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Journal: Proteomics

Year: 2022 Dec 28

Pages: e2200078

DOI: 10.1002/pmic.202200078

Blood proteome of Acute Intracranial Hemorrhage in infant victims of Abusive Head Trauma

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Received: MONTH DD, YYY; Revised: MONTH DD, YYY; Accepted: MONTH DD, YYY

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/pmic.202200078.

Abbreviations: AHT: Abusive Head Trauma; ICH: Intracranial Hemorrhage; AM: antemortem; UFM: Unit of Forensic Medicine; PM postmortem; PMB: postmortem blood; AMB antemortem blood; PMD postmortem delay; LC-MS: liquid chromatography mass spectrometry; DDA: data dependent acquisition; DIA: data-independent acquisition; SIDS: Sudden Infant Death Syndrome; MAC: Membrane Attack Complex

Statement of significance of the study

Nearly one-third of child victims of AHT with brain lesions was misdiagnosed during previous medical consultations. The current study presents non-hypothesis driven analysis of blood samples of infant victims of AHT via new generation DIA-MS proteomics. The approach is an initial step toward development of clinical screening tests of AHT cases in young children.

Abstract

Abusive head trauma (AHT) is a leading cause of mortality and morbidity in infants. While the reported incidence is close to 40 cases per 100'000 births/year, misdiagnoses are commonly observed in cases with atypical, subacute or chronic presentation. Currently, standard clinical evaluation of inflicted intracranial hemorrhagic injury (ICH) in infants urgently requires a screening test able to identify infants who need additional investigations. Blood biomarkers characteristic of AHT may assist in detecting these infants, improving prognosis through early medical care. To date, the application of innovative omics technologies in retrospective studies of AHT in infants is rare, due also to the blood serum and cerebrospinal fluid of AHT cases being scarce and not systematically accessible. Here, we explored the circulating blood proteomes of infants with severe AHT and their atraumatic controls. We discovered 165 circulating serum proteins that display differential changes in AHT cases compared with atraumatic controls. The peripheral blood proteomes of pediatric

AHT commonly reflect: i) potentially secreted proteome from injured brain, and ii) proteome dysregulated in the system's circulation by successive biological events following acute ICH. This study opens up a novel opportunity for research efforts in clinical screening of AHT cases. **1. Introduction**

Abusive head trauma (AHT), which includes Shaken Baby Syndrome (SBS), is a severe form of physical child abuse. It is caused by inflicted blunt impact on, or the violent shaking of, infants and small children, resulting in intracranial injuries and retinal hemorrhages [1,2]. AHT is the leading cause of death from traumatic brain injury (TBI) in infants under age two. Symptoms of AHT are non-specific and currently, there is no screening test to help clinicians identify infants who need additional investigations. It follows, then, that nearly one-third of child victims of AHT with brain lesions is misdiagnosed during a previous consultation [3,4], with almost three medical visits being necessary before diagnosing AHT [3,4], resulting in ongoing child abuse and increased morbidity and mortality. Pediatric AHT cases rely on medical treatments identical to those of accidental head trauma known to display a better prognosis in young infants [4]. In this context, pediatricians urgently require a screening test for infant victims of AHT.

A novel concept of data-driven discoveries integrating omics approaches is predicted to revolutionize clinical diagnostics in pediatrics [5]. A deep omics profiling of blood proteomes in infant victims of AHT is therefore a prerequisite for the discovery of: i) diagnostic and prognostic protein markers of AHT cases, and ii) medical treatments through elucidation of the molecular pathways characteristic of trauma to the developing brain. Over the past decade, developments in the performance of mass spectrometry (MS) based proteomics platforms have resulted in remarkable throughput, reproducibility, and quantification accuracy compared with past techniques [6-8]. In particular, MS platforms that rely on a data-independent acquisition (DIA-MS) strategy ensure consistency of

proteome analysis across human specimens by creating a permanent record of all ionized peptides from a tested sample [7-10]. Yet the application of omics approaches for discovery purposes is very rare in the biological specimens of child ICH and inexistent in the AHT cases. To date, postmortem blood (PMB) specimens remain largely unexplored in the biomarker discovery studies of AHT cases in infants. The serum samples of children younger than 2 with concerns regarding AHT have not been easily accessible, and the animal models that replicate the various aspects of human TBI showed considerable clinical limitations [11,12]. The recent registration of forensic biobanks via medico-legal structures covering the specimens of healthy pediatric populations underrepresented in standard clinical biobanks will open great opportunities for novel discoveries in pediatric TBI [13]. Here, we hypothesized that proteins sourced from brain tissue leakage would likely be elevated in the peripheral PMB and serum of children admitted to hospital with AHT, compared with atraumatic infants. We retrospectively explored blood proteome in AHT cases compared to child who died of sudden infant death syndrome (SIDS), via untargeted proteomics of serum samples collected before the infants' death at intensive care units (ICUs) and PMB specimens collected during medico-legal autopsies of the same infants.

In total, we analyzed 52 distinct specimens collected at two hospital units as ante– or postmortem blood and serum samples corresponding to 26 infants, 7 of whom were victims of AHT. This study is an initial step toward development of clinical screening of ICH in AHT cases in young children.

2. Materials and Methods

2.1. Clinical Samples

AHT cases were evaluated according to the guidelines of the French High Authority of Health[14]. The case-control retrospective study (Project ID 2021-01304) has been approved by the Research Ethics Committee of Geneva, Switzerland. Antemortem serum and blood collected in the ICU and PMB samples from AHT cases and atraumatic controls (infants died of SIDS) were collected from 2013 through 2018 in medico-legal context (**Table 1**). We analyzed a total of 52 samples, distributed in three study groups: i) PMB from AHT (N=7) and SIDS control cases (N=19); ii) their antemortem blood (AMB) pairs from all AHT cases (N=7) and seven SIDS controls (N=7); and iii) their antemortem sera pairs collected at ICUs from all AHT cases (N=7) and five SIDS controls (N=5, **Table1**). Proteins were isolated from the different specimens of peripheral blood and subjected to proteomic analysis. To report the results of technical variations and for objective quality control analysis of clinical samples prior to any proteomics experiment, we used repeated sampling from ante– and postmortem blood pairs of study participants and fresh blood from a single healthy volunteer (i.e., four experimental replicates of whole processes). We also performed 5 repeated injections from selected serum and blood samples across a measurement queue in the mass spectrometer (i.e., 10 technical replicates). In total, we performed 14 analytical replicates that include 4 "whole process" and 10 "MS-injection" replicates.

2.2. Proteomic analysis

Proteomic analysis of either 6 µL crude blood (post– and antemortem) or 4 µL serum samples was performed as described elsewhere [9]. Specifically, blood was collected in PET sterile vacuum tubes (BD Vacutainer, REF: 368920) by venous blood sampling. The antemortem serum samples were prepared at ICU starting from whole blood collected in sterile vacuum tubes (BD Vacutainer, REF: 366430) and left for 30 minutes at room temperature before centrifugation at 1500 g for 10 minutes. Each sample (blood or serum)

was diluted with 100mM AMBIC buffer (100mM NH4CO3, in mqH2O) at final volume of 50 µL and desalted by G-10 gel filtration cartridges (The Nest Group, Inc.). Prior to blood or serum proteome digestion to peptides, sample denaturation was performed in 10 M urea buffer followed by standard process of protein reduction and alkylation with 10 mM tris(carboxyethyl)phosphine (Sigma-Aldrich) and 20 mM iodoacetamide (Sigma-Aldrich), respectively. The urea concentration was adjusted to 1M by diluting samples with 50mM of AMBIC buffer. Overnight proteome digestion at 37°C was processed using sequencing-grade porcine trypsin (Promega) at a protease/protein ratio of 1:30. To remove undigested proteome and sample impurities, the collected peptides were purified with C18 Silica MicroSpin columns (The Nest Group, Inc.) at low-speed centrifugation (i.e., 400 g for 2 minutes) and by five successive washing steps in 0.1% aqueous formic acid (FA) with 2% acetonitrile (ACN) prior to sample LC-MS analysis. The peptides were solubilized in 20 µL of 0.1% aqueous FA with 2% ACN, adjusted to a concentration of 1 µg/µL of peptides, and an aliquot of retention time calibration peptides (i.e., 1 pmol/µL iRT-Kit Biognosys) spiked in equal amounts into each sample prior to MS injection to correct relative retention time differences between MS runs.

2.3. Liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) method

LC-ESI-MS/MS was used for performing data-independent acquisition (DIA) on an Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific) equipped with an Easy nLC1200 liquid chromatography system (Thermo Fisher Scientific). Peptides were trapped on a Acclaim pepmap100, C18, 3µm, 75µm x 20mm nano trap-column (Thermo Fisher Scientific) and separated on a 75 µm x 500 mm, C18 ReproSil-Pur (Dr. Maisch GmBH), 1.9 µm, 100 Å, home-made column.

The analytical separation was run for 135 min using a gradient of aqueous solvent A and organic solvent B. DIA was performed with MS1 full scan at a resolution of 60,000 (FWHM) followed by 30 DIA MS2 scan with variable windows. MS1 was performed in the Orbitrap with an AGC target of 1 x 106, a maximum injection time of 50 ms and a scan range from 400 to 1240 m/z. DIA MS2 was performed in the Orbitrap using HCD at 30%. Isolation windows was set to 28 *m/z* with an AGC target of 1 x 106 and a maximum injection time of 54 ms.

For generation of project specific spectral library, we performed data-dependent acquisition (DDA) of samples, with the longer analytical separation running for 180 min using a gradient of aqueous solvent A (H2O/ formic acid (FA) 99.9%/0.1%) and organic solvent B (CH3CN/H2O/FA 80.0%/19.9%/0.1%). DDA was performed with MS1 full scan at a resolution of 120'000 FWHM followed by as many subsequent MS2 scans on selected precursors as possible within 3 second maximum cycle time. MS1 was performed in the Orbitrap with an AGC target of 4 x 105, a maximum injection time of 50 ms and a scan range from 400 to 1250 m/z. MS2 was performed in the Orbitrap at a resolution of 30'000 FWHM with an AGC target at 5 x 104 and a maximum injection time of 54 ms. Isolation windows was set at 1.6 m/z and 30% normalized collision energy was used for higher-energy collisional dissociation (HCD).

2.4. Analysis of generated MS data

Analysis of infant samples recorded via DIA-MS. Raw data (i.e., 52 raw DIA-MS files) were processed by commercial proteomic software package Spectronaut (version:14.8.201029.47784, Biognosys, https://biognosys.com/software/spectronaut/) [15,16]. For differential analysis, Spectronaut DIA proteomics experiments were created either for serum or PMB cohort. Quantitative data matrices for each sample type were generated with

input of corresponding spectral library and respective DIA-MS raw data files. The proteins and peptide matrices were generated by default settings (i.e., BGS factory settings). Cross-run data normalization was performed on the whole data sets of PMB and serum based on global median normalization of precursor intensities (**Figure S1A**). Normalized protein and peptide data were exported from Spectronaut software as csv. files for further analysis. Technical replicates from selected serum and blood samples display high positive correlation (Spearman's rho ≥ 0.9) across proteins quantified between the same patient samples injected randomly across a measurement queue in the mass spectrometer (**Figure S1B**).

Generation of Spectral library. MS files acquired in parallel in DDA mode (i.e. 33 raw MS files) were used to create a crude blood- or serum- specific spectral library. For creation of spectral libraries we used default parameters against the ex_sp_9606_decoy.fasta database (the reviewed canonical Swiss-Prot complete proteome database for human, released 2014-01-24, entries 40'544) appended with common contaminants, reversed sequence decoys and iRT peptides. Included were trypsin digestion allowing 2 missed cleavages and 'Carbamidomethyl (C)' as static and 'Oxidation (M)' as variable modifications while minimum and maximum peptide length was set to 7 and 52 amino acids, respectively. The mass tolerances were set to mode "dynamic" that is software determined tolerance based on extensive mass calibration and one-time correction factor was applied for precursor- and for fragment-ions. Unique proteins identified at 1% of protein false discovery rate (FDR) were included in libraries.

2.5. The generation of protein data matrices for group comparisons

The initial protein matrices corresponding to serum (i.e., 1051 Protein Groups and 7664 tryptic peptides) or PMB (i.e., 1133 Protein Groups and 10'504 tryptic peptides) were generated at 1% protein and peptide FDR based solely on tryptic peptide quantities. For data

comparison we avoided imputation of missing values and the unique proteins identified at 1% of protein FDR were filtered from each data according to criteria that each protein (i.e., UniProt ID) be quantified in more than half of the samples per each respective condition (i.e., 4/7 and 10/19 samples, in AHT and in the control group, respectively). Based on these criteria, we found a relatively low percentage of missing values in total protein matrices, 13% and 7% of quantified data points in PMB and serum cohort, respectively (**Figure S1C**). We quantified 862 and 518 confidently detected proteins across PMB and serum cohorts, respectively.

2.6. Quantification, statistical analysis and data visualization

R software for statistical computing and graphics (version: 3.6.1) was used for the data analysis and visualization. The computation of Spearman (Spearman's rho) or Pearson correlation coefficients (Pearson's r) between respective samples was performed with package ggplot2 (version 3.3.5.) and visualizations of correlation analysis was performed with package LSD (version 4.1-0) using heatscatter function. The heat map visualization of replicates was performed with R package 'corrgram' version 1.14. The coefficient of variations (CVs) of individual proteins were computed from their standard deviation (SD) of log2-transformed protein intensities divided by mean measurement. The violin plots representing relative protein quantification variabilities between samples were generated in ggplot2 (version 3.3.5).

For protein differential analysis, we first examined data distribution of the given data set by a generation of histogram plots in R and then performed statistical testing. We performed a 2-tailed Student t-test with significance set at nominal $P<0.05$ for PMB and serum cohort. In order to adjust for sample size at minimum 80% of statistical power of the analysis, we estimated the minimal expected effect size measure (i.e., Cohen's d) for significant and

upregulated proteins. The power of the analysis for each protein was estimated based on the sample size, degree of freedom, effect of size and statistical changes by R packages library(pwr) and library(rstatix). Due to protein variability potentially related to age differences, linear regression (LR) adjusted for infant age was performed only for selected brain-specific proteins upregulated in the serum or PMB.

Protein fold change (FC) was computed from a ratio of log2-transformed value of protein intensities (i.e., Log_2FC (AHT/Ctrl)). Volcano plots were generated from log2-transformed FC and –log10 transformed P-values of proteins detected in the AHT and control comparison of specimens of interest. Boxplots of individual proteins were generated from log2-transformed protein intensities across tested samples.

The initial list of brain specific proteins was downloaded from the human protein atlas database [17]. Gene ontology (GO) candidate classification and data visualization was performed with clusterProfiler R package via DOSE (version 3.14.3). Manhattan distances were used for hierarchical clustering of log2-transformed relative protein intensities, while non-scaled original data were used in pheatmap R package (version 1.0.12). Proteins upregulated in the serum of AHT cases with statistical significance were analyzed by The KEGG (Kyoto Encyclopedia of Genes and Genomes) database [18] and KEGG map search results for "pathway" classifications with the highest number of mapped candidates (i.e., hsa04610) were exported in KGML format. The network was visualized in Cytoscape [19] by using KEGGscape applications and pathway KGML format as input file.

2.7. Validation of Brain acid soluble protein 1 (BASP1) and Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 (ENPP2) serum levels by ELISA assay

For validation experiments, we prioritize the available sera of respective infants as the gold clinical specimen for quantification of laboratory markers [20]. The serum levels of AHT and atraumatic control infants were determined by ELISA commercial assays for two selected proteins with brain-enhanced expression (Human Brain Abundant, Membrane Attached Signal Protein 1 (BASP1) ELISA Kit, AssayGenie, Dublin, Ireland, and Human Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 (ENPP2) ELISA kit Cusabio, Houston, TX 77054, USA). For both protein analyses, we used 50 µL of human serum diluted with deionized water to 100 µL. The limit of detection (LOD) or the lowest protein concentration that could be differentiated from zero was 0.112ng/mL for BASP1 and 31.25ng/ml for ENPP2. The limit of quantification (LOQ) of tested clinical samples was 0.3ng/mL for BASP1 and 430.3ng/ml for ENPP2. All samples, blank solutions and standards were measured in experimental duplicates. We performed a 2-tailed Student t-test for group comparison.

3. Results

3.1. PMB reflects antemortem blood (AMB) proteome.

We anticipated that postmortem specimens routinely collected upon medico-legal examination represent a valuable source of biological information for future research on infant victims of AHT [13] (**Figure 1**). We adapted the serum proteomic workflow as described elsewhere [9] to full blood specimens. Then, based on quantified proteome, we defined the window of postmortem delay (PMD) for blood samples eligible for proteomics.

We primarily proceeded with fresh blood from a single healthy volunteer, and which corresponds to the reference sample collected within two hours prior to protein extraction and recorded on Lumos orbitrap LC-MS platform (**Figure 1,** mid and right panel). Two independent experimental replicates of reference blood samples showed statistically

significant positive correlation between quantified proteins (Pearson's r =0.95; P<2.2e-16, **Figure 2A**, left) and with the coefficient of variation (CV) between proteins below 5% (**Figure 2A**, right). This initially suggested high reproducibility of established workflow. We then analyzed proteome depth and reproducibility of postmortem and antemortem blood cohort, distributed over 26 distinct postmortem– and 14 distinct antemortem blood samples (see Methods section 2.1). We performed a data analysis framework with a targeted spectral library of peptides within a DIA-MS proteomic platform [7,8,21] (**Figure 1,** right), ensuring reproducible signal mining and quantification accuracy across samples [10,22]. To avoid quantification of potentially degraded proteins, our spectral library was limited to peptide transitions of fully tryptic peptides [23], and contained high-quality spectra of 18'460 peptides converging to 2485 protein groups**.** Our analysis detected slightly more proteins and peptides in the AMB compared with the PMB cohort, with a high protein overlap (i.e., 923 out of 1103 PMB proteins, or 83.6%) (**Figure 2B,** left). To retain consistency of quantification in further analysis, we used only those confident proteins that had been detected in most of the individual observations of smaller case groups (see Methods section 2.5), reducing the initial list of 1103 proteins to 862 confident blood proteins in PMB cohort (i.e., 4074 stripped peptide sequence, **Figure 2B**, right). To define the time window of PMD of samples eligible for proteomic analysis, we then investigated the protein correlations and the variability between paired ante– and postmortem blood samples on normalized protein intensities (**Figures 2C-D-E**). The AMB and PMB collected from the same patient at different units showed positive proteome correlation (Pearson's r =0.85) comparable to the experimental replicates from identical PMB samples (0.85 vs 0.89, **Figure 2C,** left and mid) and slightly worse than experimental replicates of fresh blood (0.85 vs. 0.95) **(Figure 2C,** mid and **2A,** left**)**. The correlation results were similar for all postmortem specimens collected and stored at 4°C within 72 hours of delay (i.e., Pearson's r =0.84 for 72H of PMD, **Figure 2C,** mid and

right). Within 72 hours of PMD, the specific antemortem (AM) specimen identified its corresponding postmortem (PM) pair, with the highest value of correlation (i.e., color intensity, **Figure 2D**). The results show the protein CV computed across specific AM and PM blood pairs (i.e., biological replicates of same subject) was below 10% within 72 hours of PMD (**Figure 2E**, mid and right panel). This variability was not substantially different when compared with experimental variability of PMB replicates (i.e., CV: 5,8%, **Figure 2E** left), or even replicates from fresh blood specimens (**Figure 2A**, right). At this stage, we reasoned that the PMB of infant victims of AHT with a maximum PMD of 72 hours (**Table 1**) potentially represents a confident matrix for discovery purposes in proteomic analysis.

3.2. PMB cohort analysis identifies proteins that distinguish AHT cases

The differential analysis of PMB proteomes highlighted 115 proteins (2-sided $P \leq 0.05$, Student t-test) as differentially expressed in AHT cases compared with atraumatic controls (**Figure 3A,** left), of which as many as 94 were upregulated in AHT cases (**Supplementary Table S1**). Differential changes were consistent with the clinicopathological picture of AHT or TBI [1,2,24,25], revealing enrichment of proteins of acute-phase response, complement activation, hypoxia, reduced oxygen transport, catecholamine uptake, and midbrain (GO analysis for Biological process, Fisher exact test, $p<0.01$; **Figure 3A**, right).

To inspect for potentially abundant proteins related to brain trauma, we overlapped 115 differentially expressed proteins with the proteins showing brain-elevated expression. We obtained the list of organ-enriched proteins (e.g., brain-enhanced proteins, 2685, **Figure 3A**, pie chart) from The Human Protein Atlas (HPA)[17], to date, the most comprehensive resource of protein expression data across human tissues. We used The Brain Atlas (i.e., part of the HPA) containing protein profiles across 13 anatomically defined brain regions [26] and selected the subset of brain-enhanced proteins defining the genes that are either exclusively

enriched in the brain or are expressed in the brain and very few other tissues (i.e., 2–5) with at least four-fold enhanced mRNA expression compared with other body tissues (**Supplementary Table S2**.). Remarkably, we found four proteins (Volcano plot, **Figure 3A**) with brain-enhanced expression, as upregulated in the PMB of AHT cases compared with atraumatic controls: BASP1 (P=0.03, Cohen's d = 1.13), Dihydropyrimidinase-related protein 2 (DPYSL2) (P=0.03, Cohen's d = 1.46), Protein tyrosine phosphatase domain-containing protein 1(PTPDC1) (P=0.03, Cohen's d = 2.38) and Phosphoserine aminotransferase (PSAT1) (P=0.02, Cohen's d = 1.61). Notably, PMB readouts of 115 proteins accurately distinguished AHT cases from atraumatic controls reflecting biological differences between 26 samples (unsupervised sample clustering, **Figure 3B**).

3.3. Antemortem analysis of infant serum

We retrospectively analyzed the antemortem sera of the same study participants, sera available from five respective controls and all AHT cases showing a severe form of ICH (Glasgow Coma Scale score [GCS] range: 3-4, **Table 1**) upon patient hospital admission. Remarkably, we discovered 165 circulating serum proteins that display statistically significant upregulation in infants with AHT compared with atraumatic controls at 80% of statistical power (i.e., P<0.05 and Cohen's d>1.56) (**Figure 4A**).

Among them, we detected 26 upregulated proteins (i.e., out of 40 PMB proteins that we again detected with upregulated trend, **Figure S2A**) that maintain statistically significant upregulation in both PMB and sera cohort (**Figure S2A** and **Supplementary Table S3**). Importantly, 139 novel proteins were dysregulated solely in the serum samples based on the respective statistical criteria (**Supplementary Table S3**).

Our novel analysis: i) confirmed a good serum detectability of 42.5 % of proteins upregulated in the PMB of AHT cases (i.e., 40/94), and ii) discovered novel proteins dysregulated in the

serum of AHT infants collected at ICU and which were not detected in the PMB (i.e. 139). Overall, the analysis of AHT serum collected upon infant hospital admission reflects the high proteome differences compared with the serum of atraumatic cases, infants died of SIDS.

For clarity of the results, we distinguish below two types of blood serum proteome changes related to infant AHT: i) generic blood changes, and ii) brain trauma-specific proteome changes.

3.4. Generic proteome changes in the serum of infant AHT

Previous studies demonstrated that the endogenous brain coagulation cascade and complement system (CS) as major part of innate immunity are instantly activated upon mechanical brain injury [25,27]. The activation is additionally enhanced by an influx of complement components from the peripheral circulation due to increased blood brain barrier (BBB) permeability [28].

There exists, however, a lack of systematic serum proteome profiling of AHT cases in infants. As shown in **Figure 4A**, we identified 165 upregulated and statistically significant proteins at a power level of 0.80 (i.e., Cohen's d >1.56) in serum of AHT infants compared with atraumatic controls (**Supplementary Table S3**), reflecting the severe activation of components of the complement and coagulation cascade (e.g., C1S, C1R, C2, C5, C8A, C9, CFB, F2, F5, F9, F10, MASP1, **Figure 4A-B**). We observed increased serum levels of the Lectin pathway (LP) components including pathway initiators (e.g., FCN2, COLEC10, COLEC11, MASP1) and the components of the terminal complement complex (TCC) or the membrane attack complex (MAC) (C5, C7, C8A, C8G, C9) (**Figure 4A-B-C** and **Figure S2B**). Indeed, the study on animal models demonstrated that mannose binding initiators of the LP disrupt the BBB through formation of lytic MAC [29]. Moreover, LP components play a central role in adult cerebral contusions and associate with brain injury severity [30].

The highest protein difference in AHT infant serum compared with atraumatic controls was detected for Coagulation factor 5 (F5) (P<0.0001, Cohen's d = 4.39, **Figure 4A-B**), known as activated protein C (APC) cofactor, proaccelerin or labile factor. F5 is an essential part of the prothrombinase complex (i.e., F10a-F5a)[31,32] with coagulation factor 10 (i.e., F10, **Figure 4C**) that results in increased formation of thrombin from prothrombin or coagulation factor 2 (i.e., F2, **Figure 4B-C**). Thrombin already in physiological concentration exhibits C5 convertase activity and cleaves C5 complement component[33] activating the MAC by an auxiliary route (**Figure 4C**). Interestingly, a previous study revealed that a large fraction of F5 coagulation factor is secreted by the choroid plexus in the brain[34]. A number of previous studies also emphasized the importance of molecular crosstalk between coagulation and complement cascades in order to understand posttraumatic complications following TBI [35] and for the potential discovery of novel therapeutics [27].

We also detected the serum upregulation of: i) the major brain lipoprotein and one of the most studied prognostic markers after TBI Apolipoprotein E (APOE) (P=0.003, Cohen's d= 2.26) [28,36] (**Figure S2C**), ii) the proteins involved in neurogenesis such as neuropilin 1(NRP1) (P=0.02, Cohen's d= 1.87) and Semaphorin-4B (SEMA4B) (P=0.03, Cohen's d= 1.75) [37], and iii) several acute phase proteins known to be elevated within hours of severe brain trauma (e.g., APCS, ORM2, ITIH3, ITIH4, LRG1, AMBP; **Figure 4A**)[28]. In line with APOE upregulation, the enzyme Lecithin-cholesterol acyltransferase (LCAT) [38], which is the main physiological activator of APOE, was also found elevated in the serum of AHT infants (P=0.005, Cohen's d= 2.22; **Figure S2C**). Indeed, APOE is generated by astrocytes and glial cells in the cerebral cortex [39], while LCAT is highly expressed in the brainstem [38]. Overall our data suggest accordance with previously observed generic blood proteome changes following brain trauma in adult populations.

3.5. Brain trauma-specific protein changes in the serum of AHT cases

Consistent with our initial hypothesis, we confirmed several brain-enhanced proteins classified in the web-based HPA database [17] as upregulated with statistical significance in the peripheral circulation of AHT infants compared with atraumatic controls (i.e., ENPP2, CLU, MAN2A1, LANCL1, NCHL1, CRTAC1 in AHT serum, **Figure 5A**). Most of the brain proteins detected by our analysis were formerly related to the anatomical regions of brain, or to the mental and neurodegenerative disorders: Glutathione S-transferase LANCL1 (LANCL1) (P<0.02, Cohen's d=2.06) an essential protein in neuronal function [40], Clusterin (CLU) also referred to as Apolipoprotein J (P=0.01, Cohen's d= 1.80) [41], and glycolytic enzyme Alpha-mannosidase 2 (MAN2A1) (P=0.001, Cohen's d=5.19) [42,43].

Remarkably, three serum upregulated proteins were enriched in the anatomical region of the fetal brain and, according to literature, evidence showed their involvement in brain development: Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 (ENPP2), Neural cell adhesion molecule L1-like protein (NCHL1) and Brain acid soluble protein 1(BASP1).

ENPP2, known as Autotaxin (ATX), displays elevated concentration in the serum of AHT cases compared with atraumatic SIDS controls (P<0.001, Cohen's d = 3.57, **Figure 5B**). It is a major enzyme involved in the synthesis of Lysophosphatidic Acid (LPA), a bioactive lipid essential in neuronal development, as best illustrated by the early embryonic lethality of Enpp2 null mice due to neuronal tube defects [44,45]. ENPP2 expresses in the CNS and secretes in cerebrospinal fluid (CSF)[46].

Similarly to ENPP2, Neural cell adhesion molecule L1-like protein (CHL1 or NCHL1) also known as a close homolog of L1 was found elevated in AHT serum samples collected upon hospitalization (P=0.02, Cohen's d=1.79) (**Figure 5C**). CHL1 is a cell adhesion molecule of the immunoglobulin superfamily expressed by neurons and glial cells in all regions of the

brain [47] with evidence of protein enrichment in the cerebral cortex[48]. It is implicated in: i) nervous system development promoting axonal guidance and neurite outgrowth of cerebellar and hippocampal neurons [47,49] and ii) synaptic plasticity and neuronal regeneration after brain trauma[49]. Notably, the mutations in the CHL1 gene are associated with mental retardation and a broad spectrum of human mental disorders[47].

BASP1, known as Neuronal axonal membrane protein NAP-22 or CAP23, showed elevated concentration in both the serum and PMB of the AHT cases compared with atraumatic controls (**Figure 3A** and **5A**), albeit not statistically significant in the serum cohort (P=0.24, Cohens'd = 0.86, **Figure S2A**). Of note, we observed variability in BASP1 protein concentrations across the individual sera of AHT cases (**Figure 5D**). To inspect if BASP1 protein levels can be influenced by the differences, in months, in the ages of infants detected between tested groups (P=0.047, **Figure S3A**), we correlated the age and BASP1 levels across the serum and PMB of reference cases - atraumatic controls. Interestingly, our analysis demonstrated that circulating levels of BASP1 and even ENPP2 display rather negative correlation with infant age in months **(**e.g. Spearman's rho serum = -0.36**, Figure S3B-C**). This is in accordance with a recent study that showed that BASP1 displays a higher brain concentration during the development–, as opposed to adult phase [50] and even labels neuronal stem and progenitor cells (NPC) in the embryonic mouse brain similarly to known NPC markers, glial fibrillary acidic protein (GFAP) and vimentin. In line with this, the serum differences of BASP1 protein levels are more pronounced if the analysis accounts for infant age in months as a confounding factor (coefficient P (age)=0.094) between atraumatic controls and AHT cases (linear regression P=0.053, **Figure S3D**).

To validate our results by orthogonal analytical methods, we used commercially available ELISA tests of selected markers and the serum of respective infants (**Figure 5E**). We investigated BASP1 and ENPP2, two proteins with strong literature evidence of protein

expression in the developing brain. Remarkably, while both BASP1 and ENPP2 showed upregulation in AHT cases, BASP1 did not reach statistically significant results probably due to the individual variability of the samples related to patient characteristics, as observed with MS data (**Figure 5D**). Moreover, the validation results of absolute serum concentrations of both markers were in strong accordance with our large scale MS proteomics data as demonstrated by significant positive correlation across individual cases ($R_{(ENPP2)}$ =0.85; $R_{(BASP1)}$ =0.6; **Figure S3E-F**), indeed confirming higher levels of both proteins in the serum samples of AHT cases compared with atraumatic cases.

4. Discussion

In the last two decades, very few untargeted proteomic studies that employed methods based on in-gel protein quantification analyzed CSF and serum samples of pediatric AHT cases [51,52], revealing a potential for novel discoveries. These non-hypothesis-driven studies are rare, and, to our knowledge, our current study is the first recent analysis to apply "state of the art" untargeted LC-MS based proteomics to characterize the blood proteomes of infants with AHT. Previous efforts related to serum biomarkers of pediatric TBI mostly involved successive targeted studies that monitored sets of 3–5 serum biomarkers across larger sample cohorts, leading to important clinical conclusions [53-55]. However, preselected brain-specific markers based on adult data might lack specificity in very young infants, probably due to the process of rapid CNS maturation and proteome variability in the postnatal period affecting the protein composition in the serum [54-57].

Here, we generated a unique data set of blood proteomes of infants under age two recorded concurrently across the cases of pediatric AHT and atraumatic controls by large scale proteome profiling. Our differential analysis of infant postmortem and corresponding antemortem samples delivered a set of 165 accessible serum proteins upregulated in AHT, of

which 26 were also elevated in postmortem specimens, paving the way for the clinical screening of infant victims of AHT.

We observed that the several proteins upregulated in the serum of AHT cases (i.e., ENPP2, BASP1, LCAT) including the highly elevated F5 protein display an overlap of their CNS expression with the anatomical lesions found in the brain of infant victims of AHT, but not accidental head trauma [58,59].

For example, recent data on BASP1 protein levels in postmortem human brain demonstrated the highest expression in the hippocampus, brainstem and spinal cord [50], the CNS regions that display known lesions in AHT cases [58]. Along the same lines, ENPP2 displays the highest CNS expression in the corpus callosum, spinal cord and pigmented layer of the retina [60]. As mentioned above and in line with data in the HPA, F5 is dominantly expressed in the corpus callosum, the pons, and the medulla oblongata, brain regions proximate to the spinal cord, and which frequently report injuries in AHT but not in accidental head trauma in infants[2,58,59].

Moreover, we speculate that permeability of the BBB is potentially induced by the activated MAC [29] through high F5 serum levels sourced from injured neuronal regions, which results in increased formation of thrombin. This, in turn, leads to cerebral accumulation of components from the peripheral circulation, increasingly worsening the clinical picture of those infants.

Noteworthy, however, those proteome changes related to coagulopathy are rather common to all traumatic injuries and represent generic changes unspecific to AHT cases in young infants. By contrast, the serum of infants with AHT reflects proteome changes related to midbrain and brainstem (e.g., F5, BASP1, ENPP2, LCAT) and supports the statement that infant victims of abusive injuries by shaking are subjected to a specific pattern of forces [58,59]. The limitations of our study are the modest size of the cohorts and the absence of accidental ICH cases such

as are required to better estimate the proteome characteristics specific to AHT in infants with acute ICH.

Curiously, ENPP2, also enriched in brainstem, display increased protein concentration in serum of AHT cases upon hospital admission, but was almost undetectable in PMB samples, suggesting a period of protein short detectability following trauma and opening new possibilities for research on living patients. Former studies associated ENPP2 to brain trauma[61,62] and some indeed reported a transient ENPP2 increase in the brain lesions affected by trauma[63] followed by reduced mRNA levels in postmortem brain samples hours after injury[60,64].This short window of serum detectability of only several hours after TBI was seen previously for ENPP2 [64] and also for other TBI markers [57].

Here, we also performed an extensive quality control analysis on PMB, comparing proteomes from postmortem and antemortem specimens taken from the same subjects. Our study strongly encourages the future investigation of postmortem specimens from ICH cases in infants; first, because of the lack of proper animal models to study TBI in infants, and second, because we found sufficient proteome stability for all PMB specimens stored at 4°C within 72 hours of PMD. These unique proteome datasets of AHT cases and atraumatic controls together represent a remarkable opportunity to improve prognosis for these infants by early detection of AHT and medical care, and could be explored further in larger prospective studies.

5. Associated Data

Raw data files and individual spectral libraries (.kit file extension) are available via ProteomeXchange with identifier PXD028801. (Reviewer account details: Username: reviewer_pxd028801@ebi.ac.uk ;Password: pO6PhIII)

Conflict of Interest Disclosures: The authors have no example conflicts of interest to disclose.

Funding/Support: This study was supported by Geneva University Grant [MIMOSA Grant] and Swiss National Science Foundation [grant #31003A-182420]. The MIMOSA Grant and grant #31003A-182420 had no role in the design and conduct of the study.

Author Contributions: K.W., T.S, T.F., FG and A.T. designed research; K.W. and T.S. performed research; T.S. analyzed data and generated figures; A.H. acquired data; K.W and T.F obtained ethical approval; T.F, K.W, F.G. and A.T. obtained funding; T.S drafted the initial manuscript with contribution of all authors. All authors critically reviewed and revised the manuscript for important intellectual content.

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Table 1: Characteristics of infants and the samples collected for proteomic analysis.

	Abusive Head Trauma (N=7) (case group[a])	**Sudden Infant Death (N=19)** (control group[b])
Gender		
Male	5	13
Female	2	6
Age (months)		
Range (mean)	2.5-11.5 (5.8)	2.0-17.0 (3.9)
0-3	2	8
3-6	1	8
6-9	2	2
9-12	2	0
12-18	0	1
Weight (kg)		
Range (mean)	4.4-8.2 (6.6)	3.7-11 (6.6)
Size (cm)		
Range (mean)	52-75 (65.1)	56-80 (64.1)
Ethnicity		
White	6	18
Black or African American	1	1
Asian	0	0
Hispanic	0	0
Clinical state on arrival of the rescue team		
Cardiorespiratory arrest	6	18
Bradycardia	1	0
Dead	0	1
Length of hospital stay (hours)		

Range (mean)	12-190 (72.1)	1- 48 (8.6)[b]
Post mortem delay (hours)		
Range (mean)	15-69 (40)	7-42 (22.4)

[a]AHT cases display severe TBI (Glasgow Coma Scale score [GCS] range: 3-4); [b]Seven control cases were hospitalized, five of which had available ICU serum and all seven had available AMB.

Figure 1.

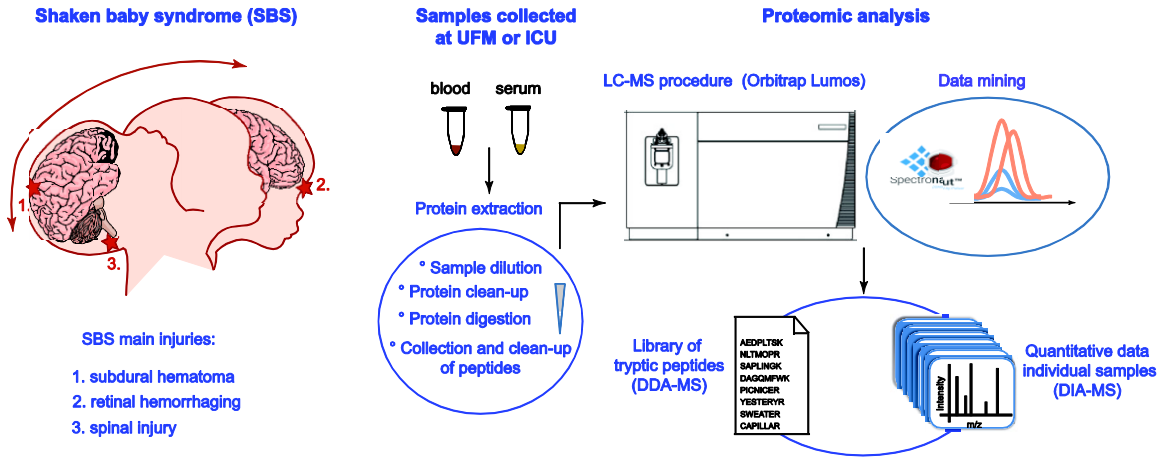


Figure 2.

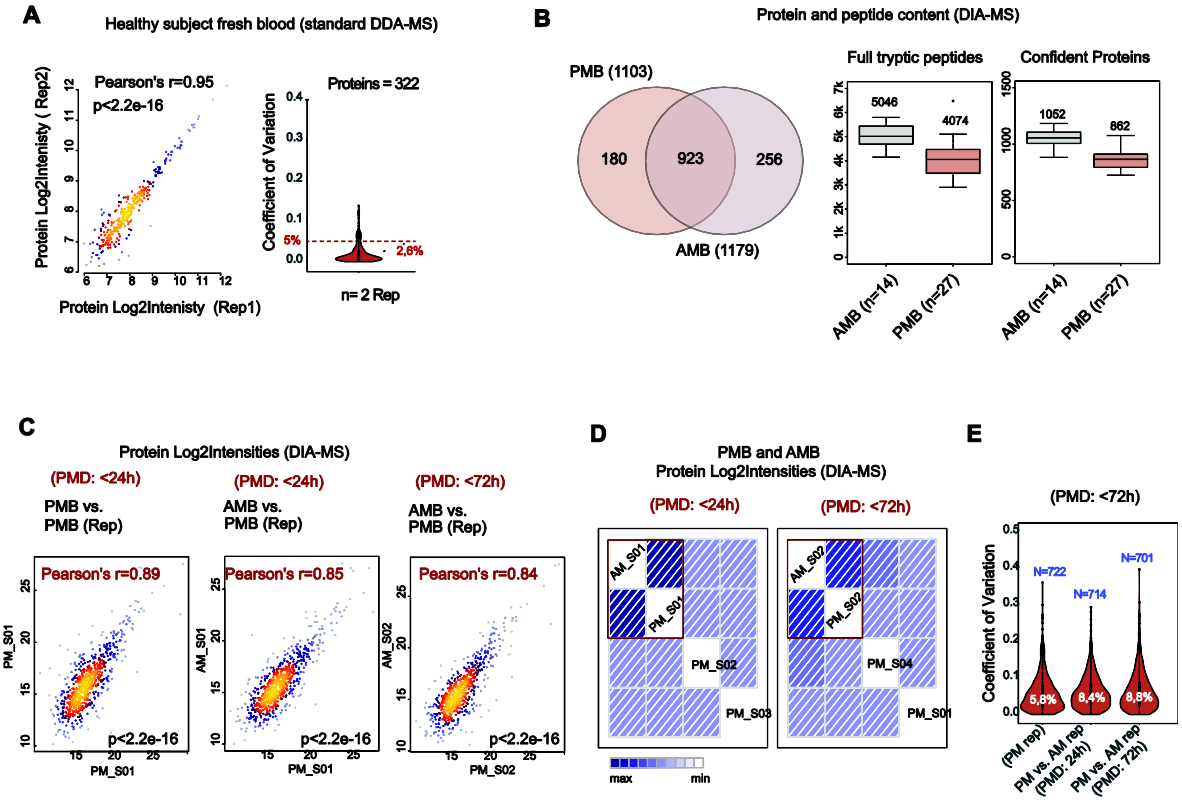


Figure 3.

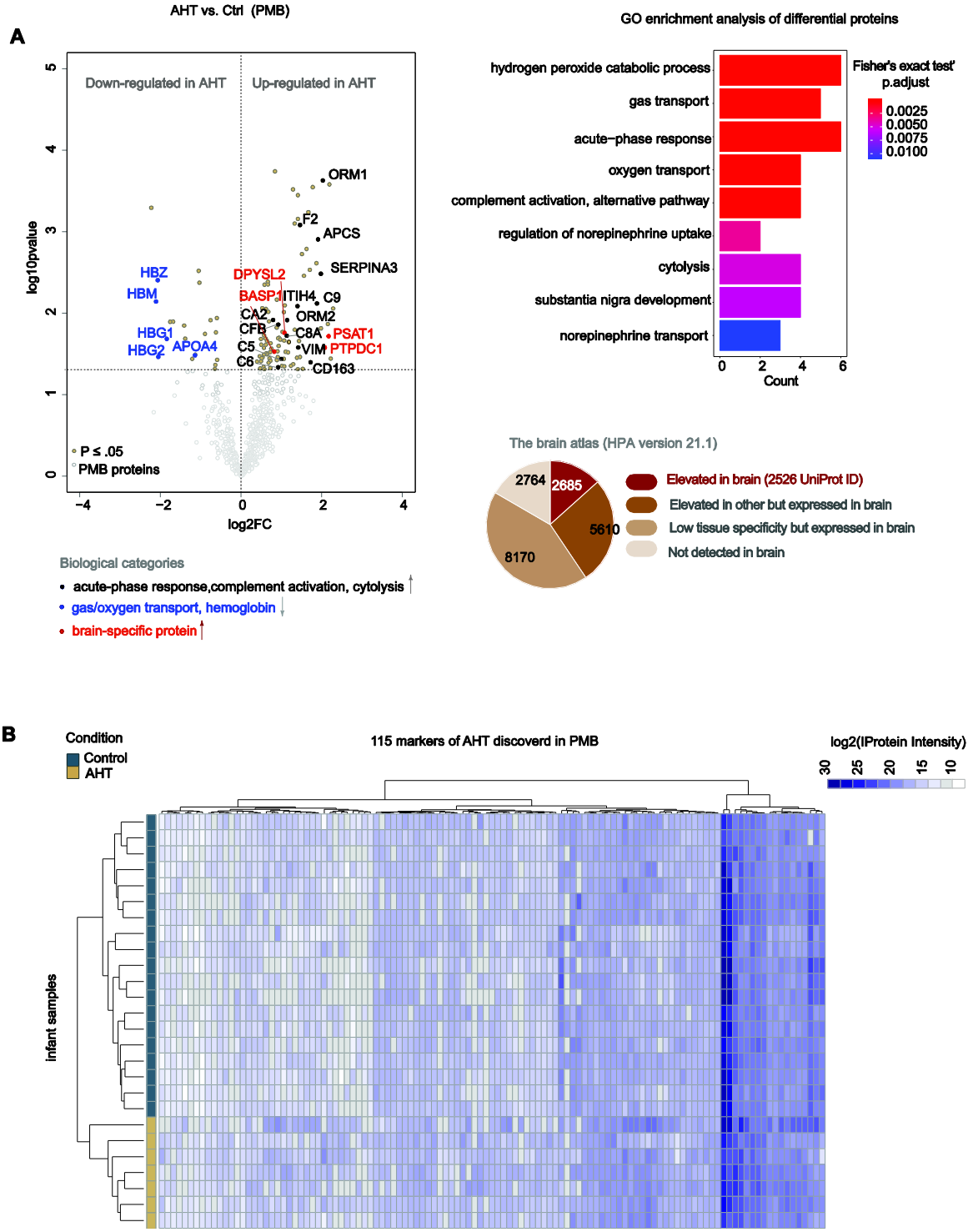


Figure 4.

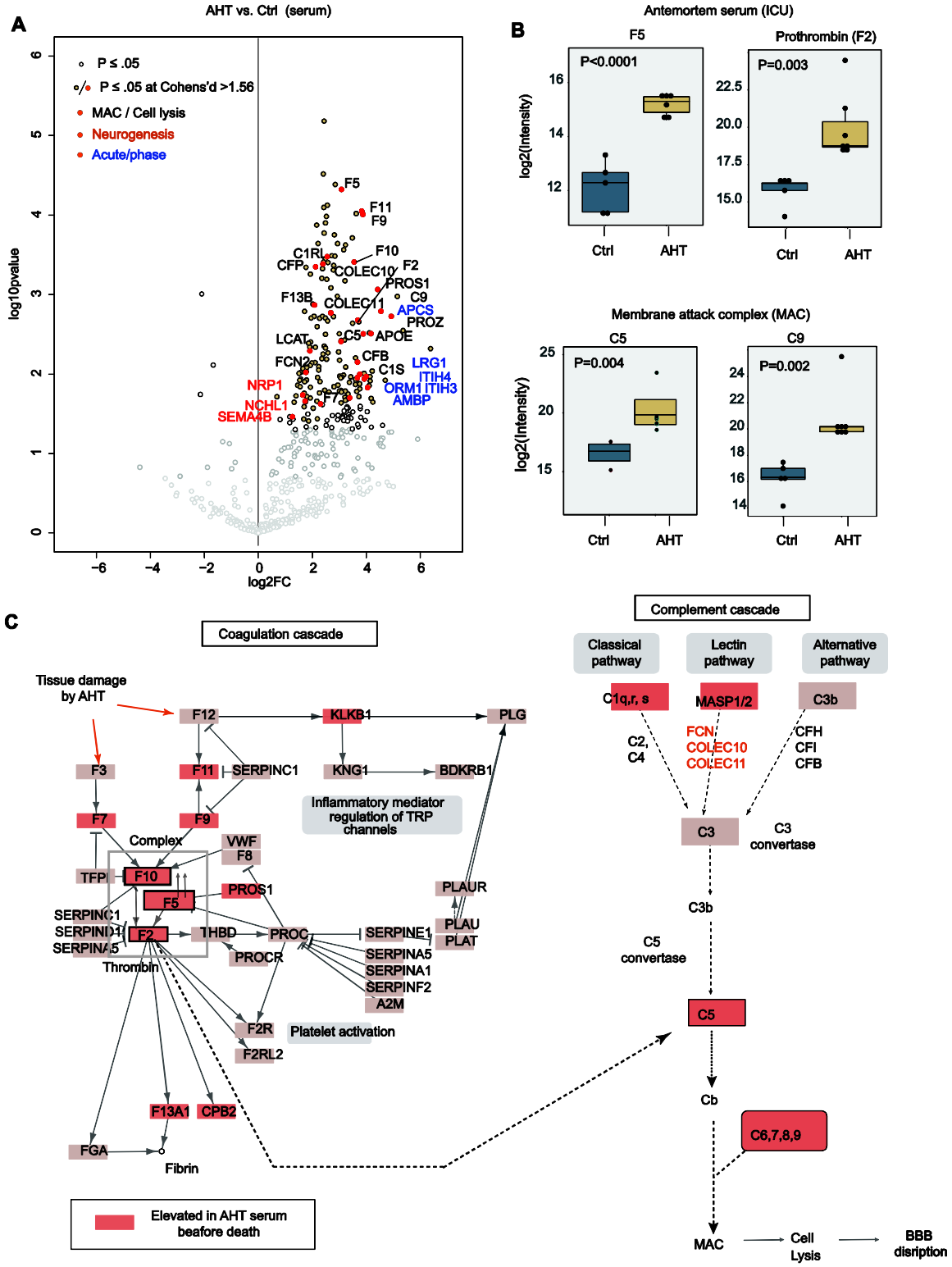
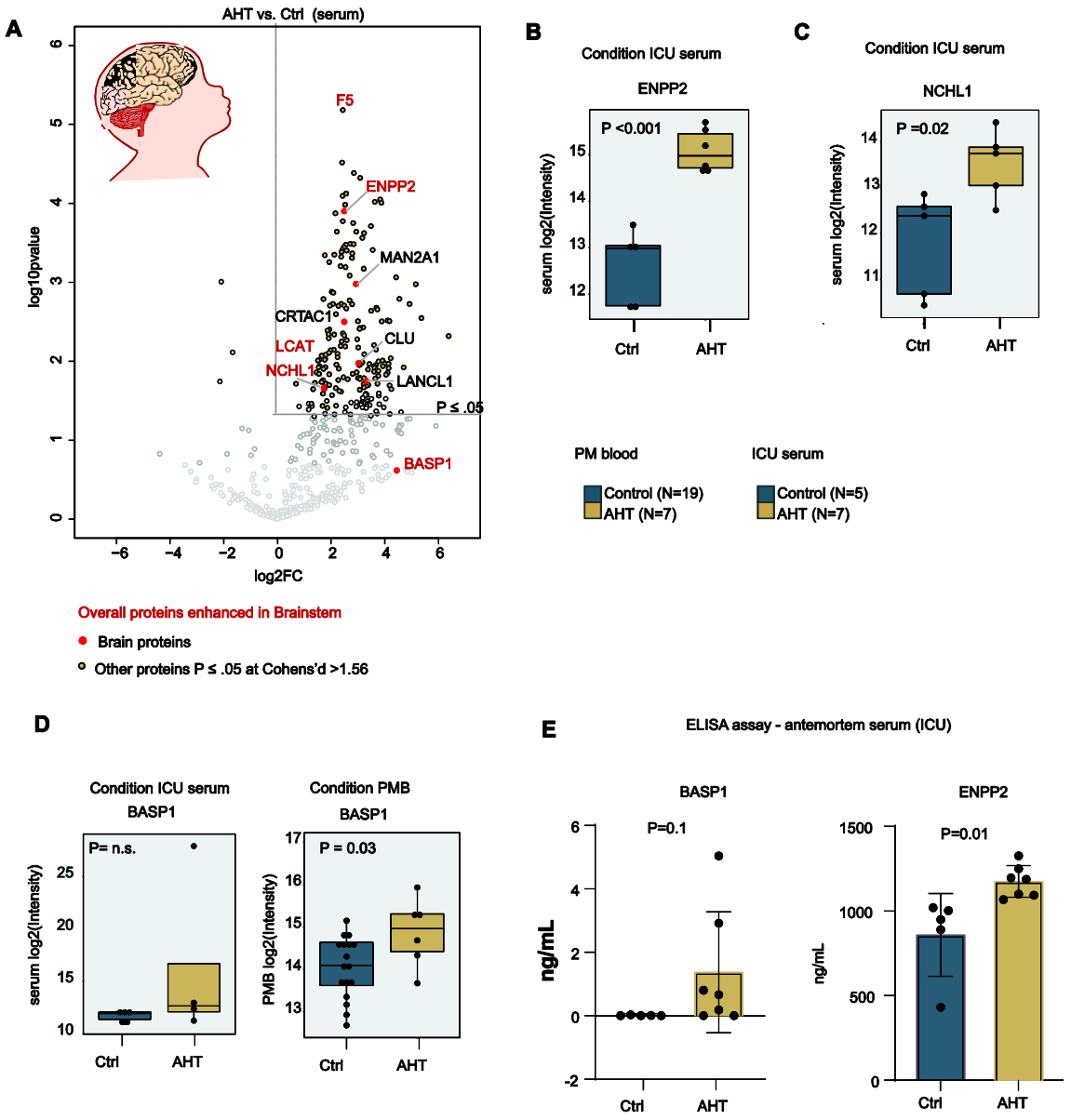


Figure 5



	Abusive Head Trauma (N=7) (case group[a])	**Sudden Infant Death (N=19)** (control group[b])
Gender		
Male	5	13
Female	2	6
Age (months)		
Range (mean)	2.5-11.5 (5.8)	2.0-17.0 (3.9)
0-3	2	8
3-6	1	8
6-9	2	2
9-12	2	0
12-18	0	1
Weight (kg)		

Range (mean)	4.4-8.2 (6.6)	3.7-11 (6.6)
Size (cm)		
Range (mean)	52-75 (65.1)	56-80 (64.1)
Ethnicity		
White	6	18
Black or African American	1	1
Asian	0	0
Hispanic	0	0
Clinical state on arrival of the rescue team		
Cardiorespiratory arrest	6	18
Bradycardia	1	0
Dead	0	1
Length of hospital stay (hours)		
Range (mean)	12-190 (72.1)	1- 48 (8.6)[b]
Post mortem delay (hours)		
Range (mean)	15-69 (40)	7-42 (22.4)