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In Vitro Oxygen Glucose Deprivation Model of Ischemic Stroke: A Proteomics-Driven Systems Biological Perspective

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Abstract

Oxygen glucose deprivation (OGD) of brain cells is the commonest in vitro model of ischemic stroke that is used extensively for basic and preclinical stroke research. Protein mass spectrometry is one of the most promising and rapidly evolving technologies in biomedical research. A systems-level understanding of cell-type-specific responses to oxygen and glucose deprivation without systemic influence is a prerequisite to delineate the response of the neurovascular unit following ischemic stroke. In this systematic review, we summarize the proteomics studies done on different OGD models. These studies have followed an expression or interaction proteomics approach. They have been primarily used to understand the cellular pathophysiology of ischemia-reperfusion injury or to assess the efficacy of interventions as potential treatment options. We compile the limitations of OGD model and downstream proteomics experiment. We further show that despite having limitations, several proteins shortlisted as altered in in vitro OGD-proteomics studies showed comparable regulation in ischemic stroke patients. This showcases the translational potential of this approach for therapeutic target and biomarker discovery. We next discuss the approaches that can be adopted for cell-type-specific validation of OGD-proteomics results in the future. Finally, we briefly present the research questions that can be addressed by OGD-proteomics studies using emerging techniques of protein mass spectrometry. We have also created a web resource compiling information from OGD-proteomics studies to facilitate data sharing for community usage. This review intends to encourage preclinical stroke community to adopt a hypothesis-free proteomics approach to understand cell-type-specific responses following ischemic stroke.

Keywords Cerebral ischemia · Proteomics · Hypoxia · Mass spectrometry · Cell-type-specific response · Clinical proteomics

Abbreviations

OGD	Oxygen glucose deprivation
CSF	Cerebrospinal fluid
MALDI	Matrix-assisted laser desorption ionization
2D-GE	Two-dimensional gel electrophoresis
ICAT	Isotope-coded affinity tag
iTRAQ	Isobaric tags for relative and absolute quantitation
TMT	Tandem mass tags
MPT	Mitochondrial permeability transition
ROS	Reactive oxygen species
EV	Extracellular vesicles
PMI	Post mortem interval
LCM	Laser-capture microdissection
PRM	Parallel reaction monitoring
SRM	Single reaction monitoring
MRM	Multiple reaction monitoring

Common abbreviations such as DMEM, MEM, HBSS, BSS, FBS, MAPK, MTT, LDH, RT-PCR, ELISA, siRNA, miRNA, and CRISPR-Cas9 are not included. In vitro OGD-proteomics studies have been done on cells of human and non-human origin. To designate proteins irrespective of the origin, non-italicized gene symbols in uppercase (corresponds to human origin) are used.

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Introduction

Oxygen glucose deprivation (OGD) of brain cells is used commonly to recapitulate the cellular pathophysiology of ischemic stroke in a simple in vitro system. It was first introduced by Goldberg and Choi in 1993 when mixed

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neocortical cultures were exposed to oxygen and glucose deprivation and reperfusion for various time intervals that showed cell-type-specific differences in mechanisms and injury severity between neurons and glial cells [1]. The in vitro OGD model can be easily adapted to various experimental conditions to recapitulate different cellular facets of ischemic stroke pathology in a reproducible manner without systemic influence. Being an in vitro model, it was faster, easier to establish compared to in vivo models, and cheaper, making it the in vitro model of choice for basic or translational stroke research [2]. Not surprisingly, since inception, many groups started to use this in vitro OGD model routinely in cultured cell lines, primary brain cells, or organotypic slice cultures to report findings that advanced the basic and preclinical stroke research for the next few decades. Most of these studies used a hypothesis-driven approach and focused on one or a few genes or proteins while trying to explain their role in the evolution of ischemic stroke pathology. These studies are grossly classified as mechanistic studies. In parallel, the second set of studies tried to determine the in vitro efficacy of various interventions, such as synthetic chemical, phytochemical, or biological entities to propose potential therapeutic options for ischemic stroke (herein called efficacy studies).

At the beginning of the new millennium, proteomics emerged as a new discipline when Fenn and Tanaka won the Nobel prize in chemistry on 2002 for the development of soft desorption ionization methods, i.e., electrospray ionization and matrix-assisted laser desorption ionization (MALDI), for mass spectrometric analyses of biological macromolecules [3, 4]. With the emergence and accelerated maturation of bioinformatics and computational biology, the larger discipline of systems biology was also taking shape as various omics technologies led to the generation of an enormous amount of biological data that needed advanced computational expertise and infrastructure for effective management (handling and storage) and interpretation [5]. All these omics technologies were conceived to investigate the system as a whole rather than targeting the "one gene-one protein-one function" paradigm. Among these omics approaches, proteomics deals with the functional unit of the cell, i.e., protein that constitutes the majority of the experimental and successful drug targets. Thus, for target discovery and hit-to-lead discovery, proteomics remains perhaps the most attractive option among all omics technologies.

On the other hand, repeated failures of investigational molecules for stroke targeting one protein or pathway in the clinical trials necessitated a paradigm shift in the conceptual framework [6]. It has recently been appreciated that rather than monotherapy, combination therapies should be tested [7]. Single-target to multi-target drugs should be developed [8]. The involvement of neurovascular unit rather than neuron alone or an involvement of extra-cranial organs in the evolution of stroke (e.g., gut-brain [9] or muscle-brain axis [10]) and intercellular interaction between neuronal and non-neuronal cell-type made it imperative to study this disease at a systems level. The stroke research community embraced proteomics as it effectively complemented the hypothesis-driven approach that was the mainstay for experimental stroke research for the last few decades. A recent review on preclinical proteomics of ischemic stroke has tried to compile the proteomics studies done primarily on rodent stroke models [11]. Due to technical limitations, studies done on rodent stroke models by us [12] and others have reported pooled response of the stroke-affected tissue that may be different from the actual response of individual cell types that constitute that portion of the brain tissue. Understanding the cell-type-specific signaling in the penumbra and their contribution towards the overall response of the affected brain during the evolution of ischemic injury remains yet another unmet need for an effective translation of preclinical knowledge into the clinics.

In this systematic review, we have summarized the proteomics studies done on the most widely used in vitro model of ischemic stroke (OGD model). The objective is to provide the community with a roadmap about how to design similar studies to address relevant pathological questions in a cell-type-specific manner. We provide a brief introduction of the OGD model and compile the pre-proteomics assays routinely used to validate the OGD model and the mass spectrometric techniques that have been used to study the OGD model followed by key results obtained from these studies. We have also commented on various limitations and challenges of the current approach. Proteomics being a rapidly evolving discipline, we have mentioned the untapped areas that can be targeted during future OGD-proteomics studies using emerging tools of protein mass spectrometry.

Methods

Two databases PubMed and Scopus were searched by combining the following terms with search-engine specific Boolean operators: cerebral ischemia, stroke, ischemic stroke, penumbra, ischemic penumbra, brain ischemia, brain infarction, cerebral infarction, brain attack, brain stroke, proteomics, neuroproteomics, protein mass spectrometry, isobaric tags for relative and absolute quantitation (iTRAQ), stable isotope labeling in cell culture (SILAC), tandem mass tags (TMT), label-free, secretome profiling, two-dimensional electrophoresis, in vitro, oxygen glucose deprivation, OGD/R, oxygen deprivation, glucose deprivation, hypoxia, hypoxia/reoxygenation, and hypoxia/reperfusion. The exact list of keywords with Boolean operators along with the number of hits and date of searching for different databases are available in Supplemental Table 1. Two reviewers (M.B.,

N.K.) have performed the electronic search and subsequent screening independently that was verified by a third person (A.D.). EndNote X9.3.3 and Mendeley v1.19.8 were used as reference managers for storing and handling the publications. A small subset of clinical proteomics studies ($n = 10$) was selected for a comparative analysis of the in vitro and bedside data.

Inclusion and Exclusion Criteria

The articles where protein mass spectrometry was used on OGD with or without intervention, reperfusion, or reoxygenation were shortlisted (Fig. 1). Proteomics analysis of hypoxia and glucose-deprived samples were also included only when OGD was present as an experimental condition in the proteomics experiment.

The articles where a proteomics approach has been used on any other sample or conditions except the ones mentioned above are excluded. The articles that are not studying cultured cells or primary cells of brain origin or organotypic cerebral culture were excluded. Studies on circulating cells (e.g., monocytes, platelets), or immune cells (e.g., $CD8^+$ T cells), or cells of cardiac origin were not included. Studies focusing on other neurological disorders such as Alzheimer's disease or Parkinson's disease or transient ischemic attack were excluded. In vitro studies that model specific facets of cellular pathophysiology of ischemic stroke such as glutamate excitotoxicity or reactive oxygen species (ROS)-induced free radical stress or apoptosis using specific chemicals (e.g., glutamate, H_2O_2, sodium nitroprusside) are not reviewed. Chemical hypoxia or ischemia is not reviewed. Articles are included only when there is a full-text version available in English.

Results

The database searching using the predetermined keyword combination identified a total of 195 unique publications from PubMed and Scopus databases after removing the duplicates. Based on the inclusion and exclusion criteria, 48 (25%) hits were filtered for further analysis. Of these 48 articles, full-text screening shortlisted 12 (25%) articles that fulfilled the inclusion criteria. Herein, these studies will be referred to as OGD-proteomics studies (Fig. 1).

The OGD-proteomics studies generally followed a consistent design (Fig. 2) whereby an OGD model is developed using primary brain cells, secondary cell lines, or organotypic slice cultures that may or may not be manipulated (e.g., transfection) before OGD, optimized, and validated using a series of pre-proteomics assays to fix a specific condition or timepoint for subsequent experiments. Next, protein mass spectrometry was performed on the OGD model and matched control(s). The results were analyzed bioinformatically, and data was validated using various molecular biological or analytical techniques to propose one or more hypothesis that is followed up by suitable proof-of-concept

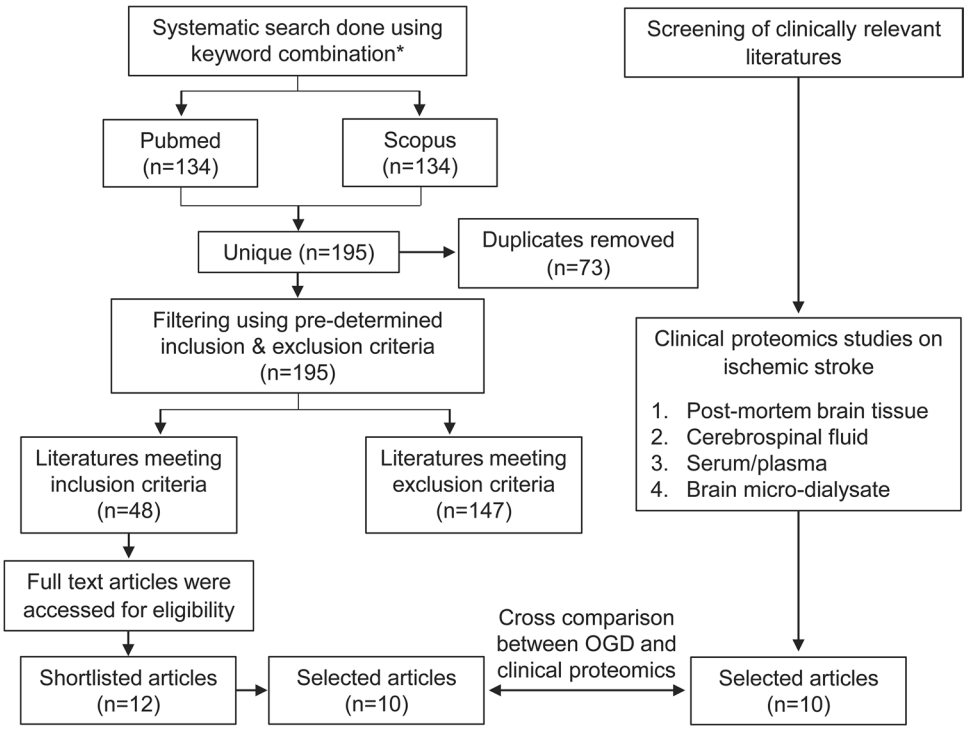

Fig. 1 Flowchart showing the strategy for systematic survey of literature. The keyword combinations can be found in Supplemental Table 1

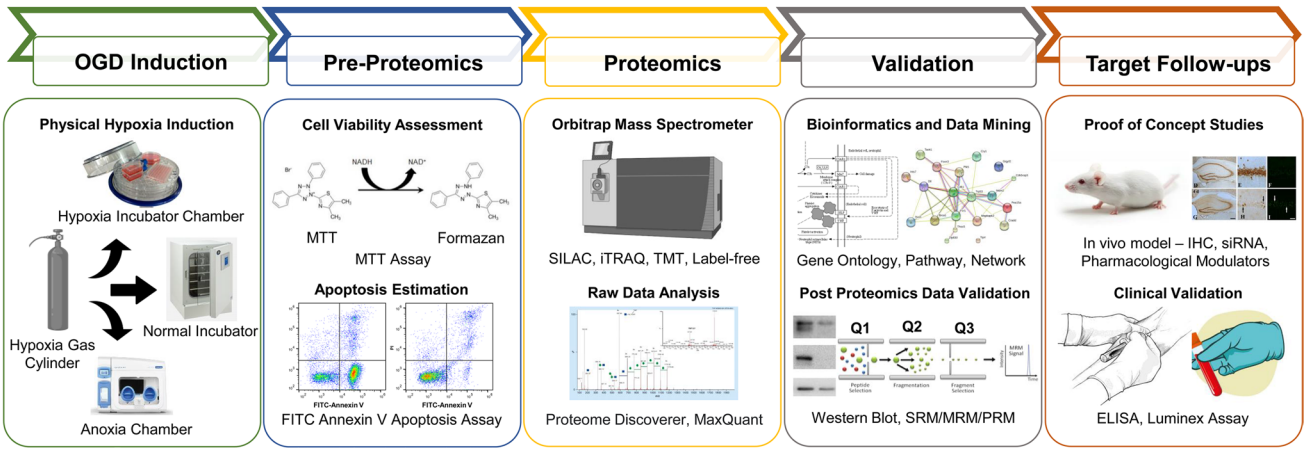

Fig. 2 Experimental design of OGD-proteomics studies

studies in rodent ischemic stroke models or is validated on patient samples of comparable pathology.

Oxygen–Glucose Deprivation: In Vitro Model of Ischemic Stroke

Brain cells, neurons, in particular, are exclusively dependent on the blood supply to sustain their oxygen and glucose demand [13]. Following the onset of ischemic stroke, blood supply is reduced below a critical level causing deprivation of oxygen and glucose to the affected brain tissue. To recapitulate this in a simple in vitro system and to define the intrinsic response of the cells devoid of systemic influence, in vitro OGD model was conceived [1].

There are various ways to induce physical hypoxia during OGD: (1) by purging a hypoxia chamber or anaerobic jar [14] with hypoxia gas, (2) using an incubator connected with hypoxia gas supply [15], or (3) using an anoxic or anaerobic chamber in presence of a palladium catalyst that removes oxygen by forming a water molecule [16, 17]. A flushed hypoxia chamber is the most commonly used method for the induction of hypoxia or OGD. Typically, the media is preequilibrated aseptically with the hypoxia gas. Different compositions of hypoxia gas have been reported, such as 95% N_2, 5% CO_2 [18] or 85% N_2, 10% H_2, 5% CO_2 [1, 17] or 90% N_2, 5% H_2, 5% CO_2 [19]. Oxygen (2%) has also been incorporated as a component in the hypoxic gas [15, 20]. The residual oxygen level in the ambient environment should be less than 3% (22.8 mm Hg) [21] or 2% [22] to consider it as a hypoxic environment.

The media generally contains low (e.g., 1% or 0.1%) or no serum to simulate reduced blood supply. The cells are exposed to a glucose-pyruvate-free [18] or low-glucose [23] culture media or balanced salt solution [17, 20]. Both primary (e.g., primary neuron, microglia, or astrocyte) and secondary (human, rat, or mouse cell lines such as SH-SY5Y, PC12, B104, N2a, IMR-32) cells have been used. Co-cultures (e.g., neuron-astrocyte), organotypic slice cultures, and acute slices of brain tissues [14] have also been utilized to partially recapitulate the complex multi-cellular environment typically studied through an in vivo model. Recently, three-dimensional cerebral organoids generated from induced pluripotent stem cells have been utilized to generate an OGD model [24]. For cultured cells, both undifferentiated and differentiated cells have been used. In general, the neuron is used most frequently among different brain cell-types. However, various non-neuronal cell types such as microglia [25], astrocytes [26], vascular endothelial cells [27, 28] have been used for generating OGD models. OGD has been used alone or in combination with re-oxygenation (OGD/R) to simulate ischemia and reperfusion injury, respectively. Variable durations of OGD (up to 72 h [29]) have been used depending on the objective of the experiment. The OGD could be intermittent or continuous.

The use of suitable controls is critical to differentiate the cellular proteomic response to OGD from only hypoxia, serum deprivation, and/or glucose deprivation. Most studies where a systems approach has been adopted used two different controls; i.e., hypoxia and low-serum/no-serum normoxic control. Cells incubated in normal growth media are used as the basal control [18]. Table 1 summarizes various parameters related to the OGD model as described in the OGD-proteomics studies. The majority of these are mechanistic studies.

Pre-proteomics Validation of OGD Model

Being hypothesis-free, proteomics studies of OGD models generally rely on developing a consistent disease model. The reproducibility of it is verified by a set of "*quality control*" assays, referred to as pre-proteomics assays, assays that are performed on the OGD model to select a condition

Table 1 Experimental parameters of hypoxia and glucose deprivation in OGD-proteomics studies

Sl. No	Cell type	Hypoxia Gas Composition[a]	Media Composition		Duration		Reference
			OGD	Normoxic Control	OGD	Reoxygenation	
1	Primary cortical neuron cultures	1	DMEM (w/o glucose)	DMEM (25 mM Glucose)	1.5 h	0.5 h	[15]
2	HT-22 cells	2	Neurobasal- A (w/o glucose)	DMEM (22 mM Glucose)	0, 4.5, 11, 17.5, 24 h	None	[23]
3	PC-12 cells	2	DMEM (w/o glucose) and 10% EV-depleted FBS	DMEM (high glucose, glucose conc. -NA)	3, 6, 12 h	None	[32]
4	Organotypic cerebral slice cultures	2	D-HBSS	DMEM (w/o serum high glucose)	1 h	7 days	[14]
5	Mouse EOC 2 microglial cells	3	EBSS (w/o glucose)	EBSS (10 mM glucose)	2 h	24, 48 h	[20]
6	Cerebral human microvascular endothelial cells	4	RPMI 1640 (w/o glucose), FBS and growth factors	RPMI 1640 (11.1 mM glucose, w/o FBS and growth factors)	6 h	None	[33]
7	EA.hy926 cells	2	HBSS (w/o glucose)	NA	1, 3, 6, 12, 24 h	None	[27]
8	SH-SY5Y cells	2	DMEM (w/o glucose) and FBS	DMEM (25 mM glucose)	3, 6, 9, 12, 18 h	None	[31]
9	B35 cells	5	BSS (w/o glucose)	DMEM (glucose conc. -NA)	6, 8 h	0–180 min	[17]
10	Primary astrocyte cells	6	DMEM/F12 (w/o glucose and w/o FBS)	DMEM/F12 (10% FBS)	1, 2, 3, 4 h	18 h	[34]
11	B104 cells	2	DMEM (w/o glucose-pyruvate) and 1% FBS	DMEM (25 mM glucose, 1 mM pyruvate, 10% serum)	2, 4, 6, and 8 h	None	[18]
12	Immortalized adult rat brain endothelial cells	NA	Media (w/o glucose and w/o serum)	1X BME amino acids, 1X BME vitamins, 0.5 g/L peptone, glucose (25 mM) and w/o serum	4 h	24 h	[28]

NA not available; *w/o* without; *BSS* Balanced Salt Solution; *EBSS* Earle's Balanced Salt Solution; *HBSS* Hank's Balanced Salt Solution; *DMEM* Dulbecco's Modified Eagle Medium; *BME* Basal Medium Eagle; *FBS* fetal bovine serum. [a]1: NA, 2% O_2; 2: 95% N_2 and 5% CO_2; 3: 95% N_2, 3% CO_2 and 2% O_2; 4: N_2, 5% CO_2 and 0.5% O_2; 5: 85% N_2, 10% H_2 and 5% CO_2; 6: 99.95% N_2 (30 min) followed by 5% CO_2 for N_2 (20 min)

or timepoint for subsequent proteomics experiment. These assays typically target biological processes, pathways or events, or specific proteins or genes that are already known to be affected in a predictable way following oxygen-glucose deprivation. A more specific objective is to determine a timepoint/condition that indicates the early or intermediate or late stage of the injury based on the results of these assays. In OGD-proteomics studies, a total of nineteen assays have been used. Figure 3 provides a pictorial compilation of some of the commonly used functional assays showing the subcellular location and mechanism behind each of these techniques (Supplemental Footnote 1) in a simplified brain cell. MTT assay followed by LDH assay is the two most frequently used techniques. A modified version of Fig. 3 along with the technical details and references is provided at https://yenepoya.res.in/database/LTN_Datta_Lab/OGD-Prot_Systematic-Review/.

Proteomics Methodologies of OGD-Proteomics Studies

The proteomics methodologies that are followed by functional characterization of the OGD model can be broadly classified into three categories; (1) expression proteomics, (2) interaction proteomics, and (3) structural proteomics. Detailed discussions about each of these methodologies are out of scope for this review and can be found elsewhere [30]. Until now, the majority of the OGD-proteomics studies have used either expression proteomics or an interaction proteomics approach. The methodological parameters of all

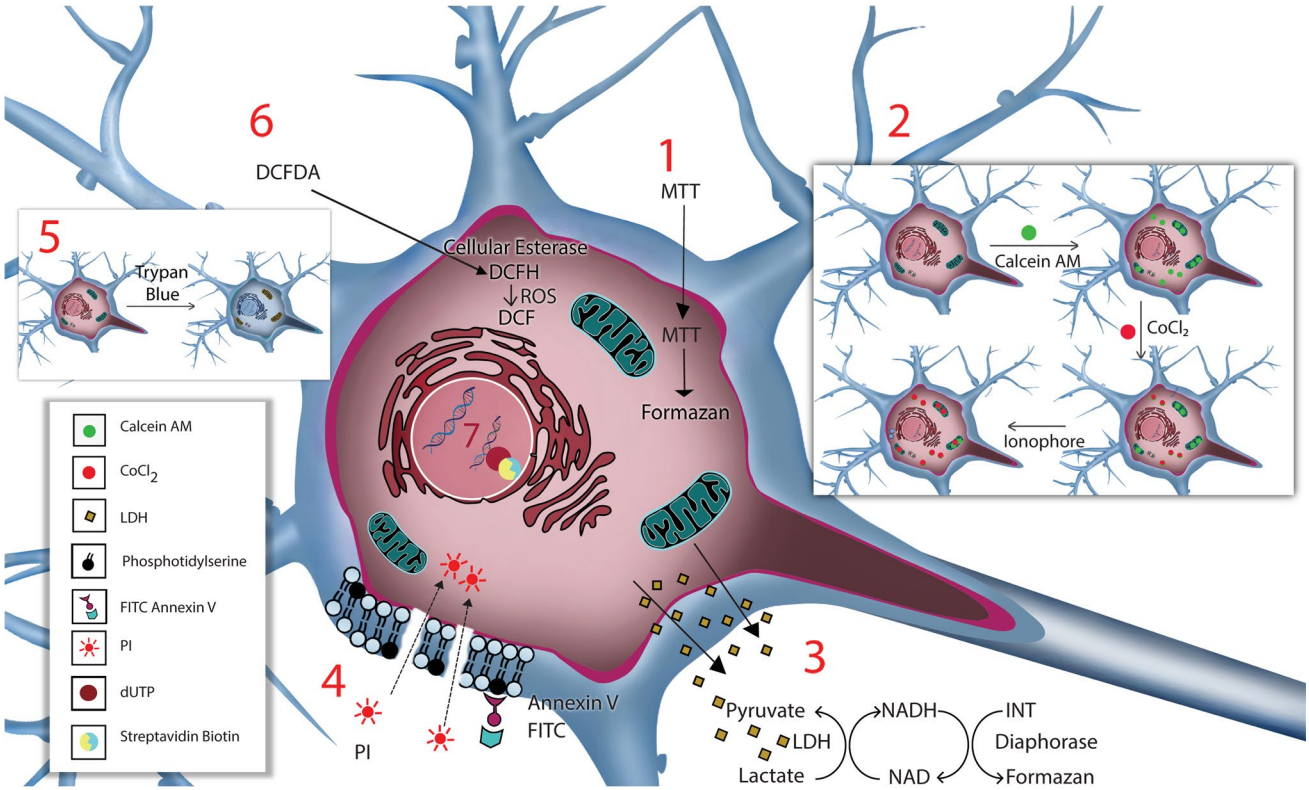

Fig. 3 Schematic diagram showing the subcellular locations and mechanisms of various pre-proteomics functional assays. (1) MTT assay, (2) MPT pore opening assay, (3) LDH assay, (4) FITC Annexin V-PI staining, (5) trypan blue dye exclusion, (6) DCFDA assay, (7) tunnel assay. The details of each of the assays can be found in Supplemental Information. A vector-graphic version is provided at https://yenepoya.res.in/database/LTN_Datta_Lab/OGD-Prot_Systematic-Review/ to aid in viewing details

OGD-proteomics studies are summarized in Supplemental Table 2 and are provided at https://yenepoya.res.in/database/LTN_Datta_Lab/OGD-Prot_Systematic-Review/OGD-Prot_Methodology_Results.htm.

Expression proteomics studies profiled OGD-treated cells or proteins secreted from the OGD-treated cells using gel-based or gel-free proteomics approaches that rely on label-free or chemical (e.g., isotope-coded affinity tag (ICAT), iTRAQ, TMT) or metabolic labeling (e.g., SILAC) techniques. In gel-free approaches, proteins are extracted from the test (i.e., OGD or OGD/R or OGD with treatment) and suitable control samples, reduced, alkylated, and digested in solution with trypsin to obtain peptides. In a label-free approach, each peptide sample is fractionated using a liquid chromatography column that is connected online with a mass spectrometer (1D-LC-MS/MS) [14, 23, 31]. Different samples are analyzed sequentially. In chemical labeling, equal quantities of peptides from different samples are labeled with isobaric tags and combined to obtain a single peptide sample. This subsequently undergoes two-dimensional liquid chromatographic separation (generally offline first and then online) followed by mass spectrometry (called 2D-LC-MS/MS) [18, 32]. In metabolic labeling, cells were grown separately in media containing either heavy or light amino acids (i.e., arginine and lysine) for consecutive passages to achieve protein labeling beyond a certain threshold (e.g., 99%). The heavy (e.g., OGD) and light (e.g., control) cells are subsequently used for the OGD experiment, where equal quantities of proteins are mixed from OGD and control samples before the proteomics sample preparation [33]. In contrast to gel-free approaches, proteins in each sample were separated by two-dimensional gel electrophoresis (2D-GE) based on the isoelectric pH and molecular weight of the proteins. The gel images obtained from test and control samples were compared and analyzed to identify the deregulated proteins and relative expression levels. These protein spots were excised and digested in-gel, and the peptides are processed by mass spectrometry to identify the protein [20, 27, 28]. Overall, gel-free approaches such as chemical or metabolic labeling were found to be superior to gel-based approaches in terms of reproducibility and proteomic coverage [28]. In addition to expression proteomics, few studies have used interaction proteomics or a mixed approach whereby interacting partners of specific target proteins (e.g., death-associated protein kinase 1 (DAPK1), small ubiquitin-like modifier 3 (SUMO3)) were identified by a combination

Table 2 Gene ontology analysis of OGD-proteomics studies to identify candidate markers

Sl. No	Cell type	Sample [a]	Approach [b]	Gene ontology analysis		Shortlisted protein(s)
				Molecular function/biological process	Cellular component	
1	Primary culture of cortical neuron	CE	M, In	Ferroptosis	Neurites, cell bodies	LRRFIP1, NR2B
2	HT-22 cells	CE	E, Ex	Extracellular matrix organization, inflammatory response, cell division, cell cycle, and ribonucleoprotein complex biogenesis	Membrane, mitochondria, endomembrane system, protein containing complex, envelope, endoplasmic reticulum, nucleus	HIF1α, HBA, HBB2, CO3, PGH2, HIG1A, P4HA2, ERO1A, PLOD1, NDRG1
3	PC-12 cells	EV	M, Ex	Oxidative stress, carbohydrate metabolism, protein synthesis and degradation, complement and coagulation, angiogenesis	Extracellular region, macromolecular complex, membrane, membrane enclosed lumen, supramolecular complex, cell junction, synapse	CAT, IDH1, PGM1, PSMC2, TCP1, PlAT, MFGE8
4	Organotypic cerebral slice cultures	SH	E, Ex	Neurite outgrowth, endocytosis, RNA transport	Cytoskeleton, membrane	CDC42, ITGAV, HRAS1, FGFR2, ITGB1, RAC1, ENAH, ACTG1, MAP2K2
5	Mouse EOC 2 microglial cells	CE	M, Ex	Signal transduction, cell proliferation, apoptosis, oxidative stress	Cytoplasm, protein complex, nucleus, extracellular region, cytoskeleton	HMGB1, CASP3, SPP1
6	Cerebral human microvascular endothelial cells	S	M, Ex	Protein folding, nucleic acid binding, glucose metabolism, extracellular matrix binding, extracellular organization	Extracellular exosomes, extracellular region, mitochondria	IGFBP2, CLU, COL1A2, ANXA1, PRDX3
7	EA.hy926 cells	CE	M, Ex	Oxidative and nitrosative stress, ubiquitination	Mitochondria, endoplasmic reticulum, cytosol	PRDX1
8	SH-SY5Y cells	CE	M, Ex	Energy production, protein folding and degradation	Mitochondria, endoplasmic reticulum, ribosomes, vesicle, cytoskeleton, spliceosome and proteasome	HSD17B10, UQCRC1, UQCRC2, RTN4, ACAT1, ACADM, ECH1, HSD17B10, VDAC1, TOM22, ACAA2
9	B35 cells	CE	M, In	Gene transcription, DNA repair, protein ubiquitination, cell proliferation, apoptosis	Mitochondria, endoplasmic reticulum, cytosol and nucleus	TIF1β, UBC9, LIG1, PIAS2, PIAS4, SOX6, SOX10
10	Primary astrocyte cells	S	M, Ex	Neurogenesis, inflammation	Extracellular matrix, basement membrane	EGF, BFGF, TGFA, VEGF, NOTCH1, APP, CSTC, NCAM1, TIMP1
11	B104 cells	CE	M, Ex	Chaperonic response, protein metabolism, oxidative defense, inflammation, apoptosis	Cytosol, mitochondria, endoplasmic reticulum, nucleus, lysosome	EEF2, EIF5A, PRDX6, GSTO1, ANXA1, VADC1, GLUD1
12	Immortalized adult rat brain endothelial cells	CE, S	M, Ex	Nucleic acid, protein and carbohydrate metabolism, signal transduction, cell structure, adhesion and motility, immunity and defense, cell cycle and apoptosis	Cytoplasmic, nuclear, secreted/extracellular matrix, endoplasmic reticulum, mitochondrial membrane, integral membrane, vesicles, Golgi	HSP1, HSP71, SOD, TIF1β

[a] *CE* cellular extract, *S* secretome, *EV* extracellular vesicle, *SH* slice harvest; [b] *M* mechanistic, *E* efficacy, *In* interaction proteomics, *Ex* expression proteomics

of immunoprecipitation and mass spectrometry [15, 17]. Figure 4 shows a timeline highlighting the introduction of main proteomics techniques in this field.

OGD-Proteomics Results

One of the key objectives of this review is to summarize the unbiased and general trends that are obtained from various OGD-proteomics studies. In 2007, Haqqani et al. reported the deregulated cellular and secreted proteome following an OGD/R (4 h/24 h) injury performed on immortalized brain vascular endothelial cells using gel-based (2D-GE) and gel-free proteomics (ICAT-2D-LC-MS/MS) approach. The deregulated proteins belonged to structural and metabolic proteins, and proteins involved in signal transduction, adhesion and motility, immunity and defense, cell cycle, and apoptosis. The in vitro results correlated well with the in vivo results obtained from laser-capture microdissection (LCM)-captured brain vessels of rats that were subjected to a 20 min transient global cerebral ischemia/reperfusion injury [28]. We had performed a series of functional assays (e.g., MTT, LDH, MPT pore opening, apoptosis) on rat B104 cells to select a timepoint (i.e., 4 h) of OGD that most closely recapitulates intracellular neuronal events in a model of cellular penumbra. There was no substantial damage of the cell membrane with no detectable apoptosis (< 5.0%) as mitochondrial permeability transition pore remained closed after 4 h of OGD. Yet quantification of the whole proteome by an iTRAQ-2D-LC-MS/MS experiment revealed down-regulation of proteins involved in protein metabolism (e.g., eukaryotic translation elongation factor 2 (EEF2), eukaryotic translation initiation factor 5A (EIF5A)) and anti-oxidative response (e.g., glutathione S-transferase omega 1 (GSTO1), peroxiredoxin 6 (PRDX6)) while certain anti-apoptotic (e.g., prohibitin 2 (PHB2)) and anti-inflammatory proteins (e.g., annexin A1 (ANXA1), annexin A2 (ANXA2)), and most of the mitochondrial proteins were increased in abundance [18]. Using co-culture of astrocytes and neuronal stem cells, Yan et al. found that short-term OGD (1-2 h) inhibits the release of regulatory factors from astrocytes that promote neuronal stem cell differentiation and proliferation. They used an iTRAQ-based quantitative proteomics analysis of astrocyte-conditioned medium following transient OGD/R (2 h/18 h) to identify these causative factors. Gene ontology analysis of the secretome revealed that these proteins are involved in neuronal development, inflammatory response, and extracellular matrix composition [34]. Llombart et al. used a SILAC based proteomics approach on human immortalized brain microvascular endothelial cell line following 6 h of OGD to quantify the differentially secreted proteins. Gene ontology analysis of these proteins revealed protein folding as the main molecular function, whereas aldosterone signaling and remodeling of epithelial adherens junctions were the most enriched canonical pathways. Five of these candidates (collagen type I alpha 2 chain (COL1A2), clusterin (CLU), insulin like growth factor binding protein 2 (IGFBP2), ANXA1, and peroxiredoxin 3 (PRDX3)) were subsequently tested in human serum samples of acute ischemic stroke and stroke mimicking conditions such as epilepsy and dementia to identify IGFBP2 as a potential diagnostic biomarker of ischemic stroke [33]. Wang et al. reported a proteomic characterization of extracellular vesicles (EV) isolated from PC12 cells that were subjected to 3, 6, and 12 h of OGD. The differentially expressed EV proteins were implicated in various biological processes such as carbohydrate metabolism, coagulation, protein synthesis and degradation, and angiogenesis. Notably, the OGD-treated PC12 cells secret EVs with increasingly greater apoptotic potential with passing durations of OGD when exposed to normal PC12 cells [32]. IL-17A is a key player in the

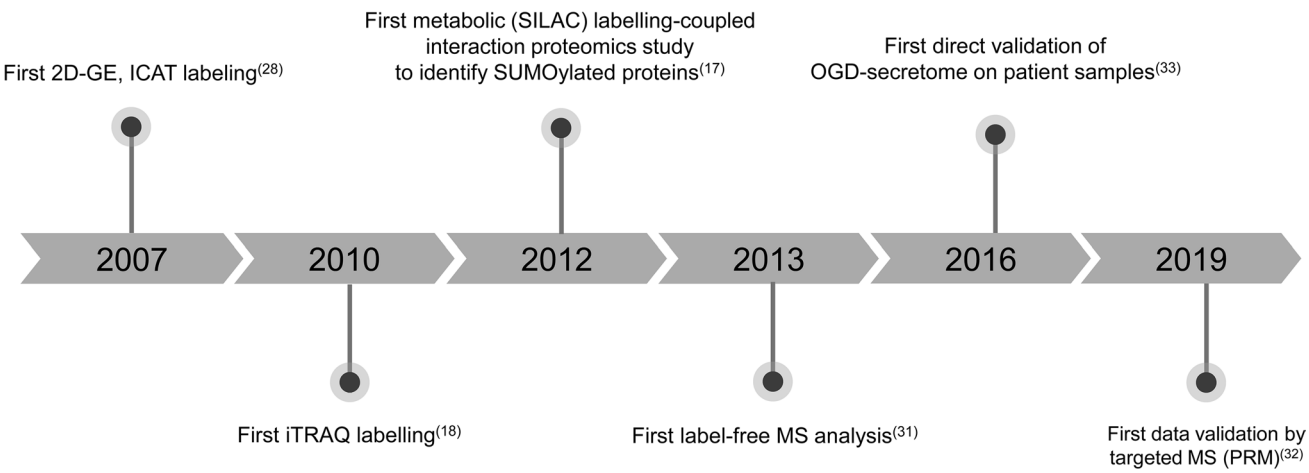

Fig. 4 Timeline representing key milestones in OGD-proteomics studies

inflammatory cascade following ischemia/reperfusion injury. Zhang et al. used a 2D-GE-based proteomics approach to compare the proteome of OGD-exposed EOC2 microglial cells with and without IL-17A siRNA treatment. This study showed an IL17A mediated upregulation of caspase 3 (CASP3), high mobility group box 1 (HMGB1), and osteopontin (SPP1) among many other altered proteins that are involved in oxidative stress, cell metabolism, apoptosis, various signaling pathways such as p53, PI3K/Akt, and Toll-like receptor signaling pathway [20]. Using label-free proteomics on organotypic cerebral slice cultures, cell division cycle 42 was reported as the key protein involved in promoting neurite outgrowth following acyl ghrelin treatment during the late phase of OGD/R (2 h/7 day) injury. Further, acyl ghrelin modified the expression of proteins of Rap1 and MAPK signaling pathways and proteins involved in processes such as regulation of actin cytoskeleton, endocytosis, and RNA transport [14]. Tao et al. performed 2D-GE of proteins from EA.hy926 endothelial cells following 6 h of OGD. Twenty-two proteins were identified using MALDI-TOF MS. Peroxiredoxin1 (PRDX1) was found to be one of the most intensely upregulated proteins providing antioxidative protection during the acute phase of injury. However, nitrosative activation of E3 ubiquitin ligase E6-associated protein causes polyubiquitination and degradation of PRDX1 during the subacute/late phase (12-24 h). This contributes to the damage of vascular endothelial cells via redox imbalance [27]. Abigail et al. performed label-free proteomic analysis on SH-SY5Y cells following prolonged OGD (18 h). About 1/3 of altered proteins were of mitochondrial and endoplasmic reticulum origin. These are involved in mitochondrial energy production, protein folding, and protein degradation as determined by Ingenuity Pathway Analysis. The upregulation of proteins involved in unfolded protein response may be linked to the progressive swelling of the endoplasmic reticulum as observed by electron microscopy [31].

DAPK1 is identified as a new component of the neuronal death signaling complex, which acts as a signaling amplifier of N-methyl-D-aspartate receptors for mediating brain damage following stroke. Using interaction proteomics, leucine-rich repeat of flightless I-interacting protein 1 (LRRFIP1) was identified as a novel neuronal DAPK1 interactor in stroke-like conditions induced on primary cultures of rat cortical neurons by OGD. The results also suggest that an increase in LRRFIP1 expression precedes neuronal death, involving increased reactive oxygen species and lipid peroxides [15]. SUMOylation increases profoundly during the acute phase of reoxygenation following OGD. Using SILAC and immunoprecipitation on a stably transfected HA-SUMO3 expressing B35 cells, SUMO3 interacting proteins were quantified following transient OGD (6 h/0.5 h, OGD/R). Of note, the OGD was generated using an anoxic chamber that was equipped with a palladium catalyst. The result demonstrates that SUMO3-conjugated proteins are involved in transcriptional regulation, DNA repair, and protein ubiquitination with ubiquitin being one of the elevated proteins. miRNA silencing of SUMO2/3 leads to complete abrogation of OGD-induced protein ubiquitination [17]. Table 2 summarizes the results of gene ontology analysis of OGD-proteomics studies along with the candidate proteins shortlisted as potential markers.

Data Validation and Follow-Up Studies

Despite rapid technical progress and increasingly wider use of proteomics approaches, general skepticism about the reliability of the proteomics data led to the use of complementary validation techniques in OGD-proteomics studies. The results were verified at the mRNA or protein level using different types of complementary molecular biological techniques such as RT-PCR [18], immunoblotting [23, 33], or ELISA [33]. Recently, in EV samples of OGD-damaged and control cells, Wang et al. has used a MS-based parallel reaction monitoring (PRM) approach to validate the TMT-LC-MS/MS results (Fig. 4) [32]. Compared to immunoassays, targeted MS/MS techniques such as single/multiple reaction monitoring (SRM/MRM) and PRM have lower developmental cost; offer far wider coverage of proteome; are faster, more flexible, amenable to multiplexing, and in principle are capable of differentiating highly similar variants such as isoforms and post-translationally modified proteins [35]. Immunocytochemistry and immunohistochemistry are used to obtain localization information of the discovered proteins [27]. The verified candidates are followed up by a variety of proof-of-concept experiments including but not limited to CRISPR-Cas9, siRNA, site-directed mutagenesis, and pharmacological modulation of the discovered proteins or pathways (Fig. 2). A more biologically relevant in vivo model (e.g., transient or permanent middle cerebral artery occlusion) [27] or comparable clinical samples (e.g., plasma or serum of patients) [33] are included in the experimental design to show the translational potential of the discovered target proteins.

Limitations and Challenges

In OGD-proteomics studies, several experimental details related to the OGD model or proteomics analysis were found missing or not easily available, e.g., the exact composition of the hypoxia gas, if reperfusion was included or not, duration of reperfusion, and ambient humidity during OGD. The dissolved oxygen content before, during, or immediately after the experiment is not available in most cases. Incidentally, the anticipated oxygen concentration inside the chamber varied widely between studies: < 0.001% [16], 0.3% [1,

36], < 0.5% [14], 1% [37]. Details about mass spectrometer, parameters used during data acquisition, and raw data analysis, a total number of identified or quantified proteins are not available for some studies (Supplemental Table 2). These factors may affect the repeatability of the experiment. A list of essential variables has been listed in Supplemental Fig. 1 to improve the overall reproducibility of future OGD-proteomics studies. It can be found at https://yenepoya.res.in/database/LTN_Datta_Lab/OGD-Prot_Systematic-Review/OGD-Prot_Additional_Variables.htm.

Beyond this, awareness about the limitations inherent to in vitro system, OGD model, and OGD-proteomics is important to avoid over-extrapolation of the results which may be misleading. First, under normal physiological conditions, cells in brain tissue experience an oxygen tension that fluctuates between 21 and 47 mmHg (2.7–6.1% (v/v)) [38]. Cultured brain cells like any other mammalian cells are routinely maintained under supra-physiological oxygen concentration (in vitro normoxia — 140 mmHg or 18.5% (v/v), at sea level, 37 ºC). Hence, the normoxic condition in vivo would be very different from in vitro normoxia. The ambient oxygen level during hypoxia or OGD experiment is generally kept below 2–3% (v/v) [21, 22]. Intuitively, the cellular response to in vitro hypoxia and OGD when compared to in vitro normoxia (~ 18.5% to < 2–3%) will not be directly comparable to in vivo OGD (ischemia). Further, during reoxygenation following hypoxia, although the aerial exchange with room air is instantaneous, oxygen dissolves at a much slower rate in the culture media than CO_2 which reaches equilibrium within minutes [39]. This may be an important consideration during the reperfusion phase following an OGD experiment especially when a short duration of reperfusion is used. Second, various humidity (e.g., 70% [24], 100% [33]) of the ambient environment has been reported during OGD-proteomics experiments that can affect the partial pressure of oxygen (pO_2) inside the hypoxia chamber or hypoxic incubator. Third, it is important to differentiate hypoxia (0.1–3% (v/v) oxygen tension) from anoxia (< 0.1% (v/v) oxygen tension) as cellular signaling is known to be different between these two conditions [40, 41]. Hypoxia would be clinically more relevant although anoxia in the context of ischemia-reperfusion injury has also been studied [16, 17]. Fourth, rather than ambient oxygen level, the concentration of oxygen that the cultured cells are experiencing, i.e., the peri-cellular oxygen concentration, is perhaps more relevant during an OGD experiment. The peri-cellular oxygen concentration can depend on multiple factors such as the geometry of the flask or well plate, type of used plasticware, volume/height of media [42], and hypoxia gas composition. Unfortunately, this is hard to predict exactly from the ambient oxygen concentration. No technologies are available that can accurately quantify and monitor intracellular oxygen levels in the living cells during routine maintenance of cell culture or an OGD experiment [39]. Fifth, brain cells, grown in high glucose (> 20 mM) and pyruvate (1 mM), are generally used as a control during OGD-proteomics studies. This is several folds greater than the glucose level seen during severe hyperglycemia in vivo. Of note, as determined by the microdialysis method, the physiological brain glucose level fluctuates between 0.82 and 2.4 mM that can be reduced to 0.16 mM during hypoglycemia [43]. Sixth, apart from glucose and oxygen, wide variation in the composition of the culture media is observed between studies (Table 1), e.g., balanced salt solution does not have amino acids or vitamins that are present in DMEM or MEM. The presence or absence of other energy sources such as pyruvate, lactate, or fatty acids can affect the cellular response to OGD.

Comparable Trends Between OGD-Proteomics and Clinical Proteomics Studies

In light of the foregoing limitations, it can be argued that being mostly done with secondary cell lines and without the presence of brain microenvironment and systemic influence, most of the molecular changes obtained from OGD-proteomics studies have little to no predictive value when compared to bedside samples following ischemic stroke. To refute this, we have compared the deregulated proteins from all OGD-proteomics studies that used an expression proteomics approach with the deregulated proteome from a set of equal number of randomly selected clinical proteomics studies done by us [44, 45] and others on human post-mortem brain samples [44, 46–48] or body fluids such as plasma [45, 49], serum [50, 51], cerebrospinal fluid (CSF) [52], or brain microdialysate [53] of stroke patients (Fig. 1). At least 49 proteins were detected in common among both types of samples (Supplemental Table 3, https://yenepoya.res.in/database/LTN_Datta_Lab/OGD-Prot_Systematic-Review/OGD-Prot_Clin-Prot_Consensus_Proteins.htm). Several proteins quantified as deregulated in OGD-proteomics studies (e.g., haptoglobin (HP), glutamic-oxaloacetic transaminase 2 (GOT2), NADH:ubiquinone oxidoreductase core subunit S1 (NDUFS1), carbonyl reductase 1 (CBR1), glial fibrillary acidic protein (GFAP)) also showed aberrant regulation in more than one clinical proteomics studies. At least ten proteins (e.g., CLU, annexin A5 (ANXA5), myosin heavy chain 9 (MYH9), galectin 3 binding protein (LGALS3BP), contactin 1 (CNTN1), ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), vimentin (VIM), serine and arginine rich splicing factor 1 (SRSF1), glutathione S-transferase pi 1 (GSTP1), PRDX1) showed identical trends between in vitro OGD samples and clinical specimens (Table 3). Among cellular components, in neurons, mitochondria appeared to be the most represented and affected organelle with key mitochondrial events (such as citric acid

Table 3 Consensus candidates between OGD-proteomics and clinical proteomics studies showing consistent trends of regulation

Sl. No	Protein[a]	Clinical proteomics			In vitro OGD-proteomics		
		Sample, disease/location[b]	Sampling time (time from index event)	Regulation	OGD sample	Sampling time [post OGD(/R)][c]	Regulation[c]
1	CLU	Serum, ischemic stroke[51]	within 24 h	Up	Human microvascular endothelial cell secretome[33]	6 h	Up
2	ANXA5	PM brain infarct, multiple locations[44]	median PMI 3.5 h	Up	EVs (PC12 cells)[32]; SH-SY5Y cells[31]	3–12 h; 18 h	Up; Up
3	MYH9	Plasma, lacunar infarction[45]	median delay: 42 days	Down	EVs (PC12 cells)[32]; SH-SY5Y cells[31]	3–12 h; 6 h, 18 h	Down; Down
4	LGALS3BP	Plasma, lacunar infarction[45]	median delay: 42 days	Down	EVs (PC12 cells)[32]	3–12 h	Down
5	CNTN1	PM brain tissue, ischemic ipsilateral hemisphere (infarct core and peri-infarct area)[48]	PMI 6 h	Up	EVs (PC12 cells)[32]	3–12 h	Up
6	UQCRC2	PM brain infarct, multiple locations[44]	median PMI 3.5 h	Down	EVs (PC12 cells)[32]	6 h, 18 h	Down
7	VIM	PM brain infarct, multiple locations[44]	median PMI 3.5 h	Up	Primary rat astrocyte cells[34]; SH-SY5Y cells[31]	2 h/18 h; 6 h	Up; Up
8	SRSF1	PM brain tissue, infarct core and contralateral areas[47]	median PMI 5.5 h	Up	SH-SY5Y cells[31]	6 h, 18 h	Up
9	GSTP1	Cerebral microdialysate, infarct core[53]	After 24 h	Up	SH-SY5Y cells[31]	6 h, 18 h	Up
10	PRDX1	Cerebral microdialysate, infarct core[53]	After 24 h	Up	SH-SY5Y cells[31]	18 h	Up

[a]Non-italicized gene symbols are used to designate proteins. [b]Location refers to a specific brain area, applicable only for tissue samples. [c] ";" semicolon is used to demarcate independent studies. "," comma indicates multiple timepoints in the same study. *PM* post-mortem; *PMI* post-mortem interval

cycle) as the most altered processes following OGD [18, 31]. This has been replicated in microdissected neurons obtained from the infarcted area of human brain post-mortem samples [47]. Not surprisingly, at times, the magnitude or direction of deregulation of commonly quantified proteins is not comparable for obvious reasons. Changing the sampling time or cell-type or modifying the associated culture conditions can improve this correlation. Overall, proteomics data generated from well-controlled OGD models can effectively complement rodent models of ischemic stroke in providing candidate biomarkers for validation in suitable clinical samples.

Cell-Type-Specific Validation of OGD-Proteomics Results in Pathologically or Clinically Relevant Samples

The OGD-proteomics data can be validated in two different ways: (1) targeted validation: by checking the cell-type-specific expression of one or more shortlisted proteins in comparable tissue samples and (2) validation of system-wide trends: by comparing the OGD-proteomics data with a similar system-wide dataset generated from cell-type-specific proteomics experiment done on comparable in vivo model or clinical samples. To perform either of these, it is important to isolate the target cell type from a predetermined location of the brain, e.g., cortical neurons could be the target cell type for validating OGD-proteomics studies done on a primary cortical neuron or secondary neuronal cell lines. LCM can be used for single-cell isolation directly from fresh, frozen, or fixed tissue samples. Fluorescence-activated cell sorting although used frequently may adversely affect the integrity of the brain cells during homogenization of the tissue. An alternate option would be to perform cell-type-specific labeling in vivo in rodent stroke models. Various techniques have been tried on brain cells such as BioOrthogonal Non-Canonical Amino Acid Tagging [54]. The cell-type-specific interaction proteomics results can be validated using in situ proximity labeling methods such as in vivo BioID [55]. The subcellular localization information can be validated using immunohistochemistry if antibodies are available or by imaging mass spectrometric techniques such as MALDI-imaging or secondary ion mass

spectrometry for candidates without validated antibodies. Of note, it will be hard to find a comparable in vivo or clinical sample for validating the trends obtained from OGD-proteomics studies done on EVs or secreted proteins (i.e., secretome or conditioned media). Proteins found in CSF or brain microdialysate are secreted by different types of brain cells, while plasma or serum contains proteins from all organs of the body. For body fluids, mass cytometry can provide cell-type-specific data which may be useful for assessing the response of immune cells which plays a central role in post-stroke inflammation. However, most of the above-mentioned cell-type-specific approaches for isolation, labeling, or mass spectrometry are technically challenging, expensive, or time-consuming [56]. A few of them such as LCM [47] and mass cytometry [57, 58] have been used in the context of ischemic stroke. Until now, none of them have been used for direct validation of OGD-proteomics results.

Looking at Systems Data through the Lens of Reductionism

The current scheme of OGD-proteomics approach (Fig. 2) where proteomics data is eventually analyzed from a reductionist standpoint following validation in a cell-type non-selective manner needs introspection. First, this allows validation of only a few proteins or pathways or processes from the whole dataset as performing targeted validation of many proteins are expensive and a time-consuming affair. Further, in a majority of the cases, the absence of good antibodies poses a problem [59]. Mass spectrometry-based validation techniques such as SRM/MRM or PRM [32] or MALDI-imaging have been used to partially circumvent this problem. Second, data interpretation remains limited to a few deregulated proteins (typically < 5% of total hits) in spite of identifying and quantifying hundreds and thousands of proteins, thereby rejecting or at least undermining the majority of the acquired data. That is reflected in part by the absence of curated data files containing the whole list of identified or quantified proteins or raw MS data in several OGD-proteomics studies (e.g., in reference 27) (Supplemental Table 2). An inevitable repercussion would be the failure to detect important general trends during a systematic review or large-scale data integration. Of note, the practice of writing systematic reviews and meta-analysis is rare in preclinical stroke research and remains one of the major reasons for the dismal bench-to-bedside translation of preclinical findings [60]. Authors should be encouraged to share curated datasets as supplementary files or web-resources, while raw data should be deposited in open directories such as PRIDE or MASSIVE (e.g., reference 23, 33) for community usage. Third, as samples obtained from in vitro OGD models are not directly comparable to in vivo samples unless a cell-type-specific validation is performed, a negative result during validation does not necessarily mean the findings are incorrect, while a positive result may be misleading as the in vivo protein level is the pooled abundance arising from different brain cell types. Similarly, when clinical samples are compared, the negative result could be due to the selection of a non-optimal cohort of patients or other patient-related unaccounted factors. It is also important to share or publish negative results to avoid a negative publication bias, as seen with hypothesis-driven studies in experimental stroke research [61].

Conclusion

The brain consists of a number of functionally and structurally distinct cell populations. Unlike cell-type or location-specific neurological disorders such as Alzheimer's disease or Parkinson's disease, ischemic stroke being a vasculature-linked disorder can affect any anatomical structure or cell type of the brain. Proteomics of in vitro OGD model forms the basis to understand the cell-type-specific global responses without systemic influence at the cellular or subcellular level that cannot be replaced by proteomics studies done on in vivo stroke models or clinical samples. Almost 14 years into the introduction of an unbiased OGD-proteomics approach [28], we have seen its relatively few yet diverse applications on the neuron [17, 18, 23, 31], astrocyte [34], microglia [20], brain vascular endothelial cells [27, 28], organotypic slice cultures [14] or on EVs [32], and secreted proteins [33] either for profiling or for determining the interacting partners of selected proteins [15, 17]. Selected candidates from secreted proteins have also been validated directly on patient samples [33]. In general, comparable trends in the expression of several proteins between OGD-proteomics and clinical proteomics of ischemic stroke samples show the unappreciated potential of this composite design during future studies on biomarker discovery (Table 3).

Future Perspective

Despite healthy progress, OGD-proteomics is far from realizing its full potential. Profiling proteomics studies targeting isolated organelle (e.g., mitochondria, nucleus) or subcellular fractions or secretome or EVs are in a nascent stage. Like hypothesis-driven studies, non-neuronal cell types such as glial or vascular endothelial cells remain poorly represented cell-type although they contribute to roughly half of the brain mass [62]. On the technical front, proteome-wide detection and quantification of cell-type-specific post-translational modifications of proteins, i.e., PTM-proteomics (such as SUMOylation, ubiquitination, phosphorylation,

glycosylation), following an ischemic stroke can only be possible using mass spectrometric techniques, not replaceable by other omics approaches including next-generation RNA sequencing. The introduction of data-independent acquisition or single-cell proteomics [63, 64] can provide an additional new dimension to the above experiments. At the molecular level, no studies are available that focused on the druggable section of the proteome such as GPCRs (GPCRomics) [65] or protein kinases (kinomics) or phosphatases in the context of ischemic stroke. On the data analysis front, the time has come to look at the proteomics data from an unbiased systems viewpoint. No stroke-specific proteomics data compendium is available for community usage. The use of statistical methodologies such as Bayesian statistics to integrate multiple system-wide datasets generated using various cell types or conditions can provide new insights into the cell-type-specific and consensus responses during the evolution of ischemic stroke. Large-scale integration of interdisciplinary data (i.e., integromics [66, 67]) is a prerequisite for the overall understanding of the regulatory processes. Some of these may offer mechanism-based targets suitable as potential therapeutic targets for proof-of-concept studies on rodent models or as biomarkers for validation on patient cohorts.

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Data Availability Supplementary materials are available. A web resource containing supplementary tables and vector graphic version of a modified Fig. 3 are available at https://yenepoya.res.in/database/LTN_Datta_Lab/OGD-Prot_Systematic-Review/

Declarations

Ethics Approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to Participate Not applicable

Consent for Publication Not applicable

Competing Interests The authors declare no competing interests.

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