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METABOLOMIC ANALYSIS IN THE PREDICTION OF ONSET AND SEVERITY OF NECROTIZING ENTEROCOLITIS

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ABSTRACT

Introduzione: L'enterocolite necrotizzante (NEC) è l'emergenza gastrointestinale più devastante del neonato pretermine. Data la sua complessa e sfaccettata fisiopatologia, la ricerca di biomarker predittivi precoci di NEC resta una sfida. La metabolomica, l'ultima delle quattro grandi scienze omiche basate sulla tecnologia, potrebbe permettere l'identificazione di metaboliti conosciuti e ancora sconosciuti coinvolti nei processi molecolari responsabili della NEC.

Materiali e metodi: Questo è uno studio caso-controllo osservazionale monocentrico che ha applicato l'approccio metabolomico untarget su campioni di urina di neonati pretermine nati con età gestazionale alla nascita <34 settimane gestazionali per identificare profili predittivi precoci di sviluppo di NEC e per discriminare i casi di NEC da controlli sani e da neonati che sviluppano perforazione intestinale spontanea (SIP). Sono stati raccolti campioni urinari di neonati alla nascita (entro 48 ore, T0), a 14 giorni (T1) e a 28 giorni di vita (T2). L'analisi metabolomica untarget ha utilizzato l'Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS). La struttura della Model Population Analysis (MPA), così come l'analisi statistica univariata e multivariata, sono state applicate per l'analisi dei dati e per confrontare le diverse popolazioni. Un valore p di 0.05 è stato considerato statisticamente significativo.

Risultati: Venti casi di NEC, 17 controlli sani, 3 soggetti con SIP e 7 soggetti con altre patologie gastrointestinali sono risultati simili per le principali caratteristiche cliniche prenatali e postnatali. Considerati i campioni urinari raccolti alla nascita, la MPA è stata in grado di predire correttamente 16 su 20 (80%) soggetti con sviluppo di NEC, 5 su 7 (71.4%) soggetti con altre patologie gastrointestinali, e tutti i soggetti con sviluppo di SIP (100%) come non appartenenti al gruppo di controlli sani. Questo sottolinea come il metaboloma urinario sia strettamente associato alla patologia sottostante. L'analisi metabolomica ha permesso di distinguere il gruppo NEC dal gruppo Controlli sia a T0 che dall'evoluzione dei campioni urinari nel tempo, dimostrando che parte delle differenze osservate a T0 si mantengono a 28 giorni. L'analisi esplorativa che ha confrontato casi di NEC medica da casi di NEC chirurgica (7 vs 7 soggetti) ha mostrato una chiara separazione dei due gruppi a T0, con ridotti livelli di acido 5-idrossiindolacetico e aumentati livelli di acido N-acetilaspartico, butirilcarnitina e propionilcarnitina nei casi di NEC chirurgica.

Conclusioni: Il nostro studio mostra the la metabolomica applicata a campioni urinari molto precoci (entro due giorni di vita) è capace di discriminare i neonati che sviluppano NEC, quelli che sviluppano SIP o altre condizioni gastrointestinali dai controlli sani. Le differenze metaboliche tra casi di NEC e controlli alla nascita persistono durante il primo mese di vita. Inoltre, i casi di NEC con necessità di intervento chirurgico sono metabolicamente diversi da quelli trattati con terapia medica già dai campioni urinari precoci. Nel nostro studio, i potenziali biomarcatori predittivi di sviluppo e di severità di NEC giocano un ruolo nelle vie di stress ossidativo. Questi risultati devono essere validati in studi futuri su coorti indipendenti utilizzando l'analisi target.

ABSTRACT

Background: Necrotizing enterocolitis (NEC) is the most devastating gastrointestinal emergency of the preterm neonate. Given its multifaceted pathophysiology, the search for early predictive biomarkers of NEC remains challenging. Metabolomics, the last of the four big technology-based omic sciences, may allow the identification of known and unknown metabolites involved in the molecular processes responsible for NEC.

Materials and Methods: This was a monocentric observational case-control study applying the untargeted metabolomic approach on urine samples of preterm infants born <34 gestational weeks (GW) to identify early predictive profiles of NEC development and to discriminate NEC cases from healthy controls and neonates developing spontaneous intestinal perforation (SIP). Neonates had their urine collected at birth (within 48 hours, T0), at 14 days (T1) and at 28 days of life (T2). Untargeted metabolomic analysis was performed with Ultra Performance Liquid Chromatography-Mass Spectrometry (UPLC-MS). The framework of Model Population Analysis (MPA), as well as univariate and multivariate statistical analyses, were used to analyze data and compare the different populations. A p value of 0.05 was considered statistically significant.

Results: Twenty NEC cases, 17 healthy controls, 3 subjects with SIP and 7 subjects with other gastrointestinal diseases were similar for the main clinical prenatal and postnatal characteristics. Considering urine samples collected at birth, MPA was able to correctly predict 16 out of 20 subjects developing NEC (80%), 5 out of 7 subjects with other gastrointestinal pathologies (71.4%), and all the subject developing SIP (100%) as not belonging to the control group. This highlights that the urinary metabolome is closely associated to the disease under investigation. Metabolomic analysis was able to distinguish the NEC Group from the Control Group both at T0 and by the evolution of urine samples over time, proving that part of the differences observed at T0 are maintained until 28 days. The exploratory analysis comparing medical vs surgical NEC cases (7 vs 7 subjects) showed a clear separation between the two groups at T0, with lower 5-hydroxyindolacetic acid and higher N-acetylaspartic acid, butyrylcarnitine and propionylcarnitine in surgical cases.

Conclusions: Our study shows that metabolomics applied on very early urine samples (first 2 days of life) is able to discriminate neonates developing NEC and those developing SIP or other gastrointestinal conditions from healthy controls. Metabolic differences between NEC cases and controls at birth persist during the first month of life. Additionally, NEC cases requiring surgery are metabolically different from those treated medically from the very early urine samples. In our study, potential predictive biomarkers of NEC development and severity mainly play a role in pathways of oxidative stress. These results need to be validated in future studies on independent cohorts using targeted analysis.

1. Background

1.1 Historical background and Definition of Necrotizing Enterocolitis

Necrotizing enterocolitis (NEC) is the most frequent and devastating gastrointestinal emergency of the preterm neonate, carrying a high burden of neonatal morbidity and mortality worldwide (1).
The disease was first described in 1888 by Paltauf (2), who reported five cases of death due to overwhelming peritonitis (3). However, it was only in 1953 that Schmid and Quaiser used the term *"necrotising enterocolitis"* for the first time (4). It is attributed to Agerty et al., instead, the first successful treatment of an infant with localized ileal perforation as a result of NEC (5).
As expressed by its name, NEC is characterized by an ischemic coagulative necrosis of the intestinal mucosa, which is associated with severe inflammation, invasion of enteric gas-forming organisms, and dissection of gas into the intestinal wall and portal venous system (6).
NEC can manifest within a wide range of severity and within various stages along a continuum of bowel disease. The first classification system for NEC was proposed in 1978 by Bell and colleagues, who included a set of clinical and radiological characteristics used to categorize infants into 1 of 3 stages of the condition (7). These criteria allowed accurate comparisons of patients with disease of similar severity and were useful in guiding therapeutic decisions. Still now, more than four decades later, Bell staging remains the most commonly utilized case definition of NEC worldwide (8).
Bell's criteria were then modified by Walsh and Kliegman in 1986 (9), who added systemic, abdominal and radiographic signs as well as indications for the management according to illness severity (**Table 1**).

Table 1. Modified Bell's staging criteria for NEC according to Walsh and Kliegman (9).

Stage	Classification	Systemic signs	Intestinal signs	Radiologic signs	Treatment
IA	Suspected	Temperature instability, apnoea, bradycardia, lethargy	Increased pre-gavage residuals, mild abdominal distention, emesis, guaiac-positive stool	Normal or intestinal dilation, mild ileus	NPO, antibiotics for 3 days pending culture
IB	Suspected	Same as IA	Same as IA plus bright red blood from rectum	Same as IA	Same as IA
IIA	Definite – mildly ill	Same as IA	Same as I, plus absent bowel sounds, with or without abdominal tenderness	Intestinal dilation, ileus, pneumatosis intestinalis	NPO, antibiotics for 7 – 10 days if exam is normal in 24-48 hours
IIB	Definite – moderately ill	Same as I, plus mild metabolic acidosis, mild thrombocytopenia	Same as I, plus absent bowel sounds, definite abdominal tenderness, with or without abdominal cellulitis or right lower quadrant mass	Same as IIA, plus portal venous gas, with or without ascites	NPO, antibiotics for 14 days, $NaHCO_3$ for acidosis
IIIA	Advanced – severely ill, bowel intact	Same as IIB, plus hypotension, bradycardia, severe apnoea, combined respiratory and metabolic acidosis, disseminated	Same as II, plus signs of generalized peritonitis, marked tenderness, and distention of abdomen	Same as IIB, plus definite ascites	NPO, antibiotics for 14 days, fluid resuscitation, inotropic support, ventilator therapy, paracentesis

		intravascular coagulation, and neutropenia			
IIIB	Advanced – severely ill, bowel perforated	Same as IIIA	Same as IIIA	Same as IIB, plus pneumoperitoneum	Same as IIA, plus surgery

Despite its widely utilization, Bell staging has several limitations, among which the inclusion of subjective criteria with variable sensitivity and specificity, the non-specific findings in stage I, and the difficult exclusion of infants with spontaneous intestinal perforation (SIP, a different entity from NEC) (10). Indeed, many of the recent observational studies include only stage II and III, however with persistent limitations related to this criteria.

After these first classifications, a number of additional definitions of NEC have been proposed in the subsequent decades. In 2020, Patel et al. reviewed 8 different definitions of the disease according to several networks and collaborative groups, highlighting the importance of a global consensus on defining NEC to improve research on the topic and its outcomes (10). Some of these suggested interpretations try to overcome limitations of Bell staging and add laboratory and radiologic criteria, as the knowledge of NEC predictive factors and advances in its radiologic characterization continue to grow.

For instance, one of these definitions, the two out of 3 rule, was proposed in 2017 by Gordon and colleagues (11) and identifies NEC infants as having abdominal distension, ileus and/or bloody stools and at least 2 of the following: pneumatosis and/or portal air by x-ray or ultrasound at presentation, persistent platelet consumption (platelet count <150.000 for 3 days after diagnosis) and postmenstrual age at disease onset more consistent with NEC than SIP. The authors, additionally, exclude patients with SIP, complex congenital anomalies, being fed <80 ml/kg/d or with > or = 36 weeks' gestation, thus focusing the diagnosis on "preterm NEC".

Two years after this cue, the result of a workgroup of experts assembled by the International Neonatal Consortium (INC) for the development of a new definition of NEC was published (12). This required 1 of 2 clinical signs (abdominal distension or hematochezia), onset between postnatal day 10 and 36 weeks postmenstrual age (PMA), and at least one among: intestinal necrosis at laparotomy, pneumatosis intestinalis or portal venous air (by x-ray or US), or evidence of vasculitis, coagulopathy or inflammation in the absence of bacterial, fungal or viral infection. Similarly to the two out of three tule, this definition also differentiates infants with "preterm NEC" from those with any differential diagnosis (SIP, feeding intolerance, congenital cyanotic heart disease or gastroschisis), "atypical NEC" or "term NEC" for reporting in clinical research.

Ideally, the gold standard for recognizing NEC from other similar conditions would be to identify necrosis of the gut at laparotomy or on histopathologic specimen, however surgery is usually required in 30-50% of those affected.

Finally, one of the more frequently used definitions of NEC is the one developed by the Vermont Oxford Network (VON), a collaborative including more than 1400 centers to improve neonatal care around the world. By VON, NEC is defined at surgery, at post-mortem examination, or based on at least one clinical finding (bilious gastric aspirate or emesis, abdominal distension or discoloration, occult or gross blood in stool without fissure) and at least one radiographic finding (pneumatosis intestinalis, hepato-biliary gas, or pneumoperitoneum)(13).

Figure 1, from the article by Patel et al. (10), summarizes the diagnostic criteria of the most important definitions of NEC at 2020.

Variable category	Bell staging			Modified Bell staging						UK	VON	CDC	2 of 3	ST	INC
	1	II	III	IA	IB	IIA	IIB	IIIA	IIIB						
Intestinal signs															
Poor feeding (intolerance)	+	+	+											+	
Emesis	+	+	+	+	+	+	+	+	+		+	+			
Pre-gavage residuals	+	+	+	+	+	+	+	+	+	+					
Bilious aspirates	+	+	+							+	+	+			
Abdominal distention (mild)	+	+	+	+	+	+	+	+	+	+	+	+	+		+
Marked distention		+	+					+	+						
Guaiac-positive stool	+	+	+	+	+	+	+	+	+						
Rectal bleeding (occult)[a]	+	+	+		+	+	+	+	+	+	+	+	+		+
Marked hemorrhage			+												
Absent bowel sounds						+	+	+	+						
Abdominal tenderness						±	+	+	+	+					
Marked tenderness								+	+						
Generalized peritonitis								+	+						
Abdominal cellulitis							±	+	+						
Right low quadrant mass							+	+	+						
Abdominal discoloration										+				+	
Radiologic findings															
Normal				+	+										
Ileus[b]	+	+	+	+	+	+	+	+	+				+		
Pneumatosis		+	+			+	+	+	+	+	+	+	+	+	+
Portal venous gas		+	+				+	+	+	+	+	+	+	+	+
Ascites							±	+	+						
Pneumoperitoneum			+						+	+	+	+			
Fixed loop[c]		+	+							+					
Small bowel separation[d]		+	+												

Figure 1. Comparison of intestinal signs and radiologic findings across NEC definitions, from Patel et al. (10)
Abbreviations: UK, United Kingdom; VON, Vermont Oxford Network; CDC, Centres for Disease Control and Prevention; ST, Stanford; INC, International Neonatal Consortium; GI, gastrointestinal.
a Includes descriptions of bright blood from rectum, haematochezia or occult bleeding (without specific mention of testing for blood such as guaiac testing).
b Includes descriptions of intestinal dilatation or distention
c Also characterized as unchanged "rigid" loops of bowel
d Caused by oedema in bowel wall

Despite the extensive research and the need for a precise, reliable and reproducible definition of NEC in order to accurately characterize its incidence, compare its outcomes, as well as ameliorate the feasibility and generalizability of studies, there is an increasing acceptance that the disease may simply be one umbrella for a spectrum of multiple conditions with different pathophysiology leading to the same outcome of intestinal necrosis (14) (**Figure 2**). The criteria used 40 years ago probably do not fit clearly into the present-day neonatal intensive care unit (NICU) care, as NEC comprises an evolving definition and appears to be not a single, homogeneous entity, but several different diseases or endotypes leading to similar symptomatology (1).
Interestingly, in fact, a recent preliminary study on 219 retrospectively-recruited infants with NEC, SIP or NEC concern found that contemporary definitions of NEC outperformed Bell staging using both standard statistics and machine learning classifiers. In particular, based on standard statistics, among six non-Bell definitions the VON definition, the 2of3, and the INC definition had better specificity but lower sensitivity than the ModBell(IIA+) whereas the 2of3 and INC definitions performed consistently better than others with machine learning scores (15). Importantly, nine features were identified as being of importance in the clinical diagnosis of NEC (among which lethargy, gestational age, volume of feeding at NEC onset, DIC and occult rectal bleeding), but these, when combined, were unable to train a better decision tree than combinations of criteria from pre-

established definitions, suggesting that interactions between features are important and may help machine learning models perform better on the data.
These considerations imply that newborn infants require a personalized approach for NEC prevention and treatment.

1.2 Incidence and NEC-related complications

The exact incidence of NEC is difficult to extrapolate as it varies depending on country, selected population and definition used. In fact, a substantial heterogeneity between studies is often found, whose explanation is probably multifactorial, but also may reflect the variation in the quality of health care systems. The estimates of the disease, despite being similar across various regions, appears to differ between high and low income countries (pooled incidence 7% vs 3%, respectively) (16), and it is inversely related to gestational age (GA) and birth weight (BW) (17). These rates may be biased by the inclusion of different NEC definitions or of various stages of NEC (i.e. all stages of NEC, only surgical NEC or Bell stage ≥II). Additionally, inclusion criteria differ among cohort studies, with some considering the whole neonatal population, others those born with a very low birth weight (VLBW, <1500 g), and others considering only the extremely low birth weight (ELBW, <1000 g) (18).
In a recent systematic review and meta-analysis of 27 cohort studies including 574,692 VLBW neonates, NEC stage II or above according to Bell criteria was estimated to affect about 6% (3-9%) of VLBW infants and 7% (2-13%) of the extremely premature (≤28 gestational weeks GW) globally. However, these numbers widely varied between studies, with some reporting an incidence as high as 17%. Furthermore, a statistically significant increase of NEC was noted over time, probably due to the rising survival of infants born at the lowest gestational ages (GA) with the great improvements in the neonatal intensive care, as well as improvement in diagnosis and reporting (16)(19)This trend was similar to the one emerged from a Swedish study published in 2013, which highlighted a J-shaped rate of NEC over time for all GA, with a concomitant reduction in mortality both for GA and for BW (20).
In Italy, data from the Italian Neonatal Network (INNSIN) show an incidence of the disease of about 4.6% in the VLBW infants (< or = 1500 g) and of 6.3% in those born extremely preterm (< or =28 GW) (*Data from Corso SIN NEC 2021*).
NEC requiring surgical intervention (surgical NEC) reaches an incidence of around 3.1% in VLBWI, but its burden accounts for up to 41.7% of all neonates with NEC, as reported by a multicenter cohort study of 820 US centers (21). However, in another recent study describing 10-year data from a Chinese center, surgical NEC represented up to 56% of NEC cases, with a lower GA, early occurrence of the disease, hemodynamically significant patent ductus arteriosus (HsPDA) and low serum bicarbonate associated with an increase probability of surgical treatment (22).
Overall, NEC is responsible for between 10% and 21% of infant mortality in premature babies, that means around 1 in 10 of all neonatal deaths (18,23,24). From the datasets of a recent systematic review, the mortality from confirmed NEC Bell 2a+ was estimated at 23.5% (18.5-28.8%) in all neonates, with the highest seen in infants with BW <1000 g and surgical disease, where it reached 50.9% (38.1%-63.5%) (18). Nevertheless, it must be highlighted than even the definition of mortality varies between studies, with some considering in-hospital mortality, others reporting death at the time of discharge or at a post-operative age of 28 days, 30 days, 6 months, 1 year or 2 years. Data from one study published in 2015 demonstrated that the greatest percent of deaths caused by NEC occurred between 29 and 60 days of life, to subsequently decreasing in the following period (>120 days) (23). In a recent study from a UK specialist center, the 30-day total mortality for NEC was 18.9% (preterm and term infants), and factors related to an increased chance of dying at 1 year,

apart from lower GA and BW, appeared to be a diffuse location of the disease (pan-NEC) and pre-operative presence of pneumoperitoneum (25). In this term cohort, an increased burden of colonic segment NEC (40%) and associated cardiac defects (21%) was seen, confirming that a cardiac origin of NEC ("cardiogenic NEC") is more common in this population.
For those who survive, NEC is related to several short- and long-term complications, chief among these intestinal failure, short bowel syndrome, cholestasis, failure to thrive, and neurodevelopmental sequalae (18,26). In particular, intestinal failure (IF, often defined as need for parenteral nutrition for >90 days post-surgery) can reach a rate up to 35% in infants with surgical NEC (18), with short-bowel syndrome (SBS) being the most common cause of IF. Post-NEC strictures occurred in about 24% of infants treated surgically at a median post-NEC age of 51 days in a cohort from the Netherlands. Nevertheless, neonates with medical NEC are not free of this risk, as a rate of 17% with a median onset of 21 days is reported for strictures in the same study (27). Dense adhesions or single bands following neonatal laparotomy for surgical NEC can also determine small bowel obstruction, and thus repeated laparotomy, delayed reinitiation of enteral feeding and prolonged parenteral nutrition (28). Other possible gastrointestinal long-term sequelae are dysmotility, cholestasis, feeding difficulties, and intestinal malabsorption with failure to thrive up to 24-36 months of age (29).
As regards neurodevelopmental impairment, the rate of this complication after NEC remains difficult to determine, as studies use variable definitions for this outcome (f.i. mental developmental index or psychomotor developmental index <70, blindness, visual or hearing impairment, moderate/severe cerebral palsy) (18,30). The review by Jones et al. describes a rate of severe neurodevelopmental delay between 24.8% and 61.1%.
Given the burden of NEC-related morbidity and mortality, it appears to be imperative to investigate for early diagnostic and predictive biomarkers of NEC, in order to adopt preventive strategies and timely medical and surgical treatment.

1.3 Presentation and differential diagnosis

1.3.1 Presentation

The usual time of NEC onset is highest between 29 and 31 weeks PMA, with an earlier onset the greater the GA at birth (31,32).
The clinical presentation of NEC can range from a subtle and insidious progression to an unexpectedly fulminant onset of gastrointestinal signs, multiorgan dysfunction and shock over a few hours (33). Several signs and symptoms are often aspecific and indicate a sudden change in feeding tolerance. These can comprise abdominal distension and tenderness, vomiting or emesis, periumbilical shining or erythema or blue discolored abdomen, increased biliary gastric residuals, diarrhea and gross/bloody stools (hematochezia). Gastric residuals are often seen in early NEC, however there is no evidence that their routine measurement in asymptomatic infants is a useful guide to prevent or detect the onset of NEC (34). Other non-abdominal non-specific systemic findings include apnea, bradycardia or tachycardia, respiratory failure, lethargy, or temperature instability, as well as hypotension in the most severe cases, probably contributed by the concomitant bacteriemia, which can be associated in up to 30% of cases (35,36).
Laboratory findings of infants presenting with NEC can involve anemia, thrombocytopenia, neutropenia, disseminated intravascular coagulopathy (DIC), metabolic acidosis with a rise in lactates, hyperglycemia, increased C-reactive protein (CRP), hyponatremia and hypoalbuminemia. The often mentioned common triad for NEC is thrombocytopenia, persistent metabolic acidosis and severe refractory hyponatremia. This latter, in particular, has been related to clinical deterioration

of NEC as it seems to reflect the inflammation through the activation of the arginine-vasopressin system resulting in water retention (37).
Notwithstanding, even these alterations are aspecific and with low sensitivity and specificity for the early detection of NEC (38).
Despite the suspect of NEC can be purely clinical, based on the most characteristic features, and the definite diagnosis made from surgical or postmortem specimens of intestine demonstrating the histological findings of inflammation, infarction and necrosis (36), abdominal x-ray (AXR) and ultrasound (AUS) are fundamental imaging surrogates.
Abdominal radiographs are routinely used as gold standard to confirm the diagnosis and follow the progression of NEC. They can detect intramural gas (pneumatosis intestinalis), ileal distension, pneumoperitoneum, or sentinel bowel loops. However, abdominal x-ray often lack sensitivity and specificity, in particular in the tiniest premature infants. In the early stages of NEC, abnormal dilated loops of bowel, consistent with ileus, can be present, pattern that can also be seen in septic paralytic ileus (36). Pneumatosis intestinalis is considered a hallmark of classical preterm NEC and consists of bubbles of gas in the small bowel wall. These can often extend to the portal venous circulation determining curvilinear lucencies over the hepatic silhouette in a plain radiograph (portal venous gas). Pneumoperitoneum, diagnostic of a perforated viscus, appears as a rounded or oval extraluminal lucency beneath the upper anterior abdominal wall in a plain or lateral view. When large, it may outline the falciform ligament giving the appearance of a football ball (33).
Bowel ultrasound (BUS) with Doppler ultrasonography is increasingly used in the evaluation for NEC and can provide additional details to plain radiographs. A recent meta-analysis highlighted that classic signs of NEC (portal venous gas, pneumatosis and free air), as well as bowel wall thinning, absent peristalsis, ascites and focal fluid collection had low sensitivity (ranging from 0.19 to 0.48) but high specificity (up to 0.99) for NEC when taken individually (39). The same authors also demonstrated that focal fluid collection, complex ascites, absent peristalsis, pneumoperitoneum, bowel wall echogenicity or thinning, absent perfusion, and dilated bowel as detected by AUS were associated with surgery or death (39).
Interestingly, a multicenter retrospective review of 96 paired studies (AXR followed by AUS within 24 hours) showed a good agreement for pneumatosis, portal venous gas and pneumoperitoneum between the two imaging modalities, with each finding present more frequently on AUS (40). Despite its increasing adoption, many barriers to BUS implementation exist, namely lack of programmed education and training for clinicians and radiologists associated with the low case volume. Recently a framework and roadmap has been provided to implement BUS in day-to-day practice (41).
Of note, it can be clearly appreciated that a combination of clinical signs and imaging findings, rather than a single predictor, can be useful in identifying patients at risk of the disease and of worse outcomes. Indeed, a multivariable predictive model incorporating four independent factors of surgical NEC (abdominal wall erythema on clinical exam, portal venous gas on AXR, echogenic free fluid and bowel wall thickening on AUS) had excellent discriminating power for surgical risk (AUC 0.937) (42).

1.3.2 Spontaneous intestinal perforation

The most common differential diagnosis of NEC in extremely preterm (≤28 GW) ELBW (<1000 g) infants is SIP, whose certain risk factor is prematurity, but that has also been associated with severe maternal chorioamnionitis, multiple delivery, and use of steroids and indomethacin close to birth (43). In the postnatal period, prophylactic indomethacin for patent ductus arteriosus (PDA) and early dexamethasone or hydrocortisone for prevention of bronchopulmonary dysplasia (BPD) also have been reported to increase the risk of this condition (44–46).

Compared to NEC, SIP is characterized by an earlier presentation (usually 7 days of life), similar clinical signs (sudden onset of abdominal distension and discoloration, hematochezia, and biliary residuals), and presence of a focal isolated perforation without inflammatory necrosis of the ileus at surgery. At AXR, pneumoperitoneum and the football sign are classical features, whereas there is absent evidence of pneumatosis intestinalis and portal venous gas. AUS shows typical A lines at the upper right quadrant that can impede the visualization of the liver or that can abruptly transit into the liver ("gut point"), as well as the enhancement of the sign of the peritoneal line as a marked iperechogenic interface between the air and the soft tissues (47). Treatment of infants with SIP and NEC is similar (see further), and despite associated with a lower mortality than NEC, SIP is still a cause of increased morbidity, among which longer length of stay (LOS), lower growth as assessed at discharge and worse neurodevelopment (48).

1.3.3 Septic ileus

Another disease which is often misdiagnosed for NEC is paralytic ileus during sepsis, or septic ileus. This entity is characterized by a functional intestinal obstruction as a result of a temporary absence of intestinal peristalsis. Hallmark features of presentation are bilious vomiting, abdominal distension and feed intolerance, therefore very similar to symptoms of NEC (49). These infants may be potentially very unstable at the beginning, with need for supportive measures and adequate resuscitation. Investigations and clinical course will subsequently guide the definitive management. Necrotizing enterocolitis itself may however be a complication of septic ileus, in particular in newborns with a severe presentation with shock, hypotension and intestinal hypoperfusion. Serial blood tests (blood gas, full blood count, urea and electrolytes, CRP, coagulation profile, blood culture), microbiological exams aimed to localize the focus of infection and a plain and a lateral AXR may be of help in the recognition of the correct condition. In an old case series of functional intestinal obstructions from the Great Ormand Street Hospital, of 51 cases, eleven had some form of infection, among the commonest bronchopneumonia, gastro-enteritis and septicemia. While features of obstruction resolved in nine, *Escherichia Coli* septicemia was fatal in two patients. However, only in three cases a pathogen was detected (50). A more recent retrospective review of ELBW cases admitted to a tertiary NICU in Korea and requiring surgery for acute abdomen due to NEC, SIP, meconium-related ileus (MRI), or meconium-non related ileus (MRNI), found that these two latter entities interested a group with higher mean gestational age compared to those with NEC or SIP. Infants received either peritoneal drainage (16.7% of infants with SIP), ostoma or primary anastomosis due to suspected bowel perforation or because of clinical deterioration despite maximal medical treatment. Of the 8 (12.3%) 30-day mortalities, 7 were NEC and one was SIP, most of which complicated by septic shock, whereas MRI and MRNI had a 100% 5-year overall survival (51).

Whether septic ileus may be related to a lack of coordinated intestinal motility due to bowel immaturity and gross deficiency of nerve fibers is still a matter of debate and ongoing research. It is also clear that sometimes it is difficult to make a definitive diagnosis without laparotomy (51,52).

The main differences between NEC, SIP and septic ileus are described in **Table 2**.

Table 2. Main differences between NEC and its major differential diagnosis (10,43,52).

Characteristic	**NEC**	**SIP**	**SEPTIC ILEUS**
Onset	Later timing of onset: 4-6 weeks of life (about 29-32 weeks of CGA)	Earlier timing of onset, within 7 days of life	Any time of onset, more common with late onset sepsis (LOS >72 hors of life)

Risk factors	Enteral feeding, especially formula milk	No or minimal enteral feeding, not necessarily related to enteral nutrition; Exposure to indomethacin for PDA; Exposure to systemic corticosteroids for BPD	Enteral feeding, normal passing of stools; Risk factors for sepsis: central or peripheral line, low birth weight, systemic corticosteroid therapy
Radiological findings	Ileus Pneumatosis Portal venous gas Ascites Pneumoperitoneum	Pneumoperitoneum	Ileus Gaseous bowel distension of the whole intestine without fluid levels
Macroscopic and Histopathological findings	Ischemic and inflammatory necrosis of distal ileus and/or colon, with or without perforation; From patchy to total coagulative intestinal necrosis of all intestinal layers (*transmuralis*), with bacterial translocation, ulcerations and submucosal air bubbles	Isolated single perforation of the terminal ileus, usually in an antimesenteric location; Remaining bowel grossly normal; Focal necrosis with demarcated margins due to the thinning of the muscolaris;	Normal intestine; Possible signs of ischemia or perforation in most severe cases;
Treatment	NPO, antibiotics, respiratory and cardiovascular support, surgery (peritoneal drainage, explorative laparotomy)	NPO, respiratory and cardiovascular support, surgery (peritoneal drainage, explorative laparotomy)	NPO, antibiotics, respiratory and cardiovascular support, blood transfusion, surgery if complication or misdiagnosis

1.4 Pathophysiological hypotheses and risk factors

1.4.1 Pathophysiological hypotheses

Despite extensive research, the etiologies of NEC continue to elude investigators. In 1975, Santulli and colleagues were the first to hypothesized that NEC was based on 3 components (53):

- Injury of the intestinal mucosa
- Presence of bacteria
- Availability of a metabolic substrate

Nowadays, it can be universally recognized that the classical NEC of the preterm infant stems from an intrinsic bowel immaturity, where enteral nutrition and other potential risk factors contribute to impair the intestinal microbiota.

The immature gut of preterm infants is marked by a functional component given by a decreased peristalsis, reduced proteolytic enzymes, increased gastric pH and laxity of the epithelial tight junctions, as well as by an immunological component with reduced lactoferrin, defensins and IgA (36,54). On this already fragile and delicate substrate, contributing factors, like prolonged antibiotic exposure, use of acid reducing or hyperosmolar agents, and bovine milk-based formulas, may trigger microbial dysbiosis and translocation of potential pathogens through the intercellular junctions. The altered microbiota determines an exaggerated inflammatory response with a major release of cytokines and chemokines and an hyperactive Toll-Like Receptors 4 (TLRs-4)-mediated signaling, progressively leading to tissue injury by reducing healing, increasing mucosal breakdown and enterocyte apoptosis. The ultimate consequence is a coagulative necrosis of variable intestinal layers, with disruption of intestinal villi and complete loss of the epithelial and muscular architecture (9). Additionally, ischemia, hypoxia-reperfusion, infection and inflammation are mechanisms

capable of producing high levels of free radicals, perturbing the normal redox balance and shifting cells to a state of oxidative stress, a major downstream component of the pathogenetic cascade in NEC (55).
Numerous animal models and, more recently, cell lines that reproduce the intestinal epitelium or stem cells producing enteroids or organoids, have been used with the intent to better understand the pathophysiology of NEC. These models are promising in evaluating treatment and preventive modalities (56).
Figure 2 represents the pathophysiology of NEC.

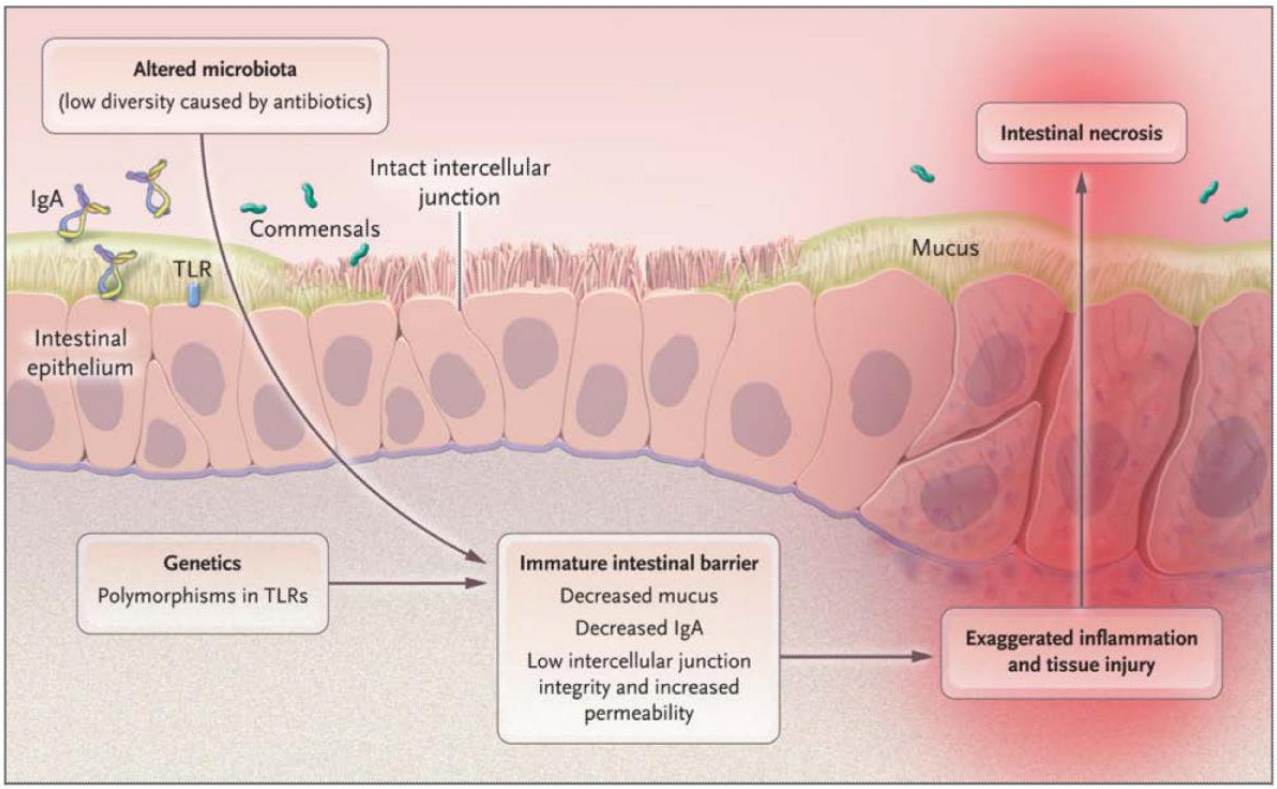


Figure 2. Pathophysiology of Necrotizing Enterocolitis. Factors conferring a predisposition to necrotizing enterocolitis include genetic factors and several immature characteristics of the fetal intestine, including altered microbiota, inadequate intestinal barrier function, and an excessive inflammatory response. These factors contribute to the severe necrosis of the small intestine that is characteristic of this disease. TLR denotes toll-like receptor. From Neu and Walker (57).

1.4.2 Antenatal and perinatal risk factors

Starting from the womb, clinical maternal chorioamnionitis seems to be significantly associated with NEC, while this does not appear to be true for histological chorioamnionitis if it does not cause vasculitis or does not involve the fetus. Despite the data are still preliminary, there is a good available evidence that supports a role of antenatal inflammation in NEC pathophysiology (58,59). It is possible that, as in bronchopulmonary dysplasia (BPD), maternal chorioamnionitis plays a different role in NEC pathophysiology depending on its onset (acute or chronic), its association with severe inflammatory response syndrome (SIRS) of the fetus, and the involved pathogen (60). Recently, *Ureaplasma* species have been acknowledged as major causative pathogens of both BPD and NEC, most likely by inducing pro-inflammatory factors and downregulating the immune system (61).
Regarding antenatal corticosteroids, these appear to be effective in NEC prevention. From randomized clinical trials (RCT), a decreased risk of NEC is seen with antenatal corticosteroids in

pregnant women at risk of preterm birth. A recent review and meta-analysis of nine observational studies, however, demonstrated that antenatal corticosteroid use before 25 weeks' gestation, does not influence the rate of NEC ≥stage II of Bell (62,63).
Despite being one of the first determinant of gut microbiota, mode of delivery seems not to be significantly associated with NEC development in neonates of women who were at imminent risk of delivery at <32 gestational weeks from a secondary analysis of data of a RCT (64,65).
A lower birth weight at delivery appears to increase the risk of NEC, with placental disease predisposing the severely growth-restricted neonate to the disease (66). Additionally, in antenatally identified pregnancies at risk for foetal growth restriction, abnormal Doppler velocimetry in the umbilical artery (absent/reverse end-diastolic flow) appeared to be a useful guide to predict NEC and mortality in the early neonatal period (67–69). A recent retrospective chart review also showed that abnormal antenatal Doppler characteristics (absent or reversed end-diastolic flow in the umbilical artery alone or also in the ductus venosus) were significantly associated with higher feeding intolerance, NEC and SIP rates (70).

1.4.3 Enteral feeding type, initiation and incremental feeding rates

Enteral feeding is a key risk factor for NEC, as only 10% of cases develop during fasting.
Bovine formula milk has been demonstrated to increase the likelihood of NEC, as it is supposed to contribute to the impairment of the normal intestinal flora and to increment the request of intestinal perfusion given its high osmolarity. Maternal breast milk is recommended for preterm and low birth weight infants as it has been demonstrated to attenuate the TLR-4 mediated pro-inflammatory response, typical hallmark in NEC pathogenesis, by activating the receptor for epidermal growth factor (EGFR) and thus resulting in enhanced mucosal healing, intestinal stem cell proliferation and decreased enterocyte apoptosis (71).
A recent systematic review and meta-analysis showed that enteral feeding with any human milk (including expressed maternal milk or donor milk) clearly reduced the risk of NEC (absolute reduction of 3.6%) in VLBW infants, with a clear dose-dependent effect (absolute risk reduction between 3.8-4.3% with high vs low dose human milk) (72). The protective effect of human milk and of its higher doses has been confirmed by another more recent study which showed a significant decline in NEC in premature infants fed mainly by HM and with the increase in HM proportion (73). Additionally, the 2019 Cochrane systematic Review comparing formula vs donor breast milk for feeding preterm or low birth weight infants demonstrated a higher risk of NEC with the former (number needed to treat for an additional harmful outcome (NNTH) 33) (74).
Human milk, especially when unpasteurized, contains distinct bioactive molecules that protect against infection and inflammation and contribute to immune maturation, organ development, and healthy microbial colonization. Nutritional components include macronutrients like proteins, fats (palmitic and oleic acids, docosahexanoic acid DHA and long chain polyunsaturated fatty acids LCPUFAs), and the disaccharide lactose, all fundamental for a correct intestinal development and growth. Macrophages, stem cells, immunoglobulins, lactoferrin, adiponectin, cytokines, chemokines and growth factors, instead, contribute in favor of intestinal maturation and repair and protect against infection and inflammation (56,75).
Early enteral feeding started within 96 hours of birth, in comparison with delayed or progressive introduction of feeds, appears to not affect the risk of NEC in preterm infants. However, currently available data are scarce and insufficient to provide consistent results on this question, especially for the extremely low birth weight and extremely preterm infants (<1000 g and <28 GW) (76–78).
Historically, observational and retrospective data supported a slower advancement of enteral feeding volumes in preterm infants to reduce the risk of NEC. However, the most important randomized controlled trial on this topic demonstrated no significant difference in NEC Bell's stage

> or = 2 if faster (30 ml/kg/day) or slower (18 ml/kg/day) incremental feeding rates were used in VLBW infants (79). This has been supported by the updated Cochrane systematic review of 14 trials, showing that daily increments up to 24 ml/kg compared with faster rates probably does not reduce the risk of NEC (moderate certainty of evidence)(80). Finally, there appears to be no difference in the incidence of NEC when infants < 37 GA and with a BW<2500 g receive bolus feeding compared to continuous ones (81).

1.4.4 Transfusion of red blood cells

Packed red blood cells (PRBC) transfusions have long been under scrutiny for their potential association with NEC and led to coin the term TANEC (transfusion-associated NEC, with NEC onset within 48-72 hours from the transfusion), with a proposed mechanism similar to the transfusion-associated gut injury (TRAGI). Whether are the transfusions or the anemia that prompted the transfusion related to NEC is still debated, and another possible hypothesis is that anemia a few days before NEC and requiring transfusion may just be the early subtle symptom of the disease before the infant starts to deteriorate(56). Confirming this theory are the results of a large multicentre cohort study of VLBWI showing that severe anemia (hemoglobin level < or = 8 g/dL) within the week of developing NEC, rather than RBC transfusion, could predispose to the disease (82). The association between NEC and RBC transfusion has been discredited by a recent review of literature as well, which found that RCT data rather suggested a protective role of transfusion, however without enough evidence for recommendation to be made (83).

The uncertainty regarding this association has often brought the neonatologists to withhold feeds during PRBC transfusion in preterm infants, and there are indeed some prospective observational data showing an increase in inflammatory markers (IL-1beta, IL-8, INF-gamma, IP-10) from 2 hours before to 48 hours after RBC transfusion, suggesting a potential contribution to gut injury. However, a relationship between levels of these inflammatory mediators and NEC was not evaluated in the study (84). Nevertheless, evidence from the literature to support withholding of feeds during transfusion is contrasting. A systematic review published in 2017 suggested that this practice during the peritransfusion period may reduce the risk of TANEC (no RCT included) (85), whereas a Cochrane Systematic Review in 2019 found insufficient data to support this practice to prevent the disease or death (only 1 RCT included, very low quality of evidence) (86). We are currently awaiting the results of a large UK pilot trial randomly assigning infants born <30 GW to two care pathways (continuing milk feeds before, during and after RBC transfusion or withholding milk feeds for 4 hours before, during and for 4 hours after RBC transfusion) and exploring the subsequent clinical outcomes (among which mortality and NEC) (87).

1.4.5 Dysbiosis and gut microbial colonization

The intestinal microbial environment has long been considered to play an important role in the development of NEC (88). The theory is supported by the fact that NEC cannot be produced in germ-free animals (89), and by the evidence of a beneficial effect of specific probiotics, in particular those composed by *Lactobacillus rhamnosus GG*, or a combination of *Bifidobacterium infantis* and *lactis* and *Streptococcus thermophilus*, in reducing the severity of NEC (90). Additionally, antibiotics act in reducing diversity progression, selecting specific bacteria (f.i. *Enterobacteriaceae*), and have been associated with an increased risk of NEC (91,92). However, which are the “good” an “bad” bacteria is still a matter of research (14). The imbalance between the microorganisms that colonize the human intestine and the host, together with the consequences of this disruption, are referred to as dysbiosis.

Data suggest that the infant's microbiome mainly depends on the maternal microbiome (both meconium and the intrauterine environment of preterm infants are not sterile), mode of delivery, enteral feeds and drug exposure, as well as the environment (54,93).
The gut microbiome can be explored by cultured-based, non-cultured based techniques, and a metagenomic approach. Ideally, the use of both culture-based and culture-independent approaches should be complimentary, as as the first allows the isolation of bacteria at low levels in samples and even when undetectable by quantitative PCR (qPCR), while the latter enables the identification of uncultivable bacteria (94).
From data deriving from microbiota analyses, mainly applying the 16S rRNA sequencing and community profiling based on the amplification of the bacterial V2–V4 regions, the intestinal microbiota of preterm infants has fewer bacterial species, less diversity and increased proportion of potential pathogens compared to term infants (89).
Near NEC onset, several studies suggest a predominance of *Proteobacteria* and a reduced abundance of *Firmicutes* in affected newborns, with a dramatic shift related to these two phyla over the week before diagnosis. Preterm infants appear to have indeed a dynamic pattern of intestinal colonization, which in NEC patients seems to be characterized by gram-positive cocci at the beginning, then overtaken by gram-negative facultative anaerobic organisms, counterbalanced by a gradually increasing abundance of anaerobes (95,96).
This evidence has also been supported by Pammi et al., who revealed a consistent trend towards higher relative abundances of *Proteobacteria (*mainly the class *Gammaproteobacteria* with the genus *Enterobacter)* and decreased relative abundances of *Firmicutes* (mainly the classes *Negativicutes* and *Bacilli)* and *Bacteroidetes* around 30 weeks of corrected GA in NEC patients (97). An opposite trend seems to occur in healthy controls, with a shift towards more gram-positive bacteria and an "adult-like" phenotype. As regards anaerobes, several studies applying culture and non-culture methods support the association of the genera *Clostridium* with NEC, in particular with a predominance of the species *Cl. Butyricum, Cl. Neonatale*, and *Cl. Perfringens*, some of which have also been related to NEC outbreaks and to fulminant NEC (i.e. NEC causing death within 48-72 hours from onset) (94,98–100).

Figure 3 describes the role of certain protective and risk factors for NEC, according to the most recent evidences from the literature.

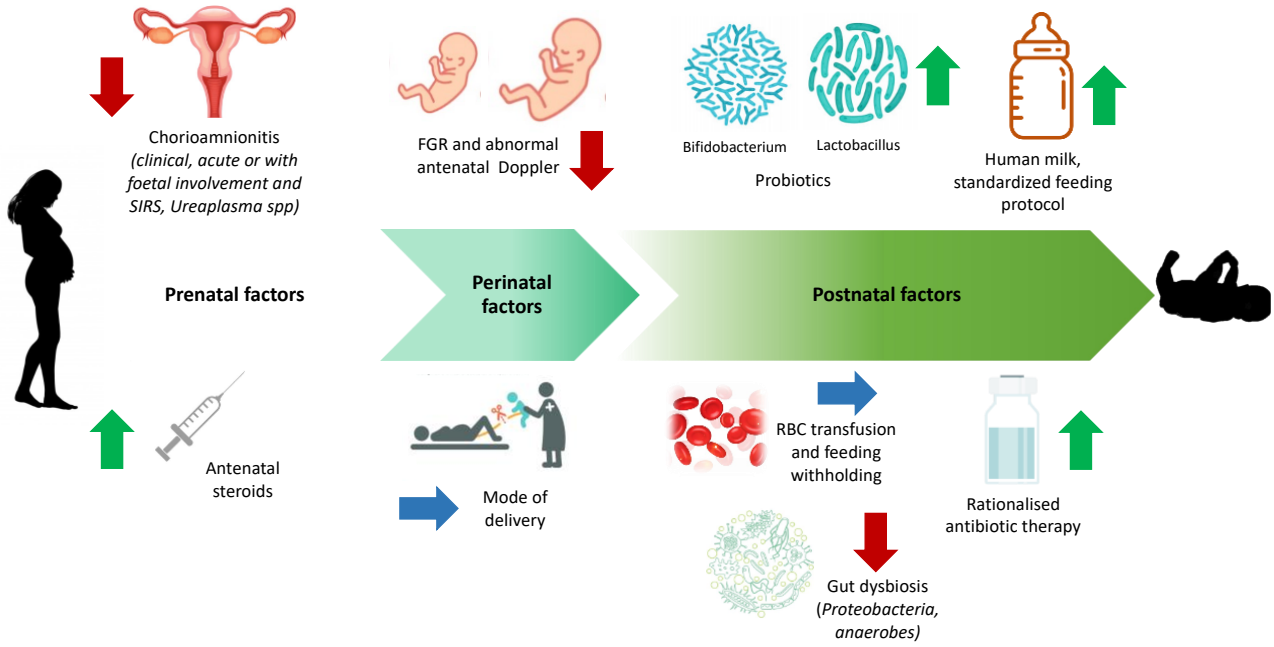


Figure 3. Role of the main protective and risk factors for NEC, according to the most recent evidences from the literature. Arrows represent potential protective effect (upward green), negative effect (downward red) and still unclear effect (rightward blue) of the factor.

1.5 Role of predictive biomarkers

Several systematic reviews have summarized the available evidence on serum, urinary and faecal biomarkers of NEC development or of NEC severity. The multifactorial pathogenesis of this complex condition, however, makes it challenging to unravel a single potential biomarker of the disease.

Depending on the timing at which they are explored, biomarkers can be divided into predictive and diagnostic. The former should be performed prior to symptoms' onset and may help in adopting preventative measures with the aim of reducing the risk of NEC development (f.i. by promoting human milk feeding, adding specific probiotics and lactoferrin, and reducing exposure to potential risk factors). The latter, instead, should be a rapid, bedside, point-of-care test to be performed at clinical suspicion of the disease and to be quickly available to help in guiding a prompt and correct management according to risk of severity (f.i. in the matter of withholding feeding and administer appropriate antibiotics for the correct duration, or to address the need for urgent surgical intervention) (94,101).

Pammi et al. (102) divided biomarkers of NEC in 4 categories: those associated with systemic inflammation, those specific of gut injury, microbial metabolites including fecal volatile organic compounds, and the composites of multiple biomarkers, that in studies are often included in a score. As recently proposed by Terrin et al. (103) for serum markers, in the early phases of the disease, when the early immune response is just elicited, the most useful markers could be the components of innate immunity response. When the cascade of inflammation is activated and the related clinical symptoms appear more evident, the markers of amplification of the inflammatory response could be a better option. Markers of tissue injury, instead, could be used in advanced stages to identify the most severe cases with necrotic damage.

In the majority of studies, these markers have been quantitatively measured through enzyme-linked immunosorbent assay (ELISA).

1.5.1 Serum biomarkers

An Italian systematic review conducted in 2016 (103) of 22 studies on serum markers of NEC Bell's stage II or advanced NEC (Bell's stage III) classified these markers according to 3 pathogenetic steps of NEC: components of the innate immune response activation, proteins amplifying the inflammatory response and tissue injury serum markers. Of the first group, only S100 A8/A9 (two proteins which constitute about 45% of the cytoplasmic proteins of neutrophils and which are upregulated during inflammation) showed high Se and Sp for NEC Bell's stage II. Other neutrophils' and monocytes' superficial proteins demonstrated low Sp and PPV. In the second group of markers, including CRP, PCT, interleukins, Serum amyloid A (SAA), IP-10, TGF-beta and TNF, only apolipoprotein CII (Apo-CII, secreted by the liver into the plasma and whose expression responds to metabolic cues) associated with a specific clinical score showed a high accuracy in the identification of definite NEC. Finally, group 3 comprised gut-associated protein levels in the blood as markers of mucosal damage (104). The review revealed high accuracy of I-FABP and IMA to identify advanced NEC. High Se and Sp were reported for IL-10 (100% and 90%), IL-1 receptor antagonist (100% and 91.7%), intestinal fatty acid binding protein (I-FABP, 100% and 91%) and ischemia-modified albumin (IMA, 94.7% and 92%) when tested to predict the evolution from NEC II to NEC III. Given the important limitations of the studies included (heterogeneity of populations, availability of data, etc.), the authors concluded that the emerged promising serum markers of NEC need to be tested in further large well-designed cohort studies.

Another meta-analysis of 7 studies on the role of serologic I-FABP for early diagnosis of NEC found a pooled sensitivity of 67%, 74% and 83% for NEC stages I, II and III, respectively, with a pooled specificity of 84% (105). A subsequent meta-analysis of 14 studies similarly demonstrated a high specificity but a medium sensitivity of plasma I-FABP (64% and 91% respectively for NEC, 71% and 76% for surgical NEC) (106).

C-reactive protein (CRP) is an acute-phase reactant protein which increases in response to inflammatory reactions. A large cohort study of 241 neonates reported an abnormal rise in CRP in those with NEC II and III. Additionally, subjects responding to treatment had decreasing serial CRP level with it returning to normal at a mean of 9 days, whereas neonates with persistently elevated CRP despite medical therapy were found to have associated complications like abscess and strictures (107). Confirming this last finding, in a retrospective monocentric study the mean maximum CRP and the mean duration of its elevation during the acute phase were greater in infants with post-NEC strictures (108). The advantage of this marker is its relatively low cost and its routine use in clinical practice. As a drawback, however, this tool is highly non-specific as it increases in multiple conditions (such as sepsis, and inflammation) other than NEC, and usually has a lag of 12-24 hours from symptoms onset. Indeed, another more recent retrospective review of 191 neonates with evidence of non-perforated NEC showed a CRP/albumin ratio > or = 3 on day 2 of NEC was associated with a significant higher likelihood for surgery and mortality, indicating CRP and CRP/ALB ratio may be more useful in determining NEC severity (109).

Procalcitonin (PCT) is the peptide precursor of calcitonin, which is synthesized by the parafollicular C cells of the thyroid. It increases within 2 to 3 hours in response to invasive infection (110).

Studies so far have reported low levels of PCT during episodes of NEC, with levels <1 ng/ml at presentation and <1.3 ng/ml thereafter, comparable to 24 healthy controls and lower than values of matched septic infants (peak 4.1 ng/ml) (111).

Serum amyloid A (SAA) is an another acute phase reactant protein expressed in several tissues including the liver and the intestine, that can be found in blood and urine after intestinal inflammation and tissue injury. From an observational study its levels were significantly higher in neonates with NEC and sepsis compared to values in those with only sepsis or no disease at the

onset of the episode, with a cut-off value of 23.2 mg/dL to differentiate NEC from sepsis. SAA levels declined from day 2 to day 10 thereafter (112).
Among other serum markers that have been investigated for NEC are cytokines (like interleukines, and TNF-alfa), growth factors (EGF, VEGF), complement activation product C5a and the platelet activating factor (PAF). However, none of these has proven to be so accurate to be included in the routine clinical practice (110). Other common laboratory parameters that have been used to predict NEC are persistent metabolic acidosis, decreasing platelet count, increasing glucose level and low leukocytes count (113), even combined in equations or in scoring systems (114).

1.5.2 Urinary biomarkers

Studies on urinary and fecal markers have produced encouraging results.
Urinary markers to study loss of integrity of enterocytes and tight junctions, as well as fecal markers of intestinal wall inflammation, have been explored in the cases of suspected NEC as these matrices can be non-invasively collected in preterm infants.
I-FABP is a small (14-15 kDa) cytosolic, water-soluble protein, limited to mature enterocytes of the small and large intestine. It is released into the circulation as soon as cell membrane integrity is compromised, and passes the glomerular filter being readily detected in urine. Therefore, it can provide specific information about the extent of intestinal epithelial cell injury. Similar to serum I-FABP, urinary I-FABP seems to be useful in predicting severe NEC, as several studies have shown higher levels in neonates with NEC stage III (115,116) or in NEC necessitating surgery or causing death compared to NEC treated conservatively (117). The molecule is also increased in those with extensive disease compared to infants with focal NEC (118). The meta-analysis by Yang et al. reported an added value using urinary I-FABP and urinary I-FABP/Cr ratio compared to plasma I-FABP (Se and Sp of 64% and 73%, and of 78% and 75% respectively) (119). This urinary marker is certainly the most studied and promising to diagnose severe cases, but future studies need to address the accurate cut-off value to consider.
Tight junctions constitute the paracellular barrier and consist of a large complex of intra- and extracellular proteins, including claudins (22 kDa). Elevated levels of claudins could serve as putative markers of tight junctions breakdown. Urinary claudin-2 and -3 have been studied in small cohorts and found to be increased in neonates with NEC compared to non-affected ones (117). A narrative review summarizing the relevance of claudin proteins in the pathophysiology of NEC has been recently published (120).
SAA can be measured in the urine as well, and its levels were found to be significantly higher in severe NEC (NEC III or surgical/fatal), with a cut-off value of 34.4 ng/mml at diagnosis and one day prior to surgery. The combination with low platelet count increased the accuracy up to a Se of 94% and a Sp of 83% a day prior to surgery (121).
Other urinary biomarkers of NEC, including proteomic biomarkers, have been extensively reviewed (122).

1.5.3 Fecal biomarkers

During intestinal inflammation, there is a sequestration of neutrophils into the gut wall, followed by their activation and release of calprotectin. Calprotectin is a calcium and zinc-binding protein, present as a heterodimeric peptide (36 kDa) and constituting 60% of the cytosolic content of neutrophils, monocytes, macrophages and submucosal epithelial cells (110). In intestinal inflammation, like in inflammatory bowel diseases, calprotectin is detectable in plasma and feces. Additionally, it is remarkably resistant to degradation by fecal bacteria and can be stable for up to 1 week in fecal matter (117,123). Numerous studies have shown elevated fecal calprotectin (FC) levels

in premature infants with NEC, but the increase of this marker did not occur so early to be useful as a screening marker. However, in a more recent study by Thibault et al., fecal lipocalin-2 and FC, both individually and in combination, were the two most reliable markers in predicting NEC (any stage) over the 10-day period prior to its development (124). The Se and Sp for both markers was >70% (cut-off value 227 μg/g for calprotectin and 6.2 μg/g for lipocain-2).
A systematic review of 13 studies described a Se range between 76 and 100% and a Sp of 39-96.4% for FC as a diagnostic test (125). However, studies proposed different cut-off values (from 280 μg/g to 792 μg/g, up to 3 mg/ml).
Interestingly, in one study including 62 neonates with clinical suspicion of NEC (29 with definite NEC), the combination of urinary I-FABP and FC significantly improved diagnostic accuracy with a Se of 94% and a Sp of 79%, which was higher that the Se and Sp of urinary I-FABP and SAA combined (121).
Another fecal biomarker that has been evaluated in the diagnosis of NEC is phagocytic-specific S100A12, a cytosolic calcium-binding protein released by phagocytes upon activation during intestinal inflammation. In a prospective study of 145 VLBW infants, levels of this marker were significantly higher at NEC onset and 4-10 days before in affected infants (n=18) compared with the unaffected ones, but a great inter- and intraindividual variability was recognized (126).

Table 3 summarizes the major serum, urinary and fecal biomarkers of NEC explored by targeted analysis (103–109,111,112,114–116,119,121–126).

	Biomarkers in NEC	
	Increased	**Decreased**
SERUM	S100 A8/A9 Apo-CII CRP SAA IP-10 TGF-β TNF-α I-FABP IL-6, -8, -10 IL-1 receptor antagonist IMA CRP/Alb	Platelet Neutrophils PCT
URINE	I-FABP I-FABP/Cr Claudin-2 and -3	
FAECES	Calprotectin Lipocalin-2 S100 A12	

Abbreviations: Alb=albumin; Cr=creatinine; CRP=C-reactive protein, I-FABP=intestinal-fatty acid binding protein; IL=interleukin; IMA=ischemia-modified albumin; IP=; PCT=procalcitonin; SAA=serum amyloid A, TGF=transforming growth factor; TNF=tumor necrosis factor.

1.6 Metabolomics and metabolomic biomarkers of NEC

The "omics" technologies allow a comprehensive and systematic detection of mediators for revealing mechanisms of diseases and host-pathogen interactions (127). In particular, metabolomics, the youngest of the four major "omics" sciences, has several advantages as it enables to depict the ultimate phenotypic expression of the ongoing biochemical response to a stimulus (128). Metabolomics allows the identification and quantification of the low-molecular weight metabolites (metabolome) present in a complex biological system, such as cells, tissues, organs and

biological fluids (129). Metabolites are building blocks of the cellular functions and can be peptides, lipids, organic acids, vitamins, minerals, drugs, amino acids, nucleic acids, carbohydrates, fatty acids, hormones and any small molecule <1000 Dalton (Da, where one hydrogen molecular atom has a molecular mass of 1 Da, so 1 Da is 1 g/mol; 1000 Da is one kilodalton) (130). The set of metabolites is not static but is continuously modified by the reciprocal interactions between the genome and environmental factors, such as nutrition, microbes, exposure to drugs or toxins (131). As last downstream products of gene transcription and enzymatic pathways, and as functional end-point of cellular reactions, metabolites provide a close picture of the organism's phenotype and its interaction with the environment (132).

1.6.1 Methodology and analytical workflow

To undertake a metabolomics study, well-defined steps need to be linked in what is called the "metabolomic workflow" (**Figure 4** from Frédérich et al. (133))

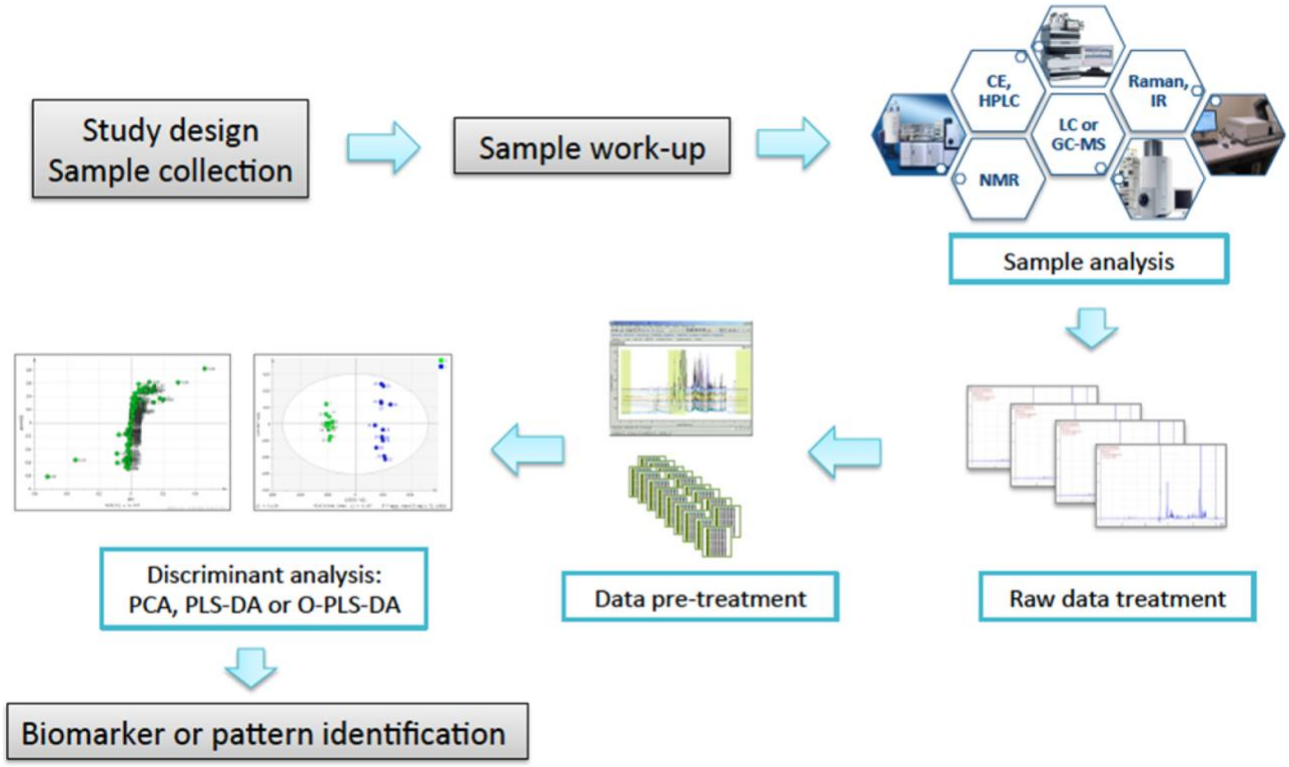


Figure 4. Classical workflow of a metabolomics analysis, with all steps carefully designed and standardized to minimize variability. The diversity of the skills required in this field highlights the complexity of the approach and its necessary multidisciplinary nature. From Frédérich et al. (133).

The experimental design and sample definitions are first important issues of metabolomics studies. Metabolomic analysis can be applied to different biological fluids, like those non-invasively collected from preterm neonates, such as umbilical cord blood, plasma, urine, stool, tracheal aspirate, etc.. The amount required for the analysis is very small (20 µL-300 µL). Sample preparation prior to analysis also represents a very important step that should be executed under standardized and validated conditions, as the intrinsic characteristics of biofluids, such as presence of water and/or proteins, require particular treatments to reduce any undesired noise (133).

Different methodological approaches are encompassed by metabolomics, such as targeted metabolomics, untargeted metabolomics, biology-driven metabolomics and pattern or fingerprint

analysis (133). By the *targeted metabolomic approach*, a limited number of biochemically annotated metabolites is identified and quantified, thus permitting of verifying previous hypotheses and of validating the metabolites discovered by the untargeted strategy. The *untargeted approach*, instead, extensively and comprehensively analyzes the entire set of metabolites present in the biological system, without an a-priori selection based on chemical class or biological activity, thus highlighting unknown and unexpected changes in response to an event (131). With its hypothesis-free hypothesis-generating global approach, the untargeted metabolomics may untangle a better understanding of the molecular processes responsible of a disease. The *metabolomic fingerprinting* is an untargeted approach that aims at recognizing a set of metabolites to discriminate 2 different groups, without necessarily identifying all metabolites present in the system. Applying it to clinical practice, this technique allows to discover a profile of biomarkers, or a metabolomic snapshot, able to distinguish between healthy patients and patients affected by a given disease and also to distinguish subgroups of different phenotypes within the same disease (131). **Table 4** summarizes the main strengths and pitfalls of the targeted and untargeted approach.

Table 4. Summary of the characteristics, strengths and pitfalls of the targeted and untargeted approaches.

METABOLOMIC METHODOLOGICAL APROACH	TARGETED	UNTARGETED
Principles	Selective approach	Complete, global, comprehensive, extensive approach
Identified metabolites	Known, biochemically annotated metabolites	Known and unknown metabolites
Technics	LC-MS, GC-MS, CE-MS	NMR, MS, FTIR, RAMAN
Strengths	Driven by a-priori hypothesis, rational approach Hypothesis-validating Absolute quantification Low detection limit	Hypothesis-free, exploratory Hypothesis-generating Applicable to pattern recognition Richness of generated data
Pitfalls	Limited number of compounds Need of pure standard for calibration	Semi-quantitative Challenging data interpretation Medium throughput

Abbreviations: CE-MS=capillary electrophoresis-mass spectrometry, FTIR= Fourier transform infrared spectroscopy, GC-MS=gas chromatography-mass spectrometry, LC-MS=liquid chromatography-mass spectrometry, NMR=nuclear magnetic resonance.

1.6.2 Analytical platforms

Due to the complexity of the matrix to be examined (e.g. osmolarity, presence of proteins, and inorganic salt concentration) and the large concentration range and types of metabolites (acidic, neutral, lipophilic, etc.), a single analytical technique cannot provide alone an exhaustive biochemical characterization of the biological system. Sophisticated analytical technologies combined with multivariate statistical methods are used for information extraction and data interpretation. Among the advanced analytical techniques for molecular identification and characterization, Nuclear Magnetic Resonance (NMR) spectroscopy and Mass spectrometry (MS) are the most largely employed (129).

The selection of the appropriate approach usually depends on the experimental objectives, biological matrix and methodological investigation (targeted vs untargeted). Nonselective approaches should be applied to obtain the maximal information for complex biological matrices, but only a partial view of the metabolome can derive from each analytical platform (133).

Historically, gas chromatography (GS) coupled with MS was the first method used to explore the metabolome. Later on, proton NMR (^{1}H-NMR) spectroscopy emerged as other powerful method

and has been extensively employed for nontargeted metabolomics. NMR spectroscopy allows the detection of molecules containing protons and different metabolites generating different signals in the NMR spectrum. NMR spectroscopy identifies the molecular structure of metabolites, both single and in complex mixtures. It is considered a universal, nondestructive detection method, indeed among its advantages are the short analytical time, the relative easy sample preparation (not requiring prior separation or chemical derivatization), and its quantitative response. Additionally, it is both highly reproducible and robust. Nevertheless, NMR exhibits two major drawbacks, the low sensitivity, prohibiting the detection of diluted metabolites, and the lack of a separation method prior to spectral measurement which often determines an extensive overlap of metabolite signals (133).

MS has rapidly become the predominant platform. It allows the identification of metabolites by generating a spectrum in which metabolites rank based on their mass. Before MS, it is necessary to perform the separation of the sample components using different types of chromatographic techniques, like liquid chromatography (LC), gas chromatography (GC) or capillary electrophoresis (CE) according to the physicochemical properties of the compounds. This makes MS very complementary to NMR, in particular for the measurement of diluted metabolites. The prior separation also permits a higher resolution and a greater sensitivity over NMR (below 1 pmol/L). Mass spectrometers can have stand-alone analyzers or various analyzers in combination within one instrument, thus taking the advantages of the strength of each instrument. Recent applications of MS in metabolomics are based on instruments like triple-quad mass spectrometer (QQQ) or Quadrupole Time-of-Flight mass spectrometer (Q-ToF). The main weaknesses of this technique remain the influence of ionization on the response, which requires a standard for quantification, and the necessity of sample preparation and separation, which decreases the reproducibility and increases the time of this platform (133).

1.6.3 Statistical analysis

The following important step after measuring and pretreating data is extracting the most relevant information to interpret the results. Given the huge amount of data generated by NMR spectroscopy and MS, this can only be managed with bioinformatic methods capable of reducing the dimension of the data via multivariate statistical analysis (134,135). Three different approaches are commonly applied to data: discriminant analyses, classification trees, and machine learning. Within discriminant analysis, two main processes can be distinguished, i.e. nonsupervised and supervised methods, according to whether there are or are not a priori group definitions. These permit the pattern recognition of data, that is to identify patterns and regularities in data.

The simplest and most extensively used nonsupervised method, principal component analysis (PCA), transforms the data obtained with analytical techniques into plots, thus making them visually understandable and easy to interpret. For this reason, PCA is generally a first-line method useful for a rapid visualization of any clustering or trends in the data structure, as well as for identifying and eliminating outliers or samples with an unusual metabolic profile.

Supervised methods are mainly used to test hypotheses and to develop predictive models. Partial least squares-discriminant analysis (PLS-DA) is one of the most widely used. It uses a training set of samples (of known classification) to create a mathematical model that is then applied to test an independent dataset. This methodology aims to optimize the separations between the classes and to identify which metabolites are most implicated in these separations (133).

1.6.4 Metabolite identification and validation

Finally, a key and challenging point of the metabolomic workflow is the structural identification, quantification and validation of the emerged biomarkers, particularly in the case of unknown metabolites. Knowledge of the implications of metabolites in different biochemical pathways is essential for understanding the impact of their variations on the physiology and/or pathology.

Many commercial and noncommercial databases and articles are currently available for molecular identification of metabolites, e.g., *MetaCyc Metabolic Pathway Database* (http://www.metacyc.org), *NMR Metabolomics Database* (http://www.liu.se/hu/mdl/main/), *Madison-Qingdao Metabolomics Consortium Database* (http://mmcd.nmrfam.wisc.edu), and the *Human Metabolome Database* (http://www.hmdb.ca). HMDB is a freely available electronic database containing detailed information about small molecule metabolites found in the human body. It is designed to contain or link three kinds of data: chemical data, clinical data and molecular biology/biochemistry data. To date, the HMDB contains 220.945 metabolite entries, including both water-soluble and lipid soluble metabolites (131,133,136).

Once "biomarker" metabolites are identified in spectral data, it is necessary to validate the relevance of these "biomarkers" via cross-validation, i.e., testing using another technique, which usually implies a targeted approach on an independent sample collection. This usually improves the validity of the findings emerged from the untargeted approach.

1.6.5 Metabolomics in biomarker discovery of NEC

Despite a continuous evolution of metabolomic studies in pediatric and neonatal research to improve in hospital and clinics practices, few are, so far, the applications to NEC and, especially, to its differential diagnostic conditions like SIP (129).

From a recent systematic review conducted by our group (94) and including studies applying untargeted metabolomics and gut microbiota analysis in the differences between NEC (Bell's stage II or III) and/or SIP cases versus healthy controls, only five studies applying metabolomics (43 cases, 95 preterm controls) were found **(Table 5)**. Few other studies were excluded due to unprecise NEC definition, inclusion of cases of suspected NEC (Bell's stage I) without a separate analysis, missing data, unavailable text or absent comparison with a control group. All included studies were cross-sectional, with a prospective collection of samples and their retrospective analysis. Three studies utilized NMR spectroscopy applied on urinary samples (137–139) while two studies applied ultra-performance LC-MS to evaluate the faecal metabolomic profiles (95,140). 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. None of the studies evaluated the differences in metabolic profiles between NEC and SIP cases.

In urine, Morrow et al. (137) demonstrated an early (between 4 and 9 days of life) urinary 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. Additionally, 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 that a close link between gut microbiota and metabolomic signatures. Two other studies, instead (138,139) reported significantly decreased metabolites, mainly amino acids and organic acids, in the urine of NEC patients collected in proximity of the disease.

In stools, NEC cases were characterized by increased sphingomyelins and decreased ceramides before disease onset in one study (140), as well as by changes in metabolites belonging to linoleate metabolism, C21-steroid hormone biosynthesis, leukotriene metabolism, and formation of prostaglandin from arachidonate (95). These pathways may confirm the involvement of an inflammatory response and an increased intestinal permeability in neonates affected by NEC.

More recently, two additional studies have been published applying untargeted metabolomics on serum samples. The first (141) used LC-MS with quadrupole ToF and demonstrated a reduction of molecules possibly identified as phosphatidylcholines or lysophosphatidylcholines at the onset of clinical presentation both in neonates with LOS and those with NEC compared to controls. L-carnitine, phosphatidylcholine, and glycerophospholipids discriminated NEC cases from healthy subjects.

In the second study (142) GC-MS applied on serum samples was able to differentiate premature infants before and after feeding, but the specificity and sensitivity of the metabolites could not achieve high accuracy to predict NEC.

In general, as already highlighted by previous authors, there is a wide variability in populations' inclusion criteria, timing of samples' collection, type of analyzed biological fluids and results among studies, and it does not appear to be a unifying metabolomic signature of NEC (129,143,144).

Other techniques have been recently developed, like metagenomics associated with targeted metabolomics, with functional *in vivo* and *in vitro* assessment, to define novel molecular mechanisms of NEC (145,146).

Table 5. Characteristics of studies applying untargeted metabolomic analysis for the predisposition/diagnosis of NEC Bell's stage II or III. Numerical data are expressed as mean (SD) or median (IQR) or as number/percentage, if not otherwise specified. Adapted from Moschino et al. (94).

Author / Year/ Country	**Study design, technique applied**	**Inclusion criteria (GA / BW) n NEC definition**	**Mean (SD) / median (IQR/range) GA (weeks)**	**Mean (SD)/ median (IQR) BW (grams)**	**Sample type and timing**	**Main findings**
Morrow 2013 Ohio USA (137)	Cross-sectional; NMR spectroscopy	<29 w, <1200 g Cases: 11 NEC Controls: 21 NEC II or III Bell's stage Subtypes of NEC based on ordination of day 4-9 samples: -NEC-I (n=4) dominated by Firmicutes (Bacilli) -NEC-II (n=5) dominated by Proteobacteria (Enterobacteriaceae)	Cases: 25.5 (1.8) Controls: 25.9 (1.5)	Cases: 791 (212) Controls: 839 (187)	Urine; T1 4-9 days; T2 10-16 days	T1: Increased Alanine and histidine normalized PI in NEC-I subtype compared to NEC-II subtype and controls; Cases: Alanine/histidine >4 (9/11, 82%) Controls: A/H >4 (5/20, 25%) Controls: decreased alanine normalized PI;
Picaud 2021 France-Italy (138)	Cross-sectional; NMR spectroscopy	VLBW Cases: 6 NEC (3 early-onset, 3 late-onset) Controls: 12 (6 with feeding intolerance FI; 6 with good digestive tolerance GDT) NEC defined by clinical evidence fulfilling Bell's stage criteria and with radiological pneumatosis intestinalis (stage ≥ II)	Cases: 27.1 (1.6) Controls 1: 27.2 (1.3) Controls 2: 27.7 (1.6)	Cases: 1016 (104) Controls 1: 920 (104) Controls 2: 950 (65)	Urine; Cases: before and at disease onset; Controls: at birth and as close as those of babies with NEC	Cases- Early-onset (<25 days): no differences; Cases - Late-onset (>40 days): increased lactate RI; Controls GDT: increased N,N-DMG, betaine, myo-inositol, creatinine, urea RI;
Rusconi 2018 MO USA (140)	Prospective; UPLC-MS/MS for BRM, then targeted analysis of 14 ceramides and 7 sohingomyelins	Inclusion criteria NI Broad range metabolomics (BRM): Cases: 9 Controls: 19 Targeted: Cases: 23; Controls: 46	BRM: Cases: 25.9 Controls: 25.1 Targeted: Cases: 25.9 (24.7–27.35) Controls: 25.5 (25–27.5)	BRM: Cases: 825.2 Controls: 787.3 Targeted: Cases: 800 (720–955) Controls: 840 (662.5–927.5)	BRM: stools closed to NEC onset (<5 days preceding it, not from the same day); Targeted: pre-event stool (1-3 days before NEC onset)	Cases (NEC II and III): increased sphingomyelins (not specified), low ceramides (not specified) Peak values expressed for the metabolites of the targeted analysis

		NEC Bell's stage II and III, no SIP				
Stewart Microbiome 2016 UK (95)	Prospective collection, retrospective analysis; UPLC-MS	<32 GW Cases: 7 NEC Controls: 28 Metabolomic analysis on 6 cases and 10 controls NEC "defined rigorously" by one senior clinician and two senior research clinicians, and classified as either surgical or medical, where pneumatosis was required for medical cases	Cases: 26 (23-30) Controls: 27 (24-30)	Cases: 760 (500-1470) Controls: 910 (545-1810)	Stools; (DOL) −14 (time point 1; TP1), −7 (TP2), 0 (TP3), +7 (TP4), and +14 (TP5)	TP3 (disease diagnosis): 5 metabolites with highest VIP scores: linoleate metabolism (2), C21-steroid hormone biosynthesis and linoleate metabolism (2), leukotriene metabolism and prostaglandin formation from arachidonate (1)
Thomaidou 2019 Greece (139)	Prospective cross-sectional; untargeted NMR spectroscopy and targeted LC-MS	Preterm neonates (GA not specified); Cases: 15 (5 NEC I, 10 NEC II/III) Controls: 15 NEC every grade; separate sub-group analysis after excluding stage I NEC cases	Cases: 34 (29-36) Controls: 33.5 (29-36)	Cases: 2030 (1100-2680) Controls: 1815 (1130-2640)	Urine at time of evaluation for NEC; Cases: 8 (4-22) d Controls: 9 (4-34) d	Cases: Low Tyrosine (FC -1.6, AUC 0.80), Proline (FC -0.26, AUC 0.83), Citrate (FC -1.3, AUC 0.85), 4-hydroxybenzoate (FC -1.09, AUC 0.86), Formate (FC -1.33, AUC 0.82), Succinate (FC -1.0, AUC 0.89), 4-hydroxyphenylacetate (FC -1.23, AUC 0.78), Fumarate (FC -1.54, AUC 0.82), Creatinine (FC -0.35, AUC 0.79), Myo inositol (FC -0.24, AUC 0.79), hippuric acid (FC -1.46, AUC 0.76)

Abbreviations: ANS=antenatal steroids; ATB=antibiotics; AU=arbitrary unit; AUC,=area under the curve; BRM=Broad range metabolomics; CAM=chorioamnionitis; CCC=complex congenital cardiopathy; CS=cesarean section; EBM=exclusive breast milk; FC= fold change; FCR=fold change ratio; F=French; FI= feeding intolerance; g=grams; GA=gestational age; GDT=good digestive tolerance; GW=gestational weeks; h=hours; MP=multiple pregnancy; NEC=necrotizing enterocolitis; NI=No information; NMR=nuclear magnetic resonance; N,N-DMG=N,N-Dimethylglycine; PI=peak intensity; RI=relative intensity; SIP=spontaneous intestinal perforation; TP=timepoint; VIP=variable importance plot; VLBW=very low birth weight; w=weeks;

1.7 Management and treatment

The cornerstone of medical intervention for NEC includes abdominal decompression with an orogastric tube, bowel rest, broad-spectrum antibiotics and intravenous nutritional support (36,57). Bowel inflammation and wall thickening during NEC cause loss of gut motility (ileus), with consequent necessity of gastric decompression and nihil per os until the ileus resolves. Fluid replacement is required to support nutritional demands and growth and to correct third space losses due to capillary leak (3). The most severely affected, especially those requiring surgical intervention, may need inotropic support and mechanical ventilation, as well RBC transfusions and correction of metabolic acidosis.

Administration of antibiotics is based on the documented bacteriemia which is present in 20-30% of NEC cases, and on the fact that pathologic specimens and peritoneal fluid from NEC cases recover from pathogenic bacteria after their use (147). There is no consensus on the appropriate antibiotic regimen to use, and the choice must be guided by the susceptibility patterns in the local NICU. Usually, a coverage for pathogens responsible for late onset sepsis (i.e. sepsis with onset >72 hours from life) is adopted, including ampicillin (or vancomycin if high prevalence of methicillin-resistant St. aureus or ampicillin-resistant enterococcus) plus gentamicin (or amikacin if resistance pattern). Antibiotics against anaerobes, like metronidazole, are often added. A 10 or 14-day course is usually sufficient and indicated in NEC Bell's stage > or = II. Antimycotics, like fluconazole or amphotericin B, are adjoined when fungal infection is suspected (36).

Surgery is required in around 40-50% of NEC cases, and common indications are intestinal perforation (Bell's stage IIIB), and deterioration of the clinical or biochemical status (e.g. shock, decreasing platelet count, neutrophil count, or both) (57).

Surgical procedures may involve drain placement, exploratory laparotomy with resection of diseased bowel, and enterostomy with creation of a stoma.

Exploratory laparotomy (ExLap) has been extensively in use since 1970s and it permits the resection of nonviable intestine. Peritoneal drainage (PD), instead, was first described in 1977 as temporary measure in the more sick infants while waiting for a clinical improvement to perform ExLap, but it has been applied as definitive therapy since 1990s-2000s (148–150).

The most traditional approach is to perform a laparotomy with diverting stoma proximal to most diseased bowel, allowing the heal of downstream bowel without translocation of stool/bacteria. Some centers consider refeeding the proximal stoma effluent through the distal mucous fistula in order to stimulate mucosal growth and minimize fluid and electrolyte losses, but there is still little evidence to support this practice (151). Nevertheless, due to the possible complications of stoma, like fluid losses, poor growth, and stoma prolapse or retraction, some groups has considered primary anastomosis as the first option to treat severe NEC (152). These two surgical techniques have been compared in a recent systematic review and meta-analysis, showing no significant difference in terms of complications or mortality rate, although the spectrum of complications was slightly different in each group (153). All in all, in 2013 the majority of surgeons (67%) seemed to opt for bowel resection and primary anastomosis in the case of focal NEC, while 75% would perform a stoma in case of multi-focal disease (154).

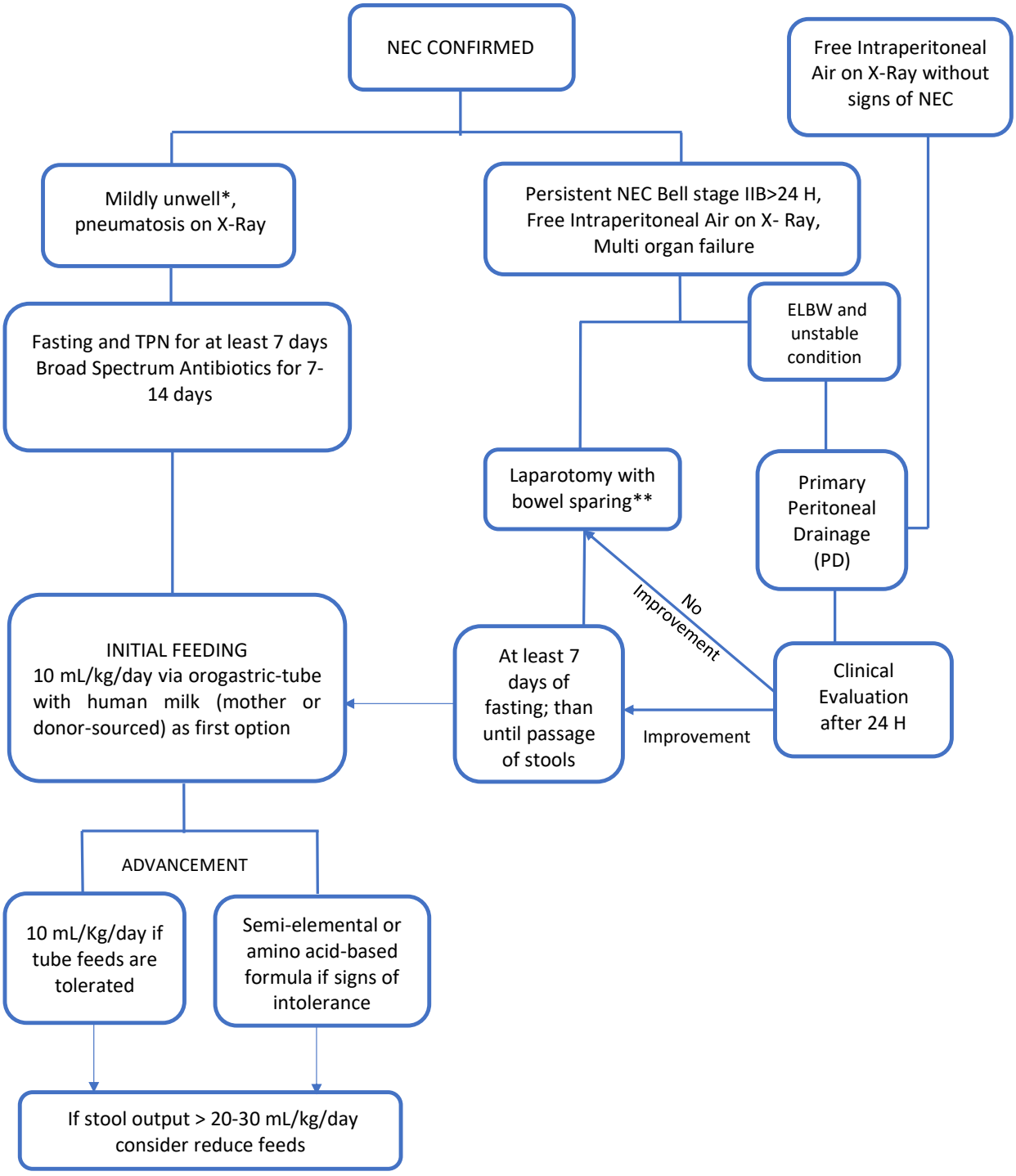
As regards PD, this consists of placement of a peritoneal drainage without exploring the intestine, and can be performed either as temporary measure or as bridge before ExLap (155). In 2013, 27% surgeons opted for primary peritoneal drainage (PPD) as definitive treatment in ELBWI with intestinal perforation, while 67% thought that PD was important for stabilization and transport (154). A recent report from 820 US centers, however, highlighted how this last approach has been progressively more used as primary and unique treatment, with an increase from 23.2% in 2006 up to 46.8% in 2017 (156).

Previous large multicenter studies revealed no significant differences in survival or clinically important outcomes between the two approaches (148–150). Nevertheless, infants treated with PD very often required a subsequent laparotomy (149). The most recent multicenter randomized clinical trial enrolling neonates from 20 centers in US and comparing initial laparotomy versus PD for NEC and SIP, demonstrated an interaction between pre-operative diagnosis and treatment group. In neonates with pre-operative diagnosis of NEC, in particular, the Bayesian probability of a beneficial laparotomy was 97%, as death or neurodevelopmental impairment developed in 69% after laparotomy and in 85% after PD (157).

Our institution policy consists of performing surgery in case of persistent NEC Bell stage IIB for more than 24 h, free intraperitoneal air detected on radiological examination, and worsening of multi organ failure. When laparotomy is performed, minimal bowel handling is recommended avoiding resection. PD is advocated in ELBWI with unstable conditions (inotrope need, maximal ventilator support) when perforation is suspected or when free intraperitoneal air is detected on X-ray without previous clinical or radiographic signs of NEC. Absence of improved condition and enteral output from PD after 24 h are indications for delayed laparotomy (**Figure 5**, from Moschino et al.(69)). this policy based on the concept of "sparing surgery" is safe and it seems to be associated with a low mortality rate (6.4%) (158).

Finally, when the entire small intestine is involved or both the small and large bowel are necrotic, often referred to as NEC totalis (NEC-T), the surgical approach is more challenging as identifying viable bowel in a pan-intestinal involvement may be difficult and lead to unnecessary extensive resection. For this devastating type of the disease, several different approaches have been proposed, among which the "patch, drain, and wait technique" (159), and the "clip and drop technique" (160). In particular, this last approach, consisting of resection of necrotic bowel and tiding off the ends of healthy bowel tracts, with a second-look laparotomy after 48–72 h, has provided positive results in terms of reduction of incidence of short-bowel syndrome (SDS) (161).

All in all, complications of surgery, whether it involves laparotomy or PD positioning, impose a close and long follow-up by a multidisciplinary team including, neonatologists, surgeons, and dieticians.



* Mildly unwell: bloody stools, food intolerance, abdominal tenderness, need for intensive care (respiratory support, inotropes)
** Proximal stomy with minimal resection; stoma reversal in planning 6-8 weeks maintaining the same amount of feeds

Figure 5. Flow chart depicting our institution policy in the approach to a neonate with suspected NEC (69).

2. Methods

2.1 Study design and setting

This is a monocentric observational case-control study conducted at the Neonatal Intensive Care Unit (NICU), Department of Women's and Children's Health, Padova University Hospital, Padova, Italy.

2.2 Objectives

Primary aim of this study was to apply the untargeted metabolomic approach on early urine samples collected within 2 days of life (DOL) from preterm infants in order to identify potential biomarker profiles able to predict NEC development.
Secondary aims of the study were:

- To distinguish neonates developing NEC from healthy controls and from those developing SIP or other gastrointestinal conditions by their early urinary metabolic profile;
- To explore the evolution of the urinary metabolome during the first month of life in neonates developing NEC;
- To characterise potential prognostic metabolic profiles of NEC severity (i.e. surgical NEC vs medical NEC);

2.3 Population

All infants admitted to the NICU with a GA <34 GW were enrolled after written informed consent for participation of a legally acceptable representative. Neonates with congenital heart disease of any gestational age were included as well, given their potential high risk for developing NEC.
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 enrolled from January 2020 to July 2022 and prospectively followed until discharge, or transfer to another Unit or Hospital, or death.
As a study applying an untargeted metabolomic approach, there is no a-priori knowledge of the number of variables characterizing each study group. Therefore, the sample size was based on the one reported by previous studies in the literature, which is of 15-20 subjects for metabolomic studies and 10-15 cases of NEC for similar studies on the topic. Additionally, the incidence of NEC reported in our center is around 10 cases/per year.

2.4 Definitions

NEC was defined according to modified Bell's stage criteria by Walsh and Kliegman (9), and cases were considered only if belonging to stage > or = II (definite NEC, **Table 1**).
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.
Definite Sepsis was defined if Vermont Oxford Network Criteria were satisfied: 1) clinical symptoms of generalized infection; 2) isolation of a pathogen in at least one blood culture; 3) treatment with antibiotics for the identified pathogen. Early onset sepsis was defined if onset <72 hours, whereas late onset sepsis if occurred > or = 72 hours from birth (162).
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 sepsis and without any sign of mechanical intestinal obstruction.
Minimal enteral feeding was defined as trophic feeds with a total volume of < or = 24 ml/kg/day, while full enteral feeding 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), haemodynamically significant patent ductus arteriosus (HSPDA), were defined according to the local protocols.

2.5 Data and Sample collection

For each patient, antenatal, perinatal and postnatal clinical and demographic characteristics, as well as laboratory data, were recorded on a preformed electronic case report form (eCRF) on the REDCap platform (https://redcap.sdb.unipd.it/redcap/). An identification code was assigned to each subject, in order to protect confidentiality according to the rules of good clinical practice and the Dlgs 196/2003.
For each patient, plasma, urine and faecal samples were collected at birth (within 48 hours, T0), and then at 14 (T14) and 28 days of life (T28), at 2 months (T2 months) and at 36 weeks of corrected gestational age (cGA) (T36). In the suspicion of NEC or SIP or gastrointestinal diseases with a presentation similar to NEC, infants had additional plasma, urine and faecal samples collected at onset and then weekly until resolution (cases).
Plasma samples were obtained from blood samples drawn through arterial or venous lines already in place, or from spare capillaries drawn for routine haematocrit and bilirubin levels.
Urine was non-invasively collected using a cotton wad and then transferred into a tube.
Faeces were non-invasively collected from the infants' nappies using a clean pad and then transferred into a tube.
Samples were stored in a freezer at -80°C until metabolomic analysis.
Only urine samples were used for the purpose of this study.

2.6 Metabolomic analysis

The analysis of urine samples was performed at the Mass Spectrometry and Metabolomics Laboratory of the Women's and Children's Health Department, Torre dell Ricerca Pediatrica "Città della Speranza" University of Padova, Padova, Italy.
Urine samples were slowly thawed overnight at +4°C and then transferred to ambient temperature for the preparation. Each sample was stirred and centrifuged at 3000 rpm (1509 g) for 15 min at 10°C, then 20 µl of the supernatant from each sample were pipetted in a well of 384 wells plate, adding 280 µL of 0.1% formic acid (FA) solution (finale volume 300 µL, dilution 1:15). All the procedures for the preparation were automatically managed by a robotic liquid handling system, Multiprobe II Ex (Perkin Elmer).
Untargeted metabolic profiling of urine samples 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.).
The mass range scan was of 20 to 1200 amu, in MS scan mode. The capillary voltage was set at 0.7 KV; and the sampling cone voltage at 40 V. The desolvation gas flow was set at 800 L/h with temperature kept at 400°C. The cone gas flow was set at 20 L/h with temperature kept at 110°C. To correct for changes in environmental or experimental condition over the course of the analysis, Leucine-Enkephalin solution at a concentration of 100 pg/ml was injected periodically (every 30 s) as internal reference (i.e. lock mass).

For LC-MS analysis a Waters Acquity UPLC HSS T3 column 2.1 mm wide and 100 mm long packed with 1.8 µm beads was used and its temperature was kept at 50°C. The mobile phase flow rate was set at 0.5 ml/min. The gradient mobile phase consisted of water with 0.1% FA (A) and methanol with acetonitrile in a 90:10 ratio with 0.1% FA (B). Each sample run lasted 12 minutes and consisted of an isocratic phase of 5% B for 1 minute, a linear increase to 30% B in 2.5 minutes, a linear increase to 95% B in 3 minutes, an isocratic phase of 95% B for 1.5 minutes, a washout phase of 5% B for 3 minutes. For each run, 3 µl of sample were injected.
Quality Control samples (QC) and Standards Solution Samples (Mix) were used to assess reproducibility and accuracy during the analysis, and examine the metabolite content of the samples.
The QCs were prepared from an aliquot (10µL) of each sample, pooled together and diluted with eight different dilution factors (1:3, 5, 7, 10, 15, 20, 50, 100) with 0.1% FA solution in water, treated as the samples. The Mix consisted of nine compounds of known exact mass and retention time. The QCs and Mixes were injected at regular intervals of 15 samples during the sequence, together with blank samples, to identify specific ions from the mobile phase, and any contaminants.
The sequence for the analysis were prebuilt in Excel to randomize the samples injections and prevent any spurious classification deriving from the position of the sample in the sequence.

2.7 Data extraction and synthesis

Raw data were extracted using Progenesis QI software (Waters Corporation, U.S.A.). The parameters were optimized through the preliminary processing of the QCs. Specifically, 0.5 was set as filter to import the raw data in positive ionization (ESI+) and 0.3 for negative ionization (ESI-), and the QC in the middle of the sequence was selected as reference for the automatic retention time alignment of the samples in the sequence. The sensitivity of the automatic algorithm for peak picking was set at 3 for both ionizations, in the time range from 0.4 to 8.0 min. As a result, the so-called time_mass variables (where “time” is the retention time and “mass” is the mass to charge ratio m/z of the spectral feature) were generated.
Features with at least one missing data in the QCs and more than 10% of missing data in the samples were eliminated. For each variable passing such a filter, missing data were imputed with a random number between zero and the minimum value measured for that variable. Data were calibrated on the basis of the local linear regression models obtained considering the trend of the QCs with the run order. Probabilistic quotient normalization was applied to take into account dilution effects.
Variables with a coefficient of variation greater than 25% in the QCs were excluded.
Data were log-transformed and autoscaled prior to performing data analysis.

2.8 Statistical analysis

Categorical clinical data were investigated by Fisher’s exact test whereas continuous clinical data by t-test for data with a normal distribution or Mann-Whitney test in the case of data with a non-normal distribution. Normal distribution was assessed by Shapiro-Wilk test. A p-value of 0.05 was considered statistically significant.
In the first step of data analysis, the urinary metabolome of the urine samples collected at birth (within the first two days of life, T0) were investigated. A preliminary data analysis was based on control charts with the aim to prove that the urinary metabolome is able to discriminate controls and subjects developing NEC, SIP and other diseases. Given the comparison of multiple groups, the control group was considered as reference and two control charts, one based on K-Nearest Neighbors (KNN) and one on Bounding Box (BB), were built in the framework of Model Population Analysis (MPA) (163). The idea underlying MPA is that information about a data set is not discovered

by a single model, but through the analysis of a large population of sub-models generated from a random sampling of the data. The control group was sampled a large number of times by Binary Matrix Sampling (BMS), sampling both observations and features, to extract a large number of subsets that were used to build the control charts, obtaining a large number of KNN and BB control charts based on different sets of controls and variables. Thus, the belonging to the reference group of the out-of-bag samples was predicted applying the set of control charts. The frequencies of right prediction were considered to estimate the thresholds to use to assess if a new sample belongs or does not belong to the control group. The main advantage of using MPA with respect to single class modelling is that it avoids models of the reference data that describe a too large space with the risk of increasing the rate of false belonging to the reference group. The chart based on KNN considers the distance matrix of the new observations with respect to the reference group, and compares the mean distances of the K-nearest reference observations with a suitable threshold calculated by MPA. The distance matrix is calculated using the scores of the PCA model (164) of the reference data. Moreover, the Q-distance of the PCA model is used as second score for comparison, and it is compared with the maximum value calculated for the reference data. Values of mean distance and Q-distance exceeding the related thresholds indicate observations that do not belong to the reference group. In the BB control chart, the data distribution of each feature in the reference group is modelled by Gaussian kernel density estimation and used to calculate the p-value of the data of the new observations. False Discovery Rate (FDR) is applied to detect the number of features for which the new data are outliers for a given significant level. If at least one feature is detected, the observation is considered not belonging to the reference group. The results obtained applying the control charts are combined in a consensus approach assuming that an observation does not belong to the reference group if at least one chart discovers the observation as outlier.

Thus, the group of subjects developing NEC was compared with the control group to discover subsets of features relevant in distinguishing the two groups. Both univariate data analysis controlling the false discovery rate by Benjamini-Hochberg procedure (165) and multivariate data analysis based on PLS for classification (PLS2C) (166) were applied. In univariate data analysis, the Mann-Whitney test with FDR was used to test the differences between the data distribution in the two groups and the volcano plot to summarise the results. PLS for classification was applied using stability selection based on variable influence on projection to highlight the relevant features of the model (167). Specifically, the number of score components of the PLS2C model was determined on the basis of the first maximum of the Matthews's correlation coefficient (MCC) calculated by repeated N-fold full cross-validation (MCCcv) under the condition to pass the randomization test on the class response. In stability selection, 200 subsets were extracted considering a probability of 0.7 and 0.5 for the observations and the variables, respectively, for the sampling procedure within the BMS strategy.

In the second step of data analysis, the time evolution of the urinary metabolome was studied considering the data available at T0, after 14 days (T1) and after 28 days (T2) for the subjects developing NEC and the matched controls. Specifically, the features resulted to be significantly different at T0 between the NEC group and the control group were investigated applying Linear Mixed Effects modelling (LME) for longitudinal data (168) controlling the false discovery rate by Benjamini-Hochberg procedure, and PLS for designed experiments. In LME modelling, the fixed effects were "time" and "group" of the subject (with the two levels NEC and control). A random effect was considered both for the coefficient of time and for the subject. In PLS analysis, the effect of the subject was removed from the data using the random effect estimated by LME and the remaining data matrix was submitted to PLS using as responses the fixed factors "time" and "group". More details about the method can be found in Peila et al. (169).

Data analysis was performed by SPSS and R-functions developed in-house using the platform R 4.0.4 (R Foundation for Statistical Computing).

2.9 Institutional Review Board Approval

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).

3. Results

3.1 Clinical characteristics of cases and controls

During the study period, 35 cases of NEC (8 NEC Bell's stage I, 13 NEC Bell's stage II, and 14 NEC Bell's stage III), 5 cases of SIP, and 113 controls were recruited. Fourteen preterm infants who were initially misdiagnosed as NEC but later revealed to have other diseases in differential diagnosis with it (i.e. septic paralytic ileus, isolated rectal bleeding) were recruited as well.

Among the 113 controls, 31 subjects with similar perinatal characteristics to cases were matched for the purpose of the analysis.

The 40 NEC/SIP patients and the 31 controls differed for gestational age at birth (higher in the controls, p=0.02), mode of delivery (more caesarean sections in the controls, p=0.02), and for days to reach FEF (as expected, more days in the NEC/SIP group, p<0.001).

For the 31 matched controls, a total of 30, 19, and 12 urine samples were collected at T0, T14 and T28, respectively.

For the 40 cases of NEC and SIP, a total of 24, 21 and 15 urine samples were collected at T0, T14, T28, respectively. At disease onset, a total of 18 urine samples were collected.

Figure 6 depicts the diagram of the study with the groups of 40 NEC/SIP cases and 31 selected controls with the relative collected samples.

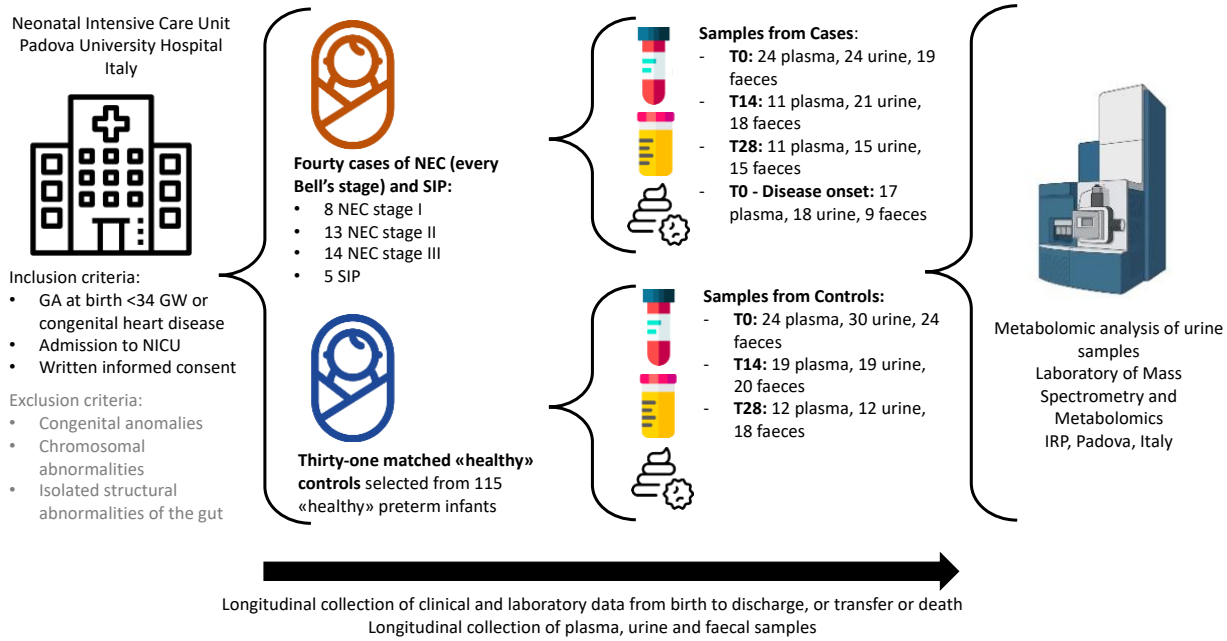


Figure 6. Diagram of the study with the groups of NEC/SIP cases and healthy controls and the relative collected samples. Designed by Freepik and BioRender.com.

Table 6 summarizes the main clinical and demographic characteristics of the whole population and of the two groups of cases and controls with their comparison.

Table 6. Clinical and demographic characteristics of the whole population and of the groups of NEC/SIP cases and healthy controls. Data are expressed as median (IQR with first and third quartiles) and categorical data as count (%). P is the p-value of the Mann-Whitney test or of the Fisher test in the comparison between NEC/SIP cases and healthy controls.

	Total (n=153)	**NEC/SIP Cases** (n=40)	**Healthy Controls** (n=31)	**p-value**
Sex n (%)				0.62
Male	90 (58.8%)	27 (67.5%)	19 (61.3%)	
Female	63 (41.2%)	13 (32.5%)	12 (38.7%)	
GA at birth (weeks)				
Median (IQR)	29 (26-31)	26 (24.7-29.2)	29 (26-30)	/
GA at birth (days)				
Median (IQR)	205 (186-221)	186 (175.5-205.5)	204 (190.5-211)	**0.02**
Birth weight (grams)				
Median (IQR)	1150 (820-1520)	835 (747.5-1200)	1040 (840-1265)	0.15
Percentile of weight at birth (%ile)				
Median (IQR)	41 (12-65)	43 (10-66.5)	32 (11-61)	0.44
IUGR n (%)	35 (22.9%)	11 (27.5%)	6 (19.3%)	0.58
SGA n (%)	35 (22.9%)	11 (27.5%)	8 (25.8%)	1.00
Apgar at 5 minutes				
Median (IQR)	8 (7-8)	7 (6-8)	8 (7-8)	0.30
Ethnicity n (%)				0.73
Caucasic	133 (86.9%)	37 (92.5%)	27 (87.1%)	
African	13 (8.5%)	2 (5%)	3 (9.7%)	
Asiatic	7 (4.6%)	1 (2.5%)	1 (3.2%)	
Mode of delivery n (%)				**0.02**
Caesarean section	133 (86.9%)	33 (82.5%)	31 (100%)	
Vaginal delivery	20 (13.1%)	7 (17.5%)	0 (0%)	
Multiple birth n (%)				1.00
Yes	60 (39.2%)	12 (30%)	10 (32.3%)	
No	93 (60.8%)	28 (70%)	21 (67.7%)	
PROM >18 hours n (%)	26 (17%)	5 (12.5%)	7 (22.6%)	0.34
Antenatal flow abnormalities n (%)	24 (17%)	6 (15%)	3 (9.7%)	0.72
Prenatal steroids n (%)				0.64
Complete	110 (71.9%)	25 (62.5%)	24 (77.4%)	
Incomplete	22 (14.4%)	6 (15%)	3 (9.7%)	
None	21 (13.7%)	6 (15%)*	4 (12.9%)	
Treatment with surfactant n (%)	90 (58.8%)	29 (72.5%)	24 (77.4%)	0.79
EOS n (%)				0.67
No	121 (79.1%)	31 (77.5%)	25 (80.6%)	
Suspected	29 (18.9%)	8 (20%)	6 (19.4%)	
Definitive	3 (2%)	1 (2.5%)	0 (0%)	
Total days of antibiotics for EOS				
Median (IQR)	5 (3-7)	5 (3-7.2)	5 (3-5.5)	0.11
HsPDA n (%)	49 (32%)	20 (50%)**	16 (51.6%)	1.00

Pharmacological treatment for PDA n (%) Paracetamol Ibuprofen Both	15 (9.8%) 20 (13.1%) 13 (8.5%)	6 (15%) 6 (15%) 7 (17.5%)	3 (9.7%) 9 (29%) 4 (12.9%)	0.51
Surgical ligation after pharmacological treatment	8 (5.2%)	5 (12.5%)	2 (6.5%)	0.46
Congenital heart disease n (%)	7 (4.6%)	3 (7.5%)	0 (0%)	0.25
PGE infusion for CHD n (%)	4 (2.6%)	1 (2.5%)	0 (0%)	1.00
Postnatal systemic steroids n (%)	21 (13.7%)	7 (17.5%)	4 (12.9%)	0.75
Human milk at start of feeding n (%)	97 (63.4%)	29 (72.5%)	22 (71%)	1.00
Total days to reach FEF Median (IQR)	16 (9-32.5)	41 (28-67)	18 (13-23.5)	**<0.001**

*Missing data of 3 cases.

**One patient not treated for HsPDA due to persistent pulmonary hypertension on echocardiography.

Abbreviations: CHD=congenital heart disease; EOS=early onset sepsis; FEF=full enteral feeding; GA=gestational age; HsPDA=haemodynamically significant patent ductus arteriosus; IQR=interquartile range; IUGR=intrauterine growth restriction; PDA=patent ductus arteriosus; PGE=prostaglandin E_2; PROM=premature rupture of membranes; SGA=small for gestational age;

3.2 Clinical and laboratory characteristics of NEC Bell's stage ≥II and SIP

Among the 40 NEC/SIP patients, 27 subjects were affected by NEC Bell's stage ≥II. Of these, 11 were medical NEC (Bell's stage IIA) and 16 surgical NEC (8 Bell's stage IIIB, 6 Bell's stage IIIA, one Bell's stage IIB and one Bell's stage IIA found to have atresia of the last ileal loop).

Five cases were affected by SIP. Due to the small size of this group, a comparison between NEC Bell's stage ≥II cases and SIP cases was not performed.

Considering only definite NEC (Bell's stage ≥II), 10 neonates (31.3%) presented with a fulminant course and required surgery or died within 48 hours from onset of symptoms.

One patient with SIP developed a NEC IIIB after recanalization.

All the cases were treated with fasting and triple antibiotic therapy (usually vancomicin or ampicillin, ceftazidime or gentamicin and metronidazole).

Twenty-one patients required surgery for NEC or SIP and were treated with PD alone, ExLap or PD followed by ExLap in 21.1%, 52.6% and 33.3% of cases, respectively.

Of the 17 patients for whom it was possible to evaluate the viability of intestinal loops (because of surgery or of autoptic findings; 14 NEC cases and 3 SIP cases), the majority had an ileal involvement (64.9%), followed by a total involvement (pan-NEC, 29.4%) and by colic involvement (5.9%).

Six and eight neonates had positive blood culture or culture of peritoneal fluid, respectively, with pathogens belonging to Firmicutes (*Clostridium spp.* and Bacilli like *St. aureus and hominis, Str. Haemolyticus and Enterococcus faecalis*), and Gamma-proteobacteria (*Enterobacter and Klebsiella spp*.).

Six neonates belonging to the NEC Bell's stage ≥II patients died.

Table 7 summarizes the main clinical and laboratory characteristics of the 32 neonates affected by NEC Bell's stage ≥II or SIP around disease onset.

Table 7. Main clinical and laboratory characteristics of the 32 neonates affected by NEC Bell's stage ≥II or SIP around disease onset (within 48 hours before and after onset), regarding the management and complications.

	NEC ≥ II/SIP Cases (n=32)	**NEC ≥ II** (n=27)	**SIP** (n=5)
Bell's stage of NEC n (%)			
IIA	12 (37.5%)	12 (37.5%)	
IIB	1 (3.1%)	1 (3.1%)	
IIIA	6 (18.8%)	6 (18.8%)	
IIIB	8 (25%)	8 (25%)	
DOL at disease onset Median (IQR)	8.5 (6-21.5)	10 (7-23.5)	4 (4-5)
Fulminant onset (less than 48 h between onset and surgery/death) n (%)	10 (31.3%)	10 (37%)	/
Feeding with only human milk at onset n (%)	11 (34.4%)	8 (29.6%)	3 (60%)
Total enteral amount at onset (ml/kg/day) Median (IQR)	127.5 (65-158,7)	140 (89.2-160)	28.5 (14.2-42.7)
Transfusion of RBC within 48 hours before onset n (%)	9 (28.1%)	7 (25.9%)	2 (40%)
Days of NPO for the disease* Median (IQR)	14.5 (10-20.5)	16 (11-21)	15 (7-10)
Days of ATB for the disease Median (IQR)	14 (10-16.3)	15 (11.5-18.5)	13 (10-14)
Haematocrit (%) Median (IQR)	33 (28.9-35.1)	31.9 (27.3-35)	34.1 (33-36.1)
Lowest WBC (/mmc) Median (IQR)	6645 (4530-10085)	6510 (4140-12620)	6820 (6250-6970)
Lowest neutrophil count (/mmc) Median (IQR)	3390 (2320-7980)	2970 (1690-7740)	5680 (4715-32725)
Lowest platelet count (/mmc) Median (IQR)	140.500 (44.500-267.750)	153000 (58000-307000)	66000 (34000-172000)
Highest CRP (mg/L) Median (IQR)	41 (8.2-79.5)	40 (8.6-80)	46.9 (8.8-52)
Lowest albumin (g/L) Median (IQR)	26 (22-31)	27.5 (22-32.5)	23 (22-27)
CRP/Albumin ratio Median (IQR)	1 (0-3)	1 (0-3.75)	2 (0-2)
Surgery for NEC/SIP n (%)	21 (65.6%)	16 (59.3%)	5 (100%)
Type of surgery for NEC n (% of those with surgery)			
PD	4 (21.1%)	2** (12.5%)	2 (20%)
ExLap	10 (52.6%)	9 (56.3%)	1 (10%)
PD followed by ExLap	7 (33.3%)	5 (31.2%)	2 (20%)

Intestinal involvement at ExLap or autopsy n (% of those explored) Ileal Colic Pan-NEC	 11 (64.7%) 1 (5.9%) 5 (29.4%)	 9 (60%) 1 (6.7%) 5 (33.3)	 2 (40%)
Haemoculture positive n (%)	6 (18.8%) *1 St. aureus, 1 Klebsiella pneumonia ESBL, 1 Ent. Cloacae, 1 Candida albicans, 1 Str. Haemolyticus, 1 E. coli*	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 n (%)	8 (25%) *1 Klebsiella pneumoniae, 1 Cl. Perfrigens and St. aureus, 3 Ent. Faecalis, 1 Candida parapsilosis, 1 St. hominis, 1 Cl. Perfrigens*	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 n (%)	6 (18.8%)	5 (18.5%)	1 (20%)
Death n (%)	6 (18.8%)	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.

3.3 Analysis of the clinical data

For the purpose of statistical analysis, only the patients with analysable urine samples were considered. These included a NEC group of 20 cases and a Control group of 17 neonates matched for main perinatal and neonatal characteristics. The NEC Group comprised 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. Patients with congenital heart diseases developing NEC were not considered for the purpose of the analysis as only three patients were enrolled in the original sample size.

Clinical data of these two groups are reported in **Table 8** and show no significant differences of the considered variables.

Moreover, 3 subjects with SIP and 7 subjects with other diseases for whom urine samples were analysable were considered and were found to have similar GA and BW to the control group.

Table 8. Clinical data of the two groups considered for the analysis. Data with a normal distribution are reported as mean (SD), data with a non-normal distribution as median [IQR] and categorical data as count.

Clinical data	NEC (N=20)	Controls (N=17)	p
Gestational age	187.2 (18)	194.9 (14)	0.16
Body weight	793 [289]	950 [250]	0.12
Sex (M/F)	15/5	9/6	0.49
Delivery mode (vaginal/caesarean)	2/18	0/17	0.49
Apgar 5 min	7 [1.5]	8 [2.3]	0.23
PPROM	3	4	0.68
IUGR	5	2	0.42
EOS	7	4	0.56

HSPDA	11	12	0.33
AED	5	1	0.19
Prenatal steroids (no/incomplete/complete)	2/5/13	2/2/13	0.59
Surfactant	16	14	1.00
Outborn/inborn	1/19	2/15	0.58

3.4 Metabolomic analysis

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.

3.4.1 Data analysis at T0

The urine sample collected at T0 for each control was considered to build the control charts used in our preliminary data analysis and the comparison between subjects developing NEC and controls.

3.4.1.1 Control charts

The control group composed of 17 observations was considered the reference in the control charts. In Model Population Analysis (MPA), 500 subsets were generated sampling the control group with a probability of 0.8 and 0.5 for the observations and the variables, respectively. The significance level and the level of FDR for the KNN and the BB-based control charts were set equal to 0.05. Considering T0 urine sample available within the first 2 days of life, 4 out of 20 subjects developing NEC were wrongly predicted to be controls, 2 out of 7 subjects with other pathologies were wrongly classified while all the subject developing SIP were correctly predicted as no controls.
The predictions of the control charts are reported in **Figure 7.** These results allowed us to conclude that the urinary metabolome is closely associated to the diseases under investigation with respect to the controls.

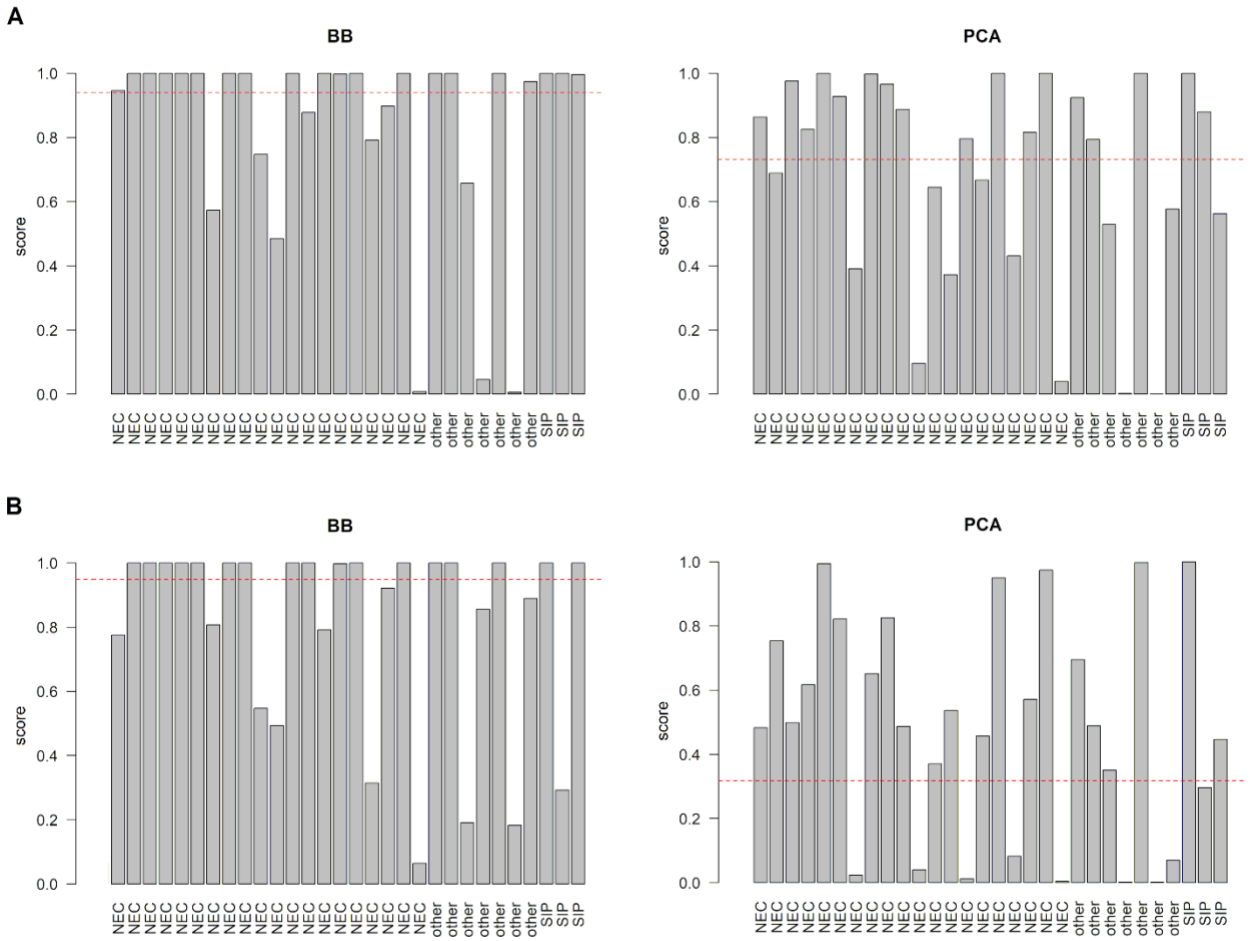


Figure 7. Predictions of the control charts obtained from the NEG (panel A) and the POS (panel B) datasets. The group of the sample are reported as labels. Samples with score greater than the threshold (dashed red line) are correctly predicted as not belonging to the control group.

3.4.1.2 NEC group vs control group

The urinary metabolome of the NEC Group and the Control Group was compared considering the first urine sample collected within the first 2 days of life.

For the NEG dataset, 8 features were discovered to be significant by Mann-Whitney test controlling FDR at level 0.05. The volcano plot of **Figure 8A** summarizes the results. The PLS2C model showed 3 score components and Matthews's correlation coefficient cross-validation MCCcv=0.782 (p=0.008). The MCC calculated with the out-of-bag predictions during stability selection (MCCoob) was 0.664. Assuming a significant level of 0.05, 111 features resulted to be relevant in distinguishing the two groups. The relevant score calculated for the dataset are reported in **Figure 8B.**

For the POS dataset, the analysis based on Mann-Whitney test controlling FDR at level 0.05 discovered 16 features as significant. The results are summarized in the volcano plot of **Figure 8C.** The PLS2C model showed 3 score components, MCCcv=0.623 (p=0.020) and MCCoob=0.596. Assuming a significant level of 0.05, 66 features resulted to be relevant in distinguishing the two groups. In **Figure 8D** are reported the relevance scores for the dataset.

Merging the results of univariate and multivariate data analysis, 111 and 67 relevant features were discovered for the NEG and the POS dataset, respectively.

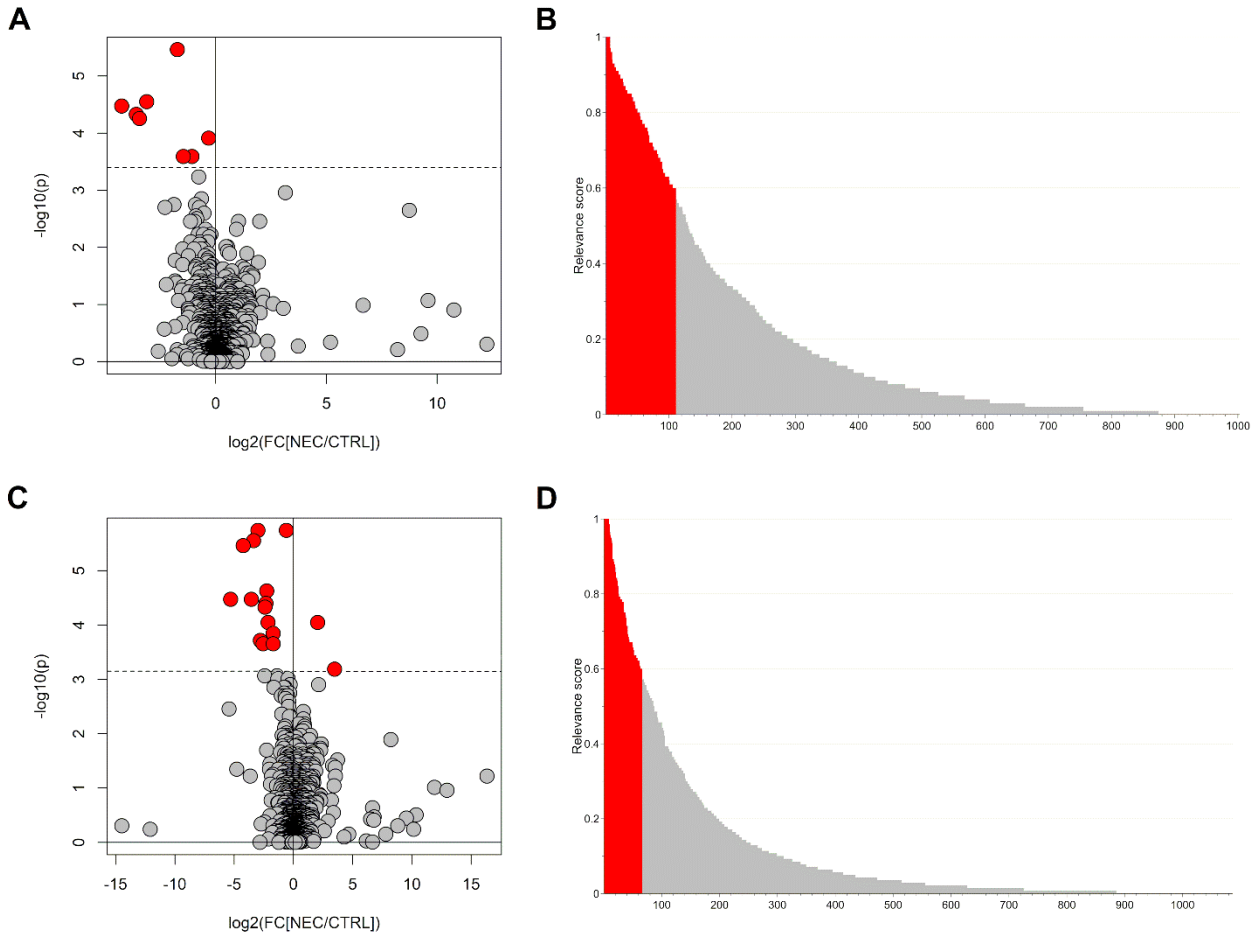


Figure 8. Metabolomics analysis at T0: volcano plot (panel A) and relevant score plot (panel B) obtained for the NEG dataset, and volcano plot (panel C) and relevant score plot (panel D) obtained for the POS dataset. The features discovered as relevant are colored in red. In the volcano plot, p is the 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.

3.4.2 Time evolution in the first 28 days of life

Eighteen out of 20 subjects of the NEC group and 14 out of 17 controls had their urine samples collected at all the three time points of the experimental design (T0, T1 corresponding to 14 days and T2 corresponding to 28 days of life). Controlling the false discovery rate at level 0.05, the analysis based on Linear Mixed Effects modelling (LME) for longitudinal data of the features discovered to be relevant at T0 highlighted 33 variables for the NEG dataset and 44 variables for the POS dataset as relevant in distinguishing the two groups within the first 28 days of life. The results are summarized in the plots of **Figure 9**.

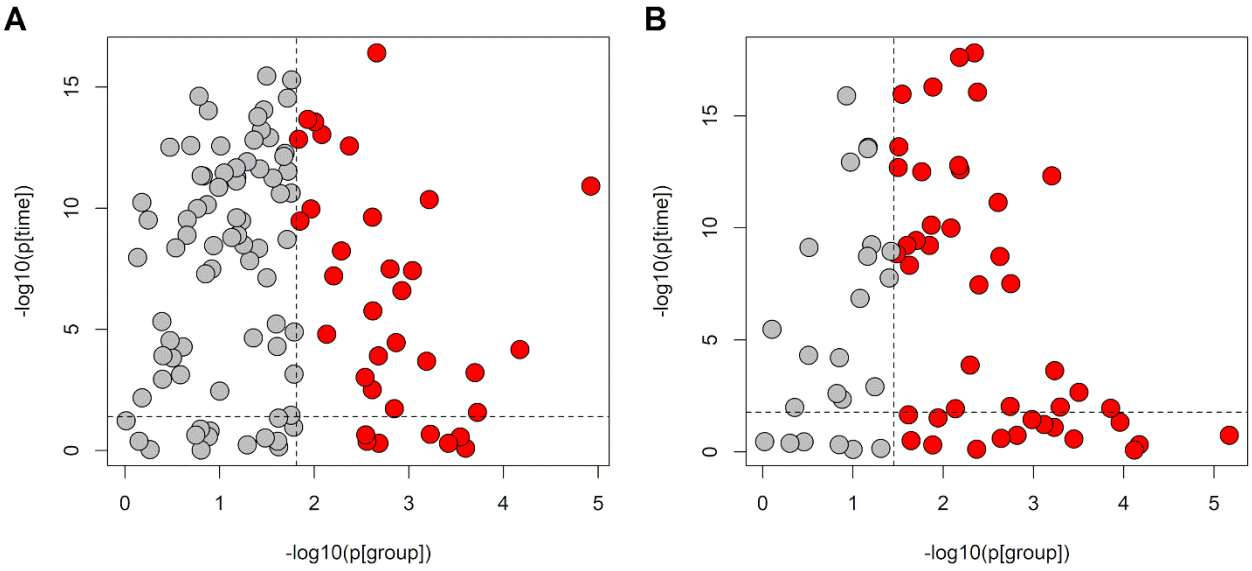


Figure 9. LME models for longitudinal data: NEG dataset (panel A) and POS dataset (panel B); p[time] and p[group] are the p-values of the fixed effects "time" and "group", respectively. Features significantly relevant to distinguish NEC and control groups are colored in red. The dashed black lines indicate the thresholds used to control the false discovery rate.

Applying PLS for designed experiments, a model with 3 score components, R2Y=0.71 ($p<0.01$) and Q2Y=0.57 ($p<0.01$), and a model with 4 score components, R2Y=0.79 ($p<0.01$) and Q2Y=0.63 ($p<0.01$) were obtained for the NEG and the POS dataset, respectively. The score scatter plots of the post-transformed models are reported in **Figure 10**. As it can be observed, samples clusterise according to the group at all time points along tp[2], while the time increases from left to right along tp[1]. The models proved that part of the differences observed at T0 at the urinary metabolic level are maintained until 28 days.

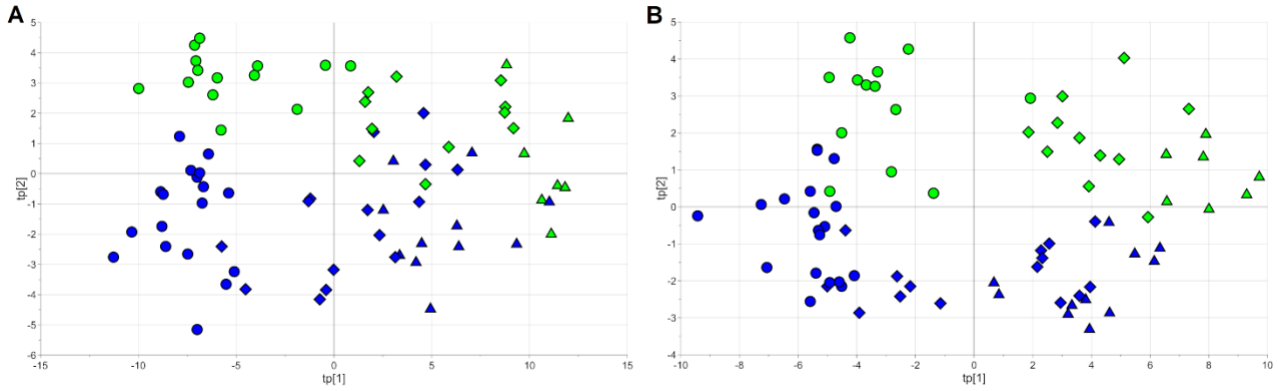


Figure 10. PLS for designed experiments: score scatter plots of the models obtained with the NEG (panel A) and the POS (panel B) datasets. Samples of the NEC group and those of the controls are colored in blue and in green, respectively. Circles are used for samples at T0, diamonds for samples at T1 and triangles for samples at T2.

The features discovered to be able to distinguish the NEC Group from the Control Group at all time points were submitted to annotation. Specifically, 12 variables were annotated at level 3 in HMDB. **Table 9** reports the annotated variables, where a FC[NEC/CTRL] >1 indicates a variable which is significantly higher in NEC patients.

Table 9. Annotated variables: ID is the time_mass identifier of the variable, ionization mode specifies the type of ionization, HMDB ID is the identifier of the compound in the Human Metabolome Database, annotation is the name of

the compound, HMDB class is the chemical class of the compound and FC[NEC/CTRL] is the fold change at T0 calculated as ratio between the median in the NEC group and the median in the control group.

ID	ionization mode	HMDB ID	Annotation	HMDB class	FC[NEC/CTRL]
2.81_319.1369m/z	ESI +	HMDB00227	Mevalonic acid	hydroxy fatty acids	0.125
0.84_264.0798n	ESI +	HMDB02381	N-Acetylcystathionine	n-acyl-alpha amino acids	0.227
2.63_319.1369m/z	ESI +	HMDB00227	Mevalonic acid isomers	hydroxy fatty acids	0.306
0.97_264.0797n	ESI +	HMDB02381	N-Acetylcystathionine isomers	n-acyl-alpha amino acids	0.381
0.62_470.1510m/z	ESI -	HMDB02061	Hyaluronic acid	glycosaminoglycan	0.474
4.68_364.2258n	ESI +	HMDB06760	11beta,17alpha,21-Trihydroxypreg-nenolone	C21-Steroid hormones	0.491
0.61_363.0722m/z	ESI +	HMDB02655	Isorhamnetin	flavonoids metabolites	0.501
2.84_267.0958m/z	ESI -	HMDB01563	1-Methylguanosine	purine nucleosides	0.691
2.84_228.1103n	ESI -	HMDB06695	Prolylhydroxyproline	dipeptide	0.767
1.00_189.0399m/z	ESI -	HMDB00393	3-Hexenedioic acid	dicarboxylic acids	0.813
1.06_258.0858n	ESI +	HMDB04813	3-Methyluridine	modified nucleoside	0.829
0.63_175.0243m/z	ESI -	HMDB02545	Galacturonic acid	organic acids	0.854

3.4.3 Surgical vs medical NEC at T0

In the NEC Group, 7 out of the 20 subjects with urine samples collected at T0 were surgically treated while 13 subjects were medically treated. To avoid bias due to differences in the clinical data at birth, a subset of 7 subjects medically treated was selected to match the subset of subjects surgically treated. **Table 10** reports the clinical data at birth of the two subsets. Assuming a significance level of 0.05, no differences were observed.

Table 10. Clinical data at birth of the two subsets of subjects developing NEC with different severity: data with a normal distribution are reported as mean (SD), data with a non-normal distribution as median [IQR] and categorical data as count.

Clinical data	Surgical NEC (N=7)	Medical NEC (N=7)	p
Gestational age	185 [21]	187 [21]	0.85
Body weight	675 [355]	1000 [380]	0.22
Sex (M/F)	5/2	7/0	0.46
Delivery mode (vaginal/caesarean)	0/7	1/6	1.00
Apgar 5 min	7.0 [1.5]	7.0 [1.5]	0.70
PPROM	1	1	1.00
IUGR	2	1	1.00
EOS	1	2	1.00
HSPDA	4	5	1.00
AED	1	1	1.00
Prenatal steroids (no/incomplete/complete)	1/2/4	0/2/5	0.57
Surfactant	6	5	1.00
Outborn/inborn	0/7	0/7	1.00

Given the small number of subjects in each group, the following data analysis must be considered exploratory and further studies with a larger number of subjects are requested to confirm the results. Moreover, to limit redundancy in the data and spurious results, we focused the analysis only

on the behavior of the annotated features. Specifically, 89 features were annotated at level 1, i.e. they were identified and used to characterize the two groups of subjects.
Univariate data analysis discovered 2 metabolites with p-value of Mann-Whitney test less than 0.10. A PLS2C model showing 1 score component, MCCcv=0.577 (p=0.036) and MCCoob=0.500 was built. Performing stability selection with a significant level of 0.05, 6 features resulted to be relevant in distinguishing the two groups. The results are summarized in **Table 11** and the profiles of the most interesting metabolites are reported in the boxplots of **Figure 11**.

Table 11. Relevant metabolites in the comparison of medical and 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 and 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

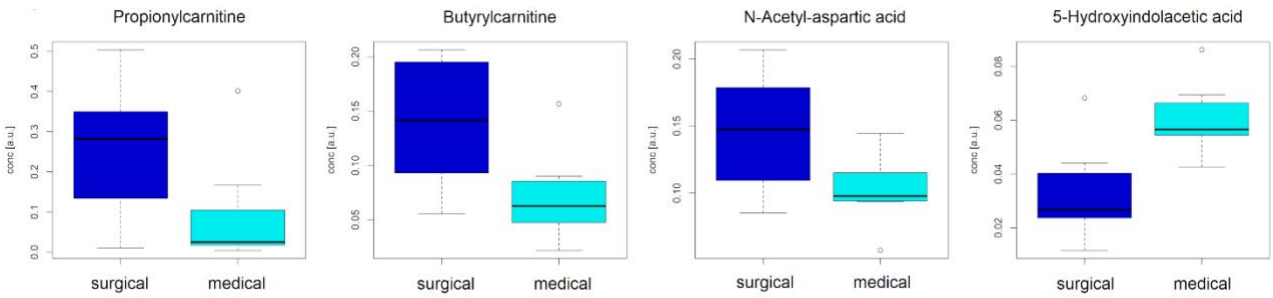


Figure 11. Boxplots representing the distributions of the most significant metabolites discovered in the comparison of surgical and medical NEC at T0.

4. Discussion

NEC remains one of the most common and yet inexplicable causes of death in preterm infants (156), therefore the search for its non-invasive prognostic and diagnostic biomarkers is one of the most intriguing topics in neonatology and has been summarized in several systematic reviews so far (103,110,144).
The multifactorial pathogenesis of this complex condition makes it challenging to unravel a single potential biomarker of the disease. The diagnosis of NEC still remains predominantly clinical, with few laboratory and radiological findings as surrogates to identify the severity. 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 (170). In fact, once the clinical and radiological picture are clearly evident, it may be too late to prevent disease progression and associated catastrophic outcomes.
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 (131).
Metabolomics can be virtually applied on any type of biological specimen and assayed by 1H-Nuclear Magnetic Resonance (1H-NMR) spectroscopy or mass spectrometry (MS). In particular, urine is a suitable sample option for a non-invasive collection in preterm neonates.
Several reviews in the last 10 years have summarized studies using metabolomics for the detection of NEC biomarkers (94,143,171), revealing a wide variability in populations' inclusion criteria (often without a clear definition of NEC, or comprising either suspected or definitive NEC or both), in timing of samples' collection (encompassing early samples or samples close to NEC onset), and type of analysed biological fluids (plasma, urine, or stools).

In our study, we applied the MPA modelling to find prediction models of diseases in respect to a healthy state. Model of Population Analysis (MPA) is a general framework for developing a new type of algorithm for modelling which uses statistical tools to extract important information from the model. The concept has attracted increasing interest in the chemometrics community in recent years. In fact, MPA has proven to be a powerful tool for modelling as it provides a better understanding of the chemical data and improves prediction and interpretation of the model. The core idea of MPA is to statistically analyse the performance of numerous sub-models generated from random sampling and to extract interesting information from outputs of the sub-models, thus permitting variable selection, outlier detection, and model comparison (163,172). The MPA framework generally contains several steps, which comprise generating sub-datasets, building sub-models on the basis of the datasets, obtaining outputs of interests, and statistical analysis. MPA-based methods can be developed changing one or more elements of the MPA framework, such as different random sampling techniques, different outputs of interest (prediction error, accuracy rate), or different statistical analysis. Major advantages of this tool are the capacity of facing large forms of data, of handling them even when there is an unbalanced classification, and the avoidance of models of the reference data describing a too large space with the risk of increasing the rate of false belonging to the reference group (163,172). 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 gastrointestinal diseases in respect to controls with a good prediction rate (80%, 100% and 71.4%, respectively).
When univariate and multivariate analysis were applied, both these techniques were able to generate good prediction models of NEC vs controls based on relevant metabolic features from the early urine samples. Indeed, the Matthews's correlation coefficient calculated with cross-validation (MCCcv) and with the out-of-bag predictions during stability selection (MCCoob) were >0.59 for models obtained from both positive and negative datasets. Out-of-bag (OOB) evaluation is a method of measuring the prediction error or the prediction score of random forests, boosted decision trees, and other machine learning models utilizing bootstrap aggregating (bagging). It is used to assess the quality of a model. Compared to cross validation, OOB evaluation uses a sample of remaining data that was not necessarily used during the model's analysis, thus considering a more random sample than the validation set of cross validation. Therefore, MCCoob score represents more accurately a good or worse prediction of the given model (173,174).
From univariate and multivariate statistical analyses, 178 significant metabolic variables emerged to be relevant at T0 in separating the NEC Group vs the Control Group. Of these, 77 were identified by Linear Mixed Effects modelling (LME) which remained differently expressed in the two groups throughout the first 28 DOL. The fact that part of the differences observed at birth at the urinary metabolic level are maintained until 28 days was confirmed also by the PLS models. Specifically, by

searching in HMDB, 12 variables were annotated at level 3, meaning that, considering the mass of the metabolite, these variables are very close to the ones identified, but further analysis with mass spectral fragmentation should be performed in order to have a more robust hypothesis of the metabolite (175). Interestingly, two metabolites had a FC[NEC/CTRL] < 0.3, indicating that they were significantly lower in NEC cases compared to controls. These were potentially identified as Mevalonic Acid (FC[NEC/CTRL 0.12) and N-Acetyl cystathionine (FC[NEC/CTRL 0.22) and their isomers.

Mevalonic acid (MVA) is a hydroxy fatty acid produced by the so called 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) pathway (or mevalonate pathway). This is an anabolic route providing metabolites for multiple cellular processes in eukaryotes, bacteria, as well as humans. MVA derived by this pathway is further involved in sterol isoprenoids production, such as biosynthesis of cholesterol (which is a precursor of bile acids, lipoproteins, steroid hormones), as well as multiple non-sterol isoprenoids (i.e. dolichol, ubiquinone, vitamin K, isopentenyl t-RNA, heme A, and farnesyl and geranylgeranyl lipid anchors)(176). Apart from being a metabolic target in both hypercholesterolemia and colorectal cancer therapy, this pathway has been hypothesized to be involved in energy input and cell proliferation (177). However, mevalonate kinase deficiency (MKD) (mevalonate aciduria) may cause hyperinflammation and autoinflammatory diseases, like Hyperimmunoglobulinemia type D, which can also present with severe early-onset colitis (178) or very early onset inflammatory bowel diseases (179). Given the scarcity of evidences, a reduction of this compound in the urine of NEC cases is difficult to interpret.

N-Acetyl cystathionine may be a byproduct of cystathionine which, instead, derives from homocysteine and is involved in the direct and reverse transulfuration pathway, where it is the precursor of cysteine and other amino acids. Cysteine is, in turn, the precursor of glutathione, the major intracellular antioxidant protecting against free radical-mediated damage. One study has shown that plasma cystathionine concentrations are higher in premature infants ≥ 32 GW than in premature infants of 33-36 GW or in full-term infants, whereas plasma cysteine concentrations are much lower in the two groups of premature infants than in mature infants, possibly indicating a cystathionase deficiency in the former (180). Additionally, it has been suggested that L-cysteine could be a conditionally essential (i.e., essential under certain conditions) amino acid for very preterm but not for borderline preterm or full-term neonates, as hepatic cystathionase activity increases with increasing gestational age and after birth (181). In our study, a lower level of N-Acetyl cystathionine in the urine of NEC patients may potentially signify a downregulated pathway in these subjects, in comparison to healthy controls, who may have higher stocks of anti-oxidant compounds like glutathione, instead. This may confirm previous findings in rats and neonates, that have hypothesize a relative deficiency of antioxidant compounds with the glutathione detoxifying system being important in maintaining intestinal barrier integrity by protecting against nitrosative stress (182,183). What is more, administration of intraperitoneal N-acetyl cysteine (NAC) in an experimental rat model of NEC seemed to significantly decrease the severity of bowel damage at histopathologic and apoptosis evaluations (184).

As discussed by previous authors (138), the number of metabolomics-based studies on NEC is small and they present a great heterogeneity of enrolled patients, sample sizes, diagnostic criteria of NEC, analytical methods and collected biofluids. This hampers an adequate comparison between our results and those previously published (94).

Morrow et al. (137) were able to classify two subtypes of NEC based on faecal microbiota ordination of samples collected between 4-9 DOL (T1), the first dominated by *Firmicutes* and the second dominated by *Proteobacteria*. In the metabolomic analysis by NMR spectroscopy on urine samples, alanine was positively associated with the first group of NEC cases preceded by *Firmicutes* dysbiosis,

whereas histidine was inversely associated with NEC cases preceded by *Proteobacteria* dysbiosis. Cases were also characterised by a urinary alanine/histidine ratio >4 more frequently than controls. Picaud et al. (138) also applied NMR spectroscopy on urine collected before, during and after diagnosis of NEC Bell's stage II (6 patients). They demonstrated that N,N-dimethylglycine (N,N-DMG) correlated in controls with good digestive tolerance, whereas lactate, betaine, myo-inositol, urea, creatinine, and N,N-dimethylglycine discriminated late-onset NEC (> 3 weeks of life) from controls with good feed tolerance.
Finally, Thoimadou et al. (139) applied untargeted NMR spectroscopy and targeted LC-MS on urine samples collected after initial evaluation for NEC in 15 cases (every stage) and at similar postnatal age for 15 controls. They found 25 discriminant metabolites belonging to amino acids, organic acids, sugars and vitamins, with NEC cases characterised by lower levels of Tyrosine, Proline, Citrate, 4-hydroxybenzoate, Formate, Succinate, 4-hydroxyphenylacetate, Fumarate, Creatinine, Myoinositol, hippuric acid.

A further interesting result of our study is the discrimination of surgical NEC vs medical NEC from the urine collected within the first 2 days of life. In particular, 6 variables resulted to be relevant in distinguishing the two groups, with N-Acetylaspartic acid, Butyrylcarnitine and Propionylcarnitine being significantly increased in neonates requiring surgery. 5-Hydroxyindolacetic acid, instead, characterised neonates who were treated medically, i.e. who had a lighter form of the disease. N-Acetylaspartic acid (NAA) is an amino acid predominantly present in brain and in lower amount in peripheral organs. Its role is still poorly understood, but an increase in its pathway has been reported in Canavan disease (a childhood leukodystrophy), Parkinson's disease and type-2 diabetes (185). Furthermore, recent studies suggest that upregulation of NAA leads to oxidative stress including upregulation of nitric oxide and reducing potential antioxidants in rats (186,187).
Butyrylcarnitine and Propionylcarnitine are acylcarnitines, i.e. esters of L-carnitine, which increase the intracellular pool of L-carnitine. Interestingly, Propionylcarnitine is supposed to exert an anti-oxidant mechanism with a dose dependent scavenging activity (188,189).
All in all, these findings, both in NEC cases and in particular in those with a severe disease requiring surgery, highlight the potential role of oxidative stress and impaired anti-oxidant mechanisms in this population.

Our study is unique for certain analyses that have been performed, compared to previous researches. The major strength of this study is the longitudinal collection of 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. Another positive consideration is the application of a robust statistical analysis able to perform a powerful validation of the obtained models. Another important strength is the inclusion of preterm infants affected not only by NEC, but also by SIP and other gastrointestinal diseases in differential diagnosis with NEC, that have being compared to healthy subjects similar for perinatal characteristics. Finally, our study also comprised the analysis for the prediction of NEC severity, identifying potential biomarkers of surgical NEC.
As regards limitations, first of all the small number of patients with NEC Bell's stage ≥II did not permit to exclude cases of suspected NEC (Bell's stage I) from the statistical analysis. Additionally, there were not enough patients with SIP or with CHD to explore the differences between classical NEC, SIP and cardiogenic NEC. Despite this, to our knowledge our cohort is the largest enrolled for a metabolomic analysis study on NEC ((94).

Finally, we have not yet confirmed our results with a targeted approach on an independent validation cohort, as previously done by another study (140).

The findings of this study are promising and pave the way for future research. Next interesting projects would be to apply untargeted metabolomic analysis on plasma samples collected from the same cohort of subjects, to proceed with the spectral fragmentation analysis to better identify in the HMDB the metabolomic variables characterising patients with NEC vs healthy subjects, and to further validate our results on an independent cohort.

5. Conclusions

This pilot explorative study demonstrates that MS metabolomics applied on non-invasively collected urine samples from birth can discriminate preterm neonates who develop NEC, SIP or other gastrointestinal conditions from healthy controls. Additionally, metabolic differences between NEC cases and controls at birth persist during the first month of life. Finally, urinary metabolome is able to differentiate patients who will require surgery from those who a medical treatment is sufficient for. In our study, potential predictive biomarkers of NEC development and NEC severity mainly play a role in pathways of oxidative stress, with N-Acetyl-aspartic acid, Butyrylcarnitine and Propionylcarnitine being potential markers of disease severity. These results need to be validated in future studies on independent cohorts and using targeted analysis.

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