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Review article

Proteomics of In Vivo models of ischemic stroke: A systematic review with a systems biological perspective

Anaekshi Gogoi [a], Manju Babu [a], Abhina Gangadhar [a], Riyama Shirin [b], Deepthi Ann Thomas [a], Jasmitha Jayaram [a], Pratik Thakkar [c], Arnab Datta [a,b,*]

[a] Laboratory of Translational Neuroscience, Division of Neuroscience, Yenepoya Research Center, Yenepoya (Deemed to be University), University Road, Deralakatte, Mangalore, Karnataka 575018, India
[b] Department of Pharmacology, Yenepoya Pharmacy College and Research Center, Yenepoya (Deemed to be University), Naringana, Deralakatte, Mangalore, Karnataka 575018, India
[c] Department of Physiology, Faculty of Medical and Health Science, The University of Auckland, 85 Park Road, Auckland 1142, New Zealand

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ABSTRACT

Background and Purpose: To discover therapeutic targets and biomarkers for ischemic stroke, a systems-level understanding of the altered brain proteome is necessary from various types of in vivo models. In the last two decades, despite accumulating numerous large-scale proteomics datasets on in vivo models of ischemic stroke, systematic classification and summarization of these data have not been attempted yet.
Methods: Following PRISMA, ARRIVE, and CAMARADES guidelines, we conducted a systematic review of proteomics studies done on various in vivo models of ischemic stroke (ivProt studies).
Results: Of 680 studies, 120 ivProt studies were shortlisted. They were classified based on TOAST stroke subtypes, model category, study duration, and sampled brain location. We collated 288 proteins deregulated in the brain tissue of MCAO-affected animals, 44 of which were reported in minimum two ivProt studies. A comparison of deregulated proteome between ivProt and clinical proteomics studies revealed consistent deregulation for 85 % of the commonly quantified proteins. The Gene Ontology analysis of the consensus stroke-perturbed proteome (n = 90) showed enrichment of annotation terms related to blood coagulation, HIF-signaling and secretory pathway. About 88 % of these proteins were found listed in an exosome database (ExoCarta), while 42 % overlapped with the therapeutic target database. Notably, this list contained eight successful drug targets (ANXA1, C3, DPYSL2, IDH1, IDH3A, PRKCE, PRKCG, and SERPINA1).
Conclusion: A web resource (ivProt_Repository) was created that may stimulate clinical validation of shortlisted proteins for biomarker discovery or proof-of-concept studies for therapeutic target discovery and help to establish novel pipelines for big data integration.

1. Introduction

Stroke is the major cause of mortality and morbidity with limited theranostic options. Ischemic stroke accounts approximately 80 % of all stroke cases. The patients with ischemic stroke get a primary treatment of intravenous thrombolytic, recombinant tissue plasminogen activator (r-tPA) (Adams et al., 1993). However, r-tPA is given in a short therapeutic window of 4.5 h from stroke onset, making it challenging to correctly diagnose the disease in time during emergency (Adams et al., 1993; Davis and Donnan, 2009). Only 2–5 % of ischemic stroke patients actually receive r-tPA due to this time constraint (Miller et al., 2011). The recently introduced mechanical embolectomy or thrombectomy (clot retrieval technique) can only be applied for cases of large vessel stroke up to 24 h of symptoms onset (Nogueira et al., 2018). Further, there is no clinically approved biomarker for stroke that can determine the risk of stroke in a healthy individual, differentiate it from hemorrhagic stroke or stroke-mimetics in the emergency, or differentiate between sub-types of ischemic stroke, or predict disease prognosis or treatment outcome after stroke. Until now, no cerebro-protective therapy has been successful in the clinical trial (Dhir et al., 2020). Hence,

* Corresponding author at: Laboratory of Translational Neuroscience, Division of Neuroscience, Yenepoya Research Center, Yenepoya (Deemed to be University), University Road, Deralakatte, Mangalore, Karnataka 575018, India.
E-mail addresses: arnabdatta@yenepoya.edu.in, arnabdattaju@gmail.com (A. Datta).

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there is an urgent need for novel approaches to discover new biomarkers for clinical validation and druggable targets for therapeutic intervention.

Of note, the majority of the successful drug-targets such as enzymes, ion channels, transporters, or receptors are protein in nature. The structural and functional diversity of the proteins coupled with a wide dynamic range of concentrations that can change in a spatiotemporal manner in tissues and cells can only be captured using proteomics approaches. This is not amenable to other omics approaches such as metabolomics, lipidomics, transcriptomics, or genomics. In the post-genomic era, with the discovery of mass spectrometry as a viable tool to identify and quantify proteins in complex biological samples, several laboratories started to apply protein mass spectrometry (herein called as proteomics) on different preclinical models of stroke. An attempt was made to collate and summarize the evolution of proteomics studies on animal models of stroke in the last few decades (Hochrainer and Yang, 2022; Li et al., 2019, 2022; Montaner et al., 2020). These reviews had used a narrative and broad approach that include clinical and in vitro proteomics studies or multi-omics approaches such as genomics, metabolomics, and transcriptomics. The hemorrhagic stroke and partial and global ischemic stroke models were also included among the in vivo studies. However, these reviews did not use a systematic way of collecting or reporting data as suggested by the Animal Research: Reporting of In Vivo Experiments (ARRIVE), Collaborative Approach to Meta-Analysis and Review of Animal Experimental Studies (CAMARADES) or Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) criteria (Macleod et al., 2004; Page et al., 2021; Percie du Sert et al., 2020). Not surprisingly, several key studies were omitted due to a selection bias as a wealth of available research data from discovery proteomics studies remained obscured or subjected to a preferential use. Due to the accumulation of huge amounts of omics data on various preclinical stroke models, there is an urgent need to devise a standardized methodology for data integration and meta-analysis of systems-level data. Unless there is a systematic review, strategies or guidelines for meta-analysis of systems-level omics data cannot be envisaged.

Herein, we have conducted a systematic review of all proteomics studies that were performed on the in vivo models of ischemic stroke (herein referred as ivProt studies) following standard guidelines such as PRISMA, ARRIVE, and CAMARADES. We have focused on ischemic stroke because the majority of the stroke cases are ischemic in nature. We have classified the proteomics studies on multiple accounts, manually curated deregulated subset of the ischemic proteome from brain tissue and body fluids, and compared their regulation with bed-side samples to propose a consolidated list of potential biomarkers and druggable targets called as "*Deregulated Proteomics Signature of Ischemic Stroke*" (DEPSIS). We have performed cheminformatics and bioinformatics analysis on DEPSIS and finally summarized the general challenges and drawbacks of these studies and identified research gaps for future studies.

2. Methods

2.1. Literature searches

Five reviewers (A.G., M.B., D.A.T, A.Ga, R.S., and J. J.) independently searched the PubMed and Web of Science databases first on 08-May-2022 and finally on 05-Mar-2024. The search encompassed studies conducted between 1979 and 2024. The search strategy was established using a combination of standardized MeSH (Medical Subject Headings) terms and keywords from relevant literature. The databases were searched by combining three categories of terms (i.e., disease-related, technique-related, and in vivo model-related) that were connected with search-engine-specific Boolean operators. The disease-related keywords are ischemic stroke, cerebral ischemia, cerebral ischaemia, brain ischemia, brain ischaemia, brain stroke, cerebral infarction, cerebral infarctions. The technique-related keywords are proteomics, neuroproteomics, quantitative proteomics, mass spectrometry, protein mass spectrometry, systems biology, Isobaric tag for relative and absolute quantitation (iTRAQ), Tandam Mass Tags (TMT), label-free, stable isotope labeling in mammals, laser-capture microdissection, MALDI imaging, data-dependent acquisition, data-independent acquisition, selected reaction monitoring, multiple reaction monitoring, parallel reaction monitoring, targeted proteomics. The in vivo model-related keywords are in vivo, middle cerebral artery occlusion model (MCAO), middle cerebral artery occlusion/ reperfusion (MCAO/R), photothrombic, endothelin-1, $FeCl_3$, ferric chloride, embolic, transient ischemia, permanent ischemia. Supplementary Table 1 contains an exact list of the terms with Boolean operators, the number of hits, and the date of the search for various databases. The information was manually retrieved, curated, and confusions were clarified with a sixth reviewer (A.D.).

2.2. Inclusion and exclusion criteria

All studies that used protein mass spectrometry on any in vivo models of focal ischemic stroke have been included. Four-vessel occlusion, a model of global cerebral ischemia and bilateral common carotid artery occlusion, a model of chronic cerebral hypoperfusion were excluded. The articles where protein mass spectrometry was not applied directly to stroke-induced brain or body fluid samples (CSF, plasma, and serum) were excluded. E.g., six articles were excluded where mass spectrometry was used for testing pharmacokinetic parameters of drugs to be tested on MCAO model. Studies involving protein arrays or DNA aptamers were excluded. Lipidomics, metabolomics, metabonomics, secretomics, and transcriptomics studies on the in vivo model were also excluded. Clinical/Case-cohort studies and review articles were not included. Full-text articles in English were only included (Fig. 1).

2.3. Study quality assessment

The methodological quality of the selected animal studies was assessed using a 15-point scale for 15 criteria derived from ARRIVE and CAMARADES guidelines with suitable modifications (Supplementary Figure 1A) (Percie du Sert et al., 2020). A score of 1 was given if the publication met the criteria, 0 if unclear, and 'NA' if not reported. E.g., if the names of the experimental groups and the number of animals per group are specified, then a score of 1 was given for the variable - 'study design'. If group details are mentioned without mentioning number of animals per group, 0 was assigned. If no details are available, then 'NA' was used.

2.4. Data extraction

For expression proteomics studies, the relative quantification information (i.e., ratios) of the altered proteins above the author-defined regulation threshold in the disease samples (i.e., ischemic stroke) compared to control samples (i.e., sham) was retrieved from the main text. For temporal proteomics studies, deregulated proteins from all time points were considered. The proteomics data for the treated or intervention group were excluded. However, the intervention details were collected and classified with a summary in the results section highlighting the interventions that were tested in the clinical trials as registered in the online database of clinical research studies (https://clinicaltrials.gov/).

The protein expression data from tissue samples and body fluid samples of in vivo models were processed separately. The regulation or ratio data were converted to categorical (i.e., up for increased regulation and down for decreased regulation) variables, and the interpretation was focused on the direction of deregulation and not on the magnitude of deregulation. The proteins that the respective authors found most important in their studies (e.g., those mentioned in the abstract or

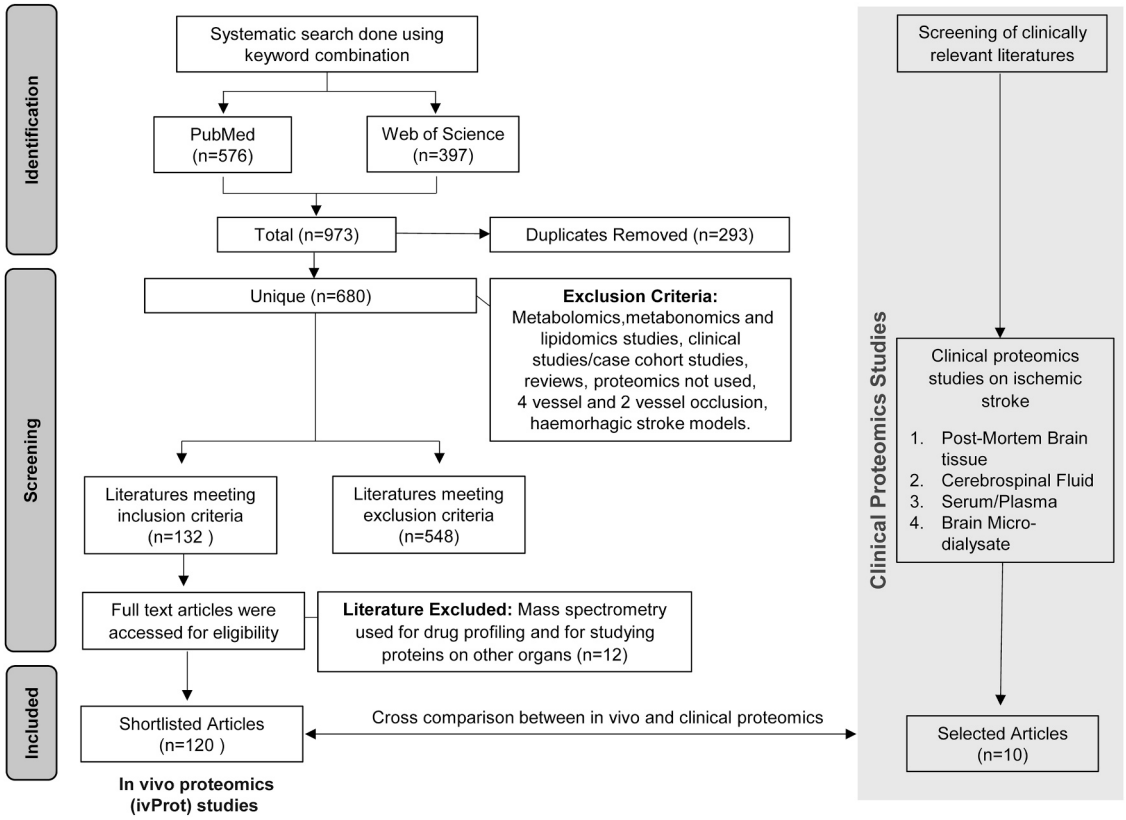


Fig. 1. PRISMA Flow Diagram showing the strategy of systematic review of literature. The combinations of keywords are available in Supplementary Table 1.

conclusion or verified by immunoblot or RT-PCR) and were identified and quantified by mass spectrometry were preferred. Any protein candidate not directly obtained from mass spectrometric measurements and studied as a follow-up target was not included. The relative quantification details of proteins that are not available in the main text and available only in the supplementary information (SI) were not used. This analysis across multiple studies was done only for the MCAO model (i.e., in Table 1) as other in vivo models such as endothelin-1, ferric chloride-induced cerebral thrombus, and photothrombic models had insufficient numbers of ivProt studies for performing manual data integration. For functional proteomics studies, the details of the proteomics technique, the names of the target protein, and their interacting partners were retrieved.

In the Table 1 and related text, when the protein names were retrieved from the shortlisted ivProt studies involving rat, mice, and monkey, it was observed that rat was the most commonly used species (73 %). Hence, all protein names were represented using NCBI official gene symbols of rat (species: *Rattus norvegicus*) to maintain uniformity. In the source article, if the protein was presented in an abbreviated form or by any other names or if rat gene symbol is not available or reviewed in the NCBI Gene or the UniProt databases, it was converted to mouse gene symbol (species: *Mus musculus*) as mentioned in the NCBI Gene. If the evidence was collected from clinical samples for cross-comparison with preclinical data (i.e., in Table 2), human gene symbols (species: *Homo sapiens*) were used. Human gene symbols were used for subsequent cheminformatics and bioinformatics analysis (i.e., Table 3, Fig. 4) as databases such as Therapeutic Target Database (TTD) and DrugBank primarily deals with human data.

2.5. Cheminformatics and bioinformatics analysis

2.5.1. TTD and DrugBank

Human gene symbols were searched into the TTD (https://idrblab.net/ttd/, RRID:SCR_006892) to retrieve target type, disease, and drug names (Zhou et al., 2024). The drug names obtained from TTD were searched in DrugBank (https://go.drugbank.com/, release version 5.1.13, RRID:SCR_002700) to retrieve information about disease, therapeutic categories, and mechanism of action (Knox et al., 2024). In case of discrepancy between two databases, entries from DrugBank were used.

2.5.2. Gene ontology analysis

The GO analysis was performed using DAVID (https://david.ncifcrf.gov/, RRID: SCR_001881, accessed on 04th Mar 2025). Official gene symbols (species: *Homo sapiens*) were used as input to perform GO analysis in the three categories; cellular component, biological process, and molecular function. A modified Fisher exact test (EASE score) was used to test the enrichment of annotation terms. Significance was considered when $P < 0.01$.

2.5.3. Protein–protein interaction (PPI) network analysis

The PPI network was constructed using STRING database (https://www.string-db.org/, RRID:SCR_005223) (Szklarczyk et al., 2017) and visualized using Cytoscape software (version 3.10.2, RRID:SCR_003032) (Shannon et al., 2003). The minimum required interaction score in STRING was set to 0.4 and *Homo sapiens* genome was used as a reference. The cytoHubba plugin (RRID:SCR_017677) was utilized to screen the top 10 genes in the PPI network for hub gene status using the degree method. The current network was constructed without additional nodes. PPI enrichment *P* value for the network was assigned based on the possibility that there are more interactions between the input list of proteins than by chance when compared to a random set of proteins.

Table 1
Deregulated proteins identified in multiple independent ivProt studies using expression proteomics approach.

Gene Symbol	Protein Name	Model Type (Study Count)	Sex (Study Count)	Occlusion/ Reperfusion (h/ h)	Location/ Regulation
Group I: Consistent Regulation – Matched Variables					
Dpysl2	Dihydropyrimidinase-Like 2	pM(7)	M(7)	24/0	C(↑)
Hspd1	Heat Shock Protein Family D (Hsp60) Member 1	pM(7)	M(6), F(1)	24/0	C(↑)
Pdxp	Pyridoxal Phosphatase	pM(2)	M(2)	24/0	C(↑)
Ahcy	Adenosylhomocysteinase	pM(8)	M(8)	24/0	C(↓)
Ak1	Adenylate Kinase 1	pM(2)	M(2)	24/0	C(↓)
Apoa1	Apolipoprotein A1	pM(4)	M(4)	24/0	C(↓)
Dnm1l	Dynamin 1-Like	pM(3)	M(3)	24/0	C(↓)
Eif4a2	Eukaryotic Translation Initiation Factor 4A2	pM(3)	M(3)	24/0	C(↓)
Eno2	Enolase 2	pM(5)	M(5)	24/0	C(↓)
Gapdh	Glyceraldehyde−3-Phosphate Dehydrogenase	pM(3)	M(3)	24/0	C(↓)
Hpca	Hippocalcin	pM(7)	M(6), F(1)	24/0	C(↓)
Idh1	Isocitrate Dehydrogenase (Nadp(+)) 1	pM(6)	M(6)	24/0	C(↓)
Idh3a	Isocitrate Dehydrogenase (Nad(+)) 3 Catalytic Subunit Alpha	pM(3)	M(3)	24/0	C(↓)
Pea15	Proliferation And Apoptosis Adaptor Protein 15	pM(6)	M(5), F(1)	24/0	C(↓)
Pkm	Pyruvate Kinase M1/2	pM(3)	M(3)	24/0	C(↓)
Ppp2r2b	Protein Phosphatase 2, Regulatory Subunit B, Beta	pM(4)	M(4)	24/0	C(↓)
Prdx2	Peroxiredoxin 2	pM(4)	M(4)	24/0	C(↓)
Pvalb	Parvalbumin	pM(5)	M(4), F(1)	24/0	C(↓)
Txn1	Thioredoxin 1	pM(3)	M(3)	24/0	C(↓)
Uchl1	Ubiquitin C-Terminal Hydrolase L1	pM(6)	M(6)	24/0	C(↓)
Group II: Consistent Regulation – Unmatched Variables					
Aco2	Aconitase 2	tM(2)	M(1), M&F(1)	0.5/48, 1/24	C(↓), IH(↓)
Alb	Albumin	tM(6)	M(6)	2/4, 2/12, 1/24, 1.5/24, 1.5/ 24, 2/24	IH(↑), C(↑), C(↑), IH(↑), C (↑), IH(↑)
Aldoc	Aldolase, Fructose-Bisphosphate C	tM(3), pM(1)	M(4)	1.5/24, 0.5/48, 2/48, 96/0	IH(↑), C(↑), C(↑), C(↑)
Anxa5	Annexin A5	tM(3)	M(3)	1.5/72, 1.5/336, 4/672	PI(↑), PI(↑), IH(↑)
C3	Complement C3	tM(3)	M(3)	2/4, 1.5/24, 2/24	IH(↑), H(↑), IH(↑)
Ctsb	Cathepsin B	tM(2)	M(2)	2/168, 2/336	C(↑), C(↑)
Dpysl2	Dihydropyrimidinase-Like 2	tM(1), pM(1)	M(2)	1.5/24, 24/0	IH(↑), S(↑)
Eno1	Enolase 1	tM(1), pM(1)	M(2)	1.5/24, 3/0	IH(↓), H(↓)
Eno2	Enolase 2	tM(1), pM(1)	M&F(1), M(1)	1/24, 24/0	IH(↓), S(↓)
Gap43	Growth Associated Protein 43	pM(1), tM(2)	M(3)	2/0, 2/4, 2/24	IH(↑), IH(↑), IH(↑)
Gfap	Glial Fibrillary Acidic Protein	tM(4)	M(4)	2/24, 2/24, 2/168, 2/336	IH(↑), C(↑), C(↑), C(↑)
Hspa1b	Heat Shock Protein Family A (Hsp70) Member 1B	tM(3)	M(3)	2/24, 2/168, 2/336	C(↑), C(↑), C(↑)
Hspd1	Heat Shock Protein Family D (Hsp60) Member 1	tM(1), pM(1)	M(2)	1.5/24, 24/0	IH(↑), S(↑)
Mdh1	Malate Dehydrogenase 1	tM(3)	M&F(2), M(1)	1/24, 1/24, 1.5/24	IH(↓), IH(↓), IH(↓)
Mdh2	Malate Dehydrogenase 2	tM(2)	M(2)	1.5/24, 2/48	IH(↑), C(↑)
P4hb	Prolyl 4-Hydroxylase Subunit Beta	tM(3)	M(3)	1/24, 0.5/48, 2/48	C and S(↑), C(↑), C(↑)
Pgam1	Phosphoglycerate Mutase 1	pM(1), tM(1), pM(1)	M(3)	2/0, 2/24, 96/0	IH(↑), IH(↑), C(↑)
Pgk1	Phosphoglycerate Kinase 1	pM(1), tM(3), pM(1)	M(6)	2/0, 2/4, 2/24, 2/48, 96/0	IH(↑), IH(↑), IH(↑), C(↑), C (↑)
Ppp2ca	Protein Phosphatase 2 Catalytic Subunit Alpha	tM(2)	M(2)	0.5/48, 2/48	C(↑), C(↑)
Sptan1	Spectrin, Alpha, Non-Erythrocytic 1	pM(1), tM(3)	M(4)	24/0, 2/12, 0.5/48, 2/48	C(↑), C(↑), C(↑), C(↑)
Stxbp1	Syntaxin Binding Protein 1	tM(2)	NA(2)	1/6, 1/20	IH(↓), IH(↓)
Syn2	Synapsin II	tM(2)	M(2)	1/24, 2/48	C(↑), C(↑)
Tf	Transferrin	tM(3)	M(3)	2/4, 1.5/24, 2/24	IH(↑), H(↑), IH(↑)
Tnfrsf11b	Tnf Receptor Superfamily Member 11B	tM(3)	NA(3)	1/3, 1/6, 1/20	IH(↑), IH(↑), IH(↑)
Tpi1	Triosephosphate Isomerase 1	pM(2)	M(2)	2/0, 96/0	IH(↑), C(↑)
Tpm3	Tropomyosin 3	pM(1), tM(2)	M(3)	2/0, 2/4, 2/24	IH(↑), IH(↑), IH(↑)
Uchl1	Ubiquitin C-Terminal Hydrolase L1	pM(1), tM(2)	M(3)	2/0, 2/4, 2/24	IH(↑), IH(↑), IH(↑)
Vim	Vimentin	tM(4)	M(4)	2/24, 2/24, 2/168, 2/336	IH(↑), C(↑), C(↑), C(↑)

* Seventy-six independent ivProt studies have been curated to shortlist unique proteins deregulated in more than one study. Study count indicates the number of times a protein was quantified in independent time-points in one or more ivProt studies. The reperfusion duration for permanent MCAO (i.e., pM) is shown as '0'. tM, tMCAO; pM, pMCAO; M, Male; F, Female; C, Cortex; IH, Ipsilateral Hemisphere; PI, Peri infarct; H, Hippocampus; S, Striatum; NA, not available. An extended html version of the table is available at Deregulated_Brain_Tissue_Proteome.

3. Results

3.1. Study selection and characteristics

We filtered a comprehensive set of 680 studies from PubMed and Web of Science databases through a targeted search on 05-Mar-2024 using a predefined combination of keywords (Supplementary Table 1). Based on the exclusion criteria, 120 (17 %) articles were shortlisted and denoted as "in vivo proteomics" (ivProt) studies (Fig. 1, extended version: **ivProt_PRISMA**).

The ivProt studies used a standardized workflow, where the animal model was established by arterial occlusion in control or comorbid conditions such as diabetes and hypertension (e.g., spontaneously hypertensive rats). Subsequently, the in vivo model underwent characterization to confirm the successful induction of ischemic stroke (herein called pre-proteomics model validation). Next, a variety of protein mass spectrometric methods was applied on target samples such as brain tissue, plasma or serum, cerebral microdialysate, or CSF. Post-proteomics experiments generally include extensive in silico data mining using tools of bioinformatics or computational biology. The objective of this in silico analysis was to shortlist candidates for experimental validation of observed expression or function in preclinical models or

Table 2
Consensus candidates between ivProt and clinical proteomics studies showing consistent trends of regulation*.

Protein	Clinical proteomics			ivProt Studies		
	Sample; Disease/Location	Sampling time (PMI/time from index event)	Regulation	Sample, Model Type	Sampling time (h)	Regulation
ATP5B	PM brain infarct; P, PL	Median PMI 3.5 h	Down	C, tM	1/24	Down
DLAT	PM brain infarct; P, PL	Median PMI 3.5 h	Down	IH, tM	1/24	Down
DPYSL2	IC, PI; TACI	PMI 6 h	Down	C, tM	1.5/3	Down
ENO1	IC, PI; TACI	PMI 6 h	Down	IH, tM	1.5/24	Down
GANAB	PI; TACI	PMI 6 h	Down	C, tM	1.5/3	Down
GAPDH	IC, PI; TACI	PMI 6 h	Down	IH, tM	1.5/24	Down
IDH3A	IC; TACI	PMI 6 h	Down	C, pM	24/0	Down
PDHB	PM brain infarct; T, PL	Median PMI 3.5 h	Down	C, tM	1.5/3	Down
VDAC2	PM brain infarct; P, PL	Median PMI 3.5 h	Down	IH, tM	1.5/24	Down
A2M	Pla; LACI	Median Delay: 42 Days	Up	Pla, pM	1.5/24	Up
ALB	IC, PI; TACI	PMI 6 h	Up	IH, tM	2/4	Up
ANXA1	PM brain infarct; P, T, PL	Median PMI 3.5 h	Up	H, tM	1.5/24	Up
ANXA2	PM brain infarct; T, PL	Median PMI 3.5 h	Up	H, tM	1.5/24	Up
ANXA5	PM brain infarct; T, PL	Median PMI 3.5 h	Up	PI, tM	1.5/72	Up
APOA1	IC, PI; TACI	PMI 6 h	Up	C, pM	24/0	Up
ARHGDIA	IC; TACI	PMI 6 h	Up	C, pM	24/0	Up
C3	IC, PI; TACI	PMI 6 h	Up	IH, tM	2/4	Up
CLU	Ser; AIS	Within 24 h	Up	H, tM	1.5/24	Up
DLG4	Ser; AIS	NA	Up	C, tM	1.5/7.5	Up
FGA	Pla; LACI	Median Delay: 42 Days	Up	H, tM	1.5/24	Up
FGB	IC; TACI	PMI 6 h	Up	H, tM	1.5/24	Up
FGG	Pla; LACI	Median Delay: 42 Days	Up	H, tM	1.5/24	Up
GFAP	IC; TACI	PMI 6 h	Up	IH, tM	2/24	Up
HBB	IC, PI; TACI	PMI 6 h	Up	IH, pM	2/0	Up
HP	IC, PI; TACI	PMI 6 h	Up	NA, tM	2/48	Up
ITIH4	Pla; LACI	Median Delay: 42 Days	Up	H, tM	1.5/24	Up
MBP	PM brain infarct; P, PL	PMI 6 h	Up	C, tM	2/72	Up
NSF	IC, PI; TACI	PMI 6 h	Up	C, pM	96/0	Up
PLG	Pla; LACI	Median Delay: 42 Days	Up	H, tM	1.5/24	Up
PRDX1	CRM; MMI	After 24 h	Up	C, tM	2/24	Up
RAB1A	IC; AIS	Median PMI 5.5 h	Up	Ser, pM	2/0	Up
TF	IC; TACI	PMI 6 h	Up	IH, tM	2/4	Up
VIM	PM brain infarct; T, PL	Median PMI 3.5 h	Up	IH, tM	2/24	Up

* An extended html version of the table is available at **Bench-to-Bedside validation**. IC, Infarct core; PI, Peri-infarct; TACI, Total anterior circulation infarcts; T, Thalamus; PL, Parietal lobe; P, Putamen; CRM, Cerebral microdialysate; MMI, Malignant MCA infarction; PMI, Post Mortem Interval; AIS, Acute ischemic stroke; LACI, Lacunar infarction; Ser, Serum; Pla, Plasma; IH, Hemisphere; C, Cortex; H, Hippocampus; tM, tMCAO; pM, pMCAO; NA, not available.

comparable clinical samples using techniques such as immunoblot, ELISA, and immunohistochemistry (Fig. 2A).

The interventions can be classified into four major categories such as novel chemical entities, novel biological entities, genetic manipulations, and others that include treadmill training, hypothermia, electro-acupuncture etc. (Supplementary Table 2**)**. Among all tested interventions in animal models, 14 interventions belonging to chemical entity or other categories have undergone one or more clinical trials. Treadmill training ($n = 210$) and recombinant tissue plasminogen activator ($n = 179$) are the two most popular interventions tested in the clinical trials **(**Supplementary Figure 2**)**.

3.2. Quality assessment

The quality of the shortlisted studies was moderate (score range, 5–15 on a 15-point scale, average score, 9 ± 2). During animal group allocation, about 69 % and 29 % of the ivProt studies reported randomization and blinding respectively. No study reported the details of sample size calculation (Supplementary Figure 1B).

3.3. ivProt studies: animal models

We have classified the ivProt studies based on the etiology of ischemic stroke per TOAST classification (Adams et al., 1993) and based on the duration of occlusion, reperfusion, and location of the sampled brain tissue for proteomics experiment (Fig. 2B-C). Clinically, a large portion of ischemic stroke originates in the large and small arteries of the middle cerebral artery (MCA) territory. Not surprisingly, the majority of ivProt studies (97 %) were focused on MCA-stroke models such as transient-MCA occlusion (with reperfusion, tMCAO) (Schanbacher et al., 2022) or permanent-MCA occlusion (without reperfusion, pMCAO) (Jiang et al., 2022), distal-hypoxia with MCAO (DH-MCAO) (Gu et al., 2021), and distal-MCAO (dMCAO) (Pan et al., 2017). The occlusion of the MCA is carried out by an intraluminal suture ligation technique to mimic large-artery atherosclerosis (LAA) (Datta et al., 2011). Another method where cardioembolic (CE) stroke was generated using an autologous venous blood clot advanced into the nearby intraluminal space to precisely occlude the MCA-M1 segment (Song et al., 2021). In another method, a filter paper soaked in 20 % ferric chloride solution was wrapped around common carotid artery for 5 min to produce intra-arterial thrombus that was pushed towards intracranial circulation by micro-forceps to create thromboembolic stroke (Yao et al., 2024). Vasoconstrictor peptide endothelin-1 is applied on the MCA surface after craniotomy to induce ischemia and recapitulate cerebral small vessel disease (SVD) (Zgavc et al., 2013). Additional information on ivProt animal models is available in the **SI Results**.

We next classified the ivProt studies into three groups based on the combined duration of occlusion and reperfusion (Fig. 2B). The ivProt studies were subdivided into acute studies when the study duration is up to 12 h (i.e., group-1) and further, group-2 (subacute) and group-3 (chronic) contained studies with a total duration that varies between 12 and 48 h and more than 48 h respectively. Among the brain locations, different parts of basal ganglia (such as striatum and substantia nigra), cerebral cortex, hippocampus, cerebellum, and the whole ischemic hemisphere (i.e. ipsilateral hemisphere) were sampled for the proteomics experiment.

The majority of the ivProt studies were performed on the LAA model MCAO (97 %) while SVD models and CE/TE models (3 %) were largely

Table 3
Protein targets from DEPSIS listed in the TTD and DrugBank Databases*.

Gene Symbol	Target Type	Drug Name	Disease	Mechanism of Action
ALB	ST	Abiraterone	Metastatic castration-resistant prostate cancer	Steroid 17-alpha-hydroxylase/17,20 lyase inhibitor
PLG	ST	Anistreplase	Myocardial infarction and Pulmonary emboli	Plasminogen activator
TTR	ST	1-Naphthylamine−5-sulfonic acid	NA	NA
C1S	ST	Sutimlimab	Cold agglutinin disease (CAD)	Complement C1s subcomponent-Inhibitor Antibody
GANAB	ST	Miglitol	Diabetes mellitus type 2	Lysosomal alpha-glucosidase antagonist
ANXA1	SDT	Difluprednate	Ocular pain	Glucocorticoid receptor agonist
C3	SDT	Pegcetacoplan	Paroxysmal nocturnal haemoglobinuria	Complement C3 inhibitor
DPYSL2	SDT	Lacosamide	Convulsion; Epilepsy, Diabetic neuropathy	Slow inactivation of voltage-gated sodium channel
IDH1	SDT	Ivosidenib	Cholangiocarcinoma; Acute myeloid leukaemia	Isocitrate dehydrogenase [NADP] cytoplasmic inhibitor
IDH3A	SDT	Enasidenib	Acute myelogenous leukaemia; Glioma	Isocitrate dehydrogenase [NADP], mitochondrial inhibitor
PRKCE	SDT	Meprobamate	Anxiety disorder; Malaria	Gamma-aminobutyric acid receptor subunit alpha−1 agonist
PRKCG	SDT	Midostaurin	Acute myeloid leukaemia; Systemic mastocytosis	Protein kinase C alpha type antagonist
SERPINA1	SDT	Pentetic acid	Emphysema; Alpha−1 antitrypsin deficiency	Transuranium elements chelators
MBP	CTT	Tiplimotide	Multiple Sclerosis	MBP Binder
HP	CTT	Zinc chloride	Zinc Deficiency syndromes	B1 bradykinin receptor agonist
GAPDH	CTT	Artenimol	*Plasmodium falciparum* infections	Actin, cytoplasmic 2 ligand
APOE	CTT	Zinc chloride	Zinc Deficiency syndromes	B1 bradykinin receptor agonist
APOA1	CTT	Copper	Occipital Horn Syndrome and Menke's disease	Amyloid-beta precursor protein binder
ALDH2	CTT	Amyl Nitrite	Angina	Atrial natriuretic peptide receptor 1 agonist
ANXA5	CTT	Copper	Occipital Horn Syndrome and Menke's disease	Amyloid-beta precursor protein binder
HSPD1	CTT	Copper	Occipital Horn Syndrome and Menke's disease	Amyloid-beta precursor protein binder
PKM	CTT	Artenimol	*Plasmodium falciparum* infections	Actin, cytoplasmic 2 ligand
HBB	CTT	Copper	Occipital Horn Syndrome and Menke's disease	Amyloid-beta precursor protein binder
CPB2	CTT	Prothrombin	Acute major bleeding	Fibrinogen alpha chain cleavage
PPP2CA	CTT	Vitamin E	Cardiovascular diseases, Diabetes mellitus	NA

* An extended html version of the table containing preclinical and literature-reported targets is available at **TTD & DrugBank Targets**. ST, successful target; SDT, successful drug target; CTT, clinical trial target; NA, not available.

underrepresented (Fig. 2B). Most ivProt studies were performed on pMCAO using subacute (12–48 h) time points compared to acute and chronic timepoints. Cerebral cortex was the most preferred location for proteomics sample preparation (Fig. 2C).

3.4. ivProt studies: pre-proteomics model validation

The animal models in ivProt studies were validated based on various predefined parameters determined through a set of "quality control" assays, referred to as pre-proteomics assays. These are classified into two categories: (1) non-terminal methods such as MRI or CT, laser Doppler imaging, or neurological scoring, and (2) terminal methods such as histological staining using TTC or H&E, TUNEL staining, and molecular biological assays such as immunoblot or RT-PCR. Details and compilation of the assays are presented in the **SI Results** and **ivProt_Preproteomics** respectively.

3.5. ivProt studies: proteomics methodologies

The ivProt studies can be broadly classified into two categories based on proteomics approaches: 1) expression proteomics which aims to identify one or more deregulated proteins from the tested samples by identifying and/or relatively quantifying the proteome, 2) functional proteomics which aims to discover the interacting partners of one or more pathologically important proteins (Fig. 3**).** The methodological details of the expression and functional proteomics are presented in the **SI Results**. An overwhelming majority of the ivProt studies (97 %) had used an expression proteomics approach. There were three primary objectives of the expression proteomics studies: 1) Mechanistic: studying the disease mechanism to identify potential therapeutic targets or biomarkers for further exploration (Datta et al., 2011), 2) Interventional: testing the efficacy of a physical (e.g., hypothermia (Zgavc et al., 2013)), mechanical (e.g., treadmill training (Mizutani et al., 2010)), chemical, or biological (e.g., small extracellular vesicles (Xia et al., 2021)) interventions, 3) proof-of-concept studies on previously identified protein targets. For example, label-free proteomics was done on different groups of mice brain samples following overexpression of ERK2 and its inhibitor Raf-kinase inhibitor protein to determine the beneficial or deleterious role of ERK1 and ERK2 in tMCAO (Schanbacher et al., 2022). Interventional (62 %) studies where various chemical entities of natural or synthetic origin were tested as potential therapy, were the most common methodological subclass among ivProt studies (Fig. 3).

3.6. ivProt studies: results - consensus deregulated proteome

A total of 288 unique deregulated proteins were manually extracted from the 76 MCAO expression proteomics studies done on tissue samples, 44 of which were reported in at least two studies. Out of these 44 targets, three proteins (i.e., Dpysl2, Hspd1, and Uchl1) were reported in ten independent studies each. These proteins were classified into two groups based on the model type, duration of occlusion-reperfusion, brain location, and the direction of regulation (Table 1**,** an extended and downloadable version is available here, **Deregulated_Brain_Tissue_Proteome**). Group I consists of the protein candidates that showed uniform regulation in multiple studies performed with identical stroke models (permanent MCAO), occlusion period (24 h), and location of sampled brain tissue (ischemic cortex). Key glycolytic and TCA cycle enzymes (e.g., Eno2, Gapdh, Pkm, Idh1, Idh3a), and redox homeostasis-related proteins (e.g., Prdx2, Txn1) were downregulated, while proteins associated with neurogenesis (e.g., Dpysl2) and chaperonic response (e.g., Hspd1) were upregulated. The proteins in group II showed identical regulation (i.e., up or down-regulation) even when key experimental variables such as model type (e.g., Aldoc, Gap43), occlusion and reperfusion duration (e.g., Gfap, Syn2), and brain location (e.g., P4hb, Tf) were not matched between independent studies. Compared to group I, proteins from group II may be more robust as potential therapeutic target. These candidates can be used as investigational markers for future studies done on the MCAO model.

A relatively few expression proteomics studies (n = 6, Fig. 2**C**)

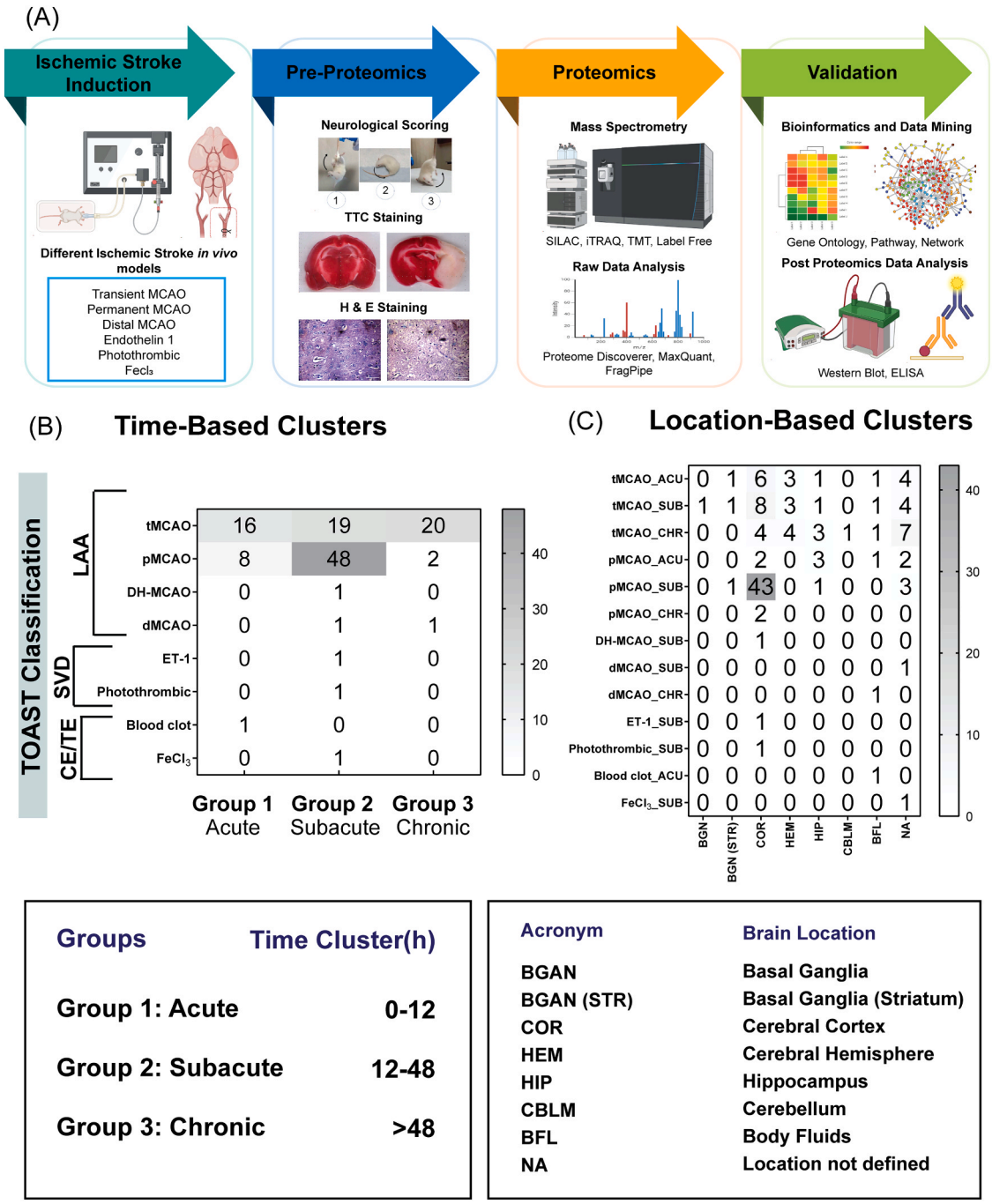


Fig. 2. (A) Experimental workflow of ivProt studies. (B) Classification of 120 ivProt studies based on sampling time into 3 groups (x-axis) and TOAST subtypes (y-axis). (C) Classification of ivProt studies based on sampled brain locations (x-axis) and TOAST subtype with combined time-based group information. In the case of tMCAO, occlusion and reperfusion durations were added. Studies that failed to report sampled brain location were marked as "NA".

performed proteomics analysis of body fluids on various in vivo models short listing 27 deregulated protein candidates in plasma, serum, or CSF from the eligible three studies (Supplementary Table 3A). Monomeric transthyretin was elevated in CSF (MCAO/R - 2/8 h) (Suzuyama et al., 2004). However, after 24 h of reperfusion, this protein was reduced in abundance in plasma when compared to baseline or 90 min occlusion condition. Most other proteins such as A2m, Itih3, C3, Alb, and Hp were elevated in plasma after 24 h of MCAO (Chen et al., 2011).

The functional proteomics studies identified the interacting partners of proteins such as Serpina3n (Zhang et al., 2022), Prkce (Feng et al., 2013), and Prkcg (Zhang et al., 2011) (Supplementary Table 3B). Incidentally, out of the interacting partners of Prkcg and Prkce, twelve (Aco2, Dpysl2, Eno1, Eno2, Gapdh, Hspd1, Idh3A, Pgk1, Pkm, Syn2, Tpi1, Uchl1) proteins were among the most commonly observed deregulated proteins found in the animal brain (Table 1).

3.7. *ivProt studies: bench-to-bedside validation*

To estimate how in vivo studies mimic clinical samples on a proteome-wide scale, we compared the deregulated ischemic brain (n = 288) and body fluid (n = 27) proteome with the altered proteome (n = 166) retrieved from a set of randomly selected ten clinical proteomics studies done on serum, plasma, and post-mortem human brain samples. These ten studies were used in our previous systematic review

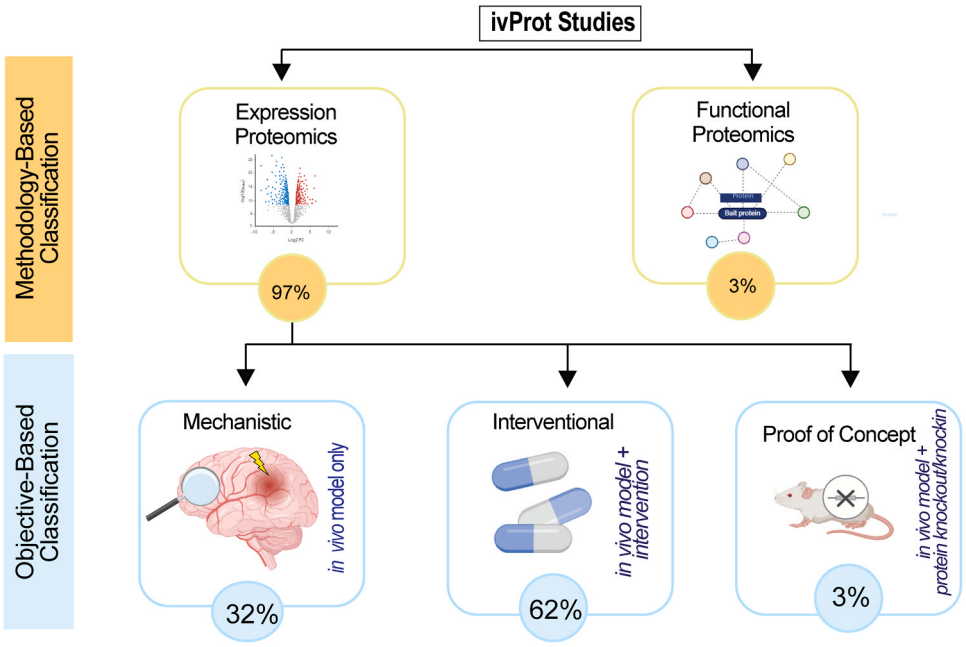


Fig. 3. Methodology and Objective-based classification of ivProt studies.

for comparing proteomics data obtained from in vitro oxygen-glucose deprivation model and clinical samples (Babu et al., 2022). Among the commonly present 39 proteins (Supplementary Table 4, Bench-to-Bedside validation), 85 % (i.e., 33) demonstrated identical patterns of regulation between in vivo samples and clinical specimens (Table 2). This was 20 % for a similar comparison between in vitro and clinical proteomics data. CLU, PRDX1, ANXA5, and VIM showed increased abundance in in vitro and in vivo models, and in clinical samples. These candidates could be tested in clinical trials as potential biomarkers on eligible subsets of ischemic stroke patients. Overall, proteomics changes observed in the animal brain and body fluids are largely similar to human stroke. Not surprisingly, in vivo model showed greater overlap with clinical samples (i.e., 85 % vs. 20 %) compared to in vitro model.

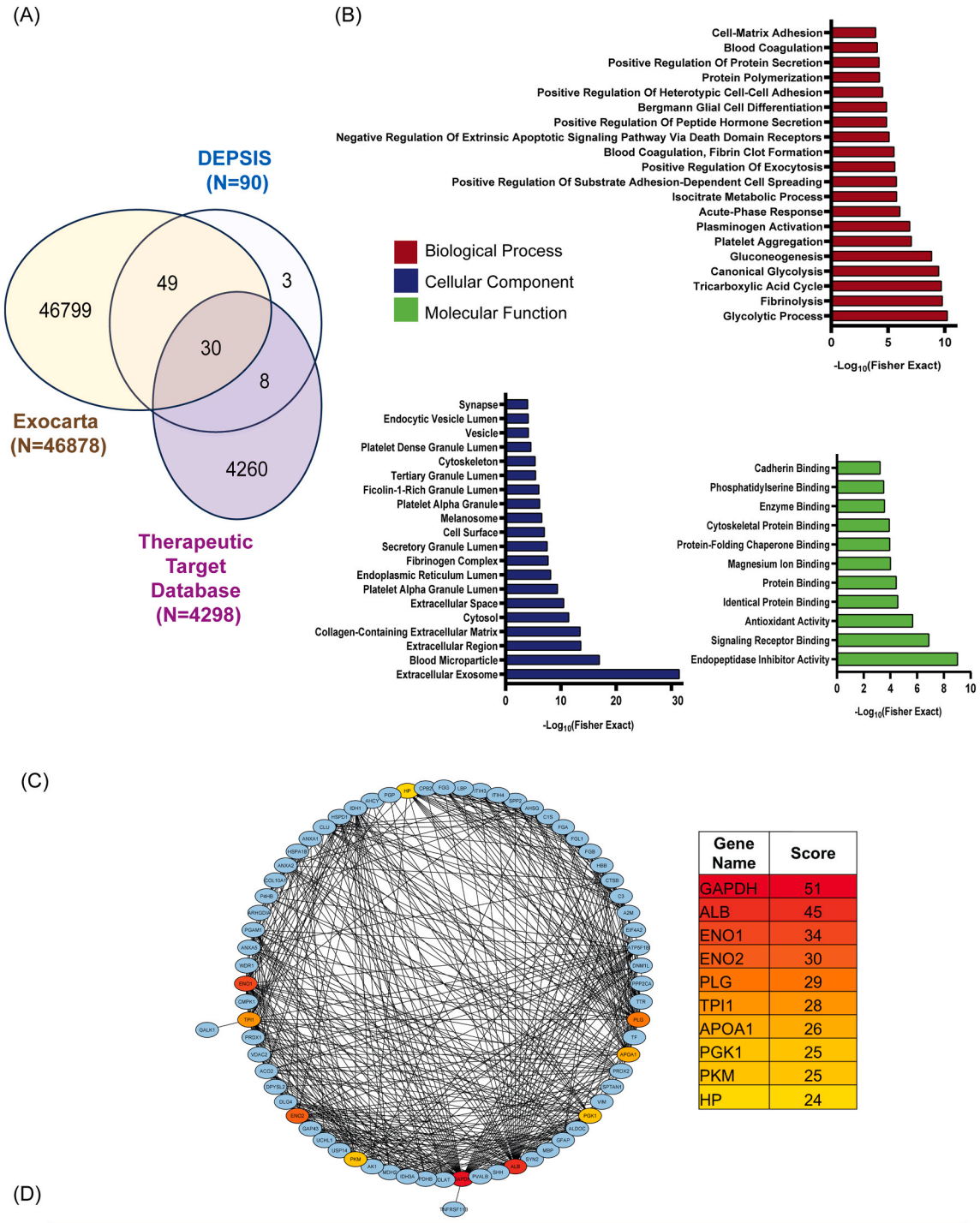
3.8. *ivProt studies: cheminformatics and bioinformatics analysis*

One of the major objectives of this systematic review is to present a consolidated list of candidate biomarkers for planning preclinical proof-of-concept or clinical validation studies on specific protein targets. We obtained 90 unique proteins by combining proteins from Tables 1–2, and Supplementary Table 3A-B and termed these proteins as DEPSIS (ivProt_Study_Design). About 42 % (n = 38) proteins from DEPSIS were present in the TTD consisting of various types of targets, such as clinical trial targets, literature-reported targets, patented-recorded targets, and successful targets (Zhou et al., 2024) (Fig. 4A). The names of the approved or investigational or experimental drugs for these targets along with corresponding diseases and the mechanisms of action were retrieved from DrugBank and TTD and presented in Supplementary Table 5 (TTD & DrugBank targets). Out of these protein targets, 13 were listed as successful targets or successful drug targets while 12 were listed as targets that are undergoing clinical trials (Table 3).

Different enriched annotation terms under GO biological process were attributed to various aspects of blood coagulation such as platelet aggregation, fibrin clot formation, plasminogen activation, and fibrinolysis. These proteins were FGA, FGB, FGG, CPB2, FGL1, ANXA2, ANXA5, HBB, PGK1, PLG, SHH, STXBP1. Notably, extracellular exosome, extracellular region, and secretory granule lumen were among the most enriched cellular component terms (Fig. 4B). Indeed, 79 out of 90 (88 %) proteins were listed in the ExoCarta database (Keerthikumar et al., 2016) (Fig. 4A). This may suggest a yet undefined role of endolysosomal secretory pathway or autophagy in ischemic stroke. The PPI network was also constructed using STRING for DEPSIS and visualized in Cytoscape to identify common interaction pathways. The PPI network showed a total of 86 nodes, 560 edges, and a significant PPI enrichment *P value*< 1.0 E-16 (Fig. 4C). Top hub proteins (GAPDH, ALB, ENO1, ENO2, PLG, TPI1, APOA1, PGK1, PKM, and HP) are proteins with a high degree of connectivity within the network. They are represented with deeper colors and higher scores. Notably, seven out of ten hub proteins were also featured as "associated proteins" in enriched KEGG pathway terms such as HIF-1 signaling pathway, complement and coagulation cascades, and glycolysis/gluconeogenesis, and pyruvate metabolism (Fig. 4D). These pathways are known to play an important role in the pathological evolution of ischemic stroke (Salaudeen et al., 2024). Moreover, five out of the top ten hub proteins (GAPDH, ALB, PLG, APOA1, HP) were deregulated in clinical proteomics studies (Table 2) and also identified as drug targets in TTD and DrugBank (Table 3).

4. Discussion

In the current review, following PRISMA criteria, we had started with 680 unique articles and shortlisted 120 of them that were classified in two different ways based on 1) study duration and location of sampled brain tissue and 2) proteomics methodology and study objectives (Fig. 2B-C, and Fig. 3). By manually curating these articles, we fetched deregulated candidates in the brain tissue and body fluid of ischemic stroke-affected animals, enlisted the interacting partners of three proteins in the MCAO model, and finally shortlisted 90 consensus proteins (i.e., DEPSIS). These proteins were mapped with TTD, DrugBank, and Exocarta databases and had undergone GO, KEGG pathway, and PPI network analysis. Although major part of the DEPSIS was detected from brain samples of MCAO-affected animals, these proteins showed a disproportionately high overlap with the ExoCarta database which is a compendium of exosome-specific proteins (88 %). Moreover, about 80 % of the proteins that are common between TTD and DEPSIS were also present in the Exocarta database (Fig. 4A), suggesting the importance of secretory pathway and autophagy in the pathophysiology and druggable target discovery for ischemic stroke. Indeed, we have observed a similar enrichment of endolysosomal proteins in the perturbed proteome of medium-sized extracellular vesicles in the plasma of



KEGG Pathway	Gene Count	FDR	Associated Proteins
Carbon metabolism	15	4.46E-15	ACO2,**TPI1**,DLAT,IDH3A,PDHB,PKM,MDH2,PGP,PGAM1,**PGK1**,ALDOC,**GAPDH**,IDH1,**ENO2**,**ENO1**
Biosynthesis of amino acids	11	1.30E-11	ACO2,**TPI1**,IDH3A,**PKM**,PGAM1,**PGK1**,ALDOC,**GAPDH**,IDH1,ENO2,**ENO1**
Glycolysis / Gluconeogenesis	10	9.01E-11	**TPI1**,DLAT,PDHB,**PKM**,PGAM1,**PGK1**,ALDOC,**GAPDH**,ENO2,**ENO1**
Complement and coagulation cascades	9	1.78E-08	CPB2,C3,FGB,**PLG**,CLU,A2M,FGG,C1S,FGA
HIF-1 signaling pathway	8	1.46E-06	PRKCG,PDHB,**PGK1**,ALDOC,**GAPDH**,TF,**ENO2**,**ENO1**
Metabolic pathways	23	1.86E-06	PDXP,ACO2,AHCY,AK1,GALK1,**TPI1**,GDA,NPL,ATP5F1B,DLAT,IDH3A,PDHB,PKM,MDH2,PGP,GANAB,PGAM1,PGK1,ALDOC,**GAPDH**,IDH1,ENO2,**ENO1**
Pyruvate metabolism	4	0.0011	DLAT,PDHB,**PKM**,MDH2

Fig. 4. Cheminformatics and Bioinformatics analysis of DEPSIS (A) Representative Venn diagram showing the consensus proteins between DEPSIS, ExoCarta, and TTD database (B) GO analysis of DEPSIS was performed using DAVID. The threshold was set as 3 for generating functional annotation chart (EASE= 0.01). (C) The PPI network was generated using STRING and visualized in Cytoscape. The hub genes were identified using colored nodes from PPI network using Cytohubba plugin in Cytoscape. The color-coded table presents the hub genes and their scores. (D) Enriched KEGG pathways containing the hub proteins from the PPI network. Proteins in bold represent the hub proteins identified in the enriched KEGG pathway. DEPSIS, Deregulated Proteomics Signature of Ischemic Stroke.

lacunar stroke patients (Datta et al., 2022).

One of the follow-up queries from our results would be how to select one or more target proteins from DEPSIS as a potential therapeutic or prophylactic target for drug discovery. We have to consider sampling time (acute or recovery phase), brain location (core or penumbra), source cell type, and subcellular location (cell surface or deep structures) of the protein target. Care should be exercised, as functional role of a specific protein or pathway (e.g., NMDA, MMP, JNK-signaling) can be biphasic depending on the sampling time and may shift from deleterious to protective or vice versa during the evolution of stroke pathology (Lo, 2008). Keeping in mind the target accessibility during drug delivery, a protein predominantly present in the exposed components of the blood-brain barrier (BBB) (i.e., vascular endothelial cell surface) may be preferred rather than a target present deep inside the brain parenchyma and across the BBB. It may be preferable to select the most deregulated protein with experimentally solved (i.e. using NMR, X-Ray crystallography or cryo-electron microscopy) or predicted structure (i.e., using artificial intelligence tools such as AlphaFold) rather than targeting a candidate from the dark proteome that is neither structured nor predicted to be disordered (Porta-Pardo et al., 2022). This will allow drug-target structural studies experimentally or by in silico docking and molecular dynamics simulation. The presence of the protein target in any of the known biomolecular classes of drug targets such as enzymes, transporter, ion channels, and receptors could be advantageous. In pharmacological terms, antagonists are generally more common as successful drugs than agonists. Once all the above parameters are considered, targeting the highly upregulated proteins for proof-of-concept studies using genetic (e.g., CRISPR-Cas9) or pharmacological approaches and developing a specific agonist or antagonist could constitute a reasonable strategy for mechanism-based therapeutic target validation and drug discovery. On the contrary, if the potential target is a downregulated enzyme (e.g., ENO1), supplementation with the enzymatic product (i.e., phosphoenolpyruvate) can alleviate the ischemic injury (Jiang et al., 2019).

One of the successful drug targets listed in DEPSIS is neurite outgrowth-promoting protein dihydropyrimidinase-like 2 (DPYSL2), also known as collapsin response mediator protein-2. This protein is targeted by the marketed anti-epileptic drug, lacosamide which has exhibited safety and efficacy in post-stroke non-convulsant status epilepticus (Belcastro et al., 2013). Lacosamide penetrates BBB and can bind DPYSL2. It selectively enhances slow inactivation of voltage-gated sodium channels without affecting fast inactivation (Rogawski et al., 2015). This indicates that the other drugs mentioned in Table 3 and Supplementary Table 5 may possibly have a beneficial role in different subsets of stroke patients. These drugs can be tested in clinical trials for therapeutic repurposing in the future.

4.1. Drawbacks and challenges

We came across several drawbacks and challenges in the ivProt studies encompassing study design, methodology, data reporting, and data interpretation. Some studies even failed to report complete details of experimental animals (e.g., strain, sex, age, weight), details of care taken after survival stroke surgery, or the composition of protein solubilisation buffer. Only a few studies have sampled deep brain structures such as hippocampus, substantia nigra, or basal ganglia for proteomics experiment (Fig. 2C). About 18 % of the ivProt studies failed to report the exact location of the brain sampled for the proteomics experiment (NA in Fig. 2C). Among the studies that reported location, several have utilized the whole ipsilateral hemisphere for proteomics sample preparation. This might lead to the pooling of the proteomics responses between distinct anatomical locations possibly providing different expression data when compared to individual substructures of the ischemic hemisphere. One study employing laser capture microdissection followed by multiple reaction monitoring on 4 h pMCAO model showed that the expression of only specific proteins such as Mbp, Ina, Plp1, Map2, Cnp, and Nefl are site-specific while Gfap, Mapt, Nefh, Tubb3, Eno2, and Uchl1 are evenly distributed among the sampled brain areas (Lian et al., 2015). Earlier, in a study involving female Japanese patients, we observed spatial variation of protein expression (e.g., SYN1, SIRT2, MBP) in post-mortem samples between adjacent infarcts in putamen, thalamus, and parietal lobe when compared to location-matched control specimens (Datta et al., 2013).

Several studies have not included sham group in the study design. Instead, comparable part of the contralateral hemisphere has been used as control for calculating relative protein expression ratios following proteomics experiment. Earlier clinical studies on strictly unilateral hemispheric infarction and animal studies on unilateral MCAO showed adaptive changes in the cerebral blood flow, electrical activity, metabolism, and gene and protein expression of the contralateral hemisphere (Filippenkov et al., 2023; Lagrèze et al., 1987). This phenomenon is called as transhemispheric diaschisis whose underlying mechanism is incompletely understood (Andrews, 1991). Hence, contralateral hemisphere may not be the ideal control and shouldn't be deemed as 'normal' tissue or a replacement of sham group.

To study post-stroke BBB damage, vasogenic edema, and inflammation, it is essential to flush out the blood from the brain vasculature by transcardiac perfusion prior to the proteomics sample preparation. This is to rule out the interference of high abundance plasma proteins when the proteins are extracted from the target brain tissue. Due to transcardiac perfusion, we could earlier detect a progressive BBB damage by sampling the ipsilateral hemisphere at different time-points (0, 4, 24 h) following 2 h MCAO showing a progressive accumulation of several plasma proteins (e.g., Alb, Tf, C3, Hpx) in the ipsilateral parenchyma (Datta et al., 2011). Unfortunately, whether the animals undergo transcardiac perfusion before tissue sampling is not explicitly mentioned in most ivProt studies. If transcardiac perfusion is not performed, it may confound the proteomics data interpretation due to the contamination of the brain proteome with the plasma proteome.

In a majority of studies, the most deregulated proteins were taken for post-proteomics validation and targeted proof-of-concept experiments. This reductionist workflow leads to the omission of the unchanged proteins. It's often not clear if a particular unlisted protein is not detected due to technical reasons or detected without showing deregulation. This will effectively exclude the possibility of performing a global integration of multiple independent datasets that are otherwise matched by demographic (e.g., age, sex, species) and experimental variables (e.g., model, time point, brain location). Only a few studies (n = 10, 8 %) have deposited raw data in open-source databases such as Proteomics IDEntification database (PRIDE), synapse, and Mass spectrometry Interactive Virtual Environment (MassIVE) (Supplementary Table 6). Due to the unavailability of the raw data, the chance of reanalyzing the data for retrieving the unchanged proteome and other biological information is permanently scrapped.

The authors have presented the protein names, gene symbols, or UniProt accession numbers in an inconsistent manner in several studies. E.g., in studies done on rat, UniProt accession numbers of mouse and human are used in the texts and tables. The protein names are sometimes abbreviated (e.g., dihydroprimidinase-related protein 2 as DRP-2) making them identical with gene symbol of a different protein (i.e., dystrophin related protein 2). Isoform or subunit information is often missing for quantified proteins. E.g., protein phosphatase 2A can be present as catalytic subunit alpha (Ppp2ca, P63331) or beta (Ppp2cb, P62716) or regulatory subunit B, beta (Ppp2r2b, P36877). Hence, presenting protein phosphatase 2A only without specific gene symbol or accession number can lead to annotation issues during meta-analysis or targeted follow-up experiments that needs specific antibody.

Finally, a particular protein is considered as differentially expressed if the ratio value (disease/control) is beyond a given fold change cut-off (cut-off generally varies from 1.2 to 2-fold in linear scale or 0.26–1 in $\log_2$ scale) together with a significant *P value* (generally <0.05). The threshold of deregulation should be study-specific and calculated to

cover up biological, experimental or technical variations between replicate samples or groups (Datta et al., 2014). However, in a majority of the studies, the fold change cut-off was not calculated from study-specific parameters. Rather they were adopted arbitrarily from other studies done by the same or other groups where a similar cut-off was used. It is possible that if the cut-off is re-estimated based on inherent variability of individual datasets, the list of deregulated proteins could change resulting in a different interpretation of the same data.

4.2. Limitations of systematic review

First, we did not include penumbra in the keyword combination as penumbra is not a fixed anatomical construct and can vary based on model variables such as model type, duration of occlusion and reperfusion, animal species, age, and sex. There is no consistent method to identify penumbra in animal brain following ischemic stroke unlike in the clinic. A recently published systematic review focusing only on the proteome of ischemic penumbra presented results of 16 ivProt studies, which can be complementary to our systematic review (Moxon et al., 2024). Secondly, we could not discuss the proteomics results of individual articles or perform a systematic review of multiple omics approaches together due to space limitation. At last, we did not perform data handling from the supplementary files or reanalyzed proteomics raw data from selected studies because the major part of these data constitutes the unchanged proteome in the respective studies.

4.3. Future recommendations

In the past two decades, proteomics has been widely used to study different animal models of ischemic stroke. Although SVD and CE/TE strokes together constitute about 50 % of all ischemic stroke and vascular dementia cases, the ivProt studies on SVD and CE/TE strokes remain underrepresented (<3 %). Future ivProt studies targeting SVD and CE/TE strokes will determine the proteomics similarities and differences between LAA and other TOAST subtypes that may pave the way for subtype-specific biomarkers or treatment protocols. Further, proteomics studies on newly developing animal models of thrombectomy will be useful to understand the tissue or clot composition (Zgavc et al., 2013).

Similar to in vivo stroke studies, there is a sex-bias seen in ivProt studies as young male animals are mostly used. Although males tend to experience a higher incidence of stroke throughout much of their lifespan, the burden of stroke is elevated among aged females. Hence, female and aged animals should be used for future ivProt studies as recommended by the STAIR guidelines (Fisher et al., 2009). Rodent brains are lissencephalic unlike primate or human brains that are gyrencephalic. Considering the higher brain complexity and homology to humans, ivProt studies on non-human primates should be promoted. A small number of ivProt studies (6 %) have used advanced imaging techniques on whole animal brains. These studies provide a unique correlation of the imaging parameters such as apparent diffusion coefficient and diffusion-perfusion mismatch with the proteome landscape. More studies correlating real-time imaging data and proteomic perturbations are needed. The use of tMCAO (46 %) was roughly equal compared to pMCAO (48 %) although tMCAO represents only 2.5–11.3 % of LAA (McBride and Zhang, 2017). In pMCAO, the therapeutic agent cannot reach to the core of the ischemic tissue. Hence, studies showing favorable outcomes on tMCAO model with a particular intervention may be misleading for future clinical trials where most of the recruited patients might represent cases of pMCAO.

On the technical front, compared to changes in the whole proteome, ivProt studies targeting various post-translational modifications such as phosphorylation, glycosylation, and acetylation remain largely underexplored (2 %). No ivProt studies applied single-cell proteomics approaches or MALDI-imaging. No studies with data-independent acquisition could be found. Among expression proteomics approaches, metabolic labeling in animal models (i.e., SILAM) of stroke has not been tried. Manual or laser-capture microdissection (Lian et al., 2015) when combined with advanced proteomics methodologies can provide better spatial resolution by focusing on specific substructures of the brain.

On the data analysis front, depositing raw protein and peptide identification and quantification data on databases such as PRIDE, MASSIVE, or Synapse and sharing the processed and analyzed data with all study variables as easily accessible web resources (e.g., **ivProt_Repository**) should be encouraged. This will assist finding, downloading, sharing, and reanalyzing big data. This in turn will create novel pipelines and methodologies for meta-analyses through multi-omics data integration using artificial intelligence and machine learning-based approaches such as Bayes' theorem and artificial neural networks. We have recently shown the successful application of Bayesian modeling (a form of supervised learning) to identify protein targets responsible for the cerebroprotective action of naringin during neuronal oxygen-glucose deprivation (Babu et al., 2024), and decipher GPCR signaling (Chen et al., 2022). Computational systems biology modeling has been used to formulate personalized treatment paradigms, and predict treatment outcomes of individual stroke patients using omics studies (Li et al., 2024).

5. Conclusions

The deregulated proteins identified in this systematic review can undergo clinical validation as potential biomarkers in suitable cohorts of ischemic stroke patients. These proteins can be tested through proof-of-concept studies to evaluate their potential as therapeutic targets. The research gaps identified through this review will encourage the stroke community to embark on interdisciplinary studies to identify mechanism-based novel therapeutic targets and biomarkers for ischemic stroke.

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CRediT authorship contribution statement

Conceptualization and Study Design: AD; Methodology: AD, AG; Data collection and analysis: AG, MB, A.Ga, DAT, RS, JJ, AD; Preparation of tables, figures, web resources: AG, MB, AD, A.Ga, DAT, RS; Writing - Original Draft: AD, AG; Writing – review & editing: AD, AG, PT; Approval of final manuscript: All authors.

Declaration of Competing Interest

The authors declare no conflict of interest.

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Supplementary materials

A web resource containing additional data with references and extended versions of the supplementary tables in easily downloadable format is available at **ivProt_Repository**.

Conflicts of interest

None

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.arr.2025.102937.

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