Introduction

Perinatal brain injury from oxygen deprivation (hypoxia-ischemia, HI) at birth occurs in 1–4 live-born babies per 1,000 deliveries at term, and is a leading cause of neonatal encephalopathy (NE), and subsequent death or disability such as vision and hearing loss, and neurocognitive and behavioral impairments including cerebral palsy (CP) [1]. Further, infants with NE who do not develop CP still have lower IQ, perceptual reasoning, and verbal comprehension [2]. Therapeutic hypothermia (TH) is now routine care for infants with moderate-to-severe NE, to reduce death or disability [3], but needs to be started within 6 h from birth for effective neuroprotec-
| Markers          | Design                                                                 | Subjects                                                                 | Finding                                                                 | Correlated outcomes                                                                 |
|------------------|------------------------------------------------------------------------|--------------------------------------------------------------------------|--------------------------------------------------------------------------|-------------------------------------------------------------------------------------|
| Apgar score      | Ancillary analysis of NICHD-NRN-RCT on TH for NE [61]                  | 174 TH and non-TH infants with SS grade II–III NE                         | Low 10-min (0–3) Apgar scores in 64/85 infants                           | Death or disability at 6–7 years of age on WPPSI-III [12], with OR of 0.68 (95% CI: 0.57–0.82) for each point increase in 10-min Apgar |
| Sarnat stage     | Retrospective cohort, 2011–2013, National Asphyxia and Cooling Register, Switzerland | 164 TH infants with SS grade II–III NE                                     | 102 (62%) infants had short-term improvement on SS, from admission to 4 days of life | OR of 0.118 (95% CI: 1.1–2.0) for disability at 18–24 months of age on BSID-II/III and GMDS [3] |
| Thompson score   | Retrospective cohort, 2007–2011, University Ulm, Germany              | 33 TH infants with SS grade II–III NE                                     | Increase in peak TS                                                      | OR of 1.5 (95% CI: 1.1–2.0) for death or cognitive impairment on WPPSI-III. Better prediction from 3 days of life [17] |
| Acid-base        | Multicenter prospective cohort                                          | 230 infants with SS grade I–II–III NE                                    | Increased BD in neonatal blood at 30–45 min after birth                  | Threshold of 14 mmol/L predictive of SS grade II–III NE; sens 73.2% and spec 82% [24] |
| *Prospective cohort |                                                                                                                                           | 174 non-TH infants with NE                                               | BD of 12–16 mmol/L and >16 mmol/L on cord blood at birth                 | Increased incidence of SS grade II–III NE and neonatal complications, respectively [23] |
| S100B            | *Retrospective cohort, 1999–2001, Skåne University, Sweden            | 13 infants with SS grade II–III NE, 21 healthy infants                   | Increased S100B levels in cord blood at birth                            | SS grade of NE, aEEG abnormalities, acidois, death or disability at 6 years of age [27] |
|                  | **Prospective cohort**                                               | 83 TH infants with SS grade II–III NE                                   | Increased neonatal or cord blood S100B at 0–72 h of life                 | Brain injury on MRI, death or BSID-II MDI (OR 2.5 [95% CI: 1.3–4.8]) or PDI (OR 2.6 [95% CI: 1.2–5.6]) scores <70 at 15 months [108, 29] |
|                  | **Prospective cohort**                                               | 176 infants with SS grade I–II–III NE                                   | Increased S100B in neonatal serum <6 h of birth                          | SS grade of NE (AUC = 0.73 for SS I/II and 0.85 for SS III, NBNA scores <35 at day 7 9–0.585, p < 0.001) [65] |
| NSE              | Prospective cohort                                                    | 43 TH infants with SS grade I–II–III NE                                  | Increased NSE concentrations in CSF at 12–72 h of life                   | Seizures, abnormal aEEG and/or MRI adverse outcomes at 2 years (death, CP, and/or BSID-II <70) (PPV 1.00 [95% CI: 0.49, 1.00], NPV 0.96 [95% CI:0.69, 1.00]) [28] |
|                  | **Prospective cohort**                                               | 83 TH infants with SS grade II–III NE                                   | Increased NSE in neonatal or cord blood at 0–72 h of life               | Brain injury on MRI, death or BSID-II MDI (OR 2.1 [95% CI: 1.2–3.6]) or PDI (OR 2.1 [95% CI: 1.2–3.6]) scores <70 at 15 months [108, 29] |
|                  | **Prospective cohort**                                               | 176 TH infants with SS grade I–II–III NE, 80 healthy infants            | Increased NSE in neonatal serum <6 h of birth                           | SS grade of NE (AUC = 0.81 for SS I/II and 0.89 for SS III, NBNA scores <35 at day 7 of life (r = –0.61, p < 0.001) [65] |
| NFL              | Brain injury biomarkers in newborns RCT [32]                          | 37 TH infants with SS grade I–II–III NE                                  | Increased NFL in neonatal plasma during and after TH                     | SS grade of NE, MRI brain injury, abnormal BSID-III at 2.7 years (after TH, median 98 h, NFL cut-off level >436 pg/mL, PPV 75%, NPV 77%) [31, 32] |
| Tau              | **Prospective cohort**                                               | 103 TH infants with SS grade II–III NE                                  | Increased tau protein in neonatal plasma at 72–96 h of life              | MRI brain injury, death or development delay at 1 year of age on BSID-III (accuracy 91%, OR 2.6 [95% CI: 1.36–4.95]) [41] |
|                  | **Prospective cohort**                                               | 41 infants with NE, 35 healthy infants                                  | Increased tau protein in neonatal serum <24 h of life                    | Abnormal GDS at 9 months of life (cut-off level) 933.04 pg/mL, AUC 0.86 (95% CI: 0.74–0.98), sens 100%, spec 70.8%, SS grade of NE [38] |
| MCP-1            | Ancillary analysis of NICHD-NRN-RCT on TH for NE [61]                 | 109 infants with SS grade II–III NE                                     | Increased MCP-1 in neonatal plasma at day 7 of life                      | Death or disability on BSID-III at 6–7 years of age (OR 3.7, 95% CI: 1.42–9.61) [62] |
### Table 1 (continued)

| Markers | Design | Subjects | Finding | Correlated outcomes |
|---------|--------|----------|---------|---------------------|
| AvA     | Prospective cohort | 24 infants with PA, 34 healthy infants | Increased AvA concentrations on cord blood at birth | PA, with cut-off 0.208 ng/mL, sens 99.1%, spec 26.7% [110] |
|         | Ancillary analysis of BiHIVE 2 RCT [66] | 28 infants with PA, 23 infants with NE, 50 healthy infants | No differences in AvA and Acvr2b mRNA on cord blood at birth between groups | Not predictive of SS grade of NE or disability up to 36 months of age on BSID-III [36] |
| UCH-L1  | Prospective cohort | 41 infants with NE, 50 infants with PA, 40 healthy infants | Increased mUCH-L1 in cord blood at birth; no difference between NE and PA groups | Not predictive of NE [44] |
|         | Prospective cohort | 20 TH infants with SS grade II–III NE | Increased UCH-L1 in neonatal blood at start of TH and 72 h | Death or brain injury on MRI. Sens 75%, spec 100% at start of TH [29] |
|         | Retrospective cohort, 1990–2001, Skåne University, Sweden | 15 infants with SS grade II–III NE, 31 healthy infants | UCH-L1 in cord blood at birth; no difference between groups | Not predictive of SS grade of NE, abnormal EEG, death or deficits up to 6 years of age [42] |
|         | *Prospective cohort | 11 non-TH infants with SS grade II NE, 15 healthy infants | UCH-L1 in neonatal serum at 24–48 h of life; no difference between groups | No outcomes reported [43] |
| MDA     | *Prospective cohort | 40 infants with SS grade I–II–III NE, 40 healthy infants | Increased MDA in cord and neonatal blood at birth and at 48 h of life | Seizure burden, mortality [46] |
|         | *Prospective cohort | 20 infants with PA, 20 healthy infants | Increased MDA in cord blood at birth | SS grade of NE, mortality [47] |
|         | Prospective cohort | 31 infants with NE, 30 healthy infants | Increased MDA in neonatal blood at day 5 of life | SS grade of NE (r 0.466, p = 0.008) [49] |
| ISOPs   | *Ancillary analysis of HYPOTOP RCT of topiramate plus TH for NE [111] | 51 TH infants with SS grade II–III NE | Increased total isoprostanoids in neonatal urine <6 h, and at 12, 24, 48, 72, and 96 h of life | Brain injury on MRI [48] |
| SOD     | Prospective cohort | 31 infants with NE, 30 healthy infants | Increased SOD in neonatal blood at day 5 of life | SS grade of NE (r 0.506, p = 0.004) [49] |
| GFAP    | Prospective cohort | 23 infants with NE, 23 healthy infants | Increased GFAP in neonatal serum <6 h and first week of life | SS grade of NE, abnormal brain MRI (GFAP at admission; sens 50%, spec 84.6%, PPV 60%, NPV 78.6%) [54] |
|         | Prospective cohort | 103 TH infants with SS grade II–III NE | Increased GFAP in neonatal serum at 96 h of life (sens 31%, spec 96% for outcome) | Death or neurodevelopmental delay up to 2.5 years of age on BSID-III assessment [41] |
|         | Prospective cohort | 11 non-TH infants with SS grade II NE, 15 healthy infants | GFAP in neonatal serum at 24–48 h of life; no difference between groups | No outcomes reported [43] |
|         | Prospective cohort | 20 TH infants with SS grade II–III NE | Increased GFAP in neonatal blood at 24 and 72 h of life | Death or MRI brain injury (GFAP at 72 h; cut-off level of 0.2 ng/mL, sens 87.9%, spec 82%) [29] |
|         | Ancillary analysis of BiHive RCT [66] | 86 TH infants with SS grade I–II–III NE, 83 healthy infants | GFAP in cord blood at birth; no difference between groups | Not predictive of PA versus NE, SS grade of NE, eligibility for TH, or abnormal BSID-III outcome at 36 months of life [56] |
|         | Retrospective cohort, 1990–2001, Skåne University, Sweden | 15 infants with SS grade II–III NE, 31 healthy infants | GFAP in cord blood at birth; no difference between groups | Not predictive of NE, SS grade of NE, acidemia, abnormal EEG, death or neurological sequelae up to 6 years of age [42] |
| Interleukins | Ancillary analysis of RCT of rEpo for neuroprotection in NE [109] | 50 infants with SS grade II–III NE | Increased IL-1ß, IL-6, IL-8, IL-10, and IL-13 in neonatal serum at day 1 of life | Global brain injury on MRI (Spearman; IL-1ß = 0.1, IL-6 = 0.13, IL-8 = 0.25, IL-10 = 0.24, IL-13 = 0.1 [39] |
|         | *Prospective cohort | 82 infants with SS grade I–II–III NE, 12 healthy controls | Increased IL-8 in neonatal serum at days 1 and 2 of life | Death and SS grade II/III NE, respectively [60] |
|         | Prospective cohort | 103 TH infants with SS grade II–III NE | Increased IL-6, IL-8, IL-10 in neonatal plasma <24 h of life | Death or severe MR brain injury (accuracy 78.7%, AUC 0.83) [41] |
| Markers | Design | Subjects | Finding | Correlated outcomes |
|---------|--------|----------|---------|---------------------|
| TNF-alpha | Ancillary analysis of NICHD-NRN RCT on TH for NE [61] | 109 infants with SS grade II–III NE | Increased TNF-α in neonatal plasma at day 0–1 of life | No association with death or CP [62] |
| Interferon-γ | Ancillary analysis of RCT of rEpo for neuroprotection in NE [109] | 50 infants with SS grade II–III NE | Increased IFNγ in neonatal plasma on day 1 of life | Global brain injury on MRI (Spearman; r = 0.08) [39] |
| GM-CSF | *Prospective cohort | 82 infants with NE, 12 healthy infants | Increased GM-CSF in neonatal serum at day 1 and days 6–7 of life | Death and composite language BSID-III scores at 18–24 months of age, respectively [64] |
| RANTES | Ancillary analysis of NICHD-NRN RCT of TH for NE [61] | 109 TH and non-TH infants with SS grade II–III NE | Lower RANTES in neonatal blood at days 0–7 of life | Death or disability at 6–7 years of life on BSID-II (OR 0.31 [95% CI: 0.13–0.74]), hearing loss, epilepsy [62] |
| miRNAs | Prospective cohort | 167 TH infants with SS grade I–II–III NE, 82 healthy infants | Reduced miR-210 and miR-374a levels in cord blood at birth | SS grade of NE, NBNA scores at day 7 of life (miR-210, r = 0.573; miR-374a, r = 0.631) and GDS scores at 48 and 52 weeks of life [64] |
| | Prospective cohort | 176 TH infants with SS grade I–II–III NE, 80 healthy infants | Reduced miR-199a levels in cord blood at birth | SS grade of NE, NBNA scores at day 7 (r = 0.644) and GDS scores at 48 (r = 0.528) and 52 weeks (r = 0.509) of life [65] |
| | *BiHiVe 2 RCT [66] | 25 infants with SS grade I–II–III NE, 26 infants with PA, 22 healthy infants | Reduced miR-374a-5p, miR-376c-3p, and miR-181b-5p levels in cord blood at birth | Presence NE and/or PA, SS grade of NE, and eligibility for TH, respectively [44, 66] |
| | BiHiVe 1/2 RCT [66] | 44 infants with SS grade I–II–III NE, 43 infants with PA, 37 healthy infants | Increased Fzd4 and Nfat5 mRNA levels in cord blood at birth | SS grade of NE and eligibility for TH (Fzd4 mRNA only), and adverse BSID-II outcomes (Nfat5; sens 71%, spec 76%, PPV 29%, NPV 95%, Fzd4 sens 71%, spec 90%, PPV 50%, NPV 96%) [67] |
| CK | *Prospective cohort | 43 TH infants with SS grade I–II–III NE | Increased CK in neonatal serum at admission | SS grade of NE [73] |
| LDH | Prospective cohort | 43 TH infants with SS grade I–II–III NE | Increased LDH in neonatal serum at day 3 of life | SS grade of NE [73] |
| ALT | Retrospective cohort, 2012–2014, Southmead Hospital, North Bristol NHS Trust, UK | 79 infants with PA | Increased ALT in cord blood <6 h of birth | SS grade II/III NE (plus TH), AUC 0.78 (0.60–0.96) [71] |
| | *Retrospective cohort, 2006–2011, 4 NICUs in the UK | 361 TH infants with SS grade I–II–III NE | Increased ALT in neonatal blood in first 7 days of life | SS grade of NE [70] |
| CRP | *Prospective cohort | 225 infants with SS grade I–II–III NE | Increased CRP in neonatal blood in first 120 h of life | SS grade of NE [72] |
| | *Retrospective cohort, 2006–2011, 4 NICUs in the UK | 361 TH infants with SS grade I–II–III NE | Increased CRP in neonatal blood in first 7 days of life, peaking at day 4 | SS grade of NE [70] |
| Troponin | Retrospective cohort, 2012–2014, Southmead Hospital, North Bristol NHS Trust, UK | 79 infants with PA | Increased Troponin I in cord blood <6 h of birth | SS grade II/III NE (with TH), AUC 0.81 (0.64–0.98) [71] |
| | Retrospective cohort, 2015–2020, Chung Shan University Hospital, Taiwan | 73 TH infants with SS grade I–II–III NE | Increased Troponin I (level ≥180 pg/mL) in neonatal serum <6 h of birth | SS grade of NE, and abnormal BSID-III or death at 1 year of age (sens 34.8%, spec 89.9%, PPV 66.7%, NPV 69.4%) [74] |
| Markers | Design | Subjects | Finding | Correlated outcomes |
|---------|--------|----------|---------|---------------------|
| (a) EEG | Meta-analysis of 26 eligible studies (2010–2019) from Embase, Cochrane and PubMed | 111 (5 studies) TH infants with PA/NE | Abnormal EEG tracing, recording times not reported | Adverse neurological outcomes up to 18 months of age; EEG pooled sens 0.63 (95% CI: 0.49–0.76), spec 0.82 (95% CI: 0.70–0.91) [76] |
| | Meta-analysis of 26 eligible studies (2010–2019) from Embase, Cochrane and PubMed | 741 (9 studies) TH infants with PA/NE | Abnormal aEEG tracing, recording times not reported | Adverse neurological outcomes up to 18 months of age; aEEG background pattern pooled sens 0.90 (95% CI: 0.86–0.94), spec 0.46 (95% CI: 0.42–0.51) [78] |
| | Prospective cohort | 74 TH and non-TH infants with SS grade II–III NE | aEEG pattern abnormalities at 3–6 h of age; PPV 84% for normothermia, 59% for TH. Abnormal voltage not predictive of outcome | Disability at 18 months of age on BSID-II, early aEEG pattern was predictive for infants with normal care but not TH [77] |
| | Meta-analysis of 9 eligible studies (2000–2014) from Medline, Cochrane, and CINAHL | 520 TH infants with SS grade II–III NE | Abnormal aEEG pattern, pooled sens 96% (95% CI: 91–98), spec 97% (95% CI: 90–100) | Death or disability on BSID-II and GMFCS at 1 year of age, hearing and / or blindness. aEEG at 48 h predicted outcome; PPV of aEEG at 6 h was poor [112] |
| | *Multicenter prospective cohort (PRIME study) | 63 non-TH infants with SS grade I NE | 24/50 infants with normal early aEEG (at 5.5 h median age) | Abnormal MRI/neuropathological exam at discharge [9, 19] |
| MRS | Prospective cohort | 223 TH infants with SS grade I–II–III NE | Reduced thalamic NAA at 7 days, sens 100% (95% CI: 74–100), spec 97% (95% CI: 90–100) | Adverse neurodevelopment at a median age of 23 months on BSID-II and GMFCS, hearing loss, blindness [91] |
| | *Prospective cohort | 14 TH infants with SS grade II–III NE | Reduced basal ganglia NAA, NAAG, and GPC-PCh, and white matter GPC-PCh | SS grade of NE [93] |
| MRI | Prospective cohort | 223 TH infants with SS grade I–II–III NE | Lower fractional anisotropy in the PLIC (n = 65) by DWI-MRI: AUC 0.92 (95% CI: 0.76–1.00) | Hearing loss, blindness, poor neurodevelopment at a median age of 23 months on BSID-III and GMFCS [91] |
| | Prospective cohort | 54 TH infants with SS grade II–III NE | Lower ADC values in thalami (PPV 84.21, NPV 87) and the PLIC (PPV 91.18, NPV 75) at 4–6 days | Death or abnormal neurodevelopment on BSID-III and GMFCS at 2 years of age [97] |
| | Retrospective cohort, 2013–2017 | 107 TH infants with SS grade II–III NE | NICHD scoring; lesion size (AUC 0.718) and count (AUC 0.705) in the PLIC, basal ganglia, thalamus on DWI-MRI | Abnormal neurodevelopment at 18–24 months on BSID-III [99] |
| | Meta-analysis of 17 eligible studies (1990–2016) from Medline, Embase, PsycINFO, and Google Scholar | 799 TH and non-TH infants with NE | Abnormal MRI at <4 weeks of life | OR 18.2 (95% CI: 9.4–34.9) for adverse neurodevelopment up to 12 months of age [98] |
This corresponds with a latent phase after reperfusion from HI that lasts $\sim 1$–$6$ h, when brain metabolism transiently recovers but pathological intracellular mechanisms can be triggered, leading to mitochondrial dysfunction and delayed bulk cell death in the brain from $\sim 12$ to $72$ h [4]. Interestingly, the rates of death or disability after TH were just $29\%$ in a recent large controlled trial, compared with $44\%$ in the first NICHD-NRN cooling trial [5]. This likely reflects multiple factors, including better monitoring, greater care to avoid pyrexia, a higher proportion of neonates with moderate NE being cooled now, greater experience with TH for these sick infants and related clinical care, and earlier initiation of TH.

Critically, the need to start TH as soon as possible from birth means that rapid identification of infants with NE has become a pressing clinical issue. Before TH, the grading of NE was made in retrospect with serial neurological assessments in the first week of life, typically using Sarnat staging, adjunct electroencephalograph (EEG) monitoring, and magnetic resonance imaging (MRI) [6]. Instead, clinical assessment of possible NE is now conducted within $6$ h from birth, in order to start cooling infants as early as possible within the therapeutic window for neuroprotection. This is challenging, as NE is a dynamic brain disorder that evolves over time [7]. Indeed, a small retrospective study showed that $7$ of $11$ of infants diagnosed with mild NE within $6$ h of birth progressed to moderate NE later [8]. However, there is compelling evidence that a proportion of infants with mild NE have high risk of poor neurodevelopmental outcomes [9]. In response, there has been therapeutic drift such that $\sim 38\%$ of these neonates are now offered TH [10], despite lack of evidence from clinical trials. An ideal biomarker would establish the severity of NE, and determine long-term prognosis in the first hours after birth. In addition, it will inform on response to treatment for clinical decision-making. In this review, we synthesize the latest literature in the field, and discuss the merits and limitations of prominent tools and potential biomarkers for predicting outcome. All studies that were sourced for this review are detailed in Table 1, including estimates of predictive value.

### Apgar Score

The Apgar clinical assessment of infants’ condition at birth is routinely performed in neonatal care, and most studies have used it as a pre-qualification criterion for TH. This score mainly reflects the extent and duration of resuscitation. The score is associated with outcome, but at $5$ min has low sensitivity and specificity [11]. Historically, need for continuing resuscitation at $10$ min was associated with worse outcomes. In the NICHD-NRN controlled cooling trial, low $10$-min Apgar scores ($0$–$3$) were associated with death or disability in $64$/85 ($75\%$) infants. Nevertheless, $\sim 21\%$ of infants with $10$-min Apgar scores of $0$ survived without disability to school age [12]. Critically, the risk of death or disability was significantly lower in cooled infants with Apgar scores of $0$–$3$, showing that prognosis of infants with even very prolonged resuscitation is improving. By comparison, profoundly depressed $10$-min Apgar scores are uncommon in infants with mild NE at birth [13].

### Neurological Exam

Modified Sarnat and Thompson scoring (SS and TS) of infant’s neurological state are very helpful to categorize severity of neurological dysfunction into $3$ stages of NE; mild (I), moderate (II), and severe (III) NE. SS is the predominant tool for infant qualification for TH [14], whereas the TS is popular in resource-limited settings, and used as adjunct to SS to confirm NE grading or as a qualifying factor for TH [15, 16]. Short-term improvement in SS or TS are predictive of long-term outcomes [17, 18], and efforts are being made to further refine SS criteria [6]. Nevertheless, the existing methods can be pragmatically useful for identifying risk of disability. In the PRIME study, a recent multicenter, prospective study of untreated mild NE defined as one or more abnormalities on the modified Sarnat examination within $6$ h of birth but not enough to meet criteria for cooling, $7$/43 ($16\%$) infants had disability at $18$–$22$ months [19]. Only one infant had CP and $2$ had autism. Notably, $17$/43 had Bayley scores $<85$ in one or more of the cognition, motor, or language domains. The median total Sarnat score (the sum of the subcategories of the modified Sarnat examination) was greater in infants who went on to develop disability than those who did not (median $7$ [IQR, $5$–$7$] vs. median $4$ [IQR, $2$–$5$]) [20]. Although not perfect, a total Sarnat score of $\geq 5$ predicted risk of disability with an area under the curve of $0.83$ ($p = 0.004$).

### Acid-Base Balance

The most common biomarkers for infants with hypoxia-ischemic NE are pH and base deficit (BD) obtained from cord or neonatal blood. Clinical guidelines for TH...
generally recommend prequalification cut-off values of a pH < 7.0 or a base deficit of ≥16 mM. Population-based studies indicate that raising the threshold pH < 7.1 is less specific [21, 22], but would capture more infants with moderate to severe NE, and presumptively more mild NE [7]. Although the extremes of BD measurement (e.g., BD > 18 mM or conversely BD < 10–12 mM) correlate with high and low risk of NE, intermediate BD values have variable outcomes [23, 24].

**Neurobiochemical Markers**

Neurobiochemical markers have been of particular interest for prediction in infants with NE [25]. Most of these biomarkers are structural proteins that provide neuronal and axonal support, and traumatic events that compromise neuronal integrity can trigger their release into interstitial cell fluid, cerebrospinal fluid (csf) and the circulation [25, 26]. Multiple markers have been examined in animal and clinical studies. The focus of this review is on the latest clinical research of bedside biomarkers of outcome.

**S100B,NSE, and Nfl Neuroproteins**

S100 calcium-binding protein-beta (S100B) is expressed in astroglial and certain neuronal populations, and neurofilament light chain protein (Nfl) and neuron-specific enolase (NSE) are a protein filament and glycolytic enzyme found in the neuronal cytoplasm. Small cohort studies in normothermia and hypothermia-treated infants show these protein concentrations become elevated in NE and correlate with outcomes [25]. For example, higher S100B levels in cord blood at birth from infants (n = 13) with moderate-severe NE correlated with acidosis, amplitude-integrated EEG (aEEG) pattern and NE severity, and death or disability at 6 years of follow-up, compared to healthy (n = 21) infants [27]. Elevated CSF-NSE levels < 72 h of birth were predictive of seizure burden and abnormal findings in aEEG, MRI, and neurodevelopment in infants with mild-severe NE, with the most accurate CSF-NSE cut-off level for poor outcome in survivors being 108 ng/mL compared with 50 ng/mL in normal infants [28]. In a clinical trial of moderate-severe NE term infants, serum S100B and NSE concentrations during whole-body cooling (at 0, 12, 24, and 72 h) also correlated with basal ganglia, global and cortical damage on MRI (at 7–10 days), and short-term neurological outcomes [29]. Overall, the findings are consistent between studies [25, 30].

Plasma Nfl levels after TH had started, and before and after rewarming, were higher in cooled infants with moderate-severe NE and unfavorable outcome on neuroimaging than in cases with mild NE and cooled infants that had a favorable outcome, with a cut-off level > 29 pg/mL at 24 h being predictive of unfavorable outcome (sensitivity 77%, specificity 69%) [31, 32]. Further, cord blood Nfl and Tau protein levels were elevated in term infants with birth asphyxia, although not just in infants that developed NE [33]. Other potential biomarkers include monocyte chemotactic protein-1, osteopontin, secretoneurin, Activin A (AvA), and neuronal exosomes [34, 35]. AvA is a member of the transforming growth-factor B super-family (TGF-B) with increased csf, serum, and urine levels in infants with moderate-to-severe NE compared with mild NE and healthy infants [30]. However, recent findings suggest that cord blood AvA protein and Acvr2b mRNA are not reliable biomarkers [36].

**Tau Protein, Ubiquitin C-terminal Hydrolase L1, and Oxidative Stress Markers**

Tau protein is expressed in neurons, astrocytes, and oligodendrocytes, and is a known neurobiomarker for neurodegenerative diseases. It has a critical role in microtubule stabilization, and so contributes to cell signaling, and synaptic and genomic regulation [26]. Common tauopathies such as Alzheimer’s and Pick’s disease are characterized by pathological deposition of tau protein in the brain [37]. Recent studies suggest that tau protein also has predictive value after perinatal HI. Plasma and serum tau protein levels within 24 h of birth were higher in infants with severe NE than infants with moderate NE, and in cases with poor neuroimaging findings and neurodevelopmental delay [38, 39]. Interestingly, lower serum tau correlated with improved short-term behavioral scores in NE infants treated with rEpo (200 IU/kg, daily from 2 to 10 days of birth) plus therapeutic cooling, compared with infants treated with hypothermia alone [40]. Finally, in cooled infants, tau protein was a late predictive marker (72–96 h) for brain damage on MRI and adverse neurological outcome [41]. Ubiquitin C-terminal hydrolase L1 is a neuron-specific, deubiquitinating enzyme that facilitates removal of pathological proteins, which helps maintain neuronal and axonal health. Like tau protein, its expression is increased after brain trauma in adult and pediatric patients. However, overall, recent neonatal evidence on outcome prediction has been disappointing [42–44].

Reactive oxygen species increase during and after HI, and can overwhelm neonatal antioxidant mechanisms
and lead to oxidative stress and brain damage [4]. Thus, oxidative stress markers may be good biomarkers for prediction of outcome [45]. In infants with perinatal asphyxia, malondialdehyde (MDA) and protein carbonyl concentrations at birth and at 48 h of life correlated with seizure burden, mortality, and degree of NE (SS grade I vs. II vs. III), but not developmental delay [46, 47]. Changes before, during, and after TH in lipid peroxidation markers such as total isoprostanoids and 15(RS)-15-F_{2}t-IsoP also correlated with lesions on MRI (at 4–8 days) in infants with NE [48]. Further, superoxide-dismutase and MDA levels were elevated at 6–14 h in infants with stage II–III NE, whereas vitamin D3 was lower compared with healthy control infants [49].

Consistent with this, serum selenium, a glutathione peroxidase constituent that is vital to anti-oxidant function, was reduced within 48 h in newborns with NE, and lowest in patients with severe NE [50]. Of note, elevated total hydroperoxides were reduced in hypothermia-treated infants with NE compared with infants that received normothermia [51]. Superoxide-dismutase and MDA levels also recovered during head cooling for 72 h, and were associated with greater scores on the Neonatal Behavioral Neurological Assessment (NBNA) at 3, 7, and 10 days of life, and Bayley-III scores at 8 months of age [52]. Of course, this functional improvement likely reflects the broad protective effects of hypothermia, rather than being mediated by anti-oxidant effects. A large prospective clinical trial in infants with grade II–III NE is recruiting (clinical trials identifier: NCT03162653) that will assess the effects of the oxygen free radical scavenger, allopurinol, in addition to TH on death or disability at 2 years of age, aEEG, and brain injury on MRI and ultrasound [53]. Peroxidation products in blood will also be measured as a marker of brain damage.

GFAP and Inflammation Markers

Glia fibrillary acidic protein (GFAP) is the main intermediate filament protein that supports the astroglial cytoskeleton. It is specific to the central nervous system and is released after HI. Evidence that it can predict neurological outcome in neonates is mixed, as reviewed [26, 30]. For example, serum GFAP concentrations were elevated within 6 h of birth and in the first week of life in infants with grade II–III NE and abnormal MRI findings [54]. Supporting this, increased serum GFAP levels were found at 24 and 72 h of life in infants who either died or had unfavorable outcome on neuroimaging (at 5–14 days), with a sensitivity of 87.5% and specificity of 82% at rewarming from TH [55]. By contrast, there was no difference in cord blood GFAP levels within 3 h of birth between infants born with perinatal asphyxia and NE (n = 86) and infants who had uncomplicated births (n = 83) [42, 56]. Serum GFAP concentration also was not predictive in infants with NE before (<6 h of life) or during TH, or in NE infants who did not qualify for TH [41, 43, 57]. Overall, these findings suggest that in isolation serum GFAP is not a reliable marker for guiding treatment or prognosis after NE.

There is consistent evidence that perinatal HI increases cytokine expression in the developing brain and circulation. Elevated levels of tumor necrosis factor-alpha, interferon-γ, and various interleukins (IL) such as IL-1β, IL-6, IL-8, IL-10, and IL-13 have been reported in infants with NE, and shown to correlate with outcome measures [39, 58]. For example, increased plasma IL-6, 8, and 10 concentrations within 24 h of life predicted death or severe MRI-defined brain damage (at 4–7 days) in infants with NE who underwent TH. The combination of these biomarkers were more predictive than each of these cytokines alone, with an accuracy of ~78% [41]. In support of this, Pang et al. [59] also reported increased IL-6 and 10 levels in infants with NE compared with non-NE controls (159 vs. 157 infants), with IL-10 elevation within 12 h of birth being predictive of NE severity, mortality, and early childhood outcomes (OR 2.28, 95% CI: 1.35–3.86) [59]. In term infants with NE, serum IL-8 levels on days 1 and 2 of life also predicted infant death and NE grade II–III, whereas changes at 2 and 6–7 days of life in granulocyte-macrophage-colony-stimulating-factor were associated with abnormal MRI findings and Bayley-III scores at 2 years [60].

Finally, in a follow-up study of the NICHD randomized controlled trial of TH for NE [61], regulated-upon-activation-normal-T-cell-expressed-and-secreted and monocyte chemotactic protein-1 levels were associated with death or impairment (OR 0.31, 95% CI: 0.13–0.74, and OR 3.70, 95% CI: 1.42–9.61). Increased tumor necrosis factor-alpha levels in the first day of life were found in infants that died or developed CP [62]. Overall, these findings indicate that certain cytokines can be useful biomarkers for prognosis, but that most cytokine changes occur too late for potential neuroprotection therapies to be viable.

microRNAs and Transcriptional Markers

Single-stranded microRNAs (miRNA) are non-coding ribonucleic acid fragments that are ~22 nucleotides in length, and silence gene expression to inhibit protein synthesis. Recent research has implicated miRNAs in
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pathological processes after perinatal HI [63]. For example, changes in hsa-miR-374a, miR-210, and miR-199 correlated with NE severity and neurological outcomes [64, 65]. Supporting this, in the Biomarker in Hypoxic-Ischemic Encephalopathy clinical trial, miR-374a-5p, miR-376c-3p, and miR-181b-5p expressions were associated with perinatal asphyxia and TH eligibility [66]. Finally, cord blood Fzd4 and Nfat5 mRNA were predictive of NE severity (SS III vs. I and II) and long-term functional outcomes [67]. Interestingly, miR-210 antagonist treatment reduced microglial inflammation and brain infarction in post-hypoxic neonatal rats [68], suggesting that miRNAs after perinatal NE are promising biomarkers and potential therapeutic targets.

Other Metabolic Markers

Multiple organ dysfunction is almost universal in infants born with NE, with high correlation between severity of NE and organ impairment during the first 3 days of life [69]. Liver enzymes, myocardial proteins, and other markers that assess end-organ damage are frequently measured in neonatal blood and may support outcome prediction. Changes in creatine and creatine kinase, lactate dehydrogenase, alanine aminotransferase, and c-reactive protein have all shown predictive value in the first 3 days of life [70–73], whereas Troponin-I levels within 6 h of birth distinguished mild NE from moderate-to-severe NE [74].

Electrophysiological Monitoring

aEEG and conventional video-EEG have a fundamental role in the monitoring and management of babies with NE. Although video-EEG is the gold standard for seizure detection, aEEG has become the method of choice for most neonatal centers as it is simpler to implement [75]. In a recent meta-analysis of 26 studies, including 1,458 near-term or term infants, multichannel EEG demonstrated sensitivity of 0.63 (95% CI: 0.49–0.76), specificity of 0.82 (95% CI: 0.70–0.91), and area under the curve (AUC) of 0.88, and for aEEG background pattern pooled sensitivity, specificity and AUC were 0.90 (95% CI: 0.86–0.94), 0.46 (95% CI: 0.42–0.51), and 0.78, respectively, for predicting an unfavorable neurological outcome in infants with NE treated with TH [76]. The times of monitoring in these studies were not reported; however, previous evidence shows that the positive predictive value of an abnormal aEEG is much less at 3–6 h after birth in TH-treated infants than normothermic infants [77], presumptively due to neuroprotection after hypothermia [78]. Moreover, aEEG monitoring has limited predictivity <6 h for mild NE [9]. Continuous and Discontinuous Normal Voltage were the predominant background patterns [73, 79], although excessive discontinuity and disrupted sleep-wake cycling are also common in these infants [79, 80]. By contrast, conventional EEG has higher accuracy, but requires resources and expertise that may not always be available in NICUs [81].

These limitations have renewed interest in novel EEG-derived biomarkers. Evoked potentials have been noted for their usefulness in improving prognosis in small cohorts of infants that received TH [82], and can be measured with standard aEEG setups [83]. Other EEG features of recent interest for prediction are cortical power bursts [84], spectral power and micro-scale transients [85–87], and neurovascular coupling [88] in moderate-to-severe NE, and sharp and diffuse delta waves, mild asymmetry, and spectral edge frequency in mild NE [7, 80].

Modern Neuroimaging

Phosphorus-31 (31P) and proton (1H) magnetic resonance spectroscopic (MRS) measurements in NE neonates are highly predictive of outcome [89–92]. In a prospective trial in term infants (n = 223) treated with TH for NE, thalamic N-acetylaspartate (NAA) levels obtained with 1H-MRS at a median age of 7 days (IQR; 5–10) had higher accuracy (AUC 0.99, 95% CI: 0.94–1.00) than other clinical measures in predicting neurological outcome at 18–24 months of age [91]. In 1H-MRS conducted 18–24 h after initiating TH, infants with severe NE also had lower basal ganglia NAA (0.62 ± 0.08 vs. 0.72 ± 0.05), NAA + N-acetylaspartylglutamate (NAAG; 0.66 ± 0.11 vs. 0.77 ± 0.06), and glycerophosphorylcholine plus phosphatidylcholine (GPC + PCh; 0.28 ± 0.05 vs. 0.38 ± 0.06) than infants with moderate NE [93].

MRI including diffusion-weighted MRI in the first week of life is the preferred method in most centers to assess HI brain injury [94]. Signal abnormalities on conventional T1/T2 weighted images are common in parasagittal regions and white matter, and basal ganglia and thalami in infants with moderate-to-severe NE. In a retrospective cohort analysis of 89 infants who had received TH, there was no difference in the overall rate of MRI injury by grade of NE (Barkovich classification; mild NE 54%, moderate NE 54%, severe NE 50%; p = 0.89), although basal ganglia/thalamic lesions were more com-
mon in severe NE \( (p = 0.03) \) [95]. However, infants with mild NE and subtle MRI abnormalities may benefit from more detailed scoring systems [96]. Signal hyperintensity in the posterior limb of the internal capsule (PLIC), thalamus, and basal ganglia in particular have high predictive value for adverse outcomes [97–99], even after TH [100].

The timing of imaging is important for prognostication. Late MRI scans at a median age of 8 days in TH-treated infants had 95% sensitivity, 94% specificity, and 91% positive predictive value for death or disability at 2 years of age [101]. By contrast, cell death is still developing in the first days of life and so early brain imaging can underestimate the severity of injury [102]. In 12 asphyxiated term infants, MRI during TH at 2–3 days of life was predictive of brain lesions at 8–13 days [103]. However, visual and quantitative evaluation were required to determine basal ganglia abnormalities [104]. Diffusion-weighted and conventional MRI also have high correlation at day 4 and 2 weeks after birth [105]. Other techniques such as diffusion tensor imaging and tractography are more advanced and may identify brain lesions earlier [94]. Cranial ultrasound and Doppler ultrasonography are adjunct technologies for diagnostic imaging, and have limited prognostic value [58]. Overall, MRI imaging is a fundamental tool for prognosis and informing clinicians on treatment response; however, it is not yet useful for guiding early neurotherapy.

Some Reflections on Biomarkers

Biomarkers can be helpful in a variety of settings, including initial recruitment for treatment within 6 h of birth, determining whether infants are responding during treatment, and finally, assessment of prognosis after the end of treatment for parental counseling and enrollment with developmental support services. Each goal has different constraints. Modern imaging and EEG have strong, established, albeit not perfect, prognostic value after the end of treatment, with a relatively wide window in which testing is reliable.

By contrast, determining prognosis shortly after perinatal HI is critical to guide clinicians in whether or not the infant should be offered potentially neuroprotective therapies such as TH. There is only a brief window for clinical decision-making and thus an optimal biomarker must be accessible and rapidly and accurately measurable. CSF biomarkers are invasive, and so not practical since most infants would not otherwise require a lumbar puncture. Similarly, urine markers will be unreliable for targeting TH in infants who commonly have oliguria due to acute kidney injury after global HI, although they may be useful for assessing treatment response and outcome prediction. Moreover, it is well known that NE evolves over time, with corresponding changes in the infant’s Sarnat stage. The pathological brain processes that occur after perinatal HI are associated with changes in biomarker response. Thus, the timing of biomarker acquisition is critical, and both their robustness and value likely depend on this. Finally, it is important to appreciate that most biomarkers have been tested within well-defined cohorts. It is likely that their predictive value would be attenuated in more diverse cohorts. Given these issues, it is not surprising that only clinical examination and EEG monitoring are widely used at present.

Conclusion

This short review of the literature shows that at present none of the proposed biomarkers has been established to be better than clinical assessment of NE for identifying infants who are likely to benefit from neuroprotective treatments. There is still only emerging evidence for biomarkers to identify mild NE, and so profiling biomarkers relative to the deleterious processes that underlie mild NE is an important area of future research. Critically, even the most promising markers show predictive values at time-points that are too late (>6 h from birth) for optimal therapeutic interventions. Speculatively, a combination of multiple modalities may offer better prognostication. Such neonatal scoring systems have shown promise in early studies, and their predictive value can likely be further improved through machine learning and deep learning algorithms to better support clinical decision-making [106, 107].

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

G.W. wrote the first draft of the MS. All authors contributed to identification of studies and writing and revising the MS.

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Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.
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