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

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Letter to the Editor

Acute lymphoblastic leukemia (ALL) afflicts 2,250 children diagnosed annually in the United States (0-14 yrs).¹ Modern treatment regimens cure approximately 90% of those afflicted, but survivors suffer from long-term sequelae.² Epidemiologic evidence for ALL development points to a role 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 ALL risk in children delivered by elective cesarean section.⁷ Emerging evidence suggests that neonatal infection susceptibility is related to active immune suppression within the neonatal environment. A key regulator within 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 at the ages of 0-14 years during 2000-2009, as well as 500 cancer-free control children who were matched to the cases by birth 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 newborns. All available cases were obtained within the catchment time and space, along with appropriate matched controls chosen via a population-based registry. Our study protocol has been 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 Guthrie card) collected via a heel-prick of the newborns, usually within two days after birth (median = 28 hours). These blood spots are stored at a central State archive under frozen (-20°C) conditions. For each subject, one third of a blood spot was excised and placed in 300 µL of extraction buffer [PBS, pH 7.4, 0.5% Tween-20 and 2x complete protease inhibitor cocktail (Roche)], shaken at 600 rpm under room temperature for 1 hour and spun 30 sec at 20,000 x g. Extracts were assayed in duplicate and block randomized on 96-well plates, with each plate containing a 7-point standard ARG2 dilution in duplicate, and the same proportion of cases and controls and racial/ethnic groups. ARG2 was measured using enzyme-linked immunosorbent assay (ELISA) (MyBioSource). Serum-protein levels were determined using Pierce BCA protein assay.

Four parameter logistic regression was used to calculate the standard curves for each batch. ARG2 levels were estimated from standard curves. ARG2 levels were averaged across duplicates and normalized to the sample-specific total serum-protein concentration. Categorical variables are shown using frequencies and percentages, and continuous variables (eg., age at neonatal blood collection) were summarized by median and interquartile range. Baseline characteristics between cases and controls were compared using chi-square test for categorical variables and 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 Figure 1 legend). All analyses were conducted using SAS Version 9.4 (SAS Institute Inc., Cary, NC) 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 due to a coefficient of variance greater than 30% between duplicate ARG2 measurements. Cases and controls were similar with regard to sex, race/ethnicity, gestational age, birth weight, and birth order (**Table 1**). Additional data on distribution of ARG2 in relationship with these covariates are presented in Supplementary Tables 1 and 2. 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 3rd or 4th quartile, compared to those whose ARG2 level was in the lowest quartile. The odds ratio was 2.20 (95% confidence interval (CI): 1.21 - 3.99, $p=0.01$) and

2.28 (95% CI: 1.28 - 4.07, $p=0.01$) for Q3 and Q4, 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 extraction levels (OR = 1.34; 95%CI:1.06-1.69, no correction for protein concentration). ARG2 levels were higher for children born with cesarean section among cases ($P = 0.06$ by Wilcoxon rank test) and controls ($P = 0.56$), and the case-control relationship with ARG2 was slightly weakened by removing all subjects with cesarean section birth, OR = 1.21(95%CI 0.98-1.52, $p = 0.07$, trend test) (Supplementary Table 3) while the full dataset retained significance when adjusting for cesarean birth (Figure 1 and Supplementary Table 4). When the analysis was restricted to B cell only (100 cases), ARG2 retained significance, OR=1.22(95%CI 1.00-1.49, $P = 0.05$, Supplementary Table 5). We therefore found that the higher neonatal ARG2 levels associated with a ALL later in childhood held true for the most common subtype of ALL: pre-B cell. Arginase has been reported to impact the immune system via its role in depletion of L-arginine, which may impair NO-mediated cytotoxicity, decrease TLR-4 mediated proinflammatory response and suppress T-cell function via downregulation of TCR-CD3 ζ .⁸ Several studies have shown increased arginase production by myeloid-derived suppressor cells (MDSCs) in adult tumors leading to a suppressed response in the tumor immune microenvironment.¹¹ Recently, a transitory presence of MDSCs has been identified in newborns, suggesting that these cells have an additional role of regulating immune suppression early in life.¹² In addition, infection susceptibility in newborn mice has been associated with temporal presence of CD71+ immunosuppressive erythroid cells producing ARG2,¹³ which could impact the response to early infections known to affect risk of ALL.^{4,5} While higher ARG2 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 naïve neonatal immune system due to lower prior microbial exposures,³ and those subjects with high ARG2 may react

inappropriately to new infections thereby stimulating leukemogenesis.^{4,5} Our group previously reported a deficit of IL-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 infectious exposures and leukemia risk.¹⁴ Recently this observation was 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. Strengths of the study include the low likelihood for 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 newborn 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 on year of birth and hence the duration of sample storage. Nondifferential 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 contributes to increasing evidence for a role of immune dysregulation at birth in the development of childhood ALL.

Disclosure of Conflicts of Interest

The authors do not have any competing financial or other interests in the contents or interpretations presented within this manuscript.

Authorship Contributions

A.B.N. performed experiments, analyzed data, and drafted the manuscript with JW; M.Z., A.D. performed experiments and A.D. conceived the study with J.W.; R.W. planned and conducted statistical analysis and co-wrote the sections on statistical analysis and results; H.H. ascertained and managed Guthrie Cards and laboratory activities; L.M., C.J., K.G., S.K., C.M., P.B. contributed to the analysis and reviewed the manuscript; X.M. and J.W. designed the overall study, planned the analysis, and helped to draft the manuscript.

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Table 1. Characteristics of Childhood Acute Lymphoblastic Leukemia Cases and Controls

	Case	Control	p
	n (%)	n (%)	
Total	137	492	
Sex			
Male	78 (57)	283 (58)	0.9
Female	59 (43)	209 (42)	
Race/ethnicity			
Hispanic	74 (54)	279 (57)	0.85
Non-Hispanic White	41 (30)	139 (28)	
Other	22 (16)	74 (15)	
Gestational age (weeks)			
<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)			
<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			
First	55 (40)	185 (38)	0.8
Second	46 (34)	165 (34)	
Third or higher	36 (26)	142 (29)	
Plurality			
Singleton	131 (96)	480 (98)	0.23
Multiple	6 (4)	12 (2)	
Delivery mode			
Vaginal delivery	84 (61)	354 (72)	0.02
Cesarean delivery	53 (39)	138 (28)	
Year of birth			
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 (hours)			
Median (interquartile range)	29 (25-40)	27(23-36)	0.01
Mother's age at delivery (years)			
<25	30 (22)	149 (30)	0.15

25-34	85 (62)	269 (55)	
≥35	22 (16)	74 (15)	
Mother's place of birth			
United States	78 (57)	262 (53)	0.44
Other	59 (43)	230 (47)	

Figure Legends:

Figure 1. Adjusted odds ratios for quartiles of neonatal Arginase 2 associated with childhood acute lymphoblastic leukemia (ALL) (n=137 cases, n=492 controls). Adjusted for sex, race/ethnicity (non-Hispanic white, Hispanic white and other), birth weight (<2500, 2500-2999, 3000-3499, 3500-3999, ≥4000), birth order (first, second, third or higher), plurality (singleton, multiple), delivery mode (vaginal, cesarean delivery), year of birth, age at neonatal blood collection, mother's age at delivery (<25, 25-34, ≥35), and mother's place of birth (United States, other). Abbreviations: OR: odds ratio; LCL – lower 95% confidence limit; UCL – upper 95% confidence limit.

Supplementary Table 1: ARG2 levels by case and control status, and within quartiles (protein-corrected)

ARG2	n	median	cases		median	controls		
			mean	range		mean	range	
overall	137	8.0	12.1	0.83-242.3	492	6.9	11.6	1.0-279.7
Q1	21	3.2	3.0	0.83-4.2	123	3.2	3.2	1.0-4.6
Q2	29	5.7	5.7	4.7-6.7	123	5.7	5.7	4.6-6.9
Q3	44	8.5	8.6	6.9-10.7	123	8.5	8.7	6.9-10.9
Q4	43	14.2	24.3	10.9-242.3	123	16.5	28.6	10.9-279.7

Supplementary Table 2: ARG2 levels (protein corrected) by demographic factors

		ARG2 cases (N = 137)			ARG2 controls (N = 492)		
		median	mean	range	median	mean	range
race	White	8.6	10.8	3.2-42.9	7.2	11.1	1.4-192.9
	Hispanic	8.0	10.0	0.8-45.3	7.5	12.6	1.0-279.7
	Other	7.7	21.4	1.1-242.3	5.7	8.4	1.5-61.9
Sex	Female	7.9	9.3	0.8-39.1	6.8	12.0	1.2-279.7
	Male	8.3	14.1	1.5-242.3	7.1	11.2	1.0-222.2
age at collection (hours)	mean (SD)						
birthweight (g)	<2500	10.9	10.2	4.8-14.2	7.3	11.1	3.2-61.9
	2500-2999	7.5	10.9	1.1-45.3	6.9	13.8	2.0-279.7
	3000-3499	9.1	16.3	2.6-242.3	6.8	11.9	1.0-49.6
	3500-3999	7.7	8.2	0.83-22.6	6.7	9.2	1.5-49.6
	4000+	7.3	7.6	4.2-13.5	7.9	13.9	2.1-222.2
gestational age	<37	10.7	32.5	4.8-242.3	6.6	15.7	2.0-279.7
	37-41	7.6	9.9	0.83-68.7	6.8	10.7	1.2-192.9
	42+	9.6	9.3	3.5-13.1	7.1	16.9	1.0-192.9
	Unknown	11.8	11.7	11.2-12.0	9.3	11.8	1.6-30.5
Plurality	No	8.0	12.0	0.83-242.3	6.9	11.6	1.0-279.7
	Yes	9.7	14.1	2.5-39.2	7.1	8.3	1.2-25.3

birth order	1	8.0	10.9	1.1-68.7	7.2	13.7	1.5-279.7
	2	8.0	14.1	0.83-242.3	6.9	9.4	1.8-84.4
	3+	8.4	11.2	1.8-45.4	6.3	11.3	1.0-192.9
C-section	No	7.6	9.2	1.1-42.9	6.8	12.7	1.5-279.7
	Yes	9.6	16.6	0.83-242.3	7.2	8.5	1.0-45.5
Mother's age	<25	9.2	10.2	2.6-22.9	6.7	11.9	1.5-192.9
	25-34	8.0	10.6	0.83-68.7	7.0	11.7	1.0-279.7
	35+	7.5	20.3	2.7-242.3	6.9	10.4	1.5-84.4
Mother's birthplace	US	8.0	9.8	1.5-42.9	6.8	10.2	1.5-192.9
	Other	8.4	15.1	0.83-242.3	6.9	13.1	1.0-279.7
Year of birth	2000	7.1	7.5	3.3-15.5	7.7	11.4	1.7-93.0
	2001	7.2	10.0	0.83-68.7	8.7	19.2	1.5-279.7
	2002	10.5	13.9	5.3-45.4	7.9	13.9	3.3-192.9
	2003	9.7	23.8	4.7-242.3	7.5	9.5	2.6-37.6
	2004	9.1	10.6	1.5-39.1	5.5	9.9	1.0-135.7
	2005	6.3	12.4	3.1-42.9	5.8	12.4	1.5-222.2
	2006	7.6	9.5	3.1-42.9	6.0	8.0	1.7-30.5
	2007	6.1	7.6	2.5-30.6	5.9	7.2	2.2-18.7
	2008	7.7	9.6	3.9-20.3	5.9	7.6	1.8-22.6
	2009				4.7	4.7	2.0-7.3

All values are expressed as ng Arginase-2 per ug protein X 10

Supplementary Table 3: Case-control results for ARG2 levels while exclude participants delivered by c-section, and quartiles cut offs based on all controls (n=84 cases, n=354 controls)

ARG2	Case n (%)	Control n (%)	OR	95% CI	p
1 st quartile	12 (14.3)	84 (23.7)	1		
2 nd quartile	21 (25.0)	95 (26.8)	1.48	0.67-3.31	0.33
3 rd quartile	29 (34.5)	84 (23.7)	2.39	1.10-5.17	0.03
4 th quartile	22 (26.2)	91 (25.7)	1.77	0.82-3.81	0.14
Trend			1.22	0.98-1.52	0.07

Supplementary Table 4: Case-control results for ARG2 levels with all participants adjusted for c-section, and without adjustment (n=137 cases, n=492 controls)

A: with C-section

ARG2	OR	95% CI	p
1 st quartile	1		
2 nd quartile	1.49	0.79-2.80	0.26
3 rd quartile	2.2	1.21-3.99	0.01
4 th quartile	2.28	1.28-4.07	0.01
Trend	1.31	1.10-1.57	<0.01

B: without C-section

OR	95% CI	p
1		
1.42	0.76-2.66	0.28
2.17	1.20-3.92	0.01
2.26	1.27-4.01	0.01
1.32	1.11-1.57	<0.01

Adjusted odds ratios for quartiles of neonatal Arginase 2 associated with childhood acute lymphoblastic leukemia (ALL) (n=137 cases, n=492 controls). Adjusted for sex, race/ethnicity (non-Hispanic white, Hispanic white and other), birth weight (<2500, 2500-2999, 3000-3499, 3500-3999, ≥4000), birth order (first, second, third or higher), plurality (singleton, multiple), delivery mode (A only: vaginal, cesarean delivery), year of birth, age at neonatal blood collection, mother's age at delivery (<25, 25-34, ≥35), and mother's place of birth (United States, other).

Supplementary Table 5: Case control results for ARG2 levels for B-cell ALL only (ICD-O-3:9836, case=100, control=492)

ARG2	Case n (%)	Control n (%)	OR	95% CI	p
1 st quartile	15(15.0)	123(25.0)	1		
2 nd quartile	25(25.0)	123(25.0)	1.82	0.88-3.75	0.11
3 rd quartile	34(34.0)	123(25.0)	2.36	1.18-4.71	0.02
4 th quartile	26(26.0)	123(25.0)	1.91	0.93-3.93	0.08
Trend			1.22	1.00-1.49	0.05

Adjusted odds ratios for quartiles of neonatal Arginase 2 associated with childhood acute lymphoblastic leukemia (ALL) (n=137 cases, n=492 controls). Adjusted for sex, race/ethnicity (non-Hispanic white, Hispanic white and other), birth weight (<2500, 2500-2999, 3000-3499, 3500-3999, ≥4000), birth order (first, second, third or higher), plurality (singleton, multiple), delivery mode (vaginal, cesarean delivery), year of birth, age at neonatal blood collection, mother's age at delivery (<25, 25-34, ≥35), and mother's place of birth (United States, other).