CLINICAL RESEARCH ARTICLE

Enquiring beneath the surface: can a gene expression assay shed light into the heterogeneity among newborns with neonatal encephalopathy?

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BACKGROUND: We aimed to assess whether a gene expression assay provided insights for understanding the heterogeneity among newborns affected by neonatal encephalopathy (NE).
METHODS: Analysis by RT-qPCR of the mRNA expression of candidate genes in whole blood from controls ($n = 34$) and NE ($n = 24$) patients at <6, 12, 24, 48, 72 and 96 h of life, followed by determination of differences in gene expression between conditions and correlation with clinical variables.
RESULTS: During the first 4 days of life, MMP9, PPARG, IL8, HSPA1A and TLR8 were more expressed and CCR5 less expressed in NE patients compared to controls. MMP9 and PPARG increased and CCR5 decreased in moderate/severe NE patients compared to mild. At 6–12 h of life, increased IL8 correlated with severe NE and death, decreased CCR5 correlated with chorioamnionitis and increased HSPA1A correlated with expanded multiorgan dysfunction, severe NE and female sex.
CONCLUSIONS: MMP9, PPARG and CCR5 mRNA expression within first days of life correlates with the severity of NE. At 6–12 h, IL8 and HSPA1A are good reporters of clinical variables in NE patients. HSPA1A may have a role in the sexual dimorphism observed in NE. CCR5 is potentially involved in the link between severe NE and chorioamnionitis.

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INTRODUCTION

Neonatal encephalopathy (NE) is the main cause of death and permanent neurological disabilities in human full-term newborn infants.[1] The incidence of NE is estimated around 1 per 1000 live births in wealthy countries[2] with a mortality rate around 25%. Identification, grading of severity, monitoring and outcome prediction are currently substantiated by clinical, neurophysiological and neuroimaging examinations.[3] Patients with moderate or severe NE are associated with a spectrum of long-term disabilities, most commonly cerebral palsy, epilepsy, sensory deficits and cognitive deficits. Recent studies indicate that a significant proportion of patients with mild grades of NE also have abnormal motor or neurodevelopmental delay.[4]

Therapeutic hypothermia (TH) remains the only neuroprotective treatment available for infants with moderate to severe NE.[5] However, its NNT is 7 (95% CI 5−10) NE patients,[5] and approximately half of children cooled still die or survive with significant functional deficits.[6] Novel therapeutic approaches (mainly focused on adjuvant therapies together with TH) demand solid molecular targets and detailed time points of administration. This accurate frame is difficult to assess due to NE's convoluted nature. Injury evolves in distinct clinical phases during hours,

weeks and even months, leading to long-term motor and cognitive deficits.

The proportion of infants treated with TH that benefit is not optimal, and the results are less than satisfactory in many patients. Newborns with NE are a complex clinical group, and therefore, there is an urgent need to establish individual profiles that identify those patients who have an increased risk of poor outcome. Data from preclinical studies highlight some of the factors underlying this heterogeneity, especially concerning sexual dimorphism, both in the pathological mechanisms and the response to TH or novel therapeutic approaches.[7] While still a yearning in current clinical practice, the assessment of individual profiles after the initial insult seems crucial. Ideally, a panel of biomarkers could broaden our perspective towards this direction.[8] It could provide information for critical decision making by the clinician and the pathological mechanisms of NE which still need to be fully elucidated.[9] Despite very important recent advances, few biomarkers correlate with relevant clinical variables at early time points and their expression time course after the injury remains largely uncharted. Changes in gene expression have been reported during and after ischemia in animal models. These changes include immediate early genes, transcription factors and heat shock proteins (HSPs), followed by

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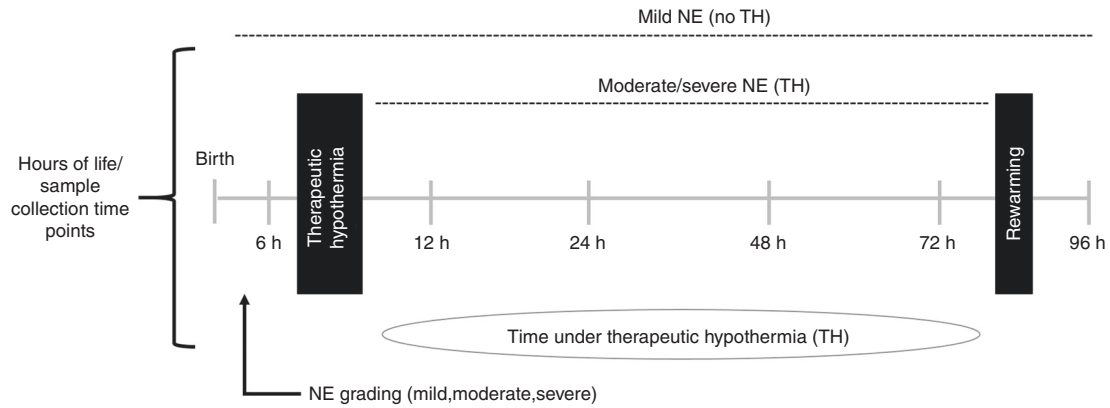R Balada et al.

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Fig. 1 Study design with time points of sample collection and TH protocol for NE patients. Controls ($n = 34$), NE patients ($n = 24$). The severity of NE was assessed immediately after admission and encephalopathy was classified in mild,[6] moderate[10] and severe.[8] Only newborns with moderate or severe NE received whole-body cooling (33.5 °C) for 72 h, when they were slowly rewarmed (<0.5 °C per hour).

genes related to inflammation, apoptosis, cytoskeletal and metabolism.[10–12] In this study, we performed a gene expression assay in NE patients' whole systemic blood at different time points after the initial insult during the first 4 days of life, and tested if the differences in gene expression correlated with relevant clinical variables at early time points (6–12 h of life). We analyzed genes related to proinflammatory response (*CCR5, CCL3, CXCR2, CXCR3, IL6, CXCL8, IL1B, TNF, NFKB1*), anti-inflammatory response (*PPARG, PTGS2, TGFB1, HSPA1A*), immune response (*TLR7, TLR8*), extracellular matrix reorganization (*MMP9*), and energy metabolism (*IGF1, IGFBP3, SIRT1, PCK2, GSK3B, EHD2*).

METHODS
Study design
This is a substudy of a prospective observational cohort study that was conducted at Sant Joan de Déu Hospital and Clinic-Maternitat Hospital, both in Barcelona, Spain, between April 2009 and July 2011. A graphical representation of the study design can be reviewed in Fig. 1.

Study populations
The study population included infants with NE ($n = 24$) consecutively born at ≥36 weeks gestational age and ≥1800 g, admitted to Hospital Sant Joan de Déu—Hospital Clinic of Barcelona, between April 2009 and December 2011. Infants were considered to have NE if they met the following criteria: (1) Apgar score ≤5 at 10 min, or need for resuscitation, including endotracheal intubation or mask ventilation for more than 10 min after birth, or acidosis (pH ≤ 7.0 and/or base deficit ≥16 mmol/l in umbilical cord blood or arterial, venous or capillary blood) within 60 min from birth; (2) neonatal encephalopathy defined as a syndrome of neurologic dysfunction manifested by a subnormal level of consciousness with or without seizures (moderate or severe NE) or palmary hyperexcitability, tremor, overactive myotatic reflexes, hypersensitivity to stimulation or startle responses (mild NE). Continuous aEEG recordings for 72 h commenced immediately after admission. MRI was performed in every patient that survived more than 72 h. MRI studies were scored using a validated scoring system.[13] The accuracy of MRI to identify brain injury in these infants has been previously published.[14]

The severity of NE was assessed immediately after admission and always before starting TH in the NICU. Encephalopathy was classified into mild ($n = 6$), moderate ($n = 10$) and severe ($n = 8$) according to a semiquantitative score, which included the aEEG findings (Supplemental Table S1 (online)). Newborns with moderate or severe NE received whole-body cooling (Techotherm TSmed 200N or Criticool, MTRE Ltd.) and were maintained at a rectal temperature of 33–33.5 °C for 72 h, followed by slow rewarming (≤0.5 °C per hour). All patients were evaluated and treated according to a strict clinical protocol for the management of NE. The severity of multiple organ dysfunctions during the first 3 days was established by means of a specific scale (MODs).[15] Patients who survived were followed up until 2 years of age and then Gross Motor Function Classification System (GMFCS) and classification of Bimanual Fine Motor Function (BFMF) were used to score functional impairment. Further, the outcome at 24 months was assessed using the Bayley Scales of Infant and Toddler Development, Third Edition (BSITD-III).

Controls ($n = 34$) consist of healthy ($n = 1$) and infants born at ≥36 weeks of gestation with birthweight ≥1800 g affected by mild polycythemia (hematocrit 60–65%) ($n = 33$), whose blood samples were extracted during the neonatal metabolic screening. Polycythemia is a condition in which there are too many red blood cells in the blood circulation and is a common finding (2–5%) of term newborns. The vast majority of newborns affected by mild polycythemia have normal development. Newborns were excluded if they presented (a) congenital abnormalities, or (b) other identifiable etiologies of neurologic dysfunction such as infection or suspicion of genetic disease, or (c) no parents' consent.

Sample collection
Systemic whole-blood samples (500 µl) were collected at <6 (before the start of TH), 12, 24, 48, 72 and 96 h of age via umbilical catheter. Patients' samples consist of serial extractions of the same individual at each time point of the study. On the other hand, control samples were obtained by one blood extraction in different individuals, providing a pool of samples for each time point. Right after collection with EDTA tubes for blood collection, 1.3 ml of RNAlater (AM7020, Ambion) was added and samples were stored at −80 °C.

RNA extraction and RT-qPCR
RNA extraction was performed using Ribopure—Blood Kit (AM1928, Ambion) following DNase I treatment (supplied by the kit). Concentration and purity were assessed by the Nanophotometer P330 (Implen, bioNova científica, S.L.). Samples with low RNA concentration or poor 260 nm/280 nm and 260 nm/230 nm ratios were discarded. Integrity of a representative group of samples was analyzed using Eukariote Total RNA Bioanalyzer (Agilent Technologies, Inc.). For each sample, 1 µg of RNA was reverse transcribed to cDNA (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems; Prime Thermal Cycler, Bibby Scientific Ltd.) and then loaded into the custom Taqman Low Density Array (TLDA, Part no. 4342249, Lot no.

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B0376) together with 50 µl of Taqman Universal PCR Master Mix No Amperase UNG (4324018, Applied Biosystems) for the gene expression assay using the 7900HT Real-Time PCR System (Applied Biosystems, no. cat. 4329001). The target *HSPA1A* was not included in the custom TLDA and was analyzed in parallel by RT-qPCR 384 using the same real-time PCR system. Each reaction was performed in duplicate. For the analysis, maximum standard deviation (SD) allowed between duplicates was 0.35. Target quantities among samples were normalized using *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) and *GUSB* (beta —glucuronidase) as endogenous controls. The complete list of genes and probes used in this study are reported in Supplemental Table S2 (online). Data output was assessed by Expression Suite Software version 1.0.3 (Life Technologies) and expressed as a (*n*)fold-change by the Relative Quantitation (RQ) method, being the calibrator the control sample of 12 h of life (reference sample), the only control not affected by mild polycythemia. Maximum Ct allowed was 35 and threshold was set at 0.2. Errors of amplification were considered when reported by the software and its data excluded from analysis.

Data analysis
Raw data graphic representation with clustered multiple comparison graphs (MedCalc Statistical Software version 13.3.3 Ostend, Belgium) was the very first step for data analysis. Genes with a discriminant graphical pattern between conditions were selected for further analysis with Linear-Mixed Effects predictive models (LMM) (Supplemental Fig. S1 (online)). For the selected genes (*n* = 6), number of individuals in each group, mean, standard deviation, median, first and third quartile and the number of missing data were described. Distribution was then studied both graphically (histogram graphics and quantile) and analytically. Each gene was statistically analyzed independently (previous logarithmic data transformation if required for model application). When using LMM, all patients' successive samples, all control samples and study time points were analyzed (except the time point of 105 h of life which was discarded since the number of NE patients was too low (*n* = 1)). For those genes with statistically significant differences of expression between groups, a second analysis was performed only within the patients group including regression models controlling for clinical variables. Only the first sample of each patient (6–12 h of life) was used for this analysis. Conditions of statistical modeling were evaluated, and derived corrective measures applied. Estimators were accompanied by a 95% confidence interval. Statistical significance was set at a probability level of <0.05. The statistical package used to process the data was the R 3.2.3 version for Windows.

RESULTS
Samples
It was not possible to extract samples from all NE patients for each time point of the study. This was mainly because some of them died before the last time point but also for other clinical issues (discharge off the NICU or umbilical catheter removal). Thus, NE samples at 96 h of life (*n* = 4) were considerably less than samples at 72 (*n* = 13), 48 (*n* = 14), 24 (*n* = 18), 12 (*n* = 17) and 6 h of life (*n* = 15). Regarding the control samples, they were extracted during a control of the polycythemia or in the neonatal metabolic screening at 48 (*n* = 13), 72 (*n* = 9) and 96 h of life (*n* = 7). Considerably fewer samples were obtained for the first time points: 6 (*n* = 2), 12 (*n* = 1) and 24 h of life (*n* = 2). This was because most parents did not accept to enroll their newborns at these time points of the study, and they are not the right time points for metabolic screening. Finally, samples were discarded if they (a) had a wrong presentation (no labeling, strange color or texture); (b) manifested problems during the RNA extraction and (c) did not perform well in the RT-qPCR. Altogether, 34 control

samples from 34 newborns and 81 NE samples from 24 patients were included in the analysis.

Demographics and clinical variables
Fifty-eight subjects (24 NE patients and 34 controls) were recruited for the study. Demographic characteristics of each population are reported in Table 1 and NE patients' clinical characteristics are summarized in Table 2.

RT-qPCR performance
Raw data were represented for all the targets except *IGF1* and *IGFBP3*, which failed to amplify during the RT-qPCR under our experimental conditions. On the other hand, *CMKLR1, CXCR3, CXCR2, TGFβ1, IL6, IL1-β, TNF-α, CCL3, GSK3β, NFKB1, PTGS2, SIRT1, TLR7, EHD2* and *PCK2* did not show a distinct graphical distribution between controls and NE patients. Finally, *MMP9, IL8, HSPA1A, PPARG, CCR5* and *TLR8* showed a clear graphical discrimination pattern between conditions and were selected for further statistical analysis with LMM (Supplemental Fig. S1 (online)).

Gene expression time course (6–96 h of life) in controls and NE patients
In order to analyze the candidate genes expression during the first 4 days of life in controls and NE patients, LMM adjusted by sex, weight, mother's age and gestation time were individually developed for each gene.

The models for *MMP9, IL8, HSPA1A* and *TLR8* predicted a higher expression of these targets in NE patients during the first 4 days of life ($+2.2 \log(RQ)$, $p < 0.001$; $+1.02 \log(RQ)$, $p = 0.004$; $+0.72 \log (RQ)$, $p = 0.005$ and $+0.62 \log(RQ)$, $p = 0.003$; respectively) with both populations (group with NE and control group) showing a gradual decrease of expression over time ($\beta = -0.17$; $p < 0.001$; $\beta = -0.02$; $p < 0.001$; $\beta = -0.17$; $p < 0.001$ and $\beta = -0.007$; $p = 0.006$; respectively) (Fig. 2a–c, f). On the other hand, the NE group expressed less *CCR5* ($-0.75 \log(RQ)$) compared to controls

Table 1. Demographic characteristics of NE patients and controls.

	Control (*n* = 34)	NE (*n* = 24)
Gender[a,b]		
Male	24 (70)	15 (65)
Female	10 (30)	8 (35)
Birthweight (g)[c]	3033 ± 557	3106 ± 567
Gestational age (weeks)[c]	38 ± 1,6	39 ± 2
Mothers age (years)[c]	31 ± 3,5	32 ± 6
Delivery[a,d]		
Cesarean (urgent)		16 (74)
Cesarean (scheduled)	8 (23)	1 (4)
Normal	25 (74)	1 (4)
Instrumental	1 (3)	4 (18)
Sentinel event[a,d]		
Yes		9 (40)
No	34 (100)	13 (60)
Apgar score[e]		
1 min	9 (7–10)	2 (0–7)
5 min	10 (9−10)	4 (0–9)
Cord arterial pH[c]	7.2 (0.04)	6.9 (0.17)

[a]Data presented as *n* (%).
[b]Documented in 23 NE patients.
[c]Mean ± SD.
[d]Documented in 22 NE patients.
[e]Median (range).

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Table 2. Clinical variables of NE patients.

	Mild (n = 6)	Moderate (n = 10)	Severe (n = 8)
Length of stay (days)[a]	5.5 (5–12)	12 (5–33)	4 (1–59)
Ventilator support[b]	5 (84)	6 (75)	8 (100)
Cardiopulmonary resuscitation[b]	5 (84)	5 (63)	7 (88)
Therapeutic Hypothermia[b,c,d]	0 (0)	8 (100)	7 (88)
Initiation of cooling (hours)[a]	Not cooled	4 (2–6)	3 (1–6)
Rewarming duration (hours)[a]	Not cooled	10 (5–12)	8 (1–47)
Intrapartum fever[b]	2 (40)	1 (12)	0 (0)
Seizures[b]	0 (0)	4 (50)	5 (63)
Chorioamnionitis[b]	5 (84)	4 (50)	3 (38)
MODs 48 h[e,f]	6 ± 12	32 ± 28	68 ± 33
MODs 72 h[e,f]	4 ± 9	30 ± 34	62 ± 36
MRI score[a]	0 (0–2)	0 (0–6)	7 (0–11)
Outcome[b]			
Dead		1 (10)	5 (62)
Survived	6 (100)	9 (90)	3 (38)

MODs 48 and 72 h refer to Multi-Organic Dysfunction Score assessed at 48 and 72 h of life.
[a]Median (range).
[b]Data presented as n (%).
[c]All variables documented in eight moderate NE patients except outcome that was documented in ten patients.
[d]One severe NE patient was not cooled since the therapeutic window for TH was missed (13 h of life).
[e]Mean ± SD.
[f]Documented in 20 NE patients.

($p < 0.001$) and time had no significant effect (Fig. 2d). Finally, NE patients expressed more *PPARG* (+0.86 log (RQ), $p = 0.002$) with apparently no significant effect of time (Fig. 2e).

Protein expression was also assessed for MMP9 and PPARγ proteins in blood leukocytes obtained from an independent cohort of control newborns ($n = 15$) at 8–96 h after birth. In agreement with the observed mRNA expression in whole blood, MMP9 and PPARγ proteins in leukocytes exhibit a significant negative correlation with time during the first 96 h after birth (MMP9, $p < 0.000$; PPARγ, $p = 0.002$. Spearman nonparametric test) (Supplemental Fig. S2, online).

Gene expression time course (6–96 h of life) according to the severity of NE (mild vs. moderate/severe)
A statistically significant interaction with time was found for *MMP9* ($\beta = 0.27$; $p = 0.007$), suggesting a faster decrease of its expression in mild vs. moderate and severe NE patients (Fig. 3a). In addition, the interaction between time and severity was at limit of statistical significance for *PPARG* in the adjusted model ($\beta = 0.24$; $p = 0.074$), suggesting a faster decrease of *PPARG* expression in mild NE patients compared to moderate and severe (Fig. 3b). On the other hand, patients with moderate and severe NE expressed less *CCR5* (-0.61 log (RQ)) compared to mild ($p = 0.003$) with no effect of time (Fig. 3c). Finally, severity of NE did not imply differences on *HSPA1A, IL8* and *TLR8* gene expression during the first 4 days of life according to the grading of NE before 6 h of life.

Correlation between gene expression and clinical variables of NE patients at early time points (6–12 h of life)
Severe NE patients expressed more *IL8* ($p < 0.01$, Fig. 4a.1) and *HSPA1A* ($p < 0.001$, Fig. 4a.2) compared to moderate and mild patients. Interestingly, the expression of *HSPA1A* at 6–12 h of life was increased in those patients with higher MODs at 48 and 72 h
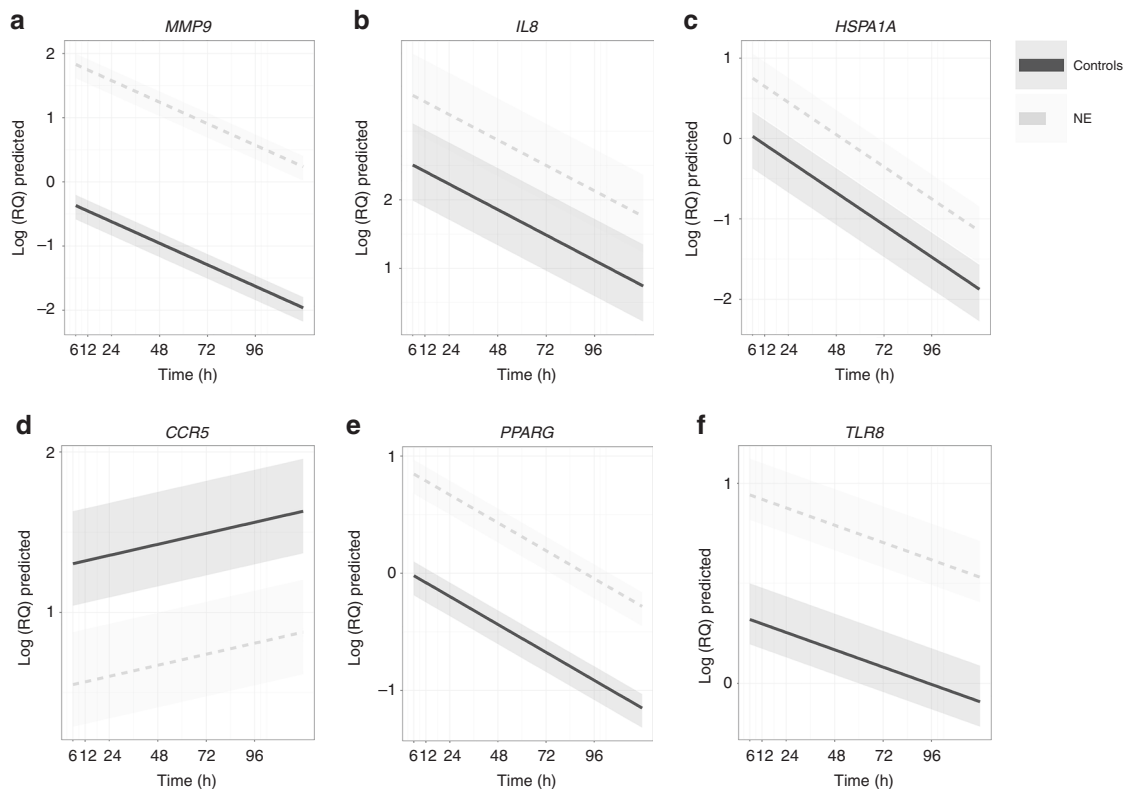

Fig. 2 Expression of the selected genes analyzed by LMM in controls and NE patients during the first 4 days of life (6−12−24−48−72 −96 h). NE patients expressed more **a** *MMP9* ($p < 0.001$), **b** *IL8* ($p = 0.004$), **c** *HSPA1A* ($p = 0.005$), **e** *PPARG* ($p = 0.002$) and **f** *TLR8* ($p = 0.003$) compared to controls. **d** *CCR5* was less expressed in NE patients ($p < 0.001$). **a–c, f** The expression of *MMP9* ($p < 0.001$), *IL8* ($p < 0.001$), *HSPA1A* ($p < 0.001$) and *TLR8* ($p < 0.006$) significantly decreased in both populations during the same period (first 4 days of life).
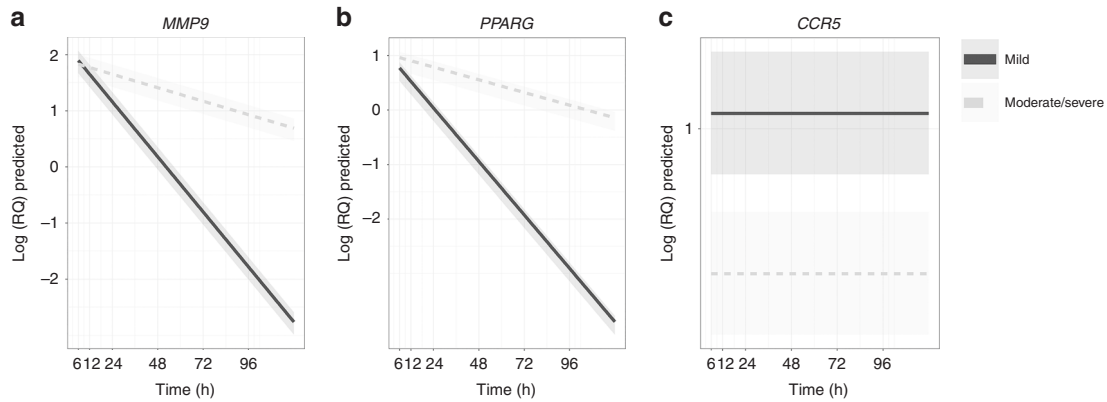
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Fig. 3 **Expression of the selected genes analyzed by LMM within NE patients (mild vs. moderate/severe) during the first 4 days of life (6−12−24−48−72−96 h). a** The expression of *MMP9* decreased faster in mild patients compared to moderate/severe patients ($p = 0.007$). **b** The expression of *PPARG* was suggested to decrease faster in mild patients compared to moderate/severe patients despite being at the limit of statistical significance in the adjusted model ($p = 0.074$). **c** Moderate and severe patients expressed less *CCR5* ($p = 0.003$) compared to mild patients with no significant effect of time.

of life ($p < 0.001$). This established a correlation of *HSPA1A* mRNA expression at early time points after birth and the degree of compromise of peripheral organs at 2−3 days of age (Fig. 4b). *IL8* expression was also correlated not only with severity of NE but also with mortality. Those patients who had a higher expression of *IL8* at 6–12 h of life had significantly higher odds (OR = 2.49) of dying during the first week of life ($p = 0.044$) (Fig. 4c.2). Regarding *CCR5*, those NE patients who suffered chorioamnionitis had a decreased expression at 6–12 h of life (-2.6 log(RQ), $p = 0.017$) (Fig. 4c.1). Among demographic variables, sex differences were found for *HSPA1A*, with NE female patients expressing more *HSPA1A* compared to NE male patients ($+1.02$ log (RQ), $p = 0.026$). Although none of the studied genes showed significant correlation with MRI score, *IL8* showed a tendency to be upregulated in those patients with higher brain damage score in the MRI (MRI score <4 had a log (RQ) median of 3.4 [2.8–4.7] and infants with score ≥4 had log (RQ) median of 5.3 [3.1–5.9]).

Finally, a 2-year follow-up study was performed with the survivors of this study. No significant statistical correlations were found between the functional outcomes and gene expression at 6–12 h of life. The functional outcomes assessed, and their values are summarized in Supplemental Table S3 (online).

DISCUSSION

New therapeutic approaches focused on adding a pharmacological agent to TH are promising tools for enhancing recovery after neonatal HI injury.[16] Nevertheless, independently of novel pharmacological strategies, it is clear that we should put a focus towards individualization among NE patients. The goal of identifying a panel of biomarkers holds the potential for achieving such purpose.

In this study we examined the gene expression of 23 targets at several time points during the first 4 days of life. Typically, studies searching for biomarkers in neonatal NE assess the protein expression of few selected targets,[8,17] the measurement in CSF or blood of brain-specific proteins[18] or using brain imaging techniques such as MRI.[19] In our study we used another approach, based on a gene expression assay. Despite the obvious gap for translational purposes (mRNA expression assays demand a greater amount of time compared to analyzing the protein level), this approach allowed us to inquire a larger number of targets ($n = 23$) and increased the possibility of finding new molecular effectors of the condition. Also, this is the first time to our knowledge that a gene expression assay is performed within a cohort of NE patients, despite changes at the mRNA level have been described in

preclinical studies.[20] Finally, our preliminary protein analysis of a few candidate genes showed similar tendencies of mRNA and protein expression during the first hours of life that, despite whole blood and leukocyte fraction can have inherent differences, support the validity of this study and its potential for clinical translation.

The expression of the selected targets was analyzed in whole systemic blood, a well-known reporter of brain function, since molecules expressed within the brain in response to the injury are often released to circulation as the breakdown of the blood brain barrier (BBB) takes place.[21] In addition, brain repair processes after neonatal HI injury may have a peripheral onset, despite studies confirming this hypothesis are still scarce.[22] Concerning the study populations, very few control samples could be obtained for the first three time points of the study. This was mainly for obvious ethical reasons. Moreover, only one control sample (the reference sample) was completely healthy (not affected by mild polycythemia). We consider that the analysis with predictive LMM based on the RQ method avoided, at least in part, these issues. Noticeably, even though our study included two centers, it only managed to recruit 24 patients. Moreover, the inability to obtain or analyze some samples further reduced the number for analysis and probably precluded potential correlations. Finally, the small number of patients with MRI and with 2-year follow-up was also an important drawback in assessing statistical correlations with gene expression at early time points.

To sum up, *MMP9*, *PPARG*, *IL8*, *HSPA1A* and *TLR8* mRNA were more expressed and *CCR5* mRNA less expressed in NE patients compared to controls during the first 4 days of life. *MMP9*, *PPARG* and *CCR5* mRNA expression were also correlated with the severity of NE during the same period. In addition, for *IL8*, *HSPA1A* and *CCR5* the levels of mRNA expression at 6–12 h of life correlated with several clinical variables. Increased IL8 and *HSPA1A* correlated with severe NE and mortality or severe NE and increased MOD at 48 and 72 h of life, respectively, with female patients showing a higher *HSPA1A* expression compared to males. Finally, decreased *CCR5* mRNA expression correlated with chorioamnionitis. While *MMP9*, *IL8* and *HSPA1A* to some extent have been widely associated with NE, it is the first time to our knowledge that *PPARG*, *CCR5* and *TLR8* are linked to this condition in a study with NE patients.

Matrix metalloproteinase 9 (gelatinase B, 92-kDa type IV collagenase) is a soluble zinc-metalloproteinase that belongs to the matrix metalloproteases superfamily (MMPs). In accordance to published studies, the sustained increase of *MMP9* mRNA expression in moderate and severe NE patients compared to mild
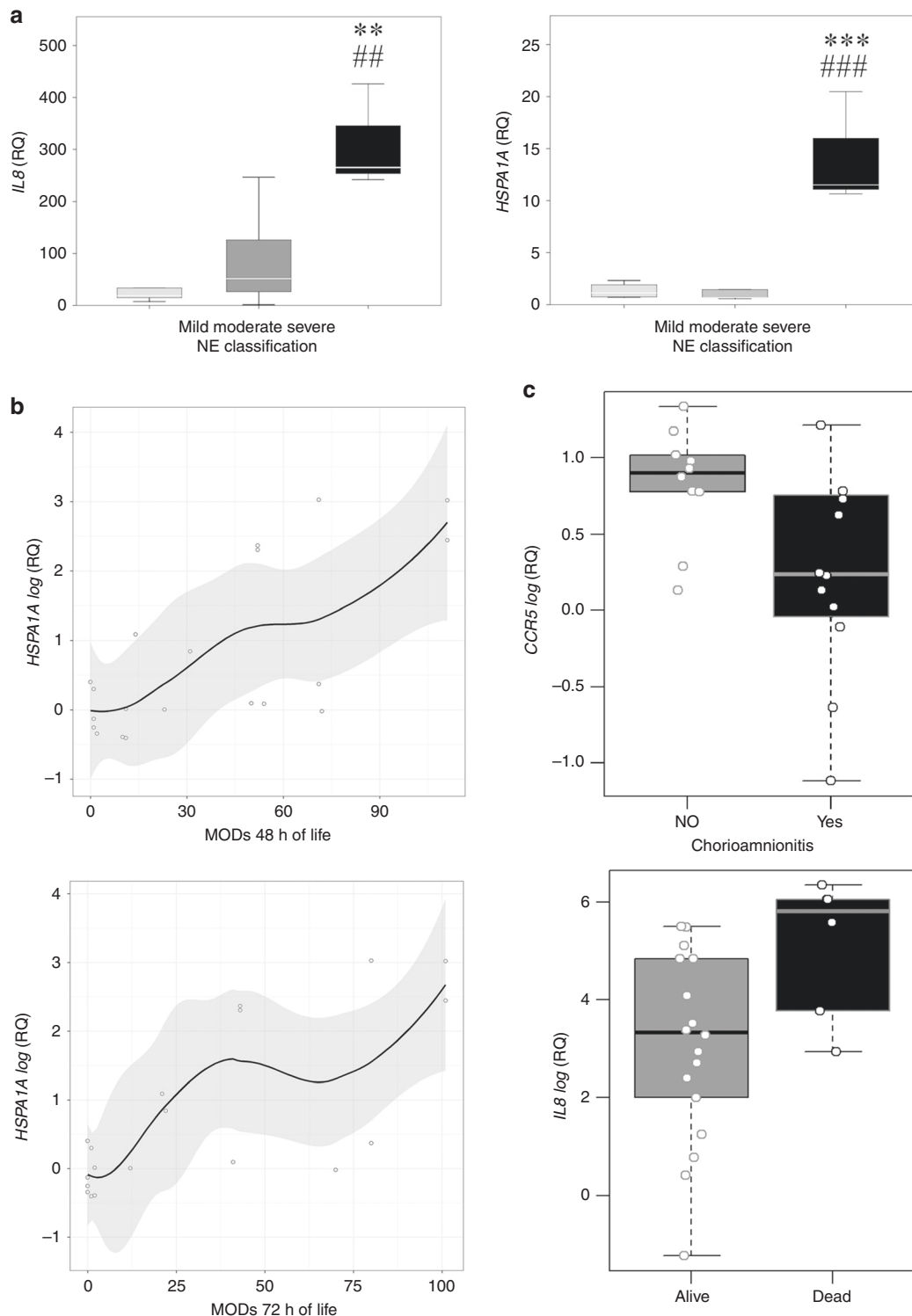
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Fig. 4 Correlations between gene expression at early time points (6–12 h of life) and clinical variables of NE patients. a.1 *IL8* mRNA expression was higher in severe patients compared to mild **($p < 0.01$) and moderate ##($p < 0.01$) patients. **a.2** *HSPA1A* mRNA expression was higher in severe NE patients compared to mild ***($p < 0.001$) and moderate ###($p < 0.001$) patients. **b** NE patients who had a higher expression of *HSPA1A* at 6–12 h of life also had a higher MODs at 48 and 72 h of life ($p < 0.001$). **c.1** NE patients whose mothers suffered chorioamnionitis expressed less *CCR5* at 6–12 h of life (-2.6 log (RQ), $p = 0.017$). **c.2** Those patients who had a higher expression of *IL8* at 6–12 h of life had significantly more odds (OR = 2.49) of having a negative outcome ($p = 0.044$).

is probably an indicator of the breakdown of the BBB.[23] On the other hand, several preclinical studies support that inhibiting MMP9 after neonatal HI injury has beneficial effects (e.g., this mechanism has been proposed to mediate erythropoietin-

induced neuroprotection)[24] but others failed to observe any benefits of this action.[25]

Interleukin-8 or Chemokine (C-X-C motif) ligand-8 (CXCL8) is a proinflammatory cytokine. Our results concur with other studies

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were an early increase (up to 24 h) in IL8 protein concentration in the serum of NE patients was associated with severity of NE and mortality.[26] Since we did not find differences in the temporal pattern of *IL8* mRNA expression, we can assume its relevance is more critical in the acute phase of the injury. Nevertheless, in one study a biphasic pattern of IL8 protein expression in serum was observed among NE patients treated with TH.[27] Those patients showing a second peak at 48 h of life had a better outcome at 12 months of life. Thus, we could be in front of a time-dependent effect of IL8, with recovery functions when its expression is increased days after the initial insult and reporting for severity when it is highly expressed at early time points.

The *HSPA1A* human gene encodes for the HSP70 protein and belongs to the HSPs superfamily. Intracellularly, HSPs are essential for basic cellular processes. Nevertheless, its transcription and translation increase fast in response to injury and are released to circulation.[28] The clinical correlations we found in the blood of NE patients concerning multiorganic damage are similar to those reported in polytraumatized patients.[29] On the other hand, the anti-inflammatory properties of HSPA1A are not fully understood yet, but a growing amount of literature is reinforcing this role.[30]

Several cohort studies have shown a higher vulnerability in human males towards hypoxic-ischemic injury in infants born near- or full-term.[31] In addition, in preclinical studies male sex was associated with limited brain repair following neonatal stroke and hypoxia-ischemia compared to females with similar brain injury.[7] Although the mechanisms underlining these differences between boys and girls remain unclear, our results might indicate that HSPA1A is one of the molecular effectors of the sexual dimorphism in NE. Also, since *HSPA1A* correlated with severity of NE and increased MODs but not mortality, it could be involved in intrinsic neuroprotective mechanisms.

Peroxisome proliferator-activated receptors (PPARs) belong to the ligand-dependent nuclear hormone transcription factor super-family. They play a critical role in the metabolism of lipids and glucose homeostasis, but PPARG is also involved in several nonmetabolic functions.[32] Since PPARG has been widely asso-ciated with anti-inflammatory properties,[33] we may assume that its correlation with the severity of NE might depict an enhanced intrinsic mechanism of neuroprotection. Similar to the current strategies involving PPARG in ischemia/reperfusion injury,[34] this provides a rationale for using PPARG agonists in neonatal HI injury. This is the first study, to our knowledge, that *PPARG* temporal pattern of mRNA expression is examined in a cohort of NE patients; further studies are needed to confirm or discard its involvement in this pathology and the potential benefits of its modulation.

CCR5 is a member of the G-protein-coupled subfamily of chemokine receptors. Cells that do not belong to the immune system can also express CCR5, especially within the CNS.[35] We found a correlation between decreased *CCR5* mRNA expression and chorioamnionitis, the inflammation of the placental tissues due to a bacterial infection. Despite results of studies examining neurodevelopmental outcomes in infants with chorioamnionitis have been inconsistent,[36] it has been associated with adverse neonatal outcomes and death.[37] Within the context of NE, chorioamnionitis is especially relevant since the benefit of TH in affected babies is unclear.[38] Furthermore, chorioamnionitis is associated with persistent acidosis in neonates with NE and may enhance tissue damage. Thus, it was not surprising to find a correlation between decreased *CCR5* mRNA expression and severity of NE. *CCR5* might report the degree of injury at early time points and be a molecular link between two relevant clinical variables in NE patients (severity of NE and chorioamnionitis). This is similar to that observed for *HSPA1A*, severity of NE and MODs, or to that observed for *IL8*, severity of NE and death. Since it has been associated both with proinflammatory and inflammation-resolving processes, the results of its modulation could not only elucidate its

role in the context of neonatal NE but also suggest a novel therapeutic target.

TLRs are innate immunity transmembrane receptors that recognize generic pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs).[39] Our results, with no correlation of *TLR8* mRNA expression with any clinical variable, provide scarce information about its role in NE other than its upregulation compared to healthy subjects. Nevertheless, the results suggest its inclusion in future clinical studies of NE.

In this study, only 6 of the initial 23 targets were selected for statistical modeling with LMM. The reason underlying this selection was the clearly distinct graphical pattern of expression between controls and NE patients (Supplemental Fig. S1 (online)). Nevertheless, several targets that were not considered in this selection phase have been involved in NE in other studies.[9,40] Since most of these studies were performed at the protein level, the reasons behind this apparent disagreement could be the different mechanisms that regulate mRNA and protein expression. Also, our decision of applying LMM analysis depending on the graphical pattern of expression could imply a certain amount of error and subjectivity.

Taking all together, over the last decade many studies focused on the search for biomarkers of brain injury in NE. While its information is crucial for understanding the molecular pathways involved, it is becoming clear that no single biomarker in blood or serum but a panel of biomarkers may substantially improve the accuracy of diagnosis, management and prognosis of NE patients. Several variables conforming the heterogeneity observed in NE may be out of clinical control (e.g., the exact timing of injury and the concomitant developmental status of the infant), but others may be considered when establishing individual profiles (gender, birth-weight, biochemical measurements, etc.). Also, levels of validated biomarkers addressing specific pathophysiological events (increased MODs, chorioamnionitis, severity of NE, etc.) would add reliable information to diminish the problem of heterogeneity and facilitate critical decision making among clinicians. More detailed studies with larger cohorts are needed to obtain accurate individualized profiles of NE patients in the near future.

CONCLUSIONS

(1) *MMP9, PPARG, IL8, HSPA1A, TLR8* and *CCR5* could be among the molecular effectors of NE pathophysiology in human patients.
(2) *IL8* (NE severity and death) and *HSPA1A* (increased MODs) perform as good reporters of relevant clinical variables in NE patients at early time points (first 12 h of life).
(3) *HSPA1A* may have a role in the sexual dimorphism observed in NE.
(4) *CCR5* is potentially involved in the link between severe NE and chorioamnionitis.

AUTHOR CONTRIBUTIONS
A.G.-A. and S.A. conceived and supervised the study. M.L., G.A. and A.G.-A. provided samples and clinical data of the patients. R.B. performed the experiments and collected the data. C.T. performed the statistical analysis. A.-A.C performed leukocyte purification and western blot analysis. R.B. wrote and S.A. and A.G.-A. critically revised the manuscript. All authors reviewed the article for intellectual content and approved the manuscript.

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ADDITIONAL INFORMATION

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