SUPPLEMENTAL MATERIAL

Supplemental Table 1 Previous studies reporting fecal calprotectin in neonates with necrotizing enterocolitis.

Year (Ref)	First Author	Country	Study Design	FCP with no NEC	FCP with medical NEC	FCP with surgical NEC	Conclusion
2003 (10)	Carroll	England	N=7 with NEC vs 7 controls	N= 7; 98 ±61 µg/g stool	N= 7; 288 ±49 µg/g stool	NR	FCP levels are higher in NEC (P=0.0006)
2007 (13)	Josefsson	Sweden	N=4 with NEC, 3 with perforation of other etiology vs 52 healthy preterm controls	N= 52; 253 µg/g stool	N= 3; Mean 13298 (3646-22513) µg/g stool	N=1, 685 µg/g stool	FCP >2000 µg/g is a useful but not an early marker of NEC
2008 (14)	Yang	USA	N=14 VLBW serial samples for first month	123±99 µg/g stool	380±246 µg/g stool*	NR	FCP may be a marker for early diagnosis of gastrointestinal illness
2010 (15)	Thuijls	Netherlands	N=35 suspected of NEC	N=21; 69.6 (1-556.4) µg/g stool	N=14; 589.2 (146.1-847.6) µg/g stool	NR	FCP is a promising diagnostic marker for NEC
2012 (16)	Aydemir	Turkey	N=25 with NEC and 25 controls	N=25; 365 (58-1006) µg/g stool	N= 16; 1285 (241-2606) µg/g stool	N= 9; 1251 (421-3337) µg/g stool	FCP >792 µg/g stool has a 76% sensitivity and 92% specificity for identifying NEC
2012 (17)	Dabritz	Germany	N= 145 (127 control and 18 with NEC). Stool samples alternate days for 4 weeks	NR	N=18; 349 (1-850) mg/kg	NR	FCP levels are elevated at onset of NEC but not correlated with disease severity
2012 (18)	Zoppelli	Germany	N= 1988 stool samples from 206 VLBW neonates	N= 38; Mean 52.7 µg/g stool	N= 14 with moderate NEC. Mean NR.	N=5 with fulminant NEC; Mean 14.4 µg/g stool	FCP is a sensitive and specific marker for NEC. However, FCP was unusually low in fulminant NEC.
2012 (19)	Selimoglu	Turkey	N=14 with NEC and 40 healthy preterm controls	N= 40; 172±171 µg/mL	N=14; 167.6±143 µg/mL	N=1; 420 µg/mL	FCP not useful in diagnosing NEC
2012 (20)	Aydemir	Turkey	N=10 with NEC and 9 controls	N= 9; Mean 104 (Range 92-123) mg/dL	N=10; Mean 185 (Range 165-198) mg/dL	NR	FCP is a useful marker for diagnosis and severity of NEC
2012	Reisinger	Netherlands	N=62 with clinical suspicion of NEC	N=19; 80 (1-625)	N=16; 402 (108-848)	NR	FCP > 286 µg/g stool has an 81% sensitivity

(21)				µg/g stool	µg/g stool		and 79% specificity for identifying NEC
2013 (22)	Jenke	Germany	N= 68 ELBW neonates. Compared gut microbiota and FCP.	NR	NR	NR	No correlation between FCP level and total stool bacterial count of any analyzed bacteria.
2014 (23)	Reisinger	Netherlands	N=29 with NEC (12 medical and 17 surgical or fatal)	NR	N= 12; 375 (146-848) µg/g stool	N=17; 479 (108-684) µg/g stool	FCP did not differentiate between medical vs surgical/fatal cases (p=0.87)
2014 (24)	Yoon	Korea	N= 16 VLBW infants. Stools serially collected.	N=12; 4.34±1.94 mg/kg	N= 4; 5.75±1.98 mg/kg	NR	FCP higher in those with NEC (p=0.02)
2014 (25)	Albanna	Egypt	N=15 with NEC and 20 controls	N=20; 73±57 mg/dL	N=12; 240±58 mg/dL	N=3; 308±4 mg/dL	FCP could be a valuable tool to investigate NEC (p<0.0001)
2015 (8)	Bin-Num	Israel	N= 15 infants, 29 stool samples. Compared bed-side FCP assay to ELISA.	195 (110-440) µg/g stool	3000 (2075-7875) µg/g stool	NR	FCP useful for rapid diagnosis of NEC (p<0.0001)

Ref, reference number; FCP, fecal calprotectin; NEC, necrotizing enterocolitis; NR, not reported. FCP values were reported variably as "µg/g stool", "mg/kg", or "mg/dL"; *infants were "sick" but not necessarily with a diagnosis of NEC.

Supplemental Figure 1. *Human NEC gastrointestinal tissue samples demonstrate NET formation.* (**A**) Using immunohistochemical techniques, we compared NET formation in gastrointestinal tissue samples collected from 5 different infants who underwent surgical treatment for NEC and 6 control infants operated on for indications other than NEC. Representative images from infants with NEC are shown in a 60x magnified image demonstrating PMNs and NETs. Calprotectin is seen in gold fluorescence. Neutrophil elastase, a protein known to be expressed on NETs, is seen in magenta fluorescence. Nuclear and NET-associated DNA is shown in blue fluorescence. White arrowheads denote NETs, and yellow arrowheads denote PMNs which express calprotectin. Bar, 10μm. (**B, C**) We quantified PMN (**B**) and NET formation (**C**) in the immunohistochemical images from infants with NEC represented in **A** and from control infants. 5 randomly selected high power fields were analyzed for each of the 5 NEC and 6 Control tissue samples. PMNs were defined as cells having neutrophil nuclear morphology and staining positive for both calprotectin and NE. NETs were defined as extracellular collections of DNA staining positive for both calprotectin and NE. ** denotes $p<0.001$. We employed the student's T-test as a statistical tool.

Supplemental Figure 2. *LPS treatment fails to induce robust expression of calprotectin mRNA or protein.* (**A**) Using real-time RT-PCR, we quantified calprotectin and IL-8 mRNA expression in control and LPS-stimulated (100 ng/mL) adult PMNs. The y-axis represents LPS-induced mRNA expression shown as a fold change over baseline normalized to β-actin. Baseline mRNA expression is arbitrarily set at 1 (dashed line). Calprotectin mRNA expression (left column) was induced 1.8 fold by LPS while IL-8 mRNA (right column) was induced 8 fold as a control for PMN stimulation. (**B**) We used immunocytochemistry to qualitatively assess calprotectin protein expression in control and LPS-stimulated (100 ng/mL, 2 hours) PMNs. Representative images from LPS-stimulated PMNs are shown here. Calprotectin protein expression is shown in gold fluorescence. Nuclear DNA as a counterstain is shown in blue fluorescence. These images are representative of 3 separate experiments using PMNs isolated from 3 different healthy adult donors. Bar, 20μm.