

Increased neonatal level of arginase 2 in cases of childhood acute lymphoblastic leukemia implicates immunosuppression in the etiology

Acute lymphoblastic leukemia (ALL) afflicts 2,250 children (0-14 years) diagnosed annually in the USA.¹ Modern treatment regimens cure approximately 90% of those affected, but survivors suffer from long-term sequelae.² Epidemiological evidence regarding the occurrence of ALL points to roles for infection and immune development.³ Clinically diagnosed infections in the first year of life have been associated with a higher risk of childhood ALL,^{4,5} whereas increased exposure to common infections based on proxy measures of childhood social contacts (e.g., daycare attendance) may reduce risk.⁶ The role of neonatal immune development in ALL is further supported by the increased risk of ALL in children delivered by elective Cesarean section.⁷ Emerging evidence suggests that susceptibility to neonatal infections is related to active immune suppression within the neonatal environment. A key regulator in perinatal immunity is arginase 2 (ARG2),^{8,9} which suppresses T cells through an anti-inflammatory cascade resulting from arginine depletion.¹⁰ Given the function of ARG2 in neonatal immune function and response to early-life infections, we investigated whether variation in ARG2 levels at birth may be associated with risk of developing ALL in childhood.

For the current study, we selected a total of 137 children who were born in seven counties of California and diagnosed with ALL between the ages of 0 and 14 years in the period from 2000 to 2009, as well as 500 cancer-free control children who were matched to the cases by year and county of birth (3 or 4 controls per case). We obtained data on cancer diagnosis from the California Cancer Registry, data on birth characteristics from the California Center for Health Statistics and Informatics, and archived neonatal blood spots from the California Biobank Program, which is part of the Genetic Disease Screening Program. The blood spots, which were used in this study to measure newborn ARG2 levels, were leftover material from statewide disease screening in neonates. All cases, along with appropriate matched controls chosen via a population-based registry, were obtained within the catchment period and area. Our study protocol was approved by the State of California Committee for the Protection of Human Subjects, and the institutional review boards at all agencies from which we obtained data or blood spots as well as the academic institutions involved (University of California, San Francisco and Berkeley, Yale University, University of Southern California). We obtained a 14-mm diameter blood spot on filter paper (also known as a Guthrie card) collected via a heel-prick of the neonates, usually within 2 days after birth (median, 28 h). These blood spots are stored frozen (-20°C) in a central State archive. For each subject, one third of a blood spot was excised and placed in 300 µL of extraction buffer [phosphate-buffered saline, pH 7.4, 0.5% Tween-20 and 2x complete protease inhibitor cocktail (Roche)], shaken at 600 rpm under room temperature for 1 h and spun for 30 sec at 20,000 x g. Extracts were assayed in duplicate and block randomized on 96-well plates, with each plate containing a seven-point standard ARG2 dilution in duplicate, and the same proportion of cases and controls and racial/ethnic groups. ARG2 was measured using an enzyme-linked immunosorbent assay (MyBioSource). Serum protein concentration was determined using a Pierce BCA protein assay.

Table 1. Characteristics of cases with childhood acute lymphoblastic leukemia and controls.

	Cases n (%)	Controls n (%)	P
Total, n.	137	492	
Sex, n. (%)			
Male	78 (57)	283 (58)	0.9
Female	59 (43)	209 (42)	
Race/ethnicity, n. (%)			
Hispanic	74 (54)	279 (57)	0.85
Non-Hispanic white	41 (30)	139 (28)	
Other	22 (16)	74 (15)	
Gestational age (weeks), n. (%)			
<37	13 (9)	46 (9)	0.88
37-41	112 (82)	397 (81)	
≥42	9 (7)	26 (5)	
Unknown	3 (2)	23 (5)	
Birth weight (grams), n. (%)			
<2500	4 (3)	19 (4)	0.63
2500-2999	23 (17)	74 (15)	
3000-3499	57 (42)	193 (39)	
3500-3999	44 (32)	153 (31)	
≥4000	9 (7)	53 (11)	
Birth order, n. (%)			
First	55 (40)	185 (38)	0.8
Second	46 (34)	165 (34)	
Third or subsequent	36 (26)	142 (29)	
Plurality, n. (%)			
Singleton	131 (96)	480 (98)	0.23
Multiple	6 (4)	12 (2)	
Delivery mode, n. (%)			
Vaginal delivery	84 (61)	354 (72)	0.02
Cesarean delivery	53 (39)	138 (28)	
Year of birth, n. (%)			
2000-2002	49 (36)	173 (35)	0.7
2003-2005	61 (45)	206 (42)	
2006-2009	27 (20)	113 (23)	
Age at neonatal blood collection (h)			
Median (interquartile range)	29 (25-40)	27 (23-36)	0.01
Mothers' age at delivery (years), n. (%)			
<25	30 (22)	149 (30)	0.15
25-34	85 (62)	269 (55)	
≥35	22 (16)	74 (15)	
Mothers' place of birth, n. (%)			
United States	78 (57)	262 (53)	0.44
Other	59 (43)	230 (47)	

Four-parameter logistic regression was used to calculate the standard curves for each batch. ARG2 levels were estimated from standard curves, averaged across duplicates and normalized to the sample-specific total serum protein concentration. Categorical variables are shown using frequencies and percentages, and continuous variables (e.g., age at neonatal blood collection) are summarized by medians and interquartile ranges. The baseline

Arginase-2 OR LCL UCL

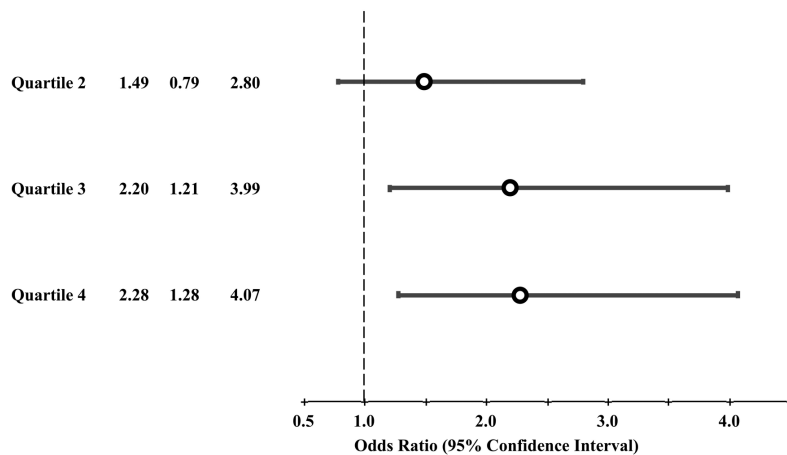


Figure 1. Adjusted odds ratios for quartiles of neonatal arginase 2 level associated with the development of childhood acute lymphoblastic leukemia. The study population consisted of 137 cases and 492 controls. Adjustments were made for sex, race/ethnicity (non-Hispanic white, Hispanic white and other), birth weight (<2500 g, 2500-2999 g, 3000-3499 g, 3500-3999 g, \geq 4000 g), birth order (first, second, third or subsequent), plurality (singleton, multiple), delivery mode (vaginal, Cesarean), year of birth, age at neonatal blood collection, mothers' age at delivery (<25 years, 25-34 years, \geq 35 years), and mothers' place of birth (USA, other). OR: odds ratio; LCL: lower 95% confidence limit; UCL: upper 95% confidence limit.

characteristics of cases and controls were compared using χ^2 tests for categorical variables and the Wilcoxon rank sum test for age at neonatal blood collection. ARG2 levels were categorized into quartiles according to the distribution among controls. An unconditional multivariable mixed-effect logistic regression model with batch as a random variable was used to estimate the association between ARG2 level and risk of ALL, adjusting for birth characteristics (listed in the legend to Figure 1). All analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA) with two-sided tests and a type I error of 5% as the threshold for statistical significance. A *P*-value for trend with increasing ARG2 was calculated by treating arginase quartile as an ordinal variable in the logistic regression model.

Eight control samples were excluded because the coefficient of variance between duplicate ARG2 measurements was greater than 30%. Cases and controls were similar with regard to sex, race/ethnicity, gestational age, birth weight, and birth order (Table 1). Additional data on the distribution of ARG2 level in relationship with these covariates are presented in *Online Supplementary Tables S1* and *S2*. Compared with controls, ALL cases were more likely to be delivered by Cesarean section (39% vs. 28%, $P=0.02$) and were older at neonatal blood collection (median: 29 vs. 27 hours, $P=0.01$). In addition, among controls, calendar age of the card was negatively correlated with ARG2 level (Spearman correlation coefficient = -0.20, $P=0.0001$). The multivariable analysis suggested that the risk of childhood ALL increased by more than two-fold in subjects whose level of ARG2 at birth was in the third or fourth quartile, compared to those whose ARG2 level was in the lowest quartile. The odds ratios (OR) were 2.20 [95% confidence interval (95% CI): 1.21-3.99, $P=0.01$] and 2.28 (95% CI: 1.28-4.07, $P=0.01$) for the third and fourth quartiles, respectively (Figure 1), with a significant trend (OR=1.31, 95% CI: 1.10-1.57, $P=0.0021$). This relationship did not change when samples were not adjusted for protein concentration (OR=1.34; 95% CI: 1.06-1.69, no correction for protein concentration). ARG2 levels were higher in children born by Cesarean section for cases ($P=0.06$ by the Wilcoxon rank test) and controls ($P=0.56$), and the case-control relationship with ARG2 level was slightly weakened by removing all subjects born by Cesarean section (OR=1.21, 95% CI: 0.98-1.52, $P=0.07$, trend test) (*Online Supplementary Table S3*) while the full dataset retained significance when adjusting for Cesarean birth (Figure 1 and *Online Supplementary Table S4*). When the analysis

was restricted to B-cell ALL only (100 cases), ARG2 level retained its significance (OR=1.22, 95% CI: 1.00-1.49, $P=0.05$) (*Online Supplementary Table S5*). We therefore found that the association between higher neonatal ARG2 levels and the development of ALL later in childhood held true for the most common subtype of ALL: pre-B cell.

Arginase has been reported to affect the immune system via its role in depletion of L-arginine, which may impair nitric oxide-mediated cytotoxicity, decrease TLR-4-mediated proinflammatory responses and suppress T-cell function via downregulation of TCR-CD3 ζ .⁸ Several studies have shown that increased arginase production by myeloid-derived suppressor cells in adult tumors leads to a suppressed response in the tumor immune microenvironment.¹¹ Recently, a transitory presence of myeloid-derived suppressor cells was identified in neonates, raising the possibility that these cells have an additional role of regulating immune suppression early in life.¹² In addition, susceptibility to infection in newborn mice has been associated with the temporal presence of CD71⁺ immunosuppressive erythroid cells producing ARG2,¹³ which could affect the response to early infections known to affect the risk of ALL.^{4,5} While a higher ARG2 level may be indicative of greater immunosuppression at birth, it is also possible that it is simply a marker of the level of early infant immune stimulation prior to Guthrie card blood sampling. Therefore, higher ARG2 levels could signify a naive neonatal immune system due to less prior exposure to microbes,³ and those subjects with high ARG2 levels may react inappropriately to new infections thereby stimulating leukemogenesis.^{4,5} Our group previously reported a deficit of interleukin-10 at birth among children who subsequently developed ALL, suggesting that a child's baseline immune function at birth may affect his/her response to subsequent exposure to infections and risk of leukemia.¹⁴ This observation was recently expanded to additional cytokines.¹⁵ Taken together with the current results, the evidence suggests that a dysregulated immune response around the time of birth may affect the responsiveness of the developing immune system and also provide a growth advantage for a preleukemic clone.

The strengths of this study include the low likelihood of selection and information bias (population-based ascertainment of study subjects, no need to contact subjects for participation, preexisting data on important covariates through birth records) and the availability of archived neonatal blood specimens for measurement of

ARG2 level at birth. While samples that have been stored for longer time appeared to have lower ARG2 levels, our cases and controls were frequency-matched for year of birth and hence the duration of sample storage. Non-differential misclassification regarding ARG2 levels would likely have biased our results towards the null.

In summary, the present study showed that a higher ARG2 level at birth was associated with statistically significant increased odds of developing ALL later in childhood. This finding suggests that immune changes related to ARG2 levels are evident long before the clinical manifestation of childhood leukemia. Our results require validation and further investigation in larger studies of neonatal ARG2 that include detailed assessment of early childhood infections and autoimmune diseases. However, this novel finding increases evidence for a role of immune dysregulation at birth in the development of childhood ALL.

Amalie B. Nielsen,^{1,2} Mi Zhou,¹ Adam J. de Smith,^{1,3} Rong Wang,⁴ Lucie McCoy,^{5,6} Helen Hansen,⁵ Libby Morimoto,⁶ Kirsten Grønbaek,² Christoffer Johansen,² Scott C. Kogan,⁷ Catherine Metayer,⁶ Paige M. Bracci,¹ Xiaomei Ma⁴ and Joseph L. Wiemels^{1,3*}

***Co-senior authors**

¹Department of Epidemiology and Biostatistics, University of California San Francisco, CA, USA; ²Department of Hematology, Rigshospitalet, Biotech Research and Innovation Centre, BRIC, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark; ³Center for Genetic Epidemiology, Keck School of Medicine, University of Southern California, CA, USA; ⁴Department of Chronic Disease Epidemiology, School of Public Health, Yale University, CT, USA; ⁵Department of Neurosurgery, University of California San Francisco, CA, USA; ⁶Division of Epidemiology, School of Public Health, University of California Berkeley, CA, USA and ⁷Department of Laboratory Medicine, University of California San Francisco, CA, USA

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*Correspondence: JOSEPH WIEMELS
wiemels@usc.edu
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