CLINICAL RESEARCH ARTICLE

Antenatal *N*-acetylcysteine to improve outcomes of premature infants with intra-amniotic infection and inflammation (Triple I): randomized clinical trial

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BACKGROUND: Intrauterine infection and/or inflammation (Triple I) is an important cause of preterm birth (PTB) and adverse newborn outcomes. *N*-acetylcysteine (NAC) is a Food and Drug Administration (FDA)-approved drug safely administered to pregnant women with acetaminophen toxicity.
METHODS: We conducted a single-center, quadruple-blind, placebo-controlled trial of pregnant women with impending PTB due to confirmed Triple I. Participants ($n = 67$) were randomized to an intravenous infusion of NAC or placebo mimicking the FDA-approved regimen. Outcomes included clinical measures and mechanistic biomarkers.
RESULTS: Newborns exposed to NAC ($n = 33$) had significantly improved status at birth and required less intensive resuscitation compared to placebo ($n = 34$). Fewer NAC-exposed newborns developed two or more prematurity-related severe morbidities [NAC: 21% vs. placebo: 47%, relative risk, 0.45; 95% confidence interval (CI) 0.21–0.95] with the strongest protection afforded against bronchopulmonary dysplasia (BPD, NAC: 3% vs. placebo: 32%, relative risk, 0.10; 95% CI: 0.01–0.73). These effects were independent of gestational age, birth weight, sex, or race. Umbilical cord plasma NAC concentration correlated directly with cysteine, but not with plasma or whole blood glutathione. NAC reduced the placental expression of histone deacetylase-2, suggesting that epigenetic mechanisms may be involved.
CONCLUSIONS: These data provide support for larger studies of intrapartum NAC to reduce prematurity-related morbidity.

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IMPACT:

- In this randomized clinical trial of 65 women and their infants, maternal intravenous NAC employing the FDA-approved dosing protocol resulted in lower composite neonatal morbidity independent of gestational age, race, sex, and birthweight.
- Administration of NAC in amniocentesis-confirmed Triple I resulted in a remarkably lower incidence of BPD. As prior studies have not shown a benefit of postnatal NAC in ventilated infants, our trial highlights the critical antenatal timing of NAC administration.
- Repurposing of NAC for intrapartum administration should be explored in larger clinical trials as a strategy to improve prematurity-related outcomes and decrease the incidence of BPD.

INTRODUCTION

In 1980, Miller et al.[1] hypothesized for the first time that bacterial colonization of the amniotic fluid could be responsible for spontaneous preterm labor and preterm prelabor rupture of membranes (PPROM). The proposed mechanism involves ascending migration of genital microorganisms, inflammation of the chorio-decidua, and passage of microbes in the gestational sac, followed by generalized fetal inflammation.[2,3]

Identification of a feasible therapy that eliminates infection, reverses the inflammatory process, and protects the fetus in utero represents a long-sought goal. The search for modalities to extend the duration of gestation and improve the premature infants' outcome included antibiotics, tocolytic drugs, and cerclage.[4,5] So far, these methods have proven largely ineffective or even harmful when preterm birth (PTB) occurs in the context of intra-amniotic inflammation and infection (Triple I).[6] Several groups, including

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ours, demonstrated that components of the antepartum, intra-partum, and postpartum exposome impact development of adverse neonatal outcomes in differential fashion.[7,8] Specifically, Triple I significantly associated with newborn's increased risk for intraventricular hemorrhage (IVH), retinopathy of prematurity (ROP), and sepsis. Conversely, the risk for bronchopulmonary dysplasia (BPD) was preferentially linked to postnatal respiratory variables such as a need for surfactant and ventilator support.[7]

N-acetylcysteine (NAC) is a cell-permeable derivative of cysteine (CYS), which is an essential precursor for endogenous glutathione (GSH) synthesis.[9] NAC and CYS can also directly scavenge free radicals and increase the pool of endogenous sulfur-containing species in the mitochondria.[10,11] Clinically, NAC is used as a mucolytic agent in patients with cystic fibrosis where it aids with the clearance of airway secretion.[12] Recently, NAC has been found to have anti-fibrotic and anti-inflammatory chemotherapeutic activities via inhibition of histone deacetylase (HDAC) and remodeling of chromatin.[13]

NAC is a known safe antidote for acetaminophen toxicity in pregnancy.[14,15] Our group was the first to test in an animal model of inflammation-induced PTB that NAC confers protection against fetal demise.[16] We reported that NAC significantly increased offspring survival by preventing stillbirth prior to birth and during spontaneous vaginal delivery.[16] Therefore, our data implied that NAC protected the fetus from the harmful effects of inflammation.[16]

Following our original publication, several groups focused their attention on the protective effect of NAC against inflammation-induced brain injury.[17–20] To date, no prior study investigated the effect of NAC on neonatal outcomes of women whose pregnancies were complicated by Triple I diagnosed using the "gold standard" analysis of the amniotic fluid. In this trial, we tested the hypothesis that in women at impending risk of PTB secondary to Triple I, antenatal administration of NAC would reduce the risk of neonatal short-term morbidity and death. As a corollary, we anticipated that NAC would preferentially lower the risk for morbidities linked to antenatal exposure to infection and/or inflammation (IVH, ROP, sepsis) and this effect would be directly related to GSH concentration in the fetal circulation.

METHODS

Participants
Women were eligible if older than 18 years of age, pregnant with a singleton, gestational age (GA) between $23^{0/7}$ and $33^{6/7}$ weeks, at high risk of PTB secondary to advanced cervical dilatation (≥3–4 cm) or PPROM and had amniocentesis results suggestive of Triple I. Exclusion criteria were anticipated delivery within 2 h, need for close medical supervision (e.g., cardiac and renal disease, congestive heart failure, history of asthma), maternal viral infection (HIV, hepatitis B or C), cord prolapse, known fetal malformation, known allergic reaction to NAC, and preeclampsia.

Assessment of Triple I
For all cases, a transabdominal amniocentesis was done at responsible clinicians' decision when mothers displayed signs and symptoms of Triple I, including fever (>100.4 °F), fundal tenderness, leukocytosis [maternal white blood cell (WBC) count >15,000 cells/µL], persistent abdominal contractions, and advanced cervical dilatation.[6] All the procedures were performed in a sterile fashion under ultrasonographic guidance and were performed independent of our study protocol.

Clinical laboratory tests performed to rapidly diagnose Triple I included amniotic fluid glucose, lactate dehydrogenase (LDH) levels, WBC count, and a Gram stain, as previously described.[21–23] Analysis of amniotic fluid included cultures for aerobe and anaerobic bacteria, *Mycoplasma* and *Ureaplasma* spp. Placenta and umbilical cord were examined for the presence of histological

chorioamnionitis and funisitis.[24] Details are provided in Supplementary Methods.

Randomization and investigational drug protocol
Eligible women were randomly assigned in a double-blind fashion to receive either NAC or placebo. The randomization process was conducted by Yale New Haven Hospital (YNHH) clinical pharmacy (O.A.) by computer-generated varying permuted blocks.[25] The NAC infusion protocol was identical to the procedure approved by Food and Drug Administration (FDA) for acetaminophen toxicity and involved a loading dose of 150 mg/kg NAC (Acetadote, Cumberland Pharmaceuticals) delivered over 1 h, followed by 50 mg/kg continuous infusion over 4 h, after which a 100 mg/kg infusion was maintained over the next 16 h or until delivery if this occurred earlier.[15,26] Study solutions (placebo and study drug) were prepared by the YNHH Clinical Pharmacy as described in Supplementary Methods. By study design, when a cesarean delivery was recommended (e.g., prior surgical scar, fetal malpresentation) and the maternal and fetal status was reassuring, we aimed to expose the fetus to the NAC bolus dose and to continue the infusion, for all cases, until cord clamping. Dextrose (5%) in water (D5W) was used as a placebo.

Adverse events associated with intravenous NAC administration were monitored for all the participants. Signs and symptoms of anaphylactic reactions (e.g., flushing, rash, hypotension, bronchospasm) were considered major adverse events and reasons for protocol interruption. Other anticipated adverse reactions, such as nausea, vomiting, urticaria, stomatitis, and rhinorrhea, were considered minor and did not require stopping of the infusion.

Neonatal outcomes were collected by certified research nurses and following completion of the study reviewed by a neonatologist team member (V.B.). Maternal demographic variables, medical history, associated treatments (e.g. antibiotics, magnesium, steroids), and results of clinical laboratory tests were retrieved from patients' medical records by members of the research team (C.S.B., I.A.B., and M.O.B.).

Definition of neonatal morbidities and study outcomes
All newborns were admitted to YNHH Newborn Intensive Care Unit (NICU) and followed prospectively until death or discharge. The type and level of delivery room resuscitation were left at the latitude of the neonatology team who also assigned the Apgar scores. Early-onset neonatal sepsis was defined as culture-confirmed and/or presumed sepsis <72 h after birth corroborated by clinical symptoms. Late-onset sepsis was defined by positive cultures ≥72 h after birth. BPD was defined as the need for supplemental oxygen at 36 weeks postmenstrual age along with characteristic radiographic changes.[27] Detailed definitions of neonatal morbidities are in Supplementary Methods.

The primary outcome of the trial was a composite score of mortality and severe short-term neonatal morbidities (IVH grades 3 and 4, ROP grades 2–4, necrotizing enterocolitis [NEC] grades 2–4, periventricular leucomalacia [PVL], BPD, any type of culture-proven sepsis,) prior to discharge or death. Each outcome occurrence was counted and the number of occurrences summed as the composite score (range 0–7). Secondary outcomes were the individual short-term morbidities and maternal and fetal levels of NAC, GSH, and cytokines. These primary and secondary outcomes were pre-specified. Due to the unexpected positive result on BPD, we extended the analysis to exploratory variables related to newborn status in the delivery room as well as immunohisto-chemical expression of HDAC2, which were not anticipated prior to unblinding.

Biological samples and mechanistic endpoints
The procedures for the collection of maternal venous blood samples, cord blood samples, and biopsies from the placenta are included in Supplementary Methods along with the laboratory

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methods used to measure concentrations of NAC, GSH, inflammatory markers, and HDAC2 expression.

Statistical analysis

At the time this trial was initiated there was no prior experience with administration of maternal NAC for fetal protection. Our sample size calculation was based on our prior human study that evaluated fetal oxidative stress before and after delivery.[28] In our original calculations, we assumed NAC administration would result in a 30% increase in fetal RBC GSH. To detect this change, we aimed to enroll 140 patients (70 in each group). However, the clinical trial was stopped earlier after randomization of 68 patients, due to the relocation of several investigators to other institutions. Statistical analysis was performed using Fisher's exact or χ^2 tests as appropriate. Multivariable logistic regression was performed to account for potential differences in GA, race, sex and birthweight. Spearman's correlations were used to analyze relationships between NAC and CYS in cord blood and other variables of interest. Data normality was tested by Shapiro–Wilk method. Data are presented as average and 95% confidence intervals (CIs) or as median and interquartile range as appropriate. A $p < 0.05$ was considered significant.

Study approval

The Yale University Human Investigation Committee approved the study protocol. Written informed consent was obtained from all participants. The consent form is available in Supplementary Material. The protocol was registered with ClinicalTrials.gov Identifier NCT00397735.

RESULTS

Demographic and maternal clinical characteristics of the study groups

This was a single-center randomized, quadruple-blinded (participant, care provider, investigator, and outcomes assessor), placebo-controlled clinical trial conducted at YNHH from November 2006 to October 2012. A flowchart of eligible women and exclusions is presented in Fig. 1. During the study, 369 women pregnant with singletons underwent amniocentesis to rule out Triple I. Of these, 288 women were found ineligible or did not meet the strict inclusion criteria. Out of the 81 consecutive women eligible for enrollment, 12 were not enrolled given the unavailability of the research team. Thus, 69 women were approached for enrollment. One patient declined participation and 68 signed consent forms. Following enrollment, 1 patient delivered before randomization, leaving 67 women randomized into the clinical trial. Following unblinding of the randomization codes, it was revealed that 33 women received NAC and 34 received placebo.

There was no statistically significant difference in the demographical and clinical characteristics between the two groups, except for more non-Hispanic white women having received NAC
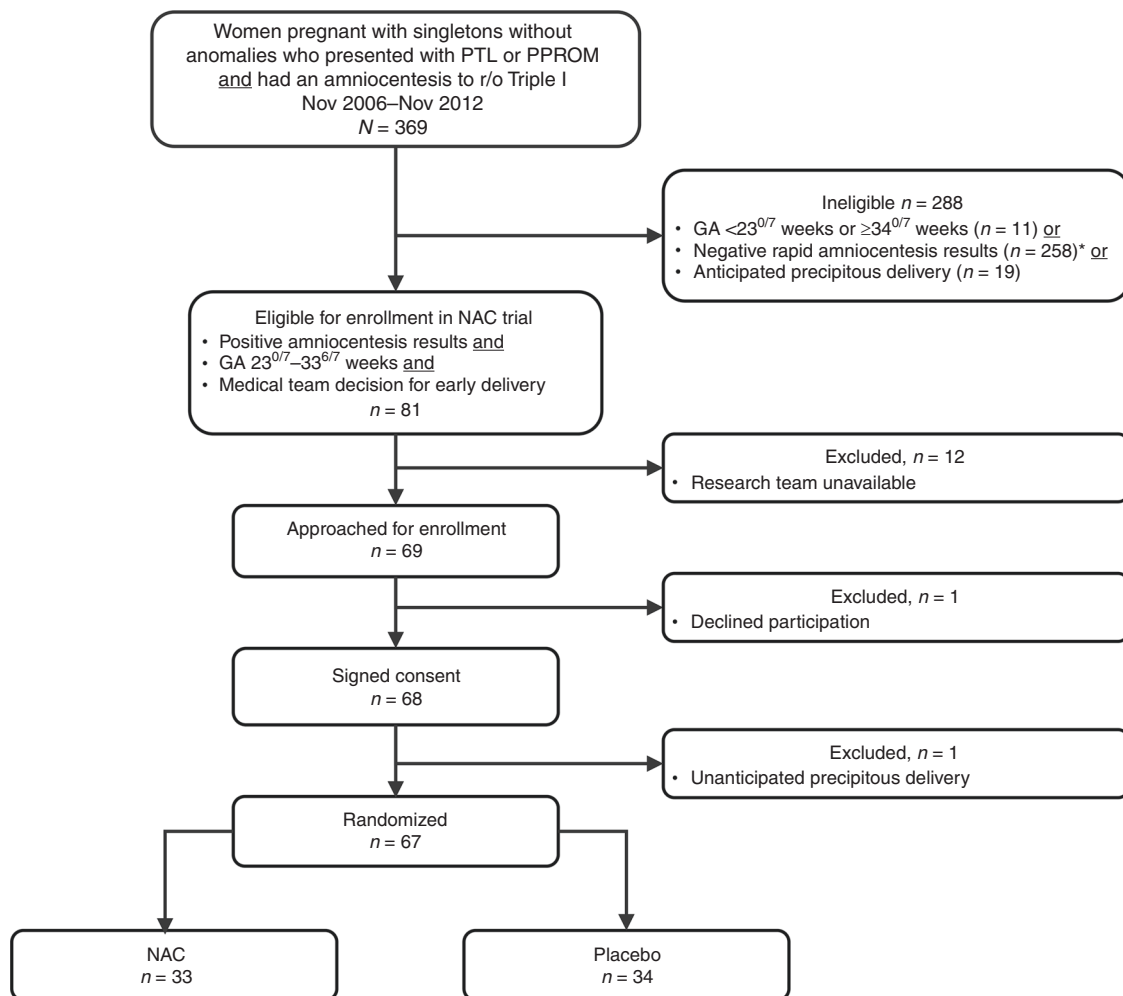
Fig. 1 Flowchart of eligible participants with inclusion and exclusion circumstances. PTL preterm labor, PPROM preterm prelabor rupture of membranes, r/o rule-out, GA gestational age, NAC *N*-acetylcysteine. *Thirteen patients who were ineligible for the trial based on negative assessment for Triple I contributed placental samples as reference for immunohistochemistry experiments.

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Table 1. Demographic and clinical characteristics of randomized women (*n* = 67).

Variables	NAC, *n* = 33	Placebo, *n* = 34	*P* value
Clinical characteristics			
Maternal age (years)*	27 [23–34]	29 [20–33]	0.400
Race/ethnicity[†]			**0.018**
Non-Hispanic White	16 (49)	5 (15)	
Non-Hispanic Black	9 (27)	18 (53)	
Hispanic	5 (15)	9 (26)	
Other	3 (9)	2 (6)	
Nulliparity[‡]	20 (60)	17 (50)	0.464
History of preterm birth[‡]	8 (47)	9 (53)	1.000
BMI (kg/m^2)*	27.5 [25.8–34.6]	28.8 [24.7–35.3]	0.927
PPROM[‡]	22 (67)	19 (56)	0.454
Tocolytics[‡]	14 (42)	12 (35)	0.621
Progesterone[‡]	6 (18)	8 (24)	0.765
Antenatal steroids[‡]	33 (100)	34 (100)	1.000
Complete steroid course[‡]	20 (60)	20 (59)	1.000
Magnesium sulfate[‡]	14 (42)	15 (44)	1.000
Antibiotics[‡]	31 (94)	32 (94)	1.000
Gestational age at amniocentesis (weeks)*	28.1 [25.8–30.5]	27.0 [25.4–30.4]	0.616
Clinical course after amniocentesis[†]			0.564
Labor induction and/or cesarean	30 (91)	29 (85)	
Labor augmentation	3 (9)	4 (12)	
Expectant management until clinical chorioamnionitis	0 (0)	1 (3)	
Clinical chorioamnionitis[‡]	3 (9)	3 (9)	1.000
Amniocentesis-delivery interval (h)*	10.1 [7.7–16.7]	8.0 [5.8–17.0]	0.146
Gestational age at delivery (weeks)*	28.1 [25.8–30.5]	27.0 [25.4–30.4]	0.616
Cesarean delivery[‡]	11 (33)	11 (33)	1.000

Significant *p* values (<0.05) are shown in bold font.
NAC N-acetylcysteine, *BMI* body mass index, *PPROM* preterm prelabor rupture of membranes.
*Data are presented as median [interquartile range] and analyzed by Mann–Whitney test.
[†]Data are presented as *n* (%) and analyzed by χ^2 test.
[‡]Data are presented as *n* (%) and analyzed by Fisher's exact test.

(Table 1, 16/33 vs. 5/34, *p* = 0.004). There were no significant differences in the results of the rapid tests for diagnosis of amniotic fluid inflammation (WBC count, LDH activity) or infection (glucose concentration, Gram stain) (Supplementary Table 1). Using traditional culture methods and/or Gram stain, >80% of cases had infection of the gestational sac, with no statistically significant difference in frequency or type of infection between NAC and placebo groups. Results of clinical placental pathology were available in 87% (52/67) of cases and showed the presence of histologic chorioamnionitis and funisits in both groups, with no difference in frequency or severity. There was no significant difference in cord blood acid–base analysis, with only one newborn in each group reported as having a cord blood pH <7. Our previously published algorithm considering haptoglobin switch-on status and cord blood interleukin-6[29] classified >70%

of newborns in the study as "exposed and haptoglobinemic," with no statistically significant difference between groups. There were no "exposed" hypoglobinemic or anhaptoglobinemic babies in this study.[30]

Investigational drug characteristics
Following randomization, all but one patient completed the loading dose (Table 2). One patient enrolled in the NAC group was started on the loading dose infusion, which ran for 18 min before delivery. Overall, women in the treatment NAC arm received 16.5 [interquartile range (IQR) 12.8–20.0] g of NAC. The total infusion volume and the median duration of the infusion were not significantly different in the NAC vs. the placebo group. Patients delivered by cesarean had a slightly nonsignificant shorter infusion duration (cesarean: 3.4 [1.8–7.4] h vs. vaginal delivery 4.9 [2.6–10.8] h, *p* = 0.147). There were five instances of antici-pated minor adverse events (nausea, vomiting, urticaria) in the NAC group that did not warrant discontinuation of drug infusion. No adverse events were reported among placebo patients. There were two protocol deviations (one in each group), which involved minor delays in the time the infusion regimens were switched to the next level. These events did not warrant exclusion of patients from the data analysis.

Effect of NAC on newborn status in the delivery room
Table 3 summarizes the newborn characteristics in the delivery room. Surprisingly, the groups were imbalanced in race and ethnicity, with more white male babies in the NAC group and more black female babies in the placebo group (*p* = 0.025). Despite this bias, the newborns of mothers who received NAC had better Apgar scores at both 1 and 5 min and required less resuscitation with positive pressure ventilation (PPV) (*p* = 0.039). The distribution of the delivery room resuscitation techniques among the newborns in the study is represented graphically in Supplementary Fig. 1. Compared to ten babies in the placebo group, only two babies in the NAC group required surfactant in the delivery room (*p* = 0.022). When modeled in multivariable linear regression, the Apgar scores and delivery room resuscitation variables were all determined by the combination of the treatment group and GA at birth. Race, sex, birth weight, cesarean delivery, complete steroid course, and concurrent exposure to magnesium sulfate were variables excluded from the model on the basis of *p* > 0.1. In multivariable logistic regression with the treatment group and GA as covariates, the odds ratio of a 5-min Apgar score ≥7 for a newborn exposed to NAC was 11.5-fold (95% CI: 2.1–63.6) greater than if exposed to placebo (*p* = 0.005), independent of GA at birth. In the same way, with each week extended in utero, the odds of a 5-min Apgar ≥7 increased by 1.6-fold (95% CI: 1.1–2.3, *p* = 0.008), independent of the treatment group.

Effect of NAC on short-term newborn outcomes
The short-term newborn outcomes at discharge from the NICU are presented in Table 4. Infusion of NAC was associated with a significantly lower frequency of our primary outcome, specifically a composite morbidity score ≥2 (*p* = 0.039). In our study, a total of eight newborns died before discharge, with six in the placebo group and two in the NAC group. Fetal exposure to NAC did not increase the risk of presumed early-onset sepsis or culture positivity in the first 3 days of life or beyond. For each individual morbidity, there were fewer diagnosed babies in the NAC group compared to the placebo group. A finding that we did not anticipate was the remarkable lower number of neonates who developed BPD in the NAC group (1/31, 3%) compared to the placebo group (10/31, 32%, *p* = 0.006). As we originally hypothe-sized and consistent with NAC's antioxidant effect, fewer new-borns exposed in utero to NAC developed IVH and/or died (*p* = 0.01), or developed BPD and/or die (*p* = 0.009).

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Table 2. Investigational drug infusion characteristics and NAC dosing.

Variables	NAC, $n = 33$	Placebo, $n = 34$	*P* value
Patient weight (kg)*	73.0 [67.8–93.9]	77.6 [64.8–95.6]	0.792
Patients completing loading dose[†]	32 (97)	34 (100)	0.239
Patients completing 4-h dose[†]	16 (48)	12 (35)	0.327
Loading dose infusion rate (mL/h)*	227 [212–296]	244 [203–288]	0.688
Loading dose volume infused (mL)*	227 [212–296]	250 [211–292]	0.543
4-h Dose infusion rate (mL/h)*, [a]	19 [17–24]	21 [18–24]	0.172
4-h Dose infusion volume (mL)*, [a]	63 [46–80]	36 [15–84]	0.319
Continuous dose infusion rate (mL/h)*, [a]	10 [9–13]	11 [9–13]	0.642
Continuous dose volume infused (mL)*, [a]	70 [38–102]	78 [19–119]	0.981
Total duration of infusion (h)*	4.9 [2.8–10.9]	3.8 [1.8–8.6]	0.284
Total infusion volume (mL)*	344 [265–419]	300 [243–411]	0.363
NAC delivered during loading dose (g)*	10.9 [10.1–14.1]	NA	NA
NAC delivered during 4-h infusion (g)*	3.0 [2.2–3.8]	NA	NA
NAC delivered during continuous infusion (g)*	3.3 [2.0–4.8]	NA	NA
Total NAC infused (g)*	16.5 [12.8–20.0]	NA	NA
Infusion and/or drug-related adverse events[†]	5 (15)[b]	0 (0)	**0.025**

Significant *p* values (<0.05) are shown in bold font.
NAC N-acetylcysteine, *mL* milliliter, *h* hour, *NA* not available.
*Data are presented as median [interquartile range] and analyzed by Mann–Whitney test.
[†]Data are presented as *n* (%) and analyzed by Fisher's exact test.
[a]Calculated only for the patients who were started on respective dose regimen.
[b]All adverse events were minor and included nausea and vomiting ($n = 2$), transitory sensation of chest tightness ($n = 2$), and hot flush ($n = 1$).

The results of logistic regression models adjusting statistically significant effects of NAC for concurrent influences of GA at birth, birth weight, sex, and race are presented in Supplementary Table 2. Exposure to NAC was protective against the development of multiple morbidities and IVH and BPD alone or assessed in combination with death. All these outcomes were predicted by the combination of the treatment group and GA at birth. A significant interaction between GA and the treatment group was observed for BPD and BPD in combination with death, but not for the other variables. Male sex was a detrimental factor in the composite risk for multiple morbidities, while the race was excluded from all models based on $p > 0.1$. The relative risks and numbers needed to treat (NNT) for each of these outcomes are presented in Table 5. The relative risk reduction for the composite morbidity score ≥2 varied from 90% (for BPD) to 55% with NNT values ranging from 3 to 4, respectively.

Effect of NAC on maternal and fetal plasma thiols, GSH, and cytokines
Next, we explored the levels of free plasma thiols (CYS, CYS-glycine [CYS-GLY], and reduced GSH), whole blood reduced (GSH) and oxidized GSH (GSSG) and plasma cytokines in the two groups, which are presented in Supplementary Tables 3, 4 and 5, respectively. Maternal levels of NAC in the treatment arm were elevated in samples collected during the drug infusion ($p < 0.001$), but undetectable in postpartum samples (Supplementary Fig. 2A). We observed significantly elevated NAC concentrations in cord blood, both in umbilical vein and artery ($p < 0.001$ for both). Although median maternal and fetal circulatory levels of CYS, CYS-GLY, and GSH did not differ between NAC and placebo groups, the NAC concentration in maternal plasma correlated with the concentration of CYS ($r = 0.908$, $p < 0.001$) and CYS-GLY ($r = 0.694$, $p = 0.012$). Significant correlations were found in umbilical vein and umbilical artery between NAC and CYS (vein: $r = 0.726$, $p < 0.001$; artery: $r = 0.850$, $p < 0.001$ Supplementary Figs. 2B, C), but not between NAC and CYS-GLY ($p > 0.05$ for both). We found

no correlation between the NAC and GSH concentrations measured in plasma or in whole blood in either maternal or fetal compartment ($p > 0.05$ for all).

Among newborns who received NAC and had at least one umbilical cord sample analyzed, only 39% (12/31) had an elevation in plasma CYS above the mean ± 2 standard deviations of the placebo group. The subgroup of newborns with elevated CYS had significantly shorter NAC median infusion time (3.4 h, 95% CI: 2.0–4.3) compared to the remainder of the babies exposed to NAC (9.5 h, 95% CI: 3.6–13.3, $p = 0.01$). No statistically significant differences in cord blood GSH/GSSG ratio between NAC and placebo groups were observed.

NAC exposure did not lower the umbilical cord blood inflammatory cytokines. We did not find any significant relationships of NAC or CYS concentrations with GSH, GSH/GSSG ratio, or cytokines in either maternal or fetal compartment.

Effect of NAC on placental HDAC2 immunoreactivity
NAC has the ability to reverse chromatin remodeling via inhibition of HDAC2 activity.[31] Alteration in lung HDAC2 activity has been strongly linked to hyperoxia-induced lung injury in mouse models of BPD.[32] Given the lack of effect of NAC on GSH concentration, we tested whether NAC exposure modified HDAC2 expression in fetal tissue. For obvious reasons, fetal lung tissues were not available for analysis. Thus, we used placenta as a surrogate, due to its fetal tissue origin. Figure 2 shows representative images of placental HDAC2 immunoreactivity from two placebos and two NAC-treated cases. Controls were placental sections from women with idiopathic spontaneous PTB (iPTB) who delivered at similar GAs in the absence of Triple I or histological chorioamnionitis. As presented in Fig. 2a, b, compared to iPTB, the placenta of Triple I pregnancies that received placebo (Fig. 2c, d) had remarkably higher HDAC2 staining compared to those without Triple I (Fig. 2e, f). In Triple I, the nuclei of cytotrophoblasts and syncytiotrophoblast were conspicuously stained in contrast with the pale pink staining of nuclei in placental villi from NAC-treated patients or iPTB

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Table 3. Newborn clinical characteristics in the delivery room.

Variables	NAC, *n* = 33	Placebo, *n* = 34	*P* value
Birth weight (g)*	1170 [810–1630]	980 [808–1448]	0.816
Newborn male sex†	15 (45)	15 (44)	1.000
Newborn race and sex‡			**0.025**
Non-Hispanic white males	9 (27)	2 (6)	
Non-Hispanic black females	5 (15)	12 (35)	
Other combinations	19 (58)	20 (59)	
1-min Apgar score*	8 [5–8]	6 [3–8]	**0.027**
1-min Apgar score <4†	2 (6)	12 (35)	**0.006**
5-min Apgar score*	9 [8–9]	8 [6–9]	**0.007**
5-min Apgar score <7†	2 (6)	13 (38)	**0.003**
Resuscitation, highest level‡,a			**0.039**
None	0 (0)	1 (3)	
Oxygen, tactile stimulation	13 (41)	5 (15)	
Mask CPAP or bag and mask ventilation	11 (34)	12 (35)	
Intubation with PPV alone	5 (16)	4 (12)	
Intubation with PPV, drugs and/or CPR	3 (9)	12 (35)	
Surfactant in the delivery room†, b	2 (6)	10 (31)	**0.022**

Significant *p* values (<0.05) are shown in bold font.
NAC N-acetylcysteine, *CPAP* continuous positive airway pressure, *PPV* positive pressure ventilation.
*Data are presented as median [interquartile range] and analyzed by Mann–Whitney test.
†Data are presented as *n* (%) and analyzed by Fisher's exact test.
‡Data are presented as *n* (%) and analyzed by χ^2 test.
aInformation is missing for one newborn in the NAC group.
bInformation is missing for two newborns in each group.

cases. The semiquantitative scoring identified significant differences among all three groups, suggesting that although NAC decreased HDAC2 staining intensity, it remained at higher than what would be expected for preterm deliveries in the absence of Triple I (Fig. 2g). These data support the notion that maternal delivery of NAC may reverse fetal epigenetic changes induced by prolonged exposure to the inflammatory intrauterine environment.

DISCUSSION
In women with amniocentesis-confirmed Triple I and imminent PTB, administration of NAC had significant clinical beneficial neonatal effects by reducing the rate of the primary composite outcome of mortality and severe short-term neonatal morbidities, with the highest protection afforded against BPD. Premature babies exposed to NAC had improved status at birth with better Apgar scores and less requirement for surfactant and PPV resuscitation. Our study demonstrates intrapartum infusion of NAC is feasible, safe, and does not increase the incidence of neonatal sepsis. Although we found that maternal administration of NAC increases the pool of endogenous CYS in the fetal compartment in 39% of fetuses, we could not substantiate a significant replenishment of plasma or RBC GSH or alterations of the maternal or fetal pro-/anti-inflammatory cytokine profiles at the dosing employed in this study.

Premature babies exposed in utero to an inflammatory insult have the poorest outcomes.[7,33,34] We conducted this trial, motivated by the wish to identify a safe and feasible therapeutic approach, to improve neonatal outcomes in the current state of clinical practice involving antibiotics, magnesium, and steroids.[35–37] Using a mouse model of inflammation-induced PTB and stillbirth, we demonstrated that antenatal administration of endotoxin killed ~60% of fetuses within 16 h, while the rate of vaginal stillbirth was 100% at 17 h following LPS exposure.[16] When we completed our initial animal studies, we posited that compromised fetuses may die secondary to a stressful laboring process. We also acknowledged our limited ability to understand the mechanism through which NAC acts to exercise its effects. In retrospect, from the perspective of the current data, NAC could have improved the fetal-to-neonatal transition process, a hypothesis that would have been impossible to confirm in premature mouse newborns.

Before this trial, there was no prior experience with intrapartum human administration of NAC in the setting of infection-induced PTB. Therefore, several assumptions were necessary, several of which were not confirmed. First, was the premise on which we calculated the target sample size assuming that NAC would raise fetal blood GSH. Second, we anticipated that NAC acts as an antioxidant. While for obvious reasons we were unable to evaluate the neonatal fetal liver GSH content, we believe that the positive results of this study with relatively short times of drug exposure support alternative mechanisms of action.

BPD is a common complication of prematurity with a stagnant or increasing incidence.[38] In premature babies born at ≤28 weeks, the prevalence of BPD is ~40%, which concurs with the 38% rate observed in our placebo group.[39] Risk factors for BPD unanimously point to exposure to invasive mechanical ventilation and supplemental oxygen after birth, which lead to volutrauma, barotrauma, and oxidative stress-induced lung injury.[40] These therapies are, however, required to ensure an adequate gas exchange of premature infants who are often unable to complete fetal–neonatal transition and breathe independently.

NAC is a well-established mucolytic agent via the supply of CYS, which induces molecular disaggregation of the mucoprotein constituents.[41] This mucolytic effect of NAC would facilitate lung fluid clearance in the immediate postnatal period. NAC may have other favorable effects on respiratory outcomes. For example, in vitro, NAC disrupts biofilm formation and affects adherence to respiratory epithelial cells of relevant pathogens.[42,43] For these reasons, several clinical trials are underway to explore the potential benefit of NAC treatment for patients with novel coronavirus infection.[44] Most preterm infants struggle to expel the lung fluid owing to weak respiratory muscles, greater chest wall flexibility, deficiency of surfactant, and immature epithelial sodium channels.[38,40] The data from this study suggests that babies who received NAC had better Apgar scores and required less invasive resuscitation and exogenous surfactant, which would be consistent with NAC facilitating the fetal–neonatal transition. Ahola et al.[45] administered NAC postnatally in premature ventilated infants. These investigators did not observe lower BPD rates despite higher circulating NAC levels than those achieved in our current trial. The authors did, however, speculate that initiation of NAC before initiation of ventilator assistance could have been more effective.

Recent studies have focused on NAC's ability to reverse chromatin remodeling via inhibition of HDAC2.[46,47] Similarly, alteration in lung HDAC2 activity has been strongly linked to hyperoxia-induced lung injury in mouse models of BPD.[32] This confers the possibility that antepartum NAC administration "restores" the fetal chromatin to a state that allows for a better adaptation of the immature lung to the postnatal environment, thus explaining the remarkably lower incidence of BPD in the treatment arm compared to placebo. By inducing a more open

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Table 4. Short-term outcomes newborn outcome assessed at discharge from NBSCU.

Outcome variables	NAC		Placebo		*P* value
	Newborns assessed	Outcome present (%)	Newborns assessed	Outcome present (%)	
Primary outcome					
Composite morbidity score ≥2*, †	33	7 (21)	34	16 (47)	**0.039**
Composite morbidity score ≥1*, †	33	13 (39)	34	18 (53)	0.330
Individual morbidities					
Death†	33	2 (6)	34	6 (18)	0.259
Early-onset sepsis, presumed and/or culture-confirmed†	33	9 (27)	34	16 (47)	1.000
Early-onset sepsis, culture-confirmed†	33	1 (3)	34	2 (6)	1.000
Late-onset sepsis, culture-confirmed†	33	8 (24)	33	10 (30)	0.783
Culture-proven sepsis, any type	33	8 (24)	34	12 (35)	0.425
Severe IVH (grades 3 and 4)†	33	2 (6)	34	4 (12)	0.673
IVH, any grade†	33	5 (15)	34	13 (38)	0.053
Severe ROP (grades 2–4)†	33	4 (12)	34	9 (26)	0.217
ROP, any grade†	33	8 (24)	34	13 (38)	0.294
Severe NEC (grades 2–4)†	33	5 (15)	33	6 (18)	1.000
NEC, any grade†	33	7 (21)	33	8 (24)	1.000
PVL†	33	1 (3)	34	2 (6)	1.000
BPD†, a	31	1 (3)	31	10 (32)	**0.006**
Other composite outcomes					
IVH and/or death†	33	6 (18)	34	17 (50)	**0.010**
BPD and/or death†	33	3 (9)	34	13 (38)	**0.009**
Length of NICU stay					
Length of stay for newborns who survived >28 days‡	31	39 [24–91]	33	62 [24–114]	0.375

Significant *p* values (<0.05) are shown in bold font.
NICU neonatal intensive care unit, *NAC N*-acetylcysteine, *IVH* intraventricular hemorrhage, *ROP* retinopathy of prematurity, *NEC* necrotizing enterocolitis, *PVL* periventricular leukomalacia, *BPD* bronchopulmonary dysplasia.
*Composite of culture-proven sepsis (any type), IVH grades 3 and 4, ROP grades 2–4, NEC grades 2–4, PVL, BPD, and/or death.
†Data are presented as *n* (%) and analyzed by Fisher's exact test.
‡Data are presented as median [interquartile range] and analyzed by Mann–Whitney test.

chromatin state, NAC may allow for clearance of transcription factors that are no longer needed postnatally. This action will favor the activity of transcription factors that control transcription of genes that enable the lung to grow and protect it from hyperoxia-induced injury. While all these mechanisms are currently just hypothesized, the results of our trial provides clues that epigenetic modification to fetal DNA could serve as biomarkers of drug exposure and therapeutic efficacy better than endpoints related to GSH replenishment.

Wiest et al.[48] performed a pharmacokinetic study of antenatal NAC in 11 pregnant women with clinical chorioamnionitis and demonstrated a rapid transfer of the drug across the placenta followed by a slower rate of fetal clearance. The dosing regimen employed by Wiest et al.[48] and by Jenkins et al.[20] in their studies differed from the NAC protocol employed in our current trial. Different from us, Wiest and Jenkins aimed to reach newborn NAC concentrations achieved in trials of postnatal NAC administration (100–300 μM). Conversely, our aim was to determine the impact of a safe FDA-approved NAC regimen that together with the identification of Triple I amniocentesis enabled longer times of fetal exposure to NAC. As shown, >50% of NAC-treated women delivered while on the lower dose of NAC continuous infusion, which could explain why the cord blood concentration on NAC achieved in our trial was lower than in the Weist et al.'s study.[48] The beneficial effect of lower NAC doses on neonatal lung outcomes concurs with Sprong et al.'s findings.[49] This group observed that lower doses of NAC were beneficial, while higher doses increased mortality in adult rats displaying endotoxin-induced lung damage. Collectively, these data support the notion that the FDA-approved NAC dosing regimen is beneficial to the premature newborn.

Adverse effects from NAC infusion usually occur during the first high dose and commonly recede with slowing down the infusion.[50] In our trial, although the NAC infusion was generally well tolerated, there were a few adverse events that could be reduced with a modified dosage. NAC's anti-inflammatory and antioxidant effect raised concerns of the potential for increasing the risk for sepsis among treated newborns. Probyn et al.[51] reported that administration of NAC to the ovine fetus exposed to endotoxin has detrimental effects by augmenting hypotension and hypoxemia. However, unlike the experimental animal model employed by Probyn, where both endotoxin and NAC were injected as a bolus in the fetal blood, the human scenario of Triple I is characterized by compartmentalization of the inflammatory process.[52] In Triple I, the fetal compartment is to some extent protected from direct inoculation of damaging associated molecular pattern proteins, at least in the initial phases of the Triple I process. In our trial, a reassuring finding was that antenatal NAC treatment did not increase the risk of either early- or late-onset neonatal sepsis. Moreover, there was no difference in umbilical blood gases while Apgar scores were improved.

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Table 5. Relative risks for short-term morbidities affected by NAC and NNTs for benefit.

Outcome	Relative risk [95% CI]	*P* value	NNT [95% CI]
Composite morbidity score[a] ≥2	0.451 [0.213–0.952]	0.037	3.869 [2.095–25.227]
IVH	0.396 [0.159–0.978]	0.047	4.332 [2.295–38.675]
IVH and/or death	0.364 [0.164–0.808]	0.013	3.143 [1.878–9.623]
BPD	0.100 [0.014–0.735]	0.024	3.444 [2.145–8.741]
BPD and/or death	0.238 [0.075–0.759]	0.015	3.431 [2.069–10.047]

NAC N-acetylcysteine, *IVH* intraventricular hemorrhage, *BPD* bronchopulmonary dysplasia, *CI* confidence interval, *NNT* number needed to treat.
[a]Composite of culture-proven sepsis (any type), IVH grades 3 and 4, ROP grades 2–4, NEC grades 2–4, PVL, BPD, and/or death.
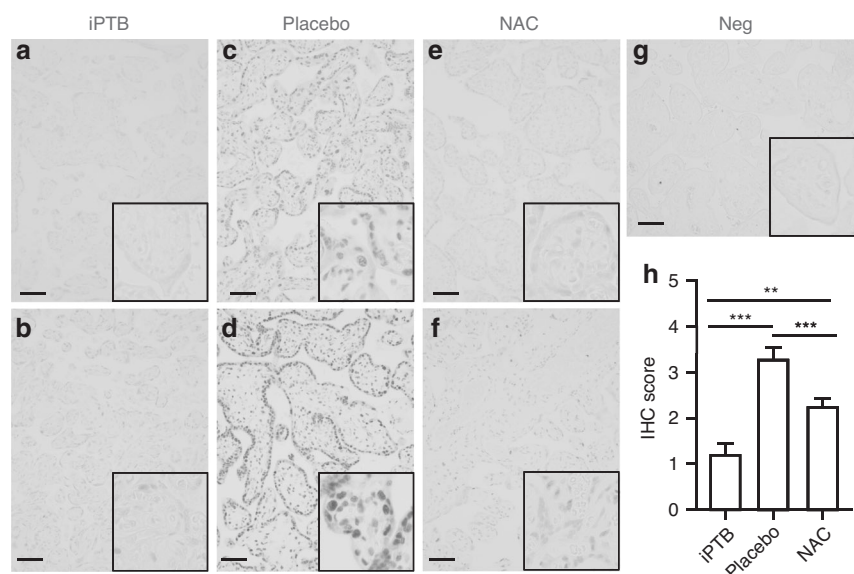
Fig. 2 Immunohistochemistry of placental villous tissue for histone deacetylase-2. Representative micrographs of immunohistochemical staining for histone deacetylase-2 (HDAC2) in placental villous tissue from women with idiopathic preterm birth (iPTB, **a**, **b**) absent Triple I or PTB in the context of Triple I who were enrolled in the trial and received either placebo (**c**, **d**) or *N*-acetylcysteine infusion (**e**, **f**). Vector NovaRed was used as peroxidase substrate and tissues were imaged and scored blindly for staining intensity without counterstaining. Negative slides (**g**) were exposed to nonimmune serum. Higher magnification inserts are shown in each lower right corner. All tissues available from the subjects in the trial were analyzed (NAC: *n* = 32; placebo: *n* = 33). ******$P < 0.01$; *******$p < 0.001$. The cases presented in **a**, **c**, **e**, and **g** were delivered at 28 weeks of gestation. The cases illustrated in **b**, **d**, and **f** were delivered at 32 weeks of gestation. The scale bar (50 μm) denotes the magnification for the panels, which were photographed at ×200. Insets were imaged at ×600 magnification.

A limitation of this study was that we could not reach our initial planned enrollment given our attempt to complete the trial at a single institution. Amniocentesis, as narrow eligibility criteria, was a significant limiting factor for the feasibility of this trial by government-sponsored, multicenter perinatal research networks. In the absence of prior experience with NAC human research at the time this trial started, the sample size was not calculated based on the primary outcome, but on a mechanistic endpoint (30% increase in fetal RBC GSH). Our assumption based on animal models proved incorrect to human biology. Nevertheless, this study provides the basis of future larger clinical trials, which now could be adequately powered for clinically relevant outcomes (delivery room resuscitation, BPD incidence), and wider inclusion criteria, which could not have been foreseen prior to this trial.

Overall, we showed that antenatal and intrapartum administration of NAC has a beneficial effect on outcomes of premature newborns born in the context of Triple I. The results of the current trial represent a clinical and translational research step forward towards understanding the mechanism by which NAC exerts its protective effects.

AUTHOR CONTRIBUTIONS
C.S.B.: conceptualization and design, methodology, protocol development, patient enrollment, acquisition of data, monitoring for possible adverse events, review of data analysis, allocation of funding and writing of the first draft of the manuscript, and revisions based on coauthor contributions. M.O.B.: conceptualization and design, methodology, protocol development, acquisition of data, patient enrollment and monitoring for possible adverse events, and revising of the manuscript. G.Z.: processing and indexing of biological specimens, acquisition and analysis of ELISA and immunohistochemistry data, and revising of the manuscript. O.A.: conceptualization and design, randomization and blinding, procurement and preparation of the

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investigational drug, obtained funding, and revising of the manuscript. L.S.: sample preparation, acquisition and analysis of HPLC data, and revising of the manuscript. S.A.-R.: protocol development, patient enrollment, monitoring for possible adverse events, acquisition of data and biological specimens, and revising of the manuscript. A.T.D.: protocol development, patient enrollment, monitoring for possible adverse events, acquisition of data and biological specimens, and revising of the manuscript. H.S.L.: protocol development, patient enrollment, monitoring for possible adverse events, acquisition of data and biological specimens, and revising of the manuscript. S.M.: sample preparation, acquisition and analysis of HPLC data, and revising of the manuscript. L.R.: development of the methodology for measurement of free thiols by HPLC, analysis of HPLC data, and revising of the manuscript. V.B.: conceptualization and design, methodology, protocol development, acquisition and analysis of neonatal outcome data, and revising of the manuscript. I.A.B.: conceptualization and design, methodology, protocol development, processing and indexing of biological specimens, acquisition of data, data analysis, allocation of funding, writing of the first draft of the manuscript, and subsequent revisions based on coauthor contributions. All authors read and approved the final submitted version.

ADDITIONAL INFORMATION

REFERENCES

1. Miller, J. M. Jr., Pupkin, M. J. & Hill, G. B. Bacterial colonization of amniotic fluid from intact fetal membranes. *Am. J. Obstet. Gynecol.* **136**, 796–804 (1980).
2. Buhimschi, I. A. et al. Advances in medical diagnosis of intra-amniotic infection. *Expert Opin. Med. Diagn.* **7**, 5–16 (2013).
3. Mendz, G. L., Kaakoush, N. O. & Quinlivan, J. A. Bacterial aetiological agents of intra-amniotic infections and preterm birth in pregnant women. *Front. Cell. Infect. Microbiol.* **3**, 58 (2013).
4. Gupta, M., Emary, K. & Impey, L. Emergency cervical cerclage: predictors of success. *J. Matern. Fetal Neonatal Med.* **23**, 670–674 (2010).
5. Medley, N., Vogel, J. P., Care, A. & Alfirevic, Z. Interventions during pregnancy to prevent preterm birth: an overview of Cochrane systematic reviews. *Cochrane Database Syst. Rev.* **11**, CD012505 (2018).
6. Higgins, R. D. et al. Evaluation and management of women and newborns with a maternal diagnosis of chorioamnionitis: summary of a workshop. *Obstet. Gynecol.* **127**, 426–436 (2016).
7. Nayeri, U. A. et al. Components of the antepartum, intrapartum, and postpartum exposome impact on distinct short-term adverse neonatal outcomes of premature infants: a prospective cohort study. *PLoS ONE* **13**, e0207298 (2018).
8. Kershenbaum, A. D. et al. Exploration of preterm birth rates using the public health exposome database and computational analysis methods. *Int. J. Environ. Res. Public Health.* **11**, 12346–12366 (2014).
9. Samuni, Y., Goldstein, S., Dean, O. M. & Berk, M. The chemistry and biological activities of *N*-acetylcysteine. *Biochim. Biophys. Acta* **1830**, 4117–4129 (2013).
10. Aldini, G. et al. *N*-Acetylcysteine as an antioxidant and disulphide breaking agent: the reasons why. *Free Radic. Res.* **52**, 751–762 (2018).
11. Ezeriņa, D., Takano, Y., Hanaoka, K., Urano, Y. & Dick, T. P. *N*-acetyl cysteine functions as a fast-acting antioxidant by triggering intracellular H(2)S and sulfane sulfur production. *Cell Chem. Biol.* **25**, 447–459.e4 (2018).
12. Tam, J., Nash, E. F., Ratjen, F., Tullis, E. & Stephenson, A. Nebulized and oral thiol derivatives for pulmonary disease in cystic fibrosis. *Cochrane Database Syst. Rev.* **7**, CD007168 (2013).
13. Liu, N. & Zhuang, S. Treatment of chronic kidney diseases with histone deacetylase inhibitors. *Front. Physiol.* **6**, 121 (2015).
14. Riggs, B. S., Bronstein, A. C., Kulig, K., Archer, P. G. & Rumack, B. H. Acute acetaminophen overdose during pregnancy. *Obstet. Gynecol.* **74**, 247–253 (1989).
15. Heard, K. J. Acetylcysteine for acetaminophen poisoning. *N. Engl. J. Med.* **359**, 285–292 (2008).
16. Buhimschi, I. A., Buhimschi, C. S. & Weiner, C. P. Protective effect of *N*-acetylcysteine against fetal death and preterm labor induced by maternal inflammation. *Am. J. Obstet. Gynecol.* **188**, 203–208 (2003).
17. Beloosesky, R., Gayle, D. A. & Ross, M. G. Maternal *N*-acetylcysteine suppresses fetal inflammatory cytokine responses to maternal lipopolysaccharide. *Am. J. Obstet. Gynecol.* **195**, 1053–1057 (2006).
18. Lanté, F. et al. Late *N*-acetylcysteine treatment prevents the deficits induced in the offspring of dams exposed to an immune stress during gestation. *Hippocampus* **18**, 602–609 (2008).
19. Wang, X. et al. *N*-acetylcysteine reduces lipopolysaccharide-sensitized hypoxic-ischemic brain injury. *Ann. Neurol.* **61**, 263–271 (2007).
20. Jenkins, D. D. et al. Fetal and neonatal effects of *N*-acetylcysteine when used for neuroprotection in maternal chorioamnionitis. *J. Pediatr.* **168**, 67–76.e6 (2016).
21. Hussey, M. J., Levy, E. S., Pombar, X., Meyer, P. & Strassner, H. T. Evaluating rapid diagnostic tests of intra-amniotic infection: Gram stain, amniotic fluid glucose level, and amniotic fluid to serum glucose level ratio. *Am. J. Obstet. Gynecol.* **179**, 650–656 (1998).
22. Abdel-Razeq, S. S. et al. Interpretation of amniotic fluid white blood cell count in "bloody tap" amniocenteses in women with symptoms of preterm labor. *Obstet. Gynecol.* **116**, 344–354 (2010).
23. Garry, D. et al. A comparison of rapid amniotic fluid markers in the prediction of microbial invasion of the uterine cavity and preterm delivery. *Am. J. Obstet. Gynecol.* **175**, 1336–1341 (1996).
24. Salafia, C. M., Weigl, C. & Silberman, L. The prevalence and distribution of acute placental inflammation in uncomplicated term pregnancies. *Obstet. Gynecol.* **73**, 383–389 (1989).
25. Matts, J. P. & Lachin, J. M. Properties of permuted-block randomization in clinical trials. *Control Clin. Trials* **9**, 327–344 (1988).
26. U.S. Food and Drug Administration. Drug Aproval Package. https://www.accessdata.fda.gov/drugsatfda_docs/nda/2004/21-539_Acetadote.cfm (2019).
27. Shennan, A. T., Dunn, M. S., Ohlsson, A., Lennox, K. & Hoskins, E. M. Abnormal pulmonary outcomes in premature infants: prediction from oxygen requirement in the neonatal period. *Pediatrics* **82**, 527–532 (1988).
28. Buhimschi, I. A., Buhimschi, C. S., Pupkin, M. & Weiner, C. P. Beneficial impact of term labor: non-enzymatic antioxidant reserve in the human fetus. *Am. J. Obstet. Gynecol.* **189**, 181–188 (2003).
29. Buhimschi, C. S. et al. Proteomics mapping of cord blood identifies haptoglobin "switch-on" pattern as biomarker of early-onset neonatal sepsis in preterm newborns. *PLoS ONE* **6**, e26111 (2011).
30. Buhimschi, C. S. et al. and Human Development Maternal-Fetal Medicine Units Network. Cord blood haptoglobin, cerebral palsy and death in infants of women at risk for preterm birth. A secondary analysis of a randomised controlled trial. *Lancet EClinicalMedicine* **9**, 11–18 (2019).
31. Rahman, I., Marwick, J. & Kirkham, P. Redox modulation of chromatin remodeling: impact on histone acetylation and deacetylation, NF-kappaB and pro-inflammatory gene expression. *Biochem. Pharmacol.* **68**, 1255–1267 (2004).
32. Zhu, L., Li, H., Tang, J., Zhu, J. & Zhang, Y. Hyperoxia arrests alveolar development through suppression of histone deacetylases in neonatal rats. *Pediatr. Pulmonol.* **47**, 264–274 (2012).
33. Dammann, O. & Leviton, A. Inflammation, brain damage and visual dysfunction in preterm infants. *Semin. Fetal Neonatal Med.* **11**, 363–368 (2006).
34. Rocha, G., Proenca, E., Quintas, C., Rodrigues, T. & Guimaraes, H. Chorioamnionitis and brain damage in the preterm newborn. *J. Matern. Fetal Neonatal Med.* **20**, 745–749 (2007).
35. Fouhy, F. et al. Perinatal factors affect the gut microbiota up to four years after birth. *Nat. Commun.* **10**, 1517 (2019).
36. Stetson, B. T. et al. Comparison of cerebral palsy severity between two eras of antenatal magnesium use. *JAMA Pediatr.* **173**, 188–190 (2019). 1.
37. American College of Obstetricians and Gynecologists' Committee on Practice Bulletins—Obstetrics. Practice Bulletin No. 171: management of preterm labor. *Obstet. Gynecol.* **128**, e155–e164 (2016).
38. Davidson, L. M. & Berkelhamer, S. K. Bronchopulmonary dysplasia: chronic lung disease of infancy and long-term pulmonary outcomes. *J. Clin. Med.* **6**, E4 (2017).
39. Stoll, B. J. et al. for the Eunice Kennedy Shriver National Institute of Child Health and Human Development Neonatal Research Network. Trends in care practices, morbidity, and mortality of extremely preterm neonates, 1993–2012. *JAMA.* **314**, 1039–1051 (2015).
40. Dumpa, V. & Bhandari, V. Surfactant, steroids and non-invasive ventilation in the prevention of BPD. *Semin. Perinatol.* **42**, 444–452 (2018).
41. Snary, D., Allen, A. & Pain, R. H. Structural studies on gastric mucoproteins: lowering of molecular weight after reduction with 2-mercaptoethanol. *Biochem. Biophys. Res. Commun.* **40**, 844–851 (1970). 24.
42. Riise, G. C., Qvarfordt, I., Larsson, S., Eliasson, V. & Andersson, B. A. Inhibitory effect of *N*-acetylcysteine on adherence of *Streptococcus pneumoniae* and *Haemophilus*

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influenzae to human oropharyngeal epithelial cells in vitro. *Respiration* **67**, 552–558 (2000).

43. Blasi, F. et al. The effect of *N*-acetylcysteine on biofilms: implications for the treatment of respiratory tract infections. *Respir. Med.* **117**, 190–197 (2016).

44. Van Hecke, O. & Lee, J. N-acetylcysteine: A rapid review of the evidence for effectiveness in treating COVID-19. https://www.cebm.net/covid-19/n-acetylcysteine-a-rapid-review-of-the-evidence-for-effectiveness-in-treating-covid-19/ (2020).

45. Ahola, T. et al. *N*-acetylcysteine does not prevent bronchopulmonary dysplasia in immature infants: a randomized controlled trial. *J. Pediatr.* **143**, 713–719 (2003).

46. Bartling, T. R. & Drumm, M. L. Loss of CFTR results in reduction of histone deacetylase 2 in airway epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **297**, L35–L43 (2009).

47. Noh, H. et al. Histone deacetylase-2 is a key regulator of diabetes- and transforming growth factor-beta1-induced renal injury. *Am. J. Physiol. Renal Physiol.* **297**, F729–F739 (2009).

48. Wiest, D. B. et al. Antenatal pharmacokinetics and placental transfer of *N*-acetylcysteine in chorioamnionitis for fetal neuroprotection. *J. Pediatr.* **165**, 672–677.e2 (2014).

49. Sprong, R. C. et al. Low-dose *N*-acetylcysteine protects rats against endotoxin-mediated oxidative stress, but high-dose increases mortality. *Am. J. Respir. Crit. Care Med.* **157**, 1283–1293 (1998).

50. Kerr, F. et al. The Australasian Clinical Toxicology Investigators Collaboration randomized trial of different loading infusion rates of *N*-acetylcysteine. *Ann. Emerg. Med.* **45**, 402–408 (2005).

51. Probyn, M. E. et al. The anti-inflammatory agent *N*-acetyl cysteine exacerbates endotoxin-induced hypoxemia and hypotension and induces polycythemia in the ovine fetus. *Neonatology* **98**, 118–127 (2010).

52. Dulay, A. T. et al. Compartmentalization of acute phase reactants interleukin-6, C-reactive protein and procalcitonin as biomarkers of intra-amniotic infection and choriomnionitis. *Cytokine* **76**, 236–243 (2015).