

CASE STUDY:



POWERED BY



HYPOXIC ISCHEMIC ENCEPHALOPATHY | DRIED BLOOD SPOT MATRIX | TWO TIMEPOINTS

Profiling dynamic severity in hypoxic ischemic encephalopathy.

Severity of presentation in neonates with suspected hypoxic ischemic encephalopathy (HIE) has a dynamic metabolomic profile. Following the initiation of therapeutic intervention, metabolite severity profiles evolve over time. These metabolite changes implicate different protein networks with potential implications for patient management and intervention protocols.

COHORT

186 dried blood spots • HIE

INSTITUTION

University of Pretoria

DATA TYPE

Untargeted metabolomics

SEARCH SPACE REDUCED

~15,000 proteins → 4-6 candidates



EXECUTIVE TAKEAWAY

Severity in HIE is dynamic. Metabolomics paired with Splash & Ripple identifies the protein networks that may be implicated.

Researchers at the University of Pretoria wanted to understand the drivers of severity in hypoxic ischemic encephalopathy (HIE). Metabolomics was used to profile the biochemical changes in these neonates at two timepoints during the application of therapeutic hypothermia. The metabolite profile revealed that severity in HIE is associated with distinct biochemical changes at different timepoints. The Splash & Ripple application mapped these changes to protein candidates and provided mechanistic arguments for the observed perturbations. If severity is dynamic, then the appropriate intervention may have temporal implications. Metabolomics paired with Splash & Ripple helped to reveal this dynamic nature of severity in HIE, and the potential mediators involved.

PROOF AT A GLANCE

186 DRIED BLOOD SPOTS 84 at 0–6 h · 102 at 48–72 h, during therapeutic hypothermia	93% METABOLITE → PROTEIN MAPPING An average of 93% of metabolite perturbations across both timepoints could be connected to at least one protein
5 ALIGNED THEMES Themes expected based on the phenotype and supported by the metabolomic data	~15,000 PROTEINS RANKED Rank based on their relative contribution to the observed metabolite perturbation profile.
0.04% BEST CANDIDATE RANK CXCL12 ranked 6th of ~15,000 at 48–72 h was best ranked protein candidate with a reported association to HIE.	2 MECHANISTIC HYPOTHESES Two mechanistic hypotheses at different timepoints reflect the temporal nature of severity in HIE.



THE SCIENTIFIC CHALLENGE

Metabolites are the readout. Proteins help explain the mechanism.

Metabolomics measures the functional end-state of biology, providing a rich readout of molecular phenotypes. That richness is exactly what makes it powerful and challenging to interpret in isolation.

A severity contrast in HIE returns dozens of significantly altered metabolites. Pathway enrichment will tell you the lipids moved, or the amino acids moved. But **enrichment stops at the category**. It cannot tell you which enzyme, transporter or regulator sits at the point where several of those compounds meet, and it cannot connect a metabolite in the blood to a signaling protein that never appears on a metabolic pathway diagram at all.

That is a real problem in neonatal encephalopathy. The injury is a cascade: primary energy failure, reperfusion, oxidative injury, inflammation, delayed cell death. Each phase may have different protein drivers. A metabolite list from a single timepoint, read on its own, cannot distinguish which phase you are looking at or whether what you are seeing is damage or the body's attempt to contain it.

ENRICHMENT ANALYSIS

Tells you the category

A categorisation of metabolite signals without any protein context is limited.

CORRELATION NETWORKS

Stay inside one omic

Metabolite-metabolite relationships reveal patterns within the measured profile. Connecting those patterns to proteins adds a route to mechanistic interpretation.

SPLASH & RIPPLE

Names the convergence point

Places every perturbed metabolite on a curated protein map, and looks for proteins where multiple metabolite signals converge.

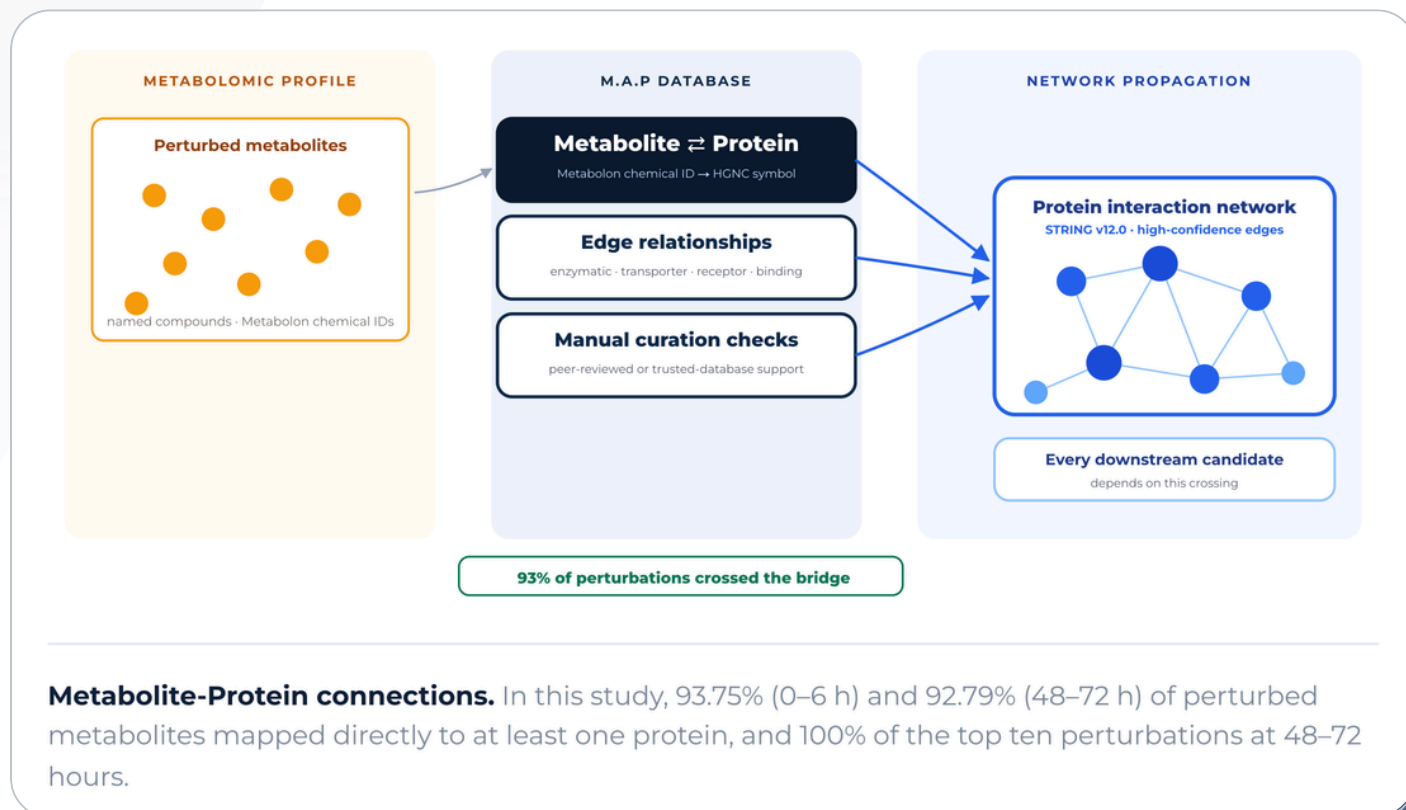


THE FOUNDATION

Metabolite Affiliated Proteins (MAP) database

Metabolon's expertly curated Metabolite Affiliated Proteins (MAP) database connects named chemical compounds to documented molecular relationships. This comprehensive resource empowers the application to deliver meaningful insights.

The MAP database leverages the Reactome database as a foundation. Metabolon has built on this foundation to cover more chemical compounds and curate additional relationships. Of the 2,820 distinct Metabolon library chemical IDs represented in the MAP database, only 25.4% have a Reactome (version 92) association. Metabolon's curated resource contains almost **three times more metabolite-protein relationships than Reactome**. Results from an internal validation (*described in accompanying white paper*) at Metabolon revealed that across 131 samples with a diagnosed mendelian disorder, Metabolon's map recovered 72.5% of diagnostic proteins compared to 57.3% using Reactome alone.





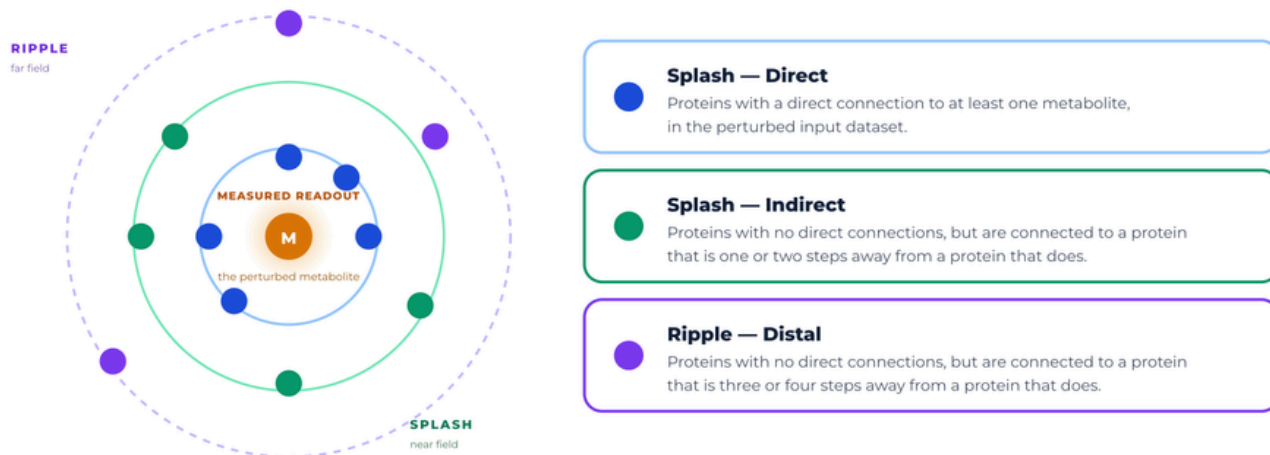
HOW SPLASH & RIPPLE WAS APPLIED

Begin with metabolomics.

Metabolomics measures the consequences of genetic and environmental insults. Splash & Ripple uses expertly curated metabolite-protein relationships to build on the metabolite measurements, leveraging a protein association network to explore neighbourhoods connected to the pattern of perturbed metabolites.

We rarely observe the insult, or the protein it acts on, directly. What we can measure are its metabolomic consequences. Splash & Ripple treats each perturbed metabolite as that measured readout and works backward across the protein network to uncover the biology behind it: the proteins whose altered behavior the metabolite reflects, and the wider neighbourhood connected to them. The proteins closest to the readout form the **Splash**; expanding the network to more proteins with higher degrees of separation gives the **Ripple**.

Splash & Ripple reduces the manual work of connecting metabolites to protein biology and makes that interpretation more accessible to researchers without specialist expertise.



The metabolite is the readout; the proteins are the candidates. At the center sits the measured metabolite. Working outward, Splash & Ripple recovers the proteins behind it and groups them by how close they are to the measured metabolites: directly mapped proteins, near neighbors at one or two interaction steps, and a separate Ripple band reached at three or four steps under stricter criteria. The groups describe network proximity, not causal certainty, and each is scored and ranked on its own terms.



HOW SPLASH & RIPPLE WAS APPLIED

STEP 1 • DATA-DRIVEN THEMES

What the metabolomic data tells us

Pathway enrichment on the perturbed metabolites produces themes grounded in the metabolite measurements with no prior assumption about the disease.

STEP 2 • EXPECTED THEMES

What the phenotype predicts

Independently, the phenotype is used to derive the biology a domain expert might anticipate.

STEP 3 • ALIGNED THEMES

Where an expected theme has support from a data-driven theme it is categorised as an **aligned theme**. Aligned themes therefore represent signal in the metabolomic dataset that has some potential relationship to the study phenotype. It acts as a filter to narrow the candidate protein list to only those most relevant to the research question.

Aligned themes, alongside highly ranked metabolites, can be used to filter the candidate protein list. This approach focuses analyses on discrete biological relationships that can be further explored using Sankey plots to visualize the potential protein-protein network pathways that connect metabolites of interest to a specific protein candidate.





STUDY AND DATA CONTEXT

A severity contrast, held at two points in the injury cascade

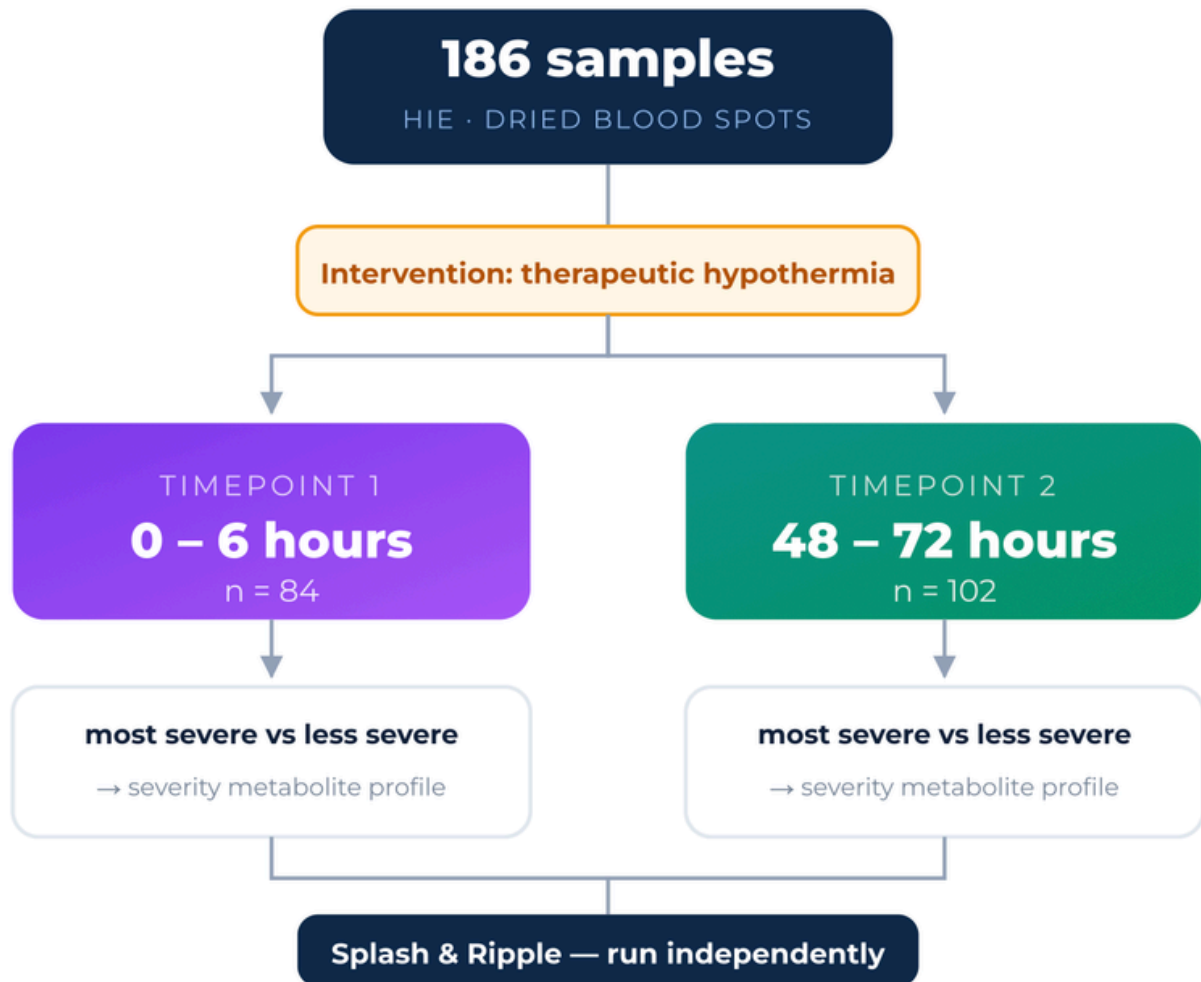
The cohort was assembled at the **University of Pretoria** as part of the **NESHIE study** under the direction of **Prof. Michael Pepper** on behalf of the **NESHIE team**, with **Dr. Caroline Foden** as primary genomics and multi-omics researcher. Dried blood spots were collected from neonates diagnosed with HIE at two timepoints over the course of the standard-of-care intervention, therapeutic hypothermia: **0–6 hours** (n = 84) and **48–72 hours** (n = 102).

Critically, the statistical design was not disease versus health. At each timepoint the analysis compared the **most severe presentations against the less severe**. Every metabolite entering Splash & Ripple is therefore a severity signal within an already-affected, already-treated population which is far more specific and far more clinically relevant question than "what does HIE look like?"

Because the two comparisons were run independently, they act as a natural internal check on each other. Anything that appears at both timepoints is a persistent feature of severity; anything that appears at only one is time-resolved.



STUDY AND DATA CONTEXT



Study design. Two independent severity contrasts, each analyzed separately, giving a time-resolved view of what distinguishes the most severely affected neonates.



WHAT IT REVEALED — TIMEPOINT 1

0-6 HOURS · N = 84

2 ALIGNED THEMES

KEY METABOLITE: PLASMALOGEN-GPE (P-16:0)

Early severity was dominated by lipid and oxidative-stress signals

Every significantly enriched pathway in the early severity contrast was a lipid pathway.

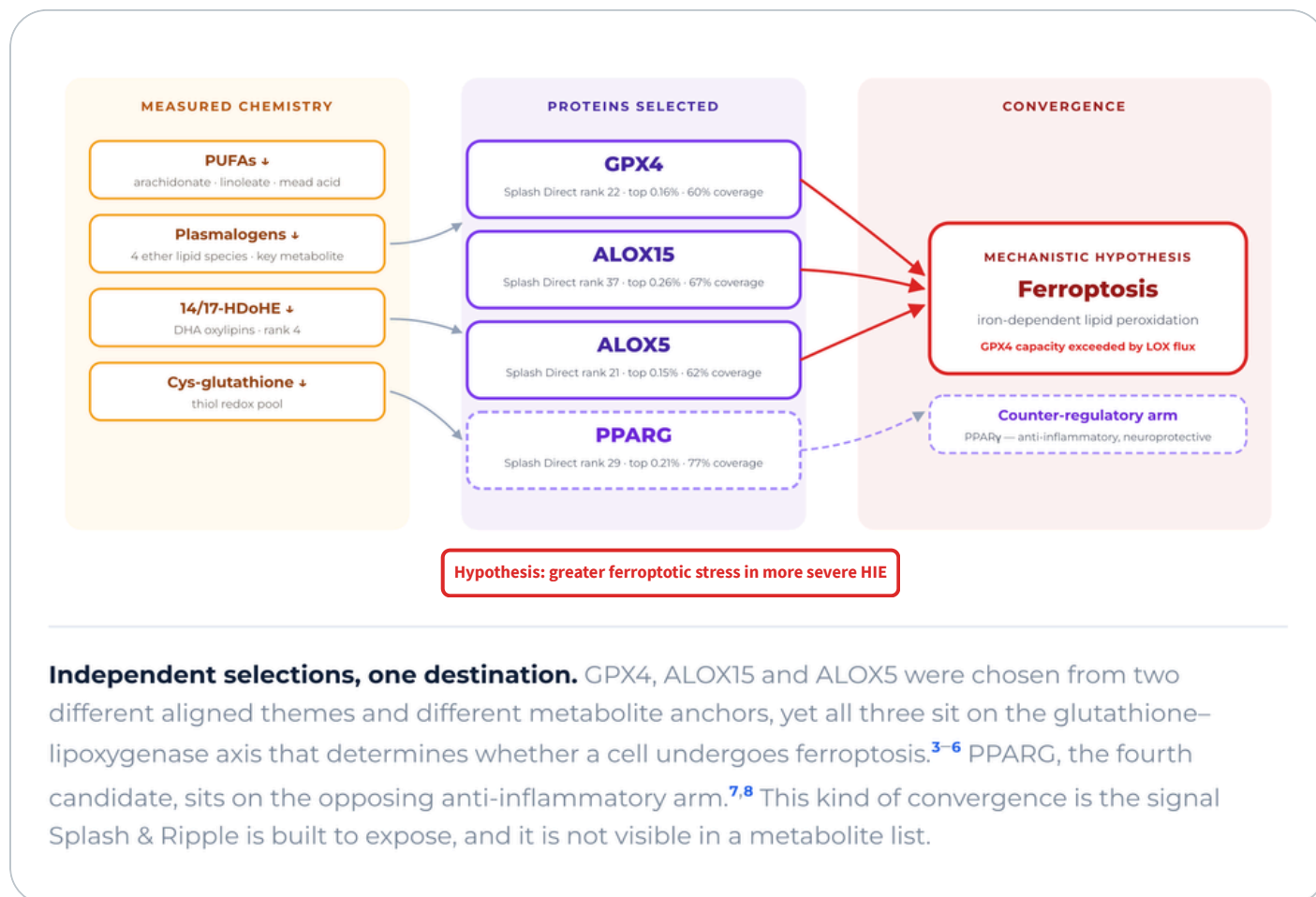
Lysoplasmalogen metabolism (adj. $p = 0.0028$) and omega-3/omega-6 fatty acid synthesis (adj. $p = 0.0032$) led the list, followed by alpha-linolenic acid metabolism, broad fatty acid metabolism and arachidonic acid oxylipin metabolism. Two of these formed aligned themes: *ether lipid/plasmalogen remodeling with oxidative stress defense*, and *PUFA imbalance with oxylipin inflammatory signaling*, because oxidative membrane injury and neuroinflammation are exactly what the HIE phenotype predicts.^{1 2}

- **OBSERVED** Polyunsaturated fatty acids depleted across the board: arachidonate, linoleate, linolenate, mead acid, dihomo-linolenate.
- **OBSERVED** Plasmalogens, the brain's endogenous membrane antioxidants, reduced at four separate species.¹
- **OBSERVED** Cysteine-glutathione disulfide reduced, alongside 14-HDoHE/17-HDoHE (rank 4), the hydroxylated DHA products.
- **INFERRED** Simultaneous loss of PUFA substrate, plasmalogen antioxidant capacity and glutathione-linked species is the signature of lipid peroxidation outpacing defense.

Applied to the ranked protein table, the two aligned themes intersected with 1-(1-enyl-palmitoyl)-GPE (P-16:0) narrowed roughly 15,000 proteins to 342 and 441 candidates respectively. From those, the analysis surfaced four proteins, and three of them converge on the same axis.



WHAT IT REVEALED — TIMEPOINT 1



The interpretation is coherent with the metabolomics: lipoxygenases consume polyunsaturated fatty acids to generate hydroperoxides; GPX4 uses glutathione to reduce them.^{4,5} If GPX4 capacity is exceeded, peroxidation propagates and the cell dies by ferroptosis.^{3,6} The measured profile shows substrate falling, plasmalogen antioxidant capacity falling, and the glutathione-linked pool falling all at once, and all more strongly in the most severe neonates.



WHAT IT REVEALED — TIMEPOINT 2

48–72 HOURS · N = 102

3 ALIGNED THEMES

KEY METABOLITE: 1-METHYLNICOTINAMIDE

The severity signal shifted to nicotinamide metabolism.

Not one lipid pathway reached significance in the later contrast. The enrichment profile had moved to amino acids, nucleotides, nicotinate and nicotinamide metabolism, tryptophan, and ascorbate.

Three aligned themes emerged, each mapping onto a recognized phase of the encephalopathy cascade: *excitotoxicity and amino acid neurotransmitter imbalance*,⁹ *mitochondrial energy failure with NAD⁺/ATP disruption*,^{10 21} and *oxidative stress with antioxidant depletion*.¹¹ And the analysis anchored all three on a single compound.

THE METABOLITE THAT CHANGED THE STORY

1-methylnicotinamide (1-MNA)

Absent from the key metabolite profile at 0–6 hours. At 48–72 hours it is the highest-ranked compound to satisfy every key-metabolite criterion: *significantly pathway-associated*, *not a xenobiotic*, *tractably connected*, and it is **significantly elevated** in the most severe cases.

SUPPORTING CHEMISTRY, SAME TIMEPOINT

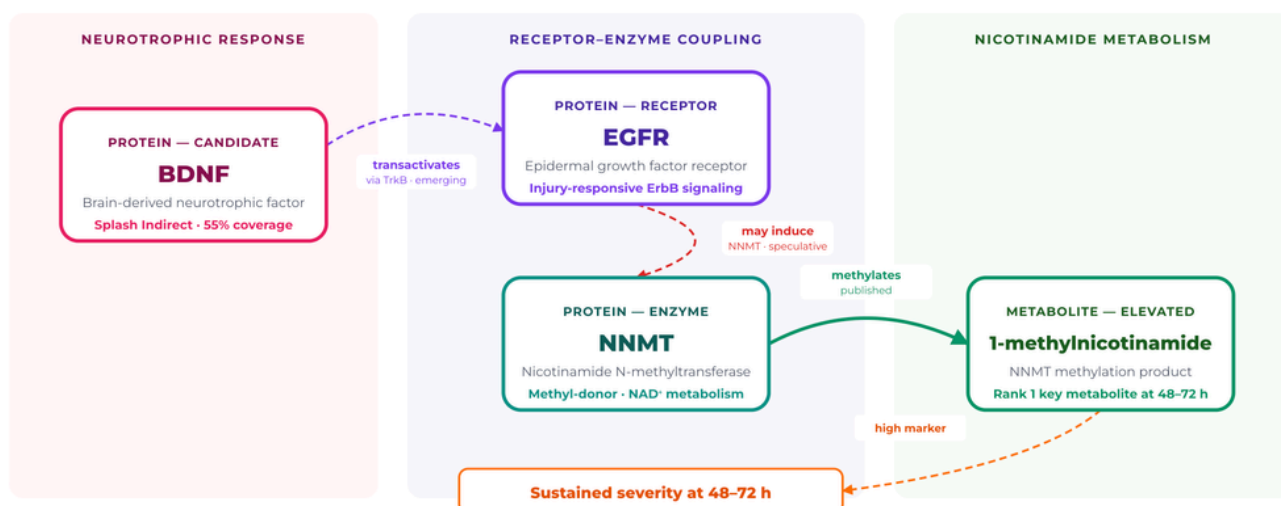
A coherent nicotinamide picture

Nicotinamide itself is **down**. Its methylated product 1-MNA is **up**. 1-MNA's own downstream catabolite, N1-methyl-2-pyridone-5-carboxamide, is **up**. NAD⁺ and NMN are **up**. Cortisol is elevated at rank 5.

Together, these changes are consistent with increased engagement of the nicotinamide methylation and clearance pathway.



WHAT IT REVEALED — TIMEPOINT 2



A path chosen and interrogated. Having ranked BDNF in Splash Indirect and 1-MNA as the top key metabolite, the analyst selected this specific protein–metabolite connection in the Sankey view and asked Splash & Ripple to reconstruct the intervening path. Each step is returned with an explicit evidence grade — **PUBLISHED** **EMERGING** **SPECULATIVE** — so the hypothesis can be evaluated rather than simply accepted.

Why interrogate BDNF in the first place?

Brain-derived neurotrophic factor is the brain's principal pro-survival, pro-repair signal, and it is directly relevant to the biology flagged here: it counters glutamate excitotoxicity, the exact mechanism behind this timepoint's excitotoxicity aligned theme, and enhancing BDNF/TrkB signalling is neuroprotective in neonatal hypoxia–ischemia models.^{12 13}

BDNF is a **promising biomarker** in this clinical space, but it is not yet clear how to read it. Studies have linked both elevated and reduced circulating BDNF to worse encephalopathy severity or outcome, and the direction appears to depend on sampling time, matrix and treatment context.^{14–17} If the relationship to prognosis remains unsettled when BDNF is considered alone, placing it inside a network with other severity-linked metabolites and proteins may help clarify how the signal should be interpreted, or point toward alternative markers that travel with the same biology.



WHAT IT REVEALED — TIMEPOINT 2

That is what makes the BDNF–1-MNA connection interesting. The link is not one-directional: 1-MNA has been reported to restore hippocampal BDNF signalling and produce TrkB-dependent effects in an independent model.¹⁸ So the elevated 1-MNA seen in the most severe neonates and the brain's neurotrophic repair program may be coupled rather than separate stories.

Compensation, or compensation that is failing?

NNMT transfers a methyl group from S-adenosylmethionine to nicotinamide, producing 1-MNA.¹⁹ As 1-MNA is produced by NNMT, its elevation is consistent with altered NNMT-linked nicotinamide metabolism, although circulating abundance alone cannot establish NNMT activity or flux.^{19 20} Read on its own, the elevation looks protective.

But the samples were taken **48 to 72 hours** after therapeutic hypothermia was first initiated, and it is the most severe neonates in whom 1-MNA is highest. A compensatory response that is still active may imply that the insult is ongoing, or that the compensation is insufficient to prevent further damage.

CANDIDATE	TIER	RANK / PERCENTILE	METABOLITE COVERAGE	WHY IT WAS SELECTED
CXCL12	Splash Direct	6 • top 0.04%	36%	CXCL12/CXCR4 signaling limits ROS-mediated damage; transiently upregulated after neonatal hypoxic–ischemic injury
APP	Splash Direct	14 • top 0.09%	50%	Regulates glutamatergic signaling; rapidly induced after neonatal HI injury
CCR2	Splash Direct	18 • top 0.12%	31%	Induced by excitotoxic injury; mediates monocyte-driven inflammatory damage



WHAT IT REVEALED — TIMEPOINT 2

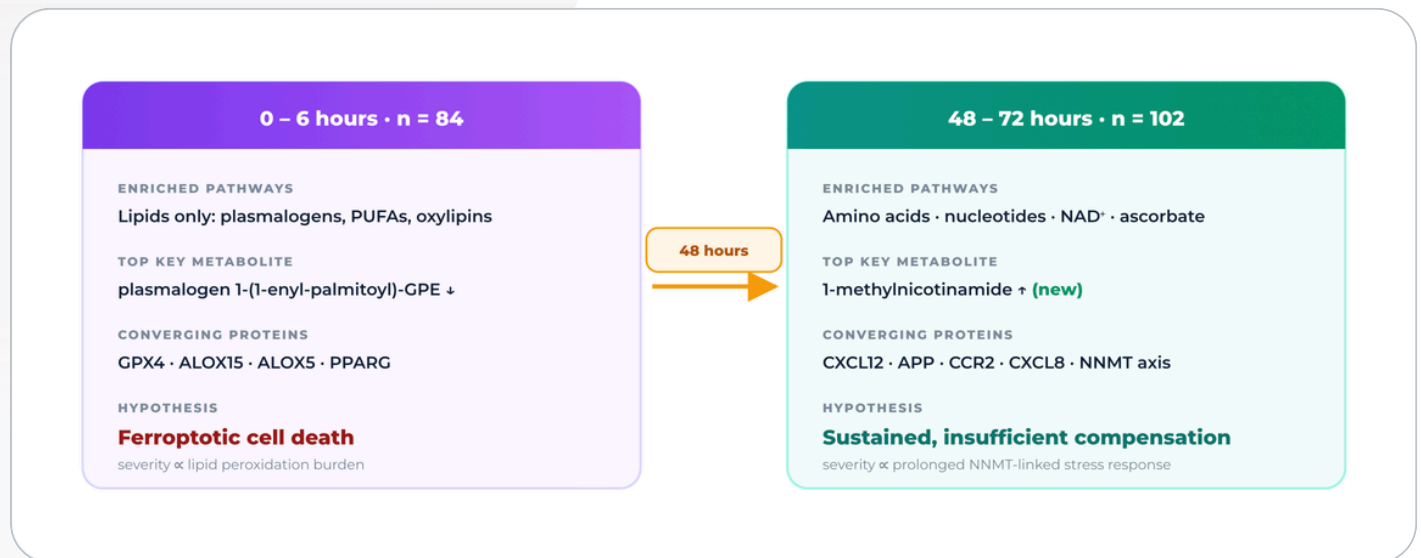
CANDIDATE	TIER	RANK / PERCENTILE	METABOLITE COVERAGE	WHY IT WAS SELECTED
CXCL8	Splash Direct	24 · top 0.16%	42%	IL-8 is elevated in newborns with HIE and correlates with injury severity
ENPP1	Splash Indirect	2,518 · top 16.5%	42%	Hydrolyzes extracellular ATP and NAD ⁺ -derived ADPR, modulating adenosine signaling
BDNF	Splash Indirect	2,526 · top 16.57%	55%	Counters glutamate excitotoxicity; promising neurotrophic biomarker in HIE whose direction remains unsettled.

CXCL8 acts like an internal control: interleukin-8 is an established plasma biomarker of HIE severity in newborns.¹⁶ The modest rank of BDNF demonstrates that multiple features, apart from raw rank, are considered during lead selection.



THE RESULT

Severity in HIE is fluid



WHERE IS THE VALUE?

If severity in HIE is fluid, i.e., it looks different at different timepoints, implicating different systems, proteins, pathways, then this has repercussions for intervention management. The first step towards addressing this challenge is a full understanding of this temporal evolution of severity. **Metabolomics paired with the Splash & Ripple application** is one method to profile *and* contextualise how severity in HIE evolves over time as a step towards better patient management protocols.



THE VALUE OF THE APPROACH

Search-space reduction reveals hypotheses to consider

Splash & Ripple helps researchers move from metabolite measurements to network-informed hypotheses.

Metabolomics revealed two sets of statistically significant compounds implicating divergent drivers of severity at different timepoints. Splash & Ripple provides greater context to each severity signal, and proposes mechanistic hypotheses for a selection of the observed changes.

By placing metabolomic data within the larger context of protein biology, researchers have an increased opportunity for identifying targetable biology. For example, GPX4 is a key regulator of ferroptosis, and understanding its activity in the early severity profile and any pressures acting on its capacity could ultimately inform novel or revised intervention strategies.





THE VALUE OF THE APPROACH

OBSERVED

Measured evidence

The metabolomic profile.

INFERRED

Network position

The selected protein candidates and their relative rank based on their relationship to the metabolomic profile.

HYPOTHESIS

Mechanistic reading

The relationship between individual protein-metabolite pairs is rationalised within the context of the study phenotype. Reasonable arguments are proposed for consideration.



LIMITATIONS AND NEXT STEPS

Informed decision making

Splash & Ripple generates hypotheses. It does not generate proof, and the outputs are explicitly built to make that distinction legible.

LIMITATIONS

What still requires validation

- Protein-level measurements were not made. Every protein here is a network inference, not an observation.
- Dried blood spots are peripheral. A circulating metabolite cannot be assigned to brain production. 1-MNA in particular has substantial renal contribution.
- Metabolite abundance is an indirect measurement of pathway flux or protein activity. The ferroptosis and NNMT interpretations remain testable mechanistic hypotheses.
- Severity associations cannot distinguish drivers of injury from downstream damage, treatment effects or compensatory responses.

NEXT STEPS

Where the hypotheses go

- Targeted quantification of 1-MNA, nicotinamide and NAD⁺ intermediates against graded outcome measures.
- Ferroptosis markers and GPX4/lipoxygenase activity in an appropriately matched model at the acute timepoint.
- Replication of the severity contrast in an independent HIE cohort.
- Longitudinal sampling to determine whether the 1-MNA elevation resolves in neonates with better outcomes.

ACKNOWLEDGMENTS

University of Pretoria

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For inquiries about the NESHIE study, please contact the University of Pretoria team:
Prof. Michael Pepper, email: michael.pepper@up.ac.za.



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Splash & Ripple — Metabolomics Interpretation · Powered by Metabolon

Case study: Profiling dynamic severity in hypoxic ischemic encephalopathy.

Software outputs are not intended for patient diagnosis or treatment. Hypotheses and interpretations require independent validation.

To learn more about how Metabolon can help your study, please **contact us** today.

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