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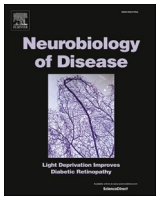

Common lipidomic signatures across distinct acute brain injuries in patient outcome prediction

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ABSTRACT

Lipidomic alterations have been associated with various neurological diseases. Examining temporal changes in serum lipidomic profiles, irrespective of injury type, reveals promising prognostic indicators. In this longitudinal prospective observational study, serum samples were collected early (46 ± 24 h) and late (142 ± 52 h) post-injury from 70 patients with ischemic stroke, aneurysmal subarachnoid hemorrhage, and traumatic brain injury that had outcomes dichotomized as favorable (modified Rankin Scores (mRS) 0–3) and unfavorable (mRS 4–6) three months post-injury. Lipidomic profiling of 1153 lipids, analyzed using statistical and machine learning methods, identified 153 lipids with late-stage significant outcome differences. Supervised machine learning pinpointed 12 key lipids, forming a combinatory prognostic equation with high discriminatory power (AUC 94.7 %, sensitivity 89 %, specificity 92 %; $p < 0.0001$). Enriched functions of the identified lipids were related to sphingolipid signaling, glycerophospholipid metabolism, and necroptosis ($p < 0.05$, FDR-corrected). The study underscores the dynamic nature of lipidomic profiles in acute brain injuries, emphasizing late-stage distinctions and proposing lipids as significant prognostic markers, transcending injury types. These findings advocate further

Abbreviations: aSAH, Aneurysmal Subarachnoid Hemorrhage; IS, Ischemic Stroke; TBI, Traumatic Brain Injury; ABI, Acute Brain Injury; SM, Sphingomyelin; CER, Ceramide Phosphate; FFA, Free Fatty Acid; AD, Alzheimer's Disease; PD, Parkinson's Disease; HD, Huntington's Disease; ALS, Amyotrophic Lateral Sclerosis; MS, Multiple Sclerosis; PAF, Platelet-Activating Factor; DHA, Docosahexaenoic Acid; LAU-0901, PAF Receptor Antagonist; NPD1, Neuroprotectin D1; AT-NPD1, Aspirin-Triggered Neuroprotectin D1; GCS, Glasgow Coma Scale; mRS, Modified Rankin Scale; LC-MS, Liquid Chromatography-Mass Spectrometry; MTBE, Methyl tert-butyl Ether; PCA, Principal Component Analysis; PLS-DA, Partial Least Squares Discriminant Analysis; ROC, Receiver Operating Characteristic; AUC, Area Under the Curve; CI, Confidence Interval; SVM, Support Vector Machine; TAG, Triacylglycerol; PC, Phosphatidylcholine; PE, Phosphatidylethanolamine; LPE, Lysophosphatidylethanolamine; GL, Glycerolipid; SL, Sphingolipid; PUFA, Polyunsaturated Fatty Acid; PLA2, Phospholipase A2; PLAC, Phospholipase C; GP, Glycerophospholipid.

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exploration of lipidomic changes for a comprehensive understanding of pathobiological roles and enhanced prediction for recovery trajectories.

1. Introduction

Subarachnoid hemorrhage (aSAH), ischemic stroke (IS), and traumatic brain injury (TBI) represent some of the most prevalent and debilitating forms of acute brain injury (ABI) (GBD 2016 Traumatic Brain Injury and Spinal Cord Injury Collaborators, 2019; GBD 2019 Stroke Collaborators, 2021). These afflictions often yield significant neurological impairment and drastically alter quality of life. Despite major strides in medical interventions and management techniques, predicting the clinical outcomes of these injuries remains a complex challenge (Dagonnier et al., 2021).

Although many facets of cellular mechanics in relation to ABI have been studied extensively, the field of lipidomics in ABI is relatively new. Lipids are known to play a significant role in cellular function including cell signaling, formation of cell membranes, energy storage, and intercellular communication (Hussain et al., 2020). Particularly in the nervous system, lipids play an extremely important role, as the nervous system, after adipose tissue, holds the body's largest lipid store (Zivko et al., 2023). In the brain, lipid concentrations in gray and white matter account for up to 40 % and 65 % of the total structural volume, respectively (Smith et al., 2022). The blood-brain barrier easily allows small lipids to pass through, which is why lipid changes in the brain are well-reflected in the circulation and can thus be detected in biofluid samples through lipidomic analyses (Hogan et al., 2018; Pardridge, 2005).

Lipidomics is the study of cellular lipid profiles at a given time, capturing a snapshot of the physiological state of an organism. Recently, the detection and quantification techniques for lipids have improved and resulted in significant interest in lipidomics (Sheth et al., 2015). These technological advancements in characterizing lipids have led to the identification of various disease-specific changes and lipid-based biomarkers (Hussain et al., 2020; Köfeler et al., 2021; Adibhatla et al., 2006). The altered metabolic pathways of lipids are associated with several neurological diseases, ranging from chronic neurodegenerative conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), and multiple sclerosis (MS) to neuropsychiatric disorders (Kao et al., 2020; Galper et al., 2022; Hunter et al., 2018; McCluskey et al., 2022; Phan et al., 2023; Tkachev et al., 2023; Yoon et al., 2022). Importantly, the lipid profile is altered in several aspects after ABI (Adibhatla et al., 2006).

Recent investigations into lipidomic profiles after ABI open promising avenues for understanding brain injury and recovery mechanisms. Lipidome profiling could provide more information about the integrity of brain connectivity and brain health. For instance, circulating lipids can be used as biomarkers for microvascular disease (Azizkhanian et al., 2019). Specific lipids such as sphingomyelins (SM) and ceramide phosphates (CER) as well as free fatty acids (FFA) are known to increase in serum after TBI and stroke in animal models, correlating with the severity of the injury (Hogan et al., 2018). A previous study demonstrated the severity of injury sustained after TBI is related to the lipidomic signature. Choline phospholipids (lysophosphatidylcholines, etherphosphatidylcholines, and SMs) levels were inversely associated with the severity of TBI and were predictors of outcome in TBI patients (Thomas et al., 2022). Of note, platelet-activating factor (PAF) is a phospholipid mediator that is known to accumulate in response to brain injury and acts as a pro-inflammatory messenger and mediator of neurotoxicity (Belayev et al., 2008). Interestingly, docosahexaenoic acid (DHA – FFA 22:6), a member of the omega-3 fatty acid family, is also shown to be enriched in the brain after ABI; however, it is involved in memory and neuroprotection via anti-inflammatory actions (Kim, 2014; Bazan, 2005). There is evidence that acute brain injuries, particularly TBI, are an inherently systemic disease, suggesting that studies of lipidomics and its trajectories may be a valuable tool in deciphering the pathophysiology of acute brain injuries (Gaddam et al., 2015; Ma et al., 2017).

Moreover, various pharmacological interventions targeting lipid metabolism have demonstrated therapeutic potential. For instance, a novel PAF receptor antagonist, LAU-0901, resulted in significant decreases in infarct volumes and was neuroprotective after cerebral ischemia in rodent model studies (Belayev et al., 2008). Also, one of the protectins based on DHA, namely DHA-derived neuroprotection D1 (NPD1), has been shown to reduce stroke-mediated tissue damage in mouse studies (Bazan et al., 2012). Similarly, novel aspirin-triggered neuroprotection D1 (AT-NPD1) reduced infarction volume significantly and protected white matter in the rat study (Bazan et al., 2012). Belayev et al. (2023) demonstrated that LAU-0901 when administered in conjunction with AT-NPD1, proved to be even more effective in stroke treatment than LAU-0901 alone (Belayev et al., 2023). These findings highlight the therapeutic potential that a deeper understanding of lipidomics may provide.

Guided by these investigations into the biological nature of lipids, our study hypothesizes that temporal changes in the serum lipidomic profile following various acute brain injuries could provide new insights into the conserved recovery process including protective changes in systemic lipid metabolism aimed at maintaining lipid homeostasis in the brain. Furthermore, our goal is to identify lipidomic alterations associated with patient outcomes that might potentially serve as prognostic biomarkers in the future and provide new target lipids for mechanistical studies in animal models.

2. Materials and methods

2.1. Study design and participants

This study was a longitudinal prospective observational cohort design that included 70 consecutive patients from the University Hospital of Turku, Finland, treated between 2016 and 2019 (Fig. 1). These patients were categorized into three groups: ischemic stroke (IS, $n = 28$), aneurysmal subarachnoid hemorrhage (aSAH, $n = 30$), and traumatic brain injury (TBI, $n = 12$) resulting in a subdural hematoma that necessitated surgical intervention.

Eligibility criteria included:

1. Diagnosis of aneurysmal subarachnoid hemorrhage, ischemic stroke (embolic, thrombotic, or cryptogenic), or traumatic brain injury leading to a subdural hematoma requiring surgical intervention.
2. Age above 18 years.
3. Provision of informed consent.

All patients received standard clinical treatment following the institution's protocols, which align with the prevailing guidelines for treating aSAH, IS, and TBI patients (Connolly et al., 2012; Carney et al., 2017; Powers et al., 2019).

Peripheral venous samples were collected twice from the participants: first, at an early timepoint averaging 46 ± 24 (SD) hours post-insult, and subsequently, at a later stage averaging 142 ± 52 (SD) hours post-insult. Three months after the initial event, the aSAH patients had their outcomes assessed during an outpatient clinic visit, while the IS and TBI patient outcomes were gauged through structured telephone interviews. The modified Rankin Scale (mRS) was employed to evaluate the outcomes, classifying patients as either favorable (mRS 0–3) or

unfavorable (mRS 4–6). If a patient died before the 3-month outcome assessment, their mRS was recorded as 6.

During the study recruitment, 11 patients opted out of the study, and one patient initially agreed but later chose to withdraw (late samples cohort: $n = 70$). Additionally, nine participants were omitted from the early lipidome biomarker detection measurements because their early samples were not available (early samples cohort $n = 61$).

2.2. Serum extraction

Standard 10 mL BD Vacutainer No Additive collection tubes (REF 364915) were utilized for venous blood serum collection. After drawing the blood, samples were left to rest at room temperature for 30 to 60 min to facilitate clot formation. Following this clotting period, the samples were centrifuged using a horizontal rotor (swing-out head) at 2200*g* for 15 min, also at room temperature. The separated serum was then distributed into three clean 10 mL BD Vacutainer No Additive tubes (REF 364915) and stored at −80 °C.

2.3. Lipidome assessment with targeted LC-MS lipidomics

Lipidomics was performed with the high-throughput targeted quantitative method by the SCIEX Lipidyzer™ Platform. Detailed materials and methods for LC-MS are provided in the supplement.

2.4. Bioinformatics and statistics

2.4.1. Analysis of lipidome

Lipidomics Workflow Manager software was used for the acquisition of samples, automated data processing, signal detection, and lipid species concentration (μM) calculations (Ghorasaini et al., 2021). The lipidomes were analyzed using Metaboanalyst 5.0 platform. Removal of features with >20 % missing values was performed in initial data preparation. Missing values were replaced by LoDs (1/5 minimum positive values of each variable), and the data was tested for skewness and handled appropriately with normalization (log10 transformation) and auto-scaled. Then the data was analyzed with *t*-test, ANOVA, partial least squares discriminant analysis (PLS-DA), heatmaps, box plots, and cross-validation (Pang et al., 2022; Pang et al., 2021; Xia et al., 2009). Advanced machine learning algorithms, including Random Forest (500 trees with 7 predictors) and linear support vector machine (SVM) (a linear kernel with recursive feature elimination with a 10-fold cross-validation to select the most relevant features), were deployed to scrutinize the significance of individual lipids in differentiating patient outcomes (Barberis et al., 2022). The algorithms were trained on a training dataset and validated on a separate set. Monte-Carlo cross-validation (MCCV) with balanced subsampling was performed, using 2/3 of the samples to evaluate lipid importance. The top lipids identified (feature importance) were then used to build classification models, which were validated on the remaining 1/3 of the samples that were initially left out. Receiver operating characteristic (ROC) analyses were implemented to evaluate the sensitivity and specificity of potential lipid biomarkers. The Youden method was used to determine the best sensitivity and specificity (Youden, 1950). Univariate and multivariate linear canonical discriminant analysis (LDA) was performed, and canonical scores were used to build combinatory biomarkers with logistic modeling predicting outcomes (Girard et al., 2018; Srinath et al., 2023). To understand the implications of the identified lipid species and their potential involvement in disease mechanisms, enrichment analyses were conducted with the Lipea platform (Acevedo et al., 2018). All the analyses were false discovery rate (FDR) corrected, and $p < 0.05$ FDR-corrected was considered to be statistically significant (Benjamini and Hochberg, 1995). Cross-validation was performed in each PLS-DA analysis with 5-fold cross-validation searching three components. Cross-validation results were based on three different measurements known as known as R-squared, Q-squared, and accuracy (Szymańska et al., 2012). Statistical software R and SAS were used in the analysis (SAS Institute Inc., 2016, Cary, NC, USA).

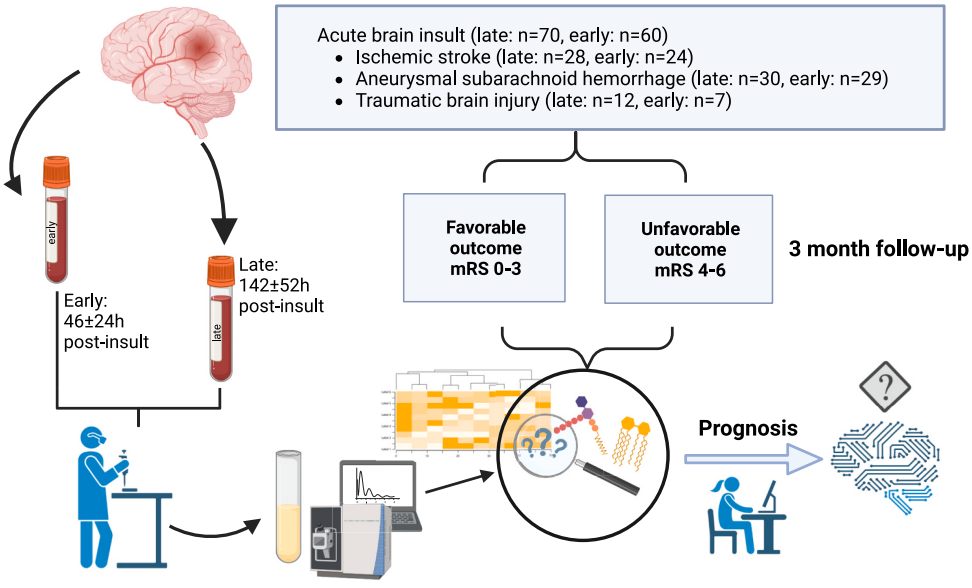

Fig. 1. Flowchart of the study In this study, serum samples from patients suffering from ischemic stroke, aneurysmal subarachnoid hemorrhage, and traumatic brain injury were collected at two timepoints post-insult and profiled using the LipidLyzer™ platform, capable of identifying 1153 lipid species. The potential of these lipids to predict patient outcomes was assessed using partial least squares discriminant analysis and *t*-tests on both early and late samples. The significance of individual lipids in differentiating outcomes was visualized and further scrutinized with machine learning methodologies. Potential biomarkers were then evaluated using receiver operating characteristic (ROC) analyses. Finally, the linear support vector machine algorithm was applied to pinpoint pivotal lipid features validated through Monte-Carlo cross-validation. Linear discriminant analysis was performed to build combinatory biomarker. Enrichment and pathway analyses were performed to understand functions of the identified lipids.

3. Results

3.1. Study population and clinical parameters

Of the enrolled cohort ($n = 70$), brain injuries were categorized into three primary etiologies: aSAH accounting for 42.9 % (30/70), TBI at 17.1 % (12/70), and IS comprising 40.0 % (28/70) (Table 1 and Supplemental table S1). Demographically, the cohort showed no statistically significant male predominance, with males constituting 54.3 % (38/70) of the study population ($p = 0.74$). The mean age was determined to be 57.7 ± 12.8 years (Table 1). Initial assessments using the Glasgow Coma Scale (GCS) yielded a mean score of 11.8 ± 4.4 (GCS minimum 3, maximum 15) (Supplemental table S1). From the enrolled cohort ($n = 70$), nine early samples were not available, resulting in 61 samples for the early cohort. Early ($n = 61$) and late (n = 70) sample cohorts were checked for balanced grouping in terms of age, gender, type of brain injury, and mRS-level ($p = 0.974$, $p = 0.936$, $p = 0.896$, and $p = 0.807$, respectively) (Table 1).

Outcomes were evaluated at the three-month mark using the modified Rankin Scale (mRS). Favorable outcomes (mRS scores 0–3) were observed in 64.3 % (45/70) of the patients. Unfavorable outcomes (mRS scores 4–6) were seen in 35.7 % (25/70) (Table 1). The cohort's overall mortality rate at three months post-injury was 20.0 % (14/70). Furthermore, favorable ($n = 45$) and unfavorable ($n = 25$) sub-cohort groups matched in terms of age, gender, and type of brain injury ($p = 0.268$, $p = 0.381$, and $p = 0.538$, respectively) (Table 2).

3.2. Lipidomic profiles of acute brain injuries

The concentrations of the circulating lipidome were quantitatively measured in serum samples at two timepoint (early = 46 ± 24 h and late = 142 ± 52 h). The entire serum lipidome after normalization, encompassing 1153 lipids screened, detected 870 lipids with no discernible clustering in the principal component analyses (PCA) across disease groups within the same outcome categories and timepoints showing homogeneity in the lipidomic profiles among aneurysmal subarachnoid hemorrhage (aSAH), ischemic stroke (IS), and traumatic brain injury (TBI) patients (Supplemental Fig. 1). Further analysis using partial least squares discriminant analysis (PLS-DA) corroborated these findings, with highly negative Q-squared values (Q-squared range = −0.06 – (−0.21)) indicating a lack of separation between the groups. (Supplemental material).

We selectively analyzed both early ($n = 61$) and late ($n = 70$) sample cohorts. Analysis of early serum samples taken approximately 46 ± 24 h post-insult had limited discriminatory ability between favorable and unfavorable outcomes and suggested model overfitting (negative Q-squared value) during cross-validation (accuracy = 0.70, R-squared = 0.27, Q-squared = −0.12) (Fig. 2A-B). In contrast, the late serum samples (142 ± 52 h post-insult) demonstrated enhanced discriminatory potential with good cross-validation performance (accuracy = 0.79, R-squared = 60.0, Q-squared = 0.24) (Fig. 2C-D). This observation guided our analysis to understand common lipidomic signatures in studied acute brain insults in the late sample cohort that could separate the favorable and unfavorable outcomes.

Table 1
Basic characteristics of early and late cohorts. Favorable modified Rankin scale (mRS) 0–3, Unfavorable mRS 4–6. aSAH = aneurysmal subarachnoid hemorrhage, TBI = traumatic brain injury, IS = ischemic stroke.

Variables	Early (n = 61)	Late (n = 70)	p-value
Age in years			0.974
Mean ± SD	58.1 ± 13.3	57.7 ± 12.8	
Min–Max	23.0–75.0	23.0–75.0	
Median (IQR)	62.5 (47.0–70.0)	62.0 (47.0–68.3)	
Sex			0.936
Male (%)	33 (54.1)	38 (54.3)	
Female (%)	28 (45.9)	32 (45.7)	
Type of brain injury			0.896
aSAH (%)	29 (43.3)	30 (42.9)	
TBI (%)	7 (10.0)	12 (17.1)	
IS (%)	25 (46.7)	28 (40.0)	
mRS			0.807
Favorable (%)	37 (60.1)	45 (64.3)	
Unfavorable (%)	24 (29.9)	25 (35.7)	

Statistical comparisons to detect group differences between early and late study cohorts (all comparisons p > 0.05). Two-sample t-test (continuous) or Chi square test or Fisher's exact test (categorical) for *p*-values.

Table 2
Basic characteristics of favorable and unfavorable patient groups in late study cohort (n = 70). Modified Rankin scale (mRS). Favorable mRS 0–3, Unfavorable mRS 4–6. aSAH = aneurysmal subarachnoid hemorrhage, TBI = traumatic brain injury, IS = ischemic stroke.

Variables	Favorable ($n = 45$)	Unfavorable ($n = 25$)	p-value
Age in years			0.268
Mean ± SD	57.0 ± 12.3	60.5 ± 13.6	
Min–Max	23.0–75.0	30.0–74.0	
Median (IQR)	59.5 (47.3–66.0)	65.0 (50.0–71.5)	
Sex			0.381
Male (%)	27 (60.0)	12 (48.0)	
Female (%)	18 (40.0)	13 (52.0)	
Type of brain injury			0.538
aSAH (%)	19 (43.3)	11 (56.3)	
TBI (%)	6 (10.0)	6 (31.3)	
IS (%)	20 (46.7)	8 (12.5)	

Statistical comparisons to detect group differences between favorable and unfavorable outcome groups (all comparisons p > 0.05). Two-sample t-test (continuous) or Chi square test or Fisher's exact test (categorical) for p-values.

3.3. Lipid signatures differentiating patients with favorable and unfavorable outcome

Temporal alteration of the lipidome after different brain insults was evident (Fig. 3A-D). We studied the presence of lipids that were statistically significant in distinguishing between the two outcome groups. No statistically significant lipids differentiated favorable from unfavorable outcomes in early samples after FDR correction (Fig. 3A, Supplemental Table S2). However, in the late sample group, 153 lipids were identified as statistically significant for differentiating favorable and unfavorable outcomes ($p < 0.05$, FDR-corrected (Fig. 3B, Table 3, Supplemental Table S3). Accounting for fold change (FC), volcano plots reiterated these findings with no significant lipids differentiating outcomes in the early samples but showed 108 significant lipids in late samples (five lipids upregulated and 103 downregulated, $p < 0.05$ FDR-corrected, $|FC| > 1.5$) (Fig. 3C-D, Supplemental Table S4–5). Machine learning random forest algorithm analysis identified the most important lipids for separating the favorable and unfavorable groups according to mean decrease accuracy including mostly different triacylglycerols (TAG) and phosphatidylcholines (PC). (Fig. 3E, Supplemental Table S6). Similarly, the heatmap generated from the PLS-DA identified the most important lipids (using variable importance projections scores) that significantly differentiated favorable and unfavorable outcomes (Fig. 3F).

We also studied temporal change in the relative abundance and distribution of lipid classes between early and late cohorts. The analysis of lipid classes between early and late cohorts reveals significant changes. Specifically, the relative abundance of DAG increased from 5.99 to 6.28 ($p = 0.0195$), TAG increased from 12.18 to 12.55 ($p = 0.0018$), and FFA decreased from 11.56 to 11.08 ($p = 0.0002$) in the late cohort. Additionally, LCER showed an increase from 2.80 to 2.95 ($p = 0.0331$), and LPC increased from 8.69 to 8.83 ($p = 0.0422$). These results suggest considerable shifts in lipid distribution over time (Supplemental Table S7).

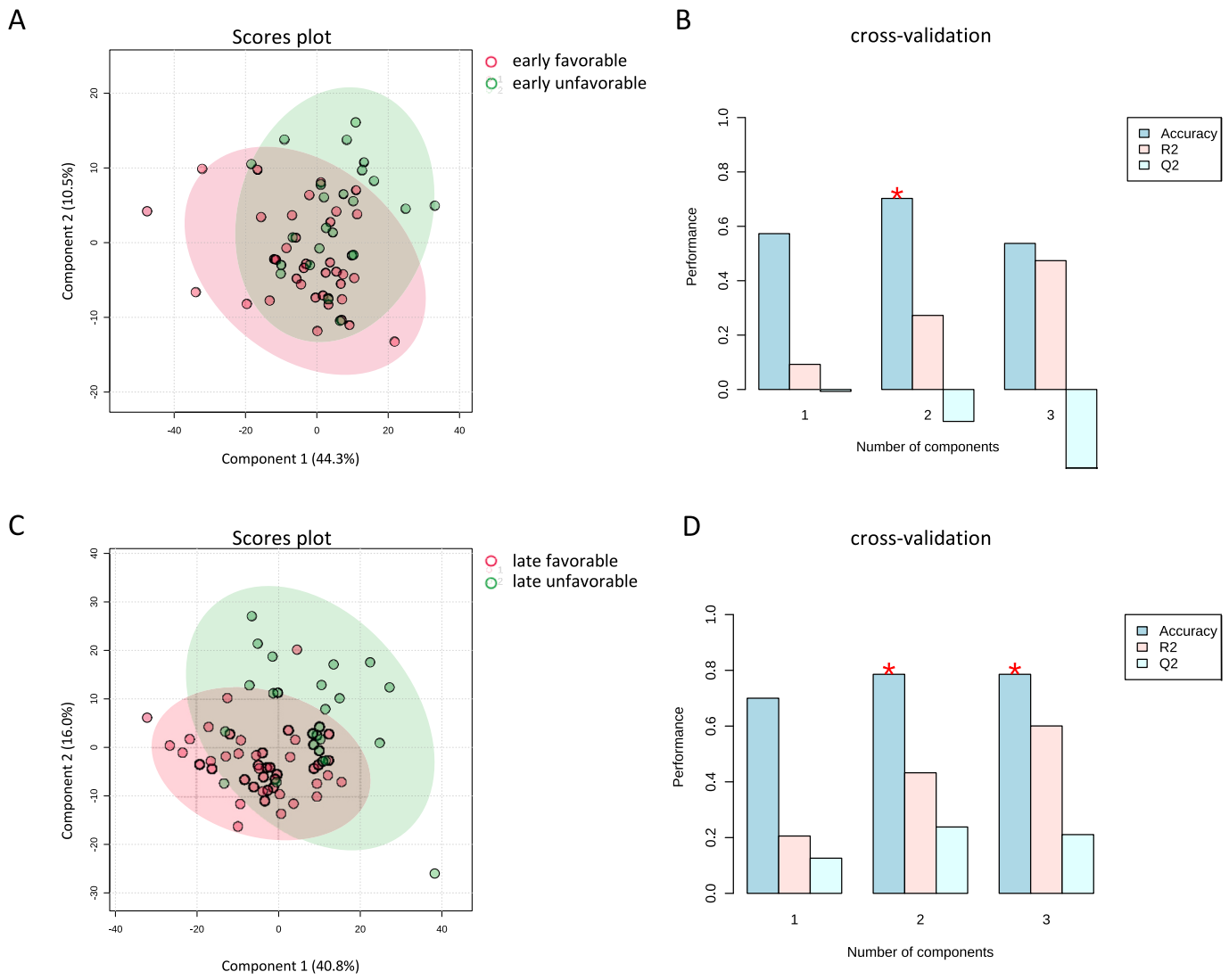

Fig. 2. Partial least squares discriminant analysis (PLS-DA) and cross-validation of the serum lipidome (1153 lipids). $N = 70$. A-B) The early serum samples (46 ± 24 h post-insult) did not indicate good discriminatory ability between outcome groups and in cross-validation negative Q-squared value propose clear overfitting the model (accuracy = 0.70, R-squared = 0.27, Q-squared = −0.12). C—D) The late serum samples (142 ± 52 h post-insult) cohort showed good discriminatory ability between favorable and unfavorable outcome groups confirmed by cross-validation (accuracy = 0.79, R-squared = 60.0, Q-squared = 0.24). aSAH = aneurysmal subarachnoid hemorrhage, IS = ischemic stroke, TBI = traumatic brain injury, favorable outcome (modified Rankin Scale 0–3), unfavorable outcome (modified Rankin Scale 4–6). * = highest accuracy. Number of patient samples in early timepoint = 61. Number of patient samples in late timepoint = 70. R2 = R-squared, Q2 = Q-squared.

3.4. *The quest for potential biomarkers*

A pivotal segment of this study, as depicted in Fig. 4, centered around leveraging the receiver operating characteristic (ROC) analyses to comprehensively evaluate the lipidome for potential biomarkers. Several lipids, specifically the most increased lipid ratio of TAG46:3-FA18:1/TAG46:3-FA18:2 and the most decreased lipid ratio of TAG44:2-FA18:1/TAG46:3-FA12:0, were identified, registering good sensitivity and specificity metrics for differentiating favorable and unfavorable outcome (84 % sensitivity and 82 % specificity (area under the curve (AUC) 90.0 % with 95 % confidence interval (CI) = 79–97 % and 88 % sensitivity and 84 % specificity (AUC 90.1 % with 95 % CI = 81–97 %), respectively. $p < 0.0001$, FDR-corrected) (Fig. 4A-B). The best performing single lipid TAG47:2-FA15:0 showed prognostication performance with an 88 % sensitivity and 67 % specificity (AUC of 80.0 % (95 % CI: 69–90 %), $p < 0.0001$, FDR-corrected) (Fig. 4C). Furthermore, the most crucial lipid identified from the machine learning algorithm random forest, PC(16:0/20:4), achieved prognostication accuracy with 91 % sensitivity and 60 % specificity AUC of 75.7 % (95 % CI: 63–87 %, $p < 0.0001$, FDR-corrected) (Fig. 4D).

3.5. *Unsupervised multivariate machine learning approach*

Utilization of machine learning further refined our lipidomic interpretations in order to gain knowledge of multivariate settings. The linear support vector machine (SVM) analysis identified the most important lipids that show differentiation between the outcome groups (Fig. 5A, Supplemental Table S8). This model identified several additional groups of lipids compared to other analyses including FFAs, PCs, TAGs, CER, SM, and PE (phosphatidylethanolamine).

3.6. *Combinatory biomarker analysis through machine learning LDA*

Building on the robust findings from our ROC analysis, the combinatorial biomarker section of our study leverages the predictive strength of a panel of 12 lipid candidates. This panel was judiciously selected

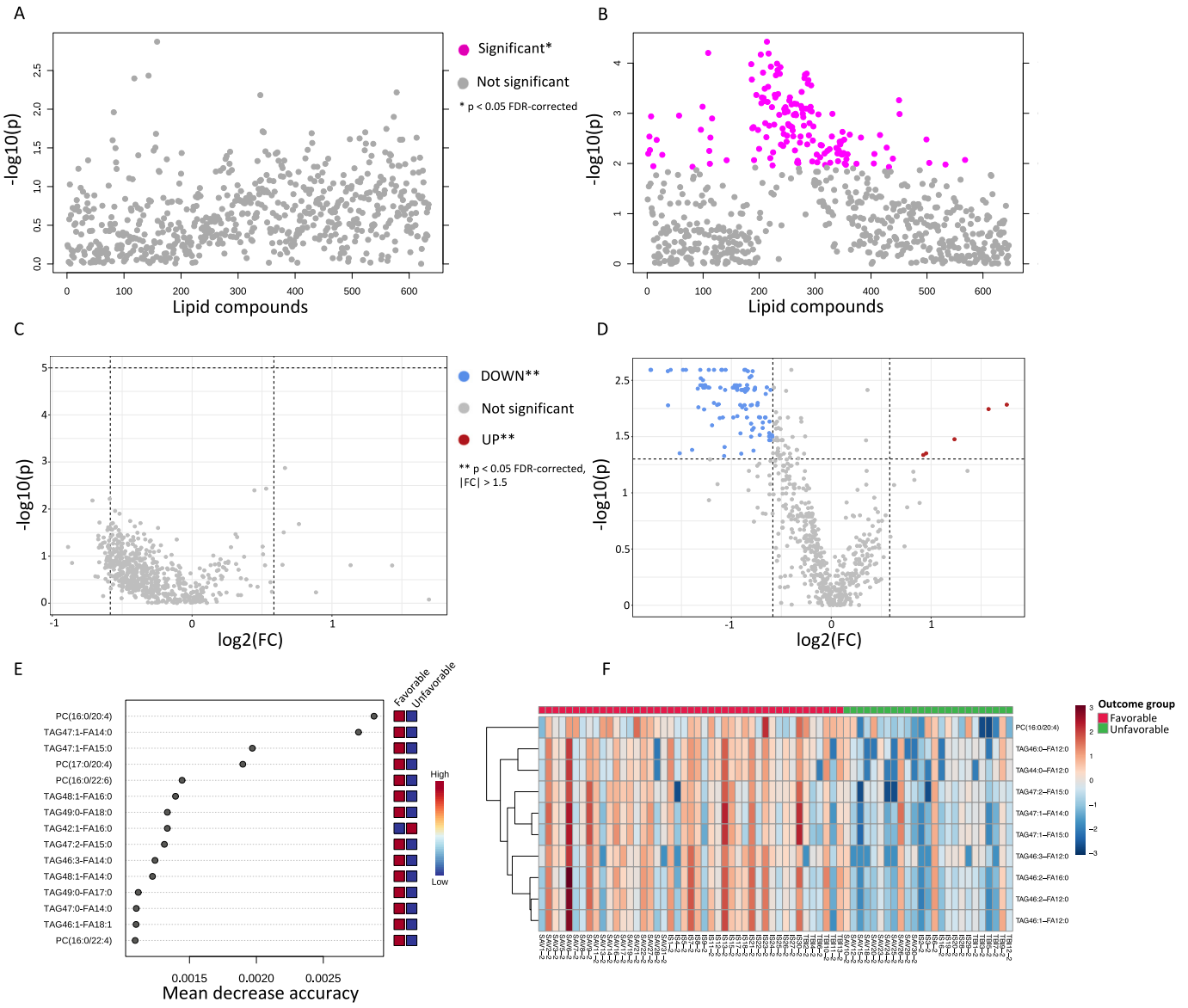

Fig. 3. Lipid signatures differentiating patients with favorable and unfavorable outcomes A) Zero statistically significant lipids were identified in early samples between favorable and unfavorable groups ($p < 0.05$ false discovery rate (FDR) corrected) B) One-hundred-fifty-three statistically significant lipids were identified in late group differentiating favorable and unfavorable outcome groups ($p < 0.05$ FDR-corrected) C) Volcano plot showing that there were not statistically significant lipids differentiating favorable and unfavorable outcome groups ($p < 0.05$ FDR-corrected, $|FC| > 1.5$). D) A volcano plot analyzing late samples showing statistically significant lipids (up 5, down 103) differentiating favorable and unfavorable outcome groups ($p < 0.05$ FDR-corrected, $|FC| > 1.5$). E) Random forest algorithm analysis identifying 15 of the most important lipids separating the favorable and unfavorable groups according to mean decrease accuracy (number of trees: 500, number of predictors 7). F) Partial least squares discriminant analysis heatmap identifying top 10 lipids calculating variable importance projections (VIP scores). Distance measure: Euclidean distance. Separation of the favorable and unfavorable groups is clearly observed in the heatmap.

based on individual performance metrics and the candidates' collective ability to enhance the prognostic accuracy for favorable outcomes in brain injury patients. The ensemble of these lipids, comprising various classes such as PCs, FFAs, SMs, TAGs, and lysophosphatidylethanolamines (LPE), was identified through a rigorous machine learning process that included feature importance ranking from a random forest algorithm and linear support vector machine (SVM) analysis.

Our refined combinatory biomarker model demonstrated high prognostic accuracy for favorable outcomes, with an odds ratio of 9.53 (95 % CI: 3.36–26.99, $p < 0.0001$). The model yielded an AUC of 94.7 % (95 % CI: 90–99 %), a sensitivity of 89 %, and a specificity of 92 % ($p < 0.0001$) (Fig. 5B). The selected lipids PC(16:0/20:4), FFA(18:4), FFA (22:6), PE(P-18:1/18:1), FFA(18:2), SM(20:1), TAG46:3-FA12:0, FFA (18:3), TAG47:1-FA14:0, CER(18:0), TAG47:2-FA15:0, and LPE(16:0) resulted an prognostic equation with canonical scores:

$$\begin{aligned}\text{Canonical score} = {} & 0.798[\text{PC}(16:0/20:4)] - 0.511[\text{FFA}(18:4)] - 0.123[\text{FFA}(22:6)] - 0.524[\text{PE}(\text{P}-18:1/18:1)] - 0.045[\text{FFA}(18:2)] + 0.356[\text{SM}(20:1)] + 0.038[\text{TAG46}:3-\text{FA12}:0] - 0.3173[\text{FFA}(18:3)] - 0.136[\text{TAG47}:1-\text{FA14}:0] - 0.800[\text{CER}(18:0)] + 0.366[\text{TAG47}:2-\text{FA15}:0] + 0.281[\text{LPE}(16:0)]\end{aligned}$$

3.7. Functions of the identified lipids

We further analyzed our lipidomic data of the 153 identified

Table 3
Statistically ten most significant lipid compounds differentiating favorable and unfavorable outcome in univariate analyses ($p < 0.05$, false discovery rate (FDR) corrected).

Lipid	p-value	p-value, FDR-corrected
TAG46:2-FA12:0	3.7×10^{-05}	<0.01
PC(16:0/20:4)	6.3×10^{-05}	<0.01
TAG46:2-FA16:0	6.4×10^{-05}	<0.01
TAG46:0-FA12:0	6.8×10^{-05}	<0.01
TAG47:1-FA14:0	1.0×10^{-04}	<0.01
TAG44:0-FA12:0	1.0×10^{-04}	<0.01
TAG46:3-FA12:0	1.1×10^{-04}	<0.01
TAG47:2-FA15:0	1.2×10^{-04}	<0.01
TAG47:1-FA15:0	1.4×10^{-04}	<0.01
TAG46:1-FA12:0	1.5×10^{-04}	<0.01

lipidome candidates ($p < 0.05$, FDR-corrected) for enriched functions. These lipids were subjected to a targeted enrichment analysis to discern potential dysregulation in metabolic pathways associated with clinical outcomes. The analysis revealed lipid classes and pathways that were significantly enriched ($p < 0.05$ FDR-corrected), suggesting a distinct lipidomic profile associated with the later phase of brain injury recovery or progression (Table 4 and Supplemental Table S9). We identified enrichments in sphingolipid metabolism and sphingolipid signaling which have been implicated in inflammatory responses and neuronal repair mechanisms. In addition, we identified enriched necroptosis functions, a form of programmed cell death, that has been increasingly recognized for its role in brain injuries.

Interestingly, each lipid in the combinatory biomarker panel contributes unique physicochemical properties and biological functions, reflecting the multifaceted nature of brain injury pathophysiology. For instance, PC(16:0/20:4) is central in cell membrane composition and signaling, while FFA(18:4) and FFA(22:6) are crucial in inflammation and resolution processes.

4. Discussion

Interest in small molecules, such as metabolites and lipids, as potential biomarkers for ABI diagnostics has increased due to limitations in the use of protein biomarkers. The brain is rich in lipids, but comprehensive analysis of molecular lipids has rarely been performed in acute brain insults in humans. In this study, we conducted an extensive analysis of the lipidomic profile in serum samples from ABI patients at both early and late timepoint. Our objective was to compare lipidomic changes with patients' mRS-measured outcomes, aiming to identify lipidomic patterns associated with outcome changes. Our goal was to identify specific lipids that could serve as potential biomarkers for outcome prognostication. Initially, we examined distinct disease groups (aSAH, IS, and TBI). Subsequently, we refined our analysis to focus on the disparities between the early and late sample sets. Our findings indicated that lipidomic changes in ABI patients, associated with outcomes, became notably significant during the later stages. We identified 12 key lipids, distinct in the late sample group, which demonstrated a robust association with patient outcomes. The development of a prognostic equation model based on these 12 lipids represents an important advancement, offering a prognostic tool for assessing recovery trajectories in ABI patients. Our study suggests that this model is applicable irrespective of the type of studied acute brain injury, indicating its potential for versatile clinical application across various ABI scenarios and conserved lipid responses in different ABIs.

4.1. Temporal nature of lipids

Lipid metabolism, known for its dynamic nature, adapts and transforms in response to varying physiological conditions (Hornburg et al., 2023). A pivotal discovery in our study is the temporal alteration of the lipidome following ABIs. This temporal dimension of lipidomic changes manifested distinctly at different stages post-injury. In the initial phase (early samples), our analysis revealed no statistically significant difference ($p < 0.05$, FDR-corrected) in lipid levels when comparing groups with favorable versus unfavorable outcomes. This initial homogeneity in lipid profiles, however, evolved markedly over time. At later stages post-injury, we observed a clear divergence: 153 lipids emerged as significant differentiators of outcomes ($p < 0.05$, FDR-corrected).

The temporal impact observed in lipidomic alterations can be attributed to a myriad of factors. Lipids, being integral to inflammation response, tissue repair, and neurological recuperation – all critical aspects of ABI pathology – begin to exhibit noticeable metabolic shifts only after the immediate aftermath of the primary injury (Adibhatla and Hatcher, 2007). This suggests that the initial impact of the primary injury may not induce immediate detectable changes in the lipidome. However, as the injury cascade progresses, these lipidomic processes might reach a threshold of detectability, thereby becoming more pronounced in later blood samples. Furthermore, the markers indicative of these recovery processes may only begin to manifest in the later stages of the acute phase. This nuanced understanding of temporal lipidomic changes underscores the complex interplay between lipid metabolism and brain injury recovery, providing valuable insights into the prognostic potential of lipidomic profiling in ABIs. Clinically, this temporal dimension suggests a valuable window in which lipidomic profiling could be most informative, offering a targeted approach for assessing recovery trajectories. By understanding when these lipid markers become predictive of outcomes, development of the prognostic and even therapeutics is enhanced. More temporal studies are required to understand evolving lipidomic state of the brain injuries.

4.2. Identified circulating lipids

Lipids can be broadly classified into five main groups, namely sterols, glycerophospholipids (GP), glycerolipids (GL), sphingolipids (SL), and fatty acids (FA). These groups further subdivide into numerous subgroups based on their structural characteristics (Hussain et al., 2020). After detecting 153 significantly outcome-differentiating lipids in the late sample group, we performed different machine learning algorithms to find the best lipid candidates for prognostic modeling. We identified lipids that included FFA, TAG, PC, PE, SM, CER, and LPE subgroups.

In prior rodent lipidomic studies addressing traumatic brain injuries, FFAs, particularly polyunsaturated fatty acids (PUFAs), have exhibited an increase post-injury. Changes in FFA levels are visible in both plasma and CSF (Hogan et al., 2018; Pilitsis et al., 2003; Martha et al., 2023). This finding aligns with our results of increasing FFAs acting as an unfavorable outcome predictor, reinforcing the significant role of FFAs in ABIs. An earlier metabolomics TBI study showed that two medium-chain fatty acids (octanoic and decanoic acid) were positively associated with the severity of TBI and with unfavorable patient outcomes (Orešič et al., 2016). In a larger prospective lipidomics study, phospholipids such as lysophosphatidylcholines, ether PCs (O-PCs), and SMs, were found to be associated with the severity of TBI and patient outcomes (Thomas et al., 2022).

Cellular damage is considered a key factor initiating the release of FFAs (Hogan et al., 2018). After a brain injury, an energy deficit ensues in the affected region, resulting in the release of glutamate and calcium-ion overload (Katayama et al., 1990; Osteen et al., 2001). These events trigger the activation of phospholipase A2 and C (PLA2 and PLAC), subsequently liberating FFAs from cell membrane phospholipids (Wieloch and Siesjö, 1982; Yang et al., 2017). Additionally, PLA2 hydrolyzes PCs, leading to a decrease in its concentration. This finding, observed in our study, has also been validated in other studies focusing on stroke, further affirming our study cohort's results (Yang et al., 2017). One of the most intriguing FFAs is FFA 22:6, also known as docosahexaenoic acid (DHA), which has demonstrated significant neuroprotective properties related to cell survival, synaptogenesis, and

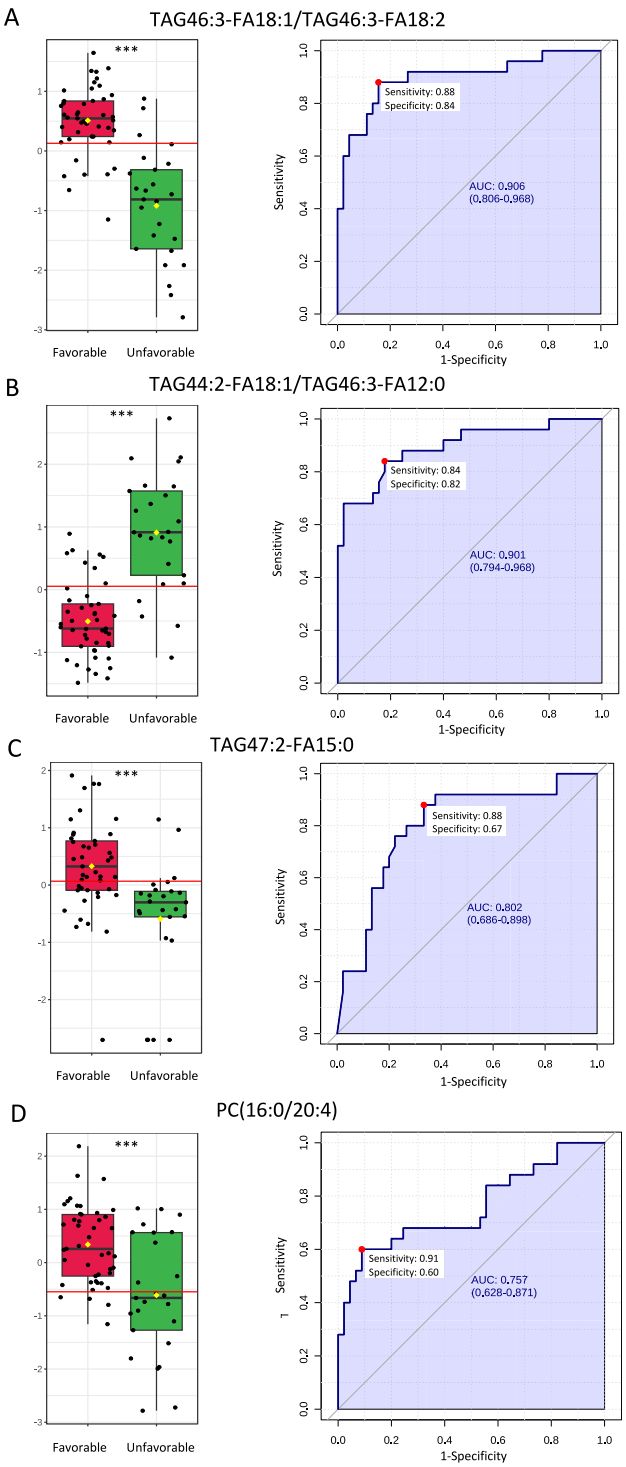

Fig. 4. Univariate and the receiver operating characteristic (ROC) analyses for biomarkers. A) The best performing increased lipid ratio TAG46:3-FA18:1/TAG46:3-FA18:2 ($p < 0.0001$ false discovery rate (FDR) corrected) differentiated favorable outcome patients from unfavorable outcome patients with 84 % sensitivity and 82 % specificity (Area under the curve (AUC) 90.0 % with 95 % confidential interval (CI) = 79–97 %). B) The best performing decreased lipid ratio TAG44:2-FA18:1/TAG46:3-FA12:0 ($p < 0.0001$ FDR-corrected) differentiated favorable outcome patients from unfavorable outcome patients with 88 % sensitivity and 84 % specificity (AUC 90.1 % with 95 % CI = 81–97 %). C) The best-performed lipid TAG47:2-FA15:0 ($p < 0.0001$ FDR-corrected) differentiated favorable outcome patients from unfavorable outcome patients with 88 % sensitivity and 67 % specificity (AUC 80.0 % with 95 % CI = 69–90 %). D) The most important lipid identified in random forest analysis PC(16:0/20:4) ($p < 0.0001$ FDR-corrected) differentiated favorable outcome patients from unfavorable outcome patients with 91 % sensitivity and 60 % specificity (AUC 75.7 % with 95 % CI = 63–87 %). Box plot presented ± IQR, yellow diamond indicates mean concentration, and the horizontal red line indicates the optimal cut-off. The optimal cut-off point for the prognostic test was determined by calculating the Youden index (red dot). The 95 % confidence intervals were calculated using 500 bootstrappings. Box plot y-axis = log2 transformed concentration (μM), x-axis: favorable (red) and unfavorable outcome (green) groups. ***$p <$ 0.0001 FDR-corrected. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

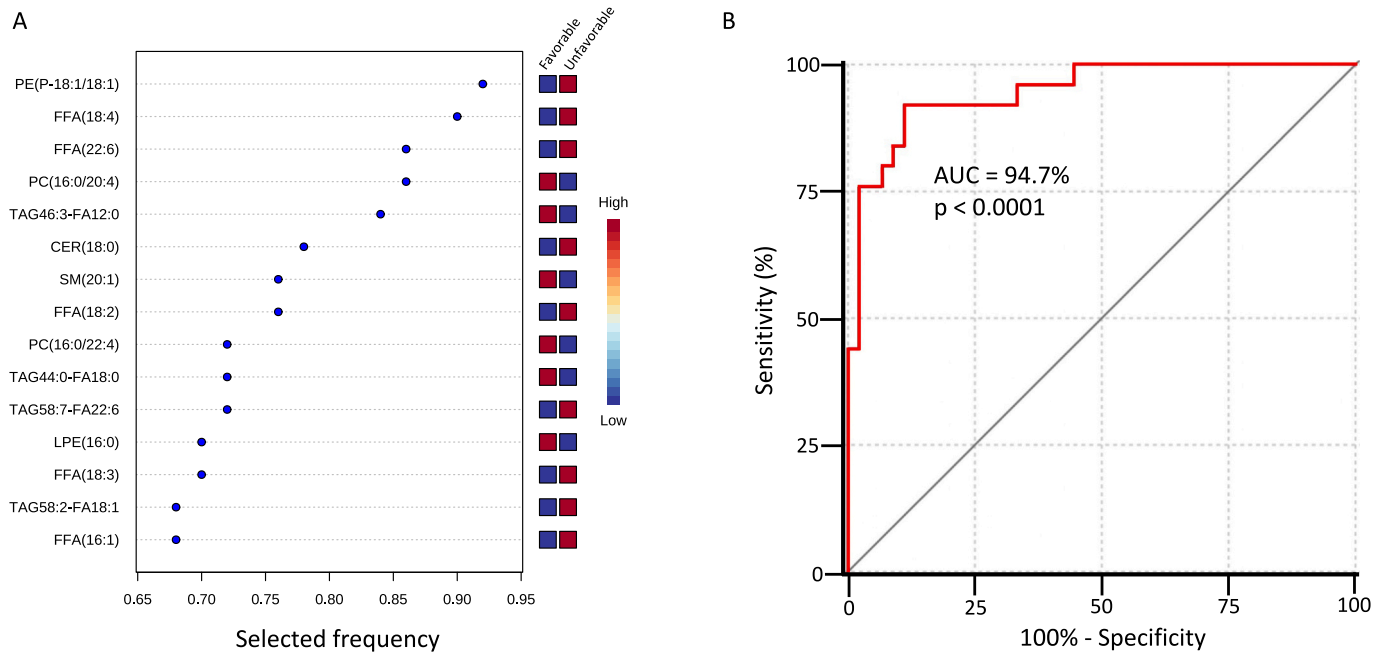

Fig. 5. Supervised linear support vector machine (SVM) algorithm: Identifying Lipid Features for Prognostic Biomarker in Late Cohort A) Linear SVM analysis showing the top 15 lipids (according to the selected frequency in the models) differentiating the favorable outcome patients from unfavorable outcome patients. B) The receiver operating characteristic curve of 12 identified candidate lipids (PC(16:0/20:4, FFA(18:4), FFA(22:6), PE(P-18:1/18:1), FFA(18:2), SM(20:1), TAG46:3-FA12:0, FFA(18:3), TAG47:1-FA14:0, CER(18:0), TAG47:2-FA15:0 and LPE(16:0)) in linear discriminant analysis prognosing favorable outcome: odds ratio 9.53 (95 % confidence interval (CI) 3.36–26.99); Area under the curve (AUC) = 94.7 %, 95 % CI = (90–99 %), $p < 0.0001$, with 89 % sensitivity and 92 % specificity. Linear discriminant analysis of these 12 lipids resulted an equation with canonical scores: 0.798[PC(16:0/20:4] – 0.511[FFA(18:4)] – 0.123[FFA(22:6)] – 0.524[PE(P-18:1/18:1)] – 0.045[FFA(18:2)] + 0.356[SM(20:1)] + 0.038[TAG46:3-FA12:0] – 0.3173[FFA(18:3)] – 0.136[TAG47:1-FA14:0] – 0.800[CER(18:0)] + 0.366[TAG47:2-FA15:0] + 0.281[LPE(16:0)].

Table 4
Pathway enrichment analysis of 153 lipids ($p < 0.05$ false discovery rate corrected (FDR)-corrected) differentiating favorable and unfavorable outcome. Three differentiating functional pathways were identified ($p < 0.05$ FDR-corrected).

Pathway name	Pathway lipids	p-value	FDR-corrected p-value*
Sphingolipid metabolism	21	6,7E-06	0,0001
Sphingolipid signaling pathway	9	4,62E-05	0,0005
Necroptosis	4	0,004	0,028

* Benjamini-Hochberg method.

neuroinflammation (Kim, 2014; Belayev et al., 2018). Positive findings from stroke-related studies have indicated the potential benefits of DHA administration (Belayev et al., 2018; Sun et al., 2022; Wu et al., 2023). Our observations revealed a notable increase in DHA concentration, particularly within the group experiencing unfavorable outcomes. This trend aligns with the hypothesis that DHA levels naturally escalate more significantly in response to severe injuries, which are often characterized by extensive damage to cell membranes (Duris et al., 2018; Mckee and Daneshvar, 2015). Consequently, the heightened DHA concentration in our study's unfavorable outcome group likely reflects this correlation, suggesting that more severe brain injuries trigger a more pronounced elevation in DHA levels.

TAGs are known to play a crucial role in neuroinflammation and neuronal apoptosis (Kuo et al., 2020). However, the exact role of TAGs in ABIs still remains unclear. In stroke studies, findings are conflicting, with inconsistent evidence regarding whether high or low TAG concentrations are associated with poorer outcomes (Yang et al., 2017; Huang et al., 2022; Weir et al., 2003). On the other hand, Kuo et al. (2020) demonstrated a significant elevation in TAG in mouse models of traumatic brain injuries, reaching peak concentrations one day post-injury and subsequently returning to baseline levels approximately three days post-injury (Kuo et al., 2020). The likely reason for the observed decrease in TAGs in our late samples of unfavorable outcome patients may be attributable to this temporal pattern. TAGs seem to act as relatively rapid markers but can still serve as effective prognostic ABI indicators by concentrating their dynamic fluctuations. Interestingly, our study revealed the best-performing individual lipid (AUC 80 %, sensitivity 88 %, and specificity 67 %) to differentiate favorable and unfavorable outcomes which was TAG47:2-FA15:0, which showed a decrease in patients with unfavorable outcomes.

Ceramides, complex lipids belonging to sphingolipids, are known to play a role in both cell membrane formation and apoptosis (Kun et al., 2020). In our study, CER 18:0 showed an increase in patients with unfavorable outcomes. Similar findings of elevated ceramide levels in stroke and traumatic brain injuries have been reported in several rodent and human studies (Sheth et al., 2015; Huang et al., 2022; Kun et al., 2020; Yuan et al., 2023). Gui et al. observed a significant correlation between the rise in ceramides and the severity of stroke (Kun et al., 2020). These consistent findings align with our results, reinforcing ceramides as robust prognostic biomarkers in acute brain injuries.

4.3. Prognostic model

We developed a prognostic model that leverages lipidomic profiling to predict outcomes in patients with ABIs. Our model, underpinned by the analysis of 12 key lipids, stands as a significant advancement in ABI research, particularly in its ability to discern between favorable and unfavorable outcomes. This achievement is underscored by the model's good performance: AUC of 94.7 %, along with an optimum performance with a sensitivity of 89 % and specificity of 92 %. The model's strength lies in its capacity to overcome the inherent heterogeneity of ABIs, encapsulating the key conserved physiological impacts of aSAH, IS, and

TBI. The temporal dimension of our lipidomic analysis, distinguishing between early and late post-injury intervals, has been pivotal in revealing the dynamic nature of lipidomic alterations. This temporal differentiation not only elucidates the evolving metabolic response following ABIs but also reinforces the credibility of our identified lipid candidates. Importantly, the model's applicability across aSAH, IS, and TBI demonstrates its versatility in handling the diverse pathophysiology of brain injuries. This model not only aids in assessing recovery trajectories but also supports personalized treatment planning. Such an approach could also help optimize and improve patient management. In future, the integration of lipidomic profiling into routine clinical practice could enable personalized treatment planning, adjusting therapeutic strategies based on each patient's unique lipidomic response post-injury. Further studies are warranted to validate these findings in larger, multicenter cohorts and to explore the utility of lipid markers at more diverse time points. Lipidomic-based models have the potential to become a part of ABI management, supporting early prognosis, tailored treatment pathways, enhanced clinical decision-making, and resource allocation.

In the context of our study, where an independent validation cohort was not available, the temporal changes serve a crucial role. They offer statistical substantiation of our findings, as the lipids that demonstrate marked alterations between the early and late post-injury stages are likely to be more directly involved in the pathophysiological processes of ABI recovery (Velten and Stegle, 2023). This temporal association strengthens the argument for these lipids as not merely correlational markers but as potential active participants in the biological response to brain injury. This suggests that the identified lipids are not random fluctuations but are consistently associated with the progression or amelioration of injury. Therefore, while external validation remains a future goal and a standard, the temporal profiling in our study provides a noteworthy argument for the reliability and relevance of the identified lipid biomarkers in ABI prognosis.

4.4. Lipid functions enriched after ABIs

The most significant findings in the enrichment and pathway analyses were related to GPs and SLs. GPs are significant components in the formation of membrane structures, functioning both in cell membranes and within mitochondria (Hishikawa et al., 2014). Brain injury causes damage to cell membranes and disrupts mitochondrial function due to oxygen deprivation, partially explaining their notable enrichment (Yang et al., 2017; Nessel and Michael-Titus, 2021). The previously mentioned CER and SM are part of the SL group and play a significant role in brain injuries (Sheth et al., 2015). Their presence in our analyses further reinforces their position as important functions depicting brain damage (Sheth et al., 2015; McCluskey et al., 2022; Yuan et al., 2023). The current scarcity of lipid studies and limited understanding in the realm of comprehensive lipid pathways and functions undoubtedly constrain the breadth of these results. Yet, this gap in knowledge opens a unique avenue for research.

5. Limitations

Our study recognizes certain limitations that should be considered when interpreting our findings. Firstly, the uneven distribution of patients with favorable and unfavorable outcomes in our sample may introduce bias into our comparative analysis, potentially impacting the reliability of observed relationships. To address this, future studies could employ strategies like propensity score matching to create balanced groups with similar baseline characteristics, stratified sampling to ensure equal representation from the outset, or weighted analysis to adjust for imbalances.

Secondly, the modest sample size of 70 participants potentially constrains the generalizability and statistical power of our findings. Although we took measures to ameliorate this by analyzing the serum lipidome at two distinct timepoints post-admission, the validity of our results would be bolstered by replicating this study with a larger, preferably multicentric cohort. A larger sample size would enhance the statistical power, enabling more robust detection of subtle lipidomic differences and increasing the reliability of our conclusions. Moreover, a multicentric cohort would allow us to assess the lipidomic profiles across diverse populations and healthcare settings, enhancing the generalizability of our findings and supporting broader application of these lipid biomarkers for prognostic use in acute brain injury.

We acknowledge that certain variables, such as the administration of propofol and nutritional support, may influence lipidomic changes in the studied individuals. However, it was not feasible to control for these factors in this study. It is important to highlight the heterogeneity in the treatment protocols for the different types of ABIs under investigation. For instance, patients with aSAH and aSDH were initially treated in the intensive care unit and frequently required longer sedation to manage complications such as high ICP, hydrocephalus, or bleeding. In contrast, IS patients were primarily managed in the stroke unit, with the vast majority not necessitating sedation. This variability in drug administration and nutritional support suggests that it is unlikely for specific treatments, such as propofol, to be the primary drivers of the significant lipidomic changes observed, given the differences in treatment protocols across patient groups. In future studies, strategies such as randomization, stratified sampling, or propensity score matching could help control for these confounding factors, minimizing their impact and strengthening the reliability of the observed lipidomic associations.

Methods of the study have certain limitations. The use of cross-validation within PLS-DA and machine learning models offers internal validation but may not fully capture external variability, potentially affecting generalizability. Additionally, feature selection in algorithms like Random Forest and SVM is influenced by parameter choices, which may impact reproducibility across different datasets. These aspects suggest that while our findings are robust within this dataset, validation with external cohorts would strengthen their reliability.

Despite these limitations, our study employed rigorous methods and leveraged the temporal dimension of lipidomic analysis, which provides strong support for the reliability of our findings. In the absence of an independent validation cohort, the observed temporal changes in lipid profiles serve a critical role in substantiating our results. Lipids that demonstrated marked alterations between early and late post-injury stages are likely to be involved in ABI recovery processes, reinforcing their relevance as prognostic biomarkers. This temporal profiling suggests that the identified lipids are not merely correlational but reflect active biological responses to brain injury. While external validation remains a future goal, the consistency of these findings across different ABI types highlights the robustness of the identified lipid biomarkers and underscores their potential for ABI prognosis.

6. Conclusions

In this study, we examined the lipidomes of patients with different acute brain injuries (aSAH, IS, TBI) with varying outcomes. Our results suggest that temporal changes in lipid levels can provide valuable information about patient outcomes across different acute brain injuries, and lipidomic changes become significantly apparent only at a later point. The developed prognostic model with combination of lipids proved to be effective in distinguishing between outcomes. The lipid patterns identified in our study not only offer new avenues for prognostication across various acute brain injuries but also present promising candidates for mechanistic studies to understand the underlying pathobiological processes. To translate these insights into clinical practice, it is imperative to validate our findings through expanded research involving independent patient cohorts.

Consent for publication

Not applicable.

Study approval and ethics

This study (T291/2016) received approval from the Turku University Hospital's Institutional Review Board and Ethics Committee and was conducted in line with the principles of the Declaration of Helsinki, including its later amendments. If a participant's severe acute illness precluded them from providing consent, written informed consent was secured from their legal representatives. The study conformed to all pertinent Finnish laws and regulations.

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CRediT authorship contribution statement

Santtu Hellström: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Antti Sajanti:** Writing – original draft, Visualization, Investigation, Data curation. **Abhinav Srinath:** Writing – original draft. **Carolyn Bennett:** Writing – original draft. **Romuald Girard:** Writing – original draft, Conceptualization. **Aditya Jhaveri:** Writing – review & editing. **Ying Cao:** Formal analysis. **Johannes Falter:** Writing – review & editing. **Janek Frantzén:** Writing – review & editing. **Fredrika Koskimäki:** Writing – review & editing, Investigation. **Seán B. Lyne:** Writing – review & editing. **Tomi Rantamäki:** Writing – review & editing, Conceptualization. **Riikka Takala:** Writing – review & editing. **Jussi P. Posti:** Writing – review & editing. **Susanna Roine:** Writing – review & editing, Investigation. **Sulo Kolehmainen:** Writing – review & editing, Project administration. **Kenneth Nazir:** Writing – review & editing, Methodology. **Miro Jänkälä:** Writing – review & editing. **Jukka Puolitaival:** Writing – review & editing. **Melissa Rahi:** Writing – review & editing, Investigation. **Jaakko Rinne:** Writing – review & editing. **Anni I. Nieminen:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Eero Castrén:** Writing – review & editing, Resources. **Janne Koskimäki:** Writing – original draft, Visualization, Supervision, Resources, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors report no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.nbd.2024.106762.

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