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QUANTITATIVE PROTEOMIC ANALYSES OF HUMAN PLASMA: APPLICATION OF MASS SPECTROMETRY FOR THE DISCOVERY OF CLINICAL DELIRIUM BIOMARKERS

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QUANTITATIVE PROTEOMIC ANALYSES OF HUMAN PLASMA: APPLICATION OF MASS SPECTROMETRY FOR THE DISCOVERY OF CLINICAL DELIRIUM BIOMARKERS

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Abstract

The biomarker discovery pipeline is a multi-step endeavor to identify potential diagnostic or prognostic markers of a disease. Although the advent of modern mass spectrometers has revolutionized the initial discovery phase, a significant bottleneck still exists when validating discovered biomarkers. In this doctoral research, I demonstrate that the discovery, verification and validation of biomarkers can all be performed using mass spectrometry and apply the biomarker pipeline to the context of clinical delirium.

First, a systematic review of recent literature provided a birds-eye view of untargeted, discovery proteomic attempts for biomarkers of delirium in the geriatric population. Here, a comprehensive search from five databases yielded 1172 publications, from which eight peer-reviewed studies met our defined inclusion criteria. Despite the paucity of published studies that applied systems-biology approaches for biomarker discovery on the subject, lessons learned and insights from this review was instrumental in the study designing and proteomics analyses of plasma sample in our cohort.

We then performed a targeted study on four biomarkers for their potential mediation role in the occurrence of delirium after high-dose intra-operative oxygen treatment. Although S100B calcium binding protein (S100B), gamma enolase (ENO2), chitinase-3-like protein 1 (CHI3L1) and ubiquitin carboxyl-

terminal hydrolase isozyme L1 (UCHL1) have well-documented associations with delirium, we did not find any such associations in our cohort. Of note, this study demonstrates that the use of targeted approaches for the purposes of biomarker discovery, rather than an untargeted, systems-biology approach, is unavoidably biased and may lead to misleading conclusions.

Lastly, we applied lessons learned and comprehensively profiled the plasma samples of delirium cases and non-delirium cases, at both pre- and post-surgical timepoints. We found 16 biomarkers as signatures of cardiopulmonary bypass, and 11 as potential diagnostic candidates of delirium (AuROC = 93%). We validated the discovered biomarkers on the same mass spectrometry platform without the use of traditional affinity-based validation methods. Our discovery of novel biomarkers with no know association with delirium such as serum amyloid A1 (SAA1) and A2 (SAA2), pepsinogen A3 (PEPA3) and cathepsin B (CATB) shed new lights on possible neuronal pathomechanisms.

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Chapter One: Introduction

Biological Mass Spectrometry for Clinical Biomarker Discovery

The use of diagnostic biomarkers is central to patient care. Biomarkers are useful, not only for the early detection of pathological changes before overt clinical manifestations, but also for monitoring treatment and for predicting outcomes. Within the omics spectrum, proteins are more proximal reporters of diseases than genes and transcripts, and most diseases manifest at the level of protein activity. It is therefore not surprising that protein-based biomarkers form a substantial proportion of laboratory tests requested in clinical practice. Despite their substantial role in diagnostics, the last few years have seen a significant decline in the number of protein biomarkers approved by the FDA for clinical use.[9-11]

Several reasons have been suggested for this down-trending observation in protein biomarker discovery. Until recently, profiling biofluids for proteomic signatures was mostly achieved using two-dimensional (2-DE) electrophoresis beginning in the 70s.[12-14] Despite the many successes with 2-DE systems,[15-19] the lack of reproducibility, the narrow dynamic range and inability to identify low abundance and/or hydrophobic proteins, among other limitations, underscored the need for high-resolution platforms. Liquid chromatography (LC) equipped with mass spectrometry (MS) overcame some of these aforementioned challenges and opened the gateway for the unbiased analyses and quantification of proteins even in complex biological samples. The advent of modern mass spectrometers, the availability of comprehensive protein sequence databases and the introduction of new peptide labeling schemes has enhanced the accuracy, sensitivity and multiplexing capabilities of mass spectrometers, allowing for the comprehensive analyses and quantification of proteins from multiple batches of samples at a time.

Notwithstanding the substantial progress in protein identification, a primary bottleneck in the biomarker workflow is the failure to validate candidate biomarkers. The use of traditional affinity-based methods to validate biomarkers discovered by MS could delay biomarker translational research and FDA approval by about a demi-decade.[20, 21] To accelerate the biomarker pipeline, we must close the translational gap between the bench and the bedside. This doctoral research contributes to the growing body of knowledge that biomarker

discovery and subsequent antibody-free validation is achievable on the same MS platform. Here, we applied MS-based discovery and validation pipeline to the specific context of post-operative delirium, using systems-biology approaches. Additionally, it is demonstrated that careful study designing, clarity regarding the intended use of the biomarkers (for screening, diagnosis or prognosis), use of the appropriate study population, choice of statistical tools and optimal sample preparation all play important roles to ensuring successful biomarker discovery.

Background

The Need for Protein-based biomarkers

Biomarkers are, by definition, objectively measurable characteristics, useful in evaluating a normal biological activity, a pathological process or a pharmacological response to some therapeutic intervention.[22] Within the omics spectrum, proteins are most preferred for diagnostic purposes. This is because genomic sequencing provides unchanging probabilistic risk with limited applicability beyond monogenic diseases such as hemophilia A, phenylketonuria and osteogenesis imperfecta.[23-25] Diagnostic assays involving transcripts (mRNA), on the other hand, has yet to gain widespread use in clinical laboratories.[26] Although proteins and metabolites are the most proximal reporters of diseases, proteins are unique in providing a functional snapshot of

the body's response to a disease process rather than metabolic products. Furthermore, most diseases manifest at the level of protein activity, given their central role in biosynthesis, signaling and structural stability of cell and tissues.[27, 28] These make proteins ideal for diagnostics, for prediction and as targets for intervention.[29] It is therefore not surprising that proteins (in particular enzymes) are the most routinely requested biomarkers in clinical laboratories[30, 31].

The broader clinical significance of protein biomarkers is also evident in routine laboratory medicine. For example, a positive post-urea breath test for $^{13}CO_2$ molecules only suggests an *H pylori* infection. However, detecting a BCR-ABL fusion protein is not only diagnostic of chronic myeloid leukemia (CML) but is also the target for Imatinib therapy and for monitoring drug response. Where the underlying pathophysiology is unknown, discovered biomarkers have been instrumental in shedding new lights on the mechanistic underpinnings of the clinical condition in question.[32-34]

Advances in measurement technology and the rising number of putative biomarkers reported in recent literature raise expectations about the ideal biomarker. Biomarkers are required to be more sensitive, reliable and accurate in identifying cases and quantifying the extent of a pathological change. Additionally, the ideal biomarker is expected (1) to detect an active pathological

change very early in the disease process, preferably before significant changes occur; (2) to be easily accessible, i.e., assaying from peripheral blood is generally preferred to taking an invasive biopsy unless it is absolutely necessary; (3) to be analytically stable and correlate well with worsening disease severity; and lastly (4) to be associated with a known disease mechanism.[22, 35]

Based on these characteristics, there is clearly a significant unmet clinical need, yet the proportion of FDA-approved *in vitro* diagnostics which are protein-based assays remains low (**Figure 1.1**). This unmet need for protein biomarkers is further reflected in the widening gap between published literature on putative biomarker candidates and the number of FDA-approved candidates for clinical use.[20, 29, 36] Attempts to fill this gap has led many authors to reimagine the biomarker discovery pipeline in terms of the choice of biological samples, the appropriateness of the study population, analytical platforms for measurement and existing approaches for clinical validation.

Ideal sample sources of biomarker discovery

Human biofluids are a rich media of diagnostic material, useful in the detection of pathological processes. They reflect the overall physiological state of an individual[37] or the disease state of a specific organ-tissue, which makes biofluids the ideal sample for biomarker discovery. Relative to other biospecimen such as tissues, the relative ease of accessibility of biofluids fulfils a major

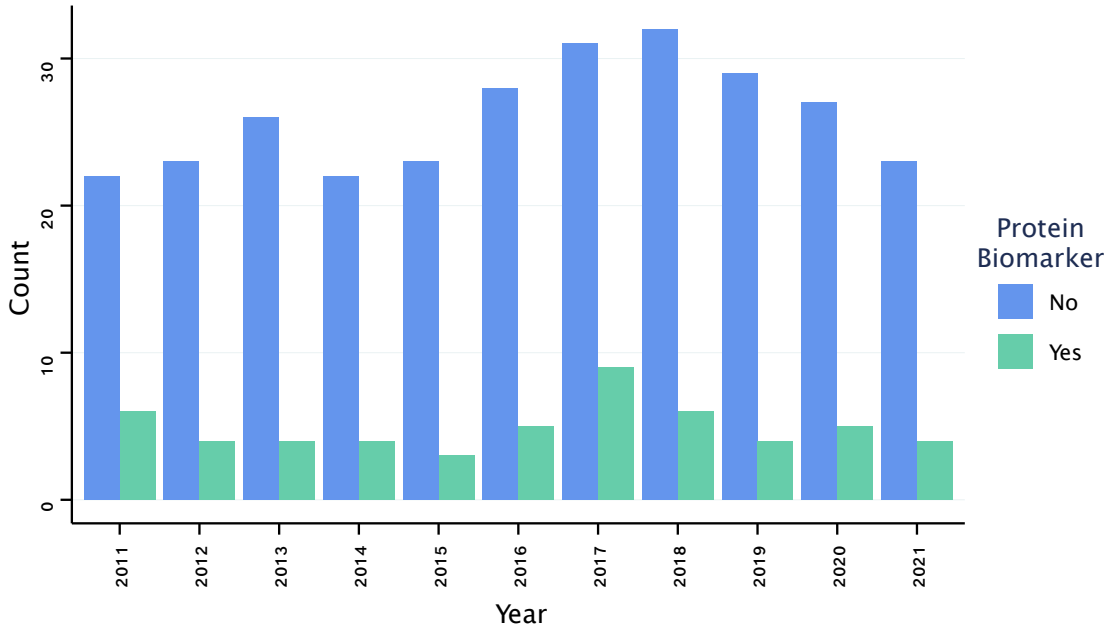


Figure 1. 1 FDA approved biomarker assays (2011 - 2021)

The number of in vitro diagnostic (IVD) tests approved by the FDA for professional use or as over the counter diagnostic, grouped into non-protein and protein-based biomarkers. Numbers include novel biomarkers as well as approvals after expiration of original patent terms. The FDA considers biomarker assays as medical devices and adhere to the same regulatory standards as other types of medical devices. Data source: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfIVD/search.cfm (last accessed: 05/25/2022)

criterion of the ideal biomarker. Use of urinary albumin to evaluate the risk of diabetic nephropathy and use of antemortem CSF tau protein to detect Alzheimer-type neuropathologic changes in the brain are well-known examples of the diagnostic potential of biofluids.[38, 39]. The proteome of human biofluids can be broadly categorized into native proteins, tissue leakage and signaling proteins.

This consistent compositional pattern is observed when comparing discovery proteomic experiments performed on blood, tears, saliva, urine, cerebrospinal and synovial fluids.[40-45]

Analytical challenges of the human plasma proteome

Of these biofluids, blood plasma/serum is the most complex by composition, and remains the most difficult proteome to characterize.[40] Proteins in plasma (or serum) represents about 20% of the entire human proteome. The wide dynamic range of protein concentrations spans about 10 – 12 orders of magnitude.[40] In addition, approximately 95% of the total protein mass in plasma (or serum) is made up of the 12 most abundant proteins (**Figure 1.2**).[46, 47] Because plasma (or serum) contains leakage proteins from many tissues in the body, this compositional complexity poses significant analytical challenges, as signal from the proteins of interest may be impaired by the dominance of albumin and other

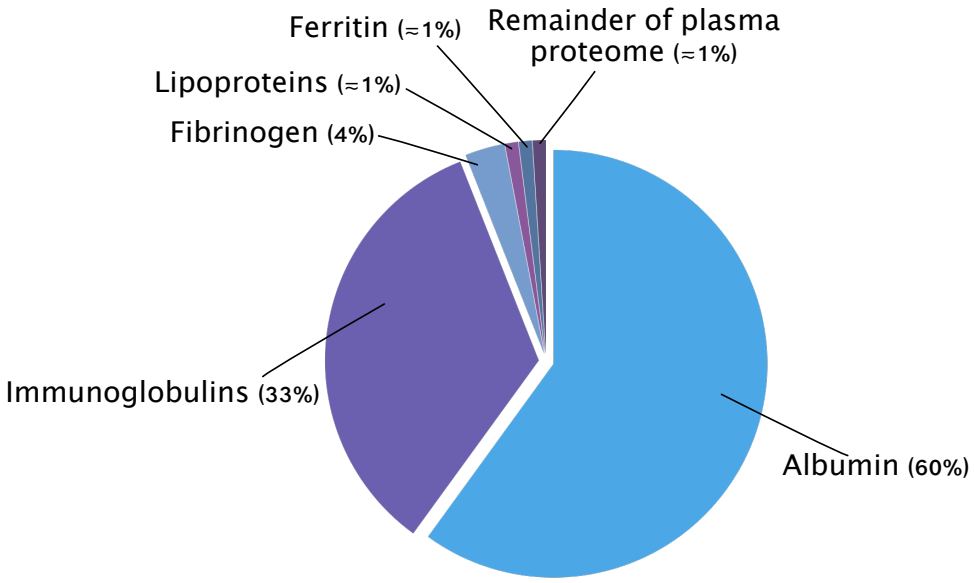


Figure 1. 2 Composition of the Plasma Proteome by Protein Mass

Data source from Pietrowska, Wlosowicz [48]

high-abundance proteins.[29, 40, 49] Furthermore, post-translational modifications and degradation mechanism by native plasma proteases in the background of lipids, salts and small molecule metabolites further reduce analytical sensitivity, reproducibility and resolution in unpredictable ways.[48, 49]

To reduce sample complexity and enhance signal from the low-abundance plasma proteome frequently requires additional experimental steps to remove the dominating high-abundance proteins. Immunodepletion, affinity enrichment and fractionation are common pre- and post-digestion options compatible with shotgun proteomics, although blood-derived exosomes have recently been explored.[50-53] By far, immunodepletion is the most commonly employed sample purification strategy, and affinity enrichment is most useful for the analysis of post-translational modifications.[54] It is worth noting that no one single approach is better than the others, and substantial removal of the high-abundance proteome may require more than one approach. Briefly, while immunodepletion by dye affinity resins rely on the covalent binding between the anionic anthraquinone dye to agarose beads to deplete albumin, immunoaffinity columns purify samples by binding to mono- or polyclonal antibodies immobilized on resin beads. The High Select Top14 Abundant Protein Depletion Mini Spin Columns (Thermo) and the Pierce™ Albumin Depletion Kit® (Thermo Fisher Scientific) are notable commercial examples.

These additional experimental steps, however important they may be in enhancing signal from the low-abundance proteome, are without limitations. A major concern about their use is the non-specific removal of proteins other than what was targeted. Because of the role of albumin in the active transport of many other proteins, Liu, Zhao [55] observed a remarkable loss of target proteins after albumin depletion. Furthermore, there is considerable degree of variation in the efficacy of the depletion step.[56] Possible reasons for this observation may include saturation of antibody binding sites and the relatively low sample capacity of antibodies used.

Following digestion of intact plasma proteins, separation strategies to reduce sample complexity is commonly achieved using electrophoresis or chromatography. Here, separation is achieved according to size, hydrophobicity, charge, isoelectric point or by affinity.[57] Post-digestion chromatographic fractionation involves the interaction of peptides with a stationary phase (e.g. reversed-phase [RP] materials) and a mobile phase gradient. Modification of properties of the mobile phase, either by changing the organic modifier concentration, pH or the salt content over time, allows for the differential elution of peptides.[58, 59] Given the benefits of extensive fractionation in reducing the dynamic range of the proteome being studied,[59-61] multidimensional strategies that combines orthogonal separation properties are often employed. Such is the approach of an in-house offline pentafluorophenyl (PFP)-RP chromatographic strategy that is orthogonal to the online C_{18}-based reversed-phase

separation.[62] Our in-house method, comparable to more commonly used approaches such as Hi-pH RP fractionation, requires fewer experimental steps, and has been demonstrated to be time-efficient and compatible with chemically-labeled peptide species.

The choice between plasma and serum remains a long-standing debate. After centrifugation of an anticoagulant-treated blood to suspend cells and cellular debris, the remaining liquid component of blood is plasma. Omitting the anticoagulant step results in serum, and the subsequent centrifugation also allows for the removal of the fibrin clots. This results in significant qualitative and quantitative differences between plasma and serum.[63, 64] In fact, the coagulation step results in a 3 – 4% lower protein concentration in serum relative to plasma.[65, 66] It is recommended that the decision to use plasma or serum be guided by the purpose for the sample draw. However, for the specific purposes of biomarker discovery requiring the unbiased profiling of blood, removal of clotting factors (as in the case of serum) may contribute to non-specific removal of other proteins of interest that may be associated with the clotting factors. This is also the recommendation by the Human Proteome Organization (HUPO), with the additional reason of a lower degree of *ex vivo* degradation during the coagulation step [67]

Mass spectrometry-based Biomarker Discovery

The biomarker discovery workflow is a series of preclinical experiments and clinical studies that aim to discover, verify and validate potential biomarkers of the clinical condition under investigation. Typically beginning with a small set of patient samples (**figure 1.3**), discovery proteomic experiments are conducted to generate the foundational hypotheses of the study. Here, samples are comprehensively profiled for all identifiable proteins that may explain the differences in the proteomic profiles of cases and controls. At this stage, experiments are designed to report protein abundance in relative terms (e.g., log fold change between cases and controls). Common options for relative quantification in shotgun proteomics include isotopic labeling (e.g., using intensities from tandem mass tags or isobaric tags for relative and absolute quantification), non-isobaric tagging (e.g., amine-specific, stable-isotope-labeled reagents) or label-free quantification (by peak area integration or spectral counting).

With a putative list of thousands of candidate biomarkers at this stage, the qualification phase ensures that the biomarker readout is independent of the discovery measurement platform and ascertains that a relationship between abundance and clinical outcome exists.[68] Often times, this step is buried in the

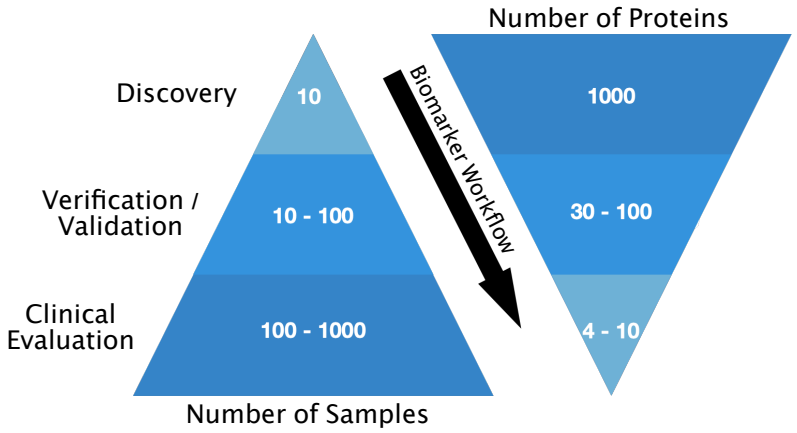


Figure 1. 3 Schematic overview of the biomarker discovery workflow

Each step forward requires substantially higher number of patient samples while focusing on a panel of very few proteins for clinical evaluation.

verification phase if the measurement platform used for verification is orthogonal to that used for the discovery. Biomarker verification then measures identified biomarker candidates in a larger independent set of samples with the primary aim of eliminating false discoveries.

To date, this phase remains the main bottleneck in the biomarker workflow.[30, 69, 70] This is because traditional affinity-based verification methods, notably ELISA, have limited multiplexing capabilities and are not suited for the high throughput setup that biomarker verification demands. Besides, measurements by ELISA are semi-quantitative.[71] ELISA verification also requires candidate biomarkers to be antigenic and often exhibit cross-reactivity.[72, 73] Where commercial antibodies are unavailable, developing high-quality assays for the many biomarkers needing verification can be time-consuming (about 1 – 2 years per antibody) and may be cost-prohibitive (over USD 100, 000 per antibody).[74-77]. As a result, many discovered biomarkers are never verified.[1, 78] Notwithstanding these, verification by ELISA is advantageous for the ability to identify proteins with the least false positive rate and measure concentrations of analytes even in low abundance.[79]

Fundamental to the biomarker pipeline is the unequivocal identification and characterization of candidate analytes from study samples. As this step sets the stage for many downstream steps at both the discovery and validation phases,

the degree of certainty required in protein identification places high demands on the quantitative assays used. Common assay types employed in the biomarker pipeline include immunohistochemistry[80], enzyme-linked immunosorbent assays (ELISA), flow cytometry[81], mass spectrometry, and more recently, proximity extension assays[82] and SomaScan[83].

Mass spectrometry and modern mass analyzers

Of these measurement platforms, mass spectrometry (MS) remains the gold standard for bioanalytical applications.[84] MS is a high throughput analytical platform with the requisite sensitivity, unprecedented selectivity and resolution to identify analytes in complex biological samples. The high mass accuracy and speed makes MS uniquely suited to quantify analytes, even at attomole concentrations.[85] At its fundamental level, MS has three functional components: (1) sample introduction, (2) ionization and (3) detection and mass analyses. The most common method for sample introduction is by liquid chromatography. In a typical proteomics setup, liquid chromatography coupled online to tandem mass spectrometers (LC-MS/MS) detects the input sample dissolved in the mobile phase and pumps eluents under very high pressures through a densely packed column containing the stationary phase. Chromatographic separation of sample at this point, typically orthogonal to

separation methods described earlier, is based on differential affinity of input material to the stationary phase.

After successful separation and elution from the column, the sample undergoes ionization and subsequent introduction into the mass spectrometer for detection according to their mass to charge (m/z) ratios. The time it takes from analytes' contact with the column material to elution off the column is the chromatographic retention time (RT). For peptides, two ionization techniques are most commonly employed: the electrospray ionization (ESI) and the matrix assisted laser desorption/ionization (MALDI). Of the two, soft ionization by ESI is achieved by applying an electric field to the sample in the capillary column. This produces charged droplets that form gaseous ions with very little fragmentation.[86] MALDI, on the other hand, achieves ionization by using short laser pulses to heat up an acidic matrix containing the sample.[87] For a LC-MS/MS setup, ESI is typically coupled to ions traps,[88] preferably because of the ease of introducing ionized samples from the columns into the mass spectrometer. Considerable gains in protein identification can be attributed to improvements in ion transmission, speed, mass accuracy, duty cycle and resolution in modern mass analyzers. Time-of-flight, Orbitraps and ion-traps are common mass analyzers, each with unique properties in terms of resolution, duty cycle and acquisition speed, among others.[89-91] Specifically for proteomics, a number of hybrid configurations exits, such as the linear ion trap-Orbitrap, that combines the

strengths of two or more mass analyzers to increase their ability to analyze samples in tandem, as briefly described below.

Generally, the first mas analyzer, MS1, separates ionized species by their mass to charge (m/s) ratio. Ions at a given m/z ratio are then selected for further fragmentation, either by collision-induced dissociation, photodissociation or ion-molecule reactions. Fragments ions introduced into the second round of mass analysis, MS2, provide the sequence information that is searched against the appropriate database to identify proteins in the sample. **Figure 1.4** shows an example of data acquired by LC-MS/MS analysis of a plasma sample. Many search algorithms exist for the identification of peptides from their respective tandem mass spectra, most common ones being SEQUEST, MASCOT, Andromeda and COMET. While SEQUEST computes cross-correlation scores by comparing the experimental spectra with theoretically derived equivalents,[92] MASCOT employs a probabilistic scoring metric, the expectation value, that ascertains the probability that the observed match between the experimental spectra and the protein database is random.[93] Conveniently integrated into the MaxQuant Environment, Andromeda uses a probability metric for scoring peptide-spectrum matches (PSM)[94]. Last but not the least, COMET works similarly to SEQUEST. It however implements a faster cross-correlation scoring by avoiding the creation, storing and indexing of theoretical spectra.[95]

Competing technologies

SomaScan (SomaLogic, Inc, Boulder, CO) and PEA (Olink Proteomics, Uppsala, Sweden) are emerging technologies, capable of simultaneously measuring thousands of plasma proteins. While SomaScan uses fully synthetic, single-stranded DNA-based molecular recognition elements to bind to proteins within a sample,[96] PEA employs oligonucleotide-labeled antibody probe pairs that bind to their respective proteins. Both are semi-targeted, high throughput analytical platforms with increased usage in recent biomarker research.[97-100] Their use requires less infrastructure, lesser number of steps in sample preparation and much less expertise when compared to MS.[101] Because they are semi-targeted, their use challenges the fundamental principle of unbiased proteomic profiling for the purposes of biomarker discovery and hypothesis generation. This, however, may be less of a concern given that newer version of SomaScan can identify 7000 different proteins from only 55µL of sample. (https://somalogic.com/panels/, last accessed 05/29/2022).

Nonetheless, MS remains the gold standard platform, although data from the different platforms may be complimentary.[101] Because SomaScan and PEA require less sample preparatory steps, the analytical challenges with the human plasma proteome, outlined earlier in this chapter, may only apply to biomarker workflows that employ MS. The extent to which additional steps such as sample fractionation and immunodepletion,

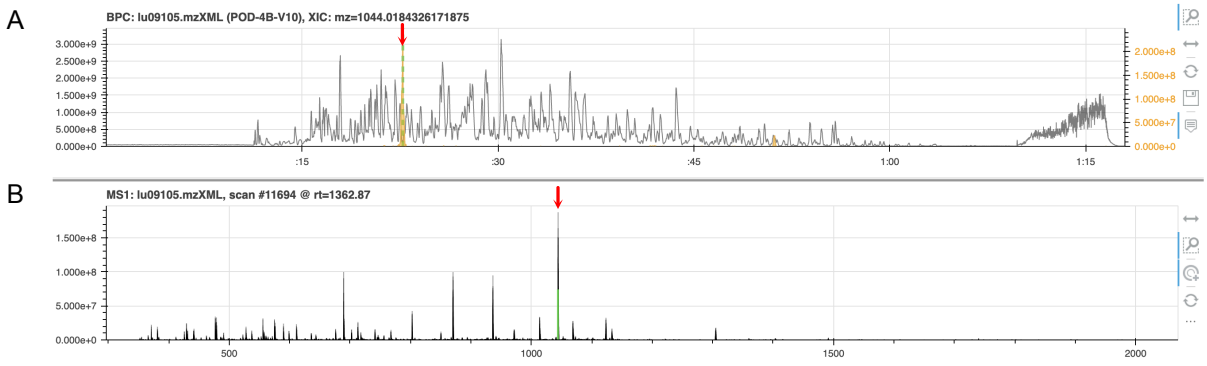


Figure 1. 4 Mass spectrometry analysis of a plasma sample

Tryptic peptides from a plasma sample spiked in with exogenous CDS1 protein was analyzed on the Orbitrap Fusion Lumos MS. Peptides were fragmented by electron transfer dissociation (ETD) and the resulting MS/MS spectra were search against the human proteome database using COMET. A: Base peak of the total ion chromatogram based on the peptide separation by liquid chromatography. B: MS1 spectra acquired at 22.9 mins retention time (red arrows). Image source: MASSIEVE, an in-house computational platform

or the lack thereof, affects data acquired by SomaScan or PEA remains to be determined.

Overcoming the validation bottleneck

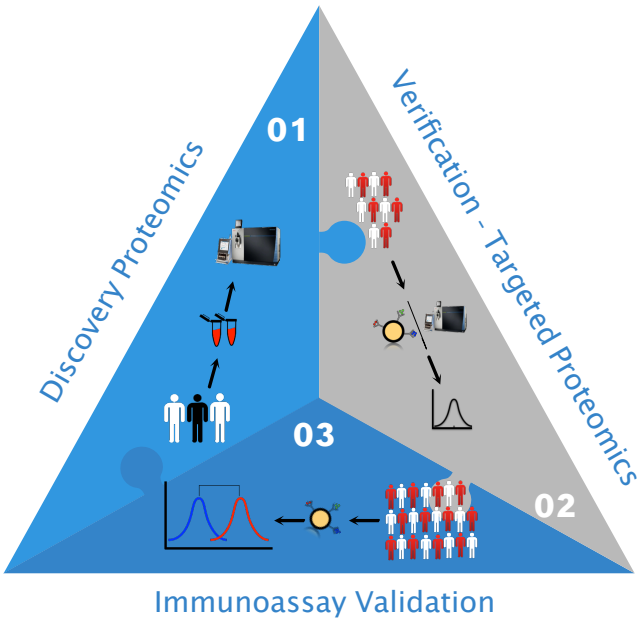
Although biomarker researchers agree that discovery, verification and validation are the major steps in the workflow (**Figure 1.3**), two major approaches to this workflow also exist. The first and more traditional approach, also called the triangular strategy, is based on discovery using MS, followed by verification and validation with immunoassays (**Figure 1.5**). Quite recently, Geyer, Holdt [30] proposed the rectangular strategy, which involves the discovery, verification and validation of candidate biomarkers all on the same MS platform. As outlined earlier regarding challenges with immunoassay-based validation schemes, the rectangular strategy seeks to eliminate this bottleneck in the biomarker workflow.

Initial steps in MS-based validation involve the development of a targeted method for the list of precursor ions discovered *a priori*. In targeted proteomics, the term "transition" refers to the pair of precursor ion – product ions following fragmentation. Broadly, selected reaction monitoring (SRM) and parallel reaction monitoring (PRM) are the available targeted proteomic methods, the choice of which is dictated by the available MS instrument and the nature of the experiment or type of information required. While SRM, primarily performed on

triple quadrupole (QqQ) MS, requires the selection of a limited number of transitions for a given peptide, PRM monitors all potential product ions of a peptide.[102] The method development is achievable directly via the MS instrument software (e.g., XCalibur) or with the assistance of specialized software (e.g. Skyline).[103] Specifically for PRM, the method file typically contains an identifier for the precursor ion, the m/z, the charge state of the precursor ion, and the corresponding retention time window, all of which can be obtained from the discovery experiments. SRM requires the additional step of specifying which transitions will be monitored at MS2.

Following data acquisition by targeted proteomics, Skyline can also facilitate additional downstream analyses, although vendor software such as QualBrowser or manually extracting the data in a programming environment are possible options. Because retention time windows are specified for any given precursor ion, particular attention on RT is required as minor changes in chromatographic conditions occurring during MS can cause significant RT shifts. **Figure 1.6** shows the RT shifts observed during an in-house PRM method development on a 1-hour gradient. We observed that for each subsequent MS run, there was a RT shift of about 30 seconds.

A


01
Discovery Proteomics
Verification - Targeted Proteomics
02
03
Immunoassay Validation

B

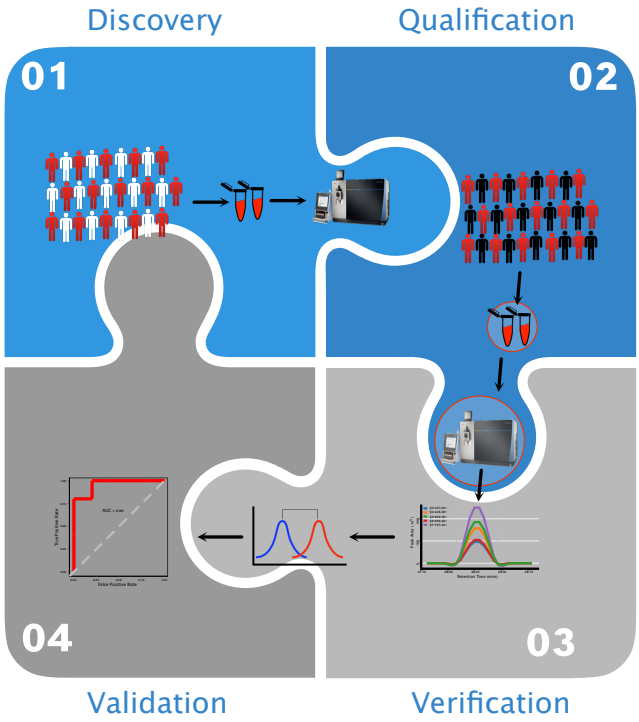

Discovery
Qualification
01
02
04
03
Validation
Verification

Figure 1. 5 Different approaches to the discovery pipeline

Consistent in both approaches is the unbiased profiling of biospecimen in the discovery phase, and an orthogonal biomarker measurement and use of independent set of samples in the subsequent phases. A: triangular strategy typically begins with a smaller set of samples, yielding 1000s of candidate biomarkers and ends with larger cohort of patients to validate a smaller panel of biomarkers; B: in the rectangular approach, both discovery and validation occur on the same MS platform without a need for affinity-based validation. Emphasis here is on orthogonality of the MS measurement techniques across the different phases of the pipeline. Qualification, verification and validation are illustrated in a continuum to demonstrate how MS eliminates bottlenecks and simplifies the biomarker pipeline

Although this problem is not well known in published literature, Heil, Remes [104] made a similar observation direct import of acquired raw data after each run in order that RTs can be adjusted for subsequent runs. Nonetheless, the advantage of MS-based validation over traditional affinity-based methods is clear, with the theoretical possibility of shortening the bench-to-bedside time gap in biomarker discovery.

Post-operative delirium

We apply the biomarker pipeline to the context of post-operative delirium. This is a debilitating clinical condition with acute onset and a fluctuating course in the immediate post-operative period. It is characterized by changes in cognition, deficits in attention, fluctuating levels of consciousness and/or disorganized thinking.[105]

It is the most common acute neuropsychiatric disorder[106, 107] and complicates post-surgical care of the elderly with worse hospital outcomes, longer hospital stays, higher risk of post-surgical strokes, increased readmission rates and higher overall mortality[108, 109]. Post-operative delirium increases hospitalization cost substantially and remains a significant healthcare burden, most especially in the geriatric population. Although incidence varies widely depending on the type of surgery,[110] the post-cardiotomy population has one

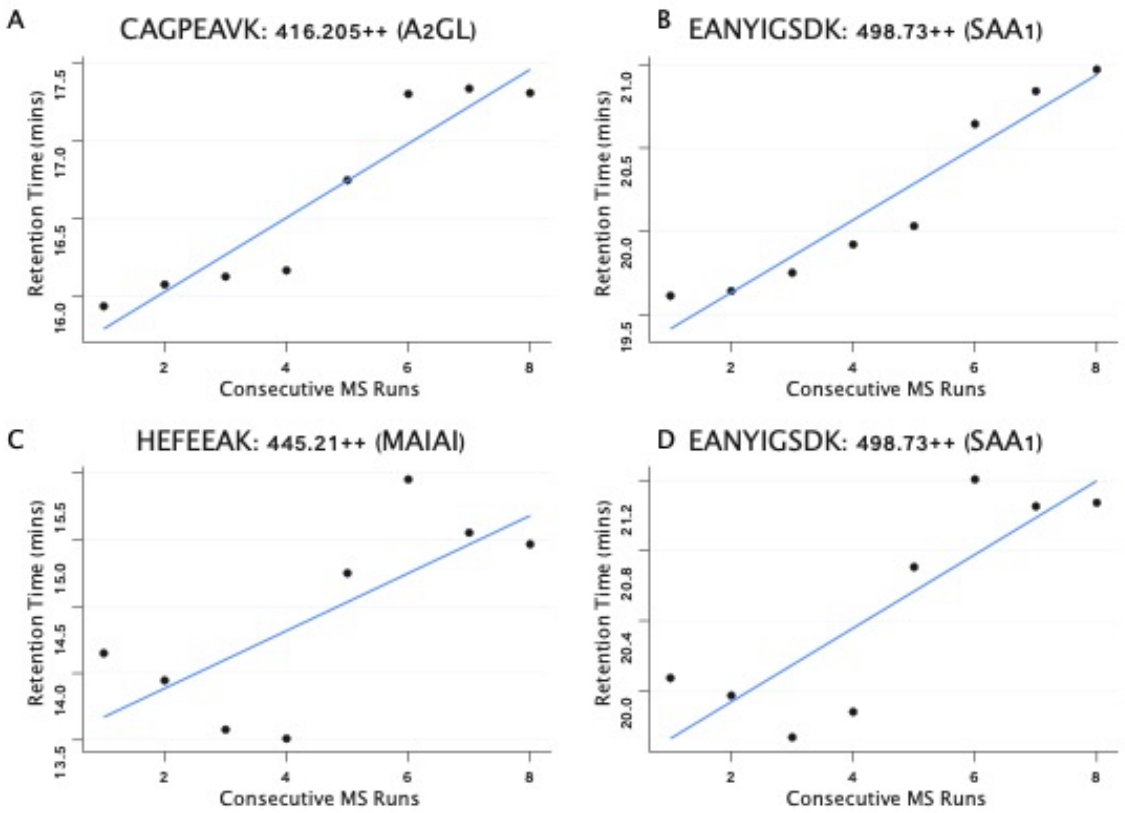


Figure 1. 6 Retention time shifts

Even when a wider RT window is specified for a given precursor, RT shifts across MS runs can be significant enough such that precursor ions may be missed. Target proteomic analyses involving many samples or runs require particular attention to this shift in RTs

of the highest incidence, documented to be over 50% in some studies.[108, 111, 112]

Complications of post-operative delirium goes farther than the immediate post-surgical period. In the balanced trial, Evered, Chan [113] observed that patients diagnosed with delirium in the immediate post-operative period experienced significant neurocognitive deficits in the one year following the surgical insult. Significant functional decline, which necessitates placement in nursing homes, has also been reported at three month following surgery.[114, 115] Although delirium is generally considered a transient condition, some patients continue to meet the criteria of diagnosis one year after discharge.[116, 117] This phenomena, conveniently termed persistent delirium, is associated with overall higher risk of mortality and delayed functional recovery.[118] Furthermore, delirium may accelerate long-term cognitive disorders such as Alzheimer's disease, although this observation has been inconsistent across studies.[119-121]

At present, no definitive treatments exist for delirium. Because patient management is largely symptomatic, there is high demand for preventative strategies. Prevention, however, requires finding subjects who are most at risk of delirium, or at least, accurately identifying patients before overt clinical manifestation. Unfortunately, the existing diagnostics tools require that patients

are already exhibiting signs of delirium. The confusion assessment method (CAM), the Intensive Care Delirium Screening Checklist (ICDSC) and the 4A's test (4AT) are common delirium screening tools. Of these, the CAM and its accompanying variants (CAM-S, CAM-ICU, 3D-CAM, etc.) are the most widely used[122] with four main components based on the 1987 DSM III-revised criteria.[123] Despite a reasonably good diagnostic accuracy of the CAM (>94% sensitivity and 90-95% specificity), there is significant diagnostic uncertainty. Assessing for confusion and disorganized thinking introduces subjectivity and arbitrariness. Even for trained users of the tool, there is poor inter-rater agreement, reported to be about 92%.[124] Furthermore, delirium remains one of the most missed- or under-diagnosed conditions in current practice. Between 32 – 72% of cases have been reported as missed diagnosis, misattribution or late diagnosis.[125-127] Additionally, the hypoactive subtype of delirium often presents with features that are not always associated with phenotypic delirium.[128, 129]

Due to aforementioned problems, the current assessment tools may not be very helpful for a condition that has no definitive treatments, and for which early case identification and prevention is desired. We therefore assert that reliable diagnostic biomarkers of delirium are urgently needed.

Research Objectives

In this doctoral research, I hypothesize that biomarkers of delirium, discovered by comprehensively profiling the plasma samples of delirium cases and non-delirium controls, may provide an objective approach to diagnosis and offer insights into possible neuronal pathomechanisms. Comprehensively profiling of patient samples requires that the initial stages of the biomarker discovery workflow is unbiased, to allow for the detection of all possibly identifiable proteomic signatures between cases and non-cases.

To achieve this overarching research objective, Chapter Two[130] is a systematic review that summarizes the major proteomic studies over the last six years that sought to discover biomarkers of delirium using unbiased, systems-biology approaches. This study provides a birds-eye view of the attempts and approaches by various scientists towards the clinical need of discovering biomarkers of delirium. Further, the review provides a thorough assessment of experimental approaches that optimize the chances of a successful discovery endeavor, lessons from previous attempts and avenues for improvement in the quest for diagnostic biomarkers of delirium.

Consistently emphasized in this doctoral thesis is the knowledge that discovery experiments be conducted in an unbiased manner, most especially for a condition such as delirium for which much less is known about the underpinning

pathomechanisms. This approach to biomarker discovery, appropriately termed as untargeted experiments, requires that selection of candidate biomarkers be guided by the data acquired. Quite surprisingly, most studies on delirium biomarkers use a targeted approach, where a list of proteins are selected for measurement in cases and controls. The use of targeted approaches, while powerful, is unavoidably biased by the *a priori* knowledge of those biomarkers. In Chapter Three[131] of this document, I describe a targeted study of plasma samples in our cohort based on select panel of four proteins. I demonstrated that use of targeted strategies for the purposes of discovery defies the fundamental logic of biomarker discovery and may potentially lead to misleading conclusions and incorrect study outcomes.

Lastly, I applied the biomarker discovery pipeline to plasma samples from subject in our study cohort. Chapter Four[132] outlines the discovery steps, the application of advanced computational tools to handle low-abundance proteins and validation strategies that ensured the accurate quantification of discovered biomarkers in an independent set of samples. It is my hope that this series of works contributes to the growing body of knowledge on clinical proteomics, biomarker discovery and the search of objective diagnostic biomarkers of post-operative delirium.

Chapter Two: Review of Proteomic Contributions to Delirium Biomarker Research

Wiredu, K., et al., *Proteomics for the discovery of clinical delirium biomarkers: A systematic review of Major Studies.* medRxiv, 2022[133]

Authors' contributions:

KW conceptualized the study, collected and analyzed data, and drafted the manuscript. EAP assisted with data collection. Both SS and SAG guided data collection and analysis and revised the manuscript.

Abstract

Delirium represents a significant healthcare burden, diagnosed in over two million elderly Americans each year. In the surgical population, delirium remains the most common complication among elderly patients and is associated with with longer hospital stays, higher costs of care, increased mortality and functional impairment. The pathomechanism of disease is poorly understood, with current diagnostic approaches somewhat subjective and arbitrary, and definitive diagnostic biomarkers are currently lacking. Despite the recent interest in delirium research, biomarker discovery for it remains new. Most attempts to discover biomarkers are targeted studies that seek to assess the involvement of one or more members of a focused panel of candidates in delirium. For a more unbiased, systems-biology view, we searched literature from MEDLINE, Cochrane Central, Web of Science, SCOPUS, and Dimensions between 2016 and 2021 for untargeted proteomic discovery studies for biomarkers of delirium conducted on human geriatric subjects. From an overall search of 1172 publications, eight peer-reviewed studies met our defined inclusion criteria. The 370 unique peri-operative biomarkers identified in these reports are enriched in pathways involving the activation of the immune system, inflammatory response, and the coagulation cascade. IL-6 was the most commonly identified biomarker. By reviewing the distribution of protein biomarker candidates from these studies, we conclude that a panel of proteins, rather than a single biomarker, would allow for discriminating delirium cases from non-cases. The paucity of hypothesis-

generating studies in the peer-reviewed literature also suggests that a systems-biology view of delirium pathomechanisms has yet to fully emerge.

Introduction

Diagnosed in over 2 million older adults each year, delirium presents a significant healthcare burden in the United States.[134, 135] Delirium is etiologically heterogenous, with many precipitating and predisposing factors.[136-138] Following surgery, it complicates geriatric hospitalizations with significant functional impairments, longer hospital stays, higher cost of care and increased overall mortality risk.[109, 139, 140] Despite the substantial impact on the quality of life in this demographic, delirium is diagnosed through subjective assessment of a constellation of signs and symptoms within the clinical history, behavioral observation and cognitive assessments.[141] As a results, commonly used tools such as the confusion assessment method (CAM) often exhibit inter-rater variability.[142, 143]

In addition, there is considerable lack of clarity regarding the pathophysiology of the condition. Given this, it is surprising to note the majority of delirium biomarker research use targeted experiments, where authors study a selected list of biomarkers. The use of targeted strategies for the purposes of discovery, while powerful, is unavoidably biased by the *a priori* knowledge of those biomarkers and the specific focus of the hypothesis under evaluation. Targeted studies may

miss as-of-yet unappreciated functional players in a condition as complex as delirium. Furthermore, the biological complexity of commonly used biofluids (such as blood and cerebrospinal fluid) necessitate the use of a measurement platform that is precise and sensitive even for biomarkers of low abundance. Mass spectrometry (MS) remains the gold standard protein discovery platform, although high-throughput platforms such as SomaScan and proximity extension assays (PEA) have recently been used.[101, 144, 145] Unlike MS, these platforms are semi-targeted and limited in the number of proteins assayable.

The inception of the Network of Investigation of Delirium: Unifying Scientists (NIDUS)[146] in 2016 to corroborate scientific evidence on delirium has encouraged a more unified nomenclature[147] and consistency in case identification for the purposes of research. However, only a small proportion of published literature since 2016 has focused on biomarker identification (**figure 2.1**).

The identification of definitive biomarkers of delirium is likely to contribute significantly to our understanding of delirium pathophysiology and to accurately identify cases of this acute and debilitating condition.[148] Here, we have summarized proteomic contributions in delirium biomarker research in the last six years (2016 – 2021), focusing on untargeted experiments that offer a systems-biology view of the condition. We examine the merits of the different

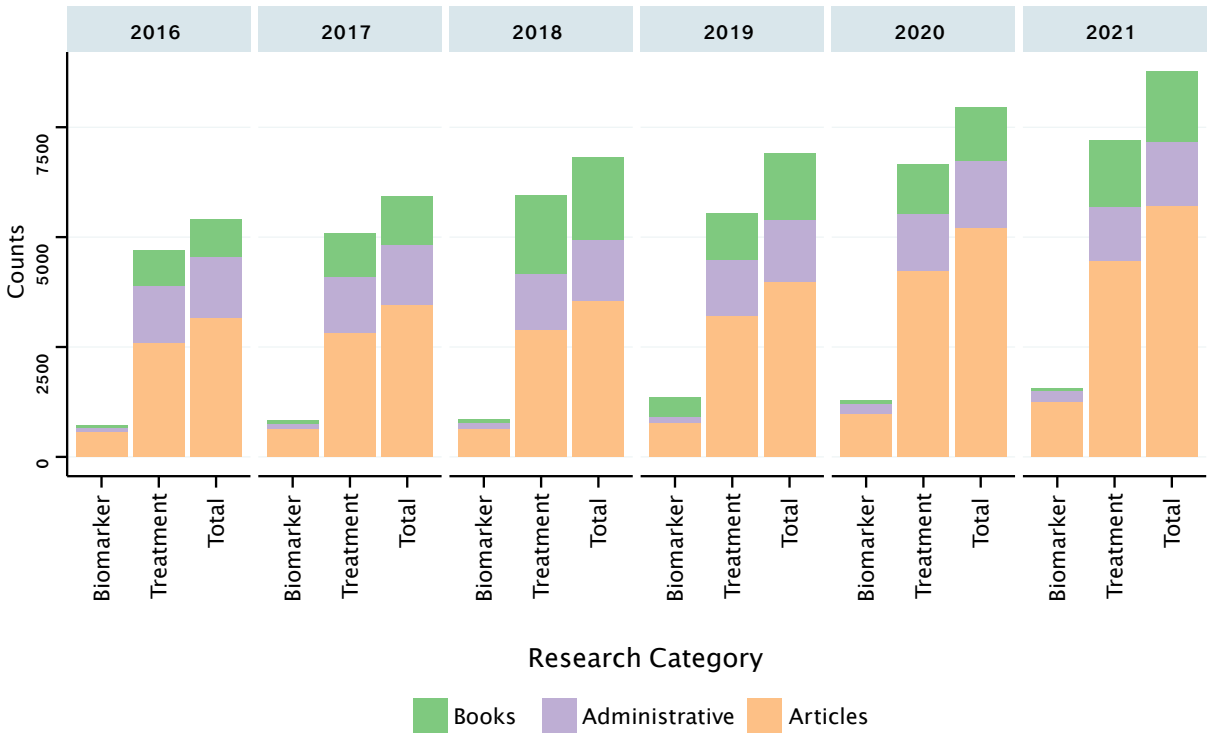


Figure 2. 1 Counts of all published documents on delirium between 2016 and 2021

Figure highlights documents involved with delirium biomarkers only, or delirium treatment and prevention. Documents described as articles include peer-reviewed original research, study protocols, preprints, poster abstracts, monographs, conference proceedings and editorials and opinions. Administrative documents include grants, patents, clinical trials and policy documents. (Source of data: Dimensions.ai[149], downloaded on 03/25/2022)

measurement platforms and experimental approaches and have offered perspectives on optimizing sample preparation for the detection of low abundance biomarkers. Lastly, we analyzed the biomarker pool from the published studies for understanding of functional themes that may be at play in the occurrence of delirium.

Methods

Following the PRISM guidelines,[150] we searched five databases (MEDLINE, SCOPUS, Central, Web of Science and Dimensions) using the key terms [delirium, acute confusion, acute brain failure] AND [biomarker, biological marker] AND [proteins, proteomics] **(Supplemental Table 1**). Search results were limited to publications written in English and published from 2016 – 2021. EndNote bibliography software version X9.3.3[151] was used for duplicate removal. All remaining publications were independently reviewed by KW and EAP in a two-stage process. Rayyan freeware, a free web-tool for systematic reviews,[152] was used to expedite the initial (title and abstract) screening. Secondary screening of remaining publications involved full text review for publications that met the inclusion criteria of (1) untargeted proteomic profiling, (2) for biomarkers of delirium, (3) conducted on human geriatric subjects. Discordance was resolved by both KW and EAP through consensus.

Results

We identified a total of 1,172 publications from 5 database searches (MEDLINE, SCOPUS, Central, Web of Science and Dimensions). The majority of articles excluded during initial screening were review articles, non-biomarker articles, poster abstracts, meeting proceedings, editorial and comments. Full text review was performed on 280 articles, many of which were either targeted biomarker studies on delirium, animal studies or involved non-protein delirium biomarkers such as brain imaging. Unique in these exclusions was one study on delirium in children. **Figure 2.2** is a flowchart of the screening steps and exclusion criteria, leading to the final eight peer-reviewed original studies summarized in **Table 1**. Of the eight studies, five were conducted in North America [1, 2, 5, 7, 8] and one each in Asia [3], Europe [6] and Sweden [4].

Study Design, Patient Selection and Choice of Controls

All but two studies had a nested, case-control design.[4, 6] Overall, the age of delirium cases averaged 73.3 years (**Table 1**). Samples from a total number of 484 subjects (cases and controls) were used for biomarker discovery alone, although there is likely an overlap in subjects selected from the same parent study (i.e., the SAGES study or the MINDDS trial). Except for one study which profiled biofluids from non-surgical patients[6], eligible subjects were all surgical patients, who underwent either cardiac[5, 8] or non-cardiac procedures.[1-4, 7] Patients' comorbidity score, either with the Charlson index or PROMIS, was established in all but for two studies[4, 6], although van Ton, Verbeek [6]

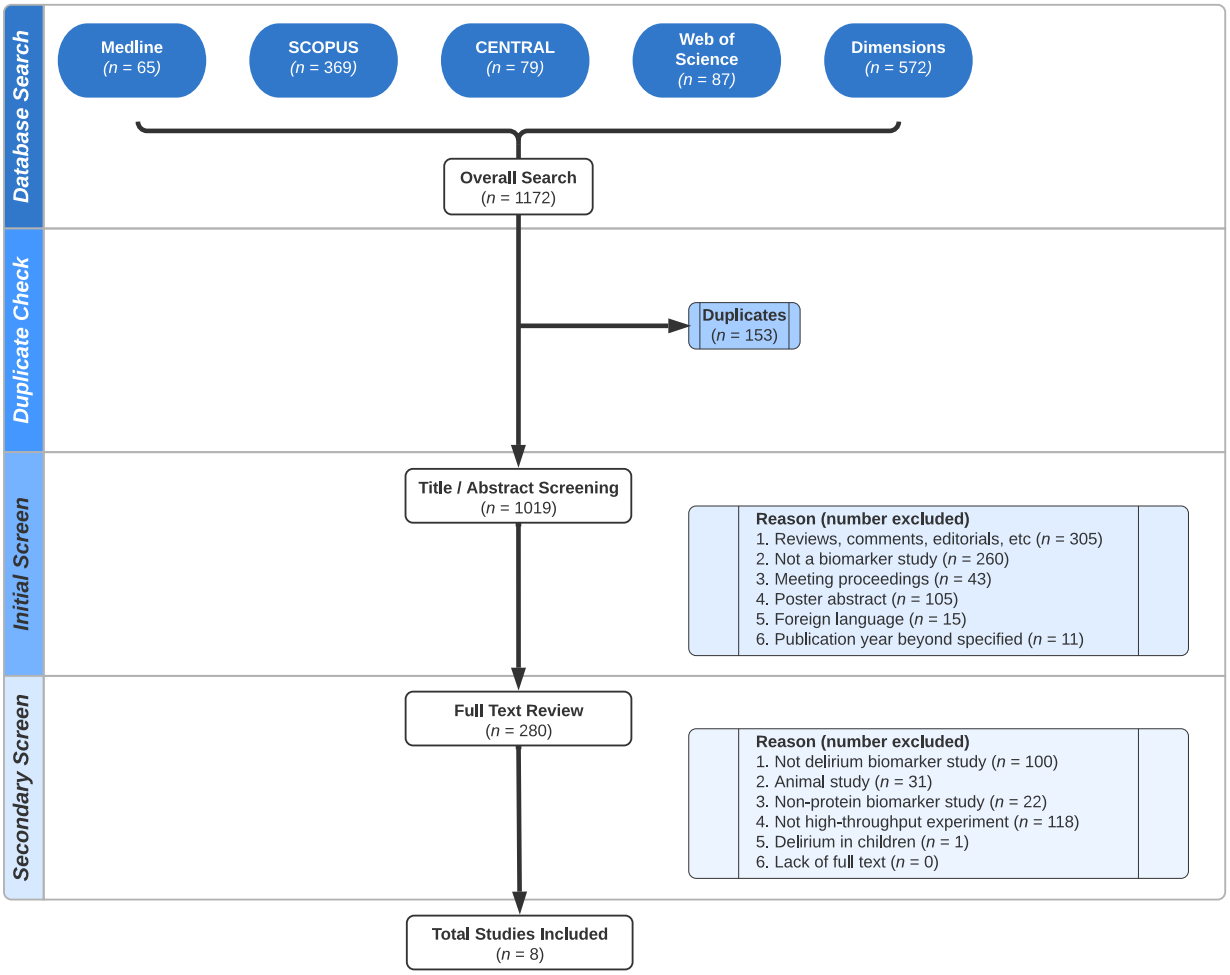


Figure 2. 2 Literature search and screening

PRISMA flow chart highlights the step-by-step process involved in the selection of the final 8 studies summarized in this review.

indicated that hypertension, diabetes, immunosuppression and cerebrovascular disorders were common in the selected cohort.

Delirium cases were identified with either the Confusion Assessment Method (CAM), the Chart-based Delirium Identification Instrument (CHART-DEL) or the Diagnostic and Statistical Manual of Mental Disorders (DSM-V) (**Table 1**). Subjects from the MINDDS trial [5, 8] received twice daily post-operative assessments for delirium occurrence, compared with once daily assessments for the SAGES cohort [1, 2, 7].

Baseline neurocognition of study participants was established in seven studies (**Table 1**). Neither the case identification method nor baseline neurocognition approach was specified by Lindblom, Shen [4]. All eight studies used non-delirium controls to establish a statistical baseline, with some variations in the choice of controls. Controls were age- and sex-matched in five studies [1, 2, 5, 7, 8], although the SAGES cohort included baseline cognitive performance as an additional matching parameter. Han, Chen [3] selected controls that matched to cases by age and by mini-mental state examination (MMSE). In the study by van Ton, Verbeek [6], two groups of controls to the post-infectious delirium cases were selected: healthy controls and controls with neurocognitive impairments

other than delirium. Similarly, Lindblom, Shen [4] selected surgical patients who suffered other neurological injuries but without the diagnosis of delirium.

Sample Preparation and Proteomic Techniques

The source of proteins for biomarker discovery included peripheral blood (five studies), cerebrospinal fluid (CSF; two studies) and both blood and CSF (one study) (**Table 1**). When CSF was used, lumbar puncture samples were collected only once. This contrasts with the serial collection of samples for the blood-based studies. Three of the six blood-based studies used plasma and the remaining three used serum. Of note, only the plasma-based studies [1, 2, 7] documented sample immunodepletion, specifically by using affinity-based depletion columns to remove the 14 most abundant proteins in an effort to detect proteins of lower abundance. Three studies used mass spectrometry (MS) as the analytical approach, three studies used proximity extension assays (PEA), and the remaining two used SomaScan technology. Two studies attempted sample multiplexing with isobaric labelling [1, 2]. There was, however, no mention of pre-analytical sample fractionation to further reduce sample complexity in any of the studies.

Reference	Study Design	Age* of cases	Age of controls	No. of cases^	No. of controls	Baseline NeuroCog	Delirium Screen	Biofluid Used	Discovery Technique	Validation Technique	No. of Proteins §	Major Findings
Dillon, Vasunilas horn [2]	Nested, case control	77.6	77.2	75¶	75	GCP	CAM or CHART-DEL	Plasma	iTRAQ LC-MS/MS	ELISA	152	Pre-operative CRP has prognostic potential, and subsequent post-operative increases in CRP levels supports a heightened neuroinflammatory model of delirium, possibly instigated by the surgical insult
Lindblom, Shen [4]	Prospective cohort	59.8†	-	8	15	-	-	Serum, CSF	PEA	-	92	Different proteomic signatures were observed in serum and CSF from the same subjects, notably TR4, EZH2 and KLF6. This

												may reflect different pathophysiological responses to surgical insult in and outside the brain
Vasunilashorn, Ngo [1]	Nested, case control	77.3	76.8	75¶	75	GCP	CAM or CHART-DEL	Plasma	iTRAQ LC-MS/MS	ELISA	80	A dynamic panel of pre-operative (CRP and AZGP1) and post-operative biomarkers (IL-6, IL-2 and CRP) can aid pre-operative risk stratification and post-operative case identification, respectively
Han, Chen [3]	Nested, case control	82.2	81.7	10	30	MMSE	CAM	CSF	LFQ LC-MS/MS	PRM-MS	1076	63 differentially abundant CSF proteins fall into two main functional

												clusters of neuronal dysgenesis and endoplasmic reticulum-related post-translational modification. Concentrations of these proteins also correlate with delirium severity
van Ton, Verbeek [6]	Retrospective cohort	64.2	49.3	15	30	-	DSM-V	CSF	PEA	-	153	Proteins involved in synaptic signaling were downregulated in both delirium and AD patients. The significant overlap in biomarkers between the two conditions hints synaptopathy (specifically

												synapse loss) may be a common early mechanism
McKay, Rhee [5]	Nested, case control	74	73	12[∂]	12	tMOCA	CAM	Serum	PEA	-	182	Post-operative differential abundance of FGF-21, FGF-23, IL-6 and MCP-3 suggests metabolic recovery as a possible pathomechanism underpinning POD
Rhee, Kuznetsov [8]	Nested, case control	75	74	8[∂]	8	CAM	CAM or Structured Chard Review	Serum	SOMAscan	-	1305	Cardiac surgery with CPB likely instigates systemic inflammation. Serum proteins with significant changes following CPB are functionally enriched for

												leukocytes recruitment and upregulation of metabolic processes (e.g., glycolysis). IL-6 and PDE3A are key examples
Vasunilashorn, Dillon [7] 44	Nested, case control	76.4†	-	18¶	18	GCP	CAM or CHART-DEL	Plasma	SOMAscan	ELISA	1305	A panel of 7 proteins identified as potential marker for preoperative risk stratification suggests a heightened pro-inflammatory state prior to occurrence of delirium. Another panel of 4 proteins was identified as post-operative diagnostic markers. CHI3L1 is

												notably the only common biomarker between the two sets of panels.

Table 2. 1 Summary of included studies

*: mean age of delirium cases and non-delirium controls (in years)

^: number of cases and controls in the discovery phases of experiment

†: authors reported a combined mean age of all subjects

§: total number of proteins identified (not necessarily what was used for downstream analysis)

¶, ∂: subjects were likely selected from the same parent study, SAGES and MINDDS, respectively

Abbreviations:

AD: Alzheimer's disease; **AZGP1**: zinc-alpha-2-glycoprotein; **CAM**: confusion assessment method; **CHART-DEL**: Chart-based Delirium Identification Instrument; **CPB**: cardiopulmonary bypass; **CRP**: c-reactive protein; **CSF**: cerebrospinal fluid; **DSM-V**: Diagnostic and Statistical Manual of Mental Disorders (5th edition); **ELISA**: enzyme-linked immunosorbent assay; **EZH2**: histone-lysine N-methyltransferase; **FGF**: fibroblast growth factor; **GCP**: general cognitive performance; **IL-2 / IL-6**: interleukin 2 / interleukin 6; **iTRAQ**: isobaric tags for relative and absolute quantification; **KLF6**: krueppel-like factor 6; **LC-MS/MS**: liquid chromatography – tandem mass spectrometry; **MCP-3**: monocyte chemotactic protein-3; **NCog**: baseline neurocognition; **PDE3A**: cGMP-inhibited 3',5'-cyclic phosphodiesterase A; **PEA**: proximity extension assay; **POD**: post-operative delirium; **PRM-MS**: parallel reaction monitoring – mass spectrometry; **tMOCA**: telephone-based Montreal cognitive assessment; **TR4**: nuclear receptor subfamily 2 group C member 2

Proteomic Biomarkers

In the union of all eight studies, 446 unique proteins were identified as candidate biomarkers for delirium. Of these biomarkers, 370 were identified peri-operatively. **Figures 2.3** and **Figures 2.4** illustrate the contribution of each study to the total pool of candidate biomarkers, and where biomarkers overlap between studies. Overall, Vasunilashorn, Dillon [7] reported the largest number of differentially abundant proteins (*n* = 128) between cases and controls. Interleukin-6 (IL-6) was the most commonly identified, differentially abundant protein among the studies [4-8]. Complement component C9, antithrombin-III (SERPINC1), the cytokine fractalkine (CX3CL1) and chitinase-3-like protein 1 (CHI3L1) are notable biomarkers that were found in three or more studies. Except for the studies done in the SAGES cohort [1, 2, 7], very few of the remaining proteins overlap between studies.

Functional analysis on the biomarker pool of 370 proteins for enriched biological processes suggests a systemic response of widespread activation and dysregulation of proteins involved in immunological reactions, inflammatory responses, and the coagulation cascade (**Figures 3A, 3D**). Furthermore, subcellular ontology annotation reveals the extracellular region as the predominant native location of these dysregulated proteins, enriched in signaling and cytokine activity (**Figures 3C, 3D**).

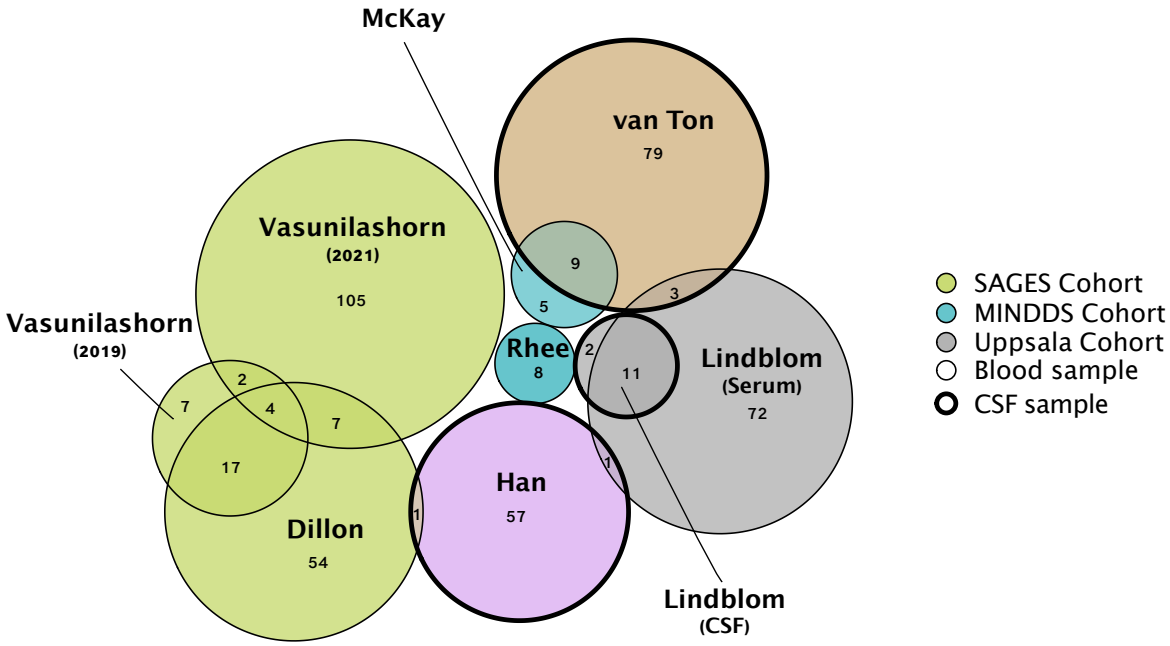


Figure 2. 3 Modified Venn (Euler) diagram

Modified Venn (Euler) diagram showing the intersecting biomarkers between the 8 studies. Size of circle is proportional to number of biomarkers. Colors represent studies from the same cohort, with likely overlap in the subjects selected for proteomic profiling. Thickness of circle outline indicates the type of biofluid used. NB: Intersections between 4 or more studies are not visualized here.

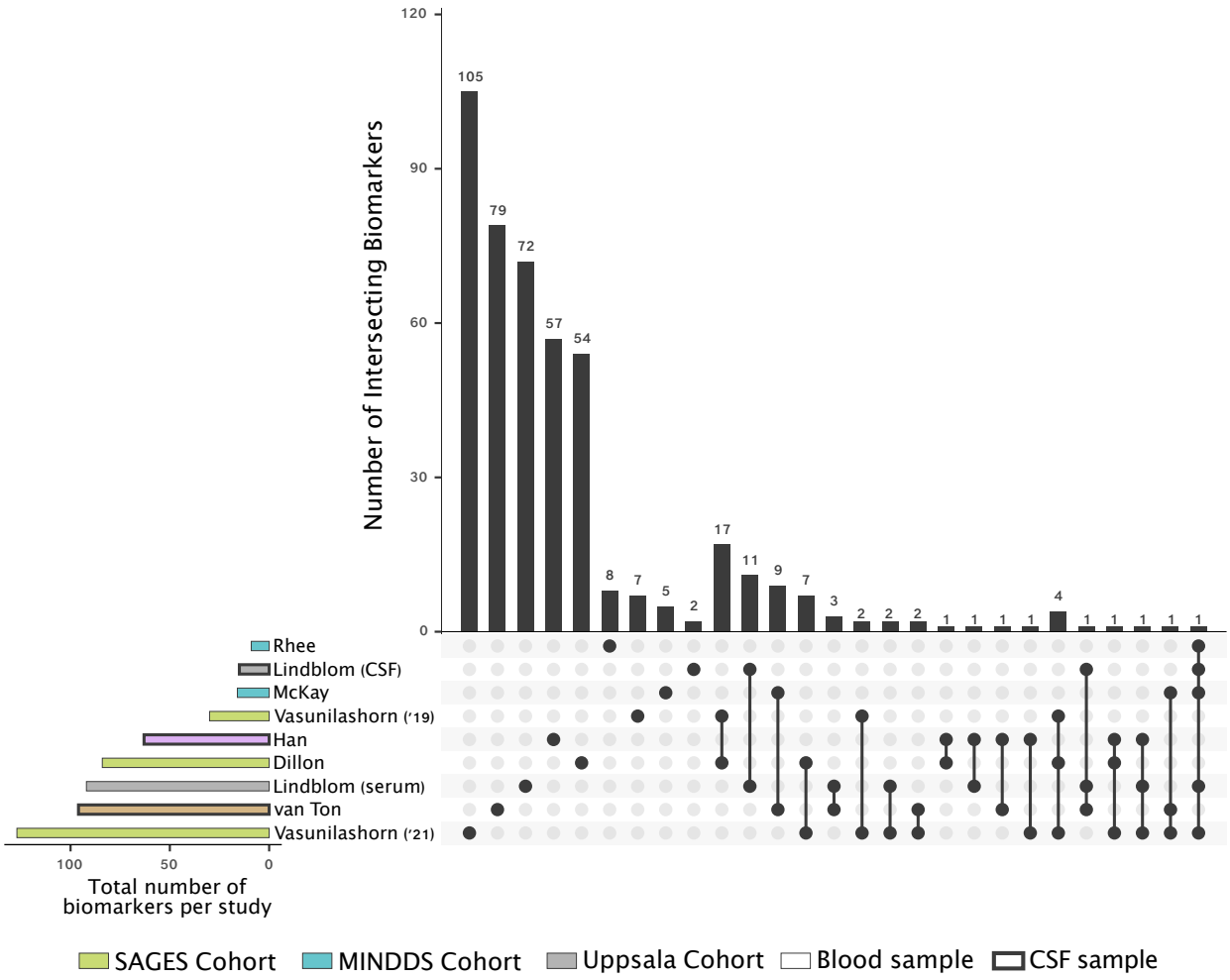


Figure 2. 4 UpSet chart

Figure shows the contributions of each study to the total pool of 446 unique biomarkers, and all the intersecting sets of proteins that could not be illustrated in figure 2 (main). Same colors represent studies from the same cohort, with likely overlap in the subjects selected for proteomic profiling. Thickness of rectangle outline indicates the type of biofluid used.

Validation Approaches

All three mass spectrometry-based studies and one SomaScan-based study performed protein verification and validation on candidate biomarkers identified by high throughput methods (**Table 1**). Of these studies, three used ELISA, an affinity-based approach[1, 2, 7] and the remaining study used a MS-based approach, specifically parallel reaction monitoring (PRM).[3] The choice of biomarkers that were validated is however varied. Of the 63 differentially abundant proteins in the study by Han, Chen [3], 20 were selected for validation by PRM based on a minimum number of peptides and transitions set by the authors. Of the remaining studies, the choice to validate CRP, SERPINA3, AZGP1 and CHI3L1 was based, partly, on the consistency of their identification in various samples, the availability of a commercial antibody and a series of binomial, signed rank and Student *t*-tests.

Discussion

We have presented an in-depth review of clinical proteomic contributions over the preceding six complete years that offered an unbiased systems-biology view of delirium. As consistency in case identification and unified nomenclature is necessary to make comparisons between studies, we began our literature search from 2016. We observed that this is also the year NIDUS was established,[146]

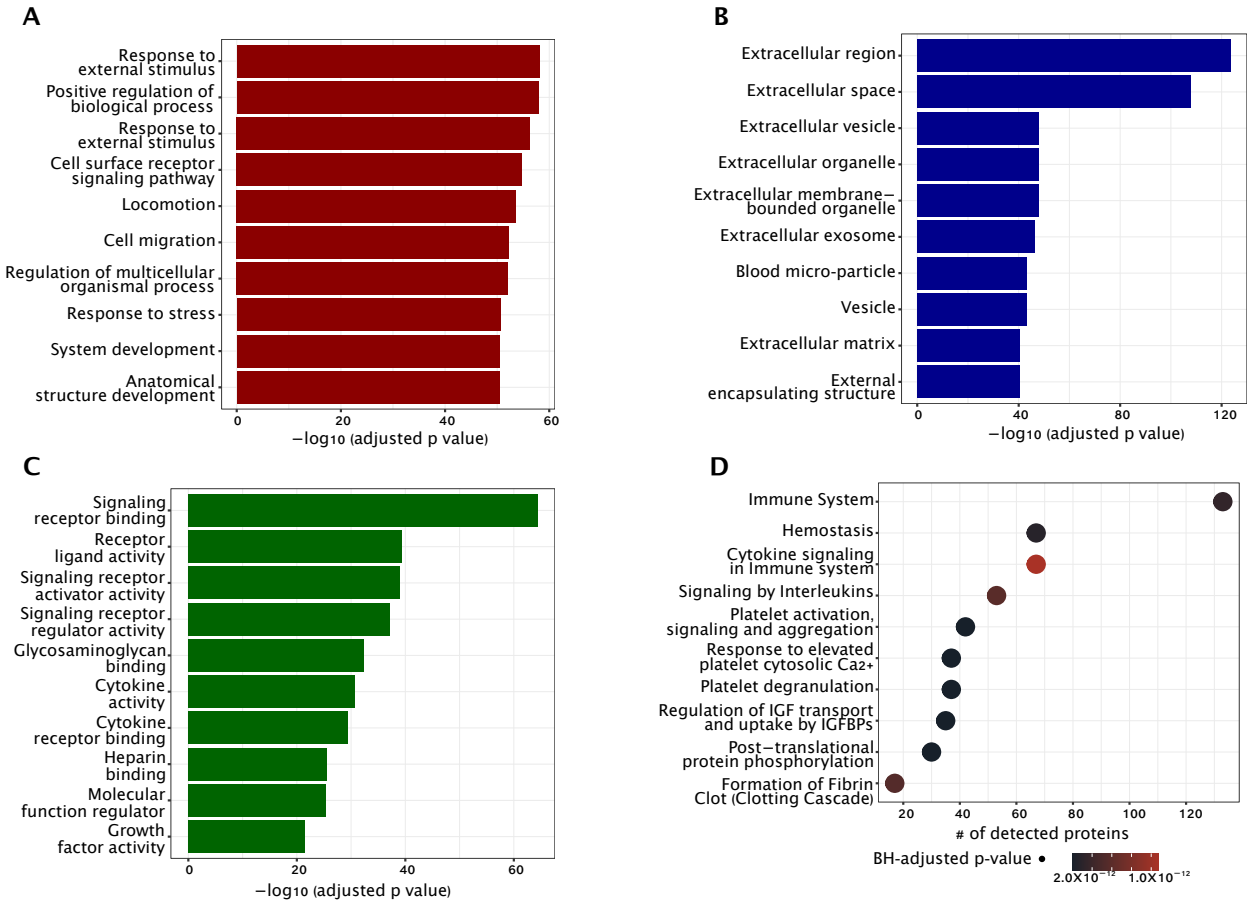


Figure 2. 5 Functional analysis of the biomarker pool

Functional analysis of the biomarker pool showing the top 10 GO terms with regards to (A) biological processes, (B) cellular component, and (C) molecular functions. The number of proteins involved in each of the major functional classes in the biomarker pool are shown in (D).

and interest in delirium research has seen a steady increase since that point. We focused on studies that measured delirium biomarkers using a discovery-based approach in the human geriatric population. The total of eight studies that met our criteria signify the paucity of literature that offers a systems-biology view of delirium in this demographic. Nonetheless, the large proportion of search results that were excluded as grants, conference abstracts, meeting proceedings, and posters suggest a growing interest in research on delirium diagnostics.

We aggregated a total of 446 biomarkers that are differentially dysregulated in human patients with delirium across eight studies. It is worth noting that subjects in one study [6] developed delirium after an infectious process. Functional analysis of the 370 peri-operative pool of biomarkers suggests a widespread activation of immunological reactions, inflammatory responses, and the coagulation cascade. We focused functional analyses only on biomarkers discovered peri-operatively, given that infectious delirium may involve a different pathophysiological process[6, 153-155], although analyses of all 446 biomarkers did not reveal any functional differences. Given that IL-2, C-X-C motif chemokine 11 and C-C motif chemokine 13 [1, 5, 7], among others, were elevated preoperatively, it is equally likely that a heightened pre-operative inflammatory state increased the risk of delirium, although functional studies would be required to rigorously test this hypothesis. This observation is consistent with prevailing knowledge that phenotypic delirium is a culmination of multiple predisposing and

precipitating factors [138, 156, 157], and predictive biomarkers may be more beneficial in certain risk groups than others.

At the cellular level, the biomarker pool is enriched in signaling and cytokine activity, predominantly in the extracellular domain. While there are many extracellular domain-containing proteins that do not localize to synapses, this observation may signify the possibility of altered synaptic functioning in the context of delirium. Synaptic dysfunction is an early event in Alzheimer's disease [158], and many researchers have suggested similar findings as a common pathophysiological starting point in the continuum of neurocognitive disorders, of which delirium and AD are a part [159-161].

Interleukin-6 remains one of the most consistently identified proteins among delirium patients. In well-functioning older patients, IL-6 is found to be prospectively associated with cognitive decline [162-164]. IL-6 is part of the core panel of frailty biomarkers [165], and has recently been suggested by Gómez-Rubio, Trapero [166] as a useful biomarker for monitoring treatment in frail individuals. The inflammatory role of IL-6 and its associations with aforementioned predisposing triggers further emphasize the neuro-inflammatory model of delirium.

Notable in the 24 overlapping proteins between Dillon, Vasunilashorn [2] and Vasunilashorn, Ngo [1] is CRP, an acute phase reactant and a non-specific marker of inflammation, infection and tissue injury [167]. Many authors have suggested elevated CRP levels to be associated with a higher risk of delirium occurrence [168-173], and could likely be used to monitor the clinical course of delirium [174]. In a recent meta-analysis of 54 observational studies, Liu, Yu [175] hinted that CRP may be a more specific marker of post-operative delirium (POD) than post-operative cognitive dysfunction (POCD). The clinical relevance of this specificity remains unclear, given that a lower baseline cognitive reserve is a precipitating factor for both POD and POCD [176].

It is well documented that elevated total cholesterol and LDL correspond to increased neuritic plaque density in Alzheimer's disease [177, 178]. One overlapping protein, apolipoprotein A-IV (APOA4) is a component of chylomicrons and HDL, synthesized mainly in the intestine and secreted into plasma [179]. Although there is some tenuous evidence of an association with cognitive impairment and Alzheimer's disease [180, 181], data on APOA4 is scarce and there has yet to be a formal interrogation of its association in delirium. This holds true for many proteins in this union of 446 biomarkers.

Furthermore, it is also unclear how the modest degree of overlap in candidate biomarkers between the eight studies reflect differences in the biofluids used or

the analytical strategies used to identify the biomarkers. While MS relies on peptide spectrum matches for protein identification, PEA (Olink Proteomics, Uppsala, Sweden) is an oligonucleotide-based immunoassay that combines quantitative real-time PCR with high-throughput quantification. SomaScan (SomaLogic, Inc, Boulder, CO), on the other hand, uses aptamers to bind specific molecular targets. These affinity technologies have recently gained attention in plasma proteomics as they are cost-effective, require less expertise, much smaller sample volumes and can quantify a little over 1000 human plasma proteins. In fact, the number of recent original publications on plasma proteomics that use PEA outnumber those that present MS-based approaches [101]. Despite reports of comparable reproducibility and complementarity of PEA and SomaScan to MS, a recent study comparing PEA to MS-based protein profiling revealed a similar modest degree of overlapping proteins as found in this review [101]. Of the 14 available PEA panels, van Ton, Verbeek [6] and McKay, Rhee [5] used the two that predominantly assay neural and inflammatory markers. It is therefore not surprising that there are nine overlapping biomarkers between these two studies. The SomaScan-based studies had only one biomarker in common, namely IL-6. Given that these affinity-based platforms are semi-targeted and predominantly assay the low abundance plasma proteome, it is our thinking that the strengths of all three analytical techniques could be viewed as complementary, offering a deeper view into the plasma proteome together than each would separately.

Three of the six blood-based studies used serum which is qualitatively and quantitatively different from plasma. Removal of clotting factors (largely fibrinogen) results in a 3 – 4% lower protein concentration in serum relative to plasma [65, 66], and may also lead to removal of proteins with specific (or non-specific) interactions with fibrin in a manner that is unpredictable. The Human Proteome Organization (HUPO) therefore endorses the use of plasma, citing a lower degree of *ex vivo* degradation and recommends that citrate or EDTA be used for anticoagulation over heparin [67]. As the sample choice should be tailored to the specific biomarker needs and the biomarker landscape of delirium is still in its infancy stages, it would be preferable that the biofluid used, their collection and sample preparation protocols permit the study of the entire plasma proteome.

Serial collection of samples in the blood-based studies allowed for the determination of temporal associations of proteomic changes with the occurrence of delirium. In all but one of the blood-based studies [4], a minimum of two samples were collected for each study participant: at baseline (pre-operative) and on post-operative day one. This is in sharp contrast to the CSF-based studies which were limited by the one-time sample collection by lumbar puncture. CSF is the proximal biofluid of choice with a greater likelihood of reflecting the immediate proteomic changes in the brain. Unfortunately, CSF access is severely limited by the invasive nature of the sampling technique (lumbar puncture). Furthermore, it is hypothesized that the relatively higher permeability

of the blood brain barrier of the elderly brain makes it possible for proteomic changes in CSF to be detected in plasma.

Immunodepletion is generally thought to be beneficial because of the wide dynamic range (~10^{10}) of protein abundances in plasma and CSF, dominated by a handful of highly abundant proteins such as albumin and immunoglobulins. This makes identifying and quantifying proteins of lower abundance otherwise difficult. Three of the eight studies reported use of immunodepletion on the samples prior to analysis. Given that PEA and SomaScan are semi-targeted, it is unclear if immunodepletion is a necessary pre-analytical step. The extent to which the use immunodepletion, or otherwise, affected the identification of candidate delirium biomarkers is unclear. In addition, fractionation strategies such as ion exchange chromatography significantly reduce sample complexity and increase the depth of proteome coverage, especially when searching for low abundance plasma proteins. With the collective results from all eight studies indicating possible neuro-inflammatory process(es) to play a prominent role in delirium pathogenesis, candidates-biomarkers of delirium are likely to be in the low abundance proteome, which makes use of immunodepletion and sample fractionation an important consideration in the experimental design. Clearly, the relative advantages and disadvantages of each sample type and the approach to sample preparation will continue to play a significant role in the design of future studies to identify protein biomarkers in delirium and neurocognitive disorders at large.

Integrating results from eight different studies was not without challenges, one of which was the use of different delirium diagnostic algorithms. Even within the same study, Dillon, Vasunilashorn [2] employed both CAM and chart review for delirium case identifications. Further, the inclusion of subsyndromal delirium (SSD) cases together with delirium cases in the studies by Dillon, Vasunilashorn [2], Vasunilashorn, Ngo [1] and Vasunilashorn, Dillon [7] may further complicate data interpretation in the context of other studies. Additionally, none of the studies formally screened for depression among the study subjects.

Lastly, nested case control is an adequate study design for biomarker studies. It is however limited in precision, inferential conclusions and power due to sampling of controls. Only associations, and not causal inferences, can be concluded from nested designs, even after adjusting for most confounding variables [182, 183]. The choice of controls to establish a statistical baseline plays a significant role in subsequent differential abundance analyses. In the study by van Ton, Verbeek [6], controls were significantly younger than delirium cases (49.3 versus 64.2 years). Nonetheless, this is the only study in which authors excluded control subjects with a known acute or chronic systemic inflammation.

Conclusion

The urgent need for diagnostic and predictive biomarkers of delirium is important not only to correctly identify cases, but also for pre-operative risk stratification and for follow-up on possible long-term neurocognitive sequelae. Interest in delirium research has seen a steady rise since the inception of NIDUS in 2016. Nonetheless, delirium biomarker research appears to be just emerging. There are only a handful of studies that offer a systems-biology view of delirium from human patient samples. For diagnostic purposes, it appears likely that a panel of biomarkers, rather than a single biomarker, has potential for discriminating delirium cases from non-cases. Collectively, biomarkers from these studies suggest an immunological and inflammatory response following surgical insult, enriched in cytokine and signaling activity in the extracellular space. Further studies are warranted to support this observation. With a greater focus on the low-abundance plasma proteome, complementary use of MS and PEA may yield a deeper plasma proteome profiling.

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Chapter Three: Perioperative Hyperoxia and Delirium after Cardiopulmonary Bypass

Wiredu, K., et al., *Perioperative Hyperoxia and Delirium after On-pump Cardiac Surgery: A Mediation Analysis.* medRxiv, 2022[131]

Authors' contributions:

KW performed data analysis, assisted with conceptualization of the study and drafted the manuscript. SV and HN assisted with literature search. MDB, SS and SAG guided study conceptualization, data analysis and manuscript revision.

Abstract

Background

Neurologic and neurobehavioural complications are common after cardiac surgery with cardiopulmonary bypass (CPB). Exposure to the artificial bypass surface, conversion to laminar flow and hypothermia likely contribute to systemic inflammation observed after CPB. To ensure adequate systemic oxygenation, the CPB patient is often exposed to supraphysiologic levels of oxygen. Relative to normoxia, perioperative hyperoxia during CPB has not been shown to impact neurocognition in the long-term. Whether this holds true for the immediate post-operative neurocognitive function is the question of this nested case-control study.

Methods

46 age- and sex-matched subjects, aged ≥ 65 years, selected for this study were randomized to receive normoxia or hyperoxia during CABG with CPB in the parent trial. Levels of four neuroinflammatory biomarkers (S100B, ENO2, CHI3L1, UCHL1) were measured at baseline and at post-bypass. Baseline neurocognition was established with the Montreal Cognitive Assessment tool and patients were assessed on each post-operative day for delirium using the confusion assessment method. Mediation analyses was conducted for the conditional effect of perioperative oxygen treatment on the occurrence of delirium, assuming mediation effect from change in biomarker levels.

Results

26 subjects (*n* = 12) demonstrated delirium. Of the four biomarkers, only S100B levels were differentially abundant post-bypass regardless of treatment (8.18

versus 10.15pg/mL, *p* value < 0.001). We found significant direct effects of treatment and the interaction of treatment and baseline neurocognition in the occurrence of delirium (effect = 0.08, *p* = 0.013). There was no significant mediating effect of S100B levels.

Conclusion

While perioperative hyperoxia may not be associated with neurocognitive dysfunction in the long-term, its immediate effects may contribute significantly to the occurrence of post-operative delirium. Taken together, our findings suggest a dose-response-time relationship between hyperoxia and neurocognitive function.

Introduction

The effects of perioperative hyperoxia on myocardial damage, acute kidney injury and long-term neurocognitive dysfunction are well documented [184-189], but the impact on the immediate post-operative neurocognitive function is less well-characterized [190]. Globally, over 1.25 million patients undergo cardiac surgery on cardiopulmonary bypass (CPB) each year [191]. Perioperatively, the CPB population are often exposed to supraphysiologic concentrations of oxygen [192]. This practice is premised on the primary goal of maintaining end-organ perfusion as hypothermia, microcirculatory heterogeneity and interstitial fluid shifts during CPB all contribute to poor tissue oxygenation [193].

At the tissue level, hyperoxia is beneficial in the ischemic preconditioning of the myocardium, reduces overall gas microemboli and provides significant oxygen

reserves in the event of interrupted ventilation [194-196]. In fact, the ability to monitor regional cerebral oxygenation in real-time has provided unequivocal evidence linking cerebral desaturation during CPB to worse clinical outcomes [197-200], further emphasizing the need for higher oxygen concentrations. On the other hand, hyperoxia may also trigger vasoconstriction that further compromises perfusion, may instigate inflammation and worsen ischemia-reperfusion injury through increased oxidative stress [201-204]. Notwithstanding these, the prevailing observation is that hyperoxia during CPB is not associated with poor long-term neurocognitive outcomes [185, 188, 205].

Delirium is etiologically heterogeneous and lower pre-surgical cognitive function is a recognized risk factor [206-208]. The extent to which perioperative oxygen treatment modifies the occurrence of delirium in a typical CPB demographic with suboptimal baseline neurocognition, however, remains largely unknown. In this nested case-control study, we examined the conditional effect of perioperative hyperoxia on the occurrence of post-operative delirium in an elderly cohort who underwent cardiac surgery on CPB. Further, we measured a panel of neuroinflammatory markers and ascertained their possible role in mediating the hyperoxia-delirium relationship. Finally, we proposed a conceptual model regarding the interaction between baseline neurocognition and perioperative hyperoxia as they relate to post-operative delirium.

Methods

Study design and Ethics approval

Subjects in this nested case-control study were selected from the parent clinical trial [188, 209] that examined the effects of intra-operative oxygen therapy on neurocognitive outcomes among cardiac surgical patients at the Beth Israel Deaconess Medical Center, Boston MA (Trial registration number NCT02591589, principal investigator: Shahzad Shaefi, registration date: October 29, 2015). Subjects were enrolled between July 2015 and July 2017, and all patients provided informed consent. Institutional review board (IRB) approval 2014P000398/33 was amended for the purposes of this current study on 09/17/2021 by the Committee on Clinical Investigations (CCI) at the BIDMC. **Figure 3.1** summarizes the design of the current study.

Patient population, Exclusion and Inclusion Criteria

Details of enrollment, exclusion criteria and treatment allocation are published elsewhere [188, 209]. Briefly, eligible participants included patients 65 years or older who were booked for elective CABG requiring CPB. Neurocognitive assessment was achieved using the telephonic Montreal Cognitive Assessment (tMoCA) as the primary endpoint. Post-operatively, subjects were assessed daily for delirium as a secondary endpoint using the confusion assessment method

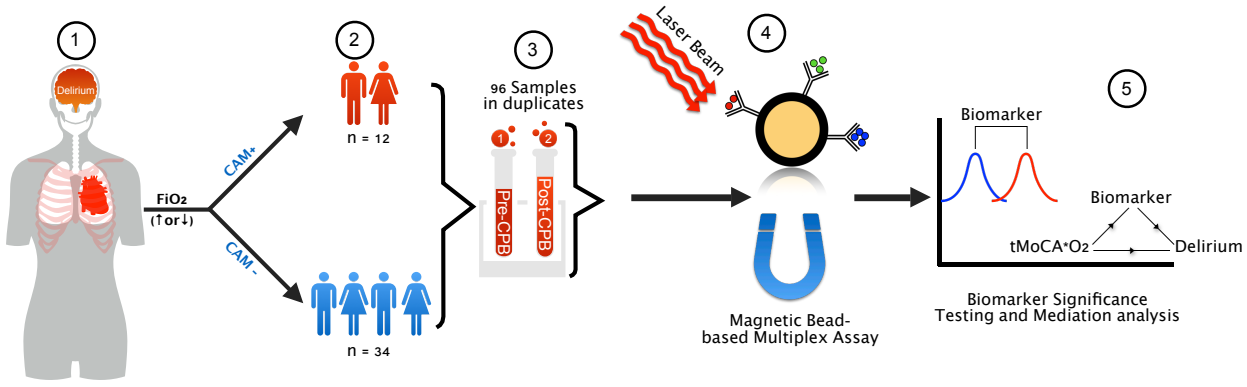


Figure 3. 1 Experimental design

Schematic illustration of the nested case-control study, plasma sampling times and biomarker measurements by bead-based multiplex assay. CAM: confusion assessment method; CPB: cardiopulmonary bypass

(CAM). Patients were excluded if they were undergoing emergent CABG, if they required single-lung ventilation, CABG without CPB, intraoperative balloon counter-pulsation or mechanical circulatory support. Subjects with MoCA scores below 10 were also excluded.

Sample size calculation

Because quantitative studies on the selected biomarkers in the context of delirium are largely unexplored, Cohen's estimation of effect size [210] was used to determine the optimal sample size at a significance level of 0.05 and a statistical power of 80%. Further, delirium cases were matched to non-delirium controls at approximately 1:3 ratio, to a total of 46 subjects in the current study.

Conduct of Study and Biomarker Measurements

Whole blood samples at baseline and post-bypass (P-BP) were collected into 4mL EDTA-treated tubes (BD Diagnostics) and centrifuged immediately. Resulting plasma was stored at -80ºC until analyses. Limits of detection, limits of quantification and linearity of biomarker signal were established using serial dilutions of patient samples and laboratory standards. Biomarker measurements were made using a custom R&D Human Premixed Multi-Analyte Panel, a magnetic bead-based multiplex assay (Catalog Number: LXSAHM-04, Lot Number: L140030). Analyte concentrations were determined by a 5-parameter logistic (PL) regression computed from the standard curves. All biomarkers were

measured in duplicates. For quality control, intra-assay variability was assessed at a cut-off of 20%.

Statistical analyses

Descriptive statistics are presented as mean (standard deviations) or count (proportion) for continuous and categorical variables, respectively. Duplicates recordings of biomarkers were averaged and compared between groups using Student's *t*-test (paired, unequal variance). Stepwise regression was used to identify clinical variables with the most predictive association to the outcome variable (delirium). Structural equation modeling (SEM) was used to construct the conceptual and statistical mediation models based on this subset of variables (**Figure 3.2**). Assuming all effects to be linear, mediation analysis was then performed to ascertain the total, indirect and direct effects of intraoperative oxygen treatment on the occurrence of delirium, with post-bypass change in biomarker levels as the assumed mediating factor. Here, the direct effect of perioperative oxygen treatment was computed as the change in the odds of developing delirium in patients receiving hyperoxia versus the odds of developing delirium in patients receiving normoxia, when baseline neurocognition is fixed (i.e., holding tMoCA score constant). Average tMoCA score was also defined as the arithmetic mean of tMoCA scores for subjects in this nested cohort. Significance of the mediation effects were computed by bootstrapping [211]. All analyses were performed in R environment for statistical computing, v4.1.1 [212] at a significance level $\alpha = 0.05$.

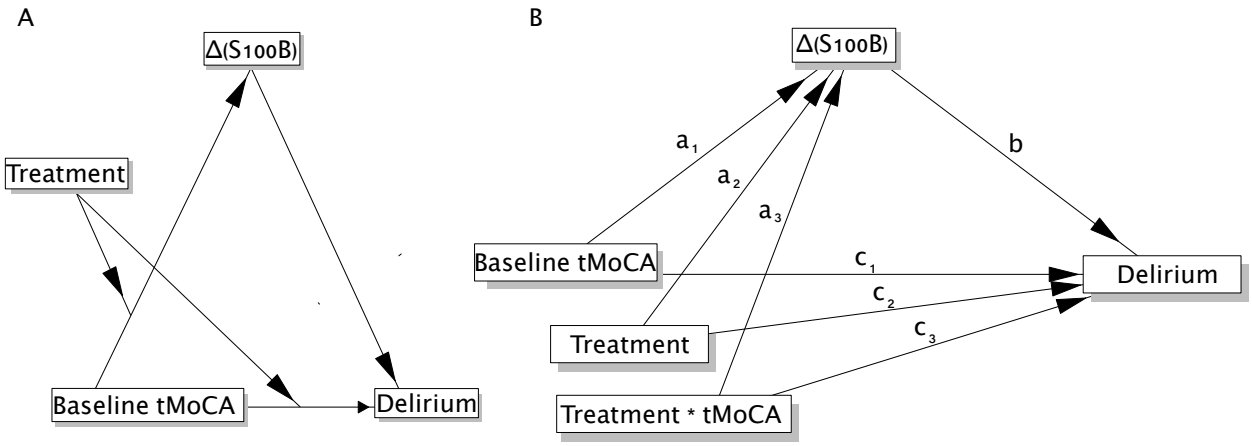


Figure 3. 2 Modelling for mediation analyses

A: Conceptual and B: Statistical models used in the mediation model to assess the conditional effects of baseline neurocognition and intra-operative oxygen treatment on delirium occurrence, and their indirect effect through change in biomarker levels.

Results

26% of subjects (n = 12) in the nested cohort demonstrated delirium postoperatively. At baseline, delirium cases and non-delirium controls were matched by age, sex and race (**Table 3.1**). Details of subjects' comorbidities and preoperative medications are reported elsewhere [188]. Intraoperatively, there was no statistically significant differences in aortic cross-clamp or cardiopulmonary bypass times between cases and non-cases. Baseline neurocognition, as measured by the Montreal Cognitive Assessment (MoCA) tool, was generally low in this cohort, and significantly lower among cases relative to controls (p = 0.02). Of the non-delirium controls, 21% (n = 7) met the criteria for subsyndromal delirium.

We measured four biomarkers well-documented to be markers of neuro-inflammation. For quality control, we set an intra-assay variability cut-off of 20%. Biomarker measurements with 20% coefficient of variation (%CV) or higher between duplicate runs were removed from all downstream analyses, although including them did not change results of our analyses. Levels of ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1) in all samples were below the limits of quantification (**Table 3.1 supplementary information**).

Table 3. 1 Baseline characteristics of study participants

	Case (N = 12)	Non-case (N = 34)	*p*-value
Demographics			
Age (years) *mean (SD)*	74 (±6.4)	70 (±4.3)	0.07
Male sex *count (%)*	9 (75%)	27 (79%)	1
BMI (*count (%)*			
Underweight	1 (8%)	1 (3%)	0.12
Normal	2 (17%)	7 (21%)	
Overweight	1 (8%)	13 (38%)	
Obese	8 (67%)	13 (38%)	
White race *count (%)*	10 (83%)	34 (100%)	0.11
Baseline neurocognitive assessment			
Pre-operative MOCA *mean (SD)*	15 (±2.7)	17 (±2.1)	0.02
Intraoperative			
Hyperoxia *count (%)*	4 (33%)	19 (56%)	0.31
Cardiopulmonary bypass time (mins) *mean (SD)*	79 (±17)	81 (±24)	0.83
Aortic Cross-clamp time (mins) *mean (SD)*	66 (±14)	67 (±21)	0.85
Postoperative			
Delirium severity score *mean (SD)*	8.9 (±3.1)	3.8 (±2.0)	< 0.01
Sub-syndromal delirium *count (%)*	0 (0%)	7 (21%)	0.22

┼4 subjects have missing data on CPBT and XCT, and 1 subject missing data on delirium severity

Table 3. 2 Measured Biomarkers and their Dynamic Range

Analyte	Dynamic Range (pg/mL)
Chitinase 3 -like 1	438.44 – 106,540
S100B	40.41 – 9,820
Enolase 2	374.44 – 90,990
UCH-L1	925.10 – 224,800

Of the remaining biomarkers, only S100B levels were significantly higher at post-bypass relative to baseline levels (p value < 0.001, **Figure 3.3**). We found no significant differences in S100B levels by sex or body mass index (BMI) at baseline or post-bypass (**Figures 3.4A, B, D, E**). Stratified analyses also showed that the absolute increase in S100B levels is not confounded by sex, BMI, patient outcomes or intra-operative oxygen treatment (**Figure 3.4C, 3F, Table 3.2**). We, however, observe that for patients with the longest CPB times (> 141 mins), the absolute change in S100B levels was not significant (p = 0.076, **Table 3.2**).

Stepwise regression analyses revealed that baseline neurocognition and hyperoxia were the most important predictors of the delirium. In our cohort, the direct effect of baseline neurocognition on the occurrence of delirium is modulated by perioperative oxygen treatment (direct effect = 0.078, p = 0.013) (**Table 3.3**). **Figure 3.5** illustrates the statistical mediation model and shows the conditional effects of perioperative oxygen treatment when holding baseline neurocognition constant. We also observe that this relationship is not mediated by the post-bypass change in S100B levels (indirect effect = 0.002, p = 0.584).

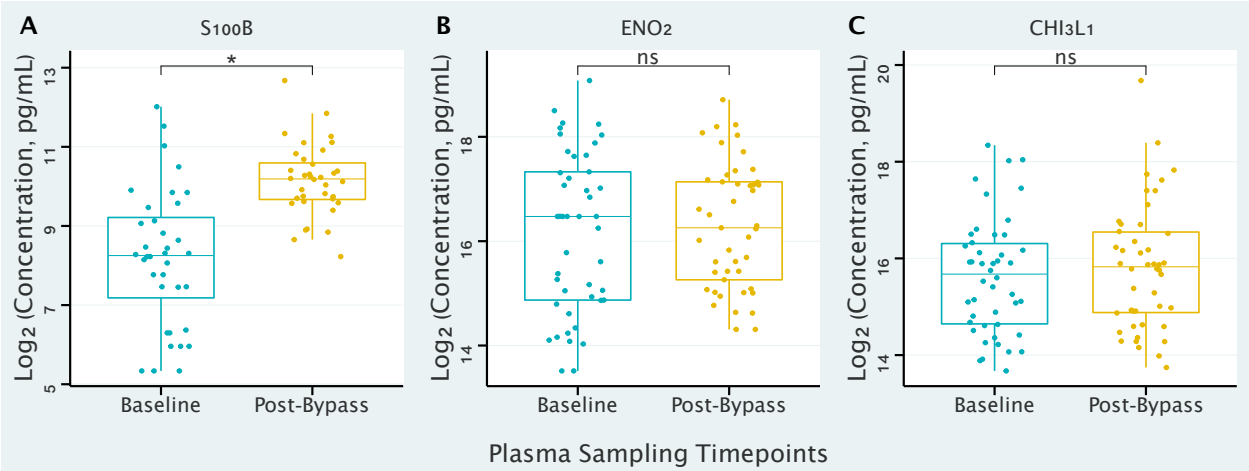


Figure 3. 3 Comparison of neuro-inflammatory biomarker levels

Boxplots show the quantile distribution (median and inter-quantile ranges) of each biomarker, and a comparison of biomarker levels at baseline and at post-bypass. Statistical tool used is the Student's *t*-test. Asterisks (*) represent statistically significant difference (i.e., *p* value < 0.05); *ns*: not significant; S100B: protein S100-B; ENO2: gamma-enolase; CHI3L1: chitinase-3-like protein 1.

Table 3. 3 Stratified analysis of baseline to post-operative change in S100B levels

	Baseline S100B (pg/mL)	Postop S100B (pg/mL)	*t*-statistic	*p*-value
Overall				
	8.18	10.15	7.68	<0.001*
Stratified by outcome				
Case	8.66	10.70	4.291	0.004*
Non-case	8.05	9.99	6.397	<0.001*
Stratified by treatment				
Hyperoxia	8.08	10.10	5.015	<0.001*
Normoxia	8.30	10.21	6.057	<0.001*
Stratified by BMI§				
Normal	8.17	10.61	4.125	0.004*
Overweight	7.81	10.03	5.907	<0.001*
Obese	8.47	10.01	3.824	0.002*
Stratified by Sex				
Female	9.09	10.65	3.372	0.015*
Male	7.96	10.03	6.922	<0.001*
Stratified by CPB Time				
< 52 mins	8.47	10.35	3.675	0.006*
52 – 112 mins	8.23	9.95	3.267	0.014*
112 – 141 mins	7.29	9.72	6.227	<0.001*
> 141 mins	8.94	10.24	2.083	0.076

*: statistically significant difference at \alpha ≤ 0.05

§: Underweight category *(n = 2)* is underpowered for parametric testing

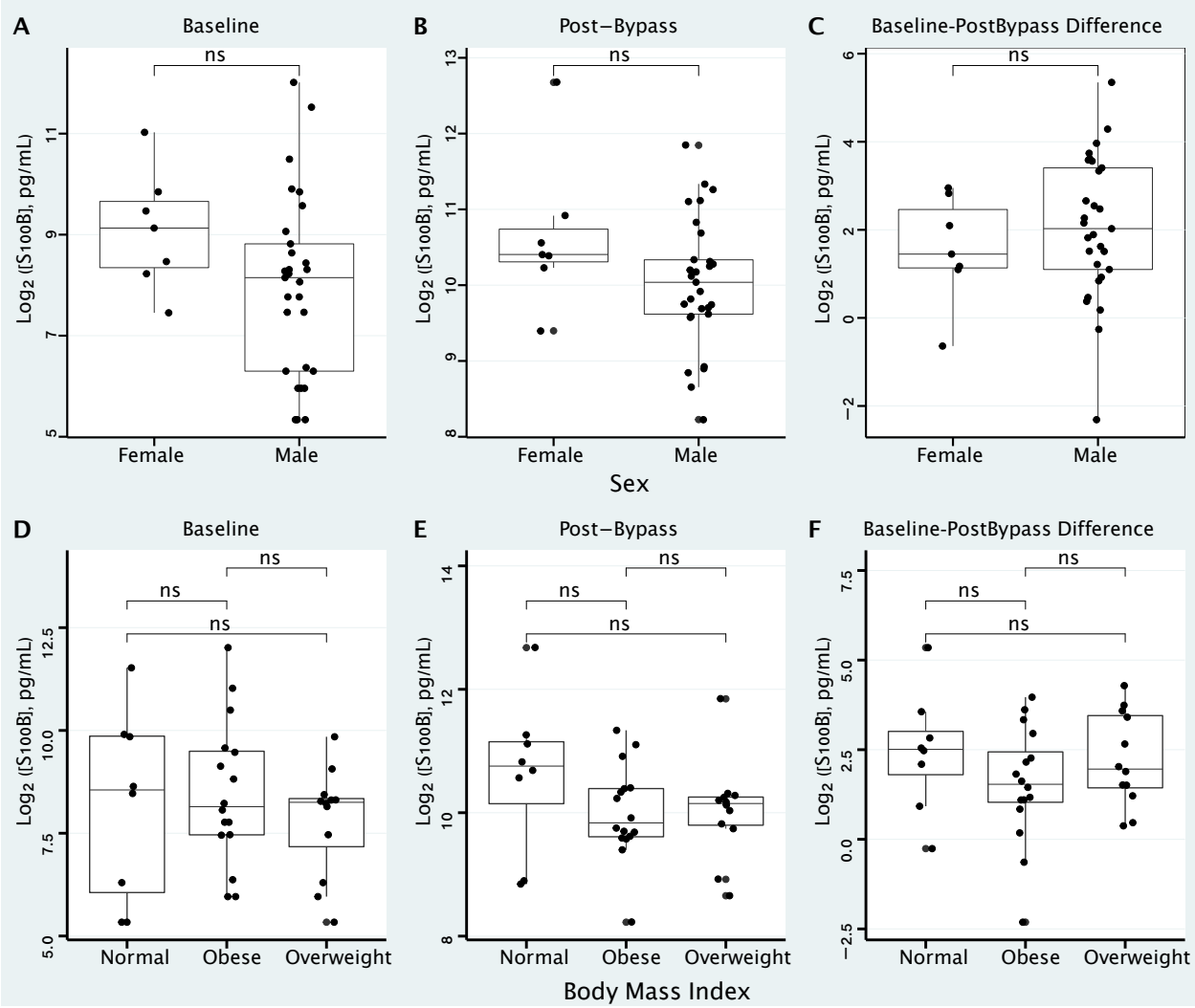


Figure 3. 4 Stratified analysis of S100B levels

by: (**A, B, C**) sex and by (**D, E, F**) body mass index.

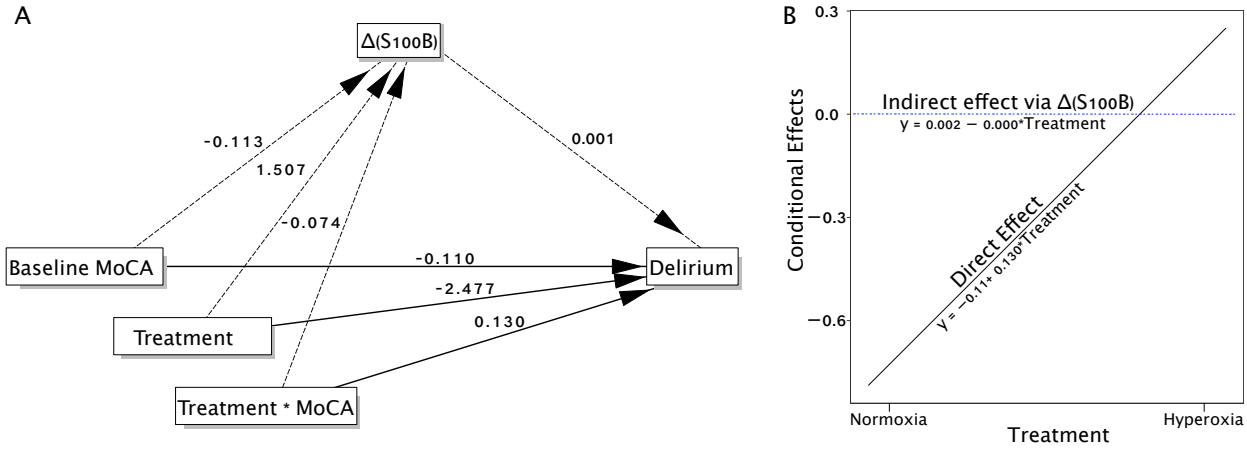


Figure 3. 5 Mediation modeling and analyses

A. Statistical model of the relationship between delirium occurrence and the interaction of baseline neurocognition and intra-operative oxygen treatment, with an assumed mediation by post-bypass increase in S100B biomarker levels. B. Conditional direct and indirect effect sizes quantified on the linear scale. Dashed lines = non-significant relationship; MoCA: Montreal Cognitive Assessment tool used to establish baseline neurocognition

Table 3. 4 Conditional effects and regression coefficients of predictors in mediation modeling

Variables	Predictors	Label	*β*	CE	SE	*p*
Δ(S100B)	tMoCA	a_1	-0.134	-0.113	0.155	0.464
Δ(S100B)	Treatment	a_2	0.412	1.507	4.638	0.745
Δ(S100B)	tMoCA * Treatment	a_3	-0.364	-0.074	0.260	0.776
Delirium	tMoCA	c_1	-0.138	- 0.110	0.042	0.009*
Delirium	Treatment	c_2	-0.724	-2.477	0.862	0.004*
Delirium	tMoCA * Treatment	c_3	0.682	0.130	0.050	0.009*
Delirium	Δ(S100B)	B	0.002	0.001	0.041	0.971

Discussion and Conclusion

We have presented a nested case-control study that sought to examine the effects of intraoperative hyperoxia on the occurrence of delirium. We found a significant direct effect between the interaction of baseline neurocognition and hyperoxia, and the odds of developing delirium. We, however, did not find a significant mediating role of neuroinflammatory biomarkers measured in our study. The causal effect of perioperative oxygen treatment on the occurrence of delirium, however small the effect size may be, reechoes the fact that delirium is etiologically heterogenous with likely many other possible pathomechanistic pathways besides our observation. Despite the paucity of literature on the relationship between perioperative hyperoxia and post-operative delirium, result of our study is consistent with recent studies investigating the matter [190, 213].

In one of the aforementioned studies, Lopez, Pandharipande [190] monitored the duration of cerebral hyperoxia with oximetry monitors intraoperatively and found that despite the considerable fluctuations in cerebral oxygenation, hyperoxia after a period of hypoxia was most strongly associated with the occurrence of delirium. Authors also observed that in delirium subjects, there was an increase in plasma concentrations of markers of oxidative stress (F_2-isoprostanes and isofurans), suggesting a possible mediation role. In the remaining study, Kupiec, Adamik [213] established a maximum PaO_2 cut-off of 33.2 kPa, beyond which post-operative delirium was more likely to occur. Notable differences between these studies and ours is the relatively smaller sample size in our study, choice of the

baseline neurocognitive assessment tool and the frequency of assessments for post-operative delirium.

Traditionally, ascertaining the mediation effect of a variable ***M*** (e.g., biomarker levels) on the relationship between a predictor ***X*** (e.g., baseline neurocognition) and outcome ***Y*** (delirium) has required that a relationship already exists between ***X*** and ***Y***. This approach is heavily debated, and proposed alternatives suggest that a prior relationship between ***X*** and ***Y***, or the lack thereof, neither proves nor rules out causal associations [214-216]. This was the observation in our study, in which we found that the relationship between ***X*** and ***Y*** is moderated by the interaction of another variable W (intra-operative oxygen therapy).

The clinical significance of the post-bypass increases in S100B levels without any significant associations with delirium remains to be determined. This is also the observation by Jönsson, Johnsson [217] and Nguyen, Huyghens [218]. Aptly described by authors as the “controversial significance of early S100B levels after cardiac surgery”, Jönsson, Johnsson [217] measured S100B levels at defined intervals from end of bypass until 48 hours post-surgery, and concluded that the predictive significance of the S100B biomarker is limited [217]. These findings are in sharp contrast to many other studies in which S100B levels were consistently increased in delirium cases regardless of the sampling time after surgery [219-221]. These conflicting findings about the S100B-delirium

relationship highlights three possibilities: (1) that plasma levels of S100B do not necessarily reflect CSF levels, (2) the relatively short half-life of S100B (60 to 120 mins) requires that blood sampling is appropriately timed, or (3) that increases in S100B levels only signal neuronal response to the surgical insult, and not because of the occurrence of delirium. With regards to the gap in plasma-to-CSF levels, S100B is also secreted by extra-neuronal tissues (e.g., adipocytes) [222, 223], although the predominant source remains in mature, perivascular astrocytes [224, 225]. To ascertain that the post-bypass increases in S100B levels in our cohort were not confounded by body fat, we performed stratified analyses and found no differences in S100B levels by sex or by BMI.

Further, although there was no association between CPB duration and incidence of delirium, we observe a downtrend in the absolute change in S100B levels with increasing CPB times and found no significant change in S100B levels for subjects with the longest CPB times (> 141 mins). We intimate that this is likely due to the short half-life of the S100B protein, although this hypothesis will require a formal interrogation from further studies.

Our study is, however, without limitations, notable among them is sample size and selection bias. Our relatively small sample size (n = 46) did not allow for the statistical adjustment of covariates such as age, sex, BMI in the structural modeling and mediation analyses. Instead, we used stepwise regression to

select the subset of variables with significant association to delirium in our cohort. Moreover, we also controlled for possible confounding during the study designing by matching cases to controls by age, sex, BMI and race (**Table 3.1**), and do not expect the lack of statistical adjustments to have any significant impact on the strength of associations in our findings. To prevent selection bias, subjects in the present study were selected to reflect the distribution of treatment (hyperoxia versus normoxia) and outcome (delirium case versus non-delirium control) in the parent trial. We acknowledge that our cohort had considerable deficits in neurocognition at baseline. While the ideal choice of controls would be subjects without any baseline deficits, controls are more appropriately sampled from, and in terms of risk, should be representative of the very population that gave rise to the cases being investigated [226, 227]. Nonetheless, we excluded subjects with extremely low tMoCA scores (< 10). In addition, patients' baseline tMoCA scores were included, and statistically adjusted for, in our models.

There are several mechanisms proposed to underlie the possible neurological damage after cardiac surgery on CPB [228]. In our study, we focused on the neuroinflammatory mechanism as a possible mediator of the exposure-to-outcome relationship. It is likely that our choice of biomarkers, albeit their recognized associations with neuroinflammation, may not be directly involved in the pathogenesis of post-bypass delirium. Given the half-lives and turnover of many inflammatory proteins, it is equally likely that our timing of blood sampling

did not permit detection of biomarker level that accurately reflects any possible neuroinflammatory process that may have been at play.

To date, our study remains the only one that has investigated the effects of perioperative hyperoxia on both the immediate and long-term neurocognitive functions [188] in the same cohort of patients. Taken together, the findings that hyperoxia increases the risk of post-operative delirium, yet with no association with long-term cognitive decline, may best be explained by a dose-response-time relationship, although further studies are required to definitely interrogate these hypotheses.

Chapter Four: Intraoperative Plasma Proteomic Changes in Cardiac Surgery:

In Search of Biomarkers of Post-Operative Delirium

Wiredu, K., et al., *Intraoperative Plasma Proteomic Changes in Cardiac Surgery: In Search of Biomarkers of Post-operative Delirium.* medRxiv, 2022[132]

Authors' contributions:

KW conceptualized the study, collected and analyzed data, and drafted the manuscript. SO and EM were instrumental in clinical sample collection. HRF guided data analysis. Both SS and SAG guided designing of the study and revised the manuscript.

Abstract

Purpose

Delirium presents a significant healthcare burden. It complicates post-operative care in up to 50% of cardiac surgical patients with worse hospital outcomes, longer hospital stays and higher overall cost of care. Moreover, the nature of delirium following cardiac surgery with cardiopulmonary bypass (CPB) remains unclear, the underlying pathobiology is poorly understood, status quo diagnostic methods are subjective, and diagnostic biomarkers are currently lacking.

Objective

To identify diagnostic biomarkers of delirium and for insights into possible neuronal pathomechanisms.

Experimental design

Comparative proteomic analyses were performed on plasma samples from a nested matched cohort of patients who underwent cardiac surgery on CPB. A targeted proteomics strategy was used for validation in an independent set of samples. Biomarkers were assessed for biological functions and diagnostic accuracy.

Results

47% of subjects demonstrated delirium. Of 3803 total proteins identified and quantified from patient plasma samples by multiplexed quantitative proteomics, 16 were identified as signatures of exposure to CPB, and 11 biomarkers

distinguished delirium cases from non-cases (AuROC = 93%). Notable among these biomarkers are C-reactive protein, serum amyloid A-1 and cathepsin-B.

Conclusions and clinical relevance

The interplay of systemic and central inflammatory markers shed new light on delirium pathogenesis. This work suggests that accurate identification of cases may be achievable using a panel of biomarkers.

Statement of Clinical Relevance

The acute implication of delirium is well-documented, yet the true extent of the consequences beyond the immediate post-operative period has yet to be fully known. Despite its impact on the geriatric population, delirium remains underdiagnosed. Correctly identifying cases remain a challenge in clinical practice: the arbitrary and subjective nature of current diagnostic tools, such as the confusion assessment method, underscores the urgent need for diagnostic biomarkers. The clinical usefulness of delirium biomarkers extent beyond the objective identification of cases. Delirium biomarkers will also be useful for risk stratification, long-term follow-up of patients and may offer insights into possible etiologies that underpin the condition. In this report, we found systemic markers of inflammation with well-established association with delirium, as well as new biomarkers that shed new light on the condition. Although validation in a larger

cohort is the necessary next step, our efforts lay the groundwork for future studies and highlight new frontiers in delirium research yet to be explored.

Introduction

Delirium remains under-diagnosed in clinical practice[125, 229, 230]. Characterized by acute fluctuations in consciousness, deficits in attention and impairments in cognition not explained by a pre-existing neurocognitive disorder, delirium is etiologically heterogenous with a particularly high incidence after cardiac surgery[154, 231]. Following cardiac surgery, it complicates post-operative care in up to 50% of patients with increased length of hospitalization, increased mortality and higher overall cost of care[232]. In the long term, post-cardiotomy delirium patients are at increased risk of many complications, including re-admissions [233], cognitive decline [176, 234-236], functional impairments [114], and stroke [237, 238], to mention a few. Clearly, delirium presents a significant healthcare burden on society. The true extent of the consequences beyond the immediate post-operative period remains unknown. Thus, the accurate identification of subjects for optimal care in the immediate post-operative period and for long-term follow-up is likely to exert a significant positive impact on patient care and costs if implemented successfully.

Unfortunately, many patients with delirium are missed [126, 239], an observation that is partly due to the subjective and variable nature of the current diagnostic approach. Efforts to improve recognition and accurate case identification has seen a steady rise in recent years, although a small fraction of these attempts

has focused on biomarker discovery. Most of these biomarker studies also employed targeted quantification strategies for a sub selected list of genes or proteins, an approach that is inherently biased and blinded to potentially novel factors involved in the etiology or consequences of delirium [130][submitted manuscript].

Challenges with delirium biomarker discovery are due, in part, to the lack of clarity regarding the underlying pathophysiology of the condition. While a one-size-fits-all explanation of delirium may be oversimplified, neuroinflammation induced by system-wide activation of an inflammatory cascade remains the prevailing mechanistic hypothesis[160, 240]. This is supported by recent untargeted and semi-targeted approaches that sought to study the proteome of human biofluids[2-8, 78], although neuroendocrine and circadian dysregulation have also been reported[160]. The emerging focus on signaling and inflammatory markers necessitate biomarker discovery approaches that focus on the low-abundance proteome, using analytical platforms with the multiplexing capability and the requisite sensitivity to detect small changes in proteomic signatures.

In the present work, we comprehensively profiled the plasma proteome of subjects at baseline and post-cardiotomy for an untargeted analysis of the plasma proteome. We included abundant protein immunodepletion and peptide fractionation to enhance signal from the low abundance plasma proteome. Using

independent set of samples, we validated candidate biomarkers at three time points (at baseline, post-bypass and post-operative) in order to understand the changing trajectories of these biomarkers over time as they relate to case identification. Finally, we demonstrate the diagnostic potential of a panel of candidate biomarkers, the accuracy of their use in discriminating cases from non-cases and the temporal association between intra-operative events and changes in biomarker levels.

Materials and Methods

Study Design and Patient Enrollment:

Subjects in this nested case-control study were selected from the parent study, a randomized double-blind trial conducted on subjects who underwent coronary artery bypass grafting (CABG) with cardiopulmonary bypass (CPB) between July 2015 and July 2017 at the Beth Israel Deaconess Medical Center (BIDMC) in Boston MA. The trial was registered with ClinicalTrials.gov (NCT02591589, https://clinicaltrials. gov/ct2/show/NCT02591589, principal investigator: Shahzad Shaefi, registration date: October 29, 2015). Institutional review board (IRB) approval 2014P000398/33 was amended for the purposes of this current study on 09/17/2021 by the Committee on Clinical Investigations at the BIDMC. Details of enrollment, subject randomization and treatment allocation in the parent study are published elsewhere [188, 209]. Briefly, patients aged 65 years or older who were booked for elective CABG requiring CPB were eligible. The primary objective was to examine the temporal relationship between intra-operative

oxygen treatment and post-operative neurocognitive function as measured by the telephone-based Montreal Cognitive Assessment (tMOCA) score. Patients were assessed for delirium as a secondary endpoint using the confusion assessment method (CAM). Patients were excluded if they were undergoing emergent CABG, if they required single-lung ventilation, CABG without CPB, intraoperative balloon counter-pulsation or mechanical circulatory support. All patients provide informed consent. 15 subjects were randomly selected for proteomic profiling in this nested case-control study. Because quantitative studies on the effect size of delirium biomarkers using mass spectrometry is largely unexplored, formal power analysis was not done.

Sample Collection:

Whole blood samples at baseline, post-bypass (P-BP) and on post-operative day one (PO1) were collected into 4mL EDTA-treated tubes (BD Diagnostics) and centrifuged immediately at 200*g* at room temperature for 10 min. Resulting plasma was stored at -80ºC until they were thawed for aliquots used here for proteomic profiling.

Chemicals and Reagents:

All LC-grade chemicals are marked with asterisk (*): Dithiothreitol (DTT), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (EPPS), Tris (hydroxymethyl) aminomethane (Tris), formic acid* and acetonitrile* were purchased from Sigma-

Aldrich. Methanol* was obtained from Fisher. Trypsin Protease, SDS, 2-iodoacetamide (IAA), High Select Top14 Abundant Protein Depletion Mini Spin Columns and TMT 11 plex kit were acquired from Thermo Fisher Scientific.

Sample Preparation analysis:

Sample Immunodepletion:

Buffer exchange on single-use High Select Top14 Abundant Protein Depletion mini-spin columns (ThermoFisher Scientific) was performed twice using 200 µL of 50mM Tris [pH 8.1] / 50mM NaCl. 10 µL of each plasma sample was applied to the mini-columns, incubated at -4ºC with gentle end-over-end mixing for 15 min, according to manufacturer's instructions. Flowthrough were collected by centrifugation at 1000*g* for 2 min into 2mL Eppendorf tubes. Concentrations of the depleted samples were obtained using the Pierce BCA Protein Assay Kit (Thermo Scientific) at 562 nm absorbance per manufacturer's instructions.

Expression and purification of recombinant CDS1 protein:

A CDS1 G-block was purchased from IDT and cloned into the pET16b plasmid. A pET13S-A plasmid containing λ-phosphatase was purchased from Addgene. CDS1 and λ-phosphatase were co-transformed with into BL-21 Rosetta *E. Coli* and colonies were grown in 5 mL LB medium (BD) with no antibiotic at 37°C overnight. The 5 mL culture was added to 400 mL LB medium with no antibiotic

and grown at 37°C until it reached an OD600 of 1. The temperature was decreased to 10°C and IPTG (UBP Bio) was added to a final concentration of 1 mM to induce CDS1 and λ-phosphatase expression. The presence of λ-phosphatase was necessary to solubilize CDS1. The culture was incubated for 24 hours before collection. Cells were frozen at -80°C in batches of 50 mL until purification.

To purify CDS1, cells were lysed in buffer (50 mM Tris pH 7.5 (Fisher Scientific), 10 mM imidazole (Fisher Scientific), 150 mM NaCl (Fisher Scientific), 1% Triton-X 100 (Sigma), 0.1 mM DTT (Amresco), and 1:500 protease inhibitor cocktail III (Research Products International)), sonicated, clarified by centrifugation, and incubated with Ni-NTA beads (Qiagen) for 3 hours at 4°C. Beads were collected, washed, and CDS1 eluted at room temperature in elution buffer (50 mM Tris-HCl pH 8.0 (Fisher Scientific) and 1 M imidazole (Fisher Scientific)). CDS1 was dialyzed overnight into dialysis buffer (25 mM Tris pH 7.5 (Fisher Scientific), 50 mM NaCl (Fisher Scientific), and 0.1 mM DTT (Amresco)), aliquoted, and stored at -80 °C. Purified CDS1 was separated on an SDS-PAGE, Coomassie stained, visualized using a BioRad Gel Doc EZ imager, and quantified against a BSA standard using BioRad Image Lab 6.1 software.

Digestion and Labelling for Biomarker Discovery

Depleted samples were treated with SDS (2% final) and DTT (2mM final) for denaturing at 75ºC for 15 min. Samples were cooled to room temperature before alkylation with IAA (7mM final) at room temperature in darkness for 30 min and quenched with DTT (additional 2mM final) for 10 minutes. Proteins were isolated by single-pot solid-phase-enhanced sample preparation (SP3) and digested to peptides in EPPS buffer overnight at 30ºC with 1:50 w/w trypsin (Promega^{TM}). Tryptic peptides were labeled with TMT-11 plex reagent for 1 hr according to manufacturer's instructions. Two channels in each set of TMT-11 plex were reserved for pooled plasma to be used as bridge samples for technical control. Labeling efficiency of at least 95% was confirmed on a 1-hr gradient before pooling. Labeled tryptic peptides were then desalted on an OASIS µHLB (Waters) and subsequently dried by vacuum centrifugation prior to off-line HPLC fractionation on a pentafluorophenyl (PFP) column as described previously [62]. 48 fractions were concatenated into 12 fractions for LC-MS/MS analysis. All samples were prepared in duplicates.

Digestion for Biomarker Validation

Equal amounts of recombinant purified CDS1 protein were added to each depleted sample before treatment with SDS (2% final) and DTT (2mM final) for denaturation and alkylation as described above. Proteins were isolated by single-pot solid-phase-enhanced sample preparation (SP3) and digested to peptides in

50mM ammonium bicarbonate buffer overnight at 30ºC with 1:50 w/w trypsin (Promega™). In a separate experiment to check for signal linearity, increasing concentrations of heavy-labeled peptides of CNDH2 condensin subunit were added to the samples at this point. Tryptic peptides were desalted on an OASIS µHLB (Waters) and dried by vacuum centrifugation. All samples were run in duplicates.

LC-MS/MS

All data were acquired on an Orbitrap Fusion Lumos Tribrid instrument (ThermoFisher Scientific, San Jose, CA) equipped with EASY-nanoLC 1200 ultra-high pressure liquid chromatograph (ThermoFisher Scientific, Waltham, MA). Dried peptides were resuspended in 5% methanol / 1.5% formic acid and injected onto a 35-cm long / 100-µm (inner diameter) in-house pulled analytical column packed with Reprosil C18 stationary phase particles. Discovery samples were separated on 120-minute gradient, and validation samples on a 60-min gradient, at 350nL/min flow rate. Acquisition parameters included 120,000-resolution at MS1, AGC target value of 5.0×10^5, scan range of 350 – 1250 m/z and maximum injection time of 100ms. For the TMT-labeled peptides, the top eight MS2 peaks were selected for further fragmentation at 55% normalized high-collision energy (HCD) via SPS-MS3 for quantification of reporter ions in the scan range of 110 – 500 m/z. For the label-free peptides in the validation phase, MS2

scans were generated at 30,000 resolution and AGC value of 2.5×10^5, using 30% normalized collision energy (HCD).

Bioinformatics

Peptide Spectral Matching:

Acquired data (in .raw format) were searched using COMET [95] against a target-decoy version of the human proteome (Uniprot, downloaded in 2020 and 2022, for the discovery and validation phases respectively). The fasta for the validation phase was appended with sequences from CDS1_SCHPO. Search parameters included a mass tolerance of 20ppm, maximum missed cleavages of 3, carbamidomethylation of cysteine as fixed modification and oxidized methionine as variable modification. In addition, the mass of 229.162932 Da was added to the N-termini and lysine residues of all peptides as fixed modification for the TMT data. A false discovery rate (FDR) of 1% was applied at the peptide level and final list of PSMs were filtered using XCorr and delta XCorr. All data were subsequently imported into R environment for statistical computing (v4.1.1) and Python programing language (v3.8) for downstream analyses [212, 241].

TMT Data Wrangling and Normalization (Discovery Phase):

After correcting for differential sample loading, the ratios of sample proteins to their respective bridge proteins were computed. Here, data from bridge samples

was used for quality control and to correct for batch-to-batch technical variations. Values were subsequently log-transformed and mean-centered. Data from all batches were combined and analyzed for possible outlier observations using OutlierDM R Package. Proteins were removed if their frequency of observation was less than half of all samples. For one-hit wonders in each batch of experiment, a retention time (RT) predicting model was built in Python using DeepRT+ as described by Ma, Ren [242]. Prediction performance was assessed with coefficient of determination (R^2) and delta-t95% ($\Delta t_{95\%}$). $\Delta t_{95\%}$ is the minimum time window containing deviations between the observed and the predicted RT for 95% of the peptides. Peptides with RT outside the $\Delta t_{95\%}$ range were excluded from downstream analysis. Missing entries in the data were imputed by making random draws from the left tail of the gaussian distribution of the entire log-transformed data matrix (using -2.5 SDs from the mean, width = 0.3).

Protein Feature Selection and Differential Abundance Analyses

To determine the subset of protein features that differentiated cases from non-cases, or postoperative expression profiles from baseline, Elastic Net algorithm was used [243]. This is a regularization and feature selection method with good performance on high-dimensional data (i.e., an $n \times p$ data with very large p proteins but small n samples). Elastic Net is insensitive to features that dominate the matrix (e.g., albumin) and likely suppress signal from low abundance predictors and skew model coefficients. In addition, Elastic Net is a good choice if overfitting and multicollinearity (or protein features that are highly correlated and

essentially communicate the same information) are a concern. Tuning parameters were achieved by grid optimization with a five-fold nested cross-validation where the last fold was held out for testing. The average of hyperparameters from all folds were computed and used to build the final model.

Using the subset of protein features, an unsupervised visualization of the data was achieved with principal component analysis (PCA). Hierarchical clustering was employed to check for reproducibility of replicate samples and inherent sample clusters, and together with a heatmap, the overall protein expression patterns. Here, clustering was achieved using Ward's clustering algorithm.[244] Briefly, Ward's minimum variance method begins with singleton clusters and recursively merges them by minimizing the total within-cluster variance as the objective function. After this point, protein values for any given biological replicates were summarized as means prior to differential abundance analyses. Two-way comparison for differential abundance was achieved by Student's *t*-test, assuming unequal variance. Differential abundance analysis was visualized with volcano plots. Because statistical comparison was done for only a subset of proteins, no correction for multiple hypothesis testing was done. Proteins were deemed differentially regulated between conditions if there was a statistically significant *t*-test (p value cutoff ≤ 0.05) and a log2 fold-change of at least ±1. This fold-change cutoff was selected to prioritize a panel of biomarkers with significant changes between conditions that is unlikely to be due to chance.

PRM Label-free Data Procession (Validation Phase):

Raw files were imported into Skyline v21.2.0.369 [103]. Precursor peptides with modifications other than carbamidomethylation of cysteine (as fixed modification) or oxidized methionine (as variable modification) were excluded. Peptide quantification criteria was defined as follows: (1) consistently identified precursors across all validation samples, (2) with maximum of two missed cleavages, (3) a consistent minimum of five transitions, and (4) at least 0.95 dop-product with the spectral library of chromatograms. All peak boundaries were manually inspected for interference-free co-eluting transitions before peak areas were integrated at the MS2 level. For any given precursor peptide, the five most intense fragment ions in the *m/z* range of 120 – 1500 were used for quantification. Final dataset was exported as .csv and analyzed in R environment for statistical computing (v4.1.2; R Core Team 2021). No imputations were required in the validation data. Data was normalized by computing peak area ratios relative to CDS1_SCHPO to correct for run-to-run variations. For each protein biomarker, Kruskal Willis global test was first used followed by post-hoc Mann-Whitney U test for pairwise comparisons of the normalized peak areas between the different sample collection timepoints (baseline, post-bypass and post-operative day 1).

Data accessibility statement:

Datasets from the discovery and validation phases are available as supplemental material.

Results

Clinical Profile of Study Participants

Subjects (*n* = 15) were selected from the parent study[188], which was a parallel group randomized controlled trial that enrolled 100 patients at Beth Israel Deaconess Medical Center (BIDMC), between July 2015 and July 2017. Delirium cases and non-delirium controls were age- and sex-matched (**Table 1**). There was no difference in baseline neurocognition between cases and non-cases, and the proportion of patients who received hyperoxic intraoperative treatment was comparable. There were no significant differences with regards to demographics, medical co-morbidities, pre-operative medications, or surgical characteristics. Details of the clinical characteristics of study subjects were reported previously[188].

Table 4. 1 Selected baseline characteristics of study subject in the discovery phase

	Delirium Cases	Non-Delirium Controls
Sample	*n* = 7	*n* = 8
Age	70 (±5.0)	71 (±4.4)
Sex (male)	7 (100%)	8 (100%)
*t*MOCA	17 (±2.3)	17 (±1.9)
Hyperoxia	4 (57%)	4 (50%)

*t*MOCA: telephone-based Montreal Cognitive Assessment test for Dementia

Discovery Phase of Biomarker Workflow

Using a multiplexed isobaric tagging (TMT)-based design, plasma samples at baseline and on post-operative day 1 from 7 delirium cases (CAM+) and 8 non-delirium controls (CAM-) were comprehensively profiled (**Figure 4.1)**. For precision, samples selected for the discovery phase of the study were analyzed in duplicates, for a total n = 60 samples, which necessitated the analysis of seven separate, batched multiplexes. To control for technical variation between batches, two channels in each of the seven 11-plex TMT sets were reserved as bridge samples using equal amounts of a pooled plasma sample. We fractionated the TMT-labeled peptides using off-line HPLC on a pentafluorophenyl (PFP) column as described previously[62] into 48 fractions, which were subsequently concatenated into 12 and analyzed by LC-MS/MS on an Orbitrap Fusion Lumos Tribrid instrument platform.

A collective total of 17,540 unique peptides from 3,803 proteins were identified from all seven multiplexes. An analysis of the number of proteins from each batch, separated into a binary group based on the corresponding number of peptides used in the identification of these proteins, demonstrates that our data are clearly dominated by so-called “one-hit proteins,” or proteins identified by a single peptide (**Figure 4.2A**). Often, single-peptide protein identifications are excluded from downstream analysis due to the increased risk of false protein identifications associated with single-peptide protein assignments. However,

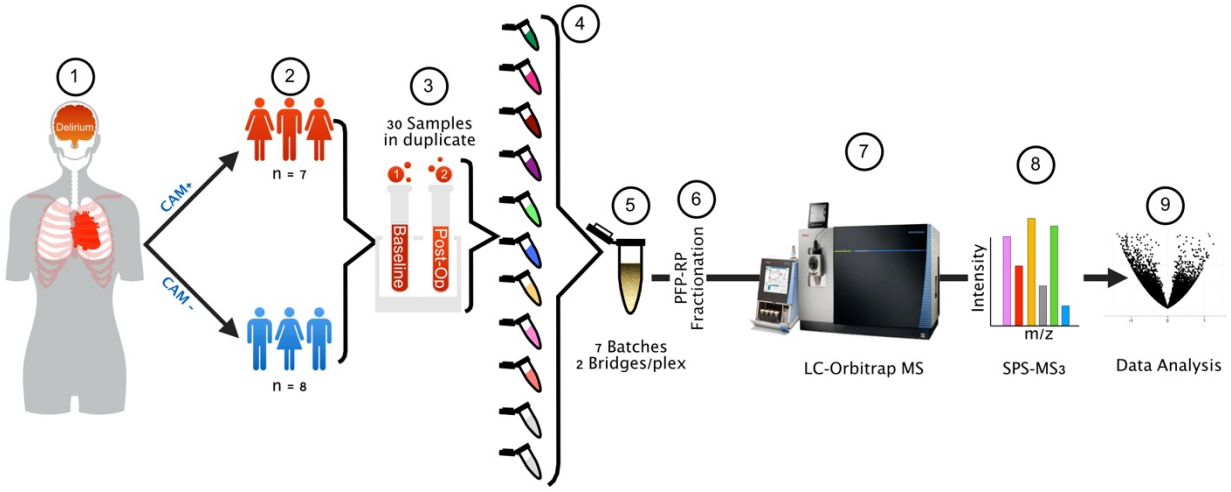


Figure 4. 1 Study Design and Biomarker Discovery Workflow

Biomarker discovery: a cohort of 15 subjects were selected from the parent study of 100 patients who underwent a non-emergent coronary artery bypass grafting (CABG) on cardio-pulmonary bypass (CPB) as part of a previously published clinical trial (1,2). Plasma samples of delirium cases (CAM+) and non-delirium controls (CAM-) were retrieved from the biorepository for subsequent proteomic analysis (3). Samples were immunodepleted, digested and labeled with multiplex isobaric quantification (TMT) reagents. For each set of TMT reagents, two channels were reserved for bridge samples for post-hoc batch correction (4). TMT-labeled samples were concatenated (5) and additionally fractionated (6) prior to LC-MS/MS (7) for quantification at MS3 (8). After peptide spectral matching and false discovery rate (FDR) curation, the final dataset of 3803 proteins was quantified and analyzed for candidate biomarkers (9).

excluding all one-hit proteins can be a huge informational cost as some of these proteins may be biomarkers of interest.

One-hit Proteins and Deep Learning for Confident Protein Identification:

To examine this further, we differentiated one-hit proteins identified only in single batches of experiments from those identified consistently across multiple batches. We reasoned that identified one-hit proteins consistently identified in multiple independent analyses are less likely to be false identifications, especially if their consistent identification is based on the same unique peptide. These one-hit proteins warrant additional peptide-centric information for protein inference beyond the sequences of the single peptides. **Figure 4.2B** displays the number of proteins identified in any given number of collective batches. Of the 3803 total proteins (**figure 4.2B**, cumulative batch ≤7), 51% (*n* = 1941 proteins) were identified based on a single peptide. While the number of proteins identified based on 2 or more peptides increased with increasing number of collective batches, the number of one-hit proteins remained fairly consistent. In particular for cumulative batches three to seven, we found 1698 one-hit proteins that were present in all of them.

To enhance the confidence in the identity of these one-hit proteins and minimize false positive identifications, we employed chromatographic retention time (RT) as additional peptide-centric information and orthogonal to their identification by tandem mass spectrometry. Here, we considered a peptide as confidently identified if, in addition to being a high-scoring peptide by PSM, the observed RT also falls within the RT window expected for that peptide and its corresponding experimental batch conditions.

For example, K.GTEAAGAMFLEAIPMSIPPEVK.F , a unique peptide from alpha-1-antitrypsin, A1AT_HUMAN (**figure 4.2C, supplemental figure 4.1**, blue rectangles) shows consistent RTs, regardless of the experimental batch or sample fraction the peptide was detected. On the other hand, K.GTEDFIVESLDASFR.Y (**figure 4.2C**, **supplemental figure 1,** red rectangles) is the only peptide-evidence that translocon-associated protein subunit alpha, SSRA_HUMAN – a one-hit protein – was detected in experimental batch 2.

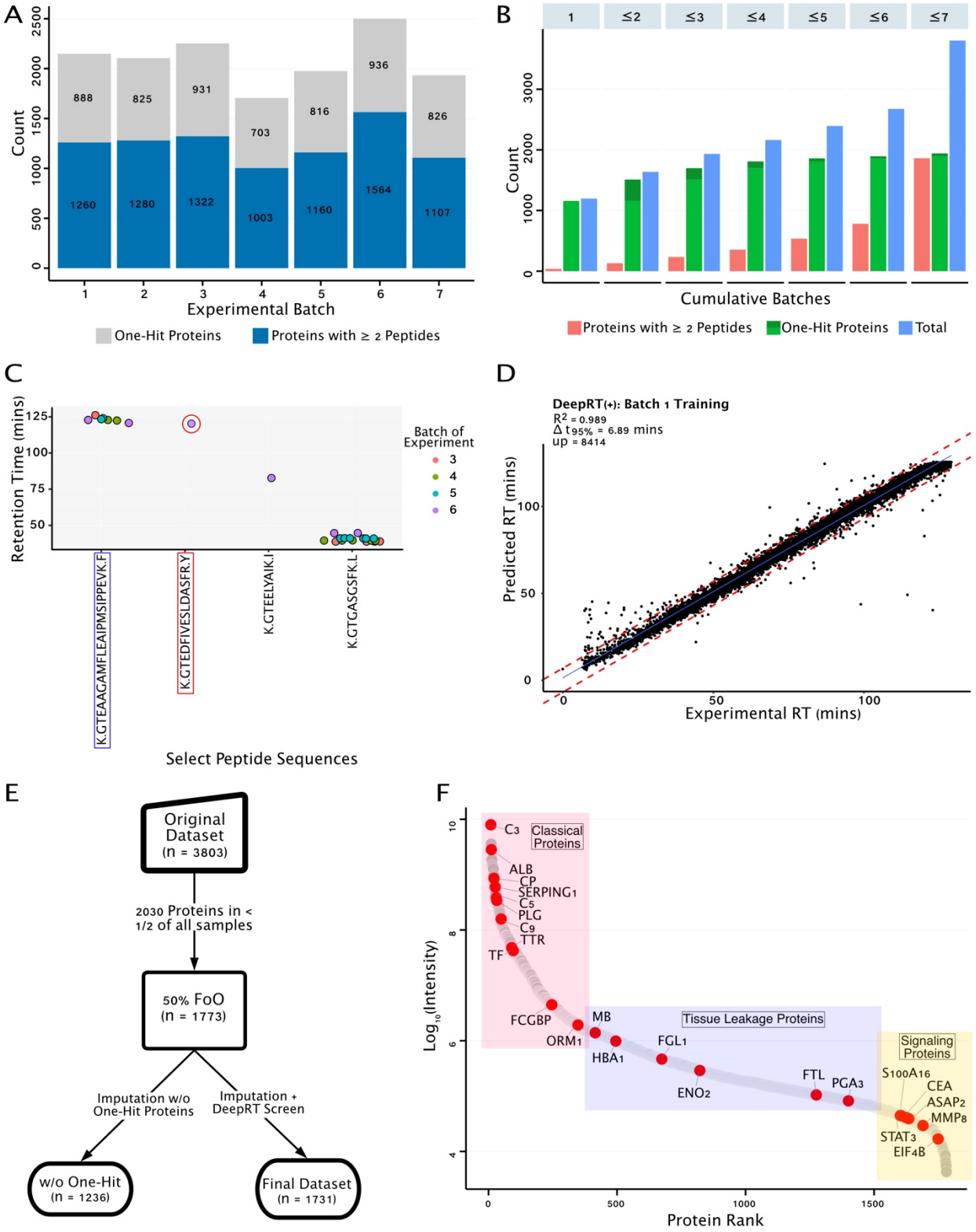

A
Count
Experimental Batch
One-Hit Proteins
Proteins with ≥ 2 Peptides
888
825
931
703
816
936
826
1260
1280
1322
1003
1160
1564
1107
B
1
≤2
≤3
≤4
≤5
≤6
≤7
Count
Cumulative Batches
Proteins with ≥ 2 Peptides
One-Hit Proteins
Total
C
Retention Time (mins)
Batch of Experiment
3
4
5
6
K.GTEAAGAMFLEAIPMSIPPEVK.F
K.GTEDFIVESLDASFR.Y
K.GTEELYAIK.I
K.GTGASGSFK.L
Select Peptide Sequences
D
DeepRT(+): Batch 1 Training
R² = 0.989
Δ t95% = 6.89 mins
up = 8414
Predicted RT (mins)
Experimental RT (mins)
E
Original Dataset (n = 3803)
2030 Proteins in < 1/2 of all samples
50% FoO (n = 1773)
Imputation w/o One-Hit Proteins
Imputation + DeepRT Screen
w/o One-Hit (n = 1236)
Final Dataset (n = 1731)
F
Log10(Intensity)
Protein Rank
Classical Proteins
Tissue Leakage Proteins
Signaling Proteins
C3
ALB
CP
SERPING1
C5
PLG
C9
TTR
TF
FCGBP
ORM1
MB
HBA1
FGL1
ENO2
FTL
PGA3
S100A16
CEA
ASAP2
MMP8
STAT3
EIF4B

Figure 4. 2: Data preparation for downstream analyses

A: Total number of proteins identified per batch. Bars are demarcated by the number of unique peptides used for protein identification. Gray portion of each bar chart represents proteins identified by only a single peptide, highlighting the scope of one-hit proteins in our analysis. **B:** Number of proteins identified in a cumulative number of experimental batches. For example, of the 1638 total proteins identified in up to two cumulative batches of experiments (cumulative batch ≤ 2), about 90% of those (n = 1470) were one-hit proteins. The number of one-hit proteins increases only marginally with increasing cumulative batches (light green portion of the green bars), in contrast to proteins identified from at least two peptides. **C:** Chromatographic retention times of select peptides from the discovery experiment. Plot shows the consistency of retention times (RT) of K.GTEAAGAMFLEAIPMSIPPEVK.F (blue rectangle), observed in two fractions from multiple LC-MS runs. K.GTEDFIVESLDASFR.Y (red rectangle), on the other hand, was only identified once. In the absence of additional peptides, these single peptides required further information to reduce false protein assignments. **D:** Scatter plot of experimental and predicted RTs of peptides from experimental batch 1. RTs were predicted by training a deep learning RT predictor, DeepRT+. Prediction performance is assessed with R^2 and $\Delta t_{95\%}$ (red dashed lines). *up* = number of unique peptides trained. **E:** Selection of the final 1731 proteins for downstream differential abundance analysis. Use of DeepRT+ salvaged 495 one-hit proteins that would otherwise be removed from downstream analysis. **F:** Dynamic range of all 1731 proteins, ranked in decreasing order of intensity. Each dot represents the median intensity of all intensity values recorded for a given protein across all samples. Intensity is plotted on the log-scale and spans 6.3 orders of magnitude between the high-abundance classical plasma proteins and the low-abundance signaling proteins. Functional groups are based on Putnam's classification. Red dots highlight representative members in each functional group. Labels are gene names of the corresponding proteins

To determine the RT window expected for these single peptides given the LC-MS conditions of their respective experimental batches, we trained a deep learning-based RT predictor, the DeepRT+ [242], using 80% of the RT of consistently identified peptides for a given experimental batch. We tested the prediction accuracy of the DeepRT+ model with the remaining 20% of the training data and subsequently used the final model to predict the RT of one-hit proteins.

We assessed performance of the RT prediction using the coefficient of determination, R^2, and Δt95%, the minimum time window containing deviations between the observed and the predicted RT for 95% of the peptides (**Figure 4.2D** and **Supplemental Figure 4.2**). We found the RT of 495 unique one-hit peptides fell within the Δt95% metric (**Table 2**) and were thus included to a final total of 1731 proteins used for downstream analysis (**Figure 4.2E**). The dynamic range of all proteins spans 6.3 orders of magnitude and confirms signal from a wide range of abundances in the plasma proteome (**Figure 4.2F**).

Table 4. 2 Summary of DeepRT+ training parameters and results of prediction assessment.

Training Parameters						Results		
Batch	RT (min)	RT (max)	max aa length	up (training)	up (predicted)	Δt95%	R^2	Eliminated
1	4.1	129.2	46	8414	776	6.89	0.989	145
2	5.2	129.4	45	8327	758	7.95	0.986	104
3	10.2	129.7	44	9240	859	12.35	0.973	127
4	9.3	129.5	43	7589	646	9.67	0.988	121
5	10.5	128.7	40	7207	749	11.24	0.975	129
6	4.4	128.6	43	10829	849	6.22	0.991	170
7	11.1	128.7	42	6406	734	9.45	0.982	120

Training. Given that each batch of sample has unique LC-MS experimental conditions that uniquely impact RT, seven different models were built for each of the seven batches of experiments. Abbreviations: RT (min): minimum RT for the batch; RT (max): maximum RT; aa: amino acid; up (training): number of unique peptides trained; up (predicted): number of unique peptides whose RTs were predicted; R^2: coefficient of determination = correlation coefficient for bivariate analysis; Δt95%: deviations between observed and predicted RT that contains 95% of peptides for a given batch of experiment.

Protein Feature Selection and Differential Abundance Analyses

To determine the subset of these 1731 proteins that are most important in discriminating plasma profiles of cases and from non-cases and between baseline and post-operative timepoints, we employed an elastic net regularized regression approach[243]. We found 47 and 64 proteins as signatures of surgical exposure and of delirium, respectively. Principal component analysis (PCA) of study subjects using the subset of protein features demonstrates that delirium cases cluster separately, with marginal overlap between non-delirium controls and baseline samples **(Figure 4.3A)**. Additionally, plasma profiles of cases and non-cases are clearly separable post-operatively, although they were indistinguishable at baseline (**Figure 4.3B**). This strongly suggests a temporal relationship between post-operative changes in proteomic signatures and subjects' surgical exposure and/or related intra-operative physiological events.

Furthermore, we quantified the extent of changes in biomarker levels before and after surgery (**Figure 4.3C**) and between cases and non-cases (**Figure 4.3D**). When using the proteins identified as a signature of delirium (**Figure 4.3D**), we observed a

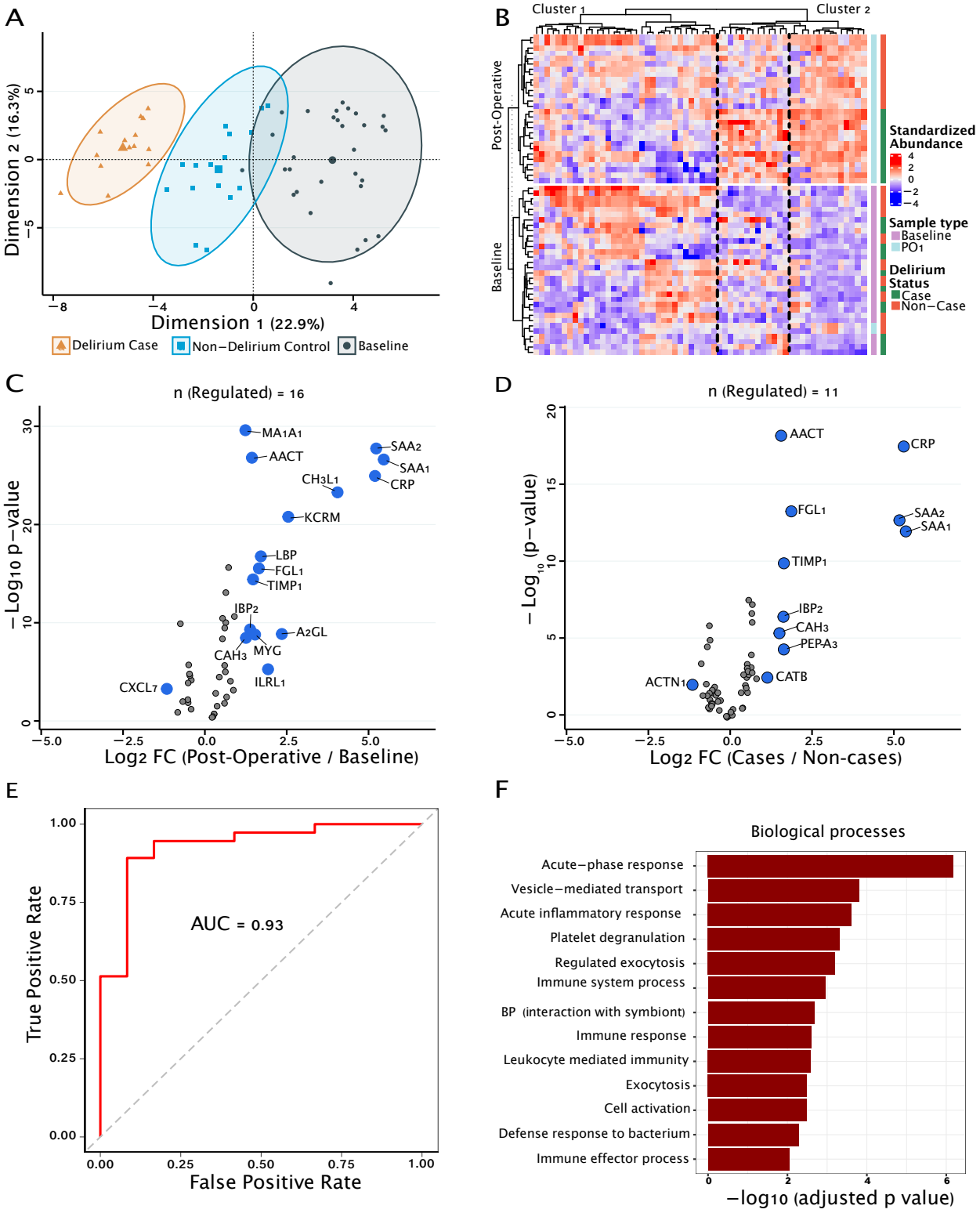

A
Dimension 2 (16.3%)
Dimension 1 (22.9%)
Delirium Case
Non−Delirium Control
Baseline
B
Cluster 1
Cluster 2
Post-Operative
Baseline
Standardized Abundance
Sample type
Baseline
PO1
Delirium Status
Case
Non−Case
C
n (Regulated) = 16
− Log10 p−value
Log2 FC (Post-Operative / Baseline)
MA1A1
SAA2
AACT
SAA1
CH3L1
CRP
KCRM
LBP
FGL1
TIMP1
IBP2
A2GL
MYG
CAH3
CXCL7
ILRL1
D
n (Regulated) = 11
− Log10 (p−value)
Log2 FC (Cases / Non-cases)
AACT
CRP
FGL1
SAA2
SAA1
TIMP1
IBP2
CAH3
PEPA3
ACTN1
CATB
E
True Positive Rate
False Positive Rate
AUC = 0.93
F
Biological processes
Acute−phase response
Vesicle−mediated transport
Acute inflammatory response
Platelet degranulation
Regulated exocytosis
Immune system process
BP (interaction with symbiont)
Immune response
Leukocyte mediated immunity
Exocytosis
Cell activation
Defense response to bacterium
Immune effector process
−log10 (adjusted p value)

Figure 4. 3 Clustering, differential abundance and functional analyses

A: Principal component analysis of all discovery samples (including replicates). Clustering is based on a subset of 64 proteins identified by the penalized regression approach (ElasticNet) for feature selection. **B:** Hierarchically clustered heatmap of proteomic signatures of delirium cases and non-delirium controls at two time points (baseline and post-operative day 1, PO1). Post-operatively, a subset of proteins (protein cluster 2, dashed lines) shows a higher expression in cases relative to non-cases, although the expression of this subset of proteins was very similar between the two groups at baseline. **C:** Volcano plot of *p*-value ($\log_{10}$ scale) vs fold-change ($\log_2$ scale) of the 47 proteins that explain most of the variation in proteomic profiles of the baseline and post-operative day 1 samples. Blue dot means protein is significantly different at PO1 relative to baseline by at least 2 folds (*p*-value cut-off = 0.05). **D:** Volcano plot of the 64 proteins that explain most of the variation in proteomic profiles between delirium cases and non-delirium controls. **E:** Diagnostic accuracy of the panel of 11 differentially abundant proteins that discriminate cases from non-cases. **F:** Functional analysis of biomarkers for biological processes enriched among the panel of 11 differentially abundant proteins that discriminate cases from non-cases.

diagnostic accuracy of 93% in discriminating cases from non-cases (**Figure 4.3E**). Functional analysis of the biomarker panel for biological processes shows acute inflammatory response and activation of the immune system as the most significantly enriched functional pathways, predominantly in the extracellular region (**Figure 4.3F** and **Supplemental Figure 4.3**).

Biomarker Verification

For further evaluation of peri-operative proteomic differences between cases and non-cases, an independent set of plasma samples was used to verify biomarkers discovered *a priori* (**Figure 4.4)**. Here, we used parallel reaction monitoring (PRM) as the targeted approach and employed label-free quantification (LFQ) as orthogonal methods different from the TMT approach used in the discovery phase. To ascertain the degree to which changes in protein concentration in the complex background of plasma are quantifiable, we artificially modified six biological replicates of a pooled plasma sample with the addition of exogenous proteins: (1) equal amounts of a non-human (*Schizosaccharomyces pombe*) homolog of the serine/threonine-protein kinase Chk2 (CDS1 in *S. pombe*); and (2) increasing concentrations of heavy-labeled AQUA peptides[245, 246] of human condensin-2 complex subunit H2 (CNDH2). From this experiment, we estimate a limit of quantification of ~1fmol on column (**Figure 4.5A**), with negligible impact on target protein quantification due to matrix effects from large (16-fold) variations in the concentration of a non-target protein in the matrix (**Supplemental Figure 4.4**).

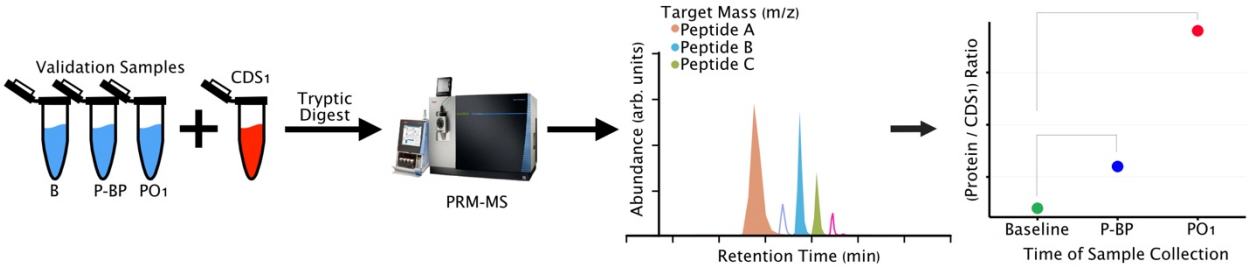


Figure 4. 4 Biomarker Validation

Validation samples included baseline (*B*), post-bypass (*P-BP*) and post-operative day 1 (*PO1*) samples. To each unlabeled validation sample, an equimolar amount of CDS1, a protein from *S. pombe* with no sequence overlap to human proteins previously expressed and purified from bacteria, was added as a reference standard to control for run-to-run variations. Select tryptic peptides of regulated proteins from the discovery phase were targeted for quantification using via PRM-MS. Concentrations of each biomarker were analyzed for changes across the sampling time points (*B*, *PB*, *PO1*). Hypothetical data are depicted as exemplars.

For candidate biomarker verification, we developed parallel reaction monitoring (PRM) methods through an iterative optimization process (**Supplemental Figure 4.5**). We monitored 153 unique peptide sequences (212 total precursor ions including the observed range of charge states) from the union of 18 differentially abundant proteins as PRMs that were distributed across the entire LC-PRM elution gradient (**Figure 4.5B)**. For example, we monitored the abundance of the peptide ESDTSYVSLK from C-reactive protein as a doubly charged ion via five individual y-ions in our PRM method via Skyline (**Figure 4.5C**) in each verification sample. The PRM methods we employed required the following minimum criteria for peptide quantification: a consistent minimum

of 5 transitions in all samples, a minimum dot-product of 95% and manual inspection of all peaks for interference-free co-eluting transitions with distinct peak boundaries. 65 precursors from 13 proteins met these criteria for downstream analysis (**Supplemental Table 4.3**). Unsupervised clustering based on the quantification of these candidate biomarkers shows that post-operative samples aggregate separately from post-bypass and baseline samples (**Figure 5D**). This is further confirmed by statistical comparison of biomarker levels between the sampling timepoints (**Figure 4.5E** and **Supplemental Figure 4.6**).

Seven biomarkers (A2GL, AACT, CH3L1, CRP, LBP, MA1A1 and SAA1/SAA2) were significantly increased at post-operative day one (PO1) relative to baseline

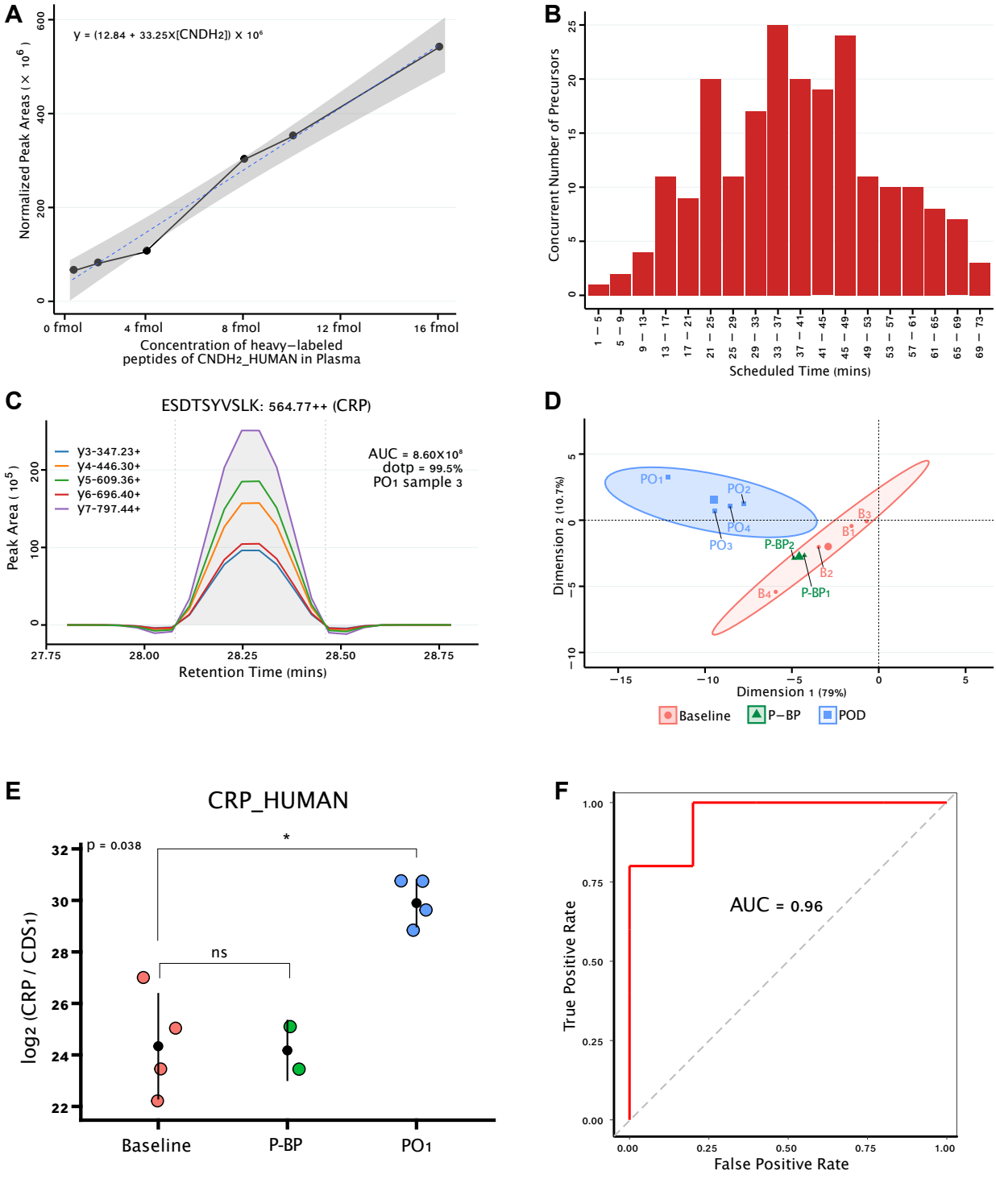

A
y = (12.84 + 33.25x[CNDH2]) x 10^6
Normalized Peak Areas (x 10^6)
0
200
400
600
0 fmol
4 fmol
8 fmol
12 fmol
16 fmol
Concentration of heavy−labeled peptides of CNDH2_HUMAN in Plasma
B
Concurrent Number of Precursors
0
5
10
15
20
25
1 — 5
5 — 9
9 — 13
13 — 17
17 — 21
21 — 25
25 — 29
29 — 33
33 — 37
37 — 41
41 — 45
45 — 49
49 — 53
53 — 57
57 — 61
61 — 65
65 — 69
69 — 73
Scheduled Time (mins)
C
ESDTSYVSLK: 564.77++ (CRP)
y3-347.23+
y4-446.30+
y5-609.36+
y6-696.40+
y7-797.44+
AUC = 8.60x10^8
dotp = 99.5%
PO1 sample 3
Peak Area (10^5)
0
100
200
27.75
28.00
28.25
28.50
28.75
Retention Time (mins)
D
PO1
PO2
PO3
PO4
P-BP2
P-BP1
B1
B2
B3
B4
Dimension 2 (10.7%)
−10
−5
0
5
−15
−10
−5
0
5
Dimension 1 (79%)
Baseline
P−BP
POD
E
CRP_HUMAN
p = 0.038
*
ns
log2 (CRP / CDS1)
22
24
26
28
30
32
Baseline
P-BP
PO1
F
AUC = 0.96
True Positive Rate
0.00
0.25
0.50
0.75
1.00
0.00
0.25
0.50
0.75
1.00
False Positive Rate

Figure 4. 5 Analyses of Validation Data

A: Normalized peak areas of CNDH2_HUMAN condensin subunit with increasing concentrations of its heavy-labeled stable isotope standards spiked into a background matrix of plasma. Grey area is the 95% confidence band of the regression line of fit: $y = (12.84 + 33.25x[CNDH2]) \times 10^6$. **B:** Number of precursors monitored concurrently during five-minute windows across the 78-minute gradient for used for validation experiments. **C:** Representative extracted ion chromatogram (XIC): the five most intense fragment ions of the CRP_HUMAN peptide ESDTSYVSLK, co-eluting at 28.3mins. All other peptides were quantified similarly with a minimum of five transitions consistent across all samples, a minimum dot product (dotp) of 95% and manual inspection for distinct peak boundaries and interference-free transitions. **D:** Principal component analysis of all validation samples. Notable here is the clustering of post-bypass samples together with the baseline, signaling similar proteomic signatures between the two timepoints. **E:** Representative plot of differential abundance analysis of validated proteins for the candidate biomarker C-reactive protein (CRP), showing changes across the three sample collection time points: baseline, post-bypass (P-BP) and post-operative day one (PO1). **F:** ROC analysis of the discriminatory power of the validated panel of biomarkers

in this validation cohort. Four razor peptides were shared between SAA1 and SAA2. However, no peptides unique to either SAA1 or SAA2 met the minimum quantification criteria for PRM verification. Similarly, none of the precursor peptides of CAH3, EFNA1, FGL1 or PEPA4 met PRM quantification criteria. Regardless of statistical significance, we observe that these candidate biomarker levels show a consistent increase in abundance between baseline and PO1 (**Supplemental Table 4.3**). This panel of differentially abundant candidate biomarkers yields a discriminatory power of 96% (84.9 – 100%) between cases and non-cases (**Figure 4.5F**).

Discussion

This unbiased proteomic analysis of samples from a prior nested case-control study is the deepest unbiased plasma proteomic profiling for potential biomarkers of delirium to date. We employed a rectangular biomarker workflow[30] to both discover and verify biomarkers of post-operative delirium on a single mass spectrometry platform without the use of traditional affinity-based verification methods. Dominated by one-hit wonders, our focus on the low-abundance proteome presented us with the challenge of protein inference, for which we applied deep learning to recover pertinent orthogonal peptide chemical information and salvage a significant number of these one-hit proteins.

We identified 3808 proteins by isobaric quantitative multiplexed proteomics, 16 of which were differentially abundant post-operatively from baseline levels, and 11 of which were differentially abundant in cases relative to controls. This

includes proteins with well-documented associations with delirium, such as CRP, CH3L1, AACT, TIMP1, as well as new ones not previously associated with delirium, including SAA, CATB and PEPA3. Using an independent set of samples, we attempted to verify the union of these candidate biomarkers and found a 96% accuracy in correctly identifying delirium patients for those for which quantification was possible. Collectively, our findings show a temporal association between intra-operative events (i.e., surgical insult, administered anesthesia, etc.) and proteomic changes associated with phenotypic delirium.

The prevailing mechanistic hypothesis of delirium is one of acute neurocognitive disruption triggered by system-wide inflammation[160, 240]. In our study, functional analysis of the post-operatively dysregulated biomarkers suggests a system-wide activation of the inflammatory cascade and related immunological reactions. Data on the associations between delirium and acute-phase reactants (APR) such as CRP is ubiquitous[2, 171, 175, 247, 248]. Although known APRs correlate well with the severity of inflammation, their usefulness as biomarkers is limited as they are not specific to delirium. We, however, found additional acute-phase reactants that may shed a new light on delirium.

Human serum amyloid A (SAA) is a collective name for a group of polymorphic proteins functionally associated with high-density lipoprotein (HDL). By the regulation of their synthesis, they are grouped into the acute phase isotypes (a-

SAA: SAA1, SAA2 and SAA3) and the constitutive isotype (c-SAA: SAA4)[249, 250]. Although predominantly secreted by the liver, extra-hepatic production occurs in the brain and may be more relevant in neurocognitive disorders such as Alzheimer's disease[251-254]. SAA has cytokine-like effects which likely provokes blood brain barrier (BBB) dysfunction, induces depressive-like behavior in mice and may impair cognition in human subjects[255-258]. In the present study, we found SAA1 and SAA2 were both upregulated post-operatively in delirium cases by over 5 folds (p value < 0.001). This is the first mention of SAA in the context of delirium and warrants further studies to formally credential this association with the condition.

The cysteine protease cathepsin B (CATB) has previously been quantified as an AD-related biomarker and correlates with mini-mental state examination (MMSE) scores [259-262], but its association with delirium is unknown. It is an inflammasome that promotes IL-1beta maturation and secretion[262]. It also has a beta-secretase activity, capable of cleaving amyloid precursor protein into amyloid beta [263]. Given that cases and non-cases in our study were matched by baseline neurocognition and tMOCA scores were statistically controlled for, upregulation of CATB in delirium cases may indicate a common pathophysiological starting point in the continuum of neurocognitive disorders, of which delirium and AD are a part. Generally recognized as the first enzyme to be discovered, pepsin (PEP-A) is the native acid protease of the stomach[264]. Blood pepsin is an established biomarker of gastric mucosal integrity, and plasma levels correlate with the degree

of mucosal damage[265-268]. Cardiac surgery and CPB places enormous physiological stress on the body. Through the cholinergic anti-inflammatory reflex, the body attempts to ameliorate the stress by increasing vagal tone[269-272] which manifests as gastric acid production. Normally, small amounts of secreted pepsin (~1%) may be found in blood and urine[273], but with increased acid production, this proportion may be higher. In the discovery phase of our study, differentially abundant PEP-A levels in cases relative to non-cases (1.64-fold increase, *p* value < 0.001) despite pre-operative proton-pump inhibitor administration in the study subjects suggests a peculiar association between plasma PEP-A levels and delirium. At present, we are unable to explain the relationship, if any, between increased vagal tone and neuroinflammation.

The independent association between CPB and delirium remains an ongoing debate and data on the relationship is conflicting. On the one hand, the use and duration of extracorporeal circulation is reported to increase the risk of delirium[274-276]. Some authors, on the other hand, have reported no associations between delirium incidence and CPD duration[277, 278]. In our cohort, there was no statistically significant difference in aortic cross-clamp time or duration of bypass between delirium cases and non-cases.[188] To determine the impact of CPB in our cohort, we compared post-operative plasma profiles to baseline regardless of the case/non-case status of subjects. We found 16 dysregulated proteins, most of which have been characterized as non-specific markers of surgical exposure[145, 279, 280]. A striking observation in our study is

the similarities in proteomic signatures between cases and non-cases at baseline, despite a clear difference at post-operative day one. Previous studies have shown that post-operative delirium cases are likely to be in a heightened pre-operative inflammatory state [2, 247, 270, 281-284], which makes them more vulnerable to intraoperative stressors. In our study, similarities in the levels of identified biomarkers at baseline suggests otherwise.

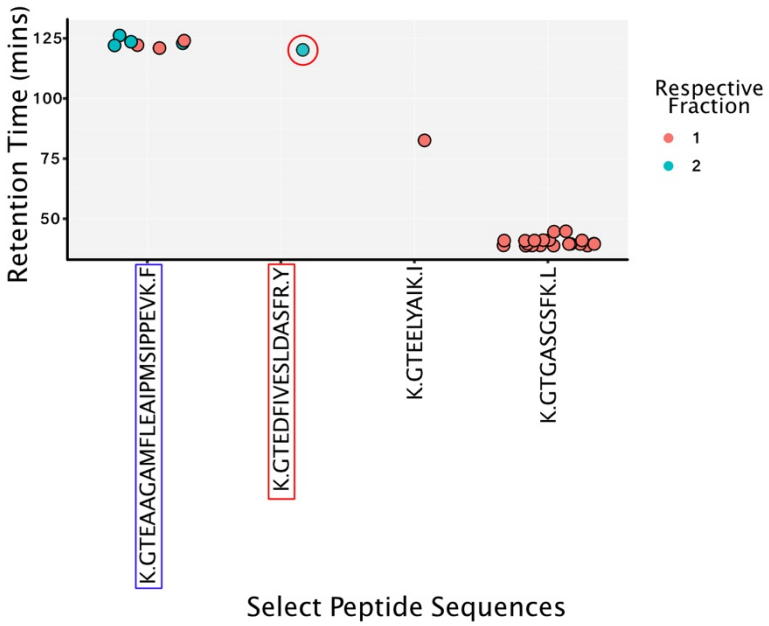
The main strength of the present study is in its unbiased, hypothesis-generating approach to identify potential biomarkers of delirium. This lays the groundwork for future studies and highlights new frontiers in delirium research yet to be explored. Translational utility from the research bench to the patients' bedside requires that the biomarker readout in the discovery phase is independent of the measurement approach used for their discovery[285]. For this reason, we validated discovered biomarkers using label-free quantification, which is orthogonal to the TMT-based measurements in the discovery phase of our study. Our choice of PRM-MS over traditional affinity methods for validation (e.g., ELISA) is further premised on the fact that affinity methods are semi-quantitative with inter-operator variability in quantification, have limited dynamic range and require larger amounts of sample. In addition to the requirement for peptide antigenicity, antibody cross-reactivity limits multiplexing (i.e., how many proteins can be validated at a time)[286]. All proteins needing validation require antibodies, a step that takes considerable amount of time to develop and can be

cost-prohibitive if commercial options are not available[69]. This, in fact, is a long-standing bottleneck in clinical biomarker workflow[287].

Our study is, however, not without limitations. First, sample sizes for both the discovery and validation phases may have limited statistical power in detecting differences in the levels of many other biomarkers. In our cohort, the CAM test was administered daily after surgery. In our statistical analysis, we did not correct for the effects of retesting on repeated test administration in this cohort. In the discovery phase, our interest in the low-abundance plasma proteome required an immunodepletion step to remove the majority of the top 14 most abundant plasma proteins. The extent to which this experimental step contributed to the removal of other proteins through their specific or non-specific binding was not ascertained. Although isotypes SAA1 and SAA2 each had unique peptides in the discovery phase, only the razor peptides met the criteria for quantification in the validation phase and were thus undistinguishable. Similarly, peptides from CAH3, EFNA1 and PEPA3 did not meet the minimum quantification criteria for verification by PRM, and peptides from FGL1 were not detected at all in any of the verification samples by PRM.

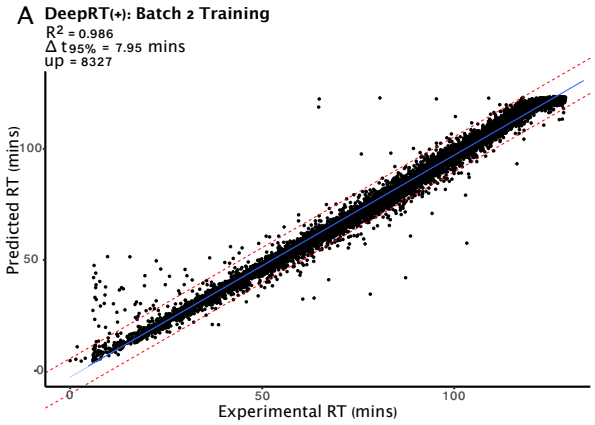
In summary, diagnostic biomarkers of delirium are urgently needed for accurate case identification, long-term risk stratification and for molecular characterization of delirium. In this study, we discovered a panel of biomarkers through the

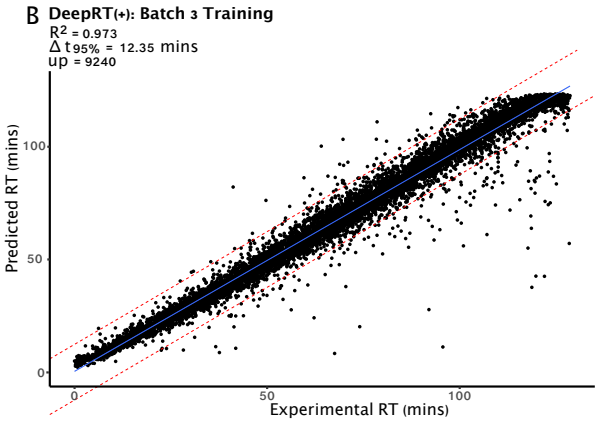
unbiased comparative analyses of baseline and post-operative plasma samples of delirium cases and non-cases. We underscored the importance of brain-specific biomarkers such as SAA and CATB and their possible role in the pathophysiology of delirium. In the long-term, it is in our research interests to rigorously test their associations with delirium and ascertain how these biomarkers change over time in a larger independent cohort.

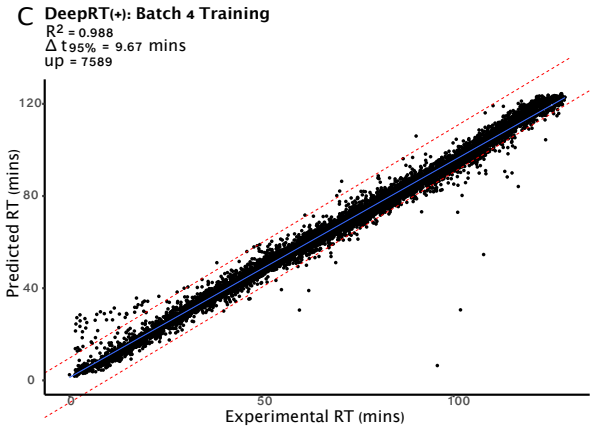


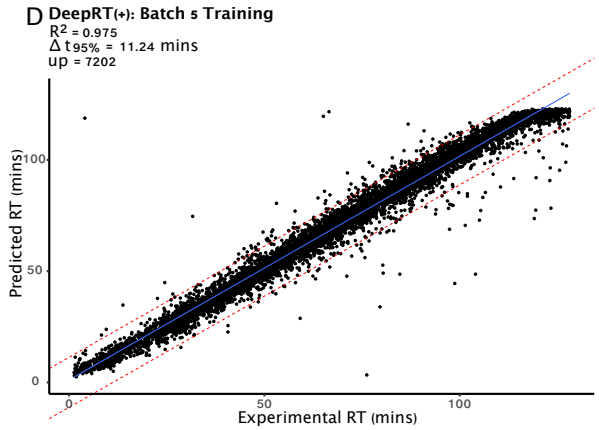
Supplemental Figure 4. 1

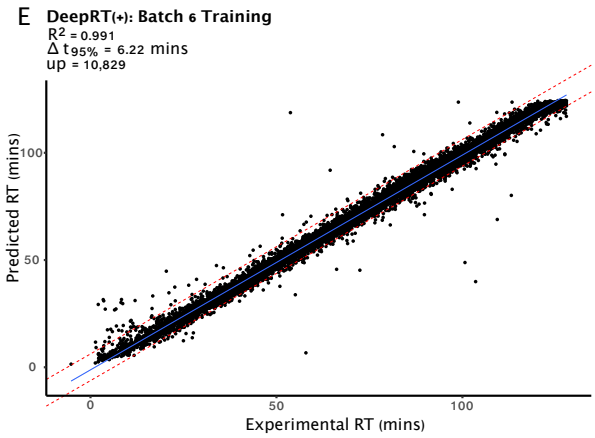
Chromatographic retention times of select peptides, showing consistency of RT and adjacency of sample fractions from which they were identified.

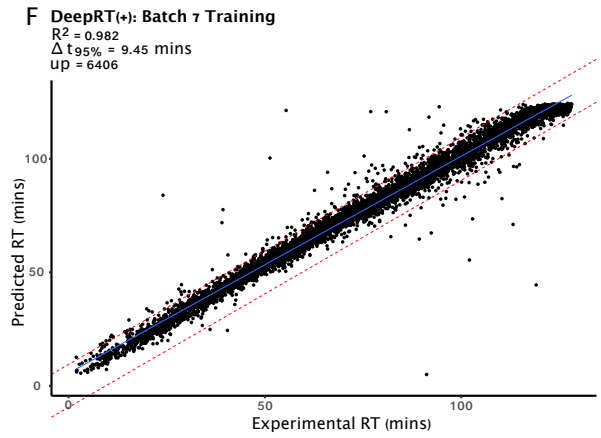

A DeepRT(+): Batch 2 Training
R2 = 0.986
Δ t95% = 7.95 mins
up = 8327
Predicted RT (mins)
Experimental RT (mins)


B DeepRT(+): Batch 3 Training
R2 = 0.973
Δ t95% = 12.35 mins
up = 9240
Predicted RT (mins)
Experimental RT (mins)


C DeepRT(+): Batch 4 Training
R2 = 0.988
Δ t95% = 9.67 mins
up = 7589
Predicted RT (mins)
Experimental RT (mins)

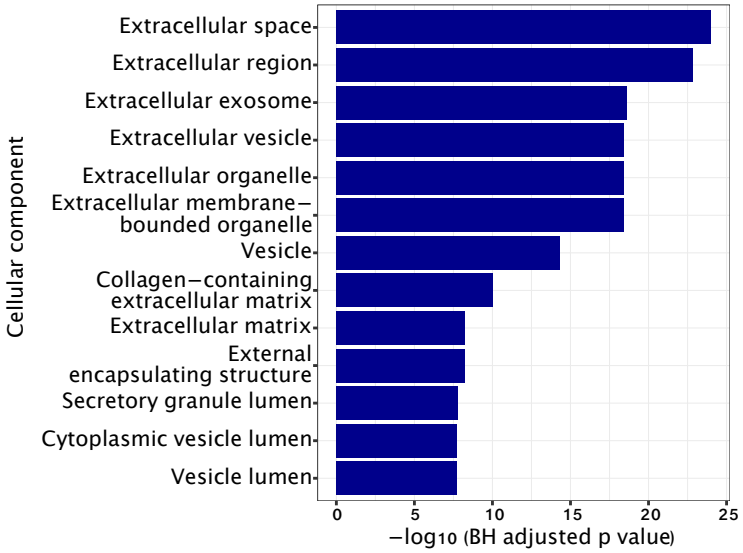

D DeepRT(+): Batch 5 Training
R2 = 0.975
Δ t95% = 11.24 mins
up = 7202
Predicted RT (mins)
Experimental RT (mins)


E DeepRT(+): Batch 6 Training
R2 = 0.991
Δ t95% = 6.22 mins
up = 10,829
Predicted RT (mins)
Experimental RT (mins)


F DeepRT(+): Batch 7 Training
R2 = 0.982
Δ t95% = 9.45 mins
up = 6406
Predicted RT (mins)
Experimental RT (mins)

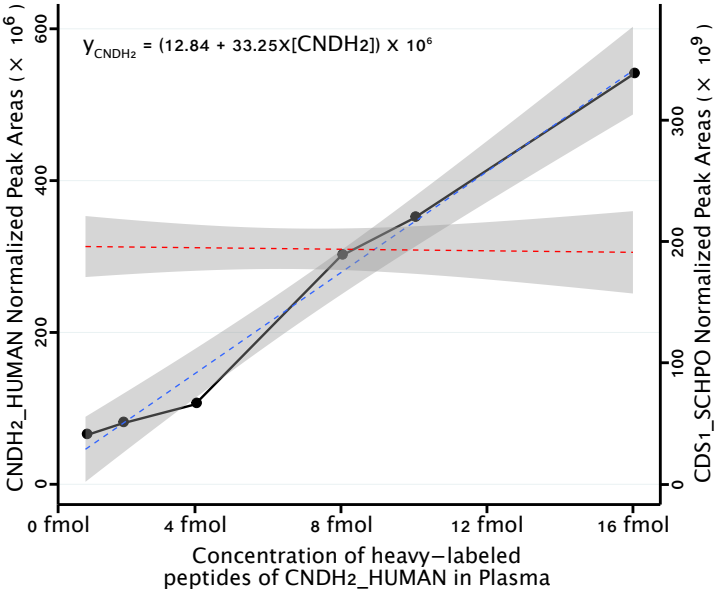
Supplemental Figure 4. 2

Scatter plot of experimental and predicted RTs of peptides from experimental batch 2 - 7. *up* = number of unique peptides trained



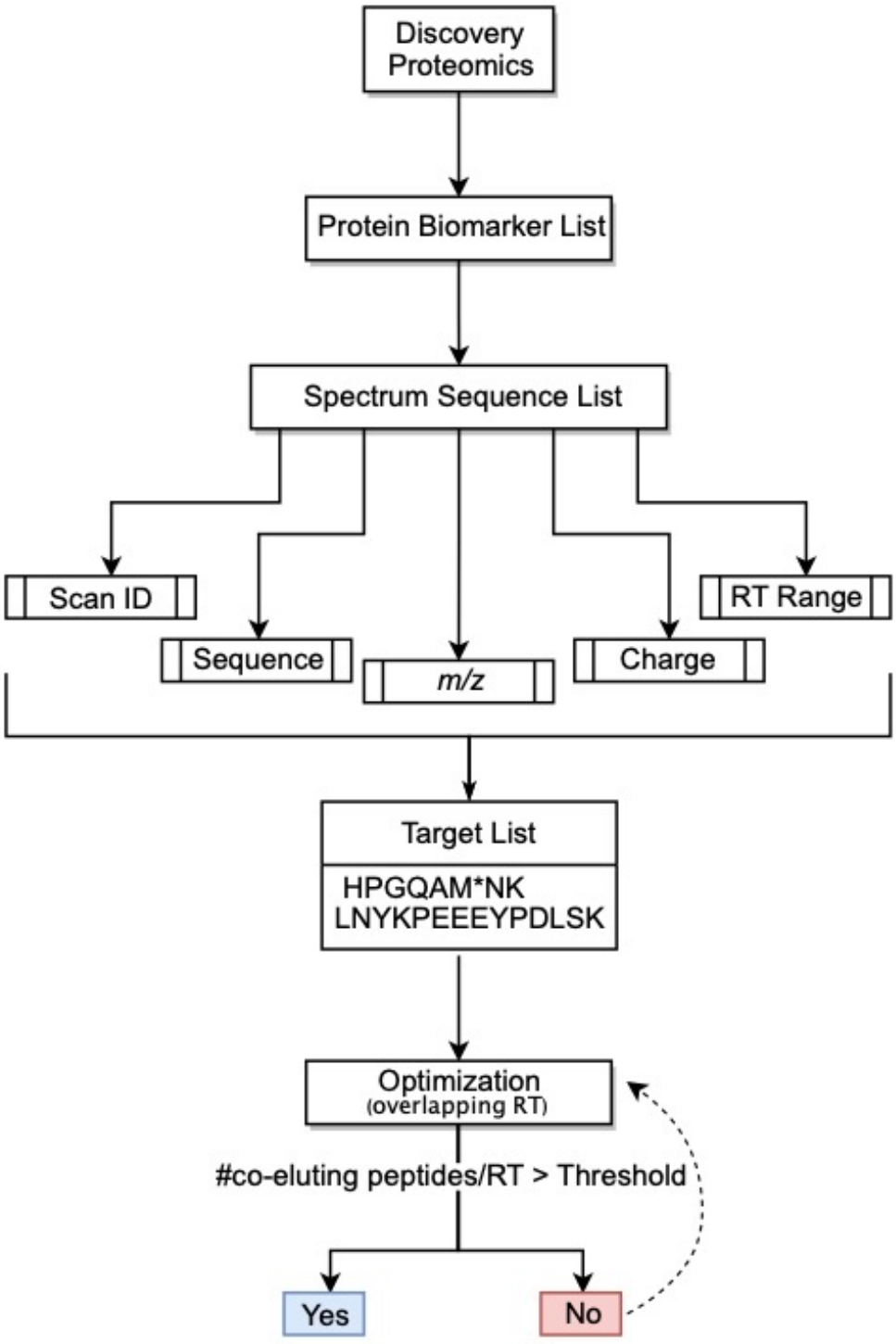
Supplemental Figure 4. 3

Functional analysis of biomarkers for enriched cellular components



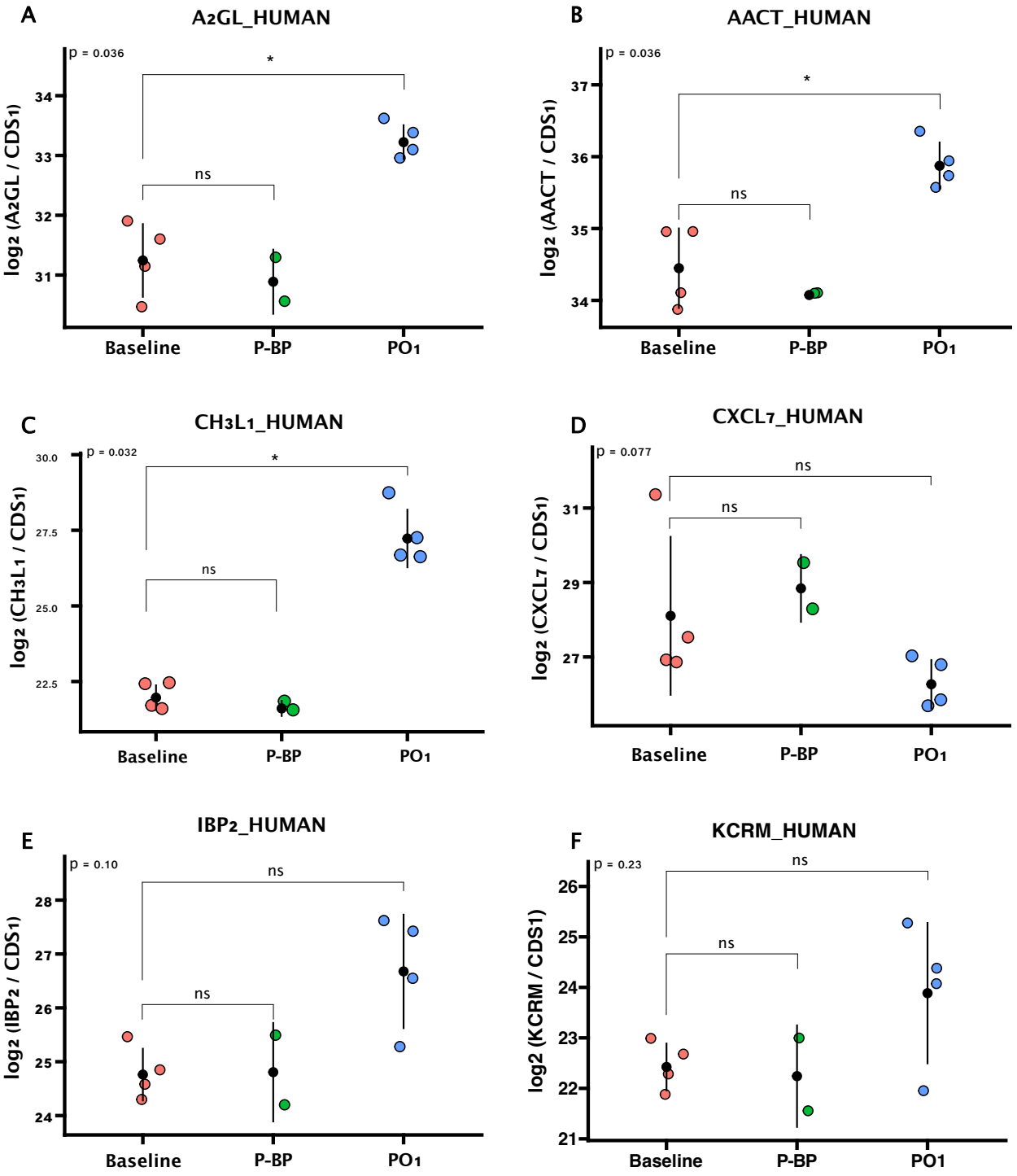
Supplemental Figure 4. 4

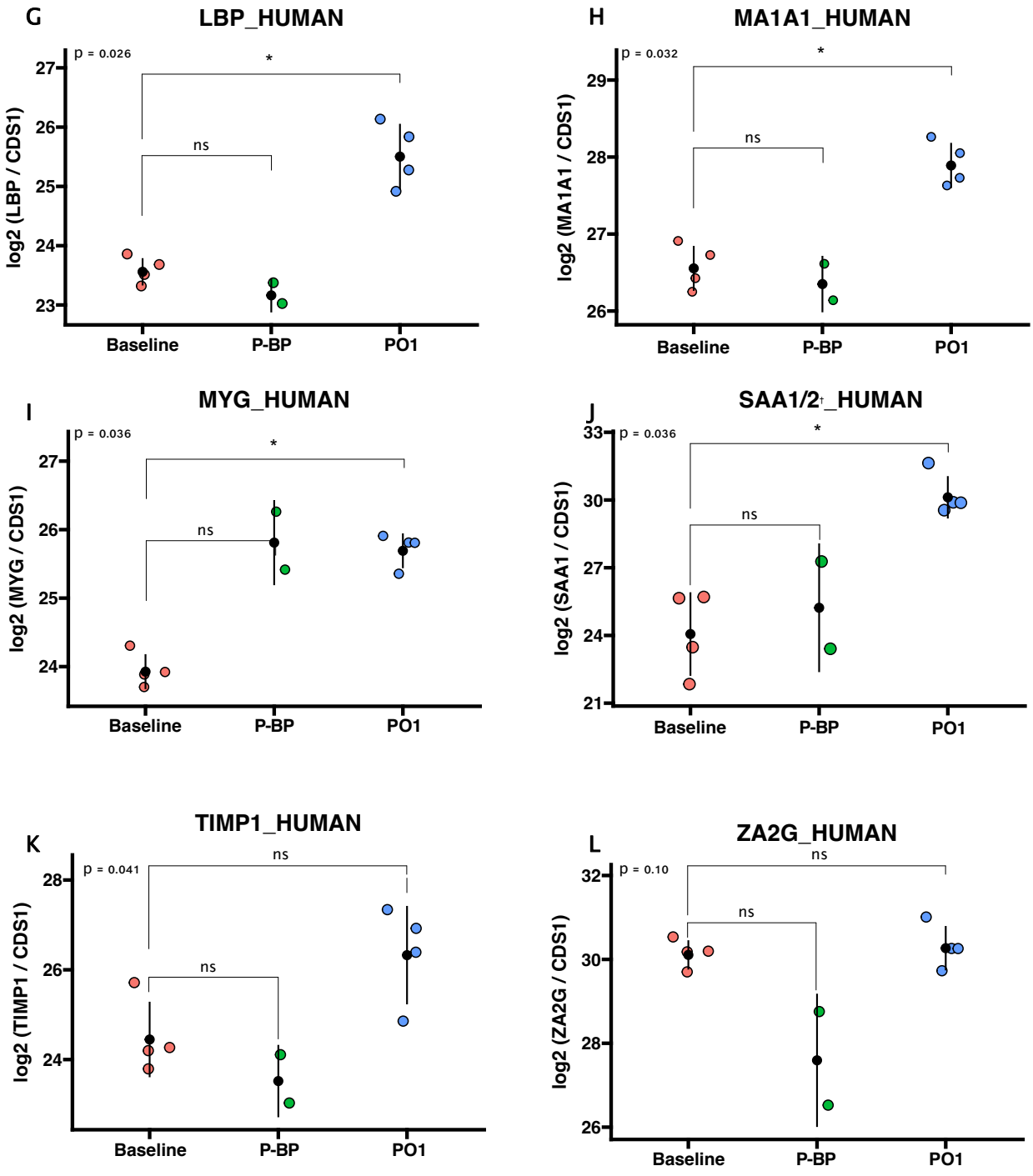
Normalized peak areas of CNDH2_HUMAN condensin subunit, superimposed with CDS1-SCHPO against increasing concentrations of its heavy-labeled stable isotope standards spiked into a background matrix of plasma.



Supplemental Figure 4. 5

Flowchart of PRM method development


A
A2GL_HUMAN
p = 0.036
*
ns
log2 (A2GL / CDS1)
34
33
32
31
Baseline
P-BP
PO1
B
AACT_HUMAN
p = 0.036
*
ns
log2 (AACT / CDS1)
37
36
35
34
Baseline
P-BP
PO1
C
CH3L1_HUMAN
p = 0.032
*
ns
log2 (CH3L1 / CDS1)
30.0
27.5
25.0
22.5
Baseline
P-BP
PO1
D
CXCL7_HUMAN
p = 0.077
ns
ns
log2 (CXCL7 / CDS1)
31
29
27
Baseline
P-BP
PO1
E
IBP2_HUMAN
p = 0.10
ns
ns
log2 (IBP2 / CDS1)
28
27
26
25
24
Baseline
P-BP
PO1
F
KCRM_HUMAN
p = 0.23
ns
ns
log2 (KCRM / CDS1)
26
25
24
23
22
21
Baseline
P-BP
PO1



Supplemental Figure 4. 6

Differential abundance analysis of validated proteins, showing changes across the three sample collection time points: baseline, post-bypass (P-BP) and post-operative day one (PO1). †: SAA1 and SAA2 could not be distinguished in the

validation phase as none of the peptides unique to them met the quantification criteria.

Chapter Five: Conclusions and Future Directions

Conclusions

This series of works contribute to the growing body of evidence regarding the possibility of discovering and using diagnostic biomarkers to diagnose delirium. We systematically reviewed recent published literature that provided a birds-eye view of untargeted, discovery proteomic experiments for biomarkers of delirium. We then demonstrated that the use of a targeted strategy for the purposes of discovery, however powerful this approach may be, can lead to misleading conclusions because of the unavoidably biased nature of targeted approaches. We subsequently applied the biomarker pipeline to plasma samples from our study cohort, and comprehensively profiled them for proteomics signatures of delirium. Summarized below are the major conclusions drawn from this thematic body of evidence.

Systematic review of proteomic contributions to delirium biomarker research

The list of potential candidate biomarkers identified in eight studies that met the study criteria suggest that a panel of proteins, rather than a single biomarker, would allow for discriminating delirium cases from non-cases. With a total of eight hypothesis generating studies over the last demi-decade, delirium biomarker research may be at its very early stages. Although functional analyses of the identified biomarker pool are consistent with the prevailing mechanistic hypothesis of neuroinflammation, a systems-biology view of delirium pathomechanisms has yet to fully emerge.

Perioperative Hyperoxia and Delirium after Cardiopulmonary Bypass

In our cohort of patients, peri-operative hyperoxia treatment was found to have no associations with long-term neurocognition at one-year post-operative. However, analysis for the impact on the immediate post-operative neurocognitive function reveals that peri-operative hyperoxia significantly contributes to the occurrence of post-operative neurocognitive dysfunction. Targeted measurements of four markers of neuroinflammation, despite their known associations with delirium in published literature, showed no associations with the outcome of delirium nor contributed any mediating role in the occurrence of delirium. Taken together, (1) our data suggests that the association between intra-operative oxygen treatment and neurocognitive function is one of a dose-

response-time relationship; and (2) use of targeted strategies for the purposes of discovery defies fundamental principles of biomarker discovery.

Intraoperative Plasma Proteomic Changes in Cardiac Surgery

Comparative analyses of proteomic profiles between delirium cases and non-cases revealed 16 biomarkers as signatures of cardiopulmonary bypass, and 11 as potential diagnostic candidates of delirium. While many of the identified biomarkers are non-specific markers of inflammation, novel identifications such as serum amyloid A1 (SAA1) and A2 (SAA2), pepsinogen A3 (PEPA3) and cathepsin B (CATB) shed new lights on delirium. Briefly, extra-hepatic production of SAA1 and SAA2 in the brain hints the possibility of brain-specific biomarkers of delirium. PEPA3, a native protease in the human stomach, found in significantly higher concentrations in the plasma of delirium cases suggest a break in the gastric mucosal integrity. This observation is consistent with an increased vagal tone as the body activates the cholinergic anti-inflammatory reflex in response to the physiological stress from cardiac surgery and CPB. Lastly, differential abundance of CATB, a well-known biomarker of Alzheimer's disease with a strong correlation, among delirium cases suggest that delirium and AD may have a common pathophysiological starting point. Equally important in this study is the potential of mass spectrometry close the time gap in translational biomarker research by eliminating the bottlenecks of biomarker verification and validation.

Future Directions

Validation in a larger independent cohort

Although biomarker validation outlines in Chapter Four was performed on an independent set of samples, it is severely underpowered. It is in our research interest to conduct a carefully designed validation experiment on discovered biomarkers, using samples from a larger independent cohort of patients.

Animal Models for Delirium Biomarker Research

Although most original biomarker studies at the moment are done with clinical samples, very few studies have attempted to study delirium in animal models. One notable study by Wang, Velagapudi [139] used older APPSwDI/mNos2$^{-/-}$ AD mice (CVN-AD) that underwent orthopedic surgery. Findings from this study was instrumental in advancing our understanding of the role of the neurovascular unit and the blood brain barrier in the pathogenesis of delirium. In this study, authors ascertained that the immune systems of the experimental mice were naïve. While animal models are simpler and relatively more controllable, with possibly less noise in acquired data, they also present with significant challenges. A major one is the correlation between cognitive assessments in humans (e.g., with the use of the confusion assessment method) and that used for animals. For animal studies to enhance our understanding of delirium, future approaches will require cognitive tests that assess specific brain functions similar to that being tested in

the humans, and for human studies to evaluate for the baseline immune or inflammatory state of subjects before recruitment into studies.

Number of scans relative of proteins identifications from plasma and the possible role of post-translational modifications

Our study focused on global proteomic changes in plasma. As a result, post-translational modifications and how they relate to the occurrence of delirium were not studied. Throughout our experiments, we observed that only a small fraction of MS2 scans provided meaningful proteomic information. Given that native plasma proteases likely modify proteins in ways that are largely unknown, and the role of PTM in the pathogenesis of delirium is only now emerging, this is an interesting question for future research in this space.

Multi-omics approach to diagnostic biomarkers of delirium

The growing body of evidence regarding delirium biomarkers underscores the complexity in identify definitive molecular signatures of delirium. In addition to proteomic approaches, many scholars have also attempted biomarker discovery at the gene, transcript and metabolite levels, all of which significantly contributes to our understanding of delirium pathomechanisms. It is likely that the different omic approaches only offer partial insights about the condition and when taken together, may offer a more complete molecular view of delirium. Interestingly, differentially abundant biomarkers such as SAA1 and APOA4 among other

plasma proteins, that functionally interact with lipoproteins suggest that lipidomics may also play a role in the pathomechanism of delirium. Taken together, a multi-omics approach may offer a more complete understanding of the molecular underpinnings of delirium.

Statistical tools for longitudinal proteomics data

Statistical analyses for longitudinal proteomics data, especially those with repeated measures is largely unexplored. When analysis required the comparison of dimensions in the same dataset (for example pre- versus post-surgery and cases versus controls), published literature is inconsistent on the right approach to analyses. While some authors have suggested the use of difference of the differences, others have used the overlap of features after performing comparison on the two dimensions separately. Yet some authors have also suggested regression modeling and the use of spectral count and other MS-centric information for variance estimation in such modeling. As proteomic biomarker research grows with the advent of modern mass analyzers, this warrants statistical method development that is tailored to the specific context of MS-derived data.

Chapter Six: Additional Works and Contributions

Affinity-based profiling of endogenous phosphoprotein phosphatases by mass spectrometry

Brooke L. Brauer, **Kwame Wiredu**, Sierra Mitchell, Greg B. Moorhead, Scott A. Gerber, Arminja N. Kettenbach

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Abstract

Phosphoprotein phosphatases (PPPs) execute >90% of serine/threonine dephosphorylation in cells and tissues. While the role of PPPs in cell biology and diseases such as cancer, cardiac hypertrophy and Alzheimer's disease is well established, the molecular mechanisms governing and governed by PPPs still await discovery. Here we describe a chemical proteomic strategy, phosphatase inhibitor beads and mass spectrometry (PIB-MS), that enables the identification and quantification of PPPs and their posttranslational modifications in as little as 12 h. Using a specific but nonselective PPP inhibitor immobilized on beads, PIB-MS enables the efficient affinity-capture, identification and quantification of endogenous PPPs and associated proteins ('PPPome') from cells and tissues. PIB-MS captures functional, endogenous PPP subunit interactions and enables discovery of new binding partners. It performs PPP enrichment without exogenous expression of tagged proteins or specific antibodies. Because PPPs

are among the most conserved proteins across evolution, PIB-MS can be employed in any cell line, tissue or organism.

Contribution

KW contributed to study conceptualization and revision of manuscript

Development and validation of inducible protein degradation and quantitative phosphoproteomics to identify kinase-substrate relationships

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Abstract

Phosphorylation signaling is an essential post-translational regulatory mechanism that governs almost all eukaryotic biological processes and is controlled by an interplay between protein kinases and phosphatases. Knowledge of direct substrates of kinases provides evidence of mechanisms that relate activity to biological function. Linking kinases to their protein substrates can be achieved by inhibiting or reducing kinase activity and quantitative comparisons of phosphoproteomes in the presence and absence of kinase activity. Unfortunately, most of the human kinases lack chemical inhibitors with selectivity required to unambiguously assign protein substrates to their respective kinases. Here, we develop and validate a chemical proteomics strategy for linking kinase activities to protein substrates via targeted protein degradation and quantitative phosphoproteomics and applied it to the well-studied, essential mitotic regulator polo-like kinase 1 (Plk1). We leveraged the Tir1/auxin system to engineer HeLa cells with endogenously homozygous auxin-inducible degron (AID)-Plk1). We HeLa cells and determined the impact of AID-tagging on Plk1

activity, localization, protein interactors, and substrate motifs. Using quantitative proteomics, we show that of over 8,000 proteins quantified, auxin addition was highly selective for degrading AID-Plk1 in mitotic cells. Comparison of phosphoproteome changes in response to chemical Plk1 inhibition to auxin-induced degradation revealed a striking degree of correlation. Finally, we explored basal protein turnover as a potential basis for clonal differences in auxin-induced degradation rates for AID-Plk1 cells. Taken together, our work provides a roadmap for the application of AID technology as a general strategy for the kinome-wide discovery of kinase-substrate relationships.

Contribution

KW contributed to comparative proteomic analyses between fast-degrading 23R3, and slow-degrading B12-11 cell lines. This involved use of unsupervised dimensionality reduction to explore inherent data structures and z-standardization to eliminate cell-line differences and allow for comparison of the rates of degradation at the global level and for specific proteins.

Quantitative survey research in anesthesiology: a field guide to interpretation

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Abstract

Background Survey research, indispensable for assessing subjective outcomes in anesthesiology, can nonetheless be challenging to undertake and interpret.

Objective To present a user-friendly guide for the appraisal of survey-derived evidence, and to apply it to published survey research in the anesthesia literature.

Methods Synthesizing published expert guidance regarding methodology and reporting, we discuss five essential criteria (with subcomponents) for evaluating survey research: (1) relevance of survey outcome to research objective, (2) trustworthiness of the instrument (testing/validation, availability), (3) collecting information well (sampling, administration), (4) representativeness (response rate), and (5) guidance towards interpretation of survey findings (generalizability, interpretation of numerical outcomes). These criteria were subsequently applied by two independent assessors to original research articles reporting survey findings, published in the five highest impact general anesthesia journals (‘Anaesthesia’, ‘Anesthesia & Analgesia’, ‘Anesthesiology’, ‘British Journal of Anaesthesia’ and ‘European Journal of Anaesthesiology’) between July 01, 2016,

and December 31, 2017, which were identified using a prespecified PubMed search strategy.

Results Among 1107 original articles published, we identified 97 reporting survey research either employing novel survey instruments (58%), established surveys (30%), or sets of single-item scores (12%). The extent to which reader-oriented benchmarks were achieved varied by component and between survey types. Results were particularly mixed for validation (mentioned for 41% of novel and 86% of established surveys) and discussion of generalizability (59% of novel survey reports, 45% of established surveys, and 17% of sets of single-item scores).

Conclusion Survey research is not uncommon in anesthesiology, frequently employs novel survey instruments, and demonstrates mixed results in terms of transparency and interpretability. We provide readers with a practical framework for critical interpretation of survey-derived outcomes.

Contribution

KW contributed to study designing, assisted in database search and literature screening, analyzed data and contributed to writing manuscript.

Brachial Artery Embolectomy in a Polytrauma Patient: A Case Report

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https://www.jce.ro/article/brachial-artery-embolectomy-in-a-polytrauma-patient-a-case-report/

Abstract

Introduction: The upper extremity is a frequent site of injury. Upper limb arterial thromboembolism, a rare complication of such injuries, may be missed if typical signs, such as pain, pulselessness, and sensory loss, cannot be ascertained or are overlooked by physicians, especially in the case of polytrauma or comatose patients. Case presentation: In this report, we present the case of a left brachial artery thromboembolism in a polytrauma patient for which brachial artery embolectomy was performed. Before surgery, the diagnosis was established with doppler ultrasonography of the upper limb vessels, performed upon suspicion of thrombus formation. Brachial artery arteriotomy and thrombo-embolectomy were performed using a size 6 Fr Fogarty catheter, after which 500 IU heparin was flushed to ensure adequate back and forward flow. Limb function and blood flow were restored immediately after the procedure. Conclusion: A high index of suspicion, timely assessment, and a prompt intervention can significantly reduce the rate of limb ischemia and/or amputations in polytrauma patients, especially in resource-limited settings.

Contribution

KW assisted in delivery of anesthesia to the patient, and collected all relevant clinical notes from the multi-disciplines to draft manuscript

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