METABOLOMIC ANALYSIS TO PREDICT THE ONSET AND SEVERITY OF NECROTIZING ENTEROCOLITIS

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ABSTRACT

Background: Necrotizing enterocolitis (NEC) is the most devastating gastrointestinal (GI) emergency of the preterm neonate. Untargeted metabolomics may allow the identification of biomarkers involved in NEC pathophysiology.

Methods: We conducted a prospective study including preterm infants born <34 gestational weeks (GW) who had their urine longitudinally collected at birth (<48 hours, T0), at 14 (T1) and 28 days (T2). Neonates were followed for their development of NEC, spontaneous intestinal perforation (SIP), or other GI conditions and compared to matched healthy controls. Urine samples were investigated by untargeted metabolomic analysis based on mass-spectrometry.

Findings: Thirty-five cases of NEC, 5 cases of SIP, 14 patients with other GI diseases and 113 controls were enrolled and then selected for the metabolomic analysis on the basis of their clinical characteristics and available samples. Considering urine samples at T0, one-class classification approach was able to correctly classify 16/20 subjects (80%) developing NEC, 3/3 (100%) subject developing SIP and 5/7 subjects (71.4%) with other GI pathologies as not belonging to the control group. Neonates with surgical NEC showed higher N-acetylaspartic acid, butyrylcarnitine and propionylcarnitine levels than those with medical NEC. Considering the time evolution of the urinary metabolome, NEC and control groups showed differences independently of the time point.

Interpretation: The urinary metabolome is closely associated with the underlying GI disease from birth. Urinary metabolic features characterize NEC cases from healthy controls until 28 days of life. The early urinary metabolome has a potential to predict surgical NEC. Future studies are needed to validate our results.

Research in context

Evidence before this study: Untargeted metabolomics has been applied for the prediction of necrotizing enterocolitis (NEC) on plasma, urine and stools. However, no studies have explored the differences in metabolic profiles of NEC and other gastrointestinal (GI) conditions of the preterm infants, nor the possibility to predict NEC severity.

Added value of this study: We demonstrate that, as early as from birth, untargeted metabolomics is able to correctly differentiate the underlying GI disease (NEC, spontaneous intestinal perforation or other conditions) in respect to a healthy state. Additionally, it is able to identify NEC cases requiring surgery, who show an impaired short-chain fatty acids metabolism.

Implications of all the available evidence: The metabolic profile of early urine samples may identify neonates at risk of NEC and who will need surgery for the disease. Further improvement of this approach may permit to apply preventive and timely therapeutic strategies to preterm infants at higher risk.

Key words: necrotizing enterocolitis, preterm neonate, biomarker, mass-spectrometry, metabolome

BACKGROUND

Necrotizing enterocolitis (NEC) is a devastating gastrointestinal emergency of the preterm neonate, affecting about 6% of very low birth weight infants (VLBWI, <1500 g) and 7% (2-13%) of extremely low gestational age neonates (ELGAN) (1,2). The mortality rate can reach 50% in surgical cases with BW <1000 g and 80% in those with a fulminant course. Additionally, NEC is associated with increased morbidity in survivors, among which intestinal failure due to short bowel syndrome, cholestasis, failure to thrive, and neurodevelopmental sequalae (3,4).

NEC can manifest within various stages along a continuum of bowel disease. The famous classification proposed in 1978 by Bell and colleagues, with the subsequent modification (5) by Walsh and Kliegman in 1986 (6), is still the most widely used by clinicians worldwide. With this staging system based on pure clinical and radiological signs, however, it is often difficult to differentiate NEC from other gastrointestinal (GI) diseases, in particular from spontaneous intestinal perforation (SIP) (7–9), self-resolving feeding intolerance, and paralytic ileus due to sepsis (10).

As clinical evaluation alone may be unreliable, neonatologists usually support their suspicion of NEC with several laboratory tests, despite the majority of alterations remain aspecific and with low sensitivity (11–14).

Metabolomics, the youngest of the four major "omics" sciences, enables to depict the ultimate phenotypic expression of the ongoing biochemical response to a stimulus (15). Indeed, as last downstream products of gene transcription and enzymatic pathways, metabolites provide a closest picture of the organism's phenotype after its interaction with the environment (16,17). This technique can be applied to different biological fluids, like those non-invasively collected from preterm neonates (umbilical cord blood, plasma, urine, stool, tracheal aspirate, etc.), as the amount required for the analysis is very small (20 μL-300 μL) (17,18). Additionally, by the *untargeted approach* the entire set of metabolites present in a biological sample can be extensively and comprehensively analyzed without any a-priori hypothesis (16). Thus, new potential *profiles* of biomarkers can be discovered, untangling unknown pathogenetic mechanisms of a disease (16).

Despite the continuous evolution of metabolomic studies in neonatal research, few are, so far, their applications to NEC and, especially, to its differential diagnostic conditions like SIP (19).

A recent systematic review conducted by our group (20) highlighted the presence of only five studies applying untargeted metabolomics (43 cases, 95 preterm controls) for NEC prediction or diagnosis (Bell's stage ≥II). The studies were cross-sectional with a prospective collection of samples and their retrospective analysis using either nuclear magnetic resonance (NMR) spectroscopy or ultra-performance liquid chromatography-mass spectrometry (UPLC-MS) applied on urinary or fecal samples. In these studies, metabolites belonging to pathways related to inflammatory response and intestinal permeability (linoleate metabolism, C21-steroid hormone biosynthesis, leukotriene metabolism, formation of prostaglandin from arachidonate, sphingomyelins, ceramides)(21,22), as well as to energy depletion (amino acids)(23–25) were hypothesized to be characteristic of affected infants. Interestingly, no studies evaluated the metabolic differences between NEC and SIP or other gastrointestinal conditions of the preterm infant, nor focused on biomarkers of NEC severity.

An updated systematic review on targeted and untargeted metabolomics in NEC also highlighted as main metabolomic alterations those involving amino acids composition (in blood and urine), fatty acid metabolism (in blood), and less homogeneous findings in feces (26).

Given the scarcity of studies on the matter, we investigated the metabolic profile of infants developing NEC in order to find early predictive biomarkers of the disease and of its severity. The primary aim of the study was to apply untargeted metabolomics based on mass spectrometry (MS) on early urine samples to identify potential biomarker profiles of NEC development in respect to healthy neonates and to those developing SIP or other GI conditions. Secondary aims were to explore the evolution of the urinary metabolome during the first month of life in neonates developing NEC vs healthy controls, and to characterise potential prognostic metabolic profiles of NEC severity (i.e. surgical NEC vs medical NEC).

MATERIAL AND METHODS

Study design and population

We conducted a single-centre study at the Neonatal Intensive Care Unit (NICU) of Padova University Hospital (Veneto, Italy) from January 2020 to July 2022. All infants admitted to the NICU born at $<34^{+0}$ gestational weeks (GW) or with congenital heart disease (CHD) were enrolled after written informed consent of a legally acceptable representative. Exclusion criteria were major congenital anomalies or chromosomal abnormality, isolated structural abnormalities of the gut (i.e. omphalos or gastroschisis), or refusal of consent. Patients were prospectively followed for their possible development of NEC, SIP, or other GI conditions (cases) until discharge, or transfer to another Unit or Hospital, or death. Infants not developing these conditions were considered as healthy controls.

This was a pilot study applying an untargeted metabolomic approach, for which there was no a-priori hypothesis of the number of variables characterizing each study group. Therefore, the sample size was based on previous studies on the topic and on the incidence of NEC in our center (around 10 cases/per year, thus making 20 NEC subjects suitable for an explorative data analysis).

For each patient clinical and demographic characteristics, as well as laboratory data, were recorded on a preformed electronic case report form (eCRF) on the REDCap platform Plasma, urine and faecal samples were non-invasively collected at birth (within 48 hours, T0), at 14 (T1) and 28 DOL (T2), at 2 months (T2 months) and at 36 weeks of corrected gestational age (cGA) (T36). In the suspicion of NEC, SIP or other GI diseases with a presentation similar to NEC, additional plasma, urine and faecal samples were collected at symptoms' onset and then weekly until resolution. More details on data and samples' collection can be found in the Supplemental Material.

NEC was defined according to the modified Bell's stage criteria by Walsh and Kliegman (6).

SIP was defined as isolated intestinal perforation with presence of free intraperitoneal air (pneumoperitoneum) on abdominal x-ray and without evidence of pneumatosis, or of necrosis if laparotomy performed.

Functional paralytic ileus due to sepsis was defined as intolerance to oral intake (increase in gastric residuals > 50% of the volume of the preceding feed, with abdominal distension, and consequent interruption of enteral feeding) with an ongoing late onset sepsis (Vermont Oxford Network VON Criteria (27)) and without any sign of mechanical intestinal obstruction.

Minimal enteral feeding (MEF) was defined as trophic feeds with a total volume of ≤24 ml/kg/day, while full enteral feeding (FEF) when enteral amount reached 150 ml/kg/day.

Other clinical variables, such as prenatal flow alterations, preterm premature rupture of membranes (PPROM), intra-uterine growth restriction (IUGR), hemodynamically significant patent ductus arteriosus (HSPDA), were defined according to the local protocols.

The protocol of this study was written according to the principles of Good Clinical Practice of the European Union and Helsinki Declaration, and was approved by the Institutional Review Board of Padova University Hospital (5128/AO/21, 4374/AO/17). No funding sources supported this study.

Figure 1 depicts the diagram of the study design.

Metabolomic and statistical analyses

Urine samples were analyzed at the Mass Spectrometry and Metabolomics Laboratory of the Women's and Children's Health Department, (Paediatric Research Institute IRP, University of Padova, Italy). Untargeted metabolic profiling was performed in positive and negative electrospray ionization (ESI+, ESI-) mode on an Acquity Ultra Performance Liquid Chromatography (UPLC) system (Waters, U.K.) coupled to a Quadrupole Time-of-Flight (QToF) Synapt XS HDMS mass spectrometer (Waters MS Technologies, Ltd., Manchester, U.K.). After raw data extraction, quality controls and blanks were used to calibrate and filter out the data. More details about metabolomics investigation can be found in the Supplemental Material.

Categorical data were investigated by Fisher's exact test, whereas t-test or Mann-Whitney test were applied to continuous data with normal or non-normal distribution, respectively. Normal distribution was assessed by Shapiro-Wilk test.
Since urinary metabolome is strongly dependent on the characteristics of the subjects, a suitable procedure of matching was applied to avoid bias due to differences in the perinatal and neonatal characteristics of the groups under investigation(28).
Different approaches were applied to investigate the urinary metabolomics data.
Specifically, one-class classification (OCC), including both univariate and multivariate methods in the framework of Model Population Analysis (MPA)(29), was applied to discriminate controls from the other subjects. In the case of two-group comparison, PLS for classification (PLSC) with stability selection(30) and Mann-Whitney test controlling the false discovery rate by Benjamini-Hochberg method(31) were used. PLS for designed experiments (PLS-doe)(32) and Linear Mixed-Effects modelling (LME) for longitudinal data(33) were applied to study the time evolution during the three time points. More details about statistical data analysis can be found in Section 1.2 of Supplemental Materials.
Data analysis was performed by R-functions developed in-house using the platform R 4.0.4 (R Foundation for Statistical Computing).
Clinical and metabolomic data have been deposited in Mendeley Data Repository (Moschino, L; Stocchero, M (2024), "METNEC Study ", Mendeley Data, V1, doi: 10.17632/83myp4xfcn.1).

RESULTS

Clinical characteristics of cases and controls

During the study period, 35 cases of NEC, 5 cases of SIP, 14 cases of other GI diseases in differential diagnosis with NEC (i.e. septic paralytic ileus, isolated rectal bleeding), and 113 healthy controls were recruited. The main clinical and laboratory characteristics of the unmatched initial groups are reported in Table 1. These show that both NEC and SIP groups carry a high burden of morbidity, with a prolonged time to reach full enteral feeding and a high risk of post-disease strictures (up to 18.5% and 20% in our cohort, respectively).

Among the 35 NEC patients, 27 were affected by NEC Bell's stage ≥II (11 medically treated and 16 surgically treated). Due to the small size of the SIP group, a statistical comparison between the NEC Bell's stage ≥II cases and the SIP cases was not performed. The main clinical and laboratory characteristics of these two groups are reported in Table 2. All these cases were treated with fasting and triple antibiotic therapy (usually vancomycin or ampicillin, ceftazidime or gentamicin and metronidazole).

Table 1. Main perinatal and neonatal characteristics of the different groups (NEC cases, SIP cases, cases with other GI conditions, controls). Data expressed as absolute number (%) or as median (IQR).

	NEC Group (n=35)	**SIP Group** (n=5)	**Other Gastrointestinal conditions** (n=14)	**Control Group** (n=113)
Type of disease	**Bell's stage of NEC** IA 6 (17.1%) IB 2 (5.7%) IIA 12 (34.3%) IIB 1 (2.9%) IIIA 6 (17.1%) IIIB 8 (22.9%)	NA (1 case with development of NEC IIIB after SIP)	**Final diagnosis** Septic paralytic ileus 2 (14.3%) Isolated rectal bleeding 8 (57.1%) Others (volvulus, obstruction) 4 (28.6%)	NA
Sex				
Male	23 (65.7%)	4 (80%)	5 (35.7%)	63 (55.8%)
Female	12 (34.3%)	1 (20%)	9 (64.3%)	50 (44.2%)
GA at birth (weeks)	26.6 (25-30.1)	26.4 (26.3-28.1)	30.3 (27.6-32.6)	30 (28.1-32)
Birth weight (grams)	855 (752.5-1230)	790 (740-825)	1353 (816-1610)	1230 (950-1550)
IUGR	9 (25.7%)	2 (40%)	2 (14.3%)	24 (21.2)
Apgar at 5 minutes	7 (6-8)	7 (6-7)	8 (7-8)	8 (7-8)
Ethnicity				
Caucasic	33 (94.3%)	4 (80%)	11 (78.6%)	96 (85%)
African	1 (2.9%)	1 (20%)	2 (14.3%)	11 (9.7%)
Asiatic	1 (2.9%)	0 (0%)	1 (7.1%)	6 5.3%)
Mode of delivery				
Caesarean section	29 (82.9%)	4 (80%)	12 (85.7%)	100 (88.5%)
Vaginal delivery	6 (17.1%)	1 (20%)	2 (14.3%)	13 (11.5%)
Prenatal steroids				
Complete	20 (57.2%)	5 (100%)	10 (71.4%)	85 (75.2%)
Incomplete	6 (17.1%)	0 (0%)	1 (7.1%)	16 (14.2%)
None	9 (25.7%)	0 (0%)	3 (21.5%)	12 (10.6%)
Treatment with surfactant	24 (68.6%)	5 (100%)	9 (64.3%)	61 (54%)
HsPDA	18 (51.4%)	2 (40%)	4 (28.6%)	29 (25.7%)
EOS				
No	26 (74.2%)	5 (100%)	10 (71.4%)	90 (79.6%)
Suspected	8 (22.9%)	0 (0%)	4 (38.6%)	21 (18.6%)
Definitive	1 (2.9%)	0 (0%)	0 (0%)	2 (1.8%)
Total days of ATBs for EOS	7 (3-8)	3 (3-5)	6 (4-7)	4 (3-6)
Postnatal systemic steroids	6 (17.1%)	1 (20%)	1 (7.1%)	14 (12.4%)
Human milk at start of feeding	25 (71.4%)	4 (80%)	9 (64.3%)	68 (60.2%)
Total days to reach FEF	37.5 (26.7-65.5)	47 (41-114)	29 (19-48)	12 (8-21)

Abbreviations: ATBs=antibioticsM; EOS=early onset sepsis; FEF=full enteral feeding; GA=gestational age; HsPDA=haemodynamically significant patent ductus arteriosus; IUGR=intrauterine growth restriction;

Table 2. Main clinical and laboratory characteristics of the neonates affected by NEC Bell's stage ≥II and by SIP within 48 hours before and after onset, the clinical management and potential complications. Data expressed as absolute number (%) or as median (IQR).

	NEC ≥ II (n=27)	**SIP** (n=5)
Type of disease	**Bell's stage of NEC** IIA 12 (37.5%) IIB 1 (3.1%) IIIA 6 (18.8%) IIIB 8 (25%)	
DOL at disease onset	10 (7-23.5)	4 (4-5)
Fulminant onset (less than 48 h between onset and surgery/death)	10 (37%)	0 (0%)
Feeding with only human milk at onset	8 (29.6%)	3 (60%)
Total enteral amount at onset (ml/kg/day)	140 (89.2-160)	28.5 (14.2-42.7)
Transfusion of RBC within 48 hours before onset	7 (25.9%)	2 (40%)
Days of NPO for the disease*	16 (11-21)	15 (7-10)
Days of ATB for the disease	15 (11.5-18.5)	13 (10-14)
Haematocrit (%)	31.9 (27.3-35)	34.1 (33-36.1)
Lowest WBC (/mmc)	6510 (4140-12620)	6820 (6250-6970)
Lowest neutrophil count (/mmc)	2970 (1690-7740)	5680 (4715-32725)
Lowest platelet count (/mmc)	153000 (58000-307000)	66000 (34000-172000)
Highest CRP (mg/L)	40 (8.6-80)	46.9 (8.8-52)
Lowest albumin (g/L)	27.5 (22-32.5)	23 (22-27)
CRP/Albumin ratio	1 (0-3.75)	2 (0-2)
Surgery for NEC/SIP	16 (59.3%)	5 (100%)
Type of surgery for NEC/SIP n (% of those with surgery) PD ExLap PD followed by ExLap	 2** (12.5%) 9 (56.3%) 5 (31.2%)	 2 (40%) 1 (20%) 2 (40%)
Intestinal involvement at ExLap or autopsy n (% of those explored) Ileal Colic Pan-NEC	 9 (60%) 1 (6.7%) 5 (33.3)	 1 (20%) 2 (40%)
Haemoculture positive	5 (18.5%) *1 St. aureus, 1 Klebsiella pneumonia ESBL, 1 Ent. Cloacae, 1 Str. Haemolyticus, 1 E. coli*	1 (20%) *Candida albicans*
Peritoneal fluid culture positive	7 (25.9%) *1 Klebsiella pneumoniae, 1 Cl. Perfrigens and St. aureus, 3 Ent. Faecalis, 1 St. hominis, 1 Cl. Perfrigens*	1 (20%) *Candida parapsilosis*
Stenosis post-NEC/SIP	5 (18.5%)	1 (20%)
Death	6 (22.2%)	0 (0%)

*Including days of NPO for subsequent surgeries or complications of NEC

**One death before performing ExLap, autopsy not performed

Abbreviations: ATB=antibiotics; CRP=C-reactive protein; ExLap=exploratory laparotomy; NEC=necrotizing enterocolitis; NPO=nihil per os; PD=peritoneal drainage; SIP=spontaneous intestinal perforation; WBC=white blood cells.

Data analysis of the urinary metabolome

Since the urinary metabolome is strongly dependent on the perinatal and neonatal characteristics of the recruited subjects, a suitable and strict matching procedure was applied to have groups of subjects with similar characteristics, in order to avoid bias in data analysis. Additionally, only neonates with enough urine for the metabolomic analysis were considered. Specifically, the NEC group comprised 20 cases and the Control group 17 neonates after matching for the main perinatal and neonatal characteristics (GA, BW, SEX, PROM, IUGR, EOS, HSPDA, delivery, Apgar 5', prenatal steroids, surfactant, outborn/inborn) (**Table S1** of the Supplemental material) and assuming a significant level of 0.05. The NEC Group included 7 neonates with NEC Bell's stage I, 6 neonates with NEC Bell's stage II and 7 neonates with NEC Bell's stage III. Additionally, 3 subjects with SIP and 7 subjects with other GI diseases with their urine samples collected at T0 were considered for the first step of the analysis. These latter groups were found to have similar GA and BW to the control group. After data pre-processing, two datasets, one composed of 1002 variables from the data acquired in negative ionization mode (*NEG dataset*) and one of 1086 variables from the data acquired by positive ionization mode (*POS dataset*), were obtained.

Urinary metabolome at birth (T0)

As a preliminary data analysis, the one-class classification (OCC) was applied to compare healthy controls with the other groups. The models were able to correctly predict 16 out of 20 (80%) subjects with NEC, 5 out of 7 (71.4%) subjects with other GI pathologies and 100% of the subject developing SIP as not belonging to the control group (**Figure S1** of the Supplemental material**).**

Considering only the comparison of the NEC group and the Control Group, Mann-Whitney test controlling the FDR at level 0.05 discovered 8 and 16 metabolic variables from the NEG and POS datasets, respectively.. The volcano plots of **Figure 2A and 2C** summarize the results. The PLS2C models showed 3 score components for each dataset with Matthews's correlation coefficients of cross-validation (MCCcv) of 0.782 ($p=0.008$) and of 0.623 ($p=0.020$) for NEG and POS datasets. The MCC calculated with the out-of-bag predictions during stability selection (MCCoob) were 0.664 and 0.596, respectively. Assuming a significant level of 0.05, 111 and 66 features from NEG and POS datasets, respectively, resulted to be relevant in distinguishing the two groups. The relevant scores calculated for the datasets are reported in **Figure 2B and 2D.**

Merging the results from the univariate and multivariate data analyses, 111 and 67 relevant features were discovered for the NEG and the POS datasets, respectively.

Metabolome evolution in the first 28 days of life

Eighteen subjects of the NEC group and 14 controls had their urine samples collected at all the three time points of the experimental design.

Exploratory data analysis based on PLS-doe showed that the urinary metabolome changed during time, but mantained a difference between NEC and control groups. Indeed, a PLS-doe model with 3 score components, $R^2=0.71$ ($p<0.01$) and $Q^2=0.57$ ($p<0.01$) was obtained for the NEG dataset and a model with 4 score components, $R^2=0.79$ ($p<0.01$) and $Q^2=0.63$ ($p<0.01$) was calculated for the POS dataset. The score scatter plots of the two model are reported in **Figure 3**.

LME analysis controlling the FDR at level 0.05 was applied to the set of relevant metabolites discovered at birth. Thirty-three and 44 variables for the NEG and POS datasets, respectively, were discovered as significant in distinguishing the two groups throughout the first 28 days of life (**Figure 4)**.

Searching the Human Metabolome Database (HMDB), variable annotation led to 12 variables annotated at level 3. Among these, mevalonic acid and N-acetylcystathionine and their isomers had

a FC[NEC/CTRL]<0.4, thus being reduced in NEC patients compared to controls (**Table S2** of the Supplemental material).

Prediction of NEC severity from urine at birth (T0)
A comparison between surgical and medical NEC cases was performed considering urine at T0. To avoid bias of the analysis due to differences in the clinical data, 7 subjects with medical NEC were matched to 7 surgical NEC cases **(Table S3** of the Supplemental material**).**
To limit redundancy in the data and spurious results, the analysis was focused on the behavior of 89 annotated metabolites at level 1 of the HMDB, which were identified and used to characterize the two groups. Univariate data analysis discovered 2 metabolites with a p-value<0.10. Multivariate analysis based on PLS2C generated a model with 1 score component, MCCcv 0.577 with p=0.036, MCCoob 0.500. Combining the two analyses, 6 metabolites were relevant in distinguishing the two groups **(Table 3, Figure 5)**. In particular, N-Acetyl-aspartic acid, Butyrylcarnitine and Propionylcarnitine resulted to be increased in patients with surgical NEC, whereas 5-Hydroxyindolacetic acid decrease in these patients.

Table 3. Relevant metabolites in the comparison of medical vs surgical NEC: annotation is the name of the compound, FC[medical/surgical] is the fold change at T0 calculated as ratio between the median in the medical NEC group and the median in the surgical NEC group; p is the p-value of the Mann-Whitney test.

Annotation	FC[medical/surgical]	p
5-Hydroxyindolacetic acid	2.12	0.017
N-Acetyl-aspartic acid	0.66	0.073
Butyrylcarnitine (C4 carnitine)	0.44	0.128
Propionylcarnitine (C3 carnitine)	0.09	0.165
Taurohyocholic acid	0.80	0.209
N-Acetyl-glycine	1.07	0.209

DISCUSSION

NEC remains one of the most common and yet inexplicable causes of death in preterm infants (2). Its multifactorial pathogenesis makes it challenging to unravel a single biomarker of the disease (13,14,34,35). Rapid, bedside, point-of-care tests, to be performed prior to or at clinical manifestations, may help in guiding the management, for instance in the matter of feeding strategies, appropriate administration of antibiotics, or the need for urgent surgical intervention.
With their hypothesis-free hypothesis-generating approach, the "omic" technologies may untangle a better understanding of the molecular processes responsible for NEC. Metabolomics has several advantages over the other omic approaches by detecting the functional end points of cellular reactions and the direct results of a biochemical response to a stimulus. As last downstream products of gene transcription and enzymatic pathways, metabolites provide a closer picture of the organism's phenotype and its interaction with the environment (16). Several reviews in the last 10 years have summarized studies using metabolomics for the detection of NEC biomarkers (20,26,35,36), revealing a wide variability in populations' inclusion criteria, NEC definition, timing of samples' collection (encompassing early samples or samples close to NEC onset), and type of analysed biological fluids (plasma, urine, or stools).

As previous similar studies, ours had a cross-sectional design with a longitudinal collection of data and samples and samples' retrospective analysis.
In order to exclude biases given by the possible influence of perinatal and postnatal factors on the individual's metabolome, a strict selection of subjects was applied and only groups matched for the major clinical characteristics were considered for the metabolomic analysis.

Firstly, we applied one-class classification using MPA to find prediction models of diseases in respect to a healthy state. MPA is a powerful tool for modelling as it permits variable selection, outlier detection, and model comparison, thus improving the prediction of the model (29). By this statistical analysis, we found that metabolomic analysis applied on early urine samples was able to correctly identify subjects developing NEC, SIP or other GI diseases in respect to controls with a good prediction rate. Therefore, the urinary metabolome appeared to be closely associated to the underlying disease from very early life.

Similarly, both univariate and multivariate analysis were able to generate good prediction models distinguishing NEC cases vs controls based on relevant metabolic features from the early urine samples. Indeed, the Matthews's correlation coefficients of the models (MCCcv and MCCoob) were >0.59 (an explanation of these scores is described in the Supplemental Material). Of these significant metabolites emerged at T0, 77 remained differently expressed in the two groups throughout the first 28 DOL. By searching in HMDB, 12 of these variables were annotated at level 3 (meaning that further analysis with mass spectral fragmentation should be performed to have a more robust hypothesis of the metabolite).

In particular, mevalonic acid (MVA) was significantly decreased in NEC cases compared to controls (FC[NEC/CTRL 0.12]). This hydroxy fatty acid, produced by the 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), is crucial in biosynthesis of cholesterol, as well as sterol and non-sterol isoprenoids (i.e. ubiquinone, vitamin K, isopentenyl t-RNA, heme A, and farnesyl and geranyl lipid anchors). Interestingly, a MVA pathway blockade/deregulation has been linked to mitochondrial dysfunction with consequent release of pro-apoptotic factors, defective autophagy, and possibly inflammasome activation with cell death (what occurs in the Mevalonate Kinase Deficiency MKD). We can hypothesize similar consequences in the gut of infants affected by NEC due to impaired MVA pathway resulting in increased inflammation (37,38).

A further interesting result of our study is the discrimination of surgical vs medical NEC from the urine collected within the first 2 DOL. In particular, 6 variables resulted to be relevant in distinguishing these two groups, with increased N-Acetylaspartic acid, Butyrylcarnitine and Propionylcarnitine in infants with severe disease needing surgery.

N-Acetylaspartic acid (NAA) is an amino acid predominantly found in the brain and in lower amount in peripheral organs. An upregulation of its pathway has been reported in Canavan disease (a childhood leukodystrophy), Parkinson's disease and type-2 diabetes (39). Recent studies suggest that an upregulation of NAA leads to oxidative stress with increased nitric oxide and reduced potential antioxidants in rats (40,41). While the role of this metabolite is difficult to interpret in NEC, an alteration in acylcarnitines' (ACs) profile, like Butyrylcarnitine and Propionylcarnitine (C4 and C3), has already been described in this condition. By targeted analysis on blood spots (newborn screening test), Sylvester et al. found 14 acylcarnitine levels as being associated with the risk of NEC, especially within the first 48 hours of life (42). The same group showed altered levels of certain ACs at day 1 as being associated with NEC development (43). Despite the debated function of ACs in the intestinal epithelium, it has being hypothesised by previous authors that a poor GI motility and a vulnerable mucosa (as in the preterm gut), in addition to carbohydrate malabsorption, presence of lactose from infant formula, and bacterial overgrowth, may cause gut fermentation of excessive carbohydrates to short chain fatty acids (SCFA), from which ACs derive. These, in turn, could impair the clearance of intraluminal content and reduce the intraluminal gut pH, favouring a microbial community imbalance, proliferation of bacteria and their translocation through the mucosa with consequent necrosis (42–45). Thus, the increase in ACs as a mirror of altered beta-oxidation of FA may be a proxy for metabolic prematurity, indicating a higher risk of abnormal responses to metabolic challenges, such as feeding (42,46). As in Sylvester et al. (42), also in our study changes in ACs concentrations appear to be chain length-specific, with increases in short-chain acylcarnitines (C3 and C4) conferring the risk of severe NEC.

Considering the small number of metabolomic studies on NEC and their great heterogeneity in respect to enrolled patients, diagnostic criteria, collected biofluids and analytical methods, an adequate comparison of our results to those previously published is difficult to make (20,25,34,35). Studies applying untargeted metabolomics for NEC utilized NMR spectroscopy (23–25) or UPLC-MS on plasma (47), urine or stools (21,22). The majority of studies collected samples in proximity to NEC diagnosis, i.e. samples were diagnostic for NEC or could be used to predict NEC severity. Few studies compared infants with NEC vs those with LOS (47–49), or with feeding intolerance (25). However, to our knowledge no studies evaluated the metabolic differences between cases of NEC and SIP, which is the main disease in differential diagnosis with it (20). Only one study assessed the differences in gut microbiota between infants with NEC and those with SIP on formalin fixed paraffin embedded tissue from the site of disease (50).

In urine, Morrow et al. (23) demonstrated an early (4-9 DOL) alanine/histidine ratio >4 to be associated with microbial characteristics and to have a sensitivity of 82% with a predictive value of 78% for NEC. Also, alanine was positively associated with NEC cases that were preceded by Firmicutes dysbiosis, and histidine was inversely associated with NEC cases preceded by Proteobacteria dysbiosis, indicating a close link between gut microbiota and metabolomic signatures. Picaud et al. (25), instead, applied NMR spectroscopy on urine collected before, during and after diagnosis of NEC Bell's stage II (6 patients) demonstrating that lactate, betaine, myo-inositol, urea, creatinine, and N,N-dimethylglycine discriminated late-onset NEC (> 3 weeks of life) from controls with good feeding tolerance.

Thoimadou et al. (24) applied both untargeted NMR spectroscopy and targeted LC-MS on urine samples collected after initial evaluation for NEC (15 cases, every stage) and at a similar postnatal age for controls. They found 25 discriminant metabolites, with NEC cases characterised by lower levels of Tyrosine, Proline, Citrate, 4-hydroxybenzoate, Formate, Succinate, 4-hydroxyphenylacetate, Fumarate, Creatinine, Myoinositol, and hippuric acid.

In 2022 the same authors (47) found significant differences in the metabolic profile of neonates with LOS or NEC compared to controls by analyzing their serum with LC-quadrupole time-of-flight MS. Phosphatidylcholines or lysophosphatidylcholines were significantly reduced both in neonates with LOS and those with NEC compared to controls, whereas L-carnitine could efficiently discriminate NEC cases from controls.

As it can be seen, methodological inconsistencies among studies, disagreement on conventions for data reporting and analysis, and the sheer magnitude of data produced are significant drawbacks to studies applying high-throughput multi-omics technologies. To overcome these challenges, machine learning and artificial intelligence are becoming popular to integrate omics data with NEC clinical features, phenotypes of progression, and predicted therapeutic targets, resulting in clinically meaningful information.

The major strength of our study is to have applied a strict selection of subjects in order to eliminate biases in the metabolomic analysis. Additionally, we have included infants affected by SIP or other GI diseases in differential diagnosis with NEC. Thirdly, we have also focused on the prediction of NEC severity, identifying potential biomarkers of surgical NEC. Finally, we have collected samples at multiple timepoints over the first month of life, starting from birth. This has permitted to evaluate the evolution of the metabolome through time, given its potential changes related to enteral and parenteral feeding, infections, administered drugs and comorbidities.

As regards limitations, the small number of patients has hampered the analysis of patients with only definite NEC (Bell's stage ≥II). Finally, we could not perform the analysis of samples collected at NEC diagnosis. Despite this, to our knowledge our cohort is one of the largest enrolled for an untargeted metabolomic study on NEC so far (20).

CONCLUSION

This explorative study demonstrates that the urinary metabolome is closely associated to the underlying gastrointestinal disease from very early life. Furthermore, urinary metabolic features characterise NEC cases vs healthy controls from birth until 28 days of life. Finally, untargeted metabolomics can identify patients who will develop surgical NEC as early as birth, with a potential role of impaired fatty acids' and acylcarnitines' metabolism.

Our study confirms the important role of metabolomics in the investigation of disease pathophysiology and in the discovery of potential diagnostic/prognostic biomarkers of NEC. Our results need to be validated in future studies on independent cohorts and using targeted analysis.

AUTHORS CONTRIBUTION

L.M. and G.V. conceptualized the study design; L.M., M.M. and S.G. performed the data collection; G.G. and P.P. were involved in the metabolomic analysis; M.S. was responsible of the statistical data analysis; L.M. and M.S. interpreted the data; L.M. wrote the first draft of the manuscript; G.V., M.S., M.D., F.F.L. and E.B. critically revised and edited the manuscript; L.M., G.V. and M.S. directly accessed and verified the data reported in this manuscript; E.B. supervised the work. All authors have had full access to the data and results of the study and have accepted responsibility to submit the manuscript for publication.

DECLARATION OF INTERESTS

The authors have no conflict of interests to declare.

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Some of our findings have been previously reported in the form of abstract (Joint of European Neonatal Society Congress 2023) and short paper (first prize Young Investigators of the Italian Neonatal Society 2023).

DATA SHARING STATEMENT

Participant data with identifiers, the study protocol and the informed consent form will be made available under specific request by email to the corresponding author.

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Figure legends

Figure 1. Diagram of the study design and flow. Images from Freepik and BioRender.com.

Figure 2. Metabolomics analysis of urine collected at birth (T0): volcano plot (panel A) and relevant score plot (panel B) obtained for the NEG dataset, and volcano plot (panel C) and relevant score plot (panel D) obtained for the POS dataset. The features discovered as relevant are colored in red. In the volcano plot, p is the p-value of the Mann-Whitney test and FC[NEC/CTRL] is the fold change calculated as ratio between the median in the NEC group and the median in the control group; the dashed black line represents the threshold used to control the false discovery rate.

Figure 3. PLS-doe: score scatter plots of the models obtained with the NEG (panel A) and the POS (panel B) datasets. Samples of the NEC group (blue) and those of the controls (green) clusterise according to the group at all time points along tp[2], while the time increases from left to right along tp[1]. Circles are used for samples at T0, diamonds for samples at T1 and triangles for samples at T2.

Figure 4. LME models for longitudinal data: NEG dataset (panel A) and POS dataset (panel B); p[time] and p[group] are the p-values of the fixed effects "time" and "group", respectively. Features significantly relevant to distinguish NEC cases and controls are in red. The dashed black lines indicate the thresholds used to control the FDR at level 0.05.

Figure 5. Boxplots representing the distributions of the most significant metabolites discovered in the comparison of medical vs surgical NEC at T0.

Neonatal Intensive Care Unit
Padova University Hospital
Italy

Ethical Committee approval
(5128/AO/21, 4374/AO/17)

Inclusion criteria:
- GA at birth <34 GW or congenital heart disease
- Admission to NICU
- Written informed consent

Exclusion criteria:
- Congenital anomalies
- Chromosomal abnormalities
- Isolated structural abnormalities of the gut

REDCap | University of Padua | CRF METABOLOMICA IN TIN

T0 (within 2 DOL)
T1 (14 DOL)
T2 (28 DOL)

Longitudinal collection of clinical data and samples

Healthy subjects
(Control group)

Discharge or transfer to other hospital

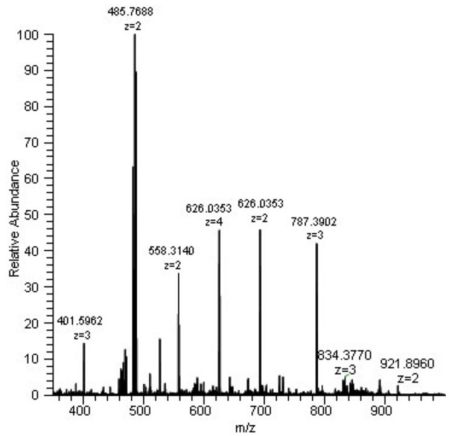
Infants with NEC, SIP, or other GI diseases

T0 (onset)
Weekly until resolution

Discharge or transfer to other hospital

Retrospective analysis of urine samples by untargeted metabolomics with UPLC-MS (QToF)

Laboratory of Mass Spectrometry and Metabolomics
Women's and Children's Health Department, Padova, Italy



Mass-to-charge spectra

Statistical analysis of clinical and metabolomic data by univariate and multivariate analyses
(Fisher's exact test, T-test, Mann-Whitney test, Shapiro-Wilk, MPA, PLS2C, LME)
($p<0.05$)

Identification of relevant metabolites (HMDB)

Hypotheses generation