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The plasma activities of lysosomal enzymes in infants with necrotizing enterocolitis: New promising class of biomarkers?

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ABSTRACT

Background: Intestinal ischemia plays a major role in the pathogenesis of necrotizing enterocolitis (NEC). The diagnosis of intestinal ischemia would be highly desirable, as it is impossible to achieve with the current diagnostic regimes. Preliminary data from an animal NEC model indicate a possible correlation between the plasma activity of the lysosomal enzyme beta-glucosidase and intestinal ischemia.
Methods: In this case–control study the plasma activities of six different lysosomal enzymes were detected by high-performance liquid-chromatography tandem mass-spectrometry in 15 infants with NEC and compared to 18 controls.
Results: The plasma activities of β-glucosidase (ABG), α-glucosidase (GAA), and galactocerebrosidase (GALC) were significantly higher in the NEC group compared with controls (ABG, $p = 0.009$; GAA, $p < 0.001$; GALC, $p < 0.001$). GAA and GALC showed the highest diagnostic value with areas under the curve of 0.91 and 0.87.
Conclusions: We identified GAA and GALC as new promising biomarkers for gut wall integrity in infants with NEC, and report first results on the plasma activity of ABG. The present study supports the hypothesis that the plasma activity of ABG might serve as a marker of intestinal ischemia in NEC. The identification of intestinal ischemia could facilitate early discrimination of infants at risk for NEC from infants with benign gastrointestinal disorders.

1. Background

Necrotizing enterocolitis (NEC) is one of the most common surgical emergencies at neonatal intensive care units [1]. Despite major effort, diagnosis and successful treatment of NEC continue to present a serious challenge, leaving mortality rates almost unchanged over the past decades. Various studies in the past have shown that clinical variables and traditional laboratory parameters are of limited prognostic accuracy [2,3]. One major impact on the expected outcome in infants with NEC is the occurrence of intestinal perforation and the need for surgical therapy. The mortality from NEC ranges between 20% and 30%, with the greatest mortality among those requiring surgery [4,5]. The identification of intestinal ischemia with consecutive loss of gut wall integrity would be highly desirable, as it is virtually impossible to achieve with the current standard diagnostic regimes (radiographic imaging and clinical appearance) [6]. In order to overcome this diagnostic dilemma, current NEC research focuses on the investigation of gut-wall specific diagnostic biomarkers [7,8]. Consequently, we sought to identify a novel set of biomarkers for intestinal ischemia, which could facilitate early diagnosis at a potentially reversible state of NEC in the future. Preliminary data from an animal model of NEC and intestinal injury indicate a possible correlation of the enzyme activity of β-glucosidase and early stages of intestinal ischemia [9,10]. However, no information regarding lysosomal plasma activities in infants with NEC is available.

We hypothesized that the decline in intestinal epithelial cells due to intestinal ischemia, as proposed in animal models, is accompanied by an increase of lysosomal enzyme activity in infants with NEC. As an extension to our previous clinical NEC research, we investigated the plasma activities of six different lysosomal hydrolases in a case–control study in infants with NEC.

2. Methods

2.1. Study design and population

This case–control study was conducted at the Department of Pediatric Surgery, Medical University of Vienna, Austria, following approval by the local ethics committee. Informed written consent was obtained from parents of all infants. Infants with NEC were recruited from January 2010 until December 2013. Only infants with the following criteria were included: exhibiting clinical signs and radiographic findings of NEC,

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informed written consent and residual blood sample for subsequent determination of the plasma activities of lysosomal enzymes. NEC stages I to III were established according to modified Bell's staging criteria [11]. In this group, blood samples were collected at the onset of clinical presentation of NEC. The control group consisted of infants with a birth weight of less than 2000 g without laboratory and clinical signs of infection at the time-point of blood sampling. Exclusion criteria were: feeding intolerance, abdominal distention, surgical intervention, or clinical signs of NEC in the medical history. Plasma from blood samples for enzyme assays was separated by centrifugation (3500×*g* for 5 min) at 4 °C and stored at −80 °C until pooled analysis could be performed.

2.2. Demographic and clinical parameters

Demographic parameters that were recorded and evaluated for all infants included birth weight, gestational age, Apgar score, age at diagnosis of NEC, and age at blood sampling in the control group. Important clinical characteristics pertaining to NEC were: frequencies of intraventricular hemorrhage, presence of a patent ductus arteriosus, administration of ibuprofen, corticosteroids and antibiotics, the need for mechanical ventilation, and application of vasopressors prior to diagnosing NEC.

2.3. Determination of lysosomal enzyme activity

The lysosomal enzyme activities of acid-sphingomyelinase (ASM), α-glucosidase (GAA), galactocerebrosidase (GALC), alpha-galactosidase (GLA), α-L-iduronidase (IDUA) and β-glucosidase (ABG), were determined in the plasma by a modified high-performance liquid-chromatography (HPLC) tandem mass-spectrometry (MS/MS) method previously described [12,13]. A total of 250 μl blood volume was collected from each study infant. In brief, 1.5 μl of plasma was diluted with 70 μl phosphate puffer (pH 7.4) and shaken for 3 min at 750 rpm on an orbital shaker. For the determination of the enzyme activities of ASM, GAA, GLA, IDUA and ABG 10 μl aliquots of the diluted plasma were each incubated with 15 μl of specific buffers containing a cocktail of MS/MS-specific substrates and internal standards for HPLC–MS/MS analysis. For the analysis of the GALC activity 1.5 μl plasma was directly incubated with 30 μl of specific buffer. All samples were incubated for 20 h at 37 °C. Enzymatic reactions were stopped by adding 100 μl of 80% acetonitrile solution containing 0.2% formic acid. All corresponding reactions were pooled to one well of a 2 ml 96-deep well plate. The plate was centrifuged at 3000×*g* for 15 min prior to HPLC–MS/MS analysis. Analysis was done in duplicate.

2.4. Statistical analysis

Continuous variables were tested for normal distribution by manual outlier analysis using boxplot analysis. Spearman rank-order correlation was used to evaluate the correlation between continuous parameters. Frequencies of parameters were compared by using Fisher's exact test. Spearman rank-order correlation was used to evaluate the association between parameters. Correlation coefficients (r_{rho}) of 0.7–1.0 or −0.7 to −1.0 were considered as strong correlations and 0.5 to 0.7 or −0.5 to −0.7 as moderate correlations. Parameters with lower correlation coefficients were not considered even when they were significant. Boxplots and the ROC analysis were generated using SigmaPlot11.0 (Systat Software GmbH, Erkrath, Germany). The midline and the given values in the graphs represent the median values. Boxes indicate the 25th and the 75th percentile; errorbar-caps indicate the 5th and the 95th percentile. The reported p-values are the results of 2-sided Mann–Whitney U tests. A p-value < 0.05 was considered as statistically significant. Calculations were done using SPSS 17.0 software (IBM; Armonk, NY, US).

3. Results

3.1. Characterization of the study groups

During the study period a total of 15 non-consecutive infants with NEC (10 received surgical and 5 received medical treatment) and 18 control infants were included. In included infants with NEC, two infants were treated with Bell stage I, three with Bell stage IIa, seven with Bell stage IIIa, and three with Bell stage IIIb. No significant differences were found in demographics (details are shown in Table 1). The clinical characteristics and laboratory parameters are summarized in Table 2. There were no significant differences found for the frequencies of intra-ventricular hemorrhage (IVH), persistent ductus arteriosus (PDA), the medical treatment of PDA and the use of steroids. Hematologic parameters (total white blood count and platelet count) and C-reactive protein (CRP) were significantly elevated in NEC infants compared to controls (details are shown in Table 2).

3.2. Significant increases of lysosomal enzyme activities(GAA, ABG, GALC) in the plasma of NEC infants

As shown in Fig. 1, three of the six tested lysosomal enzymes showed significantly elevated plasma enzyme activities in infants with NEC compared with controls (GAA, $p < 0.001$;GALC, $p < 0.001$; ABG, $p = 0.009$). The activity levels were 3.2-fold for GAA, 1.8-fold for GALC and 1.4-fold for ABG higher in the NEC compared to the control group. According to the analyzed activity levels of GAA, GALC, and ABG we were not able to discriminate surgical from medical NEC (GAA, $p = 0.734$; GALC, $p = 0.650$; ABG, $p = 0.497$). No significant differences between infants with NEC and control infants were found for the enzyme activities of ASM ($p = 0.677$), GLA ($p = 0.973$) and IDUA ($p = 0.652$). A significantly moderately positive correlation was found between GAA and GALC activities in the plasma ($r = 0.74$, $p = <0.000$). No correlations were found between the lysosomal enzyme activities in plasma and gestational age, birth-weight, age at sampling, total white blood cell count, platelet counts or CRP at the time-point of diagnosis.

3.3. Diagnostic value of GAA, ABG and GALC

In order to evaluate the diagnostic value of the three enzyme biomarkers a receiver operating characteristic (ROC) analysis was performed. The ROC analysis clearly showed that the plasma activities of GAA, GALC and ABG are potentially useful diagnostic biomarkers to differentiate between control and NEC groups (all three parameters had areas under the curve (A_{UC}) above 0.75; in detail: GAA ($A_{UC} = 0.91$, $p < 0.000$), GALC ($A_{UC} = 0.87$, $p < 0.000$) and ABG ($A_{UC} = 0.76$,

Table 1
Demographic data of infants with necrotizing enterocolitis versus control infants.

	Control (n = 18)	NEC (n = 15)	p
Gestational age, mean (range,weeks)	29 (24–33)	29 (24–40)	0.708
Birth weight, mean (range,g)	1128 (560–1994)	1232 (550–3200)	0.630
Age at blood sampling, mean (range,days)	32 (3–100)	33 (12–85)	0.845
1 min APGAR, mean (range)	8 (5–9)	8 (6–9)	0.789
5 min APGAR, mean (range)	9 (7–10)	9 (8–10)	0.421
Male sex, n (% of total)	9 (50%)	10 (67%)	0.482