Inflammatory mediators, lipoproteins and apolipoproteins in early diagnosis of amyotrophic lateral sclerosis

Hugo Alarcan a,∗, Mélanie Berthet a, Laura Suire b, Corentin Colas a, Loïc Gonzalez c, Christophe Paget c, Isabelle Benz-de Bretagne b, Eric Piver b, Patrick Vourc'h a, d, Christian Andres a, d, Philippe Corcia a, e, Hélène Blasco a, d

a Laboratoire de biochimie et biologie moléculaire, CHRU Bretonneau, 2 Boulevard Tonnellé, 37000 Tours, France
b Laboratoire de biochimie, CHRU Trousseau, avenue de la République, 37170 Chambon-les-Tours, France
c UMR1100 « Infection respiratoire & Immunité », Université de Tours, Inserm, 10 Boulevard Tonnellé, 37000 Tours, France
d UMR 1253 ilbrain, Université de Tours, Inserm, 10 Boulevard Tonnellé, 37000 Tours, France
e Service de neurologie, CHRU Bretonneau, 2 Boulevard Tonnellé, 37000 Tours, France

ABSTRACT

There is currently no diagnostic or prognostic biomarker available in clinical practice for Amyotrophic Lateral Sclerosis (ALS). The objective of this study was to monitor a combination of various inflammatory markers, lipids, and apolipoproteins alterations in ALS patients at the time of diagnosis, to assess their role as early diagnostic or prognostic biomarker candidates. C-reactive protein, orosomucoid, prealbumin, calprotectin, lipids and apolipoproteins were determined in the blood of all subjects (25 ALS patients, 23 controls) as routinely performed in our laboratory. Inflammatory mediators were evaluated by a bead-based multiplex assay. A two-step approach was used for each analytical strategy: univariate analysis followed by multivariate analysis. Eight features were significantly different between ALS patients and controls, sometimes with important fold changes. The supervised Partial least Squares Discriminant Analysis separated ALS and controls with great accuracy (94 %) and the permutation test was significant (p < 0.01), ensuring the robustness of the model. The prediction model leads to a mean sensitivity and specificity of 90 (+/- 10) and 78 (+/- 10) %, respectively, with a mean predictive positive value and negative predictive value of 80 (+/- 8.9) and 89 (+/- 11.8) %, respectively. However, the models did not discriminate subgroups of ALS patients based on ALS characteristics. This study highlights the usefulness of evaluating a combination of multiple pathways rather than focusing on a single target. These promising results suggest the need for the longitudinal monitoring of these candidates to determine their role in disease evolution.

Introduction

Amyotrophic Lateral Sclerosis (ALS) is the most common motor neuron disorder in adults. While most patients present a spinal-onset disease, bulbar or respiratory onset can also be observed. The disease evolution is rapid with death usually occurring after 3 to 5 years of evolution by respiratory paralysis and there is a lack of curative treatment [1].

To date, we still lack clinical practice diagnostic and prognostic biomarkers for ALS due to the disease’s heterogeneity and complex pathogenesis. Thus, there is an urgent need to find relevant biological analytes helpful for both diagnostic and prognostic concerns, as well as to better understand disease pathogenesis. Several mechanisms contribute to the initiation and progression of ALS, including metabolism alteration and neuroinflammation [2,3]. Interestingly, hyperlipidemia might be a possible explanation for this energetic imbalance observed in ALS patients [2]. Some studies reported increased lipid concentrations, including total cholesterol (TC), Low-Density Lipoprotein cholesterol (LDL-C) and triglycerides (TG) in the blood of ALS patients [4–6], while others found either no association or the opposite [7,8]. Lipids and apolipoproteins have also been associated with the disease’s prognosis, but again with contradictions [9]. Therefore, these controversial results hamper their definitive implication in ALS. Indeed, TC, LDL-C, High-Density Lipoprotein cholesterol (HDL-C) and TG were generally not associated with the risk of death, but some studies reported that elevated levels of TC, LDL-C and TG would be associated with longer survival time or with lower decline prognostic parameters [7,9,12].

Neuroinflammation is one of the most promising biological avenues recently explored in ALS. Many inflammatory cytokines, including interleukins (mostly IL-2, IL-6, IL-8, IL-10), Tumor necrosis factor alpha (TNF-α) or Interferon gamma (IFN-γ) have been found dysregulated in ALS patients, either in blood or cerebrospinal fluid (CSF). However, the
results were often discordant from one study to another, as illustrated with IL-6 that has been reported increased[13], but also unchanged[14] or even decreased (at mRNA level) [15]. These discrepancies might probably be due to the heterogeneity of patients included and the various timing of their exploration, as these molecules largely fluctuate within the same individuals[16,17].

Importantly, lipid alterations are linked to inflammation. Increased levels of LDL-C can lead to its retention and oxidation in oxidized LDL-C, which can interact with leucocytes innate sensors such as Toll-like receptors (TLRs) to promote inflammatory signaling pathways [18]. On the contrary, apolipoprotein A1 (apoA1), a major constituent of HDL-C, has anti-inflammatory properties by repressing TLR-mediated secretion of pro-inflammatory cytokines [19,20]. Since lipid metabolism and inflammation play a crucial role in ALS and regulate each other, they deserve an integrative analysis in this pathological context. A strategy based on the investigation of ALS pathogenicity at the same time of disease onset and considering panels of potential biomarkers in multivariate models could be more efficient than focusing on single targets [16]. In this context, the aim of this study was to perform a common investigation of various markers of inflammation, and lipid and apolipoprotein alterations in the blood of ALS patients, at diagnosis, to assess their role as early diagnostic or prognostic biomarker candidates.

Methods

Study design

At our center, blood/CSF samples of suspected ALS patients are systematically stored to constitute a biobank for further research (number CNIL 2016_080.1 and 2016_080.2). We included patients for whom a blood sample was stored within 3 months around the diagnosis and for whom ALSFR-R score, FVC and weight were recorded at that time. ALS patients knowing to present chronic inflammatory diseases (e.g inflammatory body disease) were excluded. All co-morbidity factors and treatments of included subjects were recorded and compared with the control population. All ALS patients were diagnosed as definitive or probable ALS according to the El Escorial revised criteria [21]. The control group included gender-and-age matched subjects with various diseases (supplementary Table 1). We excluded control patients having motoneurons disorders or known chronic inflammatory diseases. We assured that no control patient developed ALS or other types of motoneuron disorders. All subjects were informed in writing about the collection of their samples remaining from routine biological analyses for research aims. They were given the right to refuse such uses, but none refused. All patients were also informed about the data obtained and about their right to access these data, according to articles L.1121-1 and R1121-2 of the French Public Health Code (CNIL n’2019-071).

Blood samples were collected for routine biological exploration in ALS patients, at diagnosis, and in control patients, within the context of their disease, when a biologic exam was prescribed. The remaining volume was then stored at −20°C and all samples were then analyzed at one point. Analytical methods were carried out in accordance with relevant guidelines. C-reactive protein (CRP), orosomucoid, pre-albumin, calprotectin, TG, TC, HDL-C, apoA1, apolipoprotein B (apoB) and lipoprotein A (LpA) were determined for all patients with COBAS 6000 analyzer® (Roche Diagnostics, Meylan, France). LDL-C was estimated by the Friedewald formula. Protein electrophoresis was also determined for ALS patients.

The diagnosis, age, gender, site of onset (bulbar, respiratory or spinal), but also the date of initial symptoms were obtained for each patient. We checked that the proportion of onset forms was similar to that of the incident patients in our center (63% of spinal form, 34% of bulbar form, 3% of respiratory form). Disease progression was estimated by Body Mass Index (BMI), ALS Functional Rating Scale-Revised (ALSFRS-r) score and Forced Vital Capacity (FVC), which were documented at the time of diagnosis and at 12 months. Disease progression was analyzed according to the percentage of BMI, ALSFRS-r and FVC modification over 12 months (and expressed as groups, according to the median value of the parameter). The date of death was also collected, if available, and disease duration was defined as the time between the first symptoms of ALS and death.

Inflammatory mediator determination

Concentrations of inflammatory mediators were determined by Luminex technology with the Bio-plex™ Human Cytokine 8-plex Assay (#M50000007A) allowing measurement of Granulocyte-macrophage colony-stimulating factor, IFN-γ, IL-2, IL-4, IL-6, IL-8, IL-10 and TNF-α. We also used Bio-plex™ singleplex assays allowing the determination of IL-7 (#171B5007M), IL-12p40 (#171B6004M), IL-15 (#171B5013M), IL-17A (#171B5014M) and vascular endothelial growth factor (VEGF) (#171B5027M). Analyses were performed according to manufacturer’s instructions and samples were analyzed on a Bio-Plex™ 200 system (Bio-Rad Laboratories, Inc., Hercules, CA, USA).
**Statistical analysis**

We aimed to evaluate the role of inflammatory molecules, and lipoproteins and apolipoproteins as potential biomarkers (1) for the diagnosis of ALS, by comparing ALS patients with control patients and (2) for the prognosis by comparing subsets of ALS patients. We used a two-step approach for each analytical strategy: univariate analysis followed by multivariate analysis, which allows us to examine a panel of biomarkers rather than each variable independently. We also evaluated the correlation (pearson) of these potential biomarkers with the time between the onset of the disease and sampling.

**Univariate analysis**

Comparisons of analyte concentrations between two groups were performed using the non-parametric Wilcoxon test. We adjusted the p-values for multiple statistical tests by the false discovery rate (FDR) method. Volcano plots were constructed using R software (version 4.0.2).

**Multivariate analysis**

MetaboAnalyst was used for the multivariate analysis. Before analysis, variables were preprocessed by log transformation and autoscaling. Firstly, unsupervised analysis based on Principal Component Analyses (PCA) allowed us to observe grouping patterns, trends, and outliers. Then, we conducted Partial Least Squares Discriminant Analyses (PLS-DA). PLS-DA models were cross-validated by the k-fold method, which consists of withholding 1/10 of the samples in ten simulations (each sample being omitted once) in order to avoid overfitting. The quality of the model built from Cross Validation (CV) was assessed by prediction accuracy and permutation tests. A variable importance on projection (VIP) value was given to each variable. Features with a VIP > 0.8 were considered important to the model. Venn diagram highlighting metabolites commonly discriminant for disease evolution parameters was made with Venny 2.1 [22].

In case of significant PLS-DA models (for diagnosis or prognosis prediction), external validation was added to confirm the reliability and the relevance of the prediction. We used the whole dataset that we randomly divided into a training set (2/3 of the ALS and control subjects) and test set (the remaining 1/3 of patients). Receiver Operating Characteristic (ROC) curves were generated using Monte-Carlo cross validation (MCCV). In each MCCV, two thirds (2/3) of the samples were used to evaluate the features’ importance. The most important features were then used to build classification models which are validated on the 1/3 of the samples that were left out. Different ROC curves were built by increasing the number of selected features (2, 3, 5, 10, 20, 21). This procedure was repeated multiple times in order to calculate the performance and confidence interval of each model. We used PLS-DA as a method for ranking classification and features. Then, we chose the model that provided the highest AUC with no more than 5 features in order to keep a correct population/features, we selected the most often discriminant variables among these features and tried to predict the class of the samples from the test set. The whole procedure was independently repeated 5 times in order to estimate the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of prediction.

Multiparametric survival analysis was performed from the most commonly discriminant molecules (i.e. highlighted in at least 3 out of 4 evolution criteria). Univariate analysis was conducted using the Cox model with JMP statistical software version 13.0 (SAS Institute, Cary, NC). We also tested the known prognostic factors (site at onset, age at onset, weight loss).

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**Fig. 1.** Analysis of blood lipoproteins, apolipoproteins and inflammatory molecules in ALS and control subjects. (A) Volcano plot representing the most important features in univariate analysis. FC between ALS and control subjects are represented by the x-axis (log2 scale) and the adjusted p-value by the y-axis (negative log10 scale). We highlighted features with a FC threshold of 1.5 and an adjusted p-value threshold of 0.05. Red dots indicate an increase of the feature in ALS patients compared to controls and blue dots a decrease. (B) scores plot of the PLS-DA model. Orange dots represent ALS patients and green squares control subjects. (C) Important features (VIP > 0.8) identified by PLS-DA. Color of the line indicates the way of variation between ALS and controls: blue lines means a decrease in ALS patients, red lines an increase.
Results

Clinical characteristics

Forty-eight patients were included: 25 ALS patients and 23 age- and gender-matched control subjects. The clinical characteristics of patients are presented in Table 1. There was no difference for gender, age, hypertension, lipid-lowering drugs and diabetes between groups, despite a trend to be more diabetics patients in the control group. To note, two ALS patients took Non-Steroidal Anti-Inflammatory Drugs (NSAIDs). Mean age at onset for ALS patients was 68.5 +/- 8.7 years and 48 % were females. The site of onset was spinal in 52 % of patients, bulbar in 44 % and respiratory for one patient (4 %). The median duration of the disease was 41.4 months (22.4-87.6). Values of FVC at 12 months were missing for 6 patients and date of death was missing for 5 patients. In our cohort, no clinical characteristic correlated with the duration of disease evolution.

Lipoproteins, apolipoproteins and inflammatory molecules

Among the 13 inflammatory mediators analyzed, 9 were detected and could be quantified in patients: IL-2, IL-6, IL-7, IL-10, IL-8, IL-17A, VEGF, IFN-γ and TNF-α. The median concentrations and interquartile ranges of all features, including other markers of inflammation (CRP, oerosomucoid, prealbumin, calprotectin) and lipids are reported in Table 2. The two patient taking NSAIDs had levels of inflammatory molecules similar as those from the entire group (data not shown).

Panel of diagnosis biomarkers

According to the volcano plot, the concentrations of eight features were significantly different (adjusted p-value < 0.05) between ALS patients and control subjects, with a Fold Change (FC) threshold of 1.2: IL-8 (FC = 0.21), calprotectin (FC = 0.35), VEGF (FC = 0.41) and IL-10 (FC = 0.49) that were found in lower concentrations in ALS subjects, while TC (FC = 1.22), LDL-C (FC = 1.29), ApoA1 (FC = 1.29) and IL-7 (FC = 4.36) were found in higher concentrations (Fig. 1A). Concerning the multivariate analysis, the two groups were separated even from the unsupervised PCA analysis. The supervised PLS-DA separated ALS and controls with great accuracy (94 %) (R² = 0.78, Q² = 0.62), and the permutation test was significant (p < 0.01), ensuring the robustness of the model (Fig. 1B). In accordance with univariate analysis, the most important discrimination features (VIF value > 0.8) are those highlighted by the volcano plot, except for HDL-C and ApoB which tend to be higher in ALS patients, without reaching significance (FC = 1.18 and 1.19, adjusted p-value = 0.08 and 0.13, respectively) (Fig. 1C).

Performance of diagnosis prediction in an independent cohort

The PLS-DA models built on the 5 training sets showed performance as good as with the whole dataset (data not shown). Moreover, the ROC curves obtained by Monte Carlo analysis displayed good performance for the 5 prediction models, with AUCs ranging from 0.84 to 0.95 for the models retaining 5 features, as shown in Fig. 2 for example. Interestingly, ApoA1, IL-7, IL-8 and calprotectin were among the 5 features selected for the 5 predictions and IL-10 was selected in 3 prediction models, in consistence with their importance in the PLS-DA model of the whole data set. The prediction of the samples left out in the 5 independent test set leads to a mean sensitivity and specificity of 90 (+/- 10) and 78 (+/- 10) %, respectively. The mean PPV and NPV values were 80 (+/- 8.9) and 89 (+/- 11.8) %, respectively. Lipid-lowering drugs and comorbidities had no influence on discriminant parameters selected at least one time in the models, for both ALS and controls group (data not shown).

No panel of prognosis biomarkers

We also evaluated the ability of inflammatory molecules, lipoproteins, and apolipoproteins to discriminate subgroups of ALS patients based on ALS characteristics: age of onset, onset site, BMI, FVC and ALSFRS-r score on diagnosis. However, univariate analysis did not highlight any parameter. Using multivariate analysis, no PLS-DA model was significant according to the permutation test, so we did not build prediction models (data not shown). Moreover, for the groups based on the disease evolution parameters (variations of ALSFRS-r score, BMI and FVC over a year, survival time), no molecule was significantly different between subjects. The PLS-DA were also not significant and their performances were poor but we still built a Venn diagram highlighting the most commonly discriminant metabolites (Fig. 3). Seven features were common to at least 3 of the disease evolution parameters: ApoB, ApoB/ApoA ratio, % of alpha1 globulins, IL-2, LpA, IL-10 and TG, with the last two common to the 4 parameters. Survival analysis from these seven features revealed that only IL-10 had a p-value <0.2
Fig. 2. Procedure used for external validation. The whole dataset was divided into a training set (2/3) and a test set (1/3). Then, ROC curves built on PLS-DA (different numbers of metabolites) were obtained in the training set. The 5 features being the most frequently selected in a model with no more than 5 metabolites were used to predict the diagnostic in the test set. Predicted classes were compared to observed classes in order to calculate the number of true positives (TP), false positives (FP), true negatives (TN), and false negatives (FN). Finally, this procedure was processed 5 times in order to determine the mean sensitivity, specificity, PPV, and NPV of the models.

Fig. 3. Most important features for the disease evolution parameters. Venn diagram representing metabolites with a VIP > 0.8 with PLS-DA models between ALS patients according to the variation of BMI, FVC and ALSFRS-r score over a year, and survival time.

(raw p-value = 0.03) using the Cox model, so multiparametric survival analysis was not performed. Correlations of these molecules with the time between the onset of the disease and the sampling time are presented in supplementary Table 2. However, no molecules were statically associated with this time.

Discussion

To our knowledge, a multivariate model including inflammatory molecules and lipid parameters has never been evaluated before to predict ALS. Our results demonstrate an accurate discrimination between ALS and control patients and reveal excellent diagnosis performances from a panel of molecules, including original ones. However, these features were unsuccessful in discriminating subgroups of ALS subjects, including disease progression subgroups.

An efficient biomarker assessment strategy

One of the key concerns of this study is that most of the molecules examined here are available in numerous laboratories: lipoproteins and common inflammatory markers (CRP, orosomucoid, prealbumine) are performed in almost all clinical laboratories (and even possibly approved by an accreditation committee), and calprotectin is now a well-established biomarker for patients suffering from inflammatory body disease. As for inflammatory mediator analysis, ready-to-use multiplex approaches are now widely available and avoid time-consuming method
development. The availability of the assays will allow the translation of any potential biomarkers highlighted in this work into clinical use. We implemented a robust analytical strategy based on univariate followed by a machine-learning multivariate approach. Each model developed for diagnosis used internal validation by cross-validation and permutation tests in order to avoid overfitting. Moreover, we performed an external validation by splitting our dataset and evaluating the ability of our model built from the training set to predict the test set classes. The promising model diagnosis performances and easy determination of these variables support the construction of a prospective databank that will allow the validation of these results in a different cohort with similar clinical and demographic characteristics.

A relevant panel of diagnosis markers

Our study revealed eight molecules with blood concentrations significantly different between ALS and control subjects: LDL-C, TC, ApoA1, VEGF, IL-6, IL-8, IL-10 and calprotectin. These molecules were also discriminant (VIP value > 0.8) in our PLS-DA models and four of them (ApoA1, IL-7, IL-8 and calprotectin) were always (the 5 times) selected in our independent prediction models, which highlights their importance in the discrimination. Although the difference was not significant, the prevalence of diabetes was high in our control group, but we checked that none of these discriminant parameters was associated with the presence of diabetes. Our predictions displayed good discrimination performances, in favor of the interest of multivariate ALS analysis and the consideration of panels of biomarkers instead of single targets. However, the multivariate models did not have significant results for the prognosis of the disease.

Relevance of ApoA1 in early diagnosis of ALS

As mentioned previously, controversial results have been found concerning lipid alterations in ALS. Moreover, the progression interest of lipids is modest [9]. Surprisingly, our results suggest that ApoA1 is the most important feature in discriminating ALS patients during early diagnosis. In 2017, Mariosa et al. reported the results of the Swedish AMORIS project (636,132 people) aimed to estimate the association between glucose, lipids and lipoproteins on ALS incidence. They also observed their changes during a twenty-year follow up period [23]. They found that an increase in LDL-C, ApoB and ApoB/ApoA1 ratio was associated with a higher incidence of ALS. They also noted that ALS patients had significantly increasing levels of ApoA1 during the 10 years before the diagnosis. We did not find such an association in our cohort for the ApoB and ApoB/ApoA1 ratio, despite a tendency of ApoB to be higher in ALS patients (adjusted p-value of 0.13). This may be due to a lack of statistical power. However, we found elevated levels of ApoA1 at an early stage of the ALS disease, in consistency with its rise a few years before diagnosis. This apolipoprotein is a major constituent of HDL-C and can have other roles than lipid transport, especially an anti-inflammatory effect with the repression of pro-inflammatory cytokine secretion [19,20].

Confirmation of cytokine relevance in ALS diagnosis

Numerous studies investigated pro-inflammatory cytokines in ALS and their potential as biomarkers has recently been reviewed by Moreno-Martinez et al. [16]. Their conclusion was that it is still unclear how these inflammatory mediators can influence the progression of the disease and how they can be helpful in the diagnosis or disease progression of ALS. In our cohort, IL-8 and IL-7 were strongly discriminant according to the prediction models, being respectively five-fold lower and four-fold higher in ALS patients. IL-8 is a pro-inflammatory cytokine and has been positively associated with the disease, as reported by many works [16]. IL-7 was found elevated in the CSF of ALS patients [24,25] but blood concentrations had never been associated with the disease. This cytokine plays an important role in the survival, differentiation and proliferation of B-cell precursors [26]. It also has an important role in T-cell development since it acts as a growth and maintenance factor for thymocytes and also promotes their survival [27]. Adaptive immunity in ALS is still poorly understood (especially for B cells), but CD8+ T cells, Th1 and Th17 CD4+ T cells might be involved in late stages [28] and IL-7 could be among initiators of this response. It has been suggested that T regulators (Tregs) and Th2 CD+ T cells might be predominant in early symptomatic stages [28]. At this time, Tregs can suppress the function and proliferation of proinflammatory effector T cells by the secretion of IL-10 and TGF-β, cytosis or cytokines depletion, thus providing a neuroprotective effect [29]. Activation of macrophages linked with disease progression would impair the function of these Tregs that become dysfunctional. Consequently, they can no longer inhibit the proinflammatory T cells, enhancing the release of TNF-α and IFN-γ leading to a proinflammatory balance and a neurotoxic shift [28,30]. Importantly, discussion about cytokine findings should take into account the variations of cytokine concentrations associated with exogenous factors or factors linked to the disease. Indeed, the production of inflammatory cytokines can largely fluctuate within an individual overtime, as demonstrated by Ehrtart et al. who observed the modification of some cytokines in ALS and control subjects between two serum samples collected at six-monthly intervals [17]. The correlation between these parameters and delay between onset and sampling could provide clues about the kinetics of their modifications, but the findings are hampered by the limited size of our cohort. How and when neuroinflammation occurs in this disease remains unclear. As evoked above, it is actually hypothesized that initial pre-symptomatic and early symptomatic phases are dominated by anti-inflammatory immune responses, whereas late symptomatic and terminal stages are dominated by proinflammatory immune responses [28]. This hypothesis could explain the controversial results found between different studies in which the stage of the disease is not often mentioned. It is also consistent with our findings, which suggest a reduction of inflammatory molecules in the early stage of ALS disease. Based on its anti-inflammatory properties, apoA1 could also participate to the lower concentrations of inflammatory cytokines observed in early diagnosis of ALS. It should be noted that these inflammatory molecules had no impact on the disease’s evolution, except for IL-10 in an univariate Cox regression analysis. IL-10 is a prototypical anti-inflammatory cytokine, which can be released by the microglia to regulate the secretion of other inflammatory cytokines, culminating in a neuroprotective effect. Higher concentrations in the CSF of ALS patients were modestly associated with a higher ALSFRS-r score (r = 0.415, p = 0.035) but, to our knowledge, without p-value adjustment for multiple comparisons [24].

First observation of calprotectin performance in early ALS diagnosis

Calprotectin is derived predominantly from neutrophils, but also from monocytes and macrophages and participates to the innate immune response. It is found in many physiological fluids and its fecal concentrations are now routinely assessed for inflammatory bowel diseases as a marker of gastrointestinal inflammation [31]. Its interest has been recently extended to neurodegenerative disorders, where the disturbance of the brain-gut-microbiota axis is increasingly established in the pathophysiology of these diseases. It should be noted that there were high fecal and serum concentrations of calprotectin Parkinson’s disease [32,33]. To our knowledge, this molecule was never studied in ALS and the lower concentrations in the serum of ALS patients open new perspectives for targeted studies.

The strength of the combined analysis

All these data confirmed that inflammation and the lipid metabolism remain key mechanisms in ALS, but it is sensitive to discuss these mechanisms independently and confront our findings with the literature,
mainly because of both the heterogeneity of the analyzed biological fluids and timing of disease onset examination. The strength of our approach is to have combined some of the lipid and inflammatory markers in order to build an efficient diagnosis model. Inflammatory cytokines had to our knowledge never been studied in this context, with the evaluation of lipid alterations and calprotectin. A combination of IL-7, IL-8, calprotectin and ApoA1 were always selected in the five external validation models. All models were efficient and supported by a rigorous methodology. Accordingly, this study highlights the diagnosis value of this panel of biomarkers that are easily available in clinical laboratories.

Conclusions

To our knowledge, this is the first study to evaluate the diagnosis and prognosis value of lipids and apolipoproteins in combination with inflammatory molecules (including calprotectin) for ALS, via a multivariate approach. Our models accurately predicted the diagnosis of the disease in our cohort and support the hypothesis of an anti-inflammatory state in the early evolution of ALS. This study highlights the usefulness of evaluating combined multiple pathways rather than focusing on a single target. These results are exciting and encourage us to conduct further studies with larger cohorts in order to increase the statistical power. These promising results also suggest the interest of performing longitudinal monitoring of these candidates in order to determine their role in disease evolution.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Authors’ contributions

Hugo Alarcon: Formal Analysis (lead); Investigation (equal); Visualization (lead); Writing – Original Draft Preparation (lead); Mélanie Berthet: Formal Analysis (equal); Investigation (equal); Writing – Review & Editing (equal); Laura Suire: Investigation (equal); Writing – Review & Editing (equal); Corentin Colas: Investigation (equal). Loïc Gonzalez: Investigation (equal); Writing – Review & Editing (equal). Christophe Paget: Writing – Review & Editing (equal); Resources (equal). Isabelle Benz-de Bretegny: Writing – Review & Editing (equal). Eric Piver: Writing – Review & Editing (equal); Patrick Vourch: Writing – Review & Editing (equal). Christian Andres: Writing – Review & Editing (equal). Philippe Corcia: Investigation (equal); Resources (equal); Writing – Review & Editing (equal); Hélène Blasco: Conceptualization (lead); Supervision (lead); Writing – Review & Editing (equal).

Supplementary materials

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