

**Serial blood cytokine and chemokine mRNA and microRNA over 48h are
insult-specific in a Piglet Model of Inflammation-sensitized Hypoxia Ischemia**

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The authors declare that they have no competing interests.

Category of Study

Pre-clinical basic science article.

Consent:

Patient consent was not required for this study.

Impact

- Early stratification of infants with neonatal encephalopathy is key to provide tailored neuroprotection
- IL1A, CXCL8, IL10, CCL2 & NSE mRNA are promising biomarkers of inflammation-sensitized hypoxia
- IL10 mRNA levels differentiated all three pathological states; fold-changes from baseline highest in LPS+Hypoxia animals, followed by LPS and Hypoxia at 6h
- Mir-23, 27, 31-5p and 193-5p were significantly upregulated within 6h of a hypoxia insult
- Functional analysis highlighted the diverse roles of miRNA in cellular processes

Abstract

Background: Exposure to inflammation exacerbates injury in neonatal encephalopathy (NE). We hypothesized that brain biomarker mRNA, cytokine mRNA and microRNA differentiate inflammation (E.coli LPS), hypoxia (Hypoxia), and inflammation-sensitized hypoxia (LPS+Hypoxia) in a NE piglet model.

Methods: Sixteen piglets were randomized: (i) LPS 2µg/kg bolus; 1µg/kg infusion (LPS;n=5), (ii) Saline with hypoxia (Hypoxia;n=6), (iii) LPS commencing 4h pre-hypoxia (LPS+Hypoxia;n=5). Total RNA was acquired at baseline, 4h after LPS and 1, 3, 6, 12, 24, 48h post-insult (animals euthanized at 48h). Quantitative PCR was performed for cytokines (*IL1A*, *IL6*, *CXCL8*, *IL10*, *TNFA*) and brain biomarkers (*ENO2*, *UCHL1*, *S100B*, *GFAP*, *CRP*, *BDNF*, *MAPT*). MicroRNA was detected using GeneChip (Affymetrix) microarrays. Fold-changes from baseline were compared between groups and correlated with cell death (TUNEL) at 48h.

Results: Within 6h post-insult, we observed increased *IL1A*, *CXCL8*, *CCL2* and *ENO2* mRNA in LPS+Hypoxia and LPS compared to Hypoxia. *IL10* mRNA differentiated all groups. Four microRNAs differentiated LPS+Hypoxia and Hypoxia: hsa-miR-23a, 27a, 31-5p, 193-5p. Cell death correlated with *TNFA* ($R=0.69;p<0.01$) at 1-3h and *ENO2* ($R=-0.69;p=0.01$) at 48h.

Conclusions: mRNA and miRNA differentiated hypoxia from inflammation-sensitized hypoxia within 6h in a piglet model. This information may inform human studies to enable triage for tailored neuroprotection in NE.

Introduction

Intrapartum-related neonatal encephalopathy (NE) is a significant global health burden and the third leading cause of death in children under 5 years (1). NE has a complex and multifactorial etiology; over the last decade pre-clinical (2) and clinical (3) studies suggest that co-existing infection and inflammation with hypoxia ischemia (HI) exacerbates brain injury. A strong association exists between fetal infection/inflammation (e.g. chorioamnionitis, funisitis), perinatal brain damage and neurodisability (4).

Therapeutic hypothermia (HT) initiated within 6h of birth is now standard care for term NE in intensive care settings; cooling is only partially protective with approximately 50% of babies with adverse outcomes (5). This may be attributed, in part, to co-existing inflammation-sensitization. Indeed, in a pre-clinical neonatal rodent study, HT was ineffective in inflammation-sensitized HI (6); and in a small prospective study of placental histology relative to magnetic resonance imaging (MRI), HT was less protective in babies whose placentas showed chorioamnionitis (7). Furthermore, immune suppression associated with HT may be deleterious in infants with inflammation-sensitized brain injury (8). Given these data, the key to optimizing long-term outcomes in NE may lie in identifying biomarkers that can detect infants with prior inflammatory exposure for tailored therapeutic strategies.

A meta-analysis of brain injury biomarkers in NE identified pro-inflammatory interleukins (IL)1 β and IL6 and CSF Neuron Specific Enolase (NSE), a glycolytic enzyme found in mature neurons, as indicators of poor prognosis (9). Several other biomarkers have been investigated as indicators of injury severity and prognosis. Glial fibrillary acidic protein (GFAP), a cytoskeletal filament protein in astrocytes, has been used to stratify injury severity in NE in umbilical blood samples (10) and in CSF (11). Brain derived neurotrophic factor (BDNF) has been correlated with clinical severity of NE and poor neurological outcomes (12). Tau, a microtubule associated protein located in axons, has been isolated in CSF in traumatic brain injuries (13). Elevated S100B, a calcium-binding protein found in glial cells has been observed in infants with NE compared to controls in both urine and serum (14). Raised serum UCHL1, a neuron-specific enzyme was associated with 10min Apgar scores and abnormal MRI finding in a pilot study of infants with NE (15).

More recently, micro-ribonucleic acids (miRNAs) have generated significant interest as biomarkers of neurological disease given their relative abundance in the central nervous system (16) and stability in serum. These small non-coding RNAs regulate gene expression by binding to the 3'-untranslated region (3'-UTR) of a target messenger RNA (mRNA), triggering degradation or inhibiting protein translation. Several miRNA have been associated with NE, including miR-374a (piglet, human), miR-210 (piglet, rodents) and miR-376c (human) (17–20).

We have previously shown that exposure to *E.coli* lipopolysaccharide (LPS) administered 4h prior to hypoxia in newborn piglets increased mortality and exacerbated neuronal cell death beyond that observed by inflammation or hypoxia

alone (21). In this current study, our aim was to evaluate gene transcript expression for several brain-specific biomarkers, cytokines and miRNA in serial blood samples taken over 48h following hypoxia (Hypoxia), inflammation (LPS) and combined hypoxia and inflammation (LPS+Hypoxia). We hypothesized that (i) specific gene transcripts and miRNA can discriminate between inflammation-sensitization with and without hypoxia; and (ii) specific gene transcripts are associated with total and regional neuronal death.

Materials and methods

The study was conducted according to UK Home Office Guidelines [Animals (Scientific Procedures) Act, 1986] and complies with the ARRIVE guidelines. The study was approved by the Ethics Committee of UCL.

Animal experiments and surgical preparation

Large white piglets (male) aged <36h were sedated with intramuscular midazolam (150µg) and anesthetized with isoflurane (2-3% v/v), remaining insentient throughout experimentation. Animals were mechanically ventilated (SLE 2000, Surrey, UK) via tracheostomy and settings adjusted to maintain partial pressure of oxygen (PaO₂) and carbon dioxide (PaCO₂) at 8–13kPa and 4.5–6.5kPa, respectively. An umbilical venous catheter (Vygon, UK) was inserted for infusions including maintenance 10% dextrose (60mL/kg/day; 40mL/kg/day post-insult), fentanyl (3µg/kg/h) and antibiotics (benzylpenicillin 50mg/kg BD; gentamicin 4mg/kg OD). An umbilical arterial catheter (Vygon) was inserted for continuous monitoring of heart rate (HR) and mean arterial blood pressure (MABP) and blood sampling (pH, PaO₂, PaCO₂, Base excess (BE), electrolytes, glucose and lactate; Abbot Laboratories, UK). A peripherally inserted

central venous catheter (Vygon) was inserted for LPS or saline infusions. Post-surgery serum lactate was measured to monitor intraoperative hypoxia / hypoperfusion. Hypotension was managed by fluid boluses (0.9% saline 10mL/kg) and inotropes; dopamine 5-25µg/kg/min, dobutamine 5-20µg/kg/min, noradrenaline 0.1-1µg/kg/min and adrenaline 0.1-1µg/kg/min added sequentially as per neonatal guidelines. Heparinized saline (0.9% with 1IU/ml heparin) was infused through the umbilical arterial line.

All piglets received continuous physiological monitoring (SA instruments) and intensive care support. Complications (hyperkalemia, metabolic acidosis, seizures) were treated in accordance with neonatal guidelines. Persistent metabolic acidosis (BE > -10) was treated with sodium bicarbonate (8.4% w/v). Rectal temperature was maintained in the normothermic range (38.0-39.0°C) using a thermoregulatory water mattress (Tecotherm).

Experimental groups

Following surgery and baseline observation, piglets were randomized by computer generated sequence to either (i) LPS, n=5 (LPS 2µg/kg bolus; 1µg/kg infusion for 52h); (ii) Hypoxia, n=6 (saline with hypoxia); and (iii) LPS+Hypoxia, n=5 (same dose LPS commencing 4h prior to hypoxia) (Figure 1).

Inflammation-sensitization

E.coli lipopolysaccharide (LPS) was used to simulate endotoxemia associated with gram negative sepsis. LPS and LPS+Hypoxia animals received a 2µg/kg LPS bolus and 1µg/kg infusion started at 4h pre-hypoxia, modelling an ongoing infective process

commencing in the hours prior to birth.

Hypoxia insult

Piglets in the Hypoxia and LPS+Hypoxia groups underwent a global hypoxia insult. Inspired oxygen (FiO₂) was reduced from 21% stepwise to 4% over 3min and held for 10min. This was followed by a further 10-30min of FiO₂ 6-12% titrated to maintain moderate hypotension (target MABP 26-30mmHg). Insult length was guided by real-time measurements of the duration of isoelectric EEG (<5uV) and hypotension (MABP<30mmHg). The insult was terminated early if cardiac arrest appeared imminent as indicated by profound hypotension or bradycardia.

Blood collection

Arterial bloods were taken at baseline, 4h after the LPS bolus, immediately following insult (t=0) and at 1, 3, 6, 12, 24 and 48h time points post-hypoxia; 0.5mL of whole blood was taken for gene expression analysis and mixed with acid-phenol before being stored at -80°C in cryovials for later RNA extraction.

Brain Histology

52h following administration of an LPS bolus (48h after hypoxia in the LPS+Hypoxia and Hypoxia groups), piglets were euthanized using intravenous pentobarbital and organs fixed through transcardial perfusion with cold phosphate-buffered saline, followed by 4% paraformaldehyde in phosphate-buffered saline. The brain was dissected out and post fixed at 4°C in 4% paraformaldehyde for 7d. Coronal slices (5mm thick) of the right hemisphere, starting anterior to the optic chiasm, were embedded in paraffin wax and sectioned (5µm thick). To assess cell death, sections

were stained for nuclear DNA fragmentation using histochemistry with Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL). Two sections were taken for each animal, one through the hippocampus (R1) and another 5mm anterior (R0). Both sections were assessed for each immunohistochemical stain as described in Martinello et al (21).

RNA extraction

Total RNA was extracted from whole blood using the mirVANA miRNA Isolation Kit (Thermo Fisher Scientific, UK) in accordance with manufacturer's guidelines. RNA quantity was measured (ng/ml) using the Qubit RNA High Sensitivity (HS) Assay Kit (Thermo Fisher Scientific, UK). RNA was converted to complementary DNA (cDNA) using a high capacity reverse transcription kit (Superscript™ VILO™, Thermo Fisher Scientific, UK), according to the manufacturer's guidelines and including control samples without reverse transcriptase and without sample. Total RNA samples were then aliquoted for mRNA and miRNA analysis.

mRNA amplification and analysis

Real-time quantitative PCR (qRT-PCR) was used to amplify the cDNA sequence of interest using porcine-specific Taqman probes for IL1 α (*IL1A*), IL6 (*IL6*), IL8 (*CXCL8*), IL10 (*IL10*), Tumour Necrosis Factor α (*TNFA*), chemokine CCL2/MCP2 (*CCL2*), Brain Derived Neurotrophic Factor (*BDNF*), Glial Fibrillary Acidic Protein (*GFAP*), Ubiquitin Carboxyl-terminal Hydrolase L1 (*UCHL1*), s100b (*S100B*), Microtubule-Associated Protein Tau (*MAPT*) and Neuron Specific Enolase (*ENO2*). Three endogenous references genes were included to allow standardization; Glyceraldehyde-3-Phosphate Dehydrogenase (*GAPDH*), Tyrosine 3-Monooxygenase/Tryptophan 5-

Monooxygenase Activation Protein Zeta (*YWHAZ*) and Peptidylprolyl Isomerase A (*PPIA*) (22–24). Target gene descriptions, function and assay ID are detailed in Table 1. Reference gene expression was not significantly different between study groups. Customised 96-well plates (Thermo Fisher Scientific, UK) were pre-loaded with master mix (DNA polymerase, nucleotide triphosphates, buffers) and relevant primers. Samples were analyzed in triplicate. Relative quantification of gene expression was calculated using the $\Delta\Delta\text{CT}$ method; expression at specific time points were compared to baseline samples across all study groups.

miRNA microarray analysis

Total RNA samples were processed by UK Bioinformatics Ltd. In brief, RNA was labelled using the FlashTag™ Biotin HSR Labelling Kit (Thermo Fisher Scientific, UK) and hybridized to GeneChip miRNA 4.0 microarrays (Affymetrix). Microarrays were preloaded to interrogate all mature miRNA sequences in miRBase, Release 20. Analysis was performed using Expression Console™ software and Transcriptome Analysis Console. Fluorescent signals generated by specific miRNA were compared to baseline values from all groups. Results were filtered to identify statistically significant fold-changes from baseline (False Discovery Rate $p < 0.1$). Cross-species duplicates with identical sequences were removed and the remaining miRNA were evaluated for significance ($p < 0.05$) at specific time points between groups.

Gene Ontology Analysis

Gene ontology (GO) analysis was performed on all miRNA with significant changes from baseline to assess potential biological functions. Accession numbers of equivalent miRNA in humans were obtained from miRBase and used to build a

network with previously experimentally validated microRNA-mRNA target interactions in miRTarBase version 6.1 with the CyTargetLinker plug-in in Cytoscape version 3.2.

GO analysis for molecular function, biological process, immune system, cellular component and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways in human species were conducted with ClueGO and Bonferroni-correction for multiple testing ($p < 0.01$). A summarizing figure was constructed for specific terms with more than 5 genes/term and $p < 0.01$ after fusion of related terms.

Statistics

Statistical analysis was performed using IBM SPSS Statistics (v22.0) Armonk, NY; IBM Corp and JMP® (v14), SAS Institute Inc., Cary, NC, 1989-2019. Physiological data was analyzed using ANOVA (Tukey-Kramer multiple comparison correction); pairwise comparisons were made using a T-test or Mann-Whitney U test as appropriate. Comparisons were made between groups by calculating least mean square differences and performing an ANOVA. miRNA data was \log_{10} transformed to account for negative fold-changes. Pairwise correlation (R^2) was undertaken between mRNA/miRNA levels and TUNEL counts (\log_{10} count/ mm^2), and mean aEEG scores. In the event of a cardiac arrest during experimentation, all mRNA/miRNA data already acquired was included in the analysis.

Results

Baseline characteristics and physiological parameters

Sixteen large white male piglets aged <36h were studied. Mean piglet weight was similar between groups (1960g, range 1650-2100g; $p=0.216$). Baseline physiological parameters (HR, MBP, Temp) were within acceptable limits (Table 2). Baseline blood gases were more alkalotic in animals in the Hypoxia group. Significant differences in other parameters (core temperature, HR, MABP, BE, Lactate) were within normal limits.

Hypoxia Insult

Insult severity as determined by duration of hypoxia, hypotension (MABP<27mmHg), isoelectric EEG and area under the curve (AUC) FiO_2 , was similar between groups (Table 3). There was a trend towards shorter duration of EEG<5 μ V during insult in the LPS+Hypoxia group compared to Hypoxia ($p=0.07$). End of insult blood gases were similar between groups.

Survival and clinical illness severity

As reported in Martinello et al. (21), mortality was significantly increased in LPS+Hypoxia; 3 out of 5 LPS+Hypoxia piglets died compared to none in other groups ($p=0.022$). One piglet died within minutes of insult cessation; two piglets arrested at approximately 24h (mRNA data was acquired up to 24h). Two piglets (one LPS, one Hypoxia) required resuscitation following mechanical airway obstruction and survived to experiment completion.

Both LPS and LPS+Hypoxia animals required saline boluses and multiple inotropes to maintain hemodynamic stability, however there was no significant overall difference

between groups (Table 4). Hypoxia and LPS+Hypoxia animals required higher doses of dopamine, dobutamine, adrenaline and saline than LPS animals ($p \leq 0.046$).

Gene Expression for cytokines and chemokines

Total RNA was acquired for all LPS and Hypoxia animals. In LPS+Hypoxia, RNA was obtained in 4 out of 5 animals until 24h and 2 out of 5 animals at 48h (Supplemental file S1 (online)).

Comparisons of gene transcripts levels at specific time points identified pro-inflammatory (*IL1A*), chemotactic (*CXCL8*, *CCL2*) and anti-inflammatory (*IL10*) mRNA were significantly upregulated in LPS+Hypoxia and LPS compared to Hypoxia within 6h post-insult ($p < 0.01$; Figure 2). *IL10* mRNA levels differentiated all three pathological states; fold-changes from baseline were highest in LPS+Hypoxia, followed by LPS and Hypoxia at 6h ($p < 0.033$). Elevated *IL1A* and *IL10* mRNA levels persisted in animals receiving LPS+Hypoxia, compared to LPS and Hypoxia. *IL6* mRNA levels, thought to have both pro- and anti-inflammatory activity, was higher at 3h in LPS+Hypoxia compared to LPS ($p = 0.01$) with a trend to an increase compared to Hypoxia animals ($p = 0.06$) and a second peak at 24h post-insult ($p = 0.03$). *CXCL8* mRNA also demonstrated a biphasic response to LPS+Hypoxia, peaking at 3-6h and 24h post-insult ($p < 0.01$). Pro-inflammatory *TNFA* mRNA decreased in the first 6h in all study groups, however increased at 24h post-insult in LPS+Hypoxia and LPS compared to Hypoxia ($p < 0.01$). Complete mRNA data is listed in Supplemental file S1 (online).

Gene Expression for brain-specific biomarkers

ENO2 mRNA (neuron-specific enolase) significantly increased at 1h post-insult in LPS+Hypoxia and LPS compared to Hypoxia ($p=0.04$ and $p=0.01$, respectively). The expression of this transcript was also significantly higher at 3h in LPS+Hypoxia compared to LPS ($p=0.05$) (Figure 3).

MAPT mRNA (encoding tau protein) increased in LPS at 6h and 48h ($p=0.04$) and LPS+Hypoxia at 24h ($p<0.04$) compared to Hypoxia. *BDNF* mRNA (neurotrophic factor) was higher in LPS+Hypoxia compared to Hypoxia from 3h post-insult, reaching statistical significance at 24h ($p=0.03$).

UCHL1 mRNA (a neuron-specific enzyme) did not distinguish between groups at any time points. *S100B* mRNA, predominantly found in glial cells, varied significantly between groups at baseline and remained elevated in animals receiving LPS. *GFAP* mRNA (cytoskeletal filament protein) and *CRP* (inflammatory biomarker) were undetectable in several samples across all study groups and insufficient for analysis.

Expression of microRNA

In total, 4929 miRNAs were analyzed; we identified 22 miRNAs with significantly altered expression from baseline (FDR p -value <0.1 ; see Supplementary file S2 (online)). Significant changes from baseline levels were seen in 12 miRNAs in LPS (2 up, 10 down), 11 miRNAs in Hypoxia (all down) and 7 miRNAs in LPS+Hypoxia (2 up, 5 down).

Out of the 22 miRNA, we identified 8 promising candidates that discriminated between study groups at early ($<6h$) and late (24h) time points. In the first 6h post-insult, hsa-

mir-23a-5p and hsa-mir-27a-5p levels increased significantly in LPS and LPS+Hypoxia compared to Hypoxia at 1h, 3h and 6h (all $p < 0.01$). Also, hsa-mir-31-5p and hsa-mir-193-5p levels were significantly lower in LPS+Hypoxia and LPS compared to Hypoxia immediately post-insult ($p < 0.01$), though this difference was no longer significant by 1h ($p = 0.06$) (Figure 4A-D). There were 4 miRNAs demonstrating differential expression between groups at 24h; hsa-mir-181c-3p, hsa-mir-330-3p, hsa-mir-182-5p and hsa-mir-150-5p (Figure 4E-H).

Biomarkers of Injury Severity

Candidate biomarkers were assessed as markers of injury severity by correlating fold-changes with overall TUNEL counts (cell death) and aEEG scores.

We observed a significant positive correlation between overall TUNEL-positive cell counts and *TNFA* ($R = 0.69$, $p < 0.01$) and *S100B* ($R = 0.53$, $p = 0.04$) mRNA at 1h post-insult and a negative correlation in *ENO2* mRNA at 48h ($R = -0.69$, $p = 0.01$). Although *IL10* mRNA levels appeared to coincide with injury severity, we did not find a statistically significant correlation with cell death.

We identified 9 out of 22 significantly altered miRNAs that correlated with TUNEL-positive counts within 6h of insult; ssc-mir-31 ($R = 0.59$, $p = 0.027$), ssc-mir-199a-5p ($R = 0.79$, $p = 0.001$) and hsa-mir-214-5p ($R = 0.66$, $p = 0.01$) at 1h post insult; hsa-mir-150-5p ($R = 0.63$, $p = 0.02$) at 3h post-insult; ssc-mir-4334-3p ($R = 0.79$, $p < 0.01$) and hsa-mir-181c-3p ($R = 0.73$, $p < 0.01$) 6h post insult. Notably, hsa-mir-193a-5p and ssc-mir-99b were strongly associated with cell death at 1h, 3h and 6h post-insult ($R > 0.70$, $p < 0.01$ at 6h).

There was no significant correlation between aEEG scores and mRNA/miRNA levels (data analysis included in Supplementary File S3 (online)).

miRNA and mRNA Networks

There were several unique and overlapping predicted mRNA targets associated with the miRNA isolated at 12h in LPS, Hypoxia and LPS+Hypoxia (Figure 5). Nucleic acid metabolism, transcription and translation were affected in all three groups, though greatest in the Hypoxia group. HI is known to affect genomic integrity, triggering activation of the repair enzyme PARP-1, found only in the Hypoxia group. Whilst repair of DNA is beneficial, overactivation signals irreparable injury and triggers apoptosis. All insults affected cell-cycle control proteins, particularly p53, which lead to enrichment of cancer-related pathways. Genes related to mitochondrial membrane permeabilization were enriched in all study groups. Pathways downregulating oxidative stress-induced cell death was observed in LPS+Hypoxia only.

GO analysis also highlighted several differences in predicted biological pathways. The Hypoxia group enriched pathways related to brain development (forebrain development, regulation of neurogenesis, neurotrophin signalling pathways), cellular components (spindles, chromosomes) and protein activity (protein deacylation, serine/threonine kinase activity). Organ development pathways appeared more enriched LPS+Hypoxia and LPS, compared to Hypoxia animals; suggesting a more systemic nature to the injury mechanism. Interestingly, the signalling pathways for pro-inflammatory transcription factor NF- κ B were enriched in the LPS group, but not

LPS+Hypoxia group (Figure 6A-C). Full GO data included in Supplementary File S4 (online).

Discussion

Using a piglet model of inflammation-sensitized hypoxia, we present the analysis of serial serum inflammatory and brain-specific biomarkers that discriminate between hypoxia associated with and without inflammation within 6h of the hypoxia insult.

Cytokines

Several studies have observed an association between raised pro-inflammatory cytokines and adverse neurological sequelae in infants with NE (10). Elevated $IL1\alpha$, 6, 8 and $TNF\alpha$ in the first 24h correlate with abnormal MRI (25) and poor neurodevelopmental outcomes at 12-24 months (10,25). In our study, animals receiving an inflammatory insult had significantly higher *IL1A*, *CXCL8*, *CCL2* and *IL10* mRNA within 3h of insult compared to hypoxia injury. These biomarkers may help differentiate the predominate pathological process in infants with NE.

Cytokines and chemokines are not exclusively pro- or anti-inflammatory; for example, $IL6$ increases vascular permeability and causes cerebral oedema at high concentrations (26), however also inhibits $TNF\alpha$ and $IL1$, and promotes nerve growth factor secretion (27). $IL6$ has been reported as a poor prognostic marker in some (10,28,29), but not all studies (30). We observed a two-fold increase in *IL6* mRNA in Hypoxia and eight-fold in LPS+Hypoxia animals within 3h of injury, reflecting the increased injury associated with dual pathology. Interestingly, we noted a secondary

peak in IL6 and CXCL8 (encoding IL8) mRNA at 24h in LPS+Hypoxia. A similar biphasic profile was observed in hypothermia treated infants with good outcomes (28), suggesting this increase was not necessarily pathological. Persistent elevation of IL6 however was associated with poor prognosis. The delayed rise in IL6 and IL8 may represent activation of reparative processes, the success of which determines whether these cytokines fall or remain elevated. In our study, this biphasic pattern was observed in LPS+Hypoxia only, the group with most severe injury. Due to the limited data at 48h, it remains unclear whether these cytokines would have remained elevated had more animals survived.

The most promising differentiating biomarker among the cytokines was *IL10* mRNA, distinguishing between all 3 pathological states; transcripts were highest in LPS+Hypoxia and lowest in Hypoxia at 6h post-insult. IL10 is predominately anti-inflammatory (31) and has shown neuroprotective benefit in small animal models of NE. Raised IL10 however is associated with adverse outcomes in NE (28,32) and sepsis (33), possibly reflecting an exaggerated response to severe infection/inflammation. Although its precise role in the evolution of injury is unclear, it remains a useful marker differentiating inflammation from hypoxia injury.

Brain biomarker

Disruption to the blood brain barrier and leakage of brain-specific proteins into the extracellular space permits detection of these biomarkers in serum (34,35), urine (36) and CSF (9,37). *ENO2* mRNA was the only brain biomarker significantly altered in LPS+Hypoxia compared to Hypoxia within hours post-insult. The *ENO2* gene encodes for NSE, a glycolytic enzyme predominately found in neurons. Elevated NSE in serum

(38) and CSF (37) have been associated with increased mortality and adverse outcomes at 12 months in infants with NE. Though predominately found in neurons, NSE is also expressed in red blood cells; serum levels may therefore overestimate brain injury, particularly in the presence of haemolysis. Importantly, *ENO2* mRNA differentiated injury subtypes within the 6h therapeutic window for hypothermia, providing an opportunity to initiate alternative strategies.

Other brain-specific biomarkers investigated were not as discriminative. *MAPT* (tau) mRNA levels appeared higher in LPS+Hypoxia compared to Hypoxia throughout the experiment, though only reached statistical significance at 24h. Similarly, expression of *BDNF*, a neurotrophin secreted by neurons and astrocytes, was elevated at 24h only in the LPS+Hypoxia group. The mRNA for *UCHL1*, a gene encoding for a cysteine protease expressed in neurons, was increased in all groups however did not differentiate between different injury subtypes. Surprisingly, *GFAP* mRNA, a cytoskeletal protein in astrocytes, was undetectable in several samples despite the extensive cell death seen in some animals.

Markers of Injury Severity

Combining data from all animals, we examined whether fold-changes in cytokines or brain biomarkers were associated with TUNEL-positive cells (cell death). *TNFA* mRNA was the only cytokine that correlated with overall TUNEL counts at 1h and 3h, indicating it may be an early marker of injury severity. *ENO2* mRNA negatively correlated with cell death at 48h post-insult. Our group previously identified a significant correlation between serum IL1 β , IL10 and CSF TNF α and MRS biomarkers (Lac/NAA) and IL1 β and IL8 with TUNEL-positive cells in the thalamus in a piglet

model of NE (30). Gene expression of these cytokines however was not associated with regional or overall cell death. Patterns of aEEG are useful prognostic indicators in NE (39), we however did not find significant correlation with any cytokine or brain biomarker transcript levels.

MiRNA Biomarkers

MiRNAs are non-coding sequences of RNA that regulate gene expression and play an essential role in early neurological development (40). We identified 4 promising candidates as early differentiating biomarkers of infection-sensitized hypoxia; hsa-mir-23a, hsa-mir-27a, hsa-mir-31a-5p and hsa-mir-193-5p. Both miR-23 and mir-27, in combination with miR-24 play an important role in T-cell activation and differentiation. Mir-31, analogous to mir-31a-5p, regulates transcription factors, including hypoxia-inducible-factor 1 α (HIF1 α) and nuclear factor-kappa B (NF- κ B). Both hsa-mir-31 and hsa-mir-193-5p correlated with neuronal cell death 1h post-insult, therefore may be indicators injury severity.

Hsa-mir-181c-3p, hsa-mir-330-3p, hsa-mir-182-p and hsa-mir-150-5p were down-regulated in all groups, particularly in LPS and LPS+Hypoxia compared to Hypoxia animals at 24h. These miRNAs were significantly induced following LPS and may be useful in identifying ongoing infective or inflammatory pathologies. Mir-150-5p is highly expressed in mature B-cells and T-cells; playing a critical role in immune cell differentiation. Mir-150-5p has also been identified as a prognostic marker following acute ischaemic stroke (41), consistent with the positive correlation with overall cell death in this study. Mir-181c-3p has previously been linked to the hypoxic response (42), downstream of HIF1 α (43). We identified a positive correlation between this

miRNA and neuronal cell death, suggesting a potential role in the pathogenesis of neurological injury, which needs further study.

Several miRNAs have been identified to play a key role in HI and neuroinflammation. The most notable being miR-210, referred to as a master hypoxamiR and consistently altered in adult rodent models of ischemic stroke (19,44). Serum miR-210 was increased in pregnancies complicated by fetal growth restriction, a condition associated chronic fetal hypoxia (45). MiRNA may influence several genes simultaneously and are therefore well placed as regulators of inflammation. MiR-155 has potent pro-inflammatory properties, including the down-regulation of suppressor of cytokine signalling 1 (SOCS-1) protein and increasing cytokine and NO production (46). MiR-146a is anti-inflammatory, downregulating the TNF receptor-associated factor 6 (TRAF6) and interleukin-1 receptor-associated kinase 1 (IRAK1) adaptor molecules and thus inhibiting TLR4 activation. Interestingly, we did not observe significant changes in these miRNAs in any groups in this study.

Functional analysis of miRNAs highlighted several key pathophysiological processes, including cell cycle, signal transduction, DNA damage, transcription / translation and apoptosis. Genes related to mitochondrial membrane permeability were enriched in all groups, confirming mitochondrial injury as one of the hallmarks of secondary energy failure in NE. Downregulation of oxidative stress-induced cell death pathways was only seen in LPS+Hypoxia and may represent activation of compensatory mechanisms.

Timing of Injury

Identifying the timing of perinatal brain injury is a significant challenge in clinical practice and has significant implications on both prognosis and efficacy of treatment. Our model identified combinations of biomarkers that reached peak levels at different timepoints post-HI; ENO2 and CCL2 at 1-3h; ILA and IL10 by 6h; and TNFA and MAPT at 24h. However, these data are from a model with precise timings of inflammation and hypoxia and application in the clinical setting is much more complex.

Limitations

The main limitation of the study was the high mortality in LPS+Hypoxia, reducing available data at 48h; we were however able to obtain mRNA/miRNA for 15 out of 16 animals up to 24h. The early mortality in LPS+Hypoxia limits the interpretation of results at 48h. These animals represent the most severe spectrum of injury and therefore our results may underestimate cytokine and brain biomarker mRNA levels at this time point.

Animals in this study were not cooled. As hypothermia may be less effective in inflammation-sensitized hypoxia, we focussed on biomarkers during normothermia within the 6h treatment window to facilitate decision-making to initiate alternative neuroprotective strategies. Hypothermia may influence RNA dynamics through the induction of cold-inducible binding proteins (CIRBPs), decreasing inflammatory cytokine production (47) and attenuating caspase-dependent apoptosis (48). Hypothermia also alters miRNA regulation; miR-874 and miR-451 were significantly increased in normothermia compared to hypothermia treated adult rodents 7h post-traumatic brain injury (49). Hypothermia induces the glycine-rich RNA-binding protein (RBM3), disinhibiting Dicer, the enzyme responsible for cleaving pre-cursor pre-

miRNA into its mature counterpart (50). Further study in hypothermia-treated animals is warranted.

There are also limitations when inferring injury mechanisms using gene expression, particularly as proteins are subject to post-translational regulation and may not correlate with their respective mRNA transcripts (51).

This study raises more questions which could be investigated in future work. The following are important areas to study: evaluation of the influence of timing of LPS administration on cytokine expression and outcome, exploration of brain tissue mRNA expression to establish the specific cell lines involved, correlation of gene expression with protein levels and exploration of gender-specific differences.

Conclusions

Using a large animal model of inflammation-sensitized hypoxia, we investigate an extensive panel of biomarkers at clinically relevant time points. Within 6h of insult, *IL1A*, *CXCL8*, *IL10*, *CCL2* and *ENO2* mRNA increased in animals receiving LPS+Hypoxia and LPS compared to Hypoxia. *IL10* mRNA differentiated all three pathological states within this timeframe. These biomarkers may inform the triage of tailored treatments for infants with NE. This study also demonstrates the feasibility and clinical utility of mRNA-based biomarkers, supporting the development of gene expression testing as point-of-care diagnostics. These data require validation in the clinical setting and may facilitate tailored neuroprotective strategies to improve long term outcomes.

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Figure 1. Study Protocol. 16 piglets were randomized to (i) LPS (n=5), (ii) Hypoxia (n=6), (iii) LPS+Hypoxia (n=5). Total RNA was acquired at baseline, 4h after LPS and 1, 3, 6, 12, 24, 48h post-insult. Animals were euthanized at 48h.

Figure 2. Serum mRNA expression of inflammatory cytokines (A-D), anti-inflammatory cytokine (E) and chemokine (F) over 48h. *IL1A*, *CXCL8*, *IL10* and *CCL2* mRNA were significantly upregulated in LPS+Hypoxia compared to Hypoxia within 6h post-hypoxia.

Figure 3. Serum mRNA expression of brain-specific proteins over 48h; *ENO2* (B) was the only brain biomarker to differentiate between Hypoxia and LPS+Hypoxia within 6h. *BDNF* (A) and *MAPT* (C) mRNA were significantly upregulated in LPS+Hypoxia at 24h post-insult. Expression of *GFAP* and *CRP* mRNA was insufficient for analysis.

Figure 4. Specific miRNA that discriminate between Hypoxia and LPS+Hypoxia at early (A-D) and late time points (E-H). Hsa-mir-23a-5p and 27a were significantly upregulated in LPS+Hypoxia compared Hypoxia within 6h post-insult.

Figure 5. Unique and overlapping gene targets associated with significant miRNA isolated in LPS, Hypoxia and LPS+Hypoxia groups. The miRNA isolated in the LPS+Hypoxia group accounted for the largest proportion of unique mRNA targets.

Figure 6. Gene ontology (GO) analysis predicting mRNA targets of miRNAs identified in LPS+Hypoxia (A), Hypoxia (B) and LPS (C). High-specificity GO plots were constructed for significantly enriched terms ($p < 0.01$) with > 5 genes/term after GO-term fusion.