SUPPLEMENTAL INFORMATION

Supplementary Table 1. Incidence of postnatal CMV infection

Outcome or measure	Incidence[a]	95% CI[b]
Postnatal CMV infection in infants:	5.5 (33/596)	[4.0-7.7]
Cumulative incidence (%), week:		
4	1.8	[0.9-3.1]
8	6.1	[4.2-8.4]
12	7.3	[5.0-10.1]
- In infants born to CMV seronegative mothers	0.0 (0/139)	[0.0-1.9]
- In infants born to CMV seropositive mothers	7.2 (33/457)	[5.2-10.0]
Cumulative incidence (%), week:		
4	2.3	[1.2-4.1]
8	8.0	[5.5-11.1]
12	9.7	[6.7-13.3]
Postnatal CMV infection in infants, by source		
- Breastmilk transmitted[c]	12.4 (32/259)	[9.0-16.9]
Cumulative incidence (%), week:		
4	3.6	[1.8-6.5]
8	13.3	[9.1-18.2]
12	15.9	[11.0-21.7]
- Unknown source	0.2 (1/596)	[0.0-0.9]
CMV in breastmilk		
- CMV DNA lactia in CMV-seropositive mothers[d]	75.9 (220/290)	[70.6-80.4]
- CMV DNA lactia in CMV-seronegative mothers[e]	0.0 (0/92)	[0.0-4.0]

Abbreviations: CMV, cytomegalovirus; CI, confidence interval.

[a]Incidence reported as relative frequency with number of cases over total in parentheses. Cumulative incidence of CMV infection at given time points estimated from the cumulative incidence function for competing risks (CMV infection and death) as previously described.[1]

[b]CIs calculated using Wilson score method and are two-sided except in cases where incidence is zero wherein a one-sided upper limit confidence bound is given.

[c]Reported in 259 infants fed breast milk from 220 mothers (32 infants from multiple births) whose milk was CMV positive by NAT.

[d]325 of 392 CMV-seropositive mothers fed breast milk to their infants (83%). Breast milk samples were obtained for testing from 290 feeding mothers (89%).

[e]100 of 122 CMV-seronegative mothers fed breast milk to their infants (82%). Breast milk samples were obtained for testing from 92 feeding mothers (92%).

Supplementary Table 2. Estimates of breast milk viral load by maternal IgG categories and infant age

	Overall No. samples (No. mothers)	No. infants (No. mothers); Geometric Mean (95% CI) IU/mL	
IgG (dilution factor)		**Week 1-2**	**Week 3-7**
Low (100-200)	42 (20)	14 (11); 586 (258, 1330)	33 (17); 2182 (1122, 4244)
Medium (400)	41 (21)	14 (11); 537 (251, 1149)	23 (13); 2937 (1498, 5757)
High (800)	62 (30)	18 (12); 855 (421, 1738)	50 (25); 1669 (978, 2848)
Very high (1600)	20 (9)	4 (4); 612 (174, 2151)	18 (8); 1934 (744, 5029)

Overall, 165 breast milk samples were tested for IgG titer from 80 mothers. Breast milk samples for 7 mothers that tested negative for IgG are classified as 100.

CMV viral load values are analyzed with a repeated-measures model. Mean estimates and 95% CI were back transformed to the original scale and are reported as the geometric mean with 95% CI. IgG effect p=0.95; time effect p<0.001; IgG and time interaction effect p=0.37.

Supplementary Table 3. Characteristics of infants with postnatal CMV infection and NEC

GA (weeks)	Results of initial CMV NAT testing of blood or urine	Breastmilk CMV positive	Age at first detection of CMV in breastmilk (DOL)	Early estimate of postnatal CMV onset (DOL)[a]	Midpoint estimate of postnatal CMV onset (DOL)[a]	Onset of NEC (DOL)
27	No CMV detected	Yes	5	18	32	60
24	No CMV detected	Yes	5	19	36	30
26	No CMV detected	Yes	6	41	66	19
24	No CMV detected	Yes	6	19	28	26
29	No CMV detected	Yes	7	19	30	21
24	No CMV detected	Yes	20	5	12	20

Abbreviations: CMV, cytomegalovirus; NEC, necrotizing enterocolitis; GA, gestational age; NAT, nucleic acid testing; DOL, day of life; pCMV, postnatal CMV.

[a]Please refer to supplementary figure 1 for additional details.

Supplementary Table 4. Maximal breast milk viral load in first 14 days among infants with and without NEC

Group	N	Mean maximal CMV breast milk viral load (IU/mL)	Range (min - max IU/mL)
All mothers with detectable CMV in breastmilk during first 14 days of life[a]	136	8061	300 - 266,510
Mothers with infants who developed NEC	20	19,964	300 - 266,510
Mothers with infants who did not develop NEC	118	5919	300 - 176,600

Abbreviations: NEC, necrotizing enterocolitis; CMV, cytomegalovirus; SD, standard deviation.
[a]Two mothers with detectable CMV in breastmilk were considered in both groups as they both had two infants, one who developed NEC and one who did not.

Supplementary Table 5. Dose-response relationship between breast milk CMV viral load and NEC

Variable	Dose Exposure	CSHR For NEC (95% CI)[a]	P	Overall P[b]
Maximum CMV viral load in breast milk in first 14 days of life	≤ 1,003 IU/mL	Reference		0.04
	> 1,003-10,016 IU/mL	2.25 (0.98, 5.13)	0.05	
	> 10,016 IU/mL	3.29 (1.09, 9.89)	0.03	
Birthweight (per 100 g increase)		0.82 (0.69, 0.96)	0.01	
Center (CSHR not shown)			0.10	

Abbreviations: CMV, cytomegalovirus; NEC, necrotizing enterocolitis; CSHR, cause-specific hazard ratio; CI, confidence interval.
[a]Estimates are from multivariable analysis including all variables listed.
[b]Overall P reflects comparison of CMV viral load across 3 categorized groups.

Supplementary Table 6: Multivariable repeated measures analysis of breast milk CMV expression among seropositive mothers with infected and uninfected infants (n=220)

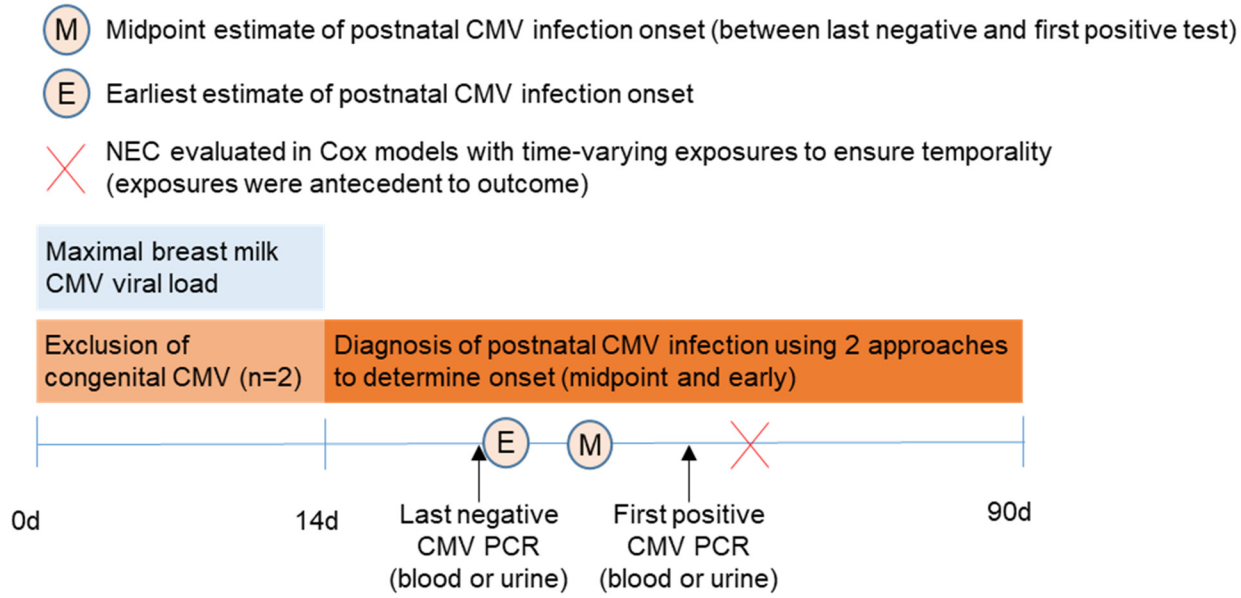
Variable	N (Samples)	Adjusted mean viral load (log_2, IU/mL) [95% CI]	BM-CMV NAT expression			% difference
			Geometric Mean	GMR (95% CI)	P	
Group					<0.001	
Transmitters	31 (78)	12.20 [11.41,12.99]	4708	2.95 (1.64, 5.29)		156%[b]
Non-transmitters	188 (393)[a]	10.64 [10.20,11.08]	1597			
Week (Postnatal age)					<0.001	
Week 1	96 (96)	9.63 [8.99,10.26]	790	Reference		
Weeks 2-3	132 (161)	11.70 [11.13,12.28]	3337	4.22 (2.74, 6.52)		208%
Weeks 4-5	116 (146)	12.52 [11.90,13.14]	5857	7.41 (4.63, 11.86)		289%
Weeks 5+	67 (69)	11.84 [11.18,12.50]	3661	4.63 (2.86, 7.51)		221%
Group by Week					0.63	
Week 1						
Transmitters	17 (17)	10.16 [9.08,11.24]	1141	2.09 (0.93, 4.66)	0.07	106%
Non-transmitters	79 (79)	9.10 [8.53, 9.66]	547			
Weeks 2-3						
Transmitters	20 (23)	12.50 [11.52,13.49]	5800	3.02 (1.45, 6.30)	0.003	160%
Non-transmitters	112 (138)	10.91 [10.40,11.41]	1920			
Weeks 4-5						
Transmitters	15 (19)	13.49 [12.40,14.57]	11483	3.84 (1.73, 8.55)	<0.001	194%[c]
Non-transmitters	101 (127)	11.55 [11.03,12.06]	2987			
Weeks 5+						
Transmitters	14 (16)	12.66 [11.54,13.77]	6465	3.12 (1.31, 7.40)	0.01	164%
Non-transmitters	53 (53)	11.02 [10.38,11.65]	2073			

Abbreviations: BM, breast milk; CMV, cytomegalovirus; VL, viral load; GMR, geometric mean ratio; CI, confidence interval.
[a]One non-transmitter mother with unknown rupture of membranes was not entered in the model due to missing data. Total of 471 observations for 219 mothers were used in the model.
[b]On average, BM CMV VL expression (IU/mL) since birth was approximately 156% higher in CMV seropositive mothers with infected infants compared to CMV seropositive mothers with uninfected infants.
[c]On average, BM CMV VL expression (IU/mL) at approximately 4-5 weeks after birth was 194% higher in CMV seropositive mothers with infected infants compared to CMV seropositive mothers with uninfected infants. The log_2 based percentage difference between the two means is: 100*13.49 - 100*11.55 = 194% (see supplementary methods for additional details and references).

Supplementary Table 6 (continued)

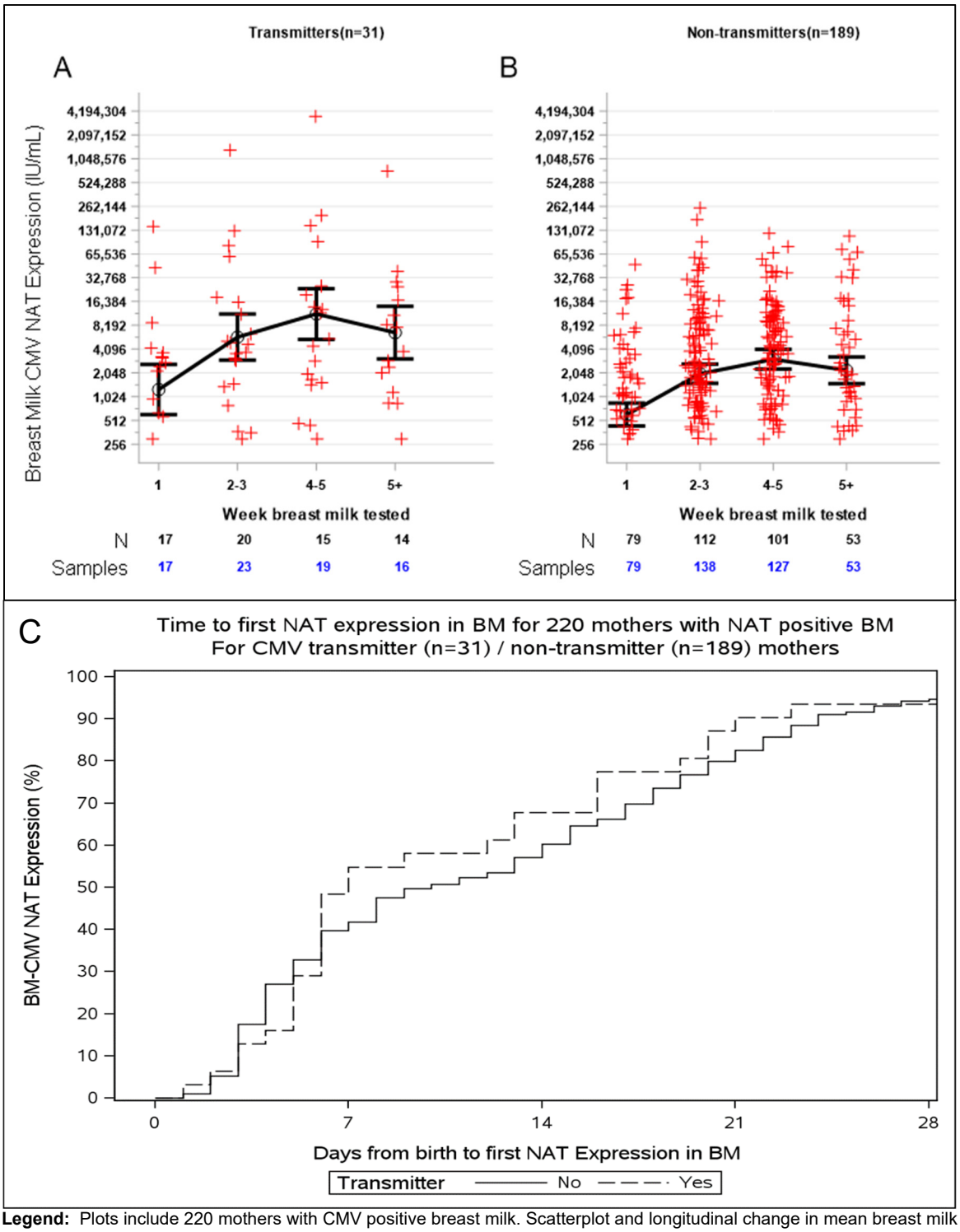
Variable	N (Samples)	Adjusted mean viral load (log_2, IU/mL) [95% CI]	BM-CMV NAT expression			% difference
			Geometric Mean	GMR (95% CI)	P	
Premature Rupture of Membranes					0.54	
Yes	89 (188)	11.33 [10.74,11.91]	2568	0.88 (0.57, 1.34)		-19%
No	130 (283)	11.52 [10.96,12.07]	2928			
Mother's race					0.75	
Black	141 (298)	11.47 [10.94,12.00]	2837	1.07 (0.71, 1.62)		10%
Non-Black	79 (174)	11.37 [10.77,11.97]	2651			
Parity					0.003	
1	118 (249)	11.87 [11.34,12.41]	3754	1.87 (1.24, 2.83)		91%
>1	101 (222)	10.97 [10.38,11.56]	2003			
Receipt of antenatal steroids					0.86	
Yes	181 (388)	11.39 [10.96,11.82]	2677	0.95 (0.56, 1.62)		-7%
No	38 (83)	11.46 [10.70,12.21]	2809			
Mother's age, years (Range 14-45)					0.02	
25		11.72 [11.18,12.25]	3363			
30		11.45 [10.97,11.93]	2795			
35		11.18 [10.66,11.70]	2323			
40		10.92 [10.28,11.55]	1932			
Mean age 31 years					0.02	
Transmitters	31 (78)	12.20 [11.41,12.99]	4708	2.95 (1.64, 5.29)		156%
Non-transmitters	188 (393)	10.64 [10.20,11.08]	1597			

Supplementary Figure 1. Schematic of assessment and model specification of study exposures and outcome.



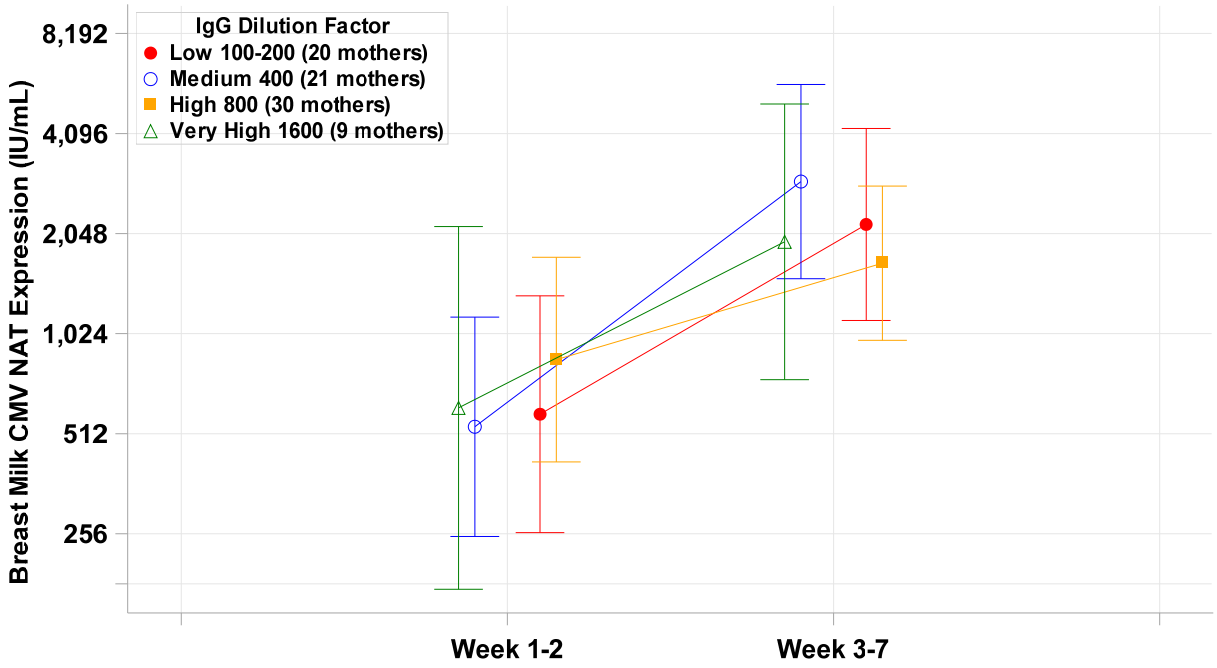
Legend: Postnatal CMV infection included as a dichotomous time-dependent covariate that can change, at most, once from uninfected to infected over 12 weeks of follow-up (i.e. once exposed always exposed). Two different approaches were used to determine the onset of postnatal CMV infection, as testing was not performed on a daily basis. The midpoint approach estimated onset based on the midpoint between the last negative and first positive test (M). The earliest estimate approach estimated onset based on the time following the last negative test (E). Both exposures were specified as time-dependent covariates, as noted above, and only NEC cases that occurred after the time-varying onset of infection were considered as events in the model.

Supplementary Figure 2. Breast milk viral load and time to first CMV detection in breast milk



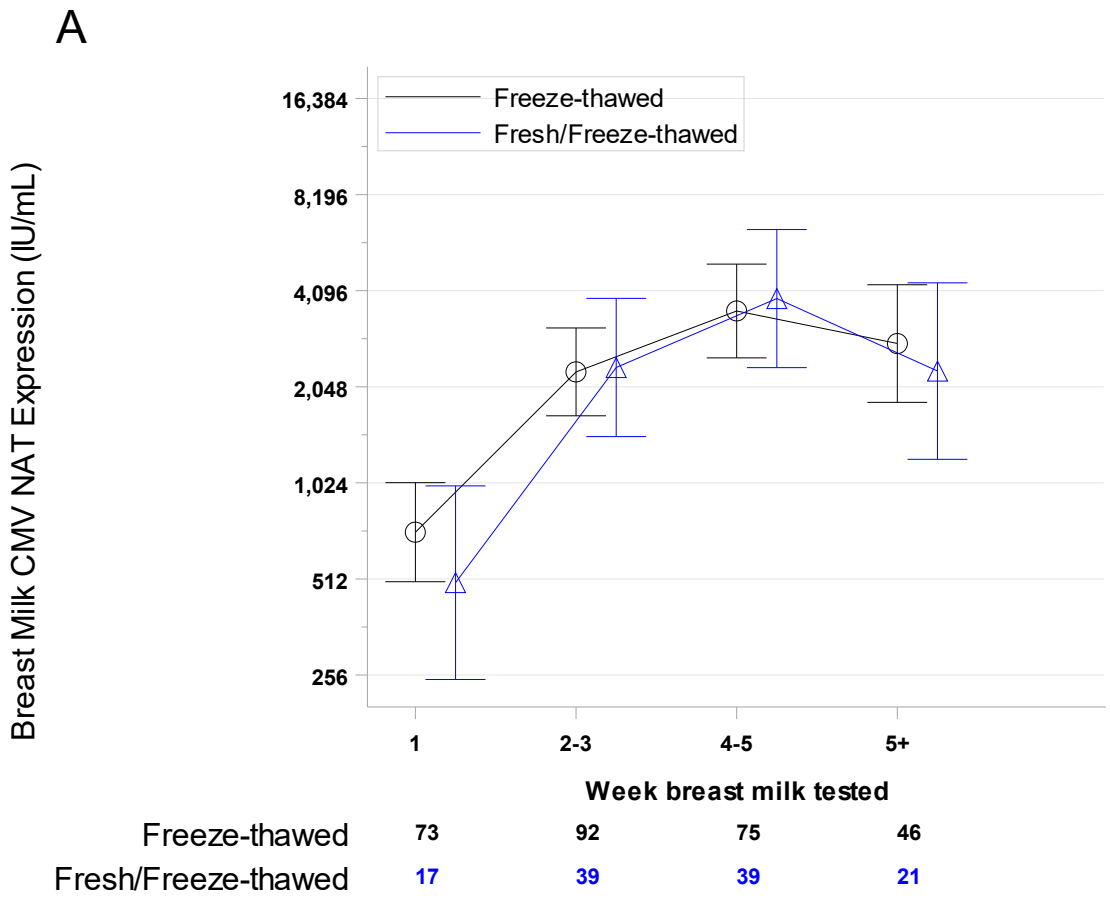
Legend: Plots include 220 mothers with CMV positive breast milk. Scatterplot and longitudinal change in mean breast milk CMV viral load expression in CMV transmitter (Panel A) and CMV non-transmitter mothers (Panel B); vertical bars indicate the 95% confidence intervals for the means. Comparison of mean breast milk CMV NAT expression for transmitter vs non-transmitters using longitudinal mixed model for repeated measures: week 1 (p=0.07); weeks 2-3 (p=0.004); weeks 4-5 (p=0.001); weeks 5+ (p=0.01). Time to first positive NAT by transmitter/non-transmitter mothers shown in Panel C.

Supplementary Figure 3. Breast milk viral load by IgG titer categories and time for CMV non-transmitter mothers (n=80)



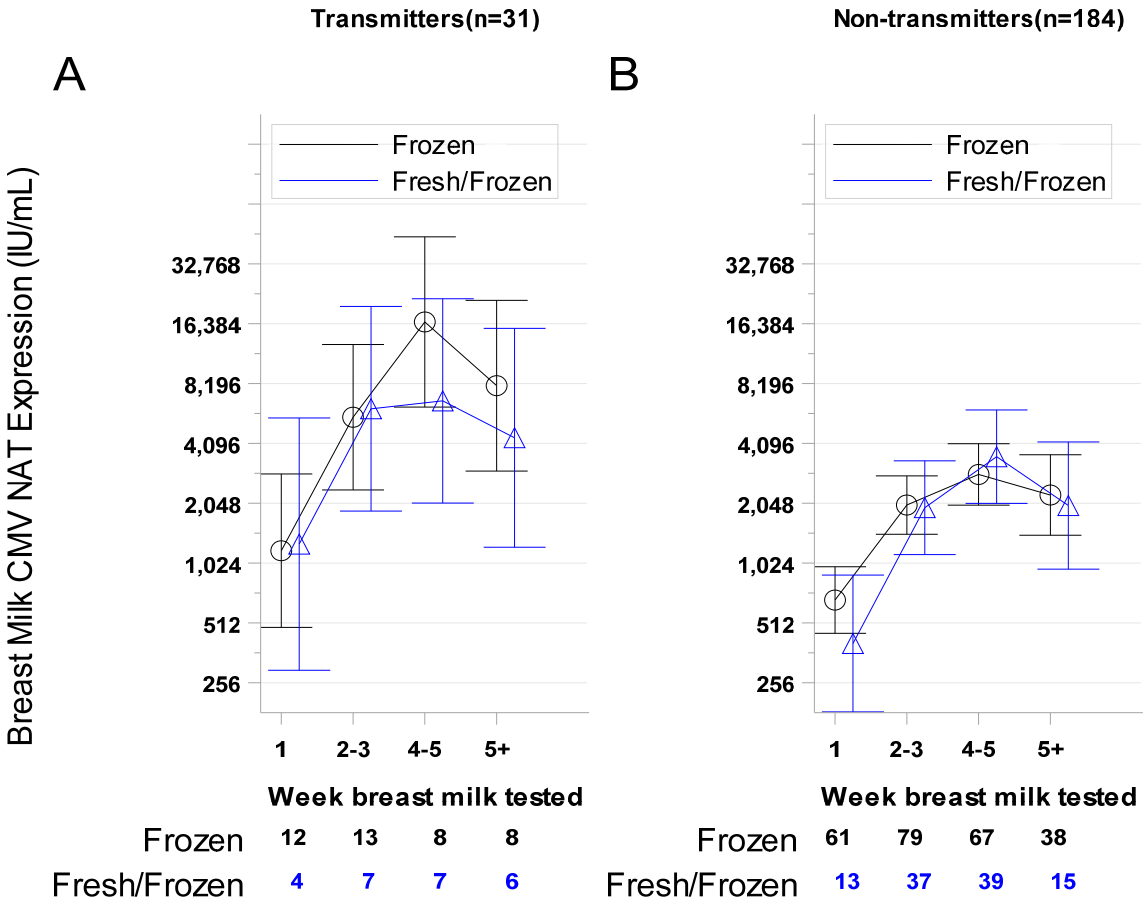
Legend: Longitudinal change in mean breast milk CMV NAT expression by IgG titer dilution factor for 80 non-transmitter mothers. Vertical bars indicate the 95% confidence intervals for the means.

Supplementary Figure 4. Breast milk CMV viral load among only freeze-thawed feeding vs. fresh/frozen feeding.



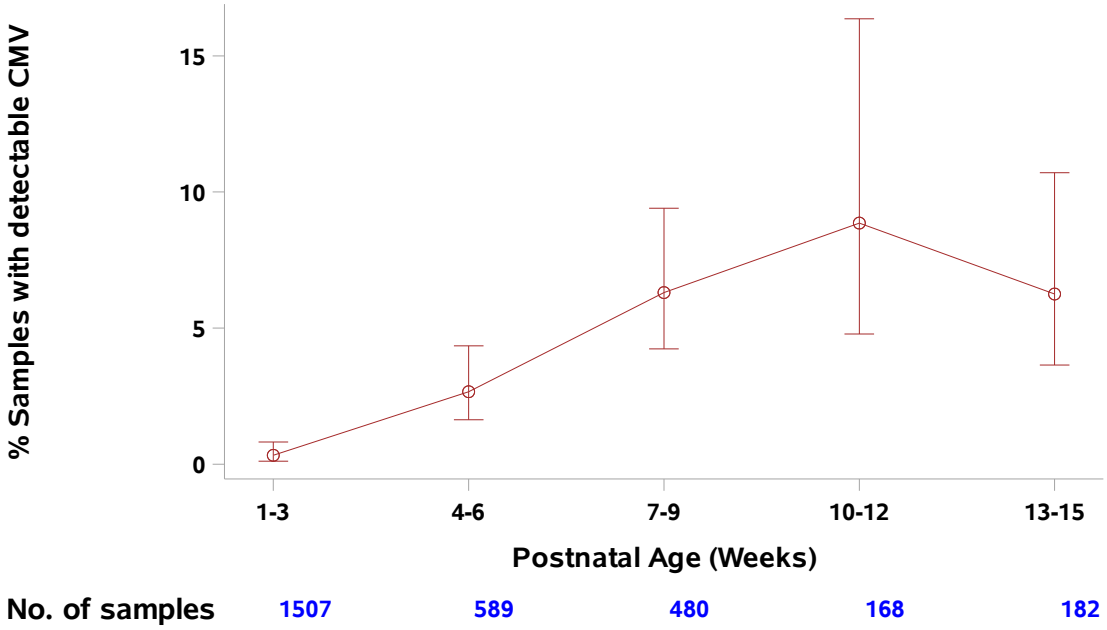
Legend: The figure depicts the breast milk CMV NAT expression over time (IU/ml) for milk obtained that was only freeze-thawed (n=156 mothers, 332 observations, black circles) or fresh and freeze-thawed (n=57 mothers, 130 observations, blue triangles). The milk type (freeze-thawed only vs. fresh and freeze-thawed) by time interaction was P=0.68. These data show no difference in viral load over time among only freeze-thawed breast milk vs. freeze-thawed and fresh milk. Two mothers not included in analysis (1 with missing data regarding milk type and the other feeding only fresh milk with no freeze-thawed milk). In analysis evaluating type of milk feeding as a risk factor for CMV infection in univariable Cox model, there was no evidence of a difference in postnatal CMV infection between infants fed only freeze-thawed milk (25 infants with postnatal CMV infection among 384 total) vs. infants fed both fresh and freeze-thawed milk (7 infants with postnatal CMV infection among 138 total): cause specific hazard ratio 1.35; 95% CI 0.59-3.11, P=0.48. Three infants who fed only fresh (unfrozen) breast milk, one of which developed postnatal CMV infection, were not included in analysis as too few to evaluate as a separate group from the fresh and freeze-thawed feeding group.

Supplementary Figure 5. Breast milk CMV viral load among only freeze-thawed feeding vs. fresh/frozen feeding to infants with and without postnatal CMV infection.



Legend: The figure depicts the breast milk CMV NAT expression over time (IU/ml) for milk obtained that was only freeze-thawed (frozen, black solid circles) or fresh and freeze-thawed (fresh/frozen, blue open squares) between mothers whose infants developed postnatal CMV infection (transmitters) and those without postnatal CMV infection (non-transmitters). The CMV status by milk type by time interaction P=0.74. These data show no evidence of a difference in viral load over time among freeze-thawed breast milk vs. fresh and freeze-thawed milk among both transmitter and non-transmitter groups. Two mothers not included in analysis (1 with missing data regarding milk type and the other feeding only fresh (unfrozen) milk).

Supplementary Figure 6. Longitudinal CMV detection in blood and urine samples



Postnatal Age (Weeks)	Postnatal Age (Days)	Number of Samples	Number of Blood Samples	Number of Urine Samples	Number of CMV positive tests	Number of infants with positive tests	Model based percentage	95% CI
1-3	0-21	1507	915	592	6	5	0.3	0.1, 0.8
4-6	22-42	589	522	67	12	11	2.7	1.7, 4.4
7-9	43-63	480	362	118	31	23	6.3	4.2, 9.4
10-12	64-84	168	81	87	18	9	8.9	4.8, 16.4
13-15	85-110	182	89	93	14	10	6.3	3.7, 10.7
		2926	1969	957	81	58		

Legend: The plot provides model-based estimates and 95% confidence intervals of the percentage of samples with detectable virus at each time interval. The analysis includes data from 2926 samples (1969 blood and 957 urine samples) from 596 infants (33 infants with CMV infection). The vertical bars are 95% confidence intervals.

Blood samples were collected for cytomegalovirus nucleic acid testing (CMV NAT) on day of birth, days 21, 40, 60, and at hospital discharge (when residual blood was available). Urine samples were collected for CMV NAT on day of birth and at hospital discharge. All subjects had at least one blood or urine sample tested using CMV NAT while on study; blood was tested for 594 infants (99.7%) and urine tested for 586 infants (98.3%).

Among 33 CMV NAT positive infants, 28 (84.8%) had positive blood results and 19 (57.5%) had positive urine results. Fourteen (2.3%) infants had positive blood results, only; 5 (0.8%) infants had positive urine results, only, and 14 infants (2.3%) had positive blood and urine results.

Supplementary Methods

CMV DNA testing of blood and breast milk

As previously described[1], CMV IgG/IgM-polyspecific antibodies in maternal blood were tested for with an US Food and Drug Administration–approved commercial serology assay (Immucor). Serum samples were tested for CMV IgM by enzyme-linked immunosorbent assay (Bio-Quant). Nucleic acid extraction for CMV NAT was performed using a commercial product (EZ1 Virus Mini Kit, version 2.0; Qiagen, Inc.). All assays were performed by following the manufacturers' protocols. Nucleic acid testing was performed with a polymerase chain reaction kit (Artus CMV TM, using the Roto-Gene instrument; Qiagen, Inc).[2] The polymerase chain reaction assay was validated on whole blood, urine, and breast milk samples and calibrated to the first World Health Organization international standard[3]. Newly diagnosed CMV in infants with a viral load of greater than 300 IU/mL was verified by repeating the first extraction as well as a new extraction. If there was an insufficient amount of a specimen for a second test, it was diluted 1:2. Any specimen that tested positive with a viral load of less than 300 IU/mL was repeated in duplicate and reported as low positive (<300 IU/mL). Specimens discordant on a second test were reported as indeterminate. Prior to testing, breast milk samples were stored at 4°C for up to 7 days and at −20°C for long-term storage. A study from our group demonstrated stability of CMV DNA in breast milk for 28 days at 4 °C and 90 days at -20 °C[4]. Blood samples were stored at 4°C and tested within 7 days of collection.

CMV serologic testing

Additional serologic testing with quantification of CMV IgG titers among seropositive mothers to determine the relationship between IgG titers and breast milk CMV viral load was performed at the Centers for Disease Control and Prevention in Atlanta, Georgia as previously described[5]. This quantitative serologic testing was performed in addition to initial qualitative assessment of CMV maternal serology performed by the primary study as previously described[1].

Statistical methods for the analysis of breast milk CMV expression

A repeated-measures analysis of the $\log_2$ breast milk CMV expression was performed with a means model via the SAS MIXED Procedure (version 9.4; SAS Institute, Cary, NC), providing separate estimates of mean breast milk CMV expression by postnatal age (weeks 1, 2-3, 4-5 and 5+ weeks) and transmitter group (CMV transmitter or CMV non-transmitter mothers). The model included 3 predictors: 1) CMV transmitter group; 2) postnatal age (categorical) and 3) the statistical interaction between CMV transmitter group and postnatal age. A compound-symmetric variance-covariance form in repeated measurements was assumed for CMV expression and robust estimates of the parameter standard errors were used to perform statistical tests and construct 95% confidence intervals[6]. The model-based means are unbiased with unbalanced and missing data, as long as missing data are non-informative. A P-value <0.05 was considered statistically significant for the main effects (CMV transmitter group and postnatal age) and for the CMV transmitter group by postnatal age interaction effect from the repeated-measures analysis.

After the univariable repeated–measures analysis, the $\log_2$ breast milk CMV expression mean and its 95% confidence interval (CI) were back transformed to the original scale and reported as the geometric mean with 95% CI. Similarly, back transformation of the difference between the mean of $\log_2$-transformed breast milk CMV expression among CMV transmitter mothers and CMV non-transmitter mothers yielded the ratio of the geometric means. Confidence intervals for the geometric mean ratio were computed by back transforming the 95% confidence bounds for the mean difference. A geometric mean ratio of 3.0 suggests the breast milk CMV expression in one group (e.g. CMV transmitter mothers) is 3 times that of another group (e.g. CMV non-transmitter mothers).

For multivariable analysis of breast milk CMV expression, the statistical model was adjusted simultaneously for CMV transmitter group, postnatal age, the statistical interaction between CMV transmitter group and postnatal age and 5 additional baseline covariates (mother's race, mother's age, premature rupture of membranes, parity and receipt of antenatal steroids). The geometric mean and its 95% confidence interval and the geometric mean ratio and its 95% confidence interval were calculated for each covariate in the presence of others in the final model. Percentage differences were also reported.[7,8]

References for Online-only Supplementary Materials

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