (22.1; 28.9), $P = 0.02$]. In the male sub-cohort (**B**), patients with high QAlb displayed a significantly shorter survival than the intermediate group [$n = 650$, 15.4 months (13.8; 22.6) versus 26.4 months (20.3; 35.0), $P = 0.001$], low QAlb was not significantly different to intermediate QAlb ($P = 0.1$); however, no effect of QAlb category was found in female patients ($n = 409$, $P = 0.22$; **C**).

Supplementary Fig. 5B and C). An exploratory analysis based on five QAlb subgroups (Supplementary Fig. 5) revealed a strong trend when male and females were considered together ($P = 0.066$; Supplementary Fig. 6A), which is mainly driven by a significant effect in males ($P = 0.035$; shorter survival in patients with higher z-score; Supplementary Fig. 6B) that does not appear in in females ($P = 0.94$; Supplementary Fig. 6C).

Sex as a variable influenced the quantitative effect of BMI on the albumin quotient with a significant positive correlation only in females (Supplementary Table 1). The albumin quotient had only a significant influence on survival in male patients, but not in female patients (Supplementary Table 2). In males, not in females, the effect of QAlb on survival was seen after 2 years (Supplementary Table 5) and it was independent from BMI and age of onset (Supplementary Tables 3 and 6).

The IgG, IgA and IgM levels were statistically significantly related with the albumin quotient in both sexes (Supplementary Table 4). The influence of diabetes on a higher QAlb was present in both sexes, but more pronounced in males (statistically significant).

Overall, QAlb at sampling appears to be significantly correlated, over several years, with survival of ALS patients; the effect of QAlb is confounded by BMI and age of onset.

Diabetes comorbidity impacts QAlb effect on survival of male ALS patients

We sought to verify that the effect of high QAlb in male ALS patients was not due to additional confounders not yet taken into consideration. As QAlb value and BCSFB may be affected by vascular, endocrine and systemic comorbidities,[20,47] we computed the prevalence of frequent comorbidities in male and female ALS patients in the three QAlb groups. The prevalence of inflammatory disorders, asthma, arterial hypertension, cardiovascular diseases and COPD was similar across all groups as well as in males and females, making it unlikely that any of these conditions may confound the relationship between QAlb and shorter survival. However, we found that prevalence of diabetes mellitus was significantly different across the groups (overall, $P = 0.0014$) and was significantly over-represented in the high QAlb subgroup of male ($P = 0.008$) but not female patients ($P = 0.35$; Supplementary Table 8).

To disentangle the possible confounding effect of the diabetes mellitus comorbidity, we performed two separate analyses. First, we expanded the multivariate Cox proportional hazard model (Supplementary Table 3, Model 5; QAlb is considered as continuous variable) to include the diagnosis of diabetes as confounder; the inclusion of diabetes did not alter the statistical significance of the QAlb (Supplementary Table 9).

Second, we performed the univariate and multivariate proportional Cox analysis using QAlb subgroups (high QAlb and low QAlb according to the z-score, as above) but excluding 92 patients with the diagnosis of diabetes mellitus. Interestingly, in the univariate Cox regression analysis of this subcohort ($n = 967$; Supplementary Table 10) the QAlb subgroups displayed no significant effect in the overall cohort (or in the female subgroup), whereas in the male patients, low QAlb was significantly associated with better prognosis (HR = 0.64, $P = 0.05$) and high QAlb with lower survival (HR = 1.77, $P = 0.003$). This remained true only for male patients with high QAlb in the multivariate model in which BMI, age of onset, age at sampling and site of onset were added as confounders (multivariate high QAlb in the overall population HR = 1.30, $P = 0.11$, in male patients HR = 1.78, $P = 0.007$).

We explored the effect of diabetes mellitus in the death certificate cohort ($n = 294$; Supplementary Table 11): after the exclusion of diabetes mellitus cases, male patients in the low QAlb group displayed a better prognosis in the univariate analysis (HR = 0.55, $P = 0.005$); however in the multivariate analysis in which BMI, age of onset, age at sampling and site of onset were included as confounders, no statistical significance remained for low QAlb in the overall population ($P = 0.95$) or in the female subgroup ($P = 0.38$) but still for the male subgroup (HR = 0.61, $P = 0.003$).

Thus, the increased HR observed in high QAlb groups, beyond BMI, is also driven by the diabetes mellitus comorbidity and does not appear in the death certificate cohort; however, even when diabetes mellitus, BMI and age of onset confounding effects are taken into account, a significant beneficial effect of a low QAlb remained in male ALS patients in the death certificate cohort.

Discussion

In the present work, based on the analysis of data from a large prospective cohort of >1000 patients with ALS, we first reproduced known sex-dependent differences in QAlb.[48-50] We then showed a correlation between QAlb and the survival of ALS patients, with low QAlb at diagnosis displaying a comparatively longer survival. This effect is influenced by BMI, age of onset and by diabetes mellitus comorbities, and it is more prominent in male than in female patients. Notably, QAlb (as well as QIgA, QIgG and QIgM, in agreement with the lack of oligoclonal IgG bands in ALS patients[51]) was comparable in ALS and in other neurological conditions. These findings support the hypothesis that some degree of disruption of the BCSFB due to metabolic, toxic or reactive mechanisms is a biomarker of worse prognosis in ALS.

Several clinical cohorts have been investigated for the relationship between BCSFB, assessed by QAlb, and clinico-prognostic features of ALS patients. Alarcan *et al.*[50] reported, in a cohort of 307 patients, an abnormal QAlb value in 30% of patients, a lack of correlation of QAlb with clinical parameters but a significant association between QAlb and survival in male, but not in female ALS patients, with male patients with QAlb above the median value displaying a shorter survival. Verde *et al.*[52] reported 10% of ALS patients with abnormal QAlb in a cohort of 328 subjects and a flat QAlb/age regression line for ALS, but no correlation with disease duration, disease severity or progression. Li *et al.*[53] reported a higher percentage (39.8%) of abnormal QAlb values in a cohort of 125 ALS-FTD patients (against a cohort of 40 controls with a different male/female ratio); a correlation between higher total CSF proteins and QAlb with poor prognosis was reported, even after accounting for clinico-demographic confounders. On the other hand, in a retrospective study of an ALS cohort of 326 patients, Zhao *et al.*[54] reported that higher CSF total proteins were associated with later onset age, a shorter disease duration and worse disease severity in females, but no effect on survival *per se*; these findings may have been influenced by an incomplete compensation for clinico-demographic factors. In a cohort of 160 patients, Prell *et al.*[31] reported a weak correlation between QAlb and indices of disease progression in univariate analyses; however, only a small minority of patients in this cohort displayed QAlb > 9, possibly reducing the sensitivity of the study. Assialioui *et al.*[32] reported a similar effect in a cohort of 246 ALS patients: in a multivariate Cox regression analysis, higher QAlb was significantly associated with worse survival with HR = 1.66 in spinal-onset ALS cases only. Earlier studies based on different techniques and smaller cohorts also reached the conclusion of limited correlation between QAlb and disease progression or severity (Leonardi *et al.*[29] studying samples from 90 patients with single radial immunodiffusion; Apostolski *et al.*[55] studying 37

subjects; Norris *et al.*[56] on 385 cases; reviewed in Winkler *et al.*[5] and Mirian *et al.*[57]).

Taken together, several of our findings are in general agreement with what was found in other, smaller cohorts supporting the view that lower QAlb is associated with longer survival but not with age at disease onset, severity at sampling, and progression rate. Our large dataset allowed the disentangling of several confounding factors, including BMI, age of onset and diabetes mellitus comorbidity that were previously not fully accounted for.

When considering the relationship between QAlb and survival, two questions should be considered. First, is the relationship real or is it due to incomplete compensation for confounding factors? Second, is there a mechanistic link between BCSFB function and pathogenic mechanisms determining survival?

Increased albumin CSF-serum quotient is regarded a measure for both (i) an increase in permeability at the blood-CSF barrier; and (ii) a slowed CSF circulation (i.e. reduced CSF flow rate) within CNS compartments, based on common conception.[58,59]

The latter could be associated with reduced elimination of CSF proteins from the CSF space into the venous circulation, which is regulated via the spinal lymphatic pathways and/or via Pacchioni granulationes at the sinus veins.[58,59] In the case of an increase in permeability, one hypothesis would be that metabolic toxic factors coupled to albumin or other carrier proteins coming from the systemic circulation accumulate in the CSF space and from there intensify damage at motor neurons. Second, with regard to slower CSF circulation, it would be a testable hypothesis that intrinsic CNS (intrathecally produced) factors are disposed more slowly from the CSF compartment and thus have longer time to participate in the neurodegenerative process.

For hypothesis testing, we suggest looking at a comprehensive spectrum of markers including inflammatory processes (innate and adaptive immune system), neurodegeneration and protection, genetic aspects (e.g. polymorphisms) and neuroimaging. A similar approach was recently applied in Alzheimer's disease, where different pathophysiological subtypes including BBB/BCSFB dysfunction could be described based on proteomic biomarkers.[60] Moreover, recent papers associated different cytokine profiles and *APOE* genotype with different levels of BBB/BCSFB permeability, suggesting that the integrity of the barriers could either influence or be influenced by central neuroinflammation and genotype.[61,62]

At least four factors may confound the prognostic role for QAlb, i.e. the flat correlation between QAlb and age, the non-linear correlation between BMI and QAlb, the correlation between BMI and QAlb being significant only for females and the impact of comorbidities. The flat correlation between QAlb and age has also recently been reported by Verde *et al.*[52]; this unpredicted effect implies that the physiological linear effect of age on QAlb upper limit, calculated according to Reiber, may not be fully meaningful when applied to ALS patients. We have, therefore, considered QAlb either as a continuous covariate or as a parameter to discriminate three patients subgroups; in both cases a relationship between QAlb and survival was observed. The low incidence of vascular and endocrine comorbidities reported in ALS[63,64] may contribute to justify the lack of age-related rise of QAlb.

QAlb may be affected by BMI and therefore the relationship between QAlb and survival may be affected by the known effect of BMI on survival.[65,66] The effect of BMI on BCSFB indexes and on CSF flow is known,[67,68] and QAlb tends to increase with increasing BMI and abdominal circumference; this effect may be non-linear, more prominent for lower BMI and modified by sex; the modelling of the BMI/QAlb relationship may therefore still be incomplete and the use of linear multivariate models may not completely compensate for it. However, lower BMI correlates with worse prognosis[65,66,69,70] but also with lower QAlb: in our findings, lower QAlb correlated with better prognosis, suggesting that this may not be a consequence of lower BMI.

Furthermore, one comorbidity, diabetes mellitus, does influence the QAlb/survival relationship. Diabetes mellitus is known to increase QAlb *per se*[20,71] and at the same time, diabetes mellitus shows a substantial trend toward decreasing survival of ALS patients, although it is associated with delayed age of onset.[15] The association between high QAlb and worse prognosis still remained after the removal of patients with diabetes mellitus but could not be detected in the sub-cohort with death certificates (in which the overall survival is precisely determined). On the other hand, the protective effect of low QAlb could still be detected in male patients without diabetes mellitus within the death certificate cohort, while also accounting for confounders (BMI, age of onset, age at lumbar puncture, site of onset).

However, if the low QAlb/longer survival relationship is not due to confounders, what are the possible mechanisms at work? BCSFB integrity may be a biomarker of ongoing metabolic or reactive processes linked to ALS, or may be causally related to the ALS pathogenic cascade. On the former, CSF flow and QAlb have previously been correlated with arterial pCO2[72]; thus, patients with low QAlb may have a better preserved respiratory function, which is an independent predictor of survival.[73] However, the protective effect of low QAlb appears 2 years after the CSF sampling and lasts at least up to 5 years later; it is unlikely (although possible) that respiratory performance may account for this prolonged effect. Likewise, BCSFB integrity may correlate with the integrity of perivascular spaces: a marker of perivascular fibroblast reactivity, osteopontin, is correlated with survival in several cohorts of ALS patients.[4]

On the latter, BCSFB and BBB integrity (although they are not subsumed into the same physiological concept[74]) may protect the CNS from blood-derived toxic products (reviewed in Ahmad *et al.*,[75] and Parker *et al.*[76]), including haemoglobin degradation products[9]: increasing extravasation of erythrocytes drives accelerated progression of ALS.[25] In experimental models, the integrity of the BCSFB and BBB has been associated with longer survival; decreasing BBB permeability by infusion of endothelial cells progenitors[77] results in the amelioration of the disease progression, whereas increasing BBB permeability by disruption of pericytes[78] results in accelerated progression. Thus, low QAlb may represent a marker of reduced activity of pathogenic pathways driven by or associated with altered BCSFB function, i.e. increased flow rate.[58]

Surprisingly, the low QAlb/survival relationship is more prominent in males than in females (also detected by Alarcan *et al.*[50]). The prognosis of ALS is influenced by sex: males have increased risk of ALS and faster progression[79,80] and males display a different risk profile based on BMI,[81] and subcutaneous fat predicts survival in males but not in females.[82] In the present study, it was shown that three confounders may influence the male/female difference: (i) BCSFB is known to be affected by sex and QAlb values are constitutively higher in males[83] (Supplementary Fig. 2) irrespective of the disease; (ii) QAlb is more affected by BMI in females than in males (in particular, in females QAlb correlates with BMI, but not in males); and (iii) the male/female divergence of the low QAlb/survival relationship is smaller in the death certificate cohort (also numerically smaller). Thus, linear models may not compensate for BMI effects to a similar extent in females and males, and furthermore some ascertainment bias in disease onset and survival cannot be discounted and could be responsible of the more prominent effect in males.

Nevertheless, rate and extent of pathogenic cascades may be sex-dependent. In murine models, males display an earlier onset and faster progression[84] and negatively respond to exercise (which may be protective for females[85]). Furthermore, response to treatment in murine models is more evident in males (DHO administration[86]; androgen receptor antagonist[87]; P2X7 antagonist[88]). Moreover, males physiologically display a higher QAlb.[47,89] Thus, the BCSFB may be more vulnerable to the pathogenic process in males than in females, and this may contribute to the reduced survival observed in males with high QAlb.

Thus, epidemiological biases may contribute to the male/female differences, together with differences in intrinsic vulnerability and baseline BCSFB function may contribute to explain the prominence of the low QAlb/survival relationship in females.

The present work has its major strengths in the size of the ALS cohort (>1000 ALS patients, >400 control subjects), which represents the largest employed so far in the study of CSF determinants of the disease, as well as in the prospective recruitment of patients and implementation of standard operating procedures over the whole duration of the recruitment period. On the other hand, two perceived limitations must be noted. First, this is a monocentric study, and therefore the extrapolation to other contexts should be performed with caution, given the reported differences due to genetic and environmental factors.[46,90] Second, as every patient is subject to a single CSF examination, the intraindividual evolution of the QAlb over time could not be studied and it is currently unknown how the QAlb may change because of disease-related events (such as hypercapnia[72]).

In conclusion, our findings, based on a large prospective cohort of ALS patients, provide evidence supporting the role of BCSFB functional integrity, measured by QAlb, as a biomarker of longer survival of ALS patients. The relationship between QAlb and survival is strongly affected by confounders and comorbidities such as BMI, age of onset, sex and diabetes mellitus. Because of its ready availability, QAlb may be considered for prognostic and stratification purposes in ALS. It remains to be elucidated if the QAlb value changes along disease progression in each patient, and longitudinal studies with multiple CSF samples are needed to address this point.

Data availability

The data that support the findings of this study are available on request from the corresponding authors. The data are not publicly available due to their containing information that could compromise the privacy of research participants.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

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3.2 Publikation II: CSF oligoclonal IgG bands are not associated with ALS progression and prognosis

Klose V., Jesse S., Lewerenz J., Kassubek J., Dorst J., Tumani H., Ludolph A. C. & Roselli F. (2023). CSF oligoclonal IgG bands are not associated with ALS progression and prognosis. Frontiers in Neurology, 14, 1170360.(Klose et al., 2023)

Fragestellung

Ziel dieser Studie war es zu herauszufinden, ob sich das OKB-Profil von ALS Patienten gegenüber anderen Krankheitsbildern wie Alzheimer Demenz, Mild Cognitive Impairment (MCI), Spannungskopfschmerz oder Fazialisparese unterscheidet. Außerdem sollte untersucht werden ob innerhalb der ALS Patienten basierend auf den OKB-Mustern Untergruppen mit verschiedenem klinischem und prognostischem Verlauf erkennbar sind, welche sich auch auf die Überlebensdauer auswirken würden.

Methodik

Auch für diese Studie wurden die extrahierten Liquordaten (2 Allgemeine Methodik) verwendet und mit den zu untersuchenden Diagnosen zusammengestellt. Anschließend wurden nur die Patienten herausgefiltert, für die OKBs gemessen wurden. Daraus ergaben sich die Studienkohorten: ALS (n=457), AD (n=516), MCI (n=91), TH (n=152) und FP (n=94). Die Studie wurde von der Ethikkommission der Universität Ulm genehmigt (Genehmigungsnummer 20/10) und in Übereinstimmung mit der Deklaration von Helsinki durchgeführt.

Die statistischen Analysen wurden wie schon die Datenbereinigung und Feinstrukturierung in der Programmiersprache R durchgeführt. Zum Vergleich der Liquorparameter zwischen den Studienkohorten wurde der Pearson's χ^2-Test für kategoriale bzw. qualitative Variablen (z.B. Geschlecht) und für quantitative bzw. kontinuierliche Variablen eine einseitige ANOVA verwendet. Falls die ANOVA signifikant war wurde mit einem post hoc Tukey HSD (Honestly Significant Difference) Test herausgefunden zwischen welchen Studienkohorten dieser Unterschied besteht (z.B. ALS vs. TH). Dieselben statistischen Tests wurden für den Vergleich der in die OKB-Typen (Tabelle 2) unterteilten ALS Patienten verwendet. Die statistischen Unterschiede in der Prävalenz der OKB-Typen wie auch die der Komorbiditäten innerhalb der ALS Kohorte wurde abermalig mit dem Pearson's χ^2 Test ermittelt. Mithilfe des Kaplan-Meyer-Log-Rank Tests wurde wurden Unterschiede im Überleben der ALS Patienten hinsichtlich ihres OKB-Typs analysiert. Das Ergebnis dieser Analyse wurde durch eine multivariante Cox Regressionsanalyse auf mögliche Störvariablen, die das Resultat beeinflussen könnten, hin untersucht.

Tabelle 3: Befunde basierend auf Oligoklonale IgG Banden (OKB) Typen (in Anlehnung an (Andersson et al., 1994), mit freundlicher Genehmigung von BMJ Publishing Group Ltd)

OKB-Typ	Befund
1	Normalbefund; keine oligoklonalen Banden im Serum und Liquor
2	Oligoklonalen Banden im Liquor, aber keine im Serum
3	Oligoklonale Banden im Liquor, zusätzlich identische Banden in Liquor und Serum
4	Identische oligoklonale Banden in Liquor und Serum
5	Monoklonale Banden in Liquor und Serum, Monoklonale Gammopathie

Ergebnisse

Zwischen den fünf Kohorten (ALS, AD, TH, FP, MCI) war das Vorkommen von OKBs nicht signifikant verschieden (OKB Typ 1: p = 0.22; Typ 2: p = 0,25; Typ 3: p = 0,22; Typ 4: p = 0,23; Typ 5: p = 0,22). Innerhalb der ALS Patienten zeigte sich, dass OKB Typ 5 älter als Patienten mit anderen OKB Typen waren. Außerdem zeigten Typ 3 Patienten einen signifikant niedrigeren ALS-FRS bei der ersten Messung im Gegensatz zu Typ 1 (p = 0,03) und 4 (p = 0,06). Bei den anderen klinischen Variablen, die aus dem ALS Schwaben Register bekannt waren, und den Liquorparametern gab es keine signifikanten Unterschiede zwischen den OKB Typen. Weiterhin zeigte sich kein signifikanter Unterschied im Überleben der jeweiligen OKB-Typen in den ALS Patienten. Infektiöse, entzündliche oder systemische Autoimmunerkrankungen in Form von Komorbiditäten der ALS traten vorwiegend bei OKB-Typen mit intrathekaler Synthese (Typ 2 & 3) auf.

Diskussion

Das OKB-Profil der ALS Patienten hat sich in unserer Studie zu den anderen neurologischen Kohorten nicht signifikant unterschieden. Basierend auf den OKB-Mustern war innerhalb der ALS Kohorte außerdem keine Unterteilung in verschiedene klinische und prognostische Verläufe auch hinsichtlich der Überlebensdauer möglich. Daher gehen wir davon aus, dass OKBs unabhängig von ALS auftreten und für den pathogenen Verlauf irrelevant sind.

Erste Arbeiten zu OKBs bei ALS (Apostolski et al., 1991) (Younger et al., 1990) berichteten, dass das Auftreten von OKBs einen Einfluss auf die Pathophysiologie der ALS habe, dies konnte durch unsere Studie mit erhöhter Population (457 statt 37 bzw. 120 Patienten) nicht bestätigt werden. Möglicherweise lagen diesem Effekt der anderen Studien die Erhöhung des Albumingehalts, Paraproteinämie (Younger et al., 1990), lymphoproliferative Störungen (Younger et al., 1991) oder auch eine alternative Diagnose zur ALS zugrunde. In unserer Arbeit konnte eine Prävalenz für das Vorkommen von OKBs des Typs 2 von 1,97% (9/457) ermittelt werden, bei Einbeziehung des Typs 3 sind es 9,63% (44/457). In vorangegangenen Studien (Süssmuth et al., 2010; Ticozzi et al., 2013) lag die Prävalenz für das Auftreten von OKB Typ 2 zwischen 1,93% und 1,61% (5/259, 2/124), bei Einschluss von Typ 3 bei 3,47% bis 6,45% (9/259, 8/124), diese Werte sind nicht signifikant verschieden von denen welche in unserer Studie ermittelt wurden (χ^2-Test: p = 0,996). Die Prävalenz von OKBs war in den neurologischen Kohorten unserer Studie vergleichbar, wie auch schon in vorangegangenen Arbeiten mit geringerer Fallzahl gezeigt (Jesse et al., 2011). Die leichte Erhöhung der Prävalenzen in unserer Studie könnten daher rühren, dass die Tests auf OKBs mittlerweile

sensitiver geworden sind. Das Auftreten der OKBs könnte außerdem durch das Vorliegen von entzündlichen und/oder autoimmunen Komorbiditäten zustande kommen wie sie in einer anderen Population bei 10,65% (28/263) der ALS Patienten kürzlich entdeckt wurden (Elisa Longinetti et al., 2022). In unserer ALS Kohorte konnte kein signifikanter Unterschied zwischen dem klinischen Verlauf von Patienten mit oder ohne Autoimmunerkrankung und positiven OKBs festgestellt werden, was darauf schließen lassen könnte, dass diese durch die Freisetzung von Antigenen im Verlauf des neurodegenerativen Prozesses entstehen oder zufällig auftreten. Jedoch wurden bei Patienten mit OKBs häufiger infektiöse oder entzündliche Komorbiditäten dokumentiert, wodurch es möglich wäre, dass die OKBs durch diese Komorbiditäten ausgelöst werden, aber die an sich ALS nicht beeinflussen. Patienten mit OKB Typ 3 weisen zwar im Gegensatz zu Typ 1, 2 und 4 einen durchschnittlich niedrigeren ALS-FRS bei der Liquorpunktion auf, was sich jedoch nicht auf die Progression oder das Überleben niederschlägt. Ein möglicher Erklärungsansatz hierfür wäre, dass eine neben der ALS bestehende entzündliche Systemerkrankung, die das Gehirn betrifft, die allgemeine körperliche Leistungsfähigkeit der Patienten und somit den ALS-FRS negativ beeinträchtigt.

Unsere Daten deuten darauf hin, dass das Vorhandensein von OKBs für den pathogenen Verlauf der ALS irrelevant ist und das gemeinsame Auftreten zufällig oder mit Begleiterkrankungen einhergeht. Daher sollten klinische Versuche mit immunmodulierenden Wirkstoffen gegen B-Zellen mit Vorsicht durchgeführt werden (eine solche wird derzeit in Deutschland geplant). Das Auftreten von OKBs bei ALS Patienten könnte jedoch zukünftig auch der Hinweis auf eine entzündliche oder infektiöse Komorbidität sein und den Anlass für eine diagnostische Untersuchung dahingehend geben. Da in unserer Kohorte die Dokumentation der Komorbiditäten eher gering ausfiel, müsste man diesen Zusammenhang eventuell an einer größeren Kohorte erneut beleuchten.

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Darstellung des Eigenanteils:
Wie bei Publikation I, wurden bei dieser Publikation auch die durch meine Arbeit nutzbar gemachten Liquordaten verwendet. Die Idee die OKB Typen der ALS Patienten zu untersuchen kam von Prof. Hayrettin Tumani, Prof. Francesco Roselli und Prof. Albert Ludolph. Ich habe diese für ALS Patienten und die Kontrollkohorten zusammengefasst und analysiert. Ebenso habe ich die klinischen Daten der ALS Patienten mit den Liquorparametern zusammengeführt, analysiert und ausgewertet. Tabellen und Abbildungen, welche in der Publikation auftauchen wurden von mir erstellt. Mit Hilfe von Prof. Francesco Roselli habe ich die erste Version des Manuskripts angefertigt . Ich habe außerdem Kommentare von Koautoren und Reviewern in die Publikation eingearbeitet.

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CSF oligoclonal IgG bands are not associated with ALS progression and prognosis

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Introduction: Amyotrophic Lateral Sclerosis (ALS) is characterized by progressive motoneuron degeneration through cell autonomous and non-cell autonomous mechanisms; and the involvement of the innate and adaptive immune system has been hypothesized based on human and murine model data. We have explored if B-cell activation and IgG responses, as detected by IgG Oligoclonal bands (OCB) in serum and cerebrospinal fluid, were associated with ALS or with a subgroup of patients with distinct clinical features.

Methods: IgG OCB were determined in patients affected by ALS (n=457), Alzheimer Disease (n=516), Mild Cognitive Impairment (n=91), Tension-type Headache (n=152) and idiopathic Facial Palsy (n=94). For ALS patients, clinico-demographic and survival data were prospectively collected in the Register Schabia.

Results: The prevalence of IgG OCB is comparable in ALS and the four neurological cohorts. When the OCB pattern was considered (highlighting either intrathecal or systemic B-cells activation), no effect of OCB pattern on clinic-demographic parameters and overall. ALS patients with intrathecal IgG synthesis (type 2 and 3) were more likely to display infectious, inflammatory or systemic autoimmune conditions.

Discussion: These data suggest that OCB are not related to ALS pathophysiology but rather are a finding possibly indicative a coincidental infectious or inflammatory comorbidity that merits further investigation.

KEYWORDS

oligoclonal bands, intrathecal synthesis, age of onset, survival, cerebrospinal fluid, amyotrophic lateral sclerosis

Introduction

Amyotrophic lateral sclerosis (ALS) has been originally conceptualized to be primarily a motoneuron disease, based on clinical, pathological and experimental evidence (1). However, recent evidence points toward a multidimensional pathophysiology (2) which may involve systemic (3–5) or CNS-restricted (6–8) immune responses. Alterations have been described in several cellular immunity compartment: disturbances in CD4+ lymphocytes (9) and Th17 (10) correlate with disease progression (11). Recently, also disturbances in B-cell dependent, humoral immunity have been suggested: meningeal tertiary lymphoid-like structures containing B-cells have been identified in a ALS murine model (12). Autoantibodies against multiple neuronal antigens (13–15) have been reported in ALS patients, and antibody-mediated toxicity in ALS has been proposed based on IgG transfer to animals (16, 17); the pathophysiological relevance of these observations remains to be ascertained. To date, the question of an ongoing B-cell response in ALS patients, or in a subset of them, remains open. Given the availability of

anti-B-cell treatments [e.g., anti CD19 and CD20 monoclonal antibodies; (18)] and the initial testing of rituximab in ALS trials, addressing this issue may shape the clinical trial landscape. More broadly, no immune response marker has gained widespread use in clinical practice for prognostication or patient stratification (19).

The detection of IgG-specific oligoclonal bands (OCB) in cerebro-spinal fluid (CSF) has been extensively employed to identify an active immune response in the form of the expansion of a limited number of B-cell clones (20) associated with extrathecal or intrathecal IgG synthesis (21). OCB is commonly associated with the diagnosis and pathophysiology of Multiple Sclerosis (22); however, OCB detection is reported also in infective, inflammatory and autoimmune conditions such as systemic lupus erythematosus, neurosyphilis, Behcet's disease, neuroborreliosis, aseptic meningitis, Sjögren's syndrome, autoimmune encephalitis and CNS vasculitis (23), and therefore OCBs are a bona-fide marker of immune activation irrespective of the etiology.

The comparison of OCB in serum and CSF allows the distinction of 5 patterns (24, 25): absence of OCB in both CSF and serum (type 1, no immune activation), presence of OCB in CSF but not in serum (type 2, indicating CNS-restricted B-cells activation), presence of some OCBs in CSF and in serum, with in addition OCB detected in CSF but not in serum (type 3, indicating a systemic B-cell activation together with a component restricted to the CNS), presence of the same OCBs in serum and CSF (type 4, pointing toward systemic B-cell activation without specific CNS components) and strong monoclonal OCB detected both in CSF and serum (type 5, usually related to monoclonal gammopathies). Thus, OCB provides a window to the ongoing extrathecal (systemic) as well as intrathecal (CNS-restricted) humoral immune activation and immunoglobulin synthesis.

Several initial studies (26, 27) suggested the possibility of increased prevalence of OCB in ALS patients, although diagnostic uncertainties at the time left the issue unresolved. More recent work (28) suggests that OCB may identify a subset of ALS patients with distinct genetic or clinical characteristics; however, a large-scale investigation of this hypothesis has not yet been performed.

The aim of our study was to address (a) whether there is any B-cell activation in ALS patients, or in a subset of them, related to the immune system activation and relevant to the pathogenic process; and (b) whether OCB patterns may identify a subset of ALS patients with distinct clinical and prognostic characteristics [as previously suggested for different immune markers-(29)]. We set out to address these questions by investigating the CSF data from a large (>450 patients) cohort of ALS patients prospectively recruited and followed-up at Ulm University.

Methods

Patients

The present study was performed in agreement to the Helsinki protocol on human experimentation and was approved by Ethics committee of the Ulm University, No. 20/10.

In the present study, five cohorts of patients, prospectively recruited at the Department of Neurology of Ulm University (Ulm, Germany) were taken into consideration.

Amyotrophic lateral sclerosis patients were diagnosed according to the revised El Escorial criteria (30). Inclusion criteria included (i) diagnosis of suspected, possible, probable and definite ALS reported by the ALS Registry Swabia (ii) age of onset >16 years (to limit the erroneous inclusion of SMA patients due to mis-reporting in the clinical charts) (iii) Exclusion criteria (i) final diagnosis of SMA or HSP (ii) age of onset <15.

The progression rate is calculated either *(ALS-FRS atfirstvisit—ALS-FRS at last visit)/months between those measurements* (250 patients) OR if only one visit was documented *(48—ALS-FRS at first visit)/months between onset and visit*, as previously reported (31–33).

Patients with tension-type headache (TH) were diagnosed according to International Classification of Headache Disorders-3rd edition (ICHD-3; https://ichd-3.org/) after clinical assessment, neuroimaging evaluation and blood and CSF parameters consideration.

Patients with Alzheimer disease (AD) and Mild cognitive impairment (MCI) were diagnosed according to current criteria (34, 35). In addition to anamnesis and third-party anamnesis, clinical neurological examination and laboratory for exclusion diagnostics, we included also neuropsychological testing and CSF determination of dementia markers total tau, pTau181, Ab42, Aβ 40/42 ratio in CSF were measured using Fujirebio, Gent, Belgium.

Patients with peripheral idiopathic facial palsy (FP) was diagnosed according to the German guidelines for the diagnosis of idiopathic facial nerve palsy (36, 37). For each patient, secondary causes of FP (37) were excluded by a combination of clinical examination and MRI; therefore, non-idiopathic FP cases (38) were excluded.

CSF sampling and processing

Cerebro-spinal fluid was obtained and analyzed as previously reported (39, 40). Upon lumbar puncture, CSF samples were collected in polypropylene tubes and subject to analysis within 1 h from sampling. Leukocyte count was performed by trained operators employing phase-contrast microscopy and a Fuchs–Rosenthal chamber as previously reported (40). CSF samples with contamination of erythrocytes were excluded in order to avoid false positive values of CSF albumin and lactate. Total protein, albumin, IgG, IgA, and IgM were measured by standard immunochemical nephelometry in CSF and serum [Dade–Behring nephelometer analyzer (ProSpec), Marburg, Germany] using a polyclonal antibody in the case of albumin and IgG as well as a latex particle-amplified antibody reaction in the cases of IgA. The inter- and intra-assay variabilities were <10% for the method-dependent absolute levels as well as for the method-independent CSF/serum quotients of albumin, IgG, IgA. CSF lactate was determined by a lactate–oxidase reaction (Greiner GmbH, Flacht, Germany).

Oligoclonal bands detection

Oligoclonal bands were determined by isoelectric focusing (IEF) on polyacrylamide gels followed by immunoblotting using an IgG-specific antibody staining, as previously reported (41). Briefly, CSF and serum samples from the same patient were adjusted for IgG concentrations and run on the same gel. Two long-standing experienced technicians and at two board-certified neurologists with extensive experience in the field of CSF analysis independently scored the immunoblots; consensus over the subtype was reached before the subtype was assigned to the dataset. The classification of the OCB subtype was performed as previously reported (21, 24, 25): type 1-no

oligoclonal IgG band, normal finding; type 2-Isolated OCB (2 or more IgG bands) in the CSF; type 3-Identical OCB (2 or more) in cerebrospinal fluid and serum, additionally (1 or more) isolated OCB in the CSF; Type-4: OCB with identical (mirror image) distribution in the CSF and serum; type 5-Monoclonal bands with identical distribution in the CSF and serum (usually suggesting a systemic gammopathy). Representative images of the five patterns as seen in ALS patients are provided in Supplementary Figure 1.

Statistical analysis

The statistical analyses were performed using the R software (version 4.0.3) on RStudio (version 1.2.5033). For the comparison of clinic-demographic and CSF parameters across the five disease cohorts (ALS, AD, MCI, TTH, and FP), the Pearson's chi-square test was used for categorical variables and one-way Analysis of Variance (ANOVA) with *post hoc* Tukey HSD (honestly significant difference) test was used for continuous variables (Table 1). Prevalence of OCB was analyzed by Pearson's chi-square (Table 2). The comparison of clinico-demographic and CSF features (Tables 3, 4) across ALS subgroups with distinct OCB subtypes was performed with the Pearson's chi-square test for categorical variables and one-way ANOVA with *post hoc* Tukey HSD (honestly significant difference) test for continuous variables. The prevalence of comorbidities (Table 5) was analyzed by Pearson's chi-square test. The difference in survival of the five subgroups of ALS patients according to the OCB subtype was analyzed by Kaplan–Meyer log-rank test (Figures 1, 2); multivariate Cox regression analysis was used for assessing the role of possible confounders. In all the analyses we considered $p = 0.05$ as the level of significance after correction for multiple comparisons.

Results

Prevalence of oligoclonal bands and their subtype patterns is comparable in ALS and AD, MCI, TTH or FP

We set out to establish if any pattern of oligoclonal bands in CSF was enriched in ALS compared to four cohorts of patients diagnosed with AD, MCI, TTH or FP. The clinico-demographic and CSF features of the five groups are reported in Table 1. Overall, ALS, AD and MCI patients were comparable in age, whereas TH and FP were significantly younger; furthermore, ALS patients were more frequently male, whereas for AD, MCI, TH and FP the opposite was true. In respect to the CSF parameters, the five groups were significantly different for total CSF Albumin and the ratio of CSF/Serum Albumin (Q-Alb; $p = 0.004$ and $p = 1.041e\text{-}05$, respectively); *post hoc* comparisons revealed that this effect was largely due to the difference between TTH and AD ($p = 8e\text{-}06$). The five groups also showed a significant difference in Q-IgG and total CSF IgA ($p = 0.0006703$ and $p = 0.004655$). The *post hoc* analysis revealed that these differences were due to the reduced Q-IgG in the TTH patients compared to AD patients ($p = 0.002$) and in the slight decrease in CSF IgA in ALS patients compared to AD patients ($p = 0.043$); all other groups were pairwise comparable. Finally, a statistically significant difference was observed for lactate ($p = 0.007$), but the *post hoc* analysis revealed that this was solely due to the difference between AD and MCI ($p = 0.026$) whereas ALS patients were not different from all others.

The five patterns of OCB were unevenly represented (Table 2): in the ALS patients, the 60% displayed a type 4 pattern (similar OCB in serum and CSF, indicating systemic immune response), whereas only 28% displayed no OCB; a minority of patients displayed a type 3 pattern (OCB in serum in addition to those found in the CSF) and a

TABLE 1 Clinico-demographic and cerebro-spinal fluid (CSF) parameters of the Amyotrophic lateral sclerosis (ALS) population and of four neurological control groups (AD, MCI, TTH, and FP).

	ALS	AD	MCI	TTH	FP	*p*-Value
Number	457	516	91	152	94	
Females/males	185/272 (40%/60%)	319/197 (62%/38%)	51/40 (56%/44%)	98/54 (64%/36%)	60/34 (64%/36%)	*1.337e-11
Mean age (years) at sampling (SD)	62.29 (12.08)	69.38 (11.19)	68.58 (9.68)	48.05 (18.41)	52.39 (18.69)	*<2.200e-16
CSF Alb (mg/L) (SD)	292.27 (117.72)	298.74 (158.48)	287.69 (97.31)	251.56 (103.17)	276.12 (130.18)	**0.0041
Q-Alb × 10^3 (SD)	6.94 (2.66)	7.54 (4.71)	6.66 (2.27)	5.89 (2.32)	6.49 (3.19)	**1.041e-05
CSF IgG (mg/L) (SD)	32.13 (14.49)	33.92 (18.53)	31.89 (14.87)	30.77 (17.24)	30.73 (15.35)	**0.1533
Q-IgG × 10^3 (SD)	3.25 (1.34)	3.51 (2.07)	3.06 (1.03)	2.91 (1.67)	3.06 (1.53)	**0.0007
CSF IgA (mg/L) (SD)	4.25 (2.91)	4.91 (4.34)	3.92 (2.80)	4.10 (3.95)	3.85 (2.91)	**0.0046
Q-IgA × 10^3 (SD)	1.90 (2.29)	1.90 (1.31)	1.60 (0.70)	1.64 (1.52)	1.69 (1.14)	**0.2349
CSF IgM (mg/L) (SD)	0.35 (0.39)	0.38 (0.70)	0.26 (0.21)	0.32 (0.40)	0.33 (0.65)	**0.3578
Q-IgM × 10^3 (SD)	0.33 (0.23)	0.40 (0.51)	0.30 (0.17)	0.34 (0.70)	0.35 (0.37)	**0.0669
Leukocytes/µl (SD)	0.86 (1.23)	4.86 (43.23)	0.88 (1.00)	1.26 (1.48)	1.27 (1.17)	**0.1759
Number of Patients Leukocytes >5/µl (%)	4 (1%)	18 (4%)	0	4 (3%)	0	** 0.2434
Lactate (mmol/L) (SD)	1.79 (0.27)	1.84 (0.38)	1.72 (0.22)	1.77 (0.42)	1.82 (0.46)	**0.0076

The TTH and FP groups are significantly younger than the AD, MCI, and ALS. TTH patients have a significantly lower CSF Albumin and Q-Alb and Q-IgG; AD patients have a significantly higher Q-IgA. Lactate levels are significantly lower in ALS, MCI and TTH. *Pearson's χ^2 test; **One-way ANOVA, Tukey *post hoc* test.

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TABLE 2 Comparable prevalence of oligoclonal bands (OCB) patterns in amyotrophic lateral sclerosis (ALS) and four neurological control populations.

	ALS	AD	MCI	TTH	FP	*p*-Value
OCB type 1 (%)	130 (28%)	106 (21%)	18 (20%)	42 (28%)	26 (28%)	*0.2237
OCB type 2 (%)	9 (2%)	8 (2%)	0	1 (1%)	0	*0.2458
OCB type 3 (%)	35 (8%)	47 (9%)	7 (8%)	13 (9%)	0	*0.2205
OCB type 4 (%)	275 (60%)	348 (67%)	65 (71%)	90 (59%)	66 (70%)	*0.2278
OCB type 5 (%)	8 (2%)	7 (1%)	1 (1%)	6 (4%)	2 (2%)	*0.2232

No difference in the prevalence of the different OCB types was detected across the five cohorts of patients. *Pearson's χ^2 test.

TABLE 3 Clinico-demographic features of amyotrophic lateral sclerosis (ALS) patients subgrouped according to the oligoclonal bands (OCB) pattern.

ALS patients (*n*=457)							
		OCB type 1	OCB type 2	OCB type 3	OCB type 4	OCB type 5	*p*-Value
Number		130 (28%)	9 (2%)	35 (8%)	275(60%)	8 (2%)	*<2.2e-16
Female/male		56/74	3/6	16/19	106/169	4/4	*0.7942
Age of onset		58.38 (11.54)	57.93 (9.92)	62.43 (14.02)	61.29 (12.65)	67.07 (7.03)	**0.0776
Age at sampling		59.94 (11.40)	59.33 (9.93)	63.65 (14.06)	63.11 (12.15)	69.63 (7.44)	**0.0378
Site of onset:	Bulbar	31	4	13	71	3	*0.2380
	Spinal	94	4	21	191	5	
	Uncertain	4	1	0	4	0	
BMI		24.81 (4.17)	25.01 (5.98)	25.86 (5.93)	24.66 (3.90)	25.60 (6.42)	**0.6301
ALS-FRS at sampling		40.7 (6.08)	42.56 (4.53)	36.97 (6.51)	40.21 (6.64)	38.63 (4.63)	**0.0369
Progression rate		0.94 (1.22)	1.08 (0.75)	1.09 (0.86)	0.81 (0.97)	1.20 (1.77)	**0.4332

Age at sampling was significantly different across groups with patients with type 5 OCB being significantly older than the others; ALS-FRS was significantly different across the groups, with *post hoc* test revealing a significant difference between OCB type 3 patients and OCB type 1 ($p = 0.03$) and OCB type 4 (with $p = 0.06$). *Pearson's χ^2 test; **One-way ANOVA, Tukey *post hoc* test.

TABLE 4 Cerebro-spinal fluid (CSF) parameters of amyotrophic lateral sclerosis (ALS) patients subgrouped according to the oligoclonal bands (OCB) pattern.

ALS patients (*n*=457)						
	OCB type 1	OCB type 2	OCB type 3	OCB type 4	OCB type 5	*p*-Value
CSF Alb (mg/L)	299.15 (118.99)	320.44 (140.75)	274.29 (108.09)	292.30 (118.46)	225.50 (69.76)	0.3666
Q-Alb $\times 10^3$	6.95 (2.58)	7.32 (3.32)	6.79 (2.78)	6.98 (2.69)	5.50 (1.33)	0.6118
CSF IgG (mg/L)	32.20 (14.21)	30.60 (13.27)	34.06 (13.98)	32.05 (14.83)	27.34 (12.39)	0.8120
Q-IgG $\times 10^3$	3.24 (1.39)	3.63 (1.72)	3.43 (1.29)	3.24 (1.33)	2.55 (0.69)	0.4814
CSF IgA (mg/L)	4.16 (2.34)	4.80 (3.89)	4.71 (3.65)	4.25 (3.04)	2.88 (1.92)	0.5453
Q-IgA $\times 10^3$	1.79 (0.91)	2.21 (1.21)	1.78 (0.92)	1.97 (2.86)	1.37 (0.61)	0.8797
CSF IgM (mg/L)	0.32 (0.31)	0.62 (0.89)	0.40 (0.47)	0.35 (0.40)	0.29 (0.22)	0.2194
Q-IgM $\times 10^3$	0.31 (0.21)	0.53 (0.42)	0.35 (0.28)	0.33 (0.22)	0.31 (0.14)	0.0809
Leukocytes/µl	0.73 (1.10)	1.11 (0.78)	1.37 (1.40)	0.84 (1.27)	1.00 (1.20)	0.0912
Lactate (mmol/L)	1.79 (0.28)	1.74 (0.14)	1.87 (0.35)	1.78 (0.26)	1.75 (0.24)	0.4034

No difference in CSF parameters was observed across OCB type subgroups. Comparison by one-way ANOVA.

very small subgroup, approx. 2%, displayed a type 2 pattern (OCB only in CSF, indicative of intrathecal immune response). Likewise, 2% of ALS patients displayed a pattern compatible with monoclonal gammopathy. Most importantly, the relative representation of the five OCB patterns was identical in the five cohorts ($p = 0.22$) and with no instance of higher-than-expected pattern representation. Thus, these data conclusively demonstrate that no pattern of OCB is enriched in ALS.

Oligoclonal band subtypes do not affect onset and progression rate of ALS

Next, we investigated if subgroups of ALS patients with distinct OCB patterns also displayed specific clinical features (Table 3). A strong trend ($p = 0.0077$) toward a difference in the age of onset was largely driven by the small subgroup with monoclonal gammopathy (type 5), which were older than the

TABLE 5 Prevalence of infectious, inflammatory, metabolic and cardiovascular comorbidities in the amyotrophic lateral sclerosis (ALS) patients subgrouped according to the oligoclonal bands (OCB) pattern.

ALS patients (n=457)						
	OCB type 1 (n=130)	OCB type 2 (n=9)	OCB type 3 (n=35)	OCB type 4 (n=274)	OCB type 5 (n=8)	p-Value
Infections % (n)	0.77% (1)	11.11% (1)	8.57% (3)	0.36% (1)	0% (0)	*0.0001
Borrelia % (n)	0% (0)	11.11% (1)	0% (0)	0% (0)	0% (0)	*4.022e-10
Meningitis % (n)	0% (0)	0% (0)	5.71% (2)	0% (0)	0% (0)	*7.408e-05
Zoster % (n)	0.77% (1)	0% (0)	3.96% (1)	0.36% (1)	0% (0)	*0.5433
Inflammation % (n)	5.38% (7)	11.11% (1)	14.29% (5)	10.94% (30)	0% (0)	*0.2812
Multiple sclerosis % (n)	0% (0)	0% (0)	3.96% (1)	0% (0)	0% (0)	*<2.2e-16
COPD % (n)	0% (0)	0% (0)	5.71% (2)	2.19% (6)	12.0% (1)	*0.0408
Asthma % (n)	3.08% (4)	11.11% (1)	0% (0)	2.19% (6)	0% (0)	*0.3723
Arterial hypertension % (n)	29.23% (38)	33.33% (3)	48.57% (17)	31.75% (87)	50% (4)	*0.2051
Heart disease % (n)	8.46% (11)	11.11% (1)	11.43% (4)	12.41% (34)	0% (0)	*0.6603
Coronary heart disease % (n)	3.85% (5)	11.11% (1)	5.71% (2)	6.20% (17)	0% (0)	*0.7422
Stroke % (n)	0.77% (1)	0% (0)	3.96% (1)	0% (0)	0% (0)	*0.1781
Diabetes mellitus % (n)	13.08% (17)	11.11% (1)	11.43% (4)	8.39% (23)	0% (0)	*0.5312

A significant over-representation of infections and inflammation in patients with intrathecal synthesis (OCB 2 and 3) is detected. *Pearson's χ^2 test.

others (in agreement with the higher incidence of gammopathies in older patients). A statistically significant difference ($p = 0.037$) was detected in the ALS-FRS at the first evaluation (sampling); *post hoc* analysis revealed that this difference was due to the significantly lower ALS-FRS score in patients with type 3 OCB vs. patients with type 1 ($p = 0.031$) and, at strong trend level, vs. patients with type 4 ($p = 0.061$). On the other hand, the five subgroups of ALS patients were comparable in terms of their site of onset and progression rate. The five groups were also largely comparable in terms of their CSF features (Table 4); strong non-significant trends were found for Q-IgM ($p = 0.08$, higher in the subtype 2) and for leukocytes ($p = 0.09$, higher in subtypes 2 and 3). Both these findings are consistent with the OCB patterns (type 2 and 3, OCB in CSF only or more OCB in CSF than in serum) indicating an immune activation affecting the intrathecal compartment. Thus, the presence and pattern of OCB is not associated with a distinct clinico-biological phenotype of ALS patients with the exception of a more severe ALF-FRS at presentation for patients with systemic and intrathecal immune activation, corresponding to OCB type 3.

OCB subtypes do not predict overall survival

We tested if the OCB pattern was associated with a different survival. We considered either the overall cohort, using the last visit as a censoring event (Figure 1), or a subgroup of patients (222 subjects) for which death certificates are available (Figure 2). In both analysis (Kaplan–Meier Log-rank method), no statistical difference was observed across the five subgroups (overall cohort $p = 0.46$; cohort with death certificates $p = 0.83$); a trend toward shorter survival of patients with the monoclonal gammopathy type was observed, in agreement with the older age and the co-existence of a neoplastic condition. We also performed a Multivariate Cox Regression analysis to confirm the lack of effect of OCB subtypes: none of the considered potential confounders, sex ($p = 0.40$), BMI ($p = 0.90$), age of onset ($p = 0.99$), onset site ($p = 0.3$) was significantly modifying the lack of correlation between survival and OCB subtype.

Isolated CSF OCB may result from unrelated comorbidities

Finally, we performed a survey of the possible sources of OCBs in ALS patients. We analyzed the prevalence of comorbidities possibly related to OCBs (infectious or inflammatory diseases) and comorbidities unrelated to OCBs (metabolic and cardiovascular conditions; Table 5). Infectious (*Borrelia Burgdoferi*, viral meningitis, herpes zoster being the most frequent) comorbidities were significantly over-represented in patients with OCB type 2 and 3 ($p < 0.001$); inflammatory conditions (systemic autoimmune diseases including thyroiditis, arthritis and, in one case, multiple sclerosis) were more prevalent in patients with OCB type 2 and 3 (11 and 14%) compared to patients without OCB, but the finding did not reach statistical significance ($p = 0.23$). Furthermore, Chronic Obstructive Pulmonary Disease (COPD) was significantly more prevalent in patients with OCB type 3 than in other subtypes (5.71%, $p = 0.040$). On the other hand, cardiovascular comorbidities, diabetes mellitus and asthma bronchiale were similarly distributed across the OCB subtype groups. Thus, OCB may originate as consequence of inflammatory or infectious comorbidities.

Discussion

In the present work, we provide compelling evidence, based on a large prospective cohort of ALS patients, that OCB do not appear in

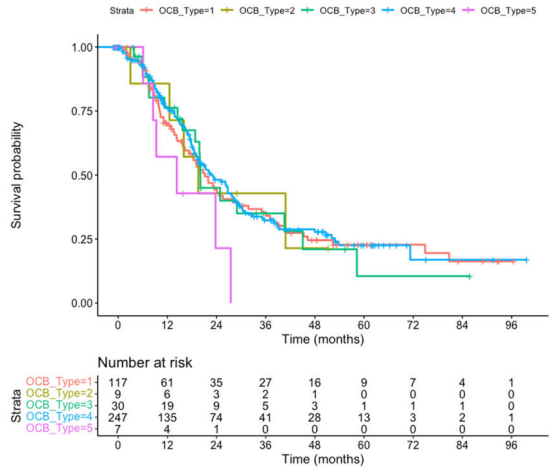

FIGURE 1
Survival of amyotrophic lateral sclerosis (ALS) patients subgrouped according to the oligoclonal bands (OCB) subtype. The whole cohort (n=457) is taken into consideration, using the date of the last visit as endpoint. No difference in survival is observed (p=0.46); patients with type 5 pattern have a trend toward shorter survival compared to type 4 (p=0.064). Kaplan–Meyer log-rank test.

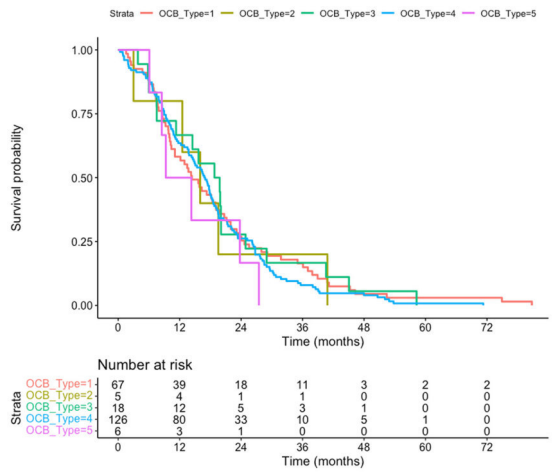

FIGURE 2
Death-certificate survival of amyotrophic lateral sclerosis (ALS) patients subgrouped according to the oligoclonal bands (OCB) subtype. The subgroup of ALS patients for whom death certificates are available (n=222) is considered; no difference of survival according to the OCB subtype is observed (p=0.83). Kaplan–Meyer log-rank test

increased frequency in ALS patients compared to other neurological controls and that OCB pattern, either related to a systemic or to a CNS immune activation, does not correlate with a distinct clinical phenotype in terms of onset, progression and overall survival. Thus, OCBs may appear in ALS as an unrelated phenomenon, irrelevant to the pathogenic process. This negative finding has implications for the clinical testing of immunomodulatory treatments for ALS and for the diagnostic interpretation of a positive OCB report.

Initial reports (26, 27) suggested that a subset of ALS patients may display OCB as part of their pathophysiology; however, these patients were also reported to have substantial increase in CSF albumin content and/or paraproteinemia (27). Thus, it remains possible that an alternative diagnosis, other than ALS, could be better suited for these cases; furthermore, some of the ALS cases with OCB displayed lymphoproliferative disorders (42). More recent work reported OCB prevalence in 9/259 [3.5%; (28)] and in 0.5–2% (40, 43). Our survey reveals a 9/457 (2%) prevalence for type 2 OCB, in line with previous reports, and a substantially higher prevalence (44/457, approx. 10%) when type 2 and type 3 are considered together. Since the prevalence appears comparable across the five conditions taken into consideration (ALS vs. AD, MCI, TTH and FP) as previously observed (40), the upward trend may be accounted for by changes in sensitivity of OCB detection and definition of positive OCB.

Our data show that ALS patients do not display higher incidence of OCB, in any pattern, than other neurological controls, suggesting that any inflammatory/autoimmune condition underlying the OCB may be coincidental rather than causal. The occurrence of inflammatory/autoimmune conditions in ALS patients has been previously subject to investigation in the Swedish nation-wide cohort; only 28 patients, out of 263 patients with purported co-existence of both conditions, were positively identified as affected by multiple sclerosis, myasthenia gravis, inflammatory polyneuropathies or dermatopolymyositis. The low rate of co-occurrence and the absence of effect on survival supported the hypothesis that the inflammatory condition did not substantially affect the progression of ALS (44) and may be due to chance or misdiagnosis (45, 46). Sporadic cases of ALS occurring together with neuromyelitis optica (47), autoimmune encephalitis (48) or multiple sclerosis (49) have been reported. Interestingly, OCB appears to be relatively more prevalent in ALS cases with distinct genetic causes [3/12 in genetic ALS cases vs. 6/247 in sporadic; the three cases presenting TDP-43 or ANG-2 mutations; (28)] and with distinctive presentation and clinical course (cerebellar involvement and hyperintense lesion in white matter). It must be noted that in these cases, the possibility of a misdiagnosis should be considered and solitary sclerosis has been identified in cases with clinical presentation compatible with motoneuron disease but positive OCB (45).

In those cases in which ALS co-occurred with an autoimmune disease and positive OCB, the clinical course of ALS was not substantially different from those of isolated ALS, suggesting that autoimmunity and OCB may originate from the release of antigens in the course of the neurodegenerative process or may occur by chance alone.

Moreover, patients with signs of intrathecal synthesis (OCB type 2 and 3) showed significantly more infectious and inflammatory comorbidities (including 1 case of a co-occurring diagnosis of Multiple Sclerosis). These findings further suggest that the detection of OCB may indicate an ongoing infectious or autoimmune disorder coincident, but unrelated to, ALS. Nevertheless, the coincident conditions determining the OCB may affect the overall performance of the patients: in fact, ALS patients with OCB type 3 display a lower ALS-FRS at CSF sampling (a proxy of ALS-FRS at diagnosis) than patients with no OCB (type 1) or intrathecal synthesis-only (type 2) or systemic-only activation (type 4; strong trend, $p = 0.06$), despite no difference in progression rate. It is possible that a co-occurring inflammatory systemic condition affecting the brain may reduce the

overall fitness of patients and lower the ALS-FRS at diagnosis; this hypothesis may be tested in dedicated studies.

Furthermore, our data show that the presence or absence of OCB, or any pattern of OCB, does not affect progression rate and survival. Thus, our data suggest that the pathophysiological process highlighted by OCB may not be relevant to the pathogenic cascade of ALS. Our findings are consistent with the inefficacy of B-cell deletion to modulate the disease burden and survival of mutant SOD1 ALS mice (50), despite the appearance of autoantibodies in this model. Thus, the observed enrichment of B-cells in meningeal tissue of the mutant SOD1 ALS mouse model (12) may constitute a response to the neurodegenerative process but not a direct player in the loss of motoneurons. It is not possible to discount, though, that the immune response and B-cell activation may be more relevant in subsets of ALS patients characterized by specific genotypes: recently, clonally expanded CD8+ T cells have been identified in familial ALS-4 cases (4), and autoimmune response characterize a murine model lacking C9Orf72 protein expression (51); furthermore, OCB appears to be disproportionately more common in ALS patients with TDP-43 mutations (28). Also, sub-grouping of ALS patients may require the consideration of markers of signaling within specific subpopulations, such as IL-23 (29) or IL-6 (52).

Finally, our data do not completely discount the involvement, in the ALS pathogenesis, of other immune processes not recaptured by OCB detection or pattern. In fact, several adaptive and innate immunity abnormalities have been reported at the level of regulatory T cells and GATA3+ Th2 lymphocytes [reduced in patients with rapid progression (53–55)] and deregulated monocytes may infiltrate the spinal cord of ALS patients and murine models (56, 57). Furthermore, local immune responses involving reactive microglia may be involved in ALS pathophysiology (2, 6, 8) but still may not be highlighted by OCB.

In conclusion, the lack of correlation between OCB and progression or survival of ALS patients, and their likely origin as epiphenomena or coincidental conditions, suggests that clinical trials with anti B-cell immunomodulatory agents should be performed with caution and may have a lower priority compared to agents addressing other cellular branches of the innate and adaptive immune system. On the other hand, the detection of OCB in the CSF of ALS patients may be considered a warning sign of a co-occurring inflammatory or infectious disease and prompt the opportune evaluation; because of the comparatively small prevalence of comorbidities in ALS patients, this investigation is to be considered exploratory and may require re-assessment in a large cohort.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving human participants were reviewed and approved by Ethics committee of the Ulm University, No. 20/10. The patients/participants provided their written informed consent to participate in this study.

Author contributions

VK organized the database, performed the statistical analysis, and contributed to the manuscript. SJ provided the clinico-demographic information about the AD and the MCI cohorts. JL provided the clinico-demographic information about the TTH and the FP cohorts. JD and JK contributed to the clinico-demographic information about the ALS cohort. HT contributed to the CSF datasets. VK, AL, and FR contributed to the conception and design of the study. AL, FR, VK, and HT contributed to the initial draft of the manuscript. VK, SJ, JL, JD, JK, HT, AL, and FR contributed to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fneur.2023.1170360/full#supplementary-material

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3.3 Publikation III: Lipids and Amyotrophic Lateral Sclerosis: A two-sample Mendelian Randomization Study

Xia K., **Klose V.**, Högel J., Huang T., Zhang L., Dorst J., Fan D. & Ludolph A. C. Lipids and Amyotrophic Lateral Sclerosis: A two-sample Mendelian Randomization Study. European Journal of Neurology.(Kailin Xia, Klose, et al., 2023)

Fragestellung

Der Zusammenhang zwischen dem Fettstoffwechsel und dem Risiko an ALS zu erkranken wurde in bisherigen Beobachtungsstudien teilweise kontrovers betrachtet. In dieser Publikation wurde daher mithilfe der Mendelschen Randomisierung (MR) untersucht, ob der Fettstoffwechsel genetisch bedingte Risikofaktoren für die ALS beinhaltet.

Methodik

Hierzu haben wir Daten aus Europäischen Kohorten für Gesamtcholesterin (n= 188.578), High-Density-Lipoprotein-Cholesterin (HDL-C) (n= 403.943), Low-Density-Lipoprotein-Cholesterin (LDL-C) (n= 440.546), Apolipoprotein A1 (ApoA1) (n= 391.193), Apolipoprotein B (ApoB) (n= 439.214) und ALS (12.577 Fälle und 23.475 Kontrollen) aus genomweiten Assoziationsstudien (GWAS) verwendet, welche öffentlich zugänglich sind (z.B. unter https://www.ebi.ac.uk/gwas/). Diese haben wir mit der Methode der MR, hinsichtlich der genetischen Zusammenhänge zwischen den genannten Lipiden und dem Risiko an ALS zu erkranken untersucht. Außerdem haben wir eine Mediationsanalyse durchgeführt, um festzustellen, ob LDL-C als potenzieller Mediator für mehrfach ungesättigte Fettsäuren (PUFAs) hin zum Risiko an ALS zu erkranken agiert.

Auf das Ethikvotum wurde in dieser Studie verzichtet, da keine Patienten direkt involviert waren und wir ausschließlich auf öffentlich verfügbare, nicht personenbezogene Daten zurückgegriffen haben. Alle mit in unsere Analyse einbezogenen Studien wurden gemäß der Deklaration von Helsinki durchgeführt.

Ergebnisse

Mithilfe der MR konnten wir feststellen, dass genetisch bedingt erhöhte Lipidwerte in der präklinischen Phase das Risiko an ALS zu erkranken erhöhen. Dieser Effekt wurde am stärksten bei erhöhten LDL-C Werten beobachtet, wobei sich das Risiko zu erkranken um 2,8% steigert (Odds Ratio (OR) 1,03, 95% KI [1,01; 1,05]; p = 0,01) pro LDL-C Anstieg um eine Standardabweichung (SD). Auch erhöhte Werte von ApoB (OR 1.02; p = 0,03) und dem Gesamtcholesterin (OR 1,02; p = 0,03) begünstigten die Entstehung der ALS. Bei HDL-C und ApoA1 konnte kein signifikanter Zusammenhang mit dem ALS Risiko ermittelt werden. Umgekehrt betrachtet bedingte das Vorliegen eines erhöhten ALS Risikos keine Veränderungen der Lipidspiegel. Des Weiteren fanden wir keinen Zusammenhang zwischen Lebensgewohnheiten, die den LDL-C-Spiegel beeinflussen und dem ALS Risiko. Durch die Mediationsanalyse konnten wir erkennen, dass LDL-C außerdem als Mediator für Linolsäure agieren könnte (Mediationseffekt 0,01).

Diskussion

Da die Rolle von Lipiden bei dem Risiko an ALS zu erkranken teilweise umstritten war, wollten wir untersuchen ob dieser Zusammenhang genetisch bedingt ist. Hierzu haben wir

das Konzept der MR angewendet und konnten erhöhte Lipidwerte in der präklinischen Phase als Risikofaktor für die ALS identifizieren.
Vorangegangene Studien, die auch die Methode der MR angewendet haben kamen zu ähnlichen Ergebnissen. Vor allem der Zusammenhang von LDL-C und dem ALS Risiko wurde oft herausgestellt. In einer Studie, welche anhand einer Europäischen und Ostasiatischen Population durchgeführt wurde konnten hingegen keine Auswirkungen von HDL-C oder dem Gesamtcholesterin auf das ALS Risiko festgestellt werden, das OR für erhöhtes LDL-C lag hingegen bei 1,14 (Zeng & Zhou, 2019). Dies wurde in einer weiteren Arbeit, welche sich auf die Europäische Population fokussiert hatte bestätigt (LDL-C OR 1,12) (Bandres-Ciga et al., 2019). Anderweitig konnte neben gesteigertem LDL-C (OR 1,16) auch erhöhtes Gesamtcholesterin (OR 1,15) als Risikofaktor für das ALS Risiko identifiziert (Chen et al., 2018). Im Vergleich zu diesen vorangegangen Studien haben wir die MR an größeren Europäischen Stichproben durchgeführt (n = 400.00 statt 190.000 bzw. 100.000), was eine höhere Anzahl von genetischen Markern und somit eine bessere statistische Aussagekraft impliziert. Außerdem untersuchten wir zusätzlich zu LDL-C, HDL-C und Gesamtcholesterin erstmalig auch die Auswirkungen von genetisch bedingt erhöhten Apolipoproteinen. In einer Follow-up Studie mit 636.132 Probanden, die über 20 Jahre hinweg gesammelt wurden, spielten auch Apolipoproteine in Bezug auf das zukünftige ALS Risiko eine Rolle. Veränderungen im Lipid- und Apolipoprotein-Stoffwechsel stünden mit dem ALS Risiko in Verbindung stehen und könnten schon Jahrzehnte vor Manifestation Hinweise auf die zukünftige Erkrankung liefern (Mariosa et al., 2017). Dass die ALS keinen Einfluss auf Gesamtcholesterin, HDL-C und LDL-C hat wurde bereits in einer weiteren Arbeit festgestellt (Chen et al., 2018) und durch unsere Untersuchungen bestätigt, wobei wir dadurch noch die zusätzliche Erkenntnis gewinnen konnten dass auch Apolipoproteine nicht von der ALS beeinflusst werden.
Da LDL-C den größten Einfluss auf das ALS Risiko hatte, haben wir uns außerdem mit LDL-C-modifizierenden Parametern des Lebensstils wie Ernährung und körperliche Aktivität auseinandergesetzt. Früchte, pflanzliches Kalzium, Beta-Karotin sollen das ALS Risiko verringern, wohingegen Fleisch, Fisch und Fast Food das Risiko an ALS zu erkranken begünstigen sollen (Jin et al., 2014). Diese modifizierenden Effekte konnten wir durch genetisch definierte Lebensstile, wobei auch die körperliche Aktivität impliziert ist, nicht in Verbindung mit dem ALS Risiko bringen. Andere MR Studien fanden in Bezug auf die genetisch prädestinierte körperliche Aktivität auch keinen Zusammenhang mit dem Risiko an ALS zu erkranken (Wu et al., 2021) (Zhang et al., 2021).
Da LDL-C beim Metabolismus von PUFAs entsteht und mit ALS in Verbindung zu stehen scheint (K. Xia et al., 2022), haben wir zusätzlich den Mediationseffekt von LDL-C zwischen PUFAs und dem ALS Risiko geprüft. Wir fanden heraus, dass dieser Mediationseffekt bei Linolsäure existiert. Mit der MR-Methode konnten wir zeigen, dass 27,3 % des Risikoeffekts von Linolsäure auf das ALS Risiko durch erhöhtes LDL-C erklärbar sind.

Originalpublikation

Referenz:
Xia K., **Klose V.**, Högel J., Huang T., Zhang L., Dorst J., Fan D. & Ludolph A. C. Lipids and Amyotrophic Lateral Sclerosis: A two-sample Mendelian Randomization Study. European Journal of Neurology, doi: 10.1111/ene.15810.(Kailin Xia, Klose, et al., 2023)

Darstellung des Eigenanteils:
Bei dieser Publikation habe ich gemeinsam mit Kailin Xia (Doktorandin von der Universität Peking) die Studie konzipiert. Die Datenanalyse/-auswertung mittels der Mendelischen Randomisierung habe in R (Version 4.0.3) durchgeführt um die Ergebnisse von Kailin Xia zu reproduzieren und validieren. Gemeinsam mit Prof. Josef Högel und Prof. Albert Ludolph haben wir die Ergebisse interpretiert. Das Manuskript habe ich gemeinsam mit Kailin Xia angefertigt und Kommentare von Koautoren und Reviewern eingearbeitet.

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ORIGINAL ARTICLE

Lipids and amyotrophic lateral sclerosis: A two-sample Mendelian randomization study

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Abstract

Objective: Previous observational studies revealed a potential but partially controversial relation between lipid metabolism and the risk of amyotrophic lateral sclerosis (ALS), potentially prone to bias. Therefore, we aimed to study whether lipid metabolism involves genetically determined risk factors for ALS through Mendelian randomization (MR) analysis.

Methods: Using genome-wide association study summary-level data for total cholesterol (TC) ($n = 188,578$), high-density lipoprotein cholesterol (HDL-C) ($n = 403,943$), low-density lipoprotein cholesterol (LDL-C) ($n = 440,546$), apolipoprotein A1 (ApoA1) ($n = 391,193$), apolipoprotein B (ApoB) ($n = 439,214$), and ALS (12,577 cases and 23,475 controls), we implemented a bidirectional MR study to evaluate a genetic relation between lipids and ALS risk. We performed a mediation analysis to assess whether LDL-C is a potential mediator on the pathway from traits of LDL-C-related polyunsaturated fatty acids (PUFAs) to ALS risk.

Results: We identified genetically predicted increased lipid levels to be associated with the risk of ALS, whereby elevated LDL-C had the most potent effect (OR 1.028, 95% CI 1.008–1.049, $p = 0.006$). The effect of increased levels of apolipoproteins on ALS was similar to their corresponding lipoproteins. ALS did not cause any changes in lipid levels. We found no relation between LDL-C-modifying lifestyles and ALS. The mediation analysis revealed that LDL-C could act as an active mediator for linoleic acid, with the mediation effect estimated to be 0.009.

Conclusions: We provided high-level genetic evidence verifying the positive link between preclinically elevated lipid and ALS risk that had been described in previous genetic and observational studies. We also demonstrated the mediating role of LDL-C in the pathway from PUFAs to ALS.

KEYWORDS
amyotrophic lateral sclerosis, genetics, instrumental variables, lipids, Mendelian randomization

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease with poor overall survival of 3–5 years due to muscle weakness and respiratory failure [1]. The causes of ALS are complex and largely unknown, among which genetic factors are the best explored. Since the causality of specific gene variants is unknown, few promising disease-modifying therapies based on genetic findings are available. Therefore, identifying genetically defined risk factors for ALS is of interest, which may provide a better understanding of ALS and reveal potential strategies in disease prevention.

Lipids constitute a fundamental energy supplier and play an essential role in many cellular processes, including apoptosis, autophagy, and intracellular signaling [2]. Apolipoproteins are indispensable constituents of lipoproteins and are responsible for maintaining structural stability and lipid transport. The main component of high-density lipoprotein cholesterol (HDL-C) is apolipoprotein A1 (ApoA1) [3], whereas apolipoprotein B (ApoB) is the primary apolipoprotein in low-density lipoprotein cholesterol (LDL-C) [4]. Circulating lipid levels are significantly determined by inheritance with heritability up to 59% [5]. Some, but not all, epidemiological studies indicate that premorbid higher levels of circulating lipids were associated with the risk of ALS [6–8]. These inconsistent results may be due to varying sample sizes, interference of confounders, and reverse causal relations. Randomized control trials (RCTs) are generally needed to overcome the limitations of observational studies. However, RCTs investigating the effects of premorbid lipid metabolism on the risk of ALS have rarely been conducted to date.

Mendelian Randomization (MR) is a statistical approach for investigating relations between an exposure and an outcome based on instrumental genetic variables (IVs) and genome-wide association studies (GWASs) of summary-level data [9]. By simulating RCTs with naturally grouped risk alleles, the MR approach can produce weighted controls to account for reverse causality and confounders [10]. Recently, Chen et al. and Zeng et al. independently found a significant association between pre-existing elevated LDL-C and the risk of ALS via MR approaches, which may be caused by shared genes [11, 12]. However, the powers of both MR studies were limited due to the relatively small sample sizes of the respective GWASs. Furthermore, the relation between apolipoproteins and ALS remains poorly explored. Similarly, the effect of LDL-C-modifying lifestyles on ALS are not clear. For example, exercise is known to have a beneficial effect on individuals with dyslipidemia, but the association between exercise and risk of ALS is controversial [13–15]. Polyunsaturated fatty acids (PUFAs) are strongly involved in the synthesis and decomposition of LDL-C and are also an essential part of lipid metabolism [16]. Both dietary intake levels and serum concentration of PUFAs were suggested to be associated with ALS risk [17–19]. Investigating the relation between PUFAs and ALS by MR and exploring the role of LDL-C may provide further support for the association between LDL-C and ALS. Therefore, to systematically evaluate the effects of genetically determined lipid and apolipoprotein levels on ALS and to assess the potential effect of lipid-modifying lifestyles on the risk of ALS, we conducted a bidirectional, two-sample, MR analysis based on the largest GWASs to date.

METHODS

Study design

We performed this study under a two-sample, bidirectional, MR analysis framework. All data used in this study were GWAS summary results from the European population. First, we calculated whether or not there was a bidirectional association between lipid levels and risk of ALS. Then we evaluated the effect of adjustable LDL-C-modifying lifestyles on ALS. Lastly, we performed a network MR analysis to assess the mediation role of LDL-C in the association between PUFAs and ALS due to the evidence that PUFAs may be relevant for ALS (Figure S1).

Data sources and instrument selection

We obtained genetic data for total cholesterol (TC) from the Global Lipids Genetics Consortium (GLGC) containing 188,578 individuals from 37 cohorts after quality control [20]. We extracted the GWASs summary-level data of LDL-C, HDL-C, ApoA1, and ApoB from the UK Biobank (UKBB), performed by Tom G. Richardson et al. separately [21] by including 393,193 to 441,016 individuals. Through adjustment for age and sex, a linear mixed model was utilized in this study for enhanced power and fewer false positives [22]. Single nucleotide polymorphisms (SNPs) independently associated with the exposures ($p < 5 \times 10^{-8}$, at $r^2 < 0.001$) were selected as candidate IVs. For those SNPs missing corresponding information in the ALS data, we searched proxies with $r^2 > 0.8$ using the publicly available online tools SNiPA [23] and LDlink [24]. If these IVs had no reported proxies, we excluded them. To make the results more robust, we additionally found proxies for those lipids related SNPs declared as palindromic. Overall, we identified 76 IVs for TC, 272 IVs for HDL-C, 139 IVs for LDL-C, 236 IVs for ApoA1, and 152 IVs for ApoB.

We used the summary statistics of ALS GWAS with the largest sample size (cases = 12,577; controls = 23,475) applying a linear mixed model, as this is considered to be more eligible in the ALS GWAS including unignorable imperfectly balanced strata than fixed-effects meta-analysis [25]. Patients were diagnosed with possible, probable, or definite ALS according to the 1994 El Escorial criteria or the revised El Escorial criteria. Seven independently associated SNPs ($p < 5 \times 10^{-8}$ at $r^2 < 0.001$) were selected to be IVs for ALS. To verify the relation between lipids (TC, HDL-C, LDL-C, ApoA1, and ApoB) and ALS, we repeated the analyses with another ALS meta-GWAS based on European ancestry (cases = 27,205; controls = 110,881) [26]. We excluded those lipid-IVs without corresponding information in the ALS data and identified 12 independent SNPs associated with ALS ($p < 5 \times 10^{-8}$ at $r^2 < 0.001$) as ALS-IVs for replicated analysis.

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According to previous recommendations [27, 28], different types of LDL-C-modifying lifestyles (nutrition and physical activity) were included. Dietary lifestyles included the intake of non-oily fish or oily fish, cereals, cooked vegetables or salad/raw vegetables (raw vegetables), fresh fruit, coffee, and tea. The exercise lifestyles were defined according to a UKBB questionnaire, based on the types of physical activity done in the past 4 weeks, including strenuous sports (sports that make one sweat or breathe hard), heavy do it yourself (DIY) (weeding, lawn mowing, carpentry, digging), light DIY (pruning, watering the lawn), walking for pleasure (not as a mean of transport), other exercises (swimming, cycling, keep fit, bowling), and none of the above. We retrieved from the IEU OpenGWAS project (https://gwas.mrcieu.ac.uk) genetic variants independently associated with corresponding lifestyles [29]. Furthermore, independently genome-wide level significant SNPs ($p < 5 \times 10^{-8}$ at $r^2 < 0.001$) were obtained for linoleic acid (LA), arachidonic acid (AA), α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), and docosahexaenoic acid (DHA) from their corresponding GWASs [30–32]. Among these, ALA, EPA, and DHA were excluded due to their insufficient numbers of IVs.

The phenotypic variance explanation (R^2, $R^2 = 2 \times \text{beta}^2 \times [1-\text{EAF}] \times \text{EAF}/\text{SD}^2$, EAF = effect allele frequency, beta = effect of each SNP on exposures) [33] and *F*-statistics ($F = R^2 \times [N-2]/[1-R^2]$) [34] of each IV were calculated to assess their strength. The summary information of IVs for lipids and PUFAs are in Table S1, and the summary statistics of instruments for LDL-C-modifying lifestyles are in Table S2.

Effect estimation with two-sample MR

We performed the primary analyses with the inverse variance-weighted (IVW) method (multiplicative random effects model). To validate the robustness of our results, the weighted median method, simple median method, MR Egger method, and MR PRESSO method were utilized as sensitivity analyses. The weighted median method yields consistent results when 50% of IVs are valid [35]. The MR PRESSO method identifies outliers with regard to IVs and assesses the effect without outliers [36].

To test for potential pleiotropy of IVs, we employed the following approaches: (1) MR Egger intercepts, to recognize the presence of pleiotropy when the intercept significantly deviates from the origin; (2) Cochran's Q test, assessing the heterogeneity of SNPs enrolled in the IVW estimates; and (3) leave-one-out analysis, to evaluate whether a single SNP drove the association by removing each IV in turn and recalculating the IVW estimates. Bonferroni correction was applied to account for multiple testing. The significance level was set at $p < 0.05/k$, where k is the number of tests using the same dataset (in the first part exploring the association between lipids and ALS, $k = 5$; in the second part exploring the association between lifestyles and ALS, $k = 14$; in the third part exploring the association between PUFAs and LDL-C or ALS, $k = 3$). A *p*-value between $0.05/k$ and 0.05 we considered as a nominal significant level. All analyses were carried out with R software (version 4.2.1), TwoSampleMR package (version 0.5.6) [37], and MR-PRESSO package (version 1.0) [36].

Mediation analysis to explore the mediation effect of LDL-C between PUFAs and risk of ALS

We analyzed the mediation effect of LDL-C on the association between PUFAs and risk of ALS via the conservative network MR method [38]. The multivariable MR method was adopted to analyze the effect of exposure on ALS conditional on other exposures in the same model. The indirect effect of PUFAs on ALS potentially mediated by LDL-C was estimated by multiplying the results from MR IVW analysis and multivariable MR analysis. The IVW approach was employed to determine the total effect of each PUFA on ALS. Thus, to explain the effect of PUFAs on risk of ALS, we divided the indirect effect by the total effect, as previously reported [39]. Since only the trait with more than two IVs can be analyzed by the multivariable MR method, EPA and AA were excluded in this mediation analysis due to their insufficient IVs.

RESULTS

Effect of lipids on risk of ALS

A genetically predicted higher level of LDL-C was associated with a 2.8% increased risk of ALS (IVW, odds ratio (OR) = 1.028, 95% confidence interval (CI) 1.008–1.049, $p = 0.006$) per one standard deviation (1-SD) unit increase of LDL-C concentration, yielded by sensitivity MR estimates (weighted median method, OR = 1.040, 95% CI 1.007–1.073, $p = 0.016$; MR Egger method, OR = 1.033, 95% CI 1.003–1.063, $p = 0.035$). The MR PRESSO method showed no outliers in IVs. Cochran's *Q* test suggested heterogeneity of IVs ($p = 0.011$), indicating the potential presence of pleiotropy (Figure 1, Table S3, Figure S2). However, the MR Egger intercept (intercept = −0.002, $p = 0.360$) showed no evidence of directional pleiotropy, suggesting that pleiotropy is unlikely to affect the main estimates. We found that no specific SNP had a decisive role regarding the association between LDL-C and ALS by applying the leave-one-out analysis. The replicated analysis confirmed the pronounced causal relation between LDL-C and ALS (IVW, OR = 1.087, 95% CI 1.010–1.170, $p = 0.025$) (Table S4).

We found evidence of a genetically predicted increase of ApoB concentration as a suggestive risk factor for ALS (IVW, OR = 1.024, 95% CI 1.003–1.046, $p = 0.027$), which is consistent with the effect of elevated LDL-C on ALS. Sensitivity analyses showed a similar trend with wider CIs and less statistical power. The validity of IVs was confirmed as neither pleiotropy (MR Egger intercept = 0.0003, $p = 0.876$) nor heterogeneity (Cochran's $Q = 164.195$, $p = 0.219$). The validated effect of ApoB on ALS through the replicated ALS GWAS yielded a positive association between genetically predicted

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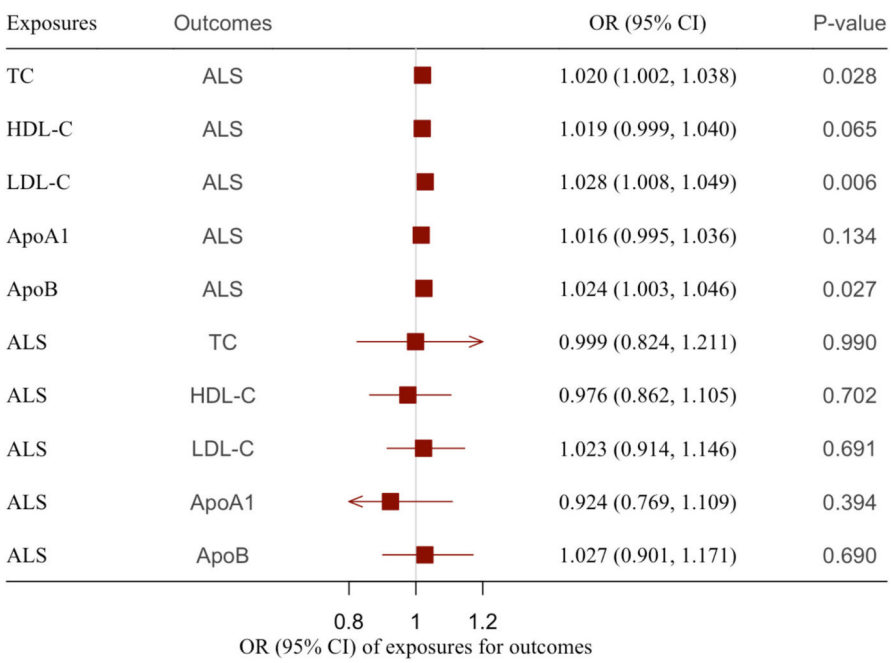

FIGURE 1 Associations of genetically predicted lipid levels and risk of amyotrophic lateral sclerosis using inverse variance-weighted Mendelian randomization analyses. 95% confidence interval beyond the range is indicated by an arrow. ALS, amyotrophic lateral sclerosis; Apo, apolipoprotein; CI, confidence interval; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; OR, odds ratio; TC, total cholesterol.

elevated ApoB and the risk of ALS (OR = 1.108, 95% CI 1.024–1.199, $p = 0.011$) (Table S4).

Genetically increased TC likewise showed a nominally significant association with the risk of ALS. The effect size of elevated TC on ALS was between LDL-C and HDL-C with an IVW OR of 1.020 (95% CI 1.002–1.038, $p = 0.028$). Cochran's Q statistic ($p = 0.019$) suggested IVs' substantial pleiotropy. The replicated analysis verified the suggestive effect of increased TC on the risk of ALS (IVW, OR = 1.088, 95% CI 1.018–1.163, $p = 0.013$).

Regarding HDL-C and ApoA1, we found insufficient evidence for a causal relation with ALS risk. The IVW method showed a 1.9% increased risk of ALS per 1 SD unit increase of HDL-C concentration (95% CI 0.999–1.040, $p = 0.065$) and a 1.6% increased risk of ALS per 1 SD unit increase of ApoA1 concentration (95% CI 0.995–1.036, $p = 0.134$). Other MR approaches yielded similar estimates (Table S3). MR Egger intercept observed apparent horizontal pleiotropy interference for HDL-C (intercept = 0.004, $p = 0.042$). Cochran's Q test revealed evident heterogeneity for both HDL-C ($p = 0.0001$) and ApoA1 ($p = 0.015$). We saw similar associations between HDL-C or ApoA1 and the risk of ALS in the replicated ALS GWAS data (Table S4).

Taken together, our results indicate that genetic factors responsible for elevated lipids (LDL-C, TC and ApoB) increase the risk of ALS, whereby LDL-C seems to play a leading role.

Effect of ALS on lipid levels

In order to examine the effect of ALS on elevated lipids, we performed the MR analyses with ALS as exposure and lipids as outcome variables. Our analysis revealed no evidence for a causal effect of ALS on lipid levels (Table S5, Figure 1, Figure S3). The IVW ORs (95% CI, p-value) were estimated to be 0.999 (0.824–1.211, $p = 0.990$) of ALS on TC, 1.023 (0.914–1.146, $p = 0.691$) on LDL-C, 1.027 (0.901–1.171, $p = 0.690$) on ApoB, 0.976 (0.862–1.105, $p = 0.702$) on HDL-C, and 0.924 (0.769–1.109, $p = 0.394$) on ApoA1. Neither obvious heterogeneity (Cochran's $Q = 4.720$, $p = 0.317$) nor directional pleiotropy (MR Egger intercept = −0.005, $p = 0.717$) were seen regarding the IVs employed in the analysis between ALS and TC. However, potentially these results were influenced by heterogeneity on the effects of ALS on LDL-C, HDL-C, ApoB, and ApoA1. After removing the outliers separately for each estimation, we still did not observe any significant results via the MR PRESSO outlier test. Likewise, no causal effect of ALS on elevated lipids was found with the replicated ALS GWAS data (Table S6).

Effect of LDL-C-modifying lifestyles on risk of ALS

We found that lifestyle (dietary interventions and physical activity) did not affect the risk of ALS. Detailed results are shown in Figure 2 and Table S7. These results were supported by sensitivity analyses. After removing outliers in IVs no significant change was observed.

LDL-C as a mediator between PUFAs and ALS

We found significant associations between genetically increased PUFAs and the risk of elevated LDL-C (Table S8). With the IVW approach, the causal effects (beta value) of elevated LA and AA on LDL-C were 0.508 ($p = 0.004$) and 0.019 ($p = 7.37 \times 10^{-19}$), respectively. Evaluating the causal relationship between PUFAs and ALS, genetically predicted elevated LA was associated with an increased risk of ALS (beta = 0.033, $p = 2.42 \times 10^{-6}$) (Table S9). Therefore, based on the principle of classical mediation inference, we deduced that

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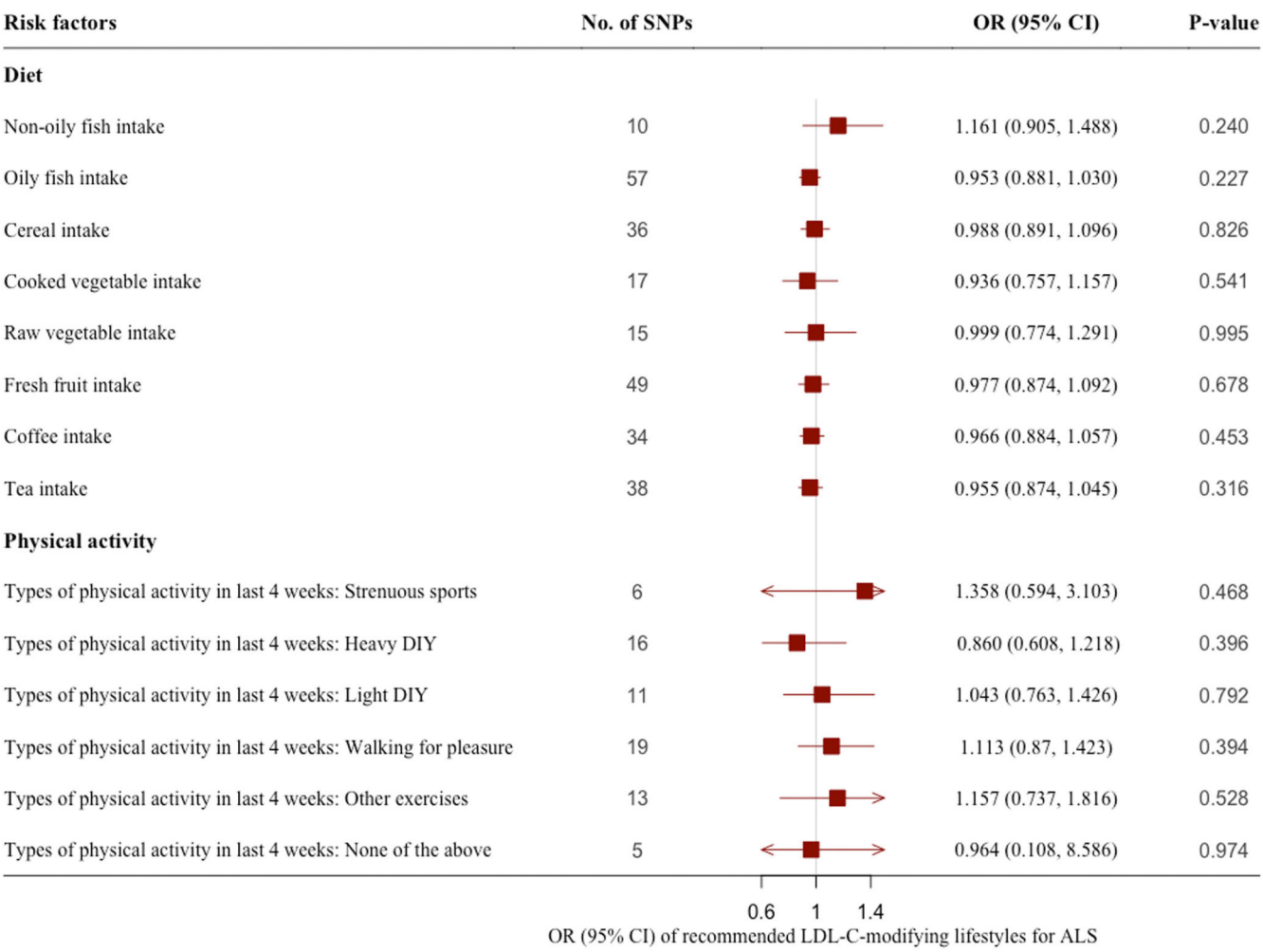

FIGURE 2 Associations of genetically predicted low-density lipoprotein cholesterol-modifying lifestyles and the risk of amyotrophic lateral sclerosis using inverse variance-weighted Mendelian randomization analyses. 95% confidence interval beyond the range is indicated by an arrow. CI, confidence interval; DIY, do it yourself; OR, odds ratio; SNP, single nucleotide polymorphism; TC, total cholesterol.

LDL-C might be a potential mediator on the pathway between LA and the risk of ALS. In multivariable MR analysis, the effect of an increased LDL-C level on the risk of ALS was 0.017. Thus, the indirect effect of elevated LA on ALS risk mediated by LDL-C was 0.009, explaining 27.3% of the total effect.

DISCUSSION

Building on the reported association between dyslipidemia and risk of ALS, we explored whether this association was genetically determined by using a bidirectional, two-sample, MR framework. Our study identified increased LDL-C levels as a genetic risk factor for ALS via various MR approaches. Consistently, elevated levels of ApoB and TC also have risk effects on ALS.

Previous MR studies have reported similar results. Zeng et al. examined the effects of TC, HDL-C, and LDL-C on the risk of ALS and found that only LDL-C and ALS were causally associated (OR = 1.14, 95% CI 1.05–1.24, $p = 1.38E{-}3$) [11]. Chen et al. reported the risk effects of TC and LDL-C on ALS using SNPs linked to both LDL-C and TC [12]. Bandres-Ciga et al. also found an independent risk role of high levels of LDL-C on ALS (OR = 1.116, 95% CI 1.03–1.20, $p = 0.003$) [40]. Compared to these studies, our study enrolled lipid-related GWASs based on significantly higher sample sizes (400,000 compared to 190,000 and 100,000, respectively), providing a higher number of IVs with improved statistical power. Besides, we have obtained high-quality European-based ALS GWAS summary data for discovery analyses and the most recent ALS GWAS statistics with the largest sample sizes for replication analyses.

Notably, for the first time, we explored the effects of apolipoproteins on the risk of ALS and revealed there was no genetically predicted association between lipid-modifying lifestyles and ALS. A Swedish study suggested that changes in lipid and apolipoprotein metabolism were associated with ALS risk and may become prodromal symptoms decades before ALS diagnosis [6]. According to our data, ALS seems not to affect lipid levels, supporting the previous polygenic risk score-based finding that ALS risk alleles were not associated with TC, HDL-C, and LDL-C [12] and providing evidence that lipid levels were indeed genetic risk factors for ALS as reported previously [11].

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Based on our data, we see LDL-C as the most potent risk factor for ALS, supporting previous evidence [11, 40]. For that reason, we focused on investigating the effects of LDL-C-modifying lifestyles such as dietary and physical activity. A previous Korean study appeared to demonstrate a protective effect of fruit intake on the risk of ALS and the risk of fish intake on ALS supported by 77 participants [41]. However, in our study population, no significant modifying effects of genetically defined lifestyles on the risk of ALS were found, supporting the null association between physical activity and ALS found in previous MR studies [14, 42]. Due to the genetic variants reflecting a lifelong exposure to a biomarker on the outcome, the effect estimated by the MR approach may be different from the effect of the short-term intervention on ALS in the real world.

Serum LDL-C is a downstream product of the metabolism of PUFAs, and an association between PUFAs and ALS has been suggested previously [43]. Therefore, we further studied the possible mediation effect of LDL-C on the pathway from PUFAs to ALS. LA is an essential omega-6 long-chain PUFA and the initial substrate of the biosynthesis of fatty acids [44]. Our findings were consistent with previous studies showing a protective effect of omega-3 PUFAs on ALS [19] and the reverse correlation between the concentrations of omega-6 PUFAs and omega-3 PUFAs [45]. With the conservative network MR method, we were able to show for the first time that 27.3% of the risk effect of LA on ALS could be explained by elevated LDL-C.

In parallel, we explored lipid profiles in asymptomatic ALS gene carriers and implied that 'worse' lipid metabolism profiles were associated with relatively benign ALS mutations [46]. Although both are preclinical studies, the MR study investigated genetic, long-term factors for the risk of disease, while the prospective cohort study focused more on the relation between lipid levels in a relatively short time before disease onset and ALS prognosis. Therefore, both studies describe different scenarios with the latter coming to similar conclusions as previous studies [6, 47].

The following limitations should be mentioned: The IVs for LDL-C-modifying lifestyles and PUFAs are limited, which may weaken the statistical power and robustness of our results. We adopted various MR approaches to test for potential pleiotropy in IVs to overcome this deficiency, including the MR Egger method, weighted median method, MR PRESSO method, and Cochran's *Q* test. Moreover, in order to provide high statistical power and broadly acceptable conclusions, ALS GWASs employed in most ALS-related MR studies involved both sporadic ALS (sALS) and familial ALS (fALS) patients. Although sALS and fALS share similar clinical and neuropathological changes and risk factors, a recent study proposed that the effects of a lifetime accumulation of risk factors on neurodegenerative disorders largely depends on genotypes [48].

CONCLUSIONS

We provided a high level of genetic evidence verifying the positive association between preclinically elevated lipid levels and the risk of developing ALS that had been described in previous genetic and observational studies. Furthermore, we explored the effects of apolipoproteins and lipid-modifying lifestyles on the risk of ALS via an MR-based approach. We also demonstrated a potential mediating role of LDL-C in the pathway from PUFAs to ALS. Therefore, in our opinion, our findings contribute to the understanding of pathogenesis and yield novel potential therapeutic targets in ALS.

AUTHOR CONTRIBUTIONS

Kailin Xia analyzed the data and wrote the manuscript. Veronika Klose validated the data. Veronika Klose and Johannes Dorst revised the draft and data analysis. Josef Högel, Tao Huang, and Linjing Zhang supervised the data analysis. Albert C. Ludolph and Dongsheng Fan designed the study and supervised the work. Albert C. Ludolph and Dongsheng Fan contributed equally to this work.

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CONFLICT OF INTEREST STATEMENT

The authors do not have any conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article and its supplementary information files.

ETHICAL APPROVAL

There were no patients directly involved in the overall process of our study. Our study is based on publicly available data only. All human studies included in this analysis were conducted according to the Declaration of Helsinki.

CONSENT FOR PUBLICATION

All authors agreed to the publication of this article.

CODE AVAILABILITY

Codes generated or used during the study are available from the corresponding author by request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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3.4 Publikation IV: Mutation-specific metabolic profiles in presymptomatic amyotrophic lateral sclerosis

Xia K., Witzel S., Witzel C., **Klose V.**, Fan D., Ludolph A. C., & Dorst J. (2023). Mutation-specific metabolic profiles in presymptomatic amyotrophic lateral sclerosis. European Journal of Neurology, 30(1), 87-95.(Kailin Xia, Witzel, et al., 2023)

Fragestellung

Da der Energiestoffwechsel bei ALS Patienten, wie schon in der vorherigen Publikation geschildert, beeinträchtigt zu sein scheint, haben wir dies in dieser vierten und letzten Publikation, die in diese Dissertation miteingebunden ist, anhand von ALS präklinischen Genträgern untersuchen wollen. Außerdem sollte auch geklärt werden, ob sich auch die metabolischen Profile der Genträger je nach Mutation unterscheiden.

Methodik

60 präsymptomatische ALS-Mutationsträger (28 C9ORF72 Mutationen, 21 SOD1 Mutationen) und 70 alters- und geschlechtsgleiche Kontrollen (Nicht-Mutationsträger aus denselben Familien z.B. Geschwister) konnten rekrutiert werden. Nüchtern wurde das metabolische Profil, der BMI, Blutdruck, Glukose, Gesamtcholesterin, HDL-C und LDL-C im Serum bestimmt. Die Studie wurde in Übereinstimmung mit der Deklaration von Helsinki durchgeführt und von der Ethikkommission der Universität Ulm genehmigt (Genehmigungsnummer 68/19).

Die statistischen Analysen beinhalteten in der deskriptiven Statistik die Bestimmung des Medians und des Interquartilsabstandes (IQR), für Gruppenvergleiche wurde bei quantitativen Variablen ein t-Test oder Wilcoxon Rang Summentest verwendet, bei qualitativen Variablen der Pearson's χ^2-Test oder der exakte Test nach Fisher. Um eine Alterskorrektur anzuwenden wurde eine lineares Regressionsmodell angewendet. Alle Analysen wurden mit SPSS (Version 26) durchgeführt.

Ergebnisse

Bei Betrachtung aller Probanden konnte kein signifikanter Unterschied zwischen Kontrollen und den ALS-Genträgern festgestellt werden. Bei Unterscheidung nach Mutation konnten wir feststellen, dass Genträger mit der C9ORF72 Mutation ein besseres kardiovaskuläres Profil als Kontrollen hatten. Dieses impliziert einen durchschnittlich niedrigeren BMI, systolischen Blutdruck, Glukosewert im Serum und erhöhtes HDL-C. Genträger mit der Mutation SOD1 hatten hingegen ein schlechteres kardiovaskuläres Profil als Kontrollen (erhöhter BMI und Glukosewert im Serum verbunden mit niedrigerem HDL-C). Beim diastolischen Blutdruck, Triglyceriden, Gesamtcholesterin und LDL-C konnten keine signifikanten Unterschiede gefunden werden.

Diskussion

Der Energiestoffwechsel bei präsymptomatischen ALS Genträgern unterschied sich in seiner Gesamtheit in unserer Studie nicht signifikant von Kontrollen, jedoch wiesen die Genträger eindeutige mutationsspezifische Unterschiede hinsichtlich des kardiovaskulären Risikoprofils auf. Hier konnten wir feststellen, dass C9ORF72 Genträger ein niedrigeres

kardiovaskuläres Risikoprofil haben als Kontrollen, wohingegen SOD1 Genträger ein höheres kardiovaskuläres Risikoprofil aufweisen.
Diese Erkenntnisse ähneln denen früherer SOD1 Mausmodelle, da SOD1 Genträger Mäuse im Vergleich zu Kontrollen niedrigere Cholesterinwerte (HDL-C inklusive) (Fergani et al., 2007) und durch langsameren Glukoseabbau einen höheren Glukosespiegel aufwiesen (Palamiuc et al., 2015). Dies kann am gestörten Stoffwechsel liegen, welchen Mäuse mit SOD1 Mutation bereits in der präklinischen Phase, durch u.a. die geringere Ansammlung von Fettgewebe und Hypermetabolismus, zeigen (L. Dupuis et al., 2004).
Ein hohes kardiovaskuläres Risikoprofil scheint während der klinischen ALS als protektiver Faktor zu agieren, wobei hauptsächlich ein höherer BMI entscheidend für längeres Überleben ist und dies bei hohen Lipiden, Blutzucker und Blutdruck nur teilweise beobachtet wurde (D'Amico et al., 2021; Ingre et al., 2020; Kirk et al., 2019; Liu et al., 2020).
Mutationsspezifisch ist beim Menschen bekannt, dass Patienten bei denen das C9ORF72-Gen betroffen ist einen späteren Krankheitsbeginn, aber einen rapiden Verlauf haben (Chiò et al., 2012). Bei SOD1 Patienten ist der Verlauf von der jeweiligen Mutation abhängig, A4V-Mutationen schreiten schneller fort, D91A und L118V hingegen langsamer (Synofzik et al., 2012).
Bei SOD1-Genträgern mit den zuletzt genannten eher benignen Mutationen wurde ein höherer nüchtern Glukosewert im Serum und ein niedrigerer HDL-C Wert festgestellt als bei anderen Mutationen des SOD1 Gens, was mit dem bisherigen Forschungsstand einhergeht. Dass jedoch auch ein niedrigerer Gesamtcholesterinspiegel und niedrigeres LDL-C bei diesen benignen Mutationen beobachtet wurde passt nicht ins bisherige Bild, dass erhöhtes Cholesterin den Krankheitsverlauf protektiv beeinflusst. Dies könnte an den mutationsspezifischen Stoffwechselprofilen liegen, sollte aber anhand einer größeren Stichprobe, als uns in dieser Studie zugänglich war noch weiter untersucht werden.

Originalpublikation

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Darstellung des Eigenanteils:
Die Idee und Konzetion dieser Publikation fand mithilfe von Prof. Albert Ludolph und Prof. Johannes Dorst statt. Ich habe die Ergebnisse von Kailin Xias Datenanalyse in R (Version 4.0.3) reproduziert und validiert. Gemeinsam mit den Koautoren haben wir die Ergebisse interpretiert. Das Manuskript wurde gemeinsam von Kailin Xia, Prof. Johannes Dorst und mir angefertigt. Desweiteren haben Kailin Xia und ich Kommentare von Koautoren und Reviewern zusammen in die Publikation eingearbeitet.

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ORIGINAL ARTICLE

Mutation-specific metabolic profiles in presymptomatic amyotrophic lateral sclerosis

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Abstract

Background and purpose: Growing evidence shows that ALS patients feature a disturbed energy metabolism. However, these features have rarely been investigated in the presymptomatic stage.

Methods: A total of 60 presymptomatic ALS mutation carriers and 70 age- and gender-matched controls (non-mutation carriers from the same families) were recruited. All subjects underwent assessments of their metabolic profiles under fasting conditions at enrollment, including body mass index (BMI), blood pressure and serum levels of blood glucose, total cholesterol, triglycerides, high-density lipoprotein (HDL) and low-density lipoprotein.

Results: All mutations combined, no differences between presymptomatic ALS gene carriers and controls were found. From a cardiovascular point of view, presymptomatic chromosome 9 open reading frame 72 (*C9ORF72*) gene carriers showed lower cardiovascular risk profiles compared to healthy controls, including lower BMI (median 22.9, interquartile range [IQR] 20.6–26.1 kg/m^2 vs. 24.9, IQR 22.7–30.5 kg/m^2; $p = 0.007$), lower systolic blood pressure (120, IQR 110–130 mmHg vs. 128, IQR 120–140 mmHg; $p = 0.02$), lower fasting serum glucose (89.0, IQR 85.0–97.0 mg/dl vs. 96.0, IQR 89.3–102.0 mg/dl; $p = 0.005$) and higher HDL (1.6, IQR 1.3–1.8 mmol/l vs. 1.2, IQR 1.0–1.4 mmol/l; $p = 0.04$). However, presymptomatic superoxide dismutase 1 (*SOD1*) gene mutation carriers showed higher cardiovascular risk profiles compared to healthy controls, including higher BMI (28.0, IQR 26.1–31.5 kg/m^2 vs. 24.9, IQR 22.7–30.5 kg/m^2; $p = 0.02$), higher fasting serum glucose (100.0, IQR 94.0–117.0 mg/dl vs. 96.0, IQR 89.3–102.0 mg/dl; $p = 0.04$) and lower HDL (1.2, IQR 1.0–1.4 mmol/l vs. 1.4, IQR 1.2–1.7 mmol/l; $p = 0.01$). These features were most prominent in patients carrying *SOD1* gene mutations associated with slow disease progression.

Conclusions: This study identified distinct metabolic profiles in presymptomatic ALS gene carriers, which might be associated with disease progression in the symptomatic phase.

KEYWORDS
amyotrophic lateral sclerosis, genetics, metabolism, presymptomatic gene carriers

Dongsheng Fan, Albert C. Ludolph and Johannes Dorst have contributed equally and they are co-corresponding authors.

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INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by a progressive loss of motor neurons leading to muscle atrophy and paresis. The involvement of respiratory muscles causes respiratory insufficiency and death after a mean survival time of only 3–5 years [1]. Despite decades of intensive research, there is still a lack of promising therapeutic strategies. The pathomechanisms behind the disease are still largely unknown. Most ALS patients have a sporadic disease, while approximately 10% of ALS patients have a positive family history (familial ALS, fALS) [2]. A repeat expansion in the chromosome 9 open reading frame 72 (*C9ORF72*) gene and various mutations in the superoxide dismutase 1 (*SOD1*) gene were identified as the most common causes for European fALS, accounting for ~60% of cases [3].

Amyotrophic lateral sclerosis patients feature distinct disturbances of energy [4], glucose [5, 6] and lipid metabolism [7, 8]. They display an increased resting energy expenditure [9, 10], catabolism and weight loss, which usually occurs early during the course of disease [11, 12], even before disease onset [13], and negatively affects prognosis [14, 15]. In the context of these metabolic changes, a mitochondrial [16, 17] and/or hypothalamic dysfunction [18, 19] have been discussed. These findings have prompted clinical trials with high-caloric and/or high-fat nutritional interventions which indicated that such interventions might beneficially influence the course of disease [20–22].

Furthermore, various studies have investigated the association between distinct metabolic profiles and the risk of developing ALS, generally indicating that ALS was associated with lower cardiovascular risk profiles [23–25]. However, these studies refer to the clinical stage of ALS, whilst the metabolic profiles of the presymptomatic stage are largely unknown. Focusing on the presymptomatic stage of ALS may provide a better understanding of the causative interactions between the observed phenomena and potentially highlight novel presymptomatic biomarkers and therapeutic targets.

Therefore, a study was performed in 60 presymptomatic ALS mutation carriers including *C9ORF72* gene, *SOD1* gene, *FUS*, *KIF5A*, *NEK1*, *SETX*, *TBK1* and *TDP43* mutations, and 70 age- and gender-matched controls (non-mutation carriers from the same families) to compare their metabolic profiles, aiming (1) to identify whether ALS patients displayed changes with regard to metabolic profiles in the presymptomatic stage and (2) to investigate whether specific ALS-causing gene mutations were associated with distinct metabolic profiles.

METHODS

Study population

A total of 130 subjects were monocentrically recruited in the Department of Neurology, University of Ulm, Germany, between 2012 and 2021. All subjects provided written informed consent. The Ethics Committee of Ulm University approved the study (approval number 20/12). Presymptomatic ALS mutation carriers ($n = 60$) were recruited from families with a positive history of ALS and a known ALS-causing mutation by genetic testing. All controls ($n = 70$) were first- or second-degree relatives (i.e., parents, children or siblings) of patients with ALS who did not carry pathological mutations.

Assessments of metabolic profiles

Amyotrophic lateral sclerosis mutation carriers and controls underwent the same standardized assessments for metabolic factors at enrollment. Body mass index (BMI) was calculated from weight and height according to the formula BMI = weight in kg/(height in m)^2. Systolic blood pressure (SBP; mmHg) and diastolic blood pressure (DBP; mmHg) were determined manually according to Riva-Rocci. Fasting serum samples were taken for measurement of glucose (mg/dl) and lipids (mmol/l), including total cholesterol (TC), triglycerides (TG), high-density lipoprotein (HDL) and low-density lipoprotein (LDL).

Statistical analysis

For descriptive statistics, median and interquartile range (IQR) were used. Group comparisons for continuous variables were performed using the two-sample *t* test or the Wilcoxon rank-sum test as appropriate. Two-sided 95% confidence intervals were calculated for mean or median group differences. Group comparisons for categorical variables were performed using a chi-squared test or Fisher's exact test as appropriate. Two-sided 95% confidence intervals were calculated for group differences of proportions. For analyses of *SOD1* gene mutations, normally distributed variables were adjusted for age via a linear regression model. A p value <0.05 (two-sided) was considered as statistically significant. The sample size was determined based on the number of presymptomatic gene carriers and respective controls collected in a monocentric setting within the given time frame. All analyses were performed using the statistical software packages SPSS version 26 (SPSS, Chicago, IL, USA) and GraphPad Prism version 9.1.1 (GraphPad, San Diego, CA, USA).

RESULTS

Between 2012 and 2021, 60 presymptomatic ALS gene mutation carriers and 70 controls (i.e., subjects from the same families as the ALS gene carriers without a disease-causing mutation) were included. Amongst the 60 mutation carriers, 28 were *C9ORF72* gene repeat expansion carriers, 21 were *SOD1* gene mutation carriers and the rest were carriers of various other mutations (Figure 1a). Demographic and clinical characteristics of each group are displayed in Table 1.

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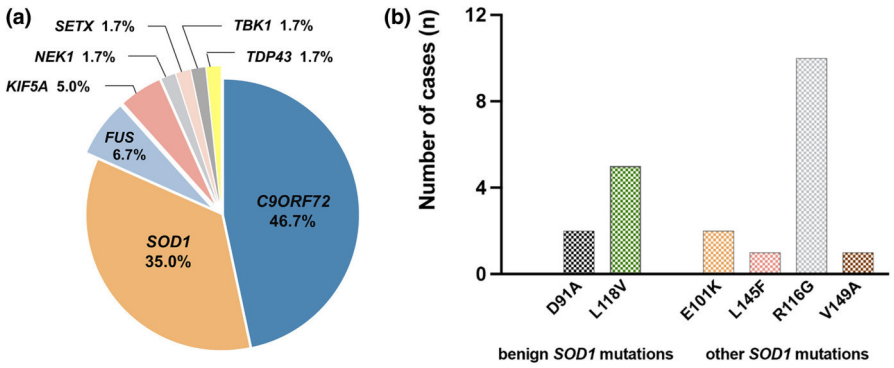

FIGURE 1 Distribution of mutations. (a) Distribution of mutations in the group of ALS gene carriers ($n = 60$). (b) Mutational spectrum of the *SOD1* gene subgroup ($n = 21$). Columns represent absolute numbers. *C9ORF72* gene, chromosome 9 open reading frame 72; *FUS*, fused in sarcoma; *NEK1*, NIMA related kinase 1; *KIF5A*, kinesin family member 5A; *SETX*, senataxin; *SOD1* gene, superoxide dismutase 1; *TBK1*, serine/threonine protein kinase; *TDP43*, transactive response DNA binding protein 43.

TABLE 1 Demographic and clinical characteristics of presymptomatic ALS gene carriers and controls from the same families without respective mutations

	ALS gene carriers (total) ($n = 60$)	*C9ORF72* gene carriers ($n = 28$)	*SOD1* gene carriers ($n = 21$)	Controls ($n = 70$)
Age[a] (years)	45 (32–54)	44 (32–51)	47 (36–65)	43 (30–52)
	$p = 0.44$	$p = 0.95$	$p = 0.10$	
Sex (male, %)	25 (42%)	7 (25%)	14 (67%)	32 (46%)
	$p = 0.64$	$p = 0.06$	$p = 0.09$	
BMI (kg/m^2)	26.1 (22.5–29.0)	22.9 (20.6–26.1)	28.0 (26.1–31.5)	24.9 (22.7–30.5)
	$p = 0.91$	$p = 0.007$	$p = 0.02$	
Fasting serum glucose (mg/dl)	94.0 (87.0–102.0)	89.0 (85.0–97.0)	100.0 (94.0–117.0)	96.0 (89.3–102.0)
	$p = 0.44$	$p = 0.005$	$p = 0.04$	
SBP (mmHg)	120 (112–135)	120 (110–130)	130 (120–140)	128 (120–140)
	$p = 0.28$	$p = 0.02$	$p = 0.73$	
DBP (mmHg)	80 (75–85)	80 (70–80)	80 (75–89)	80 (70–90)
	$p = 0.94$	$p = 0.18$	$p = 0.79$	
TG (mmol/l)	1.1 (0.7–1.7)	1.0 (0.6–1.3)	1.6 (0.9–1.9)	1.0 (0.8–1.5)
	$p = 0.64$	$p = 0.42$	$p = 0.16$	
TC[a] (mmol/l)	5.0 (4.3–6.5)	5.2 (4.6–6.9)	5.0 (3.8–6.3)	5.1 (4.5–5.8)
	$p = 0.79$	$p = 0.44$	$p = 0.61$	
HDL[a] (mmol/l)	1.3 (1.1–1.7)	1.6 (1.3–1.8)	1.2 (1.0–1.4)	1.4 (1.2–1.7)
	$p = 0.42$	$p = 0.04$	$p = 0.01$	
LDL[a] (mmol/l)	3.2 (2.5–4.6)	3.5 (2.8–4.6)	3.2 (2.0–4.6)	3.3 (2.8–4.1)
	$p = 0.89$	$p = 0.50$	$p = 0.56$	

Note: Data are *n* (%) or median (IQR). *p*-values refer to comparisons between respective gene carriers and controls.

Abbreviations: ALS, amyotrophic lateral sclerosis; BMI, body mass index; *C9ORF72* gene, chromosome 9 open reading frame 72; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; *SOD1* gene, superoxide dismutase 1; TC, total cholesterol; TG, triglycerides.

[a]Normally distributed variables.

Age and sex were equally distributed between presymptomatic ALS gene carriers and controls. Considering all mutations combined, there were no differences between mutation carriers and controls for all investigated metabolic factors, including BMI, fasting serum glucose, SBP, DBP, TG, TC, HDL and LDL (Table 1). However, distinct profiles were present for the subgroups of presymptomatic *C9ORF72* gene and *SOD1* gene mutation carriers.

Compared with controls, *C9ORF72* gene mutation carriers had a lower BMI (22.9, IQR 20.6–26.1 kg/m^2 vs. 24.9, IQR 22.7–30.5 kg/m^2; $p = 0.007$), lower fasting serum glucose (89.0, IQR 85.0–97.0 mg/

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dl vs. 96.0, IQR 89.3–102.0 mg/dl; $p = 0.005$), lower SBP (120, IQR 110–130 mmHg vs. 128, IQR 120–140 mmHg; $p = 0.02$) and higher HDL (1.6, IQR 1.3–1.8 mmol/l vs. 1.4, IQR 1.2–1.7 mmol/l; $p = 0.04$), corresponding to a lower cardiovascular risk profile (Table 1 and Figure 2a–h). In order to provide higher homogeneity between groups and limit the potential effects of genetic and environmental confounders, also the 28 *C9ORF72* gene-positive presymptomatic gene carriers were compared with 13 *C9ORF72* gene-negative controls from the same families only. This analysis confirmed the same trend for all parameters with significant differences observed when comparing *C9ORF72* gene carriers with all controls; however, due to lower numbers in this subanalysis, only HDL remained statistically significant (1.6, IQR 1.3–1.8 mmol/l vs. 1.2, IQR 1.1–1.3 mmol/l; $p = 0.004$) (Table 2).

On the other hand, compared to controls, *SOD1* gene mutation carriers featured higher BMI (28.0, IQR 26.1–31.5 kg/m^2 vs. 24.9, IQR 22.7–30.5 kg/m^2; $p = 0.02$), higher fasting serum glucose (100.0, IQR 94.0–117.0 mg/dl vs. 96.0, IQR 89.3–102.0 mg/dl; $p = 0.04$) and lower HDL (1.2, IQR 1.0–1.4 mmol/l vs. 1.4, IQR 1.2–1.7 mmol/l; $p = 0.01$) (Table 1, Figure 2a–h). The intrafamily comparison between 21 *SOD1* gene mutation carriers and 11 *SOD1* gene-negative controls from the same families, despite lower numbers, confirmed that carriers had a significantly higher BMI (28.0, IQR 26.1–31.5 kg/m^2 vs. 24.2, IQR 22.1–25.6 kg/m^2; $p = 0.007$) and lower HDL (1.2, IQR 1.0–1.4 mmol/l vs. 1.5, IQR 1.2–1.7 mmol/l; $p = 0.04$); however, in this subanalysis no difference in fasting serum glucose levels was observed (Table 3).

Due to the clinical heterogeneity of different *SOD1* gene mutations and the relatively benign prognosis of the D91A and L118V mutations, carriers of D91A and L118V mutations were also compared with other *SOD1* gene mutation carriers. Out of the 21 *SOD1* gene -positive individuals, seven were carriers of D91A and L118V, consisting of the benign *SOD1* gene mutation group. The complete mutational spectrum of the presymptomatic *SOD1* gene carrier subgroup is given in Figure 1b. It was found that the subgroup with benign *SOD1* gene mutations featured higher fasting serum glucose (111.5, IQR 106.7–116.4 mg/dl vs. 102.1, IQR 95.3–108.9 mg/dl; $p = 0.048$), lower TC (4.2, IQR 3.9–4.5 mmol/l vs. 5.6, IQR 5.3–5.9 mmol/l; $p < 0.001$), lower HDL (1.0, IQR 0.9–1.1 mmol/l vs. 1.3, IQR 1.3–1.4 mmol/l; $p < 0.001$) and lower LDL (2.7, IQR 2.3–3.0 mmol/l vs. 3.8, IQR 3.5–4.0 mmol/l; $p < 0.001$) levels compared to the other *SOD1* gene mutations combined (Table 4, Figure 3a–h).

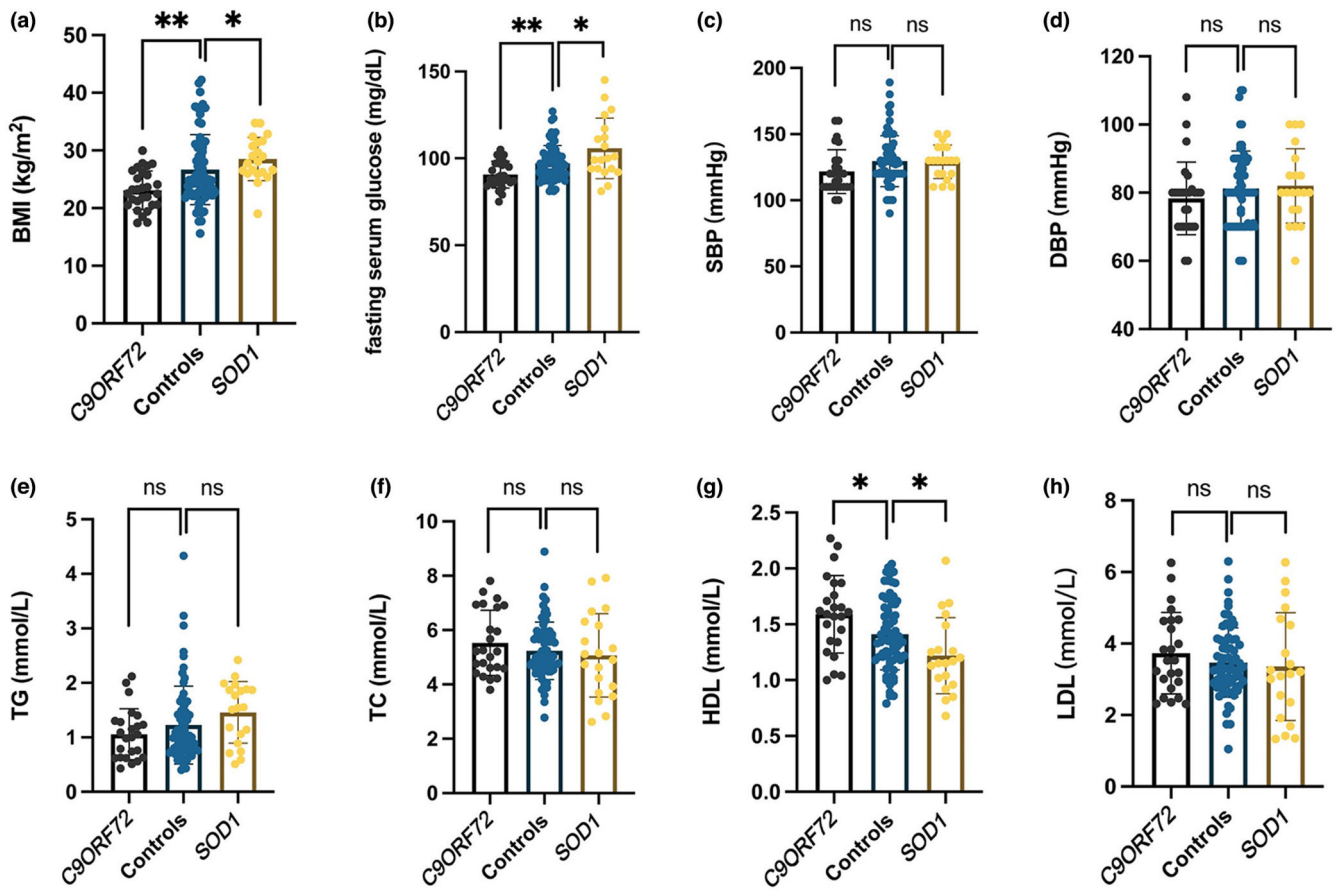

FIGURE 2 Metabolic factors in specific mutations. Plots show median, 95% confidence interval and individual values of all analyzed cardiovascular risk factors for *C9ORF72* gene mutation carriers, *SOD1* gene mutation carriers and controls. Asterisks mark significant (*$p < 0.05$) and highly significant (**$p < 0.01$) differences. ALS, amyotrophic lateral sclerosis; BMI, body mass index; *C9ORF72* gene, chromosome 9 open reading frame 72; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; *SOD1* gene, superoxide dismutase 1; TC, total cholesterol; TG, triglycerides.

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TABLE 2 Demographic and clinical characteristics of presymptomatic *C9ORF72* gene carriers and intrafamily controls without respective mutations

	C9ORF72 gene carriers ($n = 28$)	Controls from the same families ($n = 13$)	*p*-value
Age[a] (years)	44 (32–51)	43 (37–55)	0.72
Sex (male, %)	7 (25%)	7 (54%)	0.09
BMI[a] (kg/m^2)	22.9 (20.6–26.1)	25.6 (22.9–33.4)	0.05
Fasting serum glucose[a] (mg/dl)	89.0 (85.0–97.0)	94.5 (87.3–104.8)	0.32
SBP (mmHg)	120 (110–130)	130 (120–140)	0.29
DBP (mmHg)	80 (70–80)	80 (73–90)	0.47
TG[a] (mmol/l)	1.0 (0.6–1.3)	1.2 (0.9–1.8)	0.09
TC[a] (mmol/l)	5.2 (4.6–6.9)	5.7 (4.7–7.0)	0.42
HDL[a] (mmol/l)	1.6 (1.3–1.8)	1.2 (1.1–1.3)	0.004
LDL[a] (mmol/l)	3.5 (2.8–4.6)	3.9 (3.3–5.1)	0.17

Note: Data are *n* (%) or median (IQR).

Abbreviations: ALS, amyotrophic lateral sclerosis; BMI, body mass index; *C9ORF72* gene, chromosome 9 open reading frame 72; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; TC, total cholesterol; TG, triglycerides.

[a]Normally distributed variables.

TABLE 3 Demographic and clinical characteristics of presymptomatic *SOD1* gene carriers and intrafamily controls without respective mutations

	SOD1 gene carriers ($n = 21$)	Controls from the same families ($n = 11$)	*p*-value
Age[a] (years)	47 (36–65)	36 (28–54)	0.16
Sex (male, %)	14 (67%)	5 (46%)	0.28
BMI[a] (kg/m^2)	28.0 (26.1–31.5)	24.2 (22.1–25.6)	0.007
Fasting serum glucose (mg/dl)	100.0 (94.0–117.0)	99.0 (91.8–103.3)	0.37
SBP (mmHg)	130 (120–140)	130 (120–133)	0.95
DBP[a] (mmHg)	80 (75–89)	80 (80–88)	0.83
TG[a] (mmol/l)	1.6 (0.9–1.9)	1.0 (0.8–1.7)	0.32
TC[a] (mmol/l)	5.0 (3.8–6.3)	5.5 (4.9–5.9)	0.28
HDL[a] (mmol/l)	1.2 (1.0–1.4)	1.5 (1.2–1.7)	0.04
LDL[a] (mmol/l)	3.2 (2.0–4.6)	3.9 (3.3–4.2)	0.27

Note: Data are *n* (%) or median (IQR).

Abbreviations: ALS, amyotrophic lateral sclerosis; BMI, body mass index; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; *SOD1* gene, superoxide dismutase 1; TC, total cholesterol; TG, triglycerides.

[a]Normally distributed variables.

In addition, the age-adjusted metabolic profiles were compared between benign *SOD1* gene mutation carriers and healthy controls and it was found that the group with benign *SOD1* gene mutations featured lower TC (4.2, IQR 3.9–4.5 mmol/l vs. 5.2, IQR 5.0–5.5 mmol/l; $p < 0.001$), lower HDL (1.0, IQR 0.9–1.1 mmol/l vs. 1.4, IQR 1.4–1.4 mmol/l; $p < 0.001$) and lower LDL (2.7, IQR 2.3–3.0 mmol/l vs. 3.5, IQR 3.2–3.7 mmol/l; $p < 0.001$) levels.

DISCUSSION

In this study, metabolic profiles of 60 presymptomatic ALS gene carriers and 70 age- and gender-matched controls without pathological mutations were compared.

Whereas the combined analysis including all ALS mutation carriers did not reveal significant differences from controls, the mutation-specific analysis showed that *C9ORF72* gene carriers have a lower cardiovascular risk profile. In contrast, *SOD1* gene mutation carriers featured a higher cardiovascular risk profile, especially those carrying the more benign D91A and L118V mutations.

The higher cardiovascular risk profiles in *SOD1* gene patients are largely consistent with previous findings from the *SOD1* gene ALS mouse model. Fergani et al. found that presymptomatic ALS *SOD1* gene mice show lower cholesterol levels compared with wild-type mice, including HDL, very low density lipoprotein and LDL [26]. In addition, ALS mice at 65 days showed a delayed initial glucose clearance compared with wild-type mice, displaying

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TABLE 4 Demographic and clinical characteristics of benign *SOD1* gene mutation carriers and other *SOD1* gene mutation carriers

	Benign *SOD1* gene mutation carriers ($n = 7$)	Other *SOD1* gene mutation carriers ($n = 14$)	*p*-value
Age (years)	68 (37–75)	42 (33–54)	0.03
Sex (male, %)	6 (86%)	8 (57%)	0.19
BMI (kg/m^2)	28.8 (27.5–30.0)	28.5 (27.2–29.8)	0.79
Fasting serum glucose (mg/dl)	111.5 (106.7–116.4)	102.1 (95.3–108.9)	0.05
SBP (mmHg)	126.2 (119.8–132.5)	129.8 (126.7–132.9)	0.37
TG (mmol/l)	1.5 (1.3–1.7)	1.4 (1.3–1.6)	0.62
TC (mmol/l)	4.2 (3.9–4.5)	5.6 (5.3–5.9)	**<0.001**
HDL (mmol/l)	1.0 (0.9–1.1)	1.3 (1.3–1.4)	**<0.001**
LDL (mmol/l)	2.7 (2.3–3.0)	3.8 (3.5–4.0)	**<0.001**

Note: Data are age-adjusted and represented as median (IQR) or *n* (%). Bold values indicate statistically significant results.

Abbreviations: BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; *SOD1* gene, superoxide dismutase 1; TC, total cholesterol; TG, triglycerides.

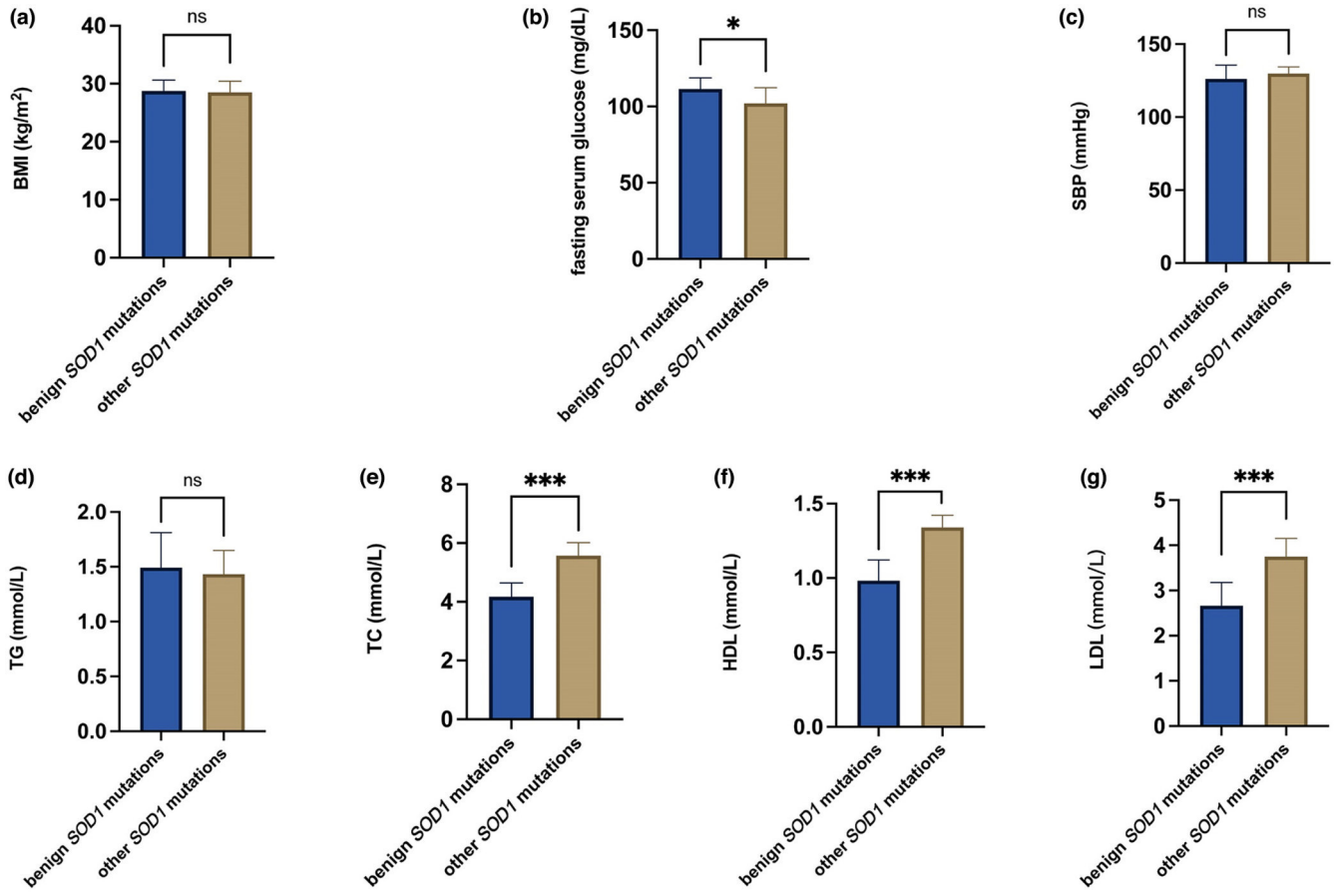

FIGURE 3 Metabolic factors in *SOD1* gene. Boxplots show mean and 95% confidence intervals for metabolic factors in presymptomatic carriers with benign *SOD1* gene mutations and presymptomatic carriers with other *SOD1* gene mutations. Asterisks mark significant (*$p < 0.05$) and highly significant (***$p < 0.001$) differences. BMI, body mass index; HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; *SOD1* gene, superoxide dismutase 1; TC, total cholesterol; TG, triglycerides.

higher glucose levels at 30 and 45 min [27]. These findings suggest that impaired glucose and lipid metabolism of *SOD1* gene mice can be detected even before the onset of symptoms. Furthermore, Dupuis et al. found that *SOD1* gene mice showed distinct metabolic alterations already during the presymptomatic stage, including reduced adipose tissue accumulation, increased energy expenditure and concomitant skeletal muscle hypermetabolism [28].

Previous studies evaluating the symptomatic phase of ALS suggested that high cardiovascular risk profiles were protective

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prognostic factors in ALS. Whilst multiple studies reported a better prognosis in patients with higher BMI, associations between disease course and high lipids, high blood glucose levels and high blood pressure were only partially observed [24, 29–31]. Although the later course of the disease in presymptomatic carriers cannot be precisely predicted, it is well known that specific mutations are associated with a more aggressive or more benign disease course. *C9ORF72* gene-associated ALS is generally characterized by a later onset and faster disease progression [32]. Amongst *SOD1* gene patients, a greater heterogeneity has been reported, which is strongly linked to the specific mutation [33]. Specifically, patients with A4V mutations show fast disease progression [34], whilst the D91A and L118V mutations are associated with a comparatively benign disease course and longer survival [35]. Therefore, it may be hypothesized that a high cardiovascular risk profile in the presymptomatic stage is associated with a better prognosis in the clinical stage of ALS.

Consistent with this hypothesis, higher fasting serum glucose and lower HDL levels were found amongst *SOD1* gene carriers of benign mutations compared to other *SOD1* gene mutation carriers. However, not consistently, lower TC and lower LDL levels in benign *SOD1* gene mutation carriers were also found. Their relative benign clinical features could be related to compensatory mechanisms leading to such metabolic phenotypes. Normal *SOD1* gene protein is involved in lipid metabolism, mitochondrial respiratory repression and redox processes. Mutant SOD1 gene has an increased affinity to the anti-apoptotic protein Bcl-2 [36], which may contribute to an impaired capability to repress mitochondrial respiration and promote aerobic fermentation. An impaired mitochondrial Ca^{2+} buffering capacity and respiratory function was also related to mutant *SOD1* gene protein [37]. One study employing a human induced pluripotent stem cell model reported that impaired misfolded *SOD1* gene protein levels were higher in A4V compared to D91A mutant motoneurons [38] which may explain varying degrees of disruption of mitochondrial respiration, intracellular calcium and redox balance, and ultimately different metabolic profiles in ALS depending on the specific mutation. Considering the low number of patients in the *SOD1* gene subgroup analysis, these findings must be further explored in future studies.

So far, the underlying mechanisms linking metabolism with disease progression have not been fully understood. Hypermetabolism, as an important factor contributing to weight loss, has been described as a common phenomenon in ALS patients. It is hypothesized to be caused by alterations in the hypothalamus [39, 40] and/or by mitochondrial dysfunction [17, 41, 42]. The lateral part of the ventromedial nucleus of the hypothalamus affects food intake and energy expenditure. It is also involved in regulating insulin sensitivity, glucose and lipid metabolism. Atrophy of the hypothalamus [18] and (compensatory) increased levels of agouti-related protein mRNA have been demonstrated in ALS and are associated with glucose intolerance and enhanced appetite [18, 40]. Significantly enhanced energy intake and thereby blood glucose and lipid levels may provide energy support for repair mechanisms in the symptomatic as well as the presymptomatic stage of ALS.

Mitochondria, on the other hand, are essential for energy metabolism, and their dysfunction has been repeatedly described in early ALS pathogenesis [42, 43]. It has been shown that there was a linear correlation between the elimination rate of serum lactic acid and disease progression [44]. Studies based on animal models of ALS suggested that decreased ATP production in the central nervous system and muscles due to mitochondrial dysfunction was associated with decreased glycolysis and a switch towards lipids as the preferred source of energy in the muscle [27]. Increased storage of energy substrates in the presymptomatic stage of ALS may therefore indicate a compensatory mechanism to provide sufficient energy levels to counteract the pathological changes of the disease.

The following limitations have to be mentioned when interpreting the results of this study. First, our conclusions were drawn from cross-sectional analyses; therefore, presymptomatic gene carriers may display a heterogeneous burden of pathological changes, including some subjects who are close to the clinical onset of disease and others still far away. As our presymptomatic gene carrier cohort is followed up prospectively, future longitudinal data may provide additional insight. Secondly, despite using data from one of the largest presymptomatic gene carrier cohorts published to date, sample sizes for specific genes were still relatively small. Therefore, the results must be verified with a larger sample size.

CONCLUSION

In this study, distinct metabolic profiles in presymptomatic ALS gene carriers were identified, indicating a lower cardiovascular risk profile in *C9ORF72* gene and a higher cardiovascular risk profile in *SOD1* gene presymptomatic gene carriers compared to controls. Considering these findings in the context of the disease course to be expected for the present mutations, metabolic profiles might have a disease-modifying effect and prognostic value for presymptomatic ALS gene carriers. Additional studies and longitudinal follow-up are needed to further evaluate these findings.

AUTHOR CONTRIBUTIONS

Study conception and design: JD, ACL. Study supervision: DSF, JD and ACL. Acquisition and analysis of data: KX and JD. Writing of the manuscript: KX. Critical revision of the manuscript: SW, DF, JD and ACL. All authors contributed to manuscript revision, read and approved the submitted version.

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CONFLICT OF INTEREST

Nothing to report.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this article.

ETHICS APPROVAL

This study was approved by the ethics committee of Ulm University, application number 68/19.

PATIENT CONSENT FOR PUBLICATION

Obtained.

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4. Zusammenfassende Beantwortung der Forschungsfragen und Ausblick

Im Rahmen dieser Arbeit untersuchten wir verschiedenste systemische Faktoren, die als Einflussfaktoren für die Amyotrophe Lateralsklerose (ALS) in Frage kamen. Hierzu habe ich Liquordaten, die bisher nicht nutzbar waren, in eine strukturierte, analysierbare Form gebracht. Für die Überprüfung des Effekts der Blut-Liquor-Schranke auf die ALS mithilfe des Liquor-Serum-Albumin-Quotient (QAlb) Wertes standen somit 1059 Patienten zur Verfügung. Verglichen mit Kontrollkohorten zweigte der QAlb bei ALS Patienten keine Altersabhängigkeit. Wir konnten feststellen, dass vorwiegend bei Männern ein hoher QAlb zu einer kürzeren Überlebenszeit führt, wohingegen ein niedriger QAlb auf eine bessere Prognose hinweist. Der Effekt des hohen QAlb wird allerdings stark durch die Einflussfaktoren von Körpermasseindex (BMI) und Diabetes geprägt, sodass ohne sie die Signifikanz verloren geht. Der Einfluss des niedrigen QAlb, sowie der geschlechterspezifische Unterschied bedingt noch weitere Forschung mit größeren Kohorten und der Einbeziehung weiterer möglicher metabolischer oder anderer Störfaktoren. Durch die von mir digitalisierten Liquordaten konnten wir als zweiten in Frage kommenden Marker der ALS die Oligoklonale IgG Banden (OKB) betrachten, diese lagen bei 457 ALS Patienten vor. Wir konnten keinen Zusammenhang zwischen den klinischen Paramenten, der Progression oder dem Überleben mit den OKB-Typen feststellen. Infektiöse, entzündliche oder systemische Autoimmunerkrankungen in Form von Komorbiditäten der ALS traten vorwiegend bei OKB-Typen mit intrathekaler Synthese (Typ 2 & 3) auf. Unsere Ergebnisse deuten daher darauf hin, dass OKBs ein möglicher Marker für eine inflammatorische oder infektiöse Komorbidität sein könnten, jedoch nicht für die ALS. In zukünftigen Studien könnte dieser Verdacht anhand größerer Stichproben mit einem höheren Vorkommen an OKBs mit intrathekaler Synthese untersucht werden. In den weiteren Publikationen lag das Augenmerk hingegen auf den Markern, die im Blut vor allem in der präklinischen Phase Einfluss auf die ALS und das Risiko an ihr zu erkranken haben. Mithilfe der MR konnten wir in der dritten Publikation erforschen, dass in der präklinischen Phase das Risiko an ALS zu erkranken durch genetisch bedingt erhöhte Lipidwerte steigt. Diese Hypothese könnte nun neben Genome-wide association study (GWAS) Daten auch anhand präklinischer ALS Genträger validiert werden. In der letzten Publikation haben wir auch den Lipid- und Glukosemetabolismus von 80 präklinischen ALS Genträgern und deren Geschwistern untersuchen können. Wir konnten hierbei bei Chromosom-9-Open-Reading-Frame-72 (C9ORF72) ALS Genträgern ein niedrigeres kardiovaskuläres Risikoprofil im Gegensatz zu Kontrollen feststellen. Superoxide Dismutase 1 (SOD1) ALS Genträger hingegen zeigten ein höheres kardiovaskuläres Risikoprofil. Dadurch konnte diese Studie die metabolischen Profile von ALS Genträgen mutationsspezifisch beschreiben, welche möglicherweise mit dem Verlauf in der klinischen Phase zusammenhängen. Beide Studien gemeinsam weisen darauf hin, dass frühe (genetisch bedingte) und späte (erworbene) Veränderungen des Glukose- und Lipidstoffwechsels keine identischen Auswirkungen auf das ALS Risiko aufweisen.

5. Zusammenfassung

Die Amyotrophe Lateralsklerose (ALS) ist eine neurodegenerative Erkrankung, die vornehmlich die Motoneurone betrifft. Durchschnittlich führt sie meist aufgrund der respiratorischen Insuffizienz im fortgeschrittenen Stadium, nach durchschnittlich drei bis sechs Jahren zum Tod. Die Pathogenese ist bisher nicht abschließend geklärt, sowie derzeit auch keine kurative Therapie vorhanden ist. Ziel der vorliegenden Arbeit war es systemische Faktoren der ALS zu identifizieren, die mit der ALS in Verbindung stehen, um die Diagnostik, Prognose und Behandlung der Patienten zu verbessern. Dazu wurden Parameter im Liquor und Blut betrachtet, um eine Aussage über die Blut-Liquor-Schranke, Inflammation, präklinische metabolische Veränderungen und präklinische kardiovaskuläre Risikoprofile der ALS Patienten zu treffen.

In zwei Publikationen konnte ich erstmals die Liquordaten von einer Kohorte mit > 1000 ALS Patienten untersuchen. Die Verbindung mit dem ALS Schwaben Register ermöglichte es außerdem klinische Parameter und Komorbiditäten mit in die Analysen einzubeziehen. Meine Ergebnisse zeigten, dass die Blut-Liquor-Schranke bei Männern mit dem Überleben zusammenhängen könnte, wobei auch Störfaktoren wie der Körpermasseindex (BMI) oder Diabetes Mellitus (DM) hier eine Rolle spielen. Es gibt Hinweise darauf, dass eine geringere Permeabilität der Blut-Liquor-Schranke bei Männern das Überleben verlängern könnte. Bei Frauen scheint die Bedeutung der Blut-Liquor-Schranke als Marker der ALS geringer zu sein. Zu dem konnte anhand der vorliegenden Daten keine Altersabhängigkeit der Blut-Liquor-Schranke bei der ALS gezeigt werden. Vor meiner Arbeit wurde das Vorhandensein von Oligoklonale Banden (OKB), als möglicher modifizierender Faktor der ALS Progression gehandelt, dies konnte ich ausschließen. OKB können jedoch ein Hinweis auf eine Komorbidität bei der ALS sein.

Darüber hinaus haben wir präklinische Marker der ALS, welche im Blut zu finden sind, betrachtet. Mithilfe von GWAS-Daten konnten wir zeigen, dass genetisch bedingt erhöhte Lipidwerte in der präklinischen Phase, das Risiko an ALS zu erkranken steigern. Anhand einer Kohorte von 80 präklinischen Genträgern konnten wir zusätzlich feststellen, dass die kardiovaskulären Risikoprofile dieser mutationsspezifisch sind. So hatten Superoxide Dismutase 1 (SOD1) Genträger ein höheres kardiovaskuläres Risikoprofil als Kontrollen, wohingegen Chromosom-9-Open-Reading-Frame-72 (C9ORF72) Genträger ein deutlich niedrigeres aufwiesen.

Durch die in dieser kumulativen Dissertation gewonnenen Erkenntnisse lassen sich sowohl neue Parameter in die Prognosestellung von ALS Patienten miteinbeziehen als auch ausschließen. In der präklinischen Phase kann eine mutationsspezifische ALS schneller erkannt und gezielter behandelt werden. Weitere Studien sind nötig um die Erkenntnisse aus meiner Arbeit zu verifizieren und in der Klinik zu erproben.

6. Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease primarily affecting motor neurons. It usually leads to death after an average of three to six years due to respiratory insufficiency in the advanced stage. The pathogenesis has not yet been conclusively elucidated, and to date there is no curative therapy available. The aim of the present study was to identify systemic factors associated with ALS in order to improve diagnosis, prognosis and treatment of patients. For this purpose, parameters in the cerebrospinal fluid (CSF) and blood were considered in order to make a statement about the Blood-CSF-Barrier, inflammation, preclinical metabolic changes and preclinical cardiovascular risk profiles of ALS patients.

In two publications, I was able to examine CSF data from a cohort of > 1000 ALS patients for the first time. The connection with the ALS Swabia Registry also allowed to include clinical parameters and comorbidities in the analyses. My results showed that the blood-CSF barrier might be related to survival in men, with confounding factors such as Body Mass Index (BMI) or diabetes mellitus (DM). There is evidence that a lower permeability of the blood-CSF barrier in men may prolong survival. In women, the function of the blood-CSF barrier as a prognostic marker for ALS is less important. Additionally, there was no age dependency of the blood-CSF barrier detected in ALS patients. Before my work, the presence of oligoclonal bands (OCB), was traded as a possible modifying factor of ALS progression, this I could exclude. However, OCB can be an indication of comorbidity in ALS.

In addition, we looked at preclinical markers of ALS in blood. Using Genome-wide association study (GWAS) data, we demonstrated that genetically elevated lipid levels in the preclinical period increase the risk of developing ALS. Using a cohort of 80 preclinical gene carriers, we additionally found that the cardiovascular risk profiles of these were mutation-specific. Thus, Superoxide Dismutase 1 (SOD1) gene carriers had a higher cardiovascular risk profile than controls, whereas Chromosom-9-Open-Reading-Frame-72 (C9ORF72) gene carriers had a significantly lower one.

Through the knowledge gained in this cumulative dissertation, it is possible to both include and exclude new parameters in the prognosis of ALS patients. In the preclinical phase, mutation-specific ALS can be detected more quickly and treated in a more targeted manner. Further studies are needed to verify the findings from my work and to test them in the clinic.

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Danksagung

Wurde aus Datenschutzgründen entfernt.