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Pharmaco-proteomic Opportunities for Individualizing Neurovascular Treatment

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Abstract

Neurovascular disease often involves multi-organ system injury. For example, patent foramen ovale (PFO) related ischemic strokes involve not just the brain, but also the heart, the lung, and the peripheral vascular circulation. For higher-risk but high-reward systemic therapy (e.g., thrombolytics, therapeutic hypothermia, PFO closure) to be implemented safely, very careful patient selection and close monitoring of disease progression and therapeutic efficacy are imperative. For example, more than a decade after the approval of therapeutic hypothermic and intravenous thrombolysis treatments, they both remain extremely underutilized, in part due to lack of clinical tools for patient selection or to follow therapeutic efficacy. Therefore, in order to understand the complexity of the global effects of clinical neurovascular diseases and their therapies, a systemic approach may offer a unique perspective and provide tools with clinical utility. Clinical proteomic approaches may be promising to monitor systemic changes in complex multi-organ diseases – especially where the disease process can be “sampled” in clinically accessible fluid such as blood, urine and CSF. Here, we describe a “pharmaco-proteomic” approach to three major challenges in translational neurovascular research directly at bedside – in order to better stratify risk, widen therapeutic windows, explore novel targets to be validated at the bench – 1) thrombolytic treatment for ischemic stroke, 2) therapeutic hypothermia for post cardiac arrest syndrome, and 3) treatment for patent foramen ovale (PFO) related paradoxical embolic stroke. In the future, this clinical proteomics approach may help to improve patient selection, ensure more precise clinical phenotyping for clinical trials, and individualize patient treatment.

Keywords

patent foramen ovale (PFO) related stroke; proteomics; cardiac arrest; therapeutic hypothermia; thrombolysis

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I. Introduction

Human neurovascular disease comprises an extremely wide range of phenotypes involving multiple cell types of the brain, the blood brain barrier (BBB), interaction with the peripheral systemic circulation, and multiple stroke subtypes. Most neurovascular disease involves multi-organ system injury – for example, focal embolism (from direct cardiac source or paradoxical emboli from the peripheral vasculature), traumatic mechanisms, or global ischemic injury from lack of perfusion, such as from sudden cardiac arrest. Decades of investigation have pushed the field forward in understanding the mechanisms of diseases, and yet the repertoire of therapeutic interventions and the tools for triaging their use have remained remarkably limited. Thrombolytic treatment (tPA) and therapeutic hypothermia (TH) are both good examples. More than a decade after the approval of these systemic treatments, demonstrated to be clinically efficacious in selected patient populations, they both remain extremely underutilized. [1-17] The major challenge preventing wider use of these higher-risk, high-reward therapies is that to be implemented safely, they require very careful patient selection and close monitoring of disease progression and therapeutic efficacy. To understand the complexity of the global effects of therapeutic targets and the organ-organ response (such as the brain and heart), a systemic rather than a targeted approach may offer a unique perspective with clinical utility.

Toward these goals, clinical proteomic approaches may be promising for complex multi-organ diseases such as neurovascular disease, especially where the disease process can be monitored in clinically accessible fluid such as blood, urine and CSF. Here, we describe a “pharmaco-proteomic” approach to three major challenges in translational neurovascular research directly at bedside, in order to better stratify risk, widen therapeutic windows, explore novel targets to be validated at the bench, and monitor therapeutic efficacy to individualize treatment.

II. Bedside clinical proteomic profiling may offer insights to individualize treatment

Unlike elegant *in vitro* and *in vivo* models, clinical samples are more heterogeneous since no two patients are quite alike – in addition to genetic variations, clinical risk factors, medication intake and even food intake can confound results. Thus, it has been challenging to monitor therapeutic efficacy clinically. To reduce confounders in an inherently complex system, the most robust comparisons are quantitative profiles taken over time from the same individual, utilizing their own baseline in healthy or pre-treatment state as “controls.” Since omics technology can afford the sensitivity of studying a multitude of markers at the same time, can one potentially leverage this power to study an individual over time and follow clinical efficacy? Might it be possible to derive an individual’s own health and disease signature and follow each individual’s unique response to a therapeutic intervention?

Applying novel proteomic techniques in specific bedside models, where measurements can be made before and after a specific intervention that triggers systemic changes in plasma signaling, helps to minimize confounders and monitor therapeutic efficacy. This new field of “pharmaco-proteomics,” matching the right technology to monitor critical clinical

interventions or drug development, has the potential to help to "individualize" treatment by maximizing benefit-risk ratio in real time. [18,19] Here we share three examples of ongoing studies with attention to: 1) reducing confounders in a complex system by using profiles taken over time; 2) applying these techniques in specific bedside models; 3) measuring a specific intervention that triggers systemic changes in plasma signaling.

A) Sifting for clues in thrombolysis "trash" for focal ischemic injuries

tPA, a serine protease, is an effective treatment for acute ischemic stroke, but is limited to a narrow time window (currently up to 4.5hr from symptom onset) due to risk of intracranial hemorrhage, resulting in very few qualifying patients. [3,6-9,20-28] There are also variable responses in patients with other risk factors such as genetic clotting states or diabetes. [7-9,25-28] Studies demonstrate that in tPA responders, there has to be a tight balance between thrombolysis and bleeding, such that the smallest amount of petechial bleeding post IV-tPA may even demonstrate efficacy. [29-36] At the most basic level - one simple question is: just what should be the individual dose? Should there be one dose for a 70kg previously healthy young man, a 70kg man with mild liver dysfunction from hepatitis, or a 70kg octogenarian with diabetes, inflammatory arthritis, and significant different body mass distribution and metabolism? Currently, they receive the same dose: 0.9mg/kg of IV tPA. Should there be tailored dosing, or can IV tPA's response be rapidly monitored in real time in order to make adjustments or call for other intervention? While there has been a large amount of good data on the interaction of tPA within the vasculature and with respect to the neurovascular unit, clinically, we can only monitor by serial clinical exams, which usually lag behind therapeutic response, or by imaging signs of recannalization, which may not indicate better ultimate clinical outcome. [29-35,37] With successful new interventional treatment under development and data demonstrating efficacy of tPA beyond the traditional time limits, it becomes even more important to find new ways to predict and follow tPA efficacy clinically. [30-51]

One method of interest, developed by the Overall group and others [52,53], is "degradomics" – the techniques for characterizing the "substrate repertoire" of a particular proteases. This method looks at the "trash" or "chewed-up bits" of various proteinases – in essence measuring composite protease degradation products – taking into account the synergistic and functional redundancy of various proteases with respect to a particular stimulus. [52,54] Degradomics is particularly well suited to the study of protein therapeutics (such as tPA – a serine protease), and other proteases important in neurovascular injury such as MMPs. [36,49,55-58] Indeed, we found that in the plasma of patients given tPA for ischemic stroke, there are robust differential protease substrate patterns over time with respect to thrombolysis status.[59] What is more interesting is that this profile persisted even up to 3-5 days after IV tPA is given – far beyond the half-life of tPA itself. In comparison to healthy control patients who had little change over time, tPA (especially in responders) triggered a sustained cascade of events that may actually be quantifiable in blood. These early findings suggest that it may be possible to monitor the systemic effects of therapy such as IV tPA in real time at the bedside. These measurable coordinated signaling responses may impact the balance between treatment efficacy and associated risks of thrombolysis-related

complications such as hemorrhage and edema, giving clinicians the opportunity to individualize therapy.

B) Hypothermic response in Post-Cardiac Arrest Syndrome – understanding multi-organ ischemic injury via circulatory signals in blood

Post-cardiac arrest syndrome is the most prevalent and devastating global ischemic condition involving multiple organ systems. In the United States alone, 350,000 to 450,000 out-of-hospital cardiac arrests occur annually.[10-17,60] Anoxic brain injury (ABI) post cardiac arrest has extremely poor prognosis: less than 10% survive to hospital discharge, and only 3-7% of survivors return to their previous level of functioning.[10-17,61] As a result of the extensive damage following cardiac arrest, this is often referred to as “Post-Cardiac Arrest Syndrome.” The general components of this syndrome are brain injury, myocardial dysfunction, and systemic ischemia/reperfusion response. Neurological injury has been shown to account for more than two-thirds of post-cardiac arrest deaths.[61]

Mild therapeutic hypothermia (TH) seems to be protective for multiple organs. Utilizing cooler temperature as treatment has long been documented in history, dating back at least to 300 BC, when Hippocrates advocated healing wounds with snow and ice.[62-64] Many other examples of TH are recorded in history for multi-organ injuries. For example, Napoleon’s surgeon general Baron Larrey noticed that wounded officers who were placed preferentially next to a fire died more quickly than the soldiers who were left hypothermic.[62] Initial interest in modern medicine for the neuroprotective value of hypothermic therapy in head trauma was dampened by major side effects of deep hypothermia, such as lethal cardiac ventricular arrythmia reported in the 1950s. [65,66] However, with multiple studies demonstrating the efficacy of mild to moderate hypothermia and the advent of critical care units to monitor major side effects, a few clinical trials have again rekindled interest in utilizing hypothermia for neuroprotection in a small population of post-cardiac arrest patients. [67-87]

The neuroprotective effects of hypothermia are mediated by a wide range of multifactorial mechanisms including promotion of cell survival signaling, suppression of programmed necrosis and apoptosis, dampening of inflammation and protection for the entire neurovascular unit. [67,69-76,78-89] These complex mechanisms of hypothermia have been extensively reviewed in diseases such as stroke [87,90-92] In the present context of cardiac arrest and brain ischemia, hypothermia could be especially relevant because it may rescue not only the CNS but also ameliorate injury in multiple organ systems affected by the global loss of blood flow. In recent clinical trials, TH nearly doubles the chance of good neurologic outcome[11,93,94] – but it remains grossly underutilized (less than 1 to 5% in most major medical centers) due to current stringent exclusion criteria to only offer this treatment to the most severely affected patients (<6 hours from time of injury, coma status etc) and fear of complications (sepsis, coagulapathy).[95,96] Hypothermia protects against multi-organ damage, but the therapy itself also alters systemic response in ways that make traditional neurologic prognostic tests ineffective. [97]

A better understanding of the extent of systemic anoxic injury and the effects of TH will help to improve both patient selection and prognosis. Current understanding of the extent of

injury relies on approximate “time down” or time for return of spontaneous circulation (ROSC) – which can be notoriously inaccurate as a result of witness accounts in a traumatic setting, and the effectiveness of first responder CPR. Most current standards of care also impose a stringent window of < 6 hours since cardiac arrest in order for patients to be treated, but time since cardiac arrest is at best an approximate substitute for an actual assessment of the specific systemic changes underlying the potential risks and benefits of TH for an individual patient. Most importantly, TH is only reserved for coma patients post cardiac arrest – those with the worst prognosis. Although these patients may benefit the most from neuroprotection, clinically, just about any patient who survives a cardiac arrest will have some type of cognitive injury, whether it be mild or severe. Thus, may non-coma patients also benefit from TH to ameliorate life-altering cognitive injury?

Especially with such a multi-organ system injury, the circulation should be a rich reservoir of information relating to both injury and repair. However, while there has been a wealth of knowledge establishing the mechanistic importance of hypothermia, most studies center on intracellular pathways and less attention has been placed on secreted or extracellular factors to translate to clinical measurements. Before hypothermic therapy was available clinically, very few markers predicted clinical outcome, with variable success for post-cardiac arrest syndrome.[98] After cooling therapy was available for cardiac arrest patients, however, even fewer markers exist: S100B is the only one studied recently, and its sensitivity of prediction is moderate at best, skewed by high levels in the most severe patients.[99,100] Thus a combined target and proteomic approach to screen for extracellular signaling may gain better understanding of the robust but complex landscape of peripheral response to hypothermic injury in patients’ blood – where all organ-organ signaling can converge in injury and response.

Post-translational modifications (PTM) may provide a promising window into systemic effects measurable in blood, because they are associated with extracellular signaling, they can occur within the short therapeutic window (<24 hours) of TH, and they are also involved in important therapeutic complications of TH such as sepsis and coagulopathy. During the hypo-metabolic state of TH, rather than looking at new production of proteins, PTMs such as glycosylation can be very important. For example, patterns of glycosylation, one of the most important extracellular PTMs involved in cell signaling/recognition, can act as plasma “zip codes” to target factors important in organ-organ interaction in the circulation. Glycosylation is pivotal in damping immune response and coagulation – two of TH’s major side effects that limit its utilization. Glycosylation, which occurs rapidly over the treatment time-window of TH, can also serve to provide “tags” for various proteomic enrichment and separation techniques. In pre-treatment blood, we found significantly different glycosylation patterns between cooled post-cardiac arrest patients with respect to clinical outcomes. After lectin enrichment, more than a thousand proteins were identified to have glycosylation PTMs in the circulation.[101-103] Analysis of this rich network of signaling cascades with respect to clinical outcome and TH efficacy and side effects is under way. Thus, TH is also an ideal bedside stimulus-response model to study enzymatic and metabolic activity to gain direct insight into host response to injury and therapeutic efficacy and complications – crucial to the ultimate prognosis for the patient.

C) Intercepting brain-heart cross-talk – studying PFO physiology and PFO closure

So far, we have provided examples of monitoring focal or global ischemic brain injury. Proteomics can also capture baseline organ-organ communication – the constant cross-talk between the brain and other organs that may affect future risk of neurovascular disease. Here we share the example of patent foramen ovale related embolic stroke - focusing on capturing interaction between the brain and the heart. Patent Foramen Ovale (PFO) is an independent embolic stroke risk factor.[104-113] A residual tunnel between the right and left atria remnant from the maternal-fetal circulation, PFO has been described as a "back door to the brain."[114] PFO related strokes result in 40% of cryptogenic strokes, affecting more than 150,000 patients yearly in the U.S. and resulting in serious long-term disabilities as they tend to occur in younger stroke victims.[108,115-117] While PFOs are highly prevalent (in 25-30% of the general population), currently there is no clear guidance on the appropriate treatment or preventative strategies. [104-107,118,119]

Just what's so complicated about PFO? Often discovered only after a stroke, in such a highly prevalent condition – one in four healthy individuals – who should be screened to prevent future strokes? How can we best prevent recurrent strokes – with PFO closure, medical therapy, or a combination of both? Individual risks vary and treatment will have to be customized.[118-125] Clinical trials have been difficult to conduct due to the heterogeneity of the patient population, and so far two small trials (CLOSURE I and RESPECT) have reported conflicting results.[126,127] The CLOSURE I trial, which included both stroke and TIA patients demonstrated no difference in PFO closure versus medical treatment. But the recently completed RESPECT trial which only included stroke patients showed potential benefit of PFO closure in a subpopulation of patients with significant risk reduction in PFO closure arm compared to medical arm in the as-treated analysis, especially when patient groups were stratified by the degree of right-to-left shunting. However, due to small sample size and patient crossover during the trial, RESPECT only demonstrated statistical significance in secondary per-protocol analysis rather than the primary intention-to-treat analysis. [126,127]

It becomes even more complicated as we examine the physiology of PFO-related stroke, commonly also known as "paradoxical embolus," as this type of stroke is the result of a venous rather than arterial clot. PFOs have traditionally been thought to facilitate paradoxical embolism by allowing venous clots to pass into arterial circulation and travel directly to the brain. However, this simple mechanism falls significantly short of explaining clinical data, as our group and others have found that only a small portion (10-17%) of patients with PFO-related stroke have a known tendency to form venous clots.[128,129] So, how do the 80-90% of PFO patients without known clot-forming conditions end up with embolisms? In the absence of known prothrombotic conditions, could there be other mechanisms at play? Might these patients have unknown "procoagulant" states related to PFO physiology? Are there markers to predict stroke in asymptomatic PFO patients? Since PFO affects more than a quarter of the world's population, the potential public health and scientific impact of understanding PFO-related neurovascular injury is enormous. Thus larger clinical trials and better translational understanding of PFO related stroke are needed in this multi-organ disease to help individualize appropriate therapy.

One central question is: while PFO's right-to-left shunting allows venous clots to enter arterial circulation, avoiding filtration by the lungs and causing ischemic stroke, can it also allow other harmful circulatory factors to travel directly from the venous to the arterial circulation? This hypothesis can be explored using discovery proteomic techniques. In order to probe the systemic effect of PFO physiology, a pharmaco-proteomic approach may be useful to study endovascular closure of PFO – an endovascular procedure that obliterates shunting by deploying a closure device. PFO endovascular closure can stop right-to-left shunting immediately during the procedure. It is an effective procedure, but requires better risk stratification, patient selection and monitoring of therapeutic efficacy. [130]

Lopez *et al.* applied a novel quantitative two-pass discovery workflow using high-resolution LC-MS/MS coupled with label-free analysis to follow protein expression in PFO patients' blood before and after PFO closure. [131] We were able to identify quantitative changes in protein expression not only before and after PFO closure, but also in long-term follow-up. [131] The resulting protein expression patterns were related to prothrombin activation, atherosclerosis signaling, acute phase response, LXR/RXR activation and coagulation pathways. In particular, post PFO closure, numerous proteins demonstrated reduced expression in stroke-related canonical pathways such as acute inflammatory response and coagulation signaling. [62] These findings support the hypothesis that the physiology of PFO itself may contribute to a potential acquired hypercoagulable state – that is, human structural anatomy may alter blood chemistry and contribute to neurovascular injury. It also demonstrates the feasibility of using a proteomic approach for biomarker discovery to help gauge PFO closure efficacy in a "proximal organ fluid" – cardiac atrial blood.

IV. Summary and Future Directions - Harvesting the power of individuality

To evaluate the change in plasma phenotype and help monitor therapeutic efficacy of various treatments, we discussed some of our early efforts to couple bedside intervention models with various proteomic technologies to help select and monitor patients for the appropriate clinical therapy in real time – a major need for new and ongoing neurovascular disease treatments. Leveraging the variation from individual patients in these bedside "models," proteomics may capture cross-talk between multiple organs through blood profiles. Advances have been made in both methodology and the fine-tuning of clinical questions, as the demand for proteomic technology in stroke research and clinical applications continues to grow. [2,18,132]

With the increasing application of proteomics to stroke, many important pathways have been identified from both pre-clinical and clinical sides. The study of multiple proteins and their interactions even predates the discovery of DNA – large-scale protein studies have been ongoing even before the word "proteome" existed in published literature. [133-136] These are only a few very limited examples of applying newer proteomic technology to clinical problem solving. Each day innovative studies are published as the field rapidly advances. Many of these have been reviewed elsewhere. [141]

However, challenges that relate to the heterogeneity of disease itself, availability of high-end instrumentation, and individual patient differences must be taken into

consideration. [18,138,139] For example, it has often been thought that clinically accessible fluids such as blood are too complex for mass spectrometry instrumentation to analyze well, mostly due to the issue of dynamic range. In complex bodily fluids such as plasma, high-abundance proteins (only about 10 or so) represent approximately 98% of the total protein content, masking the more clinically relevant lower-abundance proteins and making it difficult to assess all components of a sample. [137, 140-141] However, while this has been a limitation, advances in MS instrumentation and newer methodologies to enrich and target proteins of interest have been overcoming these obstacles. Also, emerging data show that not just the low-abundance, but high-abundance proteins too, have critical roles in human disease pathophysiology.[141] For example, existing clinical markers such as CRP (C-reactive protein) for the prediction of cardiac risk factors, PSA (prostate-specific antigen) for prostate cancer, and various immunoglobulins for collagen vascular disease, are all higher-abundance markers. And post-translational modified components such as hemoglobin A1c have revolutionized systemic disease such as diabetes to assess cumulative disease burden and follow treatment efficacy.

A bedside “stimulus-response model” to monitor therapeutic efficacy pre and post treatment may help to minimize confounders by using each patient’s pre-therapy state as a baseline. If we can carry our own blood type for a proper match in emergencies, in the future can we also carry our proteomic/genomic baseline, so that our response to therapeutic intervention can be measured against this known background to monitor therapeutic efficacy in disease states? While larger clinical trials are warranted to establish the sensitivity and specificity of biomarkers for routine use from current candidates, smaller well-designed and well-controlled pre-clinical, translational and clinical bedside models are direly needed to investigate the underlying mechanisms and expand the field to understand neurovascular disease in a dynamic state. A pharmaco-proteomic approach to obtain a “composite” signature of treatment effects may help to monitor therapeutic efficacy, improve patient selection, ensure more precise clinical phenotyping for clinical trials, and most importantly individualize treatment for our patients.

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