Cytomegalovirus proteins, maternal pregnancy cytokines, and their impact on neonatal immune cytokine profiles and acute lymphoblastic leukemogenesis in children

Early cytomegalovirus (CMV) infection and altered cytokine profiles at birth are associated with risk of childhood acute lymphoblastic leukemia (ALL).[1-5] We examined neonatal cytokine levels and CMV proteins in 130 children who contracted ALL later in life and 460 controls. We assessed the immunodominant viral coat protein (pp65) and CMV proteins that manipulate human immune function (CMV-IL-10, CMV-CXCL-1), which were detectable in most neonatal samples and correlated with specific cytokine levels (IL-10, IL12, TGF-β1, and TNFα) CMV-IL-10 was positively associated with ALL risk. Neonatal cytokines, analyzed as a principal component loaded by IL-10, IL-12, and TNFα levels, were significantly different between cases and controls. Maternal mid-pregnancy cytokine expression was weakly correlated with cytokines at birth but did not differentiate childhood ALL cases and controls. In sum, the data provide preliminary indications that CMV viral activity during pregnancy may influence the neonatal cytokine profiles linked to risk of childhood ALL.

Maternal mid-pregnancy samples and matched neonatal blood spots from five California counties were obtained from the California Biobank.[6] The study includes 137 cases born between November 1999 and 2009 and diagnosed with childhood ALL at the age of 0-14 years. Controls were frequency matched to cases on year of birth, sex, and race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, Asian/Pacific Islander, or other). Two 4.7-mm blood spot punches were treated to 160 mL of extraction buffer as described previously.[1] Extracts were randomized to 96-well plates with each plate containing similar proportions of cases and controls and racial/ethnic groups. Twelve cytokines – IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12p70, GM-CSF, TNFα, VEGF, and IFN-γ, were measured using a Luminex bead–based assay (R&D Systems) incorporating calibration standards, quality control samples, and blanks. Transforming growth factor (TGF-β1) and arginase-2 were measured individually.[7] Whole aqueous maternal sera were analyzed for cytokines by Luminex.

CMV proteins were measured using customized Luminex assays. Three capture antibodies (CMV-IL-10: AF117, R&D systems, Minneapolis, MN;[8] pp65: OAMA00562, Aviva systems Biology Corp. San Diego, CA; UL146/vCXC1: AF620, R&D systems, Minneapolis, MN) were coupled to Luminex microspheres (Bio-rad kit). CMV protein standards (CMV-IL-10:117-VL-025, R&D systems; pp65: CV001-1, Virusys Corp; UL146/vCXC1: 620-CM, R&D Systems) and blood spot extracts (10 uL/sample/test) were incubated with corresponding microspheres (5 uL/sample) at room temperature using a Curiox wash station, followed by 10 uL of 1:1,000 diluted biotinylated anti-CMV proteins antibodies (CMV-IL-10: BAF117, R&D systems; pp65: DPATB-H83463, Creative Diagnostics, New York, NY; UL146/vCXV1: BAF620, R&D Systems) and streptavidin-conjugated R-phycoerythrin 1:100 diluted stock (Bio-Rad Laboratories, Inc.). Luminex measurements were converted to concentrations using a standard protein curve, and run in duplicate and averaged. For measurements that were below the level of detection, levels were assigned as one half the lowest level of detection.

Raw data were adjusted for batch, age of the blood spot, and the level of protein extracted. Variance Stabilizing Normalization (VSN)[9] plus ComBat[10] was used to preprocess and calibrate samples. Seven cases and 40 controls were excluded (due to quality control or technical failure), resulting in 130 cases and 460 controls for the final analysis. Pearson correlation coefficients were calculated among neonatal and maternal cytokines and CMV proteins. Multivariable logistic regression models were utilized to assess associations between neonatal and maternal cytokines, CMV proteins, arginase-2 and risk of childhood ALL.

Most birth characteristics were not significantly different between cases and controls, apart from the frequency of cesarean section which was higher among cases (P=0.04; *Online Supplementary Table S1*), compatible with its status as a known risk factor for ALL.[11] Cytokines exhibited extensive correlation in children and with CMV proteins (Figure 1). CMV-IL-10 was inversely correlated with human IL-10, as well as IL-12p70, TNF-α, VEGF, arginase-2, and weakly inversely correlated with the other CMV-derived cytokine CMV-CXC-1. CMV-pp65, the coat protein of the CMV virus itself, exhibited similar correlations as CMV-IL-10. CMV-CXC-1 demonstrated significant inverse association with TGF-β1 which was not observed for the other CMV proteins (Figure 1).

While some correlations between neonatal cytokines approached rho=0.6 (Figure 1), all correlations between maternal cytokines in mid-pregnancy and child cytokines at
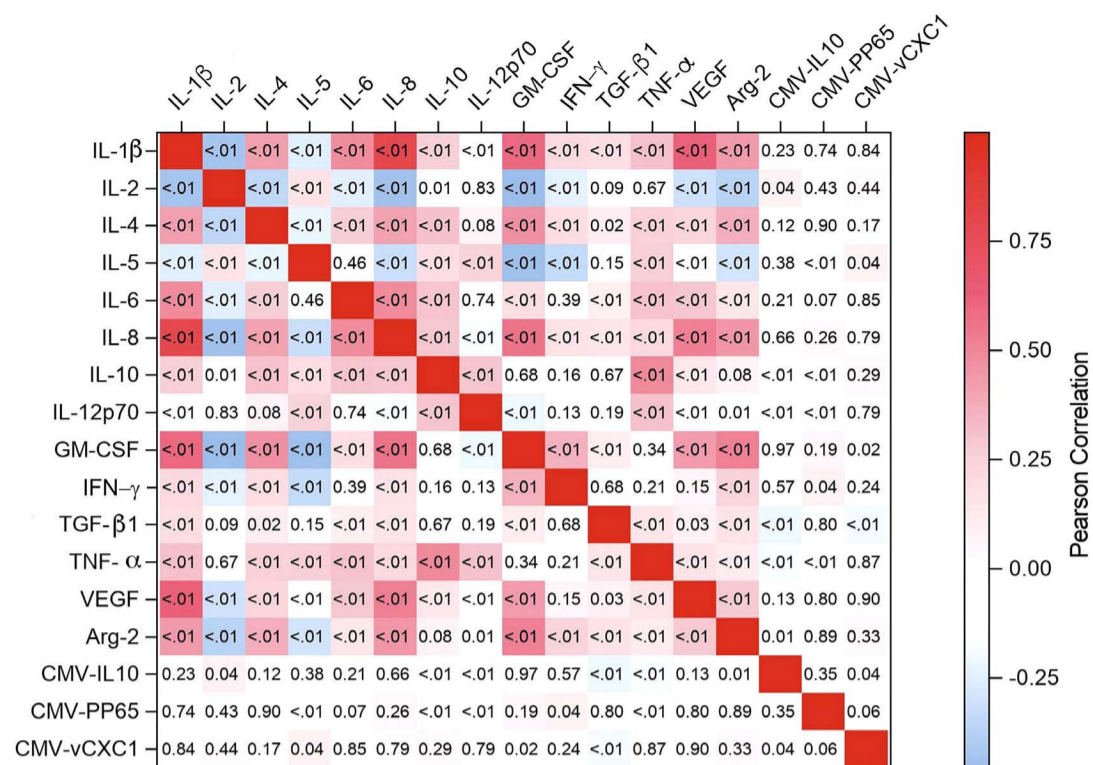
Figure 1. Pearson correlation matrix of cytokines and cytomegalovirus proteins at birth of all neonatal study subjects (n=590: 130 cases and 460 controls). The color of each square is indicative of the coefficient (noted on the scale) and the *P*-value is noted numerically. Correlations were similar among cases and control strata, and the entire sample set is displayed. The color scale is balanced towards positive correlations (red) since strong anti-correlations were less apparent.

birth were lower than absolute value of rho=0.21 (Figure 2). Many maternal-child correlations were significant with some notable differences between cases and controls (Figure 2). For instance, case-only significant positive correlations were apparent between maternal IL-6 and neonatal CMV-pp65, maternal IL-1β and neonatal CMV-CXC-1, and maternal IFN-γ and neonatal TNF-α; and inverse correlations between maternal TNF-α and neonatal TNF-αand CMV-CXC1 (Figure 2).

Like prior reports, neonatal cytokine levels were associated with case/control status. When assessed individually, IL-1β, IL-2, IL-8, and GM-CSF exhibited nominally significant associations, where only GM-CSF remained significant (odds ratio [OR] =2.38, 95% confidence interval [CI]: 1.11-5.13, comparing the third tertile to the first) in the multivariable model. When analyzed as a continuous variable, CMV-IL-10 was significantly associated with case/control status, with higher CMV-IL-10 levels linked to an increased risk of childhood ALL (OR=1.27, 95% CI: 1.01-1.59; *Online Supplementary Table S2*). Because of the high level of correlations between protein markers, we constructed summary independent variables using principal components (PC). About 60% of the variance was explained by five PC (*Online Supplementry Table S3*). The first PC, composed of IL-10, IL-12, and TNF-α, was significantly positively associated with ALL risk (Table 1; *Online Supplementary Table S3*). Principal components that were described by CMV proteins CMV-CXC-1 and pp65 (PC7 and 8) were not associated with ALL risk; however, PC9 which was predominantly loaded with CMV-IL-10 was associated with increased ALL risk (OR=1.24, 95% CI :1.01-1.54; *P*=0.04

as continuous measure, Table 1). Maternal cytokines were not related to case/control status (data not shown). We evaluated whether neonatal cytokines and CMV proteins were associated with birth characteristics, while controlling for year of birth, sex, race/ethnicity, and case-control status. Nominally significant (*P*<0.01) associations (in a positive [+], or inverse [-] direction) were apparent between IL-6 and birthweight (+), IL-1β, IL-4, and IL-8 and birth order (all -), IL-5 (+) and IFN-γ (-) with male sex, and arginase-2 (+) with cesarean section (data not shown).

In this study, cytokines and other immune markers measured at birth are associated with leukemia status later in childhood, a result supported by four previous reports.[1,3,4,7] In addition, a cytokine produced by CMV, CMV-IL-10, is associated in a positive way with ALL status. This CMV-encoded protein is 27% homologous to human IL-10 and binds with high affinity to the IL-10 receptor.[12] CMV-IL-10 was inversely correlated with human IL-10, suggesting possible feedback control, which is intriguing considering the inverse association between human IL-10 and ALL risk using a more sensitive assay performed in a prior study on a similar California-based population.[1]

We found CMV proteins in most (~90%) neonatal samples, including samples from controls. CMV infection is clinically apparent in 1 in 300 in newborns; 90% of CMV-positive neonates are clinically silent.[13] Our prior study found CMV DNA sequence in 3% of healthy California-born children and 9% in those who later contracted ALL.[2] Population prevalence of CMV in women of reproductive years is 40-60% in Western countries, and 80-100% in low resource rural areas and developing countries;[14] therefore,
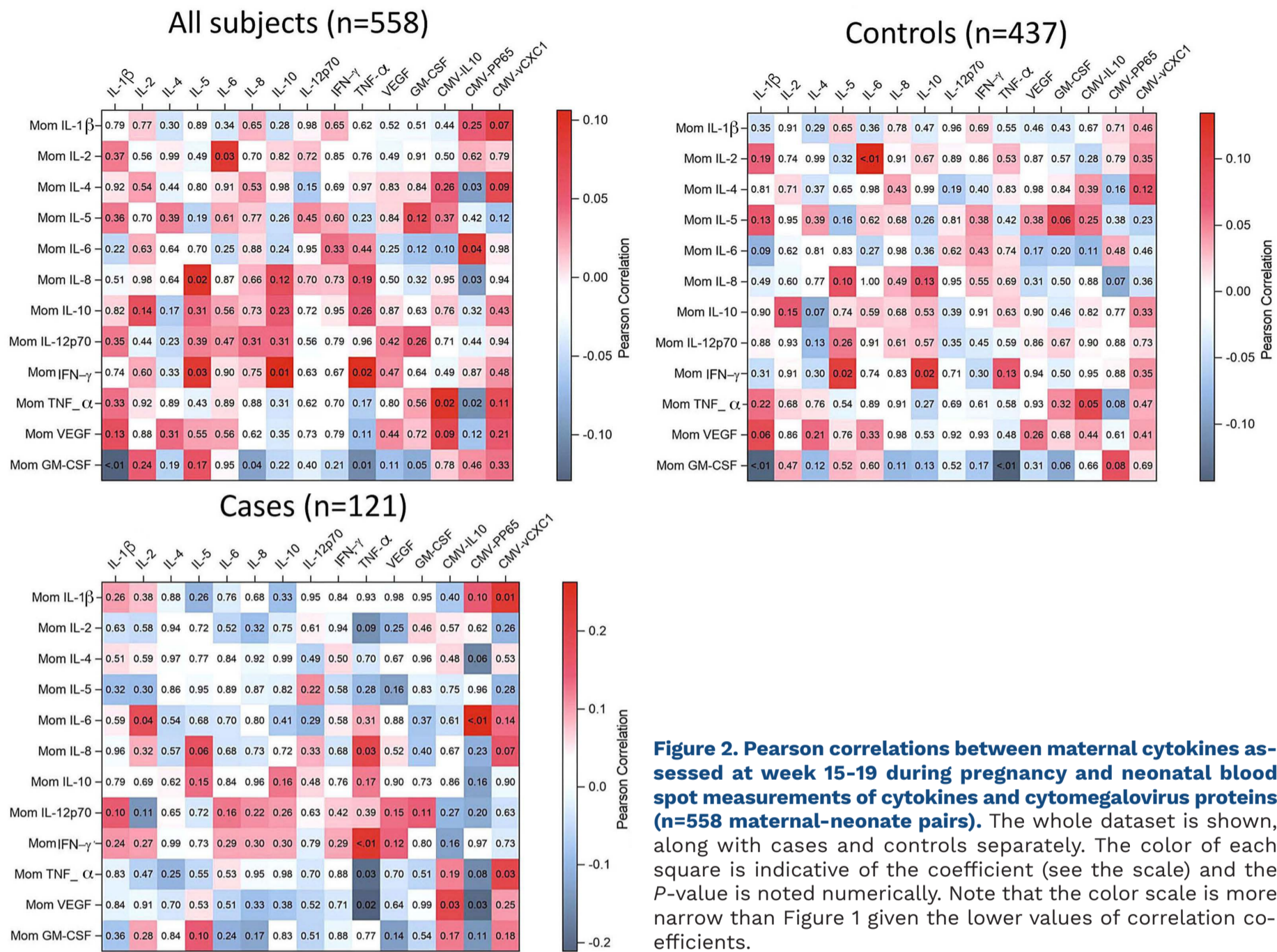
Figure 2. Pearson correlations between maternal cytokines assessed at week 15-19 during pregnancy and neonatal blood spot measurements of cytokines and cytomegalovirus proteins (n=558 maternal-neonate pairs). The whole dataset is shown, along with cases and controls separately. The color of each square is indicative of the coefficient (see the scale) and the *P*-value is noted numerically. Note that the color scale is more narrow than Figure 1 given the lower values of correlation coefficients.

Table 1. Relationship of cytokine/cytomegalovirus protein derived principal components to risk of childhood acute lymphoblastic leukemia.

PC	Continuous[a]		PC loadings[b]
	OR (95% CI)	*P*	
PC1	1.25 (1.02-1.54)	0.04	IL-10, IL-12p70, TNF-α
PC2	0.96 (0.77-1.18)	0.68	IL-1b, VEGF
PC3	1.01 (0.82-1.24)	0.96	IL-4, ARG2
PC4	0.94 (0.76-1.16)	0.58	IL-6
PC5	1.04 (0.85-1.28)	0.69	TGF-β
PC6	0.99 (0.81-1.20)	0.89	IFN-γ
PC7	0.99 (0.80-1.23)	0.95	CMV-CXC1
PC8	1.00 (0.82-1.22)	0.97	CMV-p65
PC9	1.24 (1.01-1.53)	0.04	CMV-IL-10
PC10	1.14 (0.92-1.40)	0.23	IL-2

[a]Overall risk (OR) for each unit of principal components (PC) increase. All models adjusted for age at collection, year of birth, weight (≥3,500 grams), gestational age (26-26, 27-41, 42-44 weeks, unknown) plurality (single *vs.* multiple), birth order, mode of delivery (vaginal *vs.* cesarean), mother's age at delivery (≤24, 25-34, ≥35 years), and mother's birthplace (United States *vs.* other), and all other PC. [b]Immune factors which contributed most variance to each PC. Factors are listed when they are correlated more than rho=0.6 with each PC noted (see *Online Supplementarty Table S4*).

our detection rate of CMV proteins in the current study mirrors this maternal prevalence rather than the newborn. Proteins cross the placental barrier by active transport as well as passive diffusion, the latter at a rate equivalent to the concentration of the protein.[15]

Accepting the source of CMV proteins from the pregnant mother, the correlative structure of proteins assessed here implicate that CMV infection of the mother impacts immune development of the fetus and may be the source of cytokine alterations at birth that distinguish ALL cases from controls in this and prior studies.[1,3,4,7] Significant correlations between maternal cytokines and neonatal CMV proteins (and neonatal cytokines) suggest direct or indirect manipulation of immune function, even in the absence of primary CMV infection of the neonate. Most notable here is the higher level of CMV-IL-10 found in neonates who grew up to be cases compared to controls who remained healthy. This cytokine interacts directly with the human IL-10 receptor, but is only one of several CMV genes that manipulate the immune system. The presence of CMV-IL-10 protein as a risk factor for ALL requires more analysis to examine whether it is simply a marker of primary CMV infection or is itself the factor that alters neonatal immunity impacting risk of ALL, and therefore pinpoints maternal CMV activity as consequential to ALL risk in the offspring.

To conclude, our results suggest that CMV infection is responsible at least in part for the neonatal cytokine profiles that are associated with risk of childhood ALL. Our results should be considered preliminary as the findings will not meet the more stringent threshold for statistical significance with correction for multiple comparisons, hence the potential for false discovery. CMV is, however, the first specific target for ALL prevention and potentially treatment, and its role in the pathogenesis of childhood ALL and prevention deserves further examination.

Authors

Joseph L. Wiemels,[1] Rong Wang,[2] Mi Zhou,[3] Helen Hansen,[3] Rachel Gallant,[1,4] Junghyun Jung,[1] Nicholas Mancuso,[1] Adam J. de Smith,[1] Catherine Metayer,[5] Scott C. Kogan[3] and Xiaomei Ma[2]

[1]Center for Genetic Epidemiology, Norris Comprehensive Cancer Center, and Department of Population and Public Health Sciences, University of Southern California, Los Angeles, CA; [2]Department of Chronic Disease Epidemiology, Yale School of Public Health, Yale University, New Haven, CT; [3]School of Medicine; University of California San Francisco, San Francisco, CA; [4]Children's Hospital Los Angeles, Los Angeles, CA and [5]School of Public Health, University of California, Berkeley, Berkeley, CA, USA

Correspondence:
J.L. WIEMELS – wiemels@usc.edu

Disclosures
No conflicts of interest to disclose.

Contributions
JLW and XM designed the research with assistance from CM, obtained the funding, and wrote the manuscript; RW performed the statistical analysis; MZ designed laboratory assays and with HH performed the laboratory measurements; RG, JJ, NM, AJdS, and SCG assisted with analysis and interpretation. All authors reviewed and approved the manuscript.

Data-sharing statement
We are prohibited by California statutes from publicly sharing data that are derived from biospecimens obtained from the California Biobank. We welcome questions from other investigators or request for additional analyses that are pertinent to the data presented in this Letter, and potential data sharing when permitted by the California Health and Human Services Agency Committee for the Protection of Human Subjects.

References

1. Chang JS, Zhou M, Buffler PA, Chokkalingam AP, Metayer C, Wiemels JL. Profound deficit of IL10 at birth in children who develop childhood acute lymphoblastic leukemia. Cancer Epidemiol Biomarkers Prev. 2011;20(8):1736-1740.
2. Francis SS, Wallace AD, Wendt GA, et al. In utero cytomegalovirus infection and development of childhood acute lymphoblastic leukemia. Blood. 2017;129(12):1680-1684.
3. Sooregard SH, Rostgaard K, Skogstrand K, Wiemels J, Schmiegelow K, Hjalgrim H. Neonatal inflammatory markers in children later diagnosed with B-cell precursor acute lymphoblastic leukaemia. Cancer Res. 2018;78(18):5458-5463.
4. Whitehead TP, Wiemels JL, Zhou M, et al. Cytokine levels at birth in children who developed acute lymphoblastic leukemia. Cancer Epidemiol Biomarkers Prev. 2021;30(8):1526-1535.
5. Wiemels JL, Talback M, Francis S, Feychting M. Early infection with cytomegalovirus and risk of childhood hematologic malignancies. Cancer Epidemiol Biomarkers Prev. 2019;(6):1024-1027.
6. Kharrazi M, Pearl M, Yang J, et al. California Very Preterm Birth Study: design and characteristics of the population- and biospecimen bank-based nested case-control study. Paediatr Perinat Epidemiol. 2012;(3):250-263.
7. Nielsen AB, Zhou M, de Smith AJ, et al. Increased neonatal level of arginase 2 in cases of childhood acute lymphoblastic leukemia implicates immunosuppression in the etiology. Haematologica. 2019;104(11):e514-e516.
8. Young VP, Mariano MC, Faure L, Spencer JV. Detection of cytomegalovirus interleukin 10 (cmvIL-10) by enzyme-linked immunosorbent assay (ELISA). Methods Mol Biol. 2021;2244:291-299.
9. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics. 2002;18(Suppl 1):S96-104.
10. Johnson WE, Li C, Rabinovic A. Adjusting batch effects in microarray expression data using empirical Bayes methods. Biostatistics. 2007; 8(1):118-127.
11. Marcotte EL, Thomopoulos TP, Infante-Rivard C, et al. Caesarean delivery and risk of childhood leukaemia: a pooled analysis from the Childhood Leukemia International Consortium (CLIC). Lancet Haematol. 2016;3(4):e176-185.
12. Kotenko SV, Saccani S, Izotova LS, Mirochnitchenko OV, Pestka S. Human cytomegalovirus harbors its own unique IL-10 homolog (cmvIL-10). Proc Natl Acad Sci U S A. 2000;97(4):1695-1700.
13. Manicklal S, Emery VC, Lazzarotto T, Boppana SB, Gupta RK. The "silent" global burden of congenital cytomegalovirus. Clin Microbiol Rev. 2013;26(1):86-102.
14. Buxmann H, Hamprecht K, Meyer-Wittkopf M, Friese K. Primary human cytomegalovirus (HCMV) infection in pregnancy. Dtsch Arztebl Int. 2017;114(4):45-52.
15. Malek A, Sager R, Schneider H. Transport of proteins across the human placenta. Am J Reprod Immunol. 1998;40(5):347-351.