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ORIGINAL ARTICLE

Use of serum citrulline concentrations from routine newborn screen as a biomarker for necrotizing enterocolitis

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Abstract

Purpose Necrotizing enterocolitis (NEC), a leading cause of mortality and morbidity in preterm neonates, lacks a reliable biomarker. Citrulline is primarily produced by enterocytes and correlates with intestinal function. Serum citrulline concentration (CIT) is routinely measured in routine newborn screening (NBS). The purpose of the study is to test if CIT from NBS may predict the occurrence of NEC and whether it correlates with the time to full feeds (TTFF) and length of stay (LOS), serving as a biomarker of NEC and intestinal health.

Methods In a retrospective case control study conducted on neonates with gestational age of 26–32 weeks, we compared CIT levels between cases (neonates with NEC) and controls (next-born neonate). NBS was collected within first 24 h, at day 5 and when the neonates achieved full feeds and were compared using non-parametric tests.

Results There was no difference in CIT between the controls and cases on day 1 [11.42 (7.42–14.84 vs. 11.93 (6.85–18.8) µmol/L, $p=0.55$], on day 5 [11.99 (7.99–16.55) vs. 13.70 (7.42–26.83) µmol/L, $p=0.05$], or at full feeds [14.86 (6.85–25.69) vs. 15.7 (7.42–26.26) µmol/L, $p=0.87$]. CIT on day 1 did not correlate with TTFF ($r=0.08$, $p=0.53$) or LOS ($r=0.23$, $p=0.06$), respectively).

Conclusions CIT from routine NBS does not serve as a biomarker to predict NEC in preterm neonates.

Keywords Citrulline · Newborn screen · Tandem mass spectroscopy · Necrotizing enterocolitis · Biomarker

Introduction

Necrotizing enterocolitis (NEC) is one of the leading causes of morbidity and mortality in preterm neonates. With increasing survival of preterm neonates, the incidence of necrotizing enterocolitis (NEC) is also increasing [1] affecting 1–3 neonates per 1000 live births [2] with an increased median cost in the United States of $75,000 for medical NEC and $195,000 for surgical NEC [3]. The etiology of NEC is multifactorial, with a predominant risk factor being prematurity. As preterm neonates have immature gastrointestinal tracts and immune systems, infections can trigger dysbiosis, resulting in increased growth of pathogenic bacteria and an uncontrolled inflammatory host response leading to the development of NEC. There are no sensitive or specific biomarkers that can reliably predict NEC or the development of short bowel syndrome (SBS) after surgical resection of intestine. Identification of circulating measures of enterocyte function may be potential biomarkers for the prediction of NEC and may predict the tolerance of enteral feeds after treatment of NEC and with SBS [4].

Citrulline is a non-essential amino acid produced almost exclusively by the intestine as a product of enterocyte glutamine metabolism, with minimal dietary contribution [5]. Thus, serum citrulline concentrations reflect intestinal biosynthesis and are affected by changes in enterocyte function.

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Serum citrulline concentrations have been studied as a biomarker for the loss of enterocyte mass and/or function [6–8]. In adults, an association between decreased serum citrulline concentrations has been reported with post operative intestinal length among patient with short bowel syndrome (SBS) [9], residual intestinal function in patients with chronic villous atrophy [10], and among transplant recipients to differentiate between viral and rejection-associated enteritis [11]. Additionally, increases in serum citrulline concentrations over time have predicted recovery of enterocyte function, differentiating between patients with transient as compared to permanent intestinal failure [12]. In children with SBS, higher serum citrulline concentrations predict earlier enteral tolerance of feeds and shorter time on total parenteral nutrition (TPN) [13]. Serum citrulline concentrations have been reported to be lower in preterm neonates with meconium ileus, likely due to impaired intestinal development and function [14]. However, there is a difference even among preterm neonates, with those who developed NEC having lower citrulline concentrations as compared to control preterm infants [15–17]. Since the most important risk factor for NEC is prematurity where the preterm neonates have immature intestinal function, we aimed to study the utility of citrulline levels as a biomarker of enterocyte health, in routinely collected newborn screening (NBS), for early identification of preterm neonates at risk of NEC. We hypothesized that citrulline concentrations in NBS will be lower in preterm neonates who developed NEC as compared to those who do not develop NEC. We also hypothesized that citrulline concentrations from NBS in these preterm neonates would directly correlate with the time to reach full enteral feeds and the length of hospital stay.

Methods

Patients

This retrospective case control study was conducted at the neonatal intensive care unit (NICU) at Maimonides Medical Center and included neonates admitted between January 2011 through December 2015. This study was approved by the Maimonides Medical Center Institutional Review Board as well as at the Newborn Screening Center at Wadsworth Center (New York State Department of Health (NYS DOH), Albany, NY), to release the exact citrulline concentrations from the NBS. It was exempt from informed consent. We included all preterm neonates with gestational ages from 26 to 32 weeks. Neonates with major congenital abnormalities, liver [1, 2] and kidney dysfunction [18], given its contribution to citrulline levels, inborn errors of metabolism, or those necessitating exchange transfusions, were excluded from the study.

Cases included all neonates who were diagnosed with NEC using Bells criteria [19]. The controls consisted of neonates born at GA between 26 and 32 weeks who did not develop NEC during their NICU stay and were born subsequent to the cases and met inclusion criteria. We selected controls as the baby born subsequent to the case who was within the range of GA included in the study. We took this approach, rather than matching for exact GA and weight, since the latter approach would have increased the time duration for the study, which in turn would have increased the variation in the time of introduction of feeds, extent of donor human milk use, and frequency and duration of treatment by antibiotics, given modification of clinical care in keeping with evolving guidelines.

Collection of screening samples

Blood obtained by skin puncture of the heel was collected from cases and controls by saturating marked areas on a filter paper card provided by the NYS DOH Newborn Screening Program. Specimens were dried on a flat, non-absorbent surface, away from direct heat and sunlight. These were then sent to the Newborn Screening Program at Wadsworth Center Laboratory of the NYS DOH for analysis.

Timing of collection

The sample collection was based on the New York State Department of Health guidelines [20]. The first NBS sample is routinely collected within first 24 h of life, prior to starting TPN or any blood product transfusions (DOL1) and the second NBS sample was collected on the 5th day of life (DOL5). The third sample was obtained when the infant reached full enteral feeds (DOLFF), defined as intake of 140 mL/kg/day of enteral formula or fortified breast milk while being off TPN for 24 h.

Citrulline quantification in NBS, patient data processing and reporting of results

Analytes were extracted from the dried blood spot (DBS) from NBS specimens using methanol containing stable isotope amino acid and acylcarnitine internal standards. After a derivatization step, amino acid and acylcarnitine analytes (including citrulline) in the extract were quantified using tandem mass spectrometry (MS/MS) [21]. The tandem mass spectrometry allows for the simultaneous measurement of multiple metabolites. The extraction efficiency of citrulline by MS/MS is < 100%, yet it is consistent and is sufficient for detecting citrullinemia. It is a semi-quantitative analysis and can detect concentrations of citrulline of less than 0.9 mg/dL (51.4 µmol/L) with high sensitivity. The sensitivity and the accuracy of MSMS

is comparable to that of HPLC fluorescence method for detecting citrulline concentrations less than 51 µmol/L. The coefficient of variation and standard deviation are minimal for low (< 10 µmol/L), intermediate (30 µmol/L) and high (65 µmol/L) range of citrulline [22]. Since that is the upper limit of normal citrulline production in neonates (preterm or term), it was used to interpret the data for this current study. Raw data on citrulline concentrations were measured and collected by means of the mass spectrometer, but the values were not interpreted as a part of the screening program, nor reported to clinicians as a routine.

All samples received at Wadsworth Center Laboratory Newborn Screening Center, Albany, NY were matched electronically with any prior samples from the same baby in the computer database. After imprinting both the data collection form and the blood collection card with a laboratory accession number unique for that sample (Newborn Screening number), the data form was separated from the blood collection card and sent to the data entry unit; the blood spot portion of the card was retained for processing in the laboratories. Information on all specimens were entered in a computer database by the data entry unit. Normally, concentrations that are below the established cut-off values for the analytes are reported only as "screen negative". Laboratory accession numbers for the requested samples from the neonates included in the study were provided to the Newborn Screening Program and the exact citrulline values for the samples were communicated to the principal investigator at Maimonides Medical Center as milligram/dL (mg/dL). Citrulline concentration values were converted from mg/dL to µmol/L by multiplying the value by 57.1 to compare with the studies that have reported the values of citrulline in µmol/L.

Sample size calculation

The sample size was calculated a priori based on two studies [16, 17]. Ioannou et al. [16] reported a mean difference in citrulline concentration on day 7 among neonates who developed NEC (16.8 ± 4.2 µmol/L) as compared to those who did not develop NEC (20.5 ± 4.5 µmol/L) while Celik et al. [17] reported median citrulline levels of 8.6 µmol/L (95% CI, 4.07–21.68 µmol/L) among NEC cases as compared to 20.18 µmol/L (95% CI, 10.32–42 µmol/L) among controls. Based on the findings by Iannou et al. with α of 0.05 and β of 0.2, 19 neonates were needed in each group to predict occurrence of NEC from NBS samples, while based on the findings by Celik et al. 16 neonates, including 8 cases and 8 controls, were needed to predict occurrence of NEC. We, therefore, took a conservative approach and identified 40 cases and 40 controls, two times the higher sample size calculated using these two prior studies.

Statistics

Statistical analysis was performed using SPSS (V.24 Armonk, NY) and Stata software (v15 College Station, TX). Tests for normality of data distribution were conducted. Since the data was skewed to the right, non-parametric testing was used for all analysis. Serum citrulline concentrations were reported as median with 95% confidence interval. Citrulline concentrations at DOL1, at DOL5, and DOL at full feeds were compared between groups using Mann–Whitney *U* tests, and between the three time points within each study group using the Wilcoxon signed-rank test. Additional univariate analysis was performed to compare the epidemiological and clinical variables between cases and controls. The correlation between the citrulline concentrations and time to full feeds and length of stay was performed using Spearman rho correlation.

For factors that were found to be significantly different between the two study groups, multivariable regression was conducted to elucidate the independent association of these variables with citrulline concentrations in cases and controls. The citrulline values were log-transformed prior to multivariable regression analysis.

Results

Forty cases and controls were included in the study (Fig. 1). The clinical characteristics show that GA, birth weight (BW), incidence of multiple gestation, sepsis, and IVH differed between cases and controls (Table 1). The median time for NEC diagnosis in the study group was 20 days of life (interquartile range Q1–Q3; 12.3–38.0). Incidence of NEC was inversely related to GA and BW.

Serum citrulline concentrations did not differ between cases and controls at DOL1, on DOL5, or at the time of full feeds, although there was a trend observed at DOL5 ($p = 0.054$). Moreover, citrulline levels did not differ between the 21 cases who had stage 2 or 3 NEC, including the 5

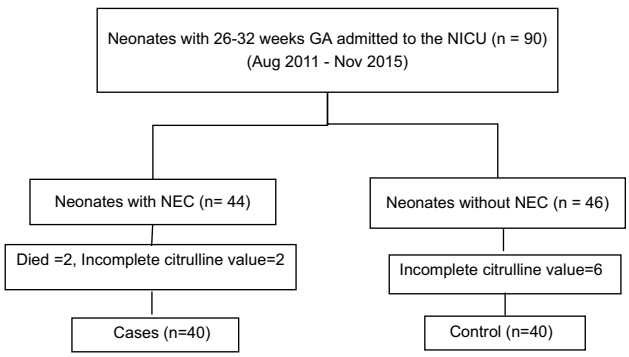

Fig. 1 Flow chart of the inclusion of cases and controls

Table 1 Patient characteristics of the cases and controls

Clinical factors and outcomes	Control ($n=40$) n(%)	Cases ($n=40$) n(%)	p value
Gestational age in weeks (mean ± SD)	30.3 ± 1.3	27.6 ± 2.5	0.001
Birth weight in grams weeks (mean ± SD)	1384.8 ± 278.8	999 ± 363.1	0.001
Ethnicity			0.214
1. Caucasian	10 (25)	7 (17.5)	
2. African American	9 (22.5)	12 (30)	
3. Hispanic	8 (20)	8 (20)	
4. Asian	13 (32.5)	12 (30)	
5. Middle eastern	0	1 (2.5)	
Multiples			0.005
1. Singleton	24 (60.0)	35 (87.5)	
2. Multiples	16 (40.0)	5(12.5)	
Gender			0.653
Male	23 (57.5)	21 (52.5)	
Female	17 (42.5)	19 (47.5)	
Small for gestational age	8 (20.0)	6 (15.0)	0.770
Sepsis	2 (5)	13 (32.5)	0.001
Patent ductus arteriosus	21 (52.5)	25 (67.5)	0.27
Intraventricular hemorrhage (any grade)	2 (5)	11 (27.5)	0.004
Retinopathy of prematurity (> stage 2)	1 (2.5)	5 (12.5)	0.09
Time to reach full feeds in days [median (25–75 percentile)]	17 (10–28)	46.5 (25–126.5)	< 0.001
Length of stay in days [median (25–75 percentile)]	41.5 (24–74)	76 (34–173.5)	< 0.001

neonates requiring surgery, and controls, or those who had stage 1 NEC (Table 2). Since GA, BW, multiple gestation and incidence of IVH differed between the study groups, we performed forward regression analysis which showed that none of these factors were independent predictors of the difference in citrulline between cases and controls. These findings were independent of feeds since the median time for starting feeds in both cases and controls was 3 days (95% CI 1–5 days) with 50% in each group starting on mothers' own milk.

We observed an increase in the citrulline concentrations in both cases and controls over time. The increase from DOL1 to full feeds approached significance for cases ($p=0.05$) and was significantly higher for controls ($p=0.002$), while the increase from birth to DOL5 ($p=0.63$ for cases and $p=0.8$ for controls), and from DOL 5 to full feeds ($p=0.8$ for cases and $p=0.52$ for controls) did not reach statistical significance (Fig. 2).

For the entire cohort, citrulline concentrations on DOL 1 ($r=0.08$; $p=0.53$) or DOL 5 ($r=0.27$; $p=0.18$) did not correlate with time to full feeds, or with length of stay [DOL1 ($r=0.23$; $p=0.06$) and DOL5 ($r=0.21$; $p=0.07$)] (Table 3). To address the effect of inflammation related to NEC on serum citrulline concentrations, the relationship between serum citrulline concentration at birth with time to full feeds and length of stay was evaluated in each study group. There was no correlation between time to reach full feeds and the citrulline concentrations at DOL1 ($r=0.01$; $p=0.95$) and DOL5 ($r=0.14$; $p=0.38$) among controls. While citrulline concentrations at DOL1 correlated with the length of stay ($r=0.39$; $p=0.03$) among controls, there was no correlation with concentrations on DOL5 ($r=-0.15$; $p=0.37$) (Table 3; Figs. 3, 4). There was

Table 2 Serum citrulline concentrations (μmol/L) at different time points for controls and cases

Serum citrulline concentration (μmol/L)	Control median (5–95% CI) ($n=40$)	Cases median (5–95% CI)			p value between controls and all cases	p value controls, stage 1 and NEC stage 2/3
Time points		All ($n=40$)	Stage 1 ($n=19$)	Stage 2/3 ($n=21$)		
At birth	11.42 (7.42–14.84)	11.93 (6.85–18.8)	11.13 (7.42–18.84)	11.13 (6.85–22.26)	0.55	0.66
At DOL 5	11.99 (7.99–16.55)	13.70 (7.42–26.83)	13.41 (7.42–26.83)	13.75 (8.56–26.83)	0.054	0.15
At full feeds	14.86 (6.85–25.69)	15.7 (7.42–26.26)	16.55 (7.99–29.12)	11.99 (5.99–21.98)	0.87	0.39

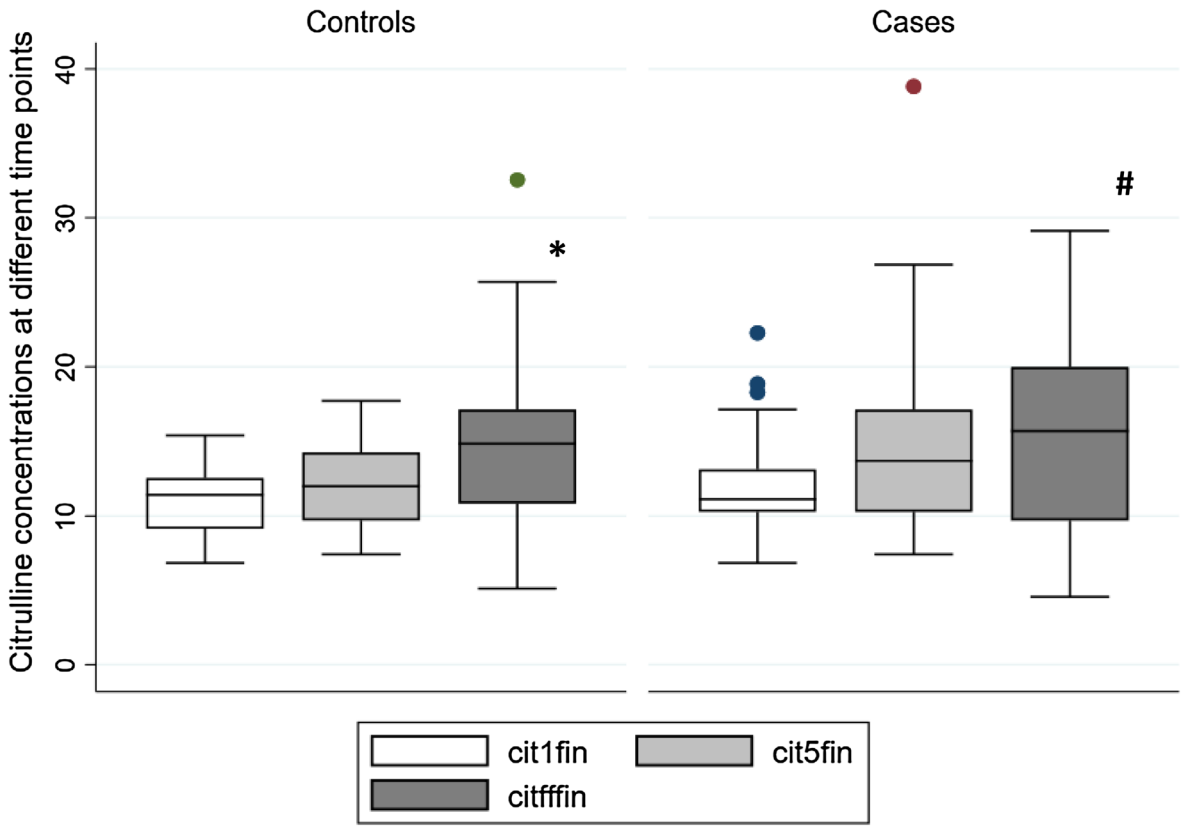

Fig. 2 Citrulline concentrations at different study time points. (*Cit-1fin* citrulline concentration on DOL1, *Cit5fin* citrulline concentration on DOL5, *Citfffin* citrulline concentration on day of full enteral feeds). *$p=0.002$ between citrulline concentrations on DOL1 and on full feeds in controls, #$p=0.05$ between citrulline concentrations on DOL1 and on full feeds in cases

Table 3 Correlation between citrulline concentrations and time to full feeds and to the length of stay for all neonates, and by case and controls

	Time to full feeds						Length of stay					
	All neonates ($n=80$)		Controls ($n=40$)		Cases ($n=40$)		All neonates ($n=80$)		Controls ($n=40$)		Cases ($n=40$)	
	r	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value	*r*	*p* value
Citrulline conc at day 1	0.08	0.53	0.01	0.95	0.07	0.69	0.23	0.06	0.39	0.03	0.17	0.33
Citrulline conc at day 5	0.27	0.18	0.14	0.38	0.22	0.17	0.21	0.07	−0.15	0.37	0.28	0.08

a similar lack of correlation between time to reach full feeds and citrulline concentrations on DOL 1 ($r=0.07$; $p=0.69$) and DOL 5 ($r=0.22$; $p=0.17$) among cases. Furthermore, the length of stay did not correlate with citrulline concentrations on DOL1 ($r=0.17$; $p=0.33$) and for DOL5 ($r=0.28$; $p=0.08$) among the cases (Table 3; Figs. 3, 4).

Discussion

In this retrospective study, we found that citrulline concentrations quantified in the NBS was not different in neonates with and without NEC both at DOL1 and at DOL5 and are, therefore, unlikely to serve as a surrogate measure of intestinal health and/or as a biomarker to predict neonates at risk of developing NEC. Although there was no correlation between the citrulline concentration on DOL1 with time to reach full feeds and length of stay among the cases, citrulline concentrations on DOL1 correlated with length of stay among controls. We also observed an increase in the serum citrulline concentration with increasing chronological age in both cases and controls from DOL1 to full feeds.

The absence of a significant difference in citrulline concentrations between cases and controls in our study supports the multifactorial nature of the disease, with a role of variability in breast milk use, and the use of antibiotics

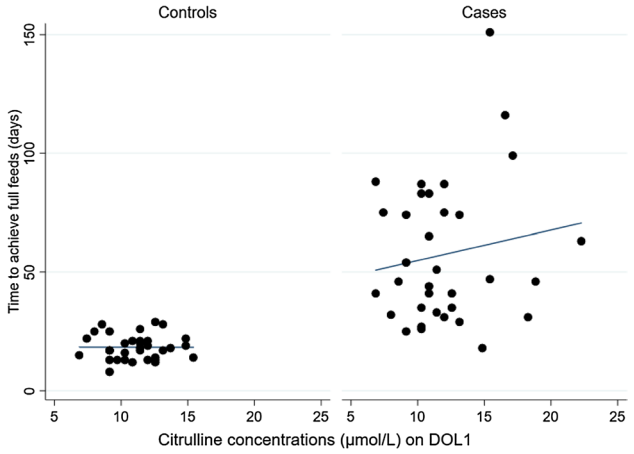

Fig. 3 Correlation between citrulline concentrations on DOL1 and time to achieve full feeds in controls and cases. For controls $r=0.01$; $p=0.95$ and for cases $r=0.07$; $p=0.69$. For the whole group it was $r=0.08$; $p=0.27$

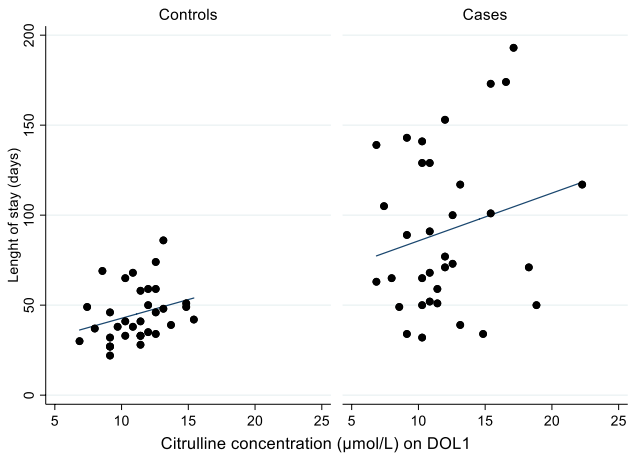

Fig. 4 Correlation between citrulline concentrations on DOL1 and length of stay in controls and cases. For controls $r=0.39$; $p=0.03$ and for cases $r=0.17$; $p=0.33$. For the whole group it was $r=0.23$; $p=0.06$

that may promote intestinal dysbiosis. Further, the timing and association of risk factors such as hypoxic episodes, polycythemia, anemia, and the presence of a PDA may have contributed to the observed lack of difference between serum citrulline concentration between cases and controls. Our findings are similar to the case–control study reported by Englund et al. [23], They measured serum citrulline from NBS obtained within the first 2 weeks of life in neonates later diagnosed with NEC and these concentrations were similar to the citrulline concentrations in the GA matched controls. Their mean citrulline concentrations were 13.1 ± 6.6 μmol/L and 13.0 ± 5.8 μmol/L among the cases and controls, respectively, which were similar to mean citrulline concentrations in our study on DOL5, which were 14.8 ± 6.2 vs. 12.1 ± 2.7 μmol/L. Englund et al. did not specify exact timing or details of feeds when NBS were obtained, which may influence serum citrulline levels given the stimulation of intestinal cells as well as the presence of parenteral amino acids, and may underlie the small differences observed between our findings and theirs. Further, our classification was more stringent, based on a chart review, than those used by Englund et al. which were related to the billing code. Additionally, our study is more representative of timing of routine NBS testing, including the first test within the first 24 h of life and prior to starting TPN, whereas ,the samples reported by Englund et al. were drawn within the first 2 weeks of life.

Becker et al. reported serial serum citrulline concentrations measured prospectively at DOL 3, 7, 14, and 21 which were lower in the cases of NEC as compared to controls, though it did not reach statistical significance [15]. On the contrary, Ioannou et al. [16] demonstrated that serial citrulline concentrations in neonates with NEC drawn at 48 h after the onset of the diagnosis of NEC, first week after diagnosis of NEC, upon reintroduction of enteral feeds, and upon advancement to full feeds, were significantly lower than age-matched controls. This is similar to the findings reported by Celik et al. [17] where citrulline concentrations among neonates with NEC were lower as compared to healthy controls immediately after the diagnosis of NEC was made. An acute decrease in serum citrulline concentrations reported immediately after the diagnosis of NEC [16, 17] reflects change in citrulline production by the intestinal cells related to inflammation related loss of function. It has been shown that plasma citrulline correlates negatively with C-reactive protein which increases acutely with inflammation or sepsis [24]. Putting our findings and those of Englund et al. in context of this literature, we speculate that citrulline concentration may be more useful for early detection of NEC, rather than as a measure of enterocyte health in preterm neonates and to predict NEC. Therefore, it may serve as a marker to differentiate NEC from ileus in a sick neonate, but may not be useful as a predictor of NEC. Since we found that citrulline levels increase from DOL 1 to the time of full feeds in both cases and controls, we speculate that serial measurements in citrulline concentrations may be more useful than a spot level to detect the drop in citrulline concentrations, although Goossens et al. [25] reported no change in the serum citrulline levels with increasing chronological age in neonates and children.

We did not observe a correlation between serum citrulline concentrations at DOL1 and DOL5 to the time to reach full feeds and to length of hospital stay in our study except where citrulline concentrations among controls correlated to the length of stay. The latter observation is a reflection of uninterrupted intestinal growth and health. Furthermore, we

observed significant and progressive increases in citrulline concentrations from birth to full feeds both among the controls and cases. This was stronger among the controls, where the increase was more and is consistent with the correlation between the citrulline concentrations on DOL1 and length of stay. These increases in concentrations over a period of time are consistent with studies where citrulline concentrations in healthy neonates steadily increase over time. Ioannou et al. [16] reported rising citrulline concentrations as enteral feeds increased to greater than 40% of total energy intake, further increasing when the infant reached full feeds, and correlating with the percent of protein fed enterally. Becker et al. reported a similar increase in median citrulline values that increased progressively over the first 3 weeks of life for controls, but not among neonates diagnosed with NEC [15]. However, Englund et al. did not observe any change in citrulline concentrations over time in those cases and controls where repeated samples were available. These observations could be due to variations in the GA of the neonates included in the study, day of life when citrulline was measured, amount and type of enteral intake, and presence of other associated comorbidities.

Our study has several limitations. First, it was a retrospective single center study with limited numbers of neonates. Though our sample size was based on prior studies [16, 17] larger prospective multicenter studies may be needed to further investigate the utility of citrulline concentration as a biomarker of intestinal health in preterm neonates. Further, although serial estimation of citrulline were done in our study, there were long time intervals between the samples, and thus the sudden decrease in the citrulline concentrations that has been associated with NEC may not have been detected. Perhaps using weekly serial serum citrulline estimations or using more sensitive methods to measure intestinal function, such as measuring increased citrulline production by stimulating intestinal cells by oral alanine or glutamine which are precursors of citrulline [26]. Additionally, as more periviable neonates of less than 25 weeks gestational age are surviving and have higher incidence and severity of NEC, as they were not included in this study they should be included in future studies. Details of amount and type of enteral intake, including the micronutrient intake amounts, are very important not only for growth but for the developing gut microbiome specially in the first few days of life [27] in preterm neonates, were not studied. These might have had an important impact on the citrulline concentrations and need to be studied further.

Conclusion

Citrulline concentrations on DOL1 or DOL5 from newborn screens were not predictive of development of NEC and did not correlate to time to reach full feeds and the length of stay except among the healthy neonates. Prospective studies are warranted to follow serial citrulline concentrations in neonates to establish norms for different gestational ages as well as chronological age including the periviable neonates of less than 25 weeks gestational age, which may be used to identify the neonates who are at risk to develop NEC and predict the time to full feeds, and length of stay.

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Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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