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Systemic and mucosal levels of lactoferrin in very low birth weight infants supplemented with bovine lactoferrin

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Abstract

Lactoferrin supplementation may help prevent infections in preterm infants but efficacy has varied with different dosing and products. We assessed the absorption and excretion of bovine lactoferrin (bLF) in thirty-one infants receiving 100, 200, or 300 mg/kg/day of enteral bLF for 30 days. BLF and human lactoferrin (hLF) in infant saliva, blood, urine, and stool and expressed or donor breast milk (EBM, DBM) collected before treatment initiation, study day 22, and one week after treatment cessation were measured by ELISA. During treatment, BLF was absorbed from the gastrointestinal tract, detected in plasma, saliva, and urine, and excreted in stool. Saliva and stool bLF levels began to decline within 12 hours after dosing and bLF was undetectable in all samples one week after treatment. HLF concentrations exceeded bLF across sample types and timepoints. Infants receiving EBM demonstrated higher saliva and stool hLF levels than DBM patients. Neither bLF nor hLF levels varied by patient characteristics, bLF dosage, or infection status. This is the first study demonstrating bLF absorption into the bloodstream and distribution to saliva and urine in preterm infants. Future studies should further explore lactoferrin pharmacokinetics as higher and more frequent dosing may improve the clinical benefit of lactoferrin supplementation.

Keywords: lactoferrin; preterm infants; bovine; supplementation; postnatal infection

Introduction

Lactoferrin (LF) supplementation has emerged as a strategy to prevent late-onset infections and associated complications in preterm, very low birth weight (<1500 grams at birth, VLBW) infants. As an iron-binding protein with a basic N-terminal domain, LF competes with pathogens for nutrients and can directly bind microbes to enhance cell lysis or modulate immune responses (Legrand, 2016; Valenti and Antonini, 2005). In the acidic environment of the stomach, the potent antimicrobial peptide lactoferricin is released from LF and can bind endotoxins on bacterial membranes to control inflammation and initiate bacterial cell death (Gifford et al., 2005; Legrand, 2016). LF also contributes to anti-inflammatory responses by downregulating the production of proinflammatory cytokines, such as TNF-alpha (Drago-Serrano et al., 2017; Otsuki et al., 2005). Due to these antimicrobial and anti-inflammatory properties, LF may help combat infection-related morbidity and mortality in VLBW infants.

Although LF is found in high concentrations in human colostrum and milk (Mastromarino et al., 2014), VLBW infants receive inadequate amounts of the protein due to their limited food tolerance. Therefore, studies have investigated the safety, tolerability, and benefits of human and bovine LF (hLF, bLF) supplementation in preterm infants during the first weeks of life (Akin et al., 2014; Barrington et al., 2016; group, 2019; Kaur and Gathwala, 2015; Manzoni et al., 2014; Manzoni et al., 2009; Martin et al., 2018; Ochoa et al., 2015). Importantly, bLF shares 77% amino acid homology with hLF (Vorland et al., 1998), has anti-inflammatory properties similar to hLF (Berlutti et al., 2006), and has demonstrated higher antimicrobial activity than hLF (Buccigrossi et al., 2007; Vorland et al., 1998). A systematic review of studies investigating enteral hLF or

bLF supplementation in preterm infants found that, overall, the interventions decreased late-onset sepsis and necrotizing enterocolitis without adverse effects (Pammi and Suresh, 2017, 2020). However, discrepancies between studies remain due to the use of varying LF products, doses, and treatment durations.

To inform bLF dosing in preterm infants, we analyzed the absorption and excretion of bLF and hLF for three escalating regimens of enteral bLF (100, 200, 300 mg/kg/day) in VLBW infants, which treatments were found to be safe and tolerable as described in our accompanying report in this issue (Kaufman et al.). While hLF levels have been examined extensively in maternal human milk, there have only been a few pharmacodynamics studies of hLF and none of bLF in preterm infants (Decembrino et al., 2017; Weimer et al., 2020). We also evaluated whether systemic and mucosal LF levels varied by postnatal infection status or by patient characteristics such as birth weight, gestational age, and nutritional source. Our goal for this pilot study was to provide insight on pharmacokinetics and dosage for future LF supplementation studies.

Material and methods

Study population

Over a 10-month period, 31 VLBW infants at the University of Virginia's (UVA) neonatal intensive care unit (NICU) to receive enteral bovine lactoferrin for 30 days (**Figure S1,** ClinicalTrials.gov Identifier: NCT02731092). VLBW infants born at UVA or transferred to the UVA NICU within 7 days of birth were eligible for enrollment. Demographic feeding information and infection-related events were collected from study participants' medical records.

87

88 *Study intervention*

89 Study intervention is detailed in our accompanying report in this issue (Kaufman
90 et al.). In brief, study participants were enrolled sequentially into one of three escalating
91 bLF supplementation groups: 100 (n=10), 200 (n=10), or 300 mg/kg/day (n=11). Bovine
92 lactoferrin (Glanbia Nutritionals, Gooding, ID, USA) was prepared by the UVA
93 Investigational Pharmacy by dissolving the product powder in sterile water for 30-60
94 minutes under aseptic conditions and was administered enterally daily. Product
95 administration began after consent, which occurred within the first 14 days of life, and
96 continued for 30 days.

97

98 *Sample collection and processing*

99 Infant saliva, blood, urine, and stool and expressed or donor breast milk (EBM,
100 DBM) were collected prior to the first lactoferrin supplement (study day 0), 22 days into
101 supplementation (study day 22), and 7 days after the last lactoferrin administration
102 (study day 37). Unstimulated saliva was collected using two swabs: 1) sterile polyester
103 tipped swab (VWR, Radnor, PA, USA) for assessment of cytomegalovirus (CMV)
104 acquisition and 2) sterile Weck-Cel swab (Beaver-Visitec International, Waltham, MA,
105 USA) for lactoferrin quantification. The polyester tipped swabs were stored at room
106 temperature and the Weck-Cel at -80°C before being transported to the laboratory in
107 batches for processing. Scavenged blood samples were collected in EDTA tubes,
108 refrigerated, and then processed within 7 days of collection by centrifugation to isolate
109 plasma. All specimens were stored at -80ºC until further processing by lab personnel, at

which time they were thawed and kept at 4ºC. Upon thawing, saliva swabs were rehydrated with 200 µL of sterile water, incubated for 20 minutes at room temperature, and spun down in a 0.22 µm Spin-X cellulose acetate filter tube (Corning, Corning, NY, USA) to extract swab contents. Stool samples were weighed and reconstituted in sterile water at a ratio of 1 mg of stool to 4 µL of water. Breast milk and reconstituted stool specimens were spun at 14,000 rpm for 20 minutes at 4ºC to remove lipids and cell debris. All laboratory personnel performing specimen processing and assays were blinded to bLF supplementation dosage.

Assessment of CMV acquisition

Postnatal CMV acquisition was determined by assessing processed day 0 and day 37 infant saliva for CMV DNA by quantitative PCR (qPCR), as previously described (Bialas et al., 2016). Prior to qPCR, the saliva samples were incubated at 95ºC for 5 minutes to denature protein. A sample was considered CMV positive by qPCR if at least two of the six technical replicates had detectable CMV DNA. Infants were considered to have postnatally acquired CMV if they tested negative by saliva qPCR at day 0 but positive at day 37.

Lactoferrin measurements

Processed saliva, plasma, urine, stool, and breast milk specimens from the three collection timepoints were assayed for bLF and hLF levels using commercial enzyme-linked immunosorbent assay (ELISA) kits, in accordance with the manufacturer's instructions (Bovine Lactoferrin ELISA Kit, Bethyl Laboratories, Montgomery, TX, USA;

Human Lactoferrin ELISA Kit, AssayPro, St. Charles, MO, USA). Prior to being assayed, samples were spun down to pellet residual debris. Point dilutions were determined for each specimen type and ELISA in order to maximize the number of samples within range of the standard curve (bLF saliva, plasma, and urine, 1:10; bLF stool, 1:1000; hLF saliva, 1:1000; hLF plasma, 1:50; hLF urine, 1:10; hLF stool, 1:50000; hLF breast milk, 1:500000). All specimens were tested in duplicate. Plates were read on a SpectraMax Plus Plate Reader (Molecular Devices, San Jose, CA, USA) to determine optical density at 450 nm. SoftMax Pro 6.3 software (Molecular Devices, San Jose, CA, USA) was used to interpolate concentrations from standard curves. Assay upper and lower limits of quantification equal the highest and lowest concentration of the standard, respectively, multiplied by the sample dilution factor. Samples outside of this range were assigned the concentration value of the quantification limit.

Importantly, product datasheets for the human and bovine LF ELISA kits we selected demonstrate that the assays yield undetectable levels of activity (0%) to bovine and human LF, respectively, indicating the lack of antigen cross-reactivity for both assays. To verify this with our study samples, we compared study day 0 hLF and bLF levels by compartment as we expected hLF at this timepoint to be very high and bLF levels to be low or undetectable. Importantly, study day 0 hLF and bLF levels did not correlate in any compartment, indicating that high levels of hLF do not yield cross-reactive bLF signal (**Figure S2**). Therefore, assay cross-reactivity was not appreciable in our hLF or bLF observations.

Statistical analysis

All statistical analyses were conducted using GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA). Non-parametric tests were used to analyze all results because the data was not normally distributed. Kruskal-Wallis Tests with Dunn's Tests for multiple comparisons were used to evaluate LF levels between bLF groups. All p-values associated with these tests are multiplicity-adjusted. Two-tailed Mann-Whitney Tests were applied to data stratified into two groups, such as LF levels by sample collection time, infant feeding source, and postnatal infections. Two-tailed Spearman Rank Tests were employed to evaluate correlations between LF levels and infant characteristics, including gestational age and birth weight. Finally, a log-linear regression model was utilized to assess the relationship between study day 22 bLF levels and sample collection timing relative to bLF dosing.

Results

Study population characteristics

Thirty-one preterm, VLBW infants were enrolled and received enteral bLF supplementation for 30 days, starting on a median day after birth of 7 (range: 2-15) (**Figure S1**). Participants were sequentially assigned to receive interventions of increasing dosage: 100 (n=10), 200 (n=10), or 300 (n=11) mg/kg/day of bLF. The gestational age and birth weight of study participants ranged from 24.1 to 34 weeks and 540 to 1480 grams, respectively **(Table S1)**. Importantly, gestational age and birth weight did not statistically vary between bLF groups (gestational age, p=0.18; birth weight, p=0.07; Kruskal-Wallis Test). During the study period, one infant in each group

acquired a bloodstream infection (BSI), caused by coagulase-negative staphylococci (CoNS), methicillin-susceptible *Staphylococcus aureus*, and polymicrobial organisms (CoNS + *Enterococcus fecalis*) on study days 9, 11, and 14, respectively by dosage group. There were no urinary tract infections nor necrotizing enterocolitis in any groups. Additionally, two infants in the 200 mg/kg/day group, who were twins, demonstrated evidence of postnatal CMV acquisition by testing negative by CMV qPCR on study day 0 (day 3 after birth) yet positive on study day 37 (day 40 after birth). These twins received only human milk (93% EBM, 7% DBM) during the study period. The majority of infants at study day 0 were fed EBM, and feeding sources diversified over the study period (**Figure S3**).

BLF levels in VLBW infants

Systemic and mucosal levels of bLF were detectable in VLBW infants during the 30-day treatment period, as indicated by study day 22 levels (**Figure 1**). The highest increases from baseline and the greatest concentrations of bLF occurred in infant urine and stool, suggesting the importance of LF in mucosal compartments. BLF levels did not statistically differ between escalating mg/kg/day bLF dosage groups in any sample type and bLF was completely cleared from all infants by one week after the last dose. To understand bLF clearance from the various anatomical compartments, we applied a log-linear regression model to evaluate the relationship between study day 22 bLF levels and sample collection time, relative to the most recent bLF dose (**Figure 2**). BLF levels in saliva, but not in other sample types, were consistent with log-linear decay within the 24 hours after bLF dosing (R^2=0.56, $p<0.001$). Accordingly, saliva levels also

demonstrated a peak a few hours after dosing in some of the patients in the 200 and 300 mg/kg/day groups, whereas bLF in other compartments did not appear to substantially fluctuate by sampling time. To further investigate the impact of sample collection time on bLF levels, we stratified study day 22 levels according to whether samples were collected within 12 hours or 12-24 hours after bLF administration (**Figure 3)**. By this comparison, both saliva and stool bLF levels were significantly decreased in samples collected 12-24 hours after dosing ($p<0.001$ and $p=0.03$ respectively, Mann-Whitney Test).

HLF levels in VLBW infants

HLF was detected in the stool, saliva, plasma, and urine of most infants across all timepoints and exceeded bLF levels in every sample type and timepoint. We found no differences in hLF concentrations between bLF dosage groups at any timepoint, in any compartment **(Figure 4)**, though we observed large variations in hLF within most compartments. As expected, maternal EBM from study day 0, which was primarily composed of colostrum, demonstrated the highest levels of hLF (median: 14.8 mg/mL; range: 8.2-26.7 mg/mL), with transitional breast milk from study day 22 having slightly decreased levels from baseline (median: 7.1 mg/mL; range: 4.1-14.7). In contrast to the high concentration of hLF observed in EBM, we were unable to detect hLF in any DBM samples **(Figure 4, Figure 5A)**.

Among the infant specimen types assessed, infant stool contained the highest concentration of hLF, with levels reaching 20 mg/mL at both study days 0 and 22 **(Figure 4)**. Despite the high concentration of hLF in breast milk at study day 0

compared to study day 22, hLF levels in infant compartments remained similar across the study period.

LF levels by patient characteristics and feeding type

Both hLF and bLF levels did not strongly correlate with infant gestational age or birth weight (**Table 1**). Moreover, these characteristics did not differ between supplementation groups and therefore cannot explain the lack of differences in bLF levels between groups (**Table S1**). We did observe differences in hLF, but not bLF, levels between EBM and DBM patients (**Figure 5**). As previously noted, EBM demonstrated consistently high levels of hLF whereas DBM had no detectable hLF **(Figure 4, Figure 5A)**. Accordingly, infants receiving EBM tended to have higher hLF levels across compartments than infants receiving DBM. Saliva and stool hLF levels were most influenced by intake of EBM compared to DBM, whereas plasma and urine levels varied less by hLF intake. Specifically, EBM patients had statistically higher study day 0 saliva and study day 22 stool hLF levels compared to DBM infants ($p=0.02$ and 0.04, Mann-Whitney Test).

LF levels by postnatal infection status

During the study period, one infant from each bLF group acquired a BSI and two infants (twins) in the 200 mg/kg/day bLF group demonstrated evidence of postnatal CMV acquisition (**Table S1**). We found no significant differences in hLF and bLF levels between infants with and without any postnatal infections, combining both postnatal

CMV and BSI cases, (**Figure 6**, **Table 2**), with the exception of elevated baseline hLF in the saliva of infants with postnatal infection (p=0.04, Mann-Whitney Test).

Discussion

This study is the first report of absorption, secretion, and excretion of bovine lactoferrin in preterm infants. While previous studies have employed hLF or bLF administration in infants (Akin et al., 2014; Asztalos et al., 2020; Barrington et al., 2016; group, 2019; Kaur and Gathwala, 2015; Manzoni et al., 2014; Manzoni et al., 2009; Ochoa et al., 2020; Ochoa et al., 2015; Sherman et al., 2016; Tarnow-Mordi et al., 2020), few have measured resultant bLF or hLF levels and none, to our knowledge, have evaluated bLF concentrations. Our study demonstrates that bLF and hLF can be detected in infant saliva, plasma, urine, and stool by commercially available ELISA kits without cross-reactivity. BLF in infant saliva and stool appears to decline to lower levels within 24 hours after dosing, whereas urine and plasma levels remain constant. These findings imply that additional pharmacokinetic studies of dosing amount, frequency, route, and duration may potentially influence levels and outcomes.

In light of the ELFIN, LIFT, and NEOLACTO studies' recent findings that enteral bLF supplementation at doses of 150 and 200 mg/kg/day did not reduce the risk of late-onset infections in preterm infants (Asztalos et al., 2020; group, 2019; Ochoa et al., 2020), it is particularly important to evaluate LF levels in order to optimize supplementation dosing and duration. Compared to the 150 mg/kg/day dosing which had no change on acquisition of BSI, there was a trend in the two studies using 200 mg/kg/day with the BSI rate being decreased by 17% and 25%, though not significantly.

The NEOLACTO study which dosed 200 mg/kg/day divided three times a day also saw a trend of 55% reduction in BSI in the sub-group of infants <1000 grams. Additionally, the one hLF study supplemented with 300 mg/kg/day divided twice a day observed a significant decrease in hospital acquired infections ($p<0.04$) with a 40% decrease in BSI ($p=0.52$) (Sherman et al 2016).

Dosage of bLF doses between 100 and 300 mg/kg/day in our small study did not statistically impact achieved bLF levels in infant compartments over the study period and infant GA, BW, and feeding type likely did not impact this result. While this result may be misleading due to the small study size, sampling time, and limited infant sample availability for some compartments, it could also indicate that excretion occurs rapidly or that maximum levels of bLF uptake are achieved by all three dosage groups after three weeks of treatment. However, a few observations in this small study would suggest higher bLF dosing may impact levels. For instance, infants receiving 200 and 300 mg/kg/day bLF achieved higher saliva bLF levels at study day 22 (30% in each group had greater than 0.05 µg/mL) compared to those receiving 100 mg/kg/day (none reached 0.05 µg/mL). This trend also applied to bLF in plasma at this timepoint, with escalating dosage groups demonstrating increasing maximum bLF levels (15.8, 16.6, 28.4 ng/mL respectively by dosage group). Additionally, all the patients in the 300 mg/kg/day group had detectable urine levels compared to around 75% of the lower doses. Another observation is that excretion via the urine and stool was relatively constant, which may imply higher doses could potentially lead to increased local gastrointestinal effect and absorption. Additional dosing studies would inform whether escalating doses impact uptake dynamics.

Beyond differences in bLF levels by supplementation group, we also observed that bLF rapidly declined after dosing, with decreased saliva and stool levels just 12 hours after administration, and was completely undetectable in all infant compartments by just one week after the last dose. Because bLF is quickly cleared from infant systemic and mucosal compartments, more frequent dosing may be needed to sustain saliva and other mucosal levels. Additionally, if a supplementation regimen is found to be beneficial, it will likely need to continue while its desired effects are observed.

During the study period, only two infants (twins) acquired postnatal CMV and three developed bloodstream infections. It is possible that the two CMV infections were perinatally acquired, as these twins were tested just 3 days after birth (their time of enrollment) and postnatal acquisition was obtained on day 40 after birth (study day 37). A sample on day 30 after birth would have more definitively established the presence or absence of perinatal infection. Timing of saliva sampling for CMV detection is also important as it is possible samples were taken after feedings and could be affected if CMV was present in maternal milk. Evaluating the urine for postnatal CMV infection would add additional identification and confirmation in some cases, especially in preterm infants. While we did not observe an association between LF levels and protection against late-onset infections, the scarce number of infection cases, timing of sample collection, and incomplete sample availability for some compartments likely limited our ability to effectively evaluate this relationship. A larger cohort, which would consequently have a higher incidence of postnatal infections, may be necessary to determine the relationship between LF levels and infection risk.

Regarding hLF levels, though we observed slightly higher hLF levels in colostrum and transitional milk compared to some previous reports (Hirai et al., 1990; Mastromarino et al., 2014; Ronayne de Ferrer et al., 2000; Yang et al., 2018), our findings are very similar to those found in a large clinical trial in Peru of 346 mothers of low birth weight infants (Villavicencio et al., 2017). Also similar to other studies, we did not detect any measurable amount of hLF in DBM (**Figure 5**). The presence of hLF in saliva, plasma, urine, and stool samples from patients not receiving EBM suggests endogenous production of hLF in these preterm infants. Moreover, it appears that hLF levels in saliva and stool, but not plasma and urine, are affected by hLF intake, as the levels in saliva and stool corresponded with whether infants received EBM or DBM. This may suggest that plasma and urine levels are mostly influenced by local production, whereas saliva and stool levels may result from both local production and secretion of hLF acquired through feedings. Gastroesophageal reflux events may also affect saliva levels.

Our study is also limited in that we only dosed once a day, primarily administered via a nasogastric or orogastric tube. Given our findings of decreased saliva and stool bLF levels just 12 hours after bLF treatment, with saliva levels demonstrating log-linear decay, dosing multiple times a day may meaningfully impact resultant bLF levels and clinical outcomes. The NEOLACTO trial administered bLF three times a day compared to daily and observed promising trends, noted above. Future studies with more frequent collection timepoints will need to examine LF pharmacokinetics in more detail to inform optimal dosing regimens. Animal data also suggests that absorption may be improved by administering part of the LF dose via the sublingual route (Hayashi et al., 2017). HLF

levels detected in the DBM patients may have been affected by EBM received prior to the study day testing.

Together, our data highlight both the feasibility of quantifying bovine and human lactoferrin levels in systemic and mucosal compartments of VLBW preterm infants and also the insight that these measurements provide on supplementation protocol design. We are the first study to demonstrate that supplemental bovine lactoferrin can be absorbed by the gastrointestinal tract into the blood, secreted into saliva and urine, and potentially cleared more quickly from the saliva and stool than blood and urine. Quantification of bovine and human lactoferrin levels will hopefully allow future studies to determine the optimal dosing to elucidate whether lactoferrin treatment is protective against late-onset infections and other neonatal outcomes.

References

Akin, I.M., Atasay, B., Dogu, F., Okulu, E., Arsan, S., Karatas, H.D., Ikinciogullari, A., Turmen, T., 2014. Oral lactoferrin to prevent nosocomial sepsis and necrotizing enterocolitis of premature neonates and effect on T-regulatory cells. Am J Perinatol 31, 1111-1120.

Asztalos, E.V., Barrington, K., Lodha, A., Tarnow-Mordi, W., Martin, A., 2020. Lactoferrin infant feeding trial_Canada (LIFT_Canada): protocol for a randomized trial of adding lactoferrin to feeds of very-low-birth-weight preterm infants. BMC pediatrics 20, 40.

Barrington, K.J., Assaad, M.A., Janvier, A., 2016. The Lacuna Trial: a double-blind randomized controlled pilot trial of lactoferrin supplementation in the very preterm infant. J Perinatol 36, 666-669.

Berlutti, F., Schippa, S., Morea, C., Sarli, S., Perfetto, B., Donnarumma, G., Valenti, P., 2006. Lactoferrin downregulates pro-inflammatory cytokines upexpressed in intestinal epithelial cells infected with invasive or noninvasive Escherichia coli strains. Biochemistry and cell biology = Biochimie et biologie cellulaire 84, 351-357.

Bialas, K.M., Westreich, D., Cisneros de la Rosa, E., Nelson, C.S., Kauvar, L.M., Fu, T.M., Permar, S.R., 2016. Maternal Antibody Responses and Nonprimary Congenital Cytomegalovirus Infection of HIV-1-Exposed Infants. J Infect Dis 214, 1916-1923.

368 Buccigrossi, V., de Marco, G., Bruzzese, E., Ombrato, L., Bracale, I., Polito, G.,
369 Guarino, A., 2007. Lactoferrin induces concentration-dependent functional modulation
370 of intestinal proliferation and differentiation. Pediatric research 61, 410-414.

371 Decembrino, L., DeAmici, M., De Silvestri, A., Manzoni, P., Paolillo, P., Stronati, M.,
372 2017. Plasma lactoferrin levels in newborn preterm infants with sepsis. J Matern Fetal
373 Neonatal Med 30, 2890-2893.

374 Drago-Serrano, M.E., Campos-Rodriguez, R., Carrero, J.C., de la Garza, M., 2017.
375 Lactoferrin: Balancing Ups and Downs of Inflammation Due to Microbial Infections. Int J
376 Mol Sci 18.

377 Gifford, J.L., Hunter, H.N., Vogel, H.J., 2005. Lactoferricin: a lactoferrin-derived peptide
378 with antimicrobial, antiviral, antitumor and immunological properties. Cell Mol Life Sci
379 62, 2588-2598.

380 group, E.t.i., 2019. Enteral lactoferrin supplementation for very preterm infants: a
381 randomised placebo-controlled trial. Lancet 393, 423-433.

382 Hayashi, T., To, M., Saruta, J., Sato, C., Yamamoto, Y., Kondo, Y., Shimizu, T.,
383 Kamata, Y., Tsukinoki, K., 2017. Salivary lactoferrin is transferred into the brain via the
384 sublingual route. Biosci Biotechnol Biochem 81, 1300-1304.

385 Hirai, Y., Kawakata, N., Satoh, K., Ikeda, Y., Hisayasu, S., Orimo, H., Yoshino, Y., 1990.
386 Concentrations of lactoferrin and iron in human milk at different stages of lactation. J
387 Nutr Sci Vitaminol (Tokyo) 36, 531-544.

388 Kaufman, D.A., Berenz, A., Itell, H.L., Conaway, M., Blackman, A., Nataro, J.P.,
389 Permar, S.R., Dose Escalation Study of Bovine Lactoferrin in Preterm Infants: Getting
390 the Dose Right. Biochemistry and Cell Biology.

391 Kaur, G., Gathwala, G., 2015. Efficacy of Bovine Lactoferrin Supplementation in
392 Preventing Late-onset Sepsis in low Birth Weight Neonates: A Randomized Placebo-
393 Controlled Clinical Trial. J Trop Pediatr 61, 370-376.

394 Legrand, D., 2016. Overview of Lactoferrin as a Natural Immune Modulator. J Pediatr
395 173 Suppl, S10-15.

396 Manzoni, P., Meyer, M., Stolfi, I., Rinaldi, M., Cattani, S., Pugni, L., Romeo, M.G.,
397 Messner, H., Decembrino, L., Laforgia, N., Vagnarelli, F., Memo, L., Bordignon, L.,
398 Maule, M., Gallo, E., Mostert, M., Quercia, M., Bollani, L., Pedicino, R., Renzullo, L.,
399 Betta, P., Ferrari, F., Alexander, T., Magaldi, R., Farina, D., Mosca, F., Stronati, M.,
400 2014. Bovine lactoferrin supplementation for prevention of necrotizing enterocolitis in
401 very-low-birth-weight neonates: a randomized clinical trial. Early Hum Dev 90 Suppl 1,
402 S60-65.

403 Manzoni, P., Rinaldi, M., Cattani, S., Pugni, L., Romeo, M.G., Messner, H., Stolfi, I.,
404 Decembrino, L., Laforgia, N., Vagnarelli, F., Memo, L., Bordignon, L., Saia, O.S., Maule,
405 M., Gallo, E., Mostert, M., Magnani, C., Quercia, M., Bollani, L., Pedicino, R., Renzullo,
406 L., Betta, P., Mosca, F., Ferrari, F., Magaldi, R., Stronati, M., Farina, D., Italian Task
407 Force for the Study and Prevention of Neonatal Fungal Infections, I.a.S.o.N., 2009.
408 Bovine lactoferrin supplementation for prevention of late-onset sepsis in very low-birth-
409 weight neonates: a randomized trial. JAMA 302, 1421-1428.

Martin, A., Ghadge, A., Manzoni, P., Lui, K., Brown, R., Tarnow-Mordi, W., Group, L.C.S., 2018. Protocol for the Lactoferrin Infant Feeding Trial (LIFT): a randomised trial of adding lactoferrin to the feeds of very-low birthweight babies prior to hospital discharge. BMJ Open 8, e023044.

Mastromarino, P., Capobianco, D., Campagna, G., Laforgia, N., Drimaco, P., Dileone, A., Baldassarre, M.E., 2014. Correlation between lactoferrin and beneficial microbiota in breast milk and infant's feces. Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine 27, 1077-1086.

Ochoa, T.J., Zegarra, J., Bellomo, S., Carcamo, C.P., Cam, L., Castaneda, A., Villavicencio, A., Gonzales, J., Rueda, M.S., Turin, C.G., Zea-Vera, A., Guillen, D., Campos, M., Ewing-Cobbs, L., Group, N.R., 2020. Randomized Controlled Trial of Bovine Lactoferrin for Prevention of Sepsis and Neurodevelopment Impairment in Infants Weighing Less Than 2000 Grams. J Pediatr 219, 118-125 e115.

Ochoa, T.J., Zegarra, J., Cam, L., Llanos, R., Pezo, A., Cruz, K., Zea-Vera, A., Cárcamo, C., Campos, M., Bellomo, S., Group, N.R., 2015. Randomized controlled trial of lactoferrin for prevention of sepsis in peruvian neonates less than 2500 g. Pediatr Infect Dis J 34, 571-576.

Otsuki, K., Yakuwa, K., Sawada, M., Hasegawa, A., Sasaki, Y., Mitsukawa, K., Chiba, H., Nagatsuka, M., Saito, H., Okai, T., 2005. Recombinant human lactoferrin has preventive effects on lipopolysaccharide-induced preterm delivery and production of inflammatory cytokines in mice. Journal of perinatal medicine 33, 320-323.

Pammi, M., Suresh, G., 2017. Enteral lactoferrin supplementation for prevention of sepsis and necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev 6, CD007137.

Pammi, M., Suresh, G., 2020. Enteral lactoferrin supplementation for prevention of sepsis and necrotizing enterocolitis in preterm infants. Cochrane Database Syst Rev 3, CD007137.

Ronayne de Ferrer, P.A., Baroni, A., Sambucetti, M.E., Lopez, N.E., Ceriani Cernadas, J.M., 2000. Lactoferrin levels in term and preterm milk. J Am Coll Nutr 19, 370-373.

Sherman, M.P., Sherman, J., Arcinue, R., Niklas, V., 2016. Randomized Control Trial of Human Recombinant Lactoferrin: A Substudy Reveals Effects on the Fecal Microbiome of Very Low Birth Weight Infants. J Pediatr 173 Suppl, S37-42.

Tarnow-Mordi, W.O., Abdel-Latif, M.E., Martin, A., Pammi, M., Robledo, K., Manzoni, P., Osborn, D., Lui, K., Keech, A., Hague, W., Ghadge, A., Travadi, J., Brown, R., Darlow, B.A., Liley, H., Pritchard, M., Kochar, A., Isaacs, D., Gordon, A., Askie, L., Cruz, M., Schindler, T., Dixon, K., Deshpande, G., Tracy, M., Schofield, D., Austin, N., Sinn, J., Simes, R.J., collaborators, L., 2020. The effect of lactoferrin supplementation on death or major morbidity in very low birthweight infants (LIFT): a multicentre, double-blind, randomised controlled trial. Lancet Child Adolesc Health.

Valenti, P., Antonini, G., 2005. Lactoferrin: an important host defence against microbial and viral attack. Cell Mol Life Sci 62, 2576-2587.

Villavicencio, A., Rueda, M.S., Turin, C.G., Ochoa, T.J., 2017. Factors affecting lactoferrin concentration in human milk: how much do we know? Biochemistry and cell biology = Biochimie et biologie cellulaire 95, 12-21.

Vorland, L.H., Ulvatne, H., Andersen, J., Haukland, H., Rekdal, O., Svendsen, J.S., Gutteberg, T.J., 1998. Lactoferricin of bovine origin is more active than lactoferricins of human, murine and caprine origin. Scandinavian journal of infectious diseases 30, 513-517.

Weimer, K.E.D., Roark, H., Fisher, K., Cotten, C.M., Kaufman, D.A., Bidegain, M., Permar, S.R., 2020. Breast Milk and Saliva Lactoferrin Levels and Postnatal Cytomegalovirus Infection. Am J Perinatol.

Yang, Z., Jiang, R., Chen, Q., Wang, J., Duan, Y., Pang, X., Jiang, S., Bi, Y., Zhang, H., Lonnerdal, B., Lai, J., Yin, S., 2018. Concentration of Lactoferrin in Human Milk and Its Variation during Lactation in Different Chinese Populations. Nutrients 10.

466 **Table 1.** Human and bovine LF levels do not vary by infant gestational age or birth

467 weight.

		Gestational Age		Birth Weight	
	n	Spearman R	P-value	Spearman R	P-value
Study Day 0 Human LF					
EBM	19	-0.07	0.77	-0.05	0.84
Saliva	20	-0.24	0.31	-0.03	0.90
Plasma	21	-0.01	0.96	-0.002	>0.99
Urine	21	-0.34	0.13	-0.29	0.21
Stool	19	-0.07	0.78	-0.09	0.72
Study Day 22 Human LF					
EBM	16	-0.34	0.19	-0.38	0.15
Saliva	28	-0.03	0.88	0.08	0.68
Plasma	11	0.19	0.56	0.12	0.72
Urine	24	**-0.56**	**0.01***	**-0.56**	**0.004***
Stool	29	0.01	0.96	0.05	0.82
Study Day 22 Bovine LF					
Saliva	28	**-0.41**	**0.03***	**-0.39**	**0.04***
Plasma	11	0.04	0.90	0.18	0.59
Urine	24	-0.34	0.10	-0.23	0.27
Stool	30	0.03	0.87	0.11	0.56
Study Day 37 Human LF					
Saliva	24	-0.30	0.15	-0.28	0.19
Plasma	3	0.87	0.67	0.50	>0.99
Urine	19	-0.38	0.11	-0.19	0.43
Stool	19	-0.36	0.13	**-0.58**	**0.01***

468

469 * Spearman p-value < 0.05

470 **Table 2**. Human and bovine LF levels do not explain VLBW infant infection risk.

	No Infections	**Postnatal Infections**		
	(n=26)	CMV (n=2)	BSI (n=3)	Combined (n=5)
Study Day 0 Human LF				
EBM (mg/mL)	14.88 (8.89-26.69)	*No Samples*	8.2 (8.2-8.2)	8.2 (8.2-8.2)
Saliva (µg/mL)	**3.62*** **(0.63-80)**	80 (80-80)	15.19 (15.19)	**80*** **(15.19-80)**
Plasma (µg/mL)	0.64 (0.16-4)	0.56 (0.56-0.56)	0.41 (0.22-0.63)	0.49 (0.22-0.63)
Urine (µg/mL)	0.05 (0.01-0.80)	*No Samples*	0.43 (0.07-0.80)	0.43 (0.07-0.80)
Stool (µg/mL)	1459 (156.3-20000)	156.3 (156.3-156.3)	1461 (1461)	156.3 (156.3-1461)
Study Day 22 Human LF				
EBM (mg/mL)	7.32 (4.13-14.55)	5.48 (5.34-5.63)	14.69 (14.69-14.69)	5.63 (5.34-14.69)
Saliva (µg/mL)	9.82 (0.63-80)	40.3 (0.63-80)	6.26 (0.63-8.56)	6.26 (0.63-80)
Plasma (µg/mL)	0.89 (0.36-4)	*No Samples*	0.95 (0.95)	0.95 (0.95)
Urine (µg/mL)	0.8 (0.01-0.80)	0.24 (0.24)	0.47 (0.15-0.80)	0.24 (0.15-0.80)
Stool (µg/mL)	2142 (156.3-20000)	10749 (1497-20000)	156.3 (156.3-992.9)	992.9 (156.3-20000)
Study Day 22 Bovine LF				
Saliva (µg/mL)	0.01 (0.01-1.44)	0.49 (0.01-0.97)	0.09 (0.01-3.27)	0.09 (0.01-3.27)
Plasma (µg/mL)	0.01 (0.01-0.03)	*No Samples*	0.01 (0.01)	0.01 (0.01)
Urine (µg/mL)	0.25 (0.01-1.83)	0.01 (0.01)	1.63 (0.17-3.10)	0.17 (0.01-3.10)
Stool (µg/mL)	34.11 (5.48-123.7)	63.27 (60.09-66.45)	38.85 (34.11-51.28)	51.28 (34.11-66.45)

471

472 Median (range)

473 * Mann-Whitney Test p-value < 0.05 (no infections vs. combined postnatal infections)

Figure 1. **BLF levels in VLBW infants did not vary by bLF supplementation dose**. BLF levels were measured in infant saliva, plasma, urine, and stool (A-D, µg/mL) at the three study timepoints. All compartments had undetectable levels of bLF within 7 days of treatment cessation (day 37). BLF levels did not vary significantly between bLF supplementation groups within each timepoint ($p>0.05$, Kruskal-Wallis Test). The number of samples varies by compartment due to sample criteria, availability, and volume restrictions. Dotted lines denote assay limits of quantification. Solid lines indicate median concentrations.

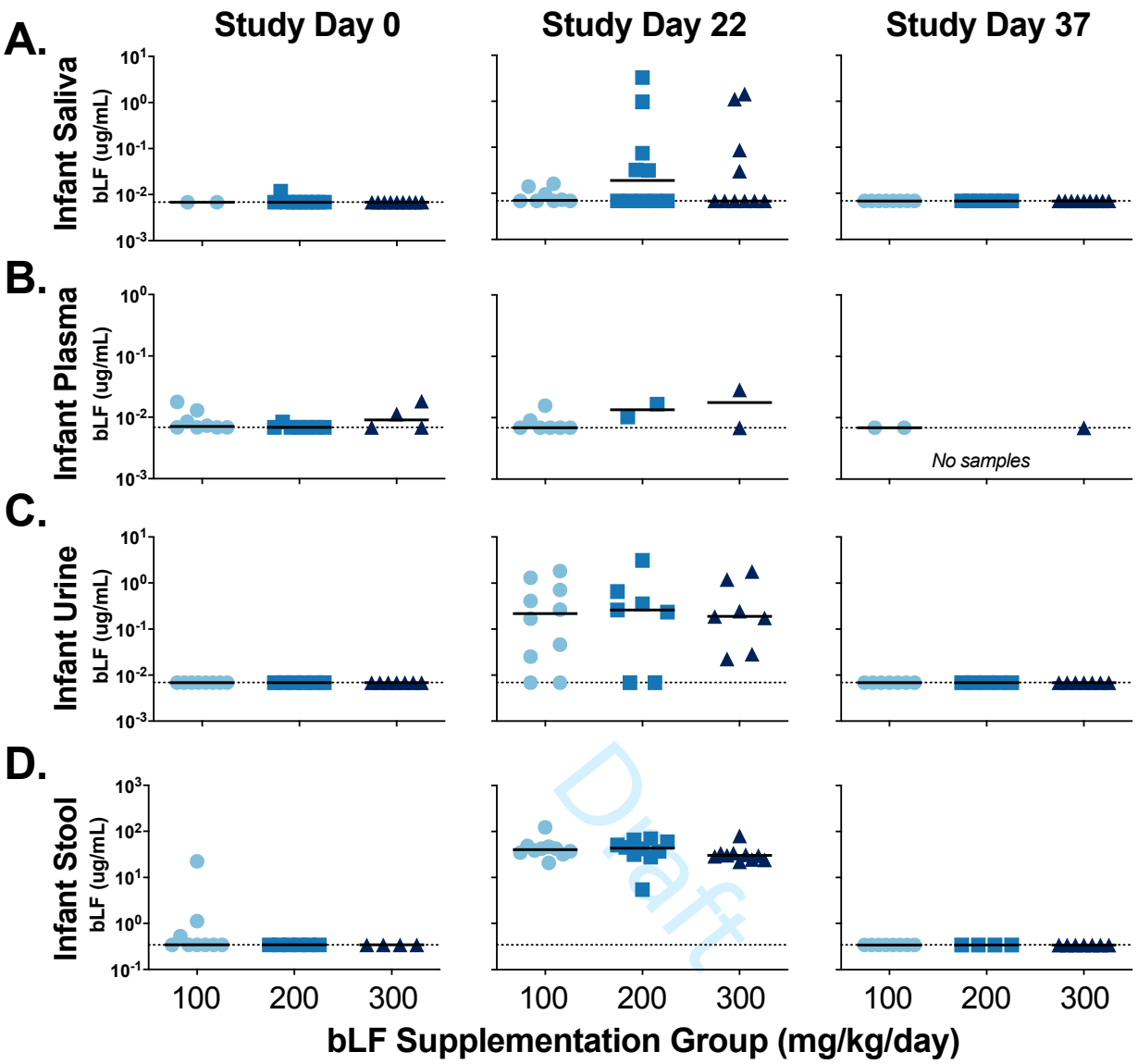
Figure 2. Saliva bLF levels demonstrate log-linear decay within 24 hours of dosing. Infant study day 22 (D22) bLF levels were assessed according to sample collection time in hours after the most recent bLF administration (A-D). After $\log_{10}$ transformation, bLF levels from samples collected up to 24 hours post treatment were modeled using a linear regression. The R^2 model for linear fit is depicted by gray lines.

Figure 3. BLF levels in both saliva and stool decline after 12 hours post bLF administration. D22 bLF levels in each infant compartment measured were stratified according to whether samples were collected within 12 hours or 12-24 hours after bLF treatment (A-D). The difference between stratified samples, combined across supplementation groups, was evaluated by Mann-Whitney Tests ($p<0.05$ denoted in figure). The number of samples varies by compartment due to sample criteria, availability, and volume restrictions.

Figure 4. **HLF is detectable in most breast milk and VLBW infant samples**. HLF levels were measured in EBM and DBM (A, mg/mL, DBM data points in shaded region) and in infant saliva, plasma, urine, and stool (B-E, µg/mL) on study days 0, 22, and 37. HLF levels did not vary significantly between bLF dosage groups within each timepoint ($p>0.05$, Kruskal-Wallis Test).

Figure 5. **Infants receiving expressed breast milk (EBM) have higher saliva and stool hLF levels.** Infant feeding types were recorded on study days 0 and 22. Breast milk hLF levels and hLF and bLF levels in infant saliva, plasma, urine, and stool (A-E and F-I, respectively) on study days 0 and 22 were compared between EBM and DBM patients. All comparisons were evaluated with Mann-Whitney tests ($p<0.05$ denoted in figure).

Figure 6. **LF levels do not explain VLBW infant infection risk**. HLF levels in EBM and DBM (A, DBM data points in shaded region) and infant saliva, plasma, urine, and stool (B-E) on study days 0 and 22 were compared between infants without postnatal infection and those that acquired a postnatal infection during the study period. BLF levels in infant saliva, plasma, urine, and stool (F-I) on study day 22 were also compared between these groups. All comparisons were evaluated with Mann-Whitney tests ($p<0.05$ denoted in figure).



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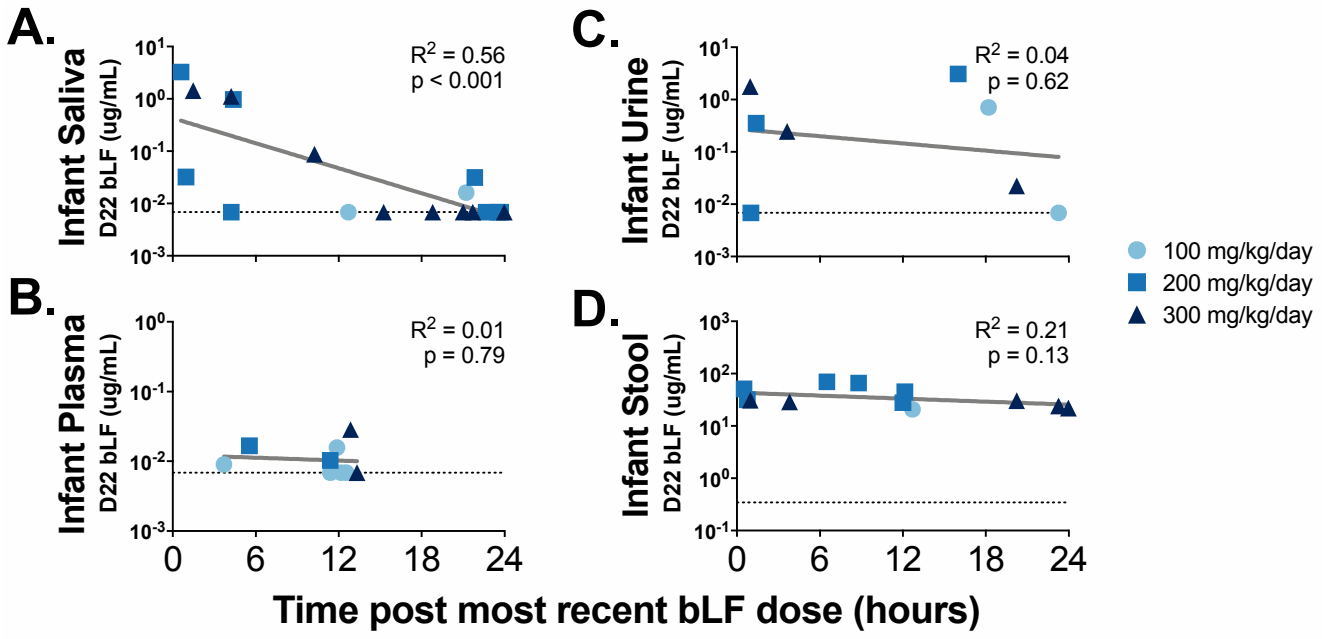

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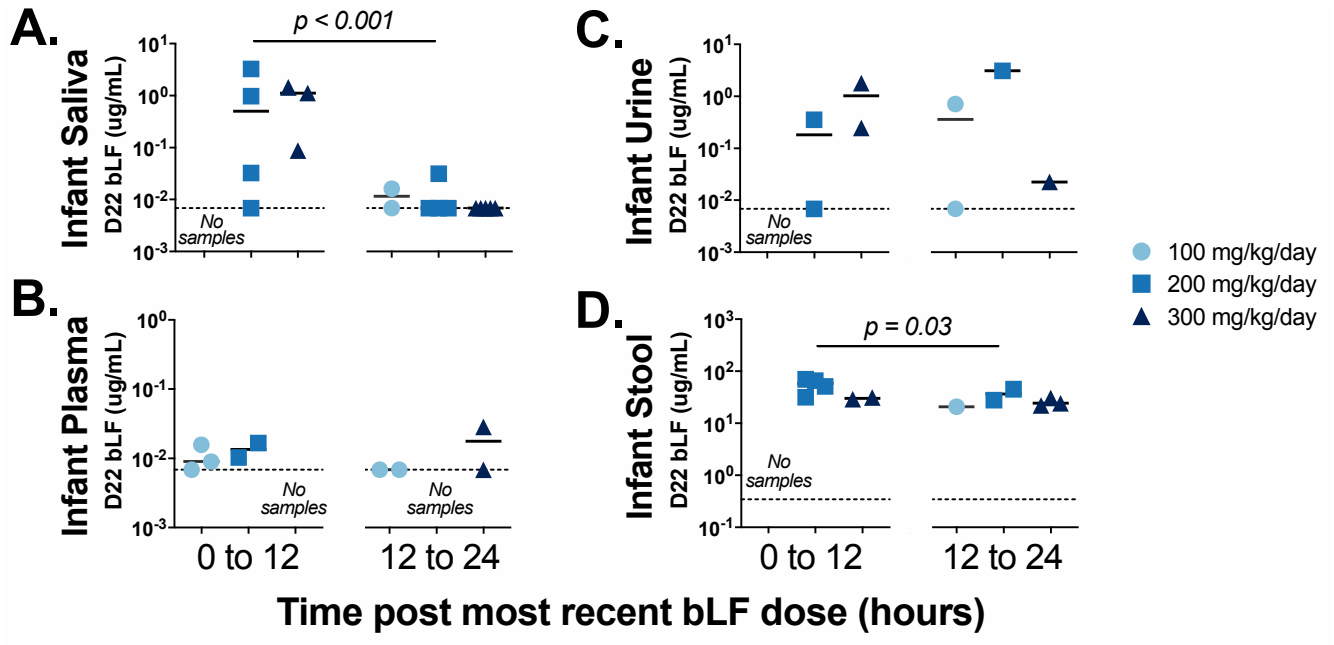

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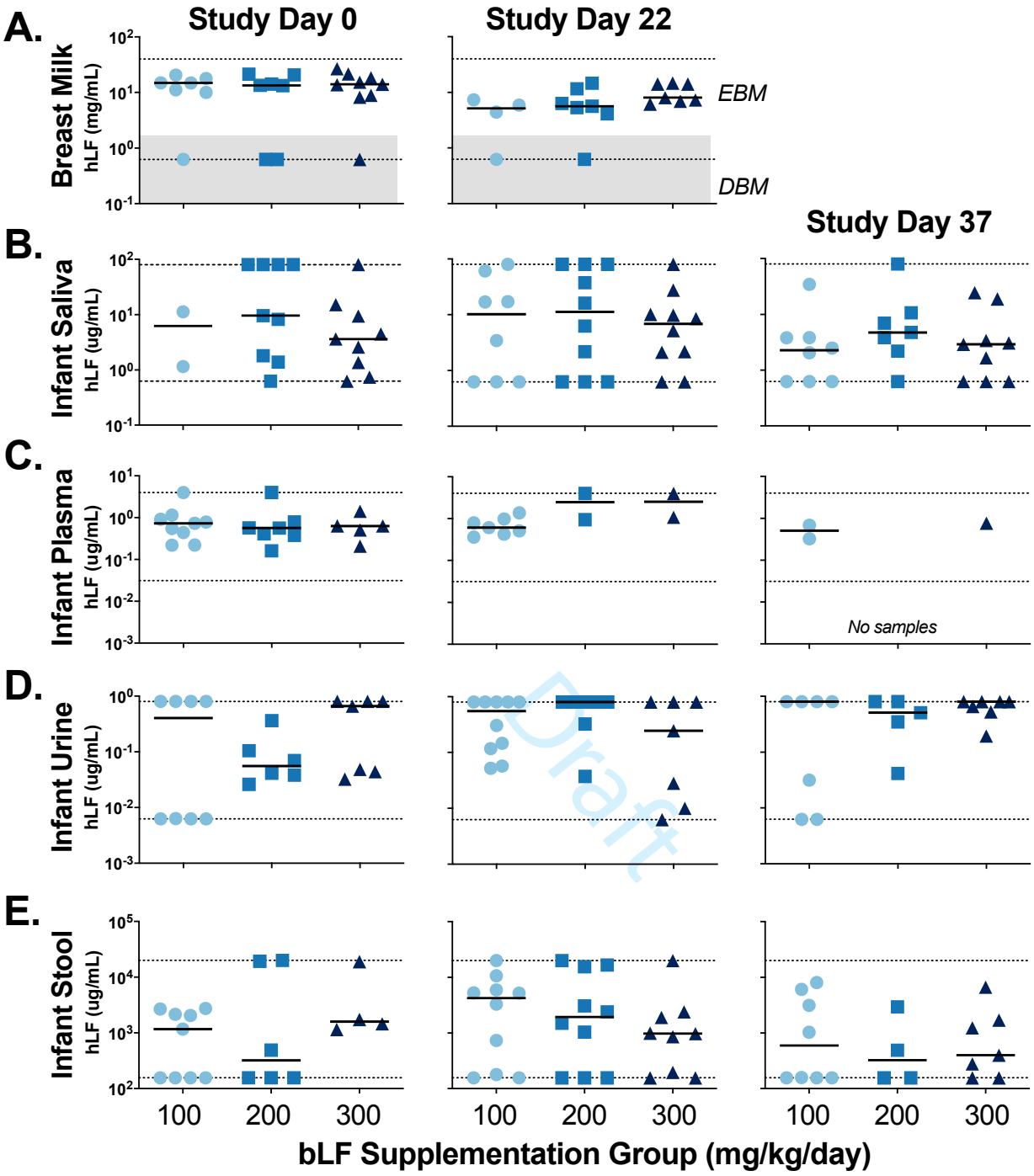

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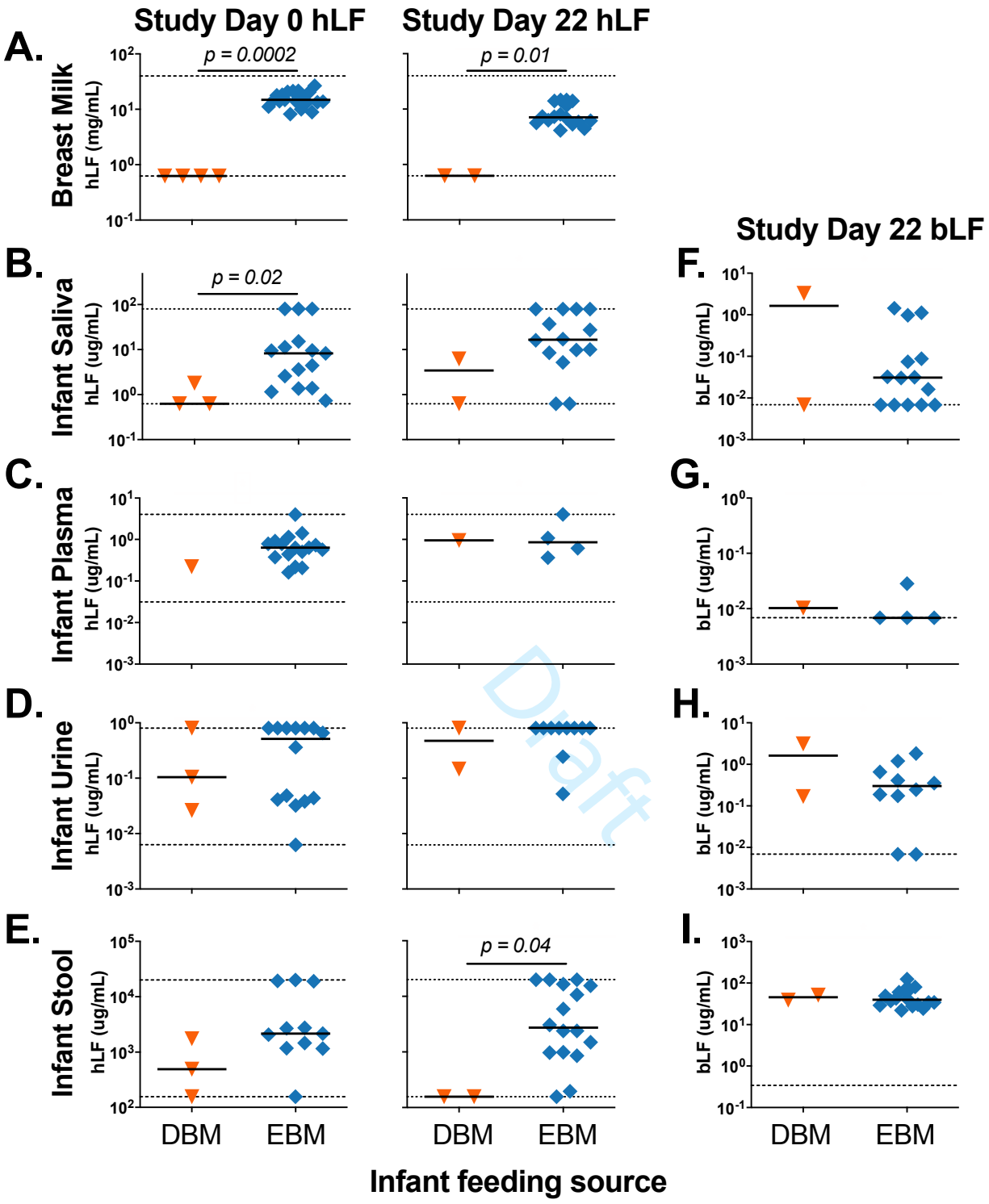

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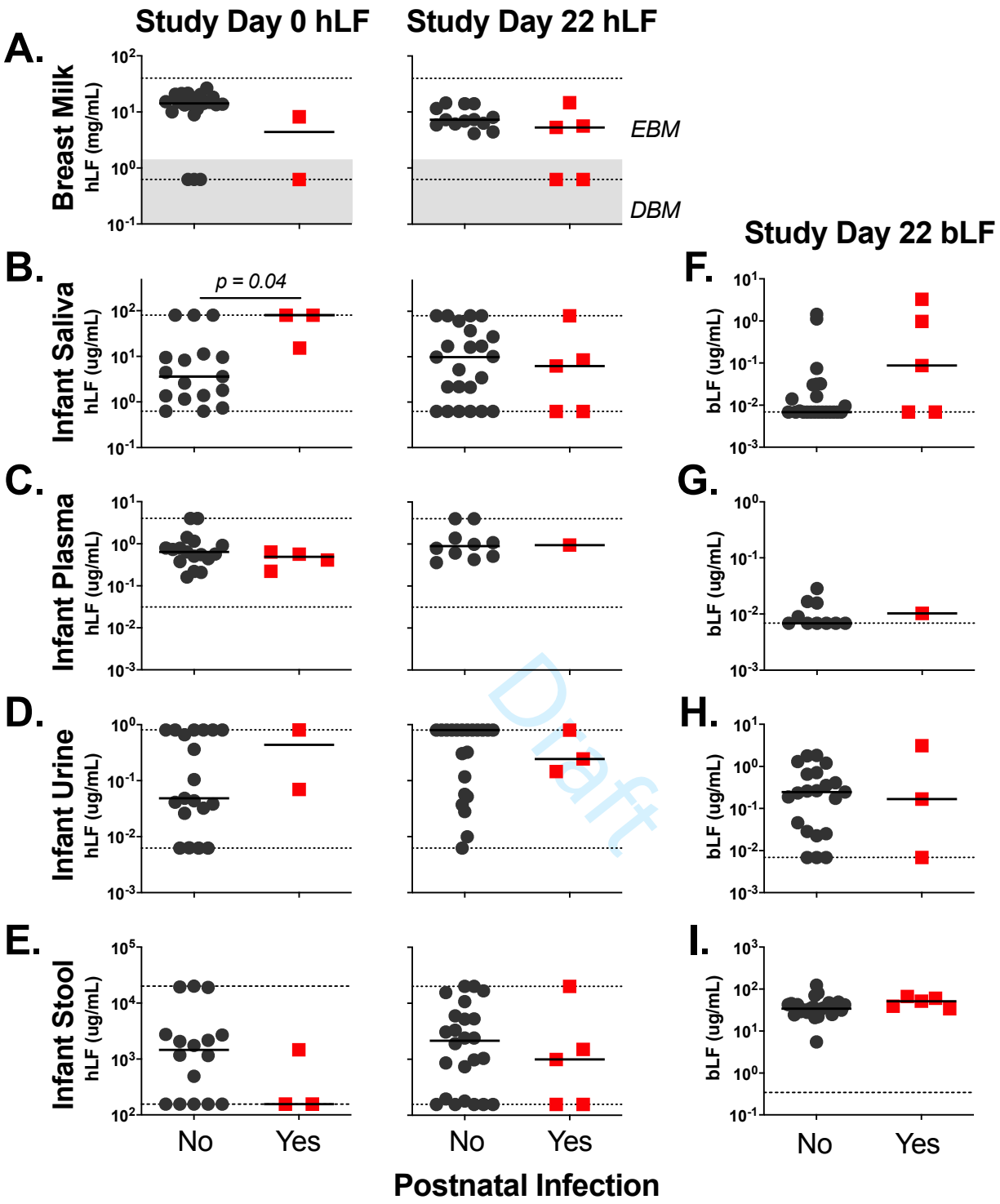

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