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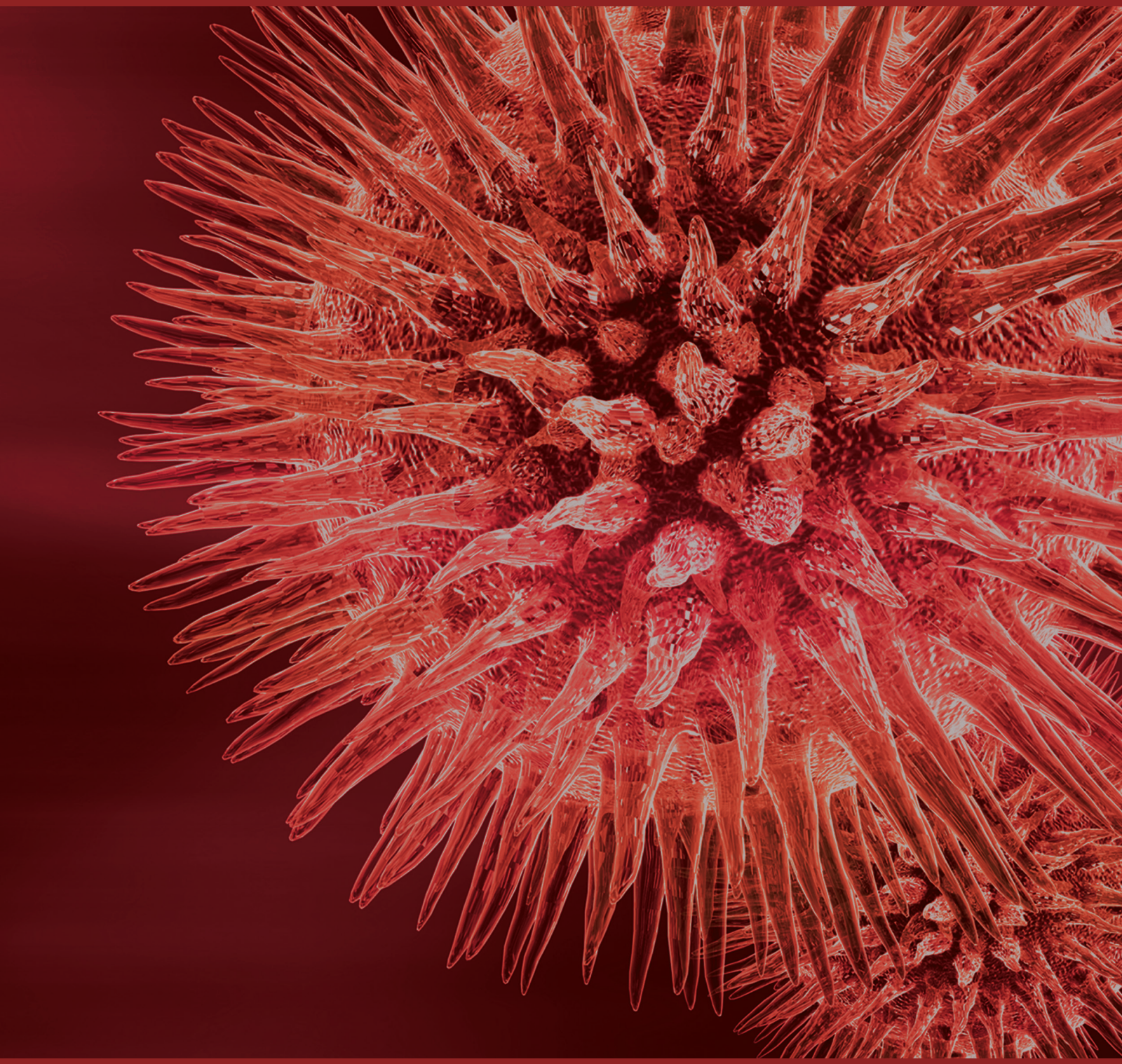
Acute Leukemia in Children

Guest Editors: Juan Manuel Mejía-Aranguré and Richard J. Q. McNally



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Editorial

Acute Leukemia in Children

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Acute lymphoblastic leukemia (ALL) is the most common cancer during childhood. Nevertheless, in recent years there have been marked improvements in treatment and consequent survival. There are a number of patients that experience relapses and patients that cannot be cured. In this special issue different aspects relating to factors influencing the best response to treatment are described. M. Czogała et al. showed that plasma ammonia concentration reflexes L-asparaginase activity may be a useful tool for assessing treatment in children with ALL. In another study A. Medina-Sanson et al. identified the importance of a genetic polymorphism of deoxycytidine kinase and cytidine deaminase in assessing the toxicity by cytarabine in children with acute myeloid leukemia and found that this polymorphism might predict death in affected children.

A. Vilchis-Ordoñez et al. addressed a very interesting topic about subpopulations of leukemic cells that contribute to a proinflammatory microenvironment within B-ALL bone marrow that may cause a delay in the normal hematopoiesis.

Two studies assessed the participation of environmental risk factors in the development of acute leukemia. A. Morales-Sánchez et al. did not find that EBV, HCMV, HHV6, and HHV7 were related to the development of ALL. Furthermore, J. D. Ferreira et al. did not show that maternal alcohol consumption during pregnancy was associated with leukemia in young children.

Finally a study by V. C. Bekker-Méndez et al. demonstrated differences in the frequency of gene rearrangement between the Mexican population and populations from developed countries.

From these articles it is clear that throughout the world the survival of children with acute leukemia is a great concern, especially in less developed countries. It is very important to search for factors associated with treatment compliance in children and those factors that may be related to treatment response.

The microenvironment in the leukemic bone marrow is a very important factor in the etiology of acute leukemia. Recovery of the bone marrow during treatment is also very important and further studies are needed to address this important aspect.

The environmental etiology of acute leukemia is a challenge that still has not been resolved. To identify environmental risk factors is not easy. It is important to identify both the environmental risk factors and also the gene-environment interactions that increase the susceptibility to development of leukemia.

The presentation of acute kinds of leukemia in Hispanic children is different from other populations; notably clinical features, the age incidence peak, and molecular factors are different.

In this special issue the complexity of the molecular and clinical epidemiology of acute leukemia in children has been shown. There are countries where success has been clearly demonstrated, but in other countries this is not apparent. This disease kills more children in developed countries and some emerging economies. There is a need to strengthen the research groups that are working to identify the factors that affect the adherence to treatment and the response to treatment. However, there is also a need for integration with

those research groups that are working on the etiology of leukemia, especially including epidemiology and cellular and molecular biology.

We need to understand more about leukemia, in order to be able to cure and prevent it. The challenge continues because it is not a dream to think that one day almost all kinds of leukemia in children could be cured and that one day this disease could be prevented.

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Research Article

Maternal Alcohol Consumption during Pregnancy and Early Age Leukemia Risk in Brazil

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Objectives. To investigate the association between the maternal alcohol consumption during pregnancy and early age leukemia (EAL) in offspring. *Methods*. Datasets were analyzed from a case-control study carried out in Brazil during 1999–2007. Data were obtained by maternal interviews using a standardized questionnaire. The present study included 675 children (193 acute lymphoid leukemia (ALL), 59 acute myeloid leukemia (AML), and 423 controls). Unconditional logistic regression was performed, and adjusted odds ratios (adj. OR) on the association between alcohol consumption and EAL were ascertained. *Results*. Alcohol consumption was reported by 43% of ALL and 39% of AML case mothers and 35.5% of controls'. Beer consumption before and during pregnancy was associated with ALL in crude analysis (OR = 1.54, 95% CI, 1.08–2.19), although in adjusted analysis no statistical significance was found. For weekly intake of ≤1 glass (adj. OR = 1.30, 95% CI, 0.71–2.36) and ≥1 glass/week (adj. OR = 1.47, 95% CI, 0.88–2.46) a potential dose-response was observed (P trend < 0.03). *Conclusion*. This study failed to support the hypothesis of an increased risk of EAL associated with maternal alcohol intake during pregnancy, neither with the interaction with tobacco nor with alcohol consumption.

1. Introduction

Acute leukemia in early childhood is seldom, mainly those cases diagnosed within the first year of life—infant leukemia (IL) [1, 2]. IL deserves special attention because this group is biologically and clinically distinct from leukemia in older children [3]. The somatic gene mutations in clonal cells, for instance, *MLL* gene rearrangements (*MLL-r*), constitute the biological basis of this hematopoietic malignancy that arises during fetal life [4, 5].

Although the etiology of the majority hematopoietic malignancies in children remains largely unknown, Down syndrome [6, 7] and exposure to ionizing radiation [8] and certain chemotherapeutic agents [9, 10] are associated with increased risk of childhood acute leukemia. Previous researches demonstrated that for some childhood leukemia types the causality factors are likely to be multiple and associated with leukemia subtype-specific, combining environmental exposures and genetic susceptibility modulation risk [11, 12].

Several studies conducted in the last decade demonstrated a positive association between childhood leukemia and maternal alcohol consumption during pregnancy [13–17] leading to the premises that maternal alcohol drinking during pregnancy could cause DNA damage during the preconceptions in gametes cells or during pregnancy in fetal cells. Ethanol was established as a teratogen substance that produces pre- and postnatal growth deficiency according to experimental models [18]. Recently, additional epidemiological observations confirmed the previous studies, but findings

with low risk association estimates could be a consequence of methodological approaches [19–24].

Several chemical and biological mechanisms likely contribute to the damaging effects of alcohol exposure on the developing fetus. The toxic metabolite acetaldehyde resulting by the break-down of alcohol in the liver and other tissues plays a major role in the tumor-promoting effect demonstrated by animal models [25, 26]. Transplacental crossover alcohol metabolites were measured and similar fetal and mother alcohol concentration rates were observed, leading to the conclusive evidence that the amniotic fluid acts as a reservoir of alcohol toxic metabolites [27, 28]. Other suggested mechanisms for carcinogenic pathways include cell death by apoptosis, increased oxidative stress, and facilitation of cellular entry for other carcinogens [29].

In order to test the hypothesis that maternal exposure to alcohol consumption would be associated with early age leukemia (EAL), we analyzed acute lymphoblastic (ALL) and myeloid (AML) cases included in the Brazilian Collaborative Study Group of Infant Leukemia (BCSGIAL). The joint effects of maternal smoking exposure and alcohol consumption as addictive effects were also tested.

2. Materials and Methods

2.1. Study Population. Cases and controls were assessed throughout a multicenter study "Multi-Institutional Study of Infant Leukemia: Contribution of Immunomolecular Markers in Distinguishing Different Etiopathogenic Factors" that focuses on the investigation of EAL. It was a hospital-based study in which the participants were ascertained from different Brazilian regions [31, 32].

2.2. Case and Controls Ascertainment. Eligible cases were children with acute leukemia (ALL or AML) aged ≤24 months at the diagnosis, confirmed by cell morphology, immunophenotyping profile, and standard cytogenetic-molecular methods [33]. The controls were selected children with nonmalignant diseases that were attended in hospitals where cases were recruited and also from pediatric care centers in the same cities. They were frequency matched with leukemia cases according to age (≤24 months) and enrolled from the same geographic areas where cases were diagnosed. The reasons for clinical assistances were viral infections and parasitic diseases (n = 124, 29.4%); nonmalignant hematological diseases (n = 83, 19.6%); asthma and bronchitis (n = 43, 10.2%); hemangioma (n = 40, 9.4%); severe diarrhea (n = 39, 9.2%); cardiovascular diseases (n = 25, 5.8%); and other nonmalignant conditions (n = 69, 16.4%).

The variables elected for the present analysis were obtained in a dataset built from 1999 to 2007. As soon as the diagnosis of acute leukemia was established, the maternal exposure information was obtained through questionnaires. After the written informed consent was signed, a face-to-face interview was applied to mother of cases and controls. Previous data about different maternal exposures and smoking during pregnancy used herein as a basis for interaction analysis are described elsewhere [30, 32]. The content of questionnaires included data about family income, maternal age at child birth, education level, illness previous history to conception, medication use, occupation, personal habits, and the child's birth characteristics. The exposure assessment to smoking exposure was determined by the qualitative analysis (yes/no) during the three months before the index pregnancy and the three trimesters of the pregnancy, as well as after birth during the breastfeeding period. Levels of pregnancy smoking were categorized as nonsmokers; moderate smokers (1–20 cigarettes/day); heavy smokers (≥20 cigarettes/day) [30].

Like the exposure assessment to maternal alcohol consumption, mothers were asked whether they had ever drunk alcohol (yes/no), occasionally, on a regular basis, during the three months before the index pregnancy, and during pregnancy. Then, further questions were also collected to elucidate the frequency and amount of beverage consumption by number of wine glasses, beer, or spirit drinks per week. Answers were classified as abstainers (0 glasses/week), occasional drinkers (by alcohol consumption less than one glass/week), and frequent drinkers (alcoholic intake more than one glass/week).

2.3. Exclusion Criteria. Children with genetic syndromes, myelodysplasia, malignant tumor, adoptive parents, or unknown biological mothers were not eligible to the study (cases and/or controls groups). The frequency rate of acceptance of invited mothers (cases and controls) was 96% and 95%, respectively [32].

2.4. Ethical Aspect. This study used primary data obtained from the project "Multi-Institutional Study of Infant Leukemia: Contribution of Immunomolecular Markers in Distinguishing Different Etiopathogenic Factors." This investigation was approved by the Research Ethics Committee of the Instituto Nacional de Câncer (CEP #005/06).

2.5. Statistical Analysis. The sample size calculation was performed considering the percentage of exposed controls of the same exposure to alcohol casual women in childbearing age (36%, as in the Brazilian National Antidrug Secretary Survey), the ratio of 2 controls per case, 80% power of study, with a confidence interval 95%. Unconditional logistic regression was performed to estimate the magnitude of association between maternal alcohol consumption and EAL, being the respective odds ratios (OR) and their 95% confidence intervals (CI) ascertained after adjustment for birth weight (<4,000 g, ≥4,000 g), child's ethnicity (whites or non-whites), maternal age at index birth (<35 years, ≥35 years), maternal education level (≤8 years, >8 years), and oral contraceptive intake during pregnancy (no use, use during pregnancy), previously identified as confounders in the studied dataset [32]. To test the interaction between maternal alcohol consumption and tobacco smoking and the risk of EAL, statistical assessment of effect modification was performed on a multiplicative model by fitting models containing both main effects (smoking and alcohol consumption) and their cross-product terms nested models

adjusted for the confounders mentioned above. Assuming independence for both maternal smoking and drinking, the periods during pregnancy were considered as independent variables in the model, referring to a baseline category of abstainers' drinkers and nonsmokers [17].

3. Results

The demography distribution of cases and controls is shown in Table 1. There were 116 IL cases (46.0%), a higher proportion of whites observed among all EAL cases (67.5%) than controls (36.2%), $P < 0.01$. The majority of EAL cases (61.95%) and controls (56.0%) were enrolled in the Southeastern cities, with the Northeast cities running second, respectively, 20.6% and 24.1%. Mothers of cases were older than mothers of controls ($P < 0.01$). Maternal levels of education were higher among cases ($P < 0.01$).

Maternal alcohol consumption before (3 months of preconception) and during pregnancy was evaluated as potential risk factor for EAL as shown in Table 2; 150 out of 423 mothers of controls (35.5%) had reported alcohol consumption, either preconception or during pregnancy, whereas 106 out of 252 EAL mothers (42.1%) reported use of alcoholic beverages without differences between mothers of ALL and mother of AML. Maternal alcohol intake both before and during pregnancy was observed in 55% of ALL and 48% of AML cases (kappa P value < 0.001 for both). Maternal beer consumption less than 1 glass/week during preconception significantly increased the risk for ALL as crude OR = 1.84, 95% CI (1.12–3.04). All adjusted OR analysis performed to test alcohol exposure during pregnancy and EAL demonstrated no statistically significant results. However, an adj. OR = 1.30 (95% CI 0.71–2.36) for weekly beer intake of ≤1 glass and adj. OR = 1.47 (95% CI 0.88–2.46) for >1 glass show a P trend < 0.03 in the ALL subtype. Mothers of ALL cases have not reported spirits consumption during pregnancy. Depending on the type of reported alcoholic beverage consumption in the preconception, risk estimates were slightly more pronounced for spirits in AML, adj. OR = 3.61 (95% CI 0.83–15.7), than for beer, adj. OR = 1.13 (95% CI 0.60–2.14), and other beverages, adj. OR = 2.16 (95% CI 0.74–6.35). According to maternal alcoholic consumption in the same period and ALL development in the offspring, an adj. OR = 1.36 (95% CI 0.91–2.03) was observed for reported beer consumption, adj. OR = 0.84 (95% CI 0.22–3.29) for spirits consumption, and adj. OR = 1.48 (95% CI 0.74–2.96) for other beverages consumption.

For mothers of children with ALL (age stratum ≤ 11 months) who reported consumption of any alcoholic beverages before or during pregnancy an increased OR was observed (adj. OR = 1.29, 95% CI 0.73–2.27), although not statistically significant (Table 3). For alcoholic consumption in the preconception period, the risk estimate was an adj. OR = 1.56, 95% CI 0.88–2.79, and an adj. OR = 1.49, 95% CI 0.77–2.89, during pregnancy. In the stratum of children aged 12-23 months, the highest estimate in cases of AML, was observed for the maternal intake of any alcoholic beverages before or during pregnancy (adj OR = 1.49, 95% CI 0.61 to 3.61).

To evaluate the interaction between maternal alcohol consumption and tobacco smoking and the risk of EAL, a logistic regression analysis was performed and results are shown in Table 4. The OR magnitude of exposure did not vary between models, which resulted in nonstatistically significant results to the overall models analyzed.

Table 1: Distribution of selected maternal and child demography of early age leukemia and controls, Brazil, 1999–2007*.

	Cases** *N* (%)	Controls *N* (%)	*P* value
Age			
≤11 mo.	116 (46.0)	255 (60.2)	<0.01
12–24 mo.	136 (54.0)	168 (39.8)	
Gender			
Males	130 (51.6)	226 (53.4)	0.643
Females	122 (48.4)	197 (46.6)	
Birth weight			
<4,000 g	234 (92.8)	393 (93.0)	0.470
>4,000 g	16 (6.2)	21 (5.0)	
Missing	2 (0.8)	9 (2.0)	
Ethnicity			
Whites	170 (67.5)	153 (36.2)	<0.01
Non-whites	77 (30.5)	256 (60.5)	
Missing	5 (2.0)	14 (3.3)	
Place of birth			
Northeast	52 (20.7)	101 (24.0)	0.552
Midwest	18 (7.1)	31 (7.3)	
Southeast	155 (61.5)	238 (56.2)	
South	27 (10.7)	53 (12.5)	
Maternal age[a]			
<18 years	8 (3.2)	60 (14.2)	<0.01
18–24 years	91 (36.1)	182 (43.0)	
25–34 years	117 (46.4)	145 (34.2)	
>35 years	36 (14.3)	36 (8.5)	
Maternal education			
<8 years	81 (32.1)	206 (48.6)	<0.01
>8 years	146 (57.9)	209 (49.4)	
Total	252 (100)	423 (100)	—

[a] Maternal age at child delivery; *N*, number of cases; mo. = months.
*Data presented in Ferreira et al., 2012 [30]; **including acute lymphoblastic leukemia, $n = 193$, and acute myeloid leukemia, $n = 59$.

4. Discussion

Several studies demonstrated a positive association between maternal alcohol consumption during prenatal or pregnancy and childhood leukemia [15–17, 21, 22, 34]. Alcohol consumption during pregnancy may affect fetal cells and is

Table 2: Maternal alcohol consumption preconception and/or during pregnancy, early age leukemia, and control mothers, Brazil, 1999–2007.

Maternal alcohol drinking	Controls *n* (%)	ALL *n* (%)	AML *n* (%)	ALL		AML	
				OR (95% CI)	Adj. OR[a] (95% CI)	OR (95% CI)	Adj. OR[a] (95% CI)
No	261 (61.7)	107 (55.4)	36 (61.0)	1.00	1.00	1.00	1.00
Yes	150 (35.5)	83 (43.0)	23 (39.0)	1.35 (0.95–1.92)	1.21 (0.81–1.80)	1.11 (0.64–1.95)	1.17 (0.63–2.18)
Missing	12 (2.8)	3 (1.6)	0 (0.0)				
Beer							
Preconception							
No	273 (64.5)	108 (56.0)	38 (64.4)	1.00	1.00	1.00	1.00
Yes	135 (32.0)	82 (42.5)	21 (35.6)	1.54 (1.08–2.19)	1.36 (0.91–2.03)	1.12 (0.63–1.98)	1.13 (0.60–2.14)
Missing	15 (3.5)	3 (1.5)	0 (0.0)				
≤1 glass/week	46 (10.9)	32 (16.6)	12 (20.3)	1.84 (1.12–3.04)	1.30 (0.71–2.36)	1.87 (0.91–3.84)	2.06 (0.90–4.76)
>1 glass/week	66 (15.6)	39 (20.2)	6 (10.2)	1.44 (0.91–2.28)	1.47 (0.88–2.46)	0.65 (0.26–1.60)	0.75 (0.29–1.94)
Missing	38 (9.0)	14 (7.2)	3 (5.1)				
				P trend = 0.03		*P* trend = 0.68	
During pregnancy							
No	337 (79.7)	146 (75.6)	52 (88.1)	1.00	1.00	1.00	1.00
Yes	73 (17.3)	44 (22.8)	7 (11.9)	1.39 (0.91–2.12)	1.29 (0.78–2.14)	0.62 (0.27–1.43)	0.84 (0.35–2.00)
Missing	13 (3.0)	3 (1.5)	0 (0.0)				
≤1 glass/week	27 (6.4)	21 (16.6)	4 (6.8)	1.80 (0.98–3.28)	1.13 (0.50–2.52)	0.96 (0.32–2.86)	1.38 (0.43–4.42)
>1 glass/week	34 (8.0)	19 (20.2)	1 (1.7)	1.29 (0.71–2.34)	1.43 (0.74–2.77)	0.19 (0.03–1.42)	0.21 (0.28–1.63)
Missing	25 (5.9)	7 (3.6)	2 (3.4)				
				P trend = 0.17		*P* trend = 0.07	
Spirits							
Preconception							
No	397 (93.9)	178 (92.2)	51 (86.4)	1.00	1.00	1.00	1.00
Yes	12 (2.8)	4 (2.1)	3 (5.1)	0.74 (0.24–2.34)	0.84 (0.22–3.29)	1.95 (0.53–7.13)	3.61 (0.83–15.7)
Missing	14 (3.3)	11 (5.7)	5 (8.5)				
≤1 glass/week	4 (0.9)	2 (1.1)	1 (1.7)	1.11 (0.20–6.14)	0.79 (0.08–7.90)	1.95 (0.21–17.8)	2.06 (0.17–24.4)
>1 glass/week	5 (1.2)	0 (0.0)	2 (3.4)	0 (0.0)	0 (0.0)	3.11 (0.59–16.5)	7.18 (1.09–47.3)
Missing	17 (4.0)	13 (6.7)	5 (8.5)				
				P trend = 0.14		*P* trend = 0.21	
During pregnancy							
No	397 (93.9)	182 (94.3)	53 (89.8)	1.00	1.00	1.00	1.00
Yes	12 (2.8)	0 (0.0)	1 (1.7)	0 (0.0)	0 (0.0)	0.62 (0.08–4.90)	0.87 (0.10–7.55)
Missing	14 (3.3)	11 (5.7)	4 (6.8)				
≤1 glass/week	5 (1.2)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
>1 glass/week	2 (0.4)	0 (0.0)	1 (1.7)	0 (0.0)	0 (0.0)	3.75 (0.33–42.0)	9.13 (0.61–135)
Missing	19 (4.5)	0 (0.0)	5 (8.5)				
						P trend = 0.88	
Other beverage[b]							
Preconception							
No	376 (88.9)	164 (85.0)	49 (83.0)	1.00	1.00	1.00	1.00
Yes	33 (7.8)	18 (9.3)	5 (8.5)	1.25 (0.68–2.29)	1.48 (0.74–2.96)	1.16 (0.43–3.12)	2.16 (0.74–6.35)
Missing	14 (3.3)	11 (5.7)	5 (8.5)				
≤1 glass/week	18 (4.2)	13 (6.7)	4 (6.8)	1.65 (0.79–3.46)	1.93 (0.84–4.44)	1.70 (0.55–5.24)	3.42 (0.81–14.3)
>1 glass/week	13 (3.1)	4 (2.1)	1 (1.7)	0.71 (0.23–2.20)	0.93 (0.28–3.16)	0.59 (0.07–4.61)	1.26 (0.14–11.5)
Missing	14 (3.3)	12 (6.2)	5 (8.5)				
				P trend = 0.91		*P* trend = 0.89	

Table 2: Continued.

Maternal alcohol drinking	Controls *n* (%)	ALL *n* (%)	AML *n* (%)	ALL		AML	
				OR (95% CI)	Adj. OR[a] (95% CI)	OR (95% CI)	Adj. OR[a] (95% CI)
During pregnancy							
No	385 (91.0)	174 (90.2)	54 (91.5)	1.00	1.00	1.00	1.00
Yes	24 (5.7)	8 (4.1)	4 (6.8)	0.74 (0.36–1.67)	0.94 (0.37–2.40)	1.28 (0.43–3.85)	2.26 (0.68–7.51)
Missing	14 (3.3)	11 (5.7)	1 (1.7)				
≤1 glass/week	13 (3.1)	4 (2.1)	3 (5.1)	0.68 (0.22–2.12)	0.99 (0.28–3.46)	1.78 (0.49–6.45)	3.41 (0.81–14.3)
>1 glass/week	7 (1.7)	3 (1.5)	1 (1.7)	0.95 (0.24–3.71)	1.19 (0.27–5.19)	1.10 (0.13–9.13)	1.26 (0.14–11.6)
Missing	18 (4.2)	12 (6.2)	1 (1.7)				
					P trend = 0.57		*P* trend = 0.82

[a]Adjusted OR by use of oral contraceptives during pregnancy, maternal age at child birth, maternal education, birth weight, and infant ethnicity, three months before pregnancy, including wine consumption; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; *n* = number of cases; Adj. OR, adjusted odds ratio; CI, confidence interval; one glass = 200 mL.

[b]Preconception (legend: three months before pregnancy) and [c]other beverages (legend: including wine consumption).

Table 3: Maternal alcohol drinking preconception and/or during pregnancy risk according to offspring age strata, leukemia subtypes, and control mothers, Brazil, 1999–2007.

Maternal drinking	Controls *n* (%)	ALL *n* (%)	AML *n* (%)	ALL		AML	
				OR (95% CI)	Adj.[a] OR (95% CI)	OR (95% CI)	Adj.[a] OR (95% CI)
Any beverages							
≤11 mo.							
No	158 (37.3)	47 (24.3)	18 (30.5)	1.00	1.00	1.00	1.00
Yes	89 (21.1)	40 (20.7)	10 (17.0)	1.52 (0.92–2.48)	1.29 (0.73–2.27)	0.99 (0.44–2.23)	0.97 (0.38–2.46)
Missing	8 (1.9)	1 (0.5)	0 (0.0)				
12–23 mo.							
No	103 (24.4)	60 (31.1)	18 (30.5)	1.00	1.00	1.00	1.00
Yes	61 (14.4)	43 (22.3)	13 (22.0)	1.21 (0.73–2.00)	1.07 (0.61–1.89)	1.22 (0.56–2.66)	1.49 (0.61–3.61)
Missing	4 (0.9)	2 (1.1)	0 (0.0)				
Preconception							
≤11 mo.							
No	158 (37.3)	45 (23.3)	18 (30.5)	1.00	1.00	1.00	1.00
Yes	86 (20.3)	42 (21.7)	10 (17.0)	1.72 (1.04–2.82)	1.56 (0.88–2.79)	1.02 (0.45–2.31)	1.01 (0.39–2.61)
Missing	11 (2.7)	1 (0.5)	0 (0.0)				
12–23 mo.							
No	103 (24.4)	60 (31.1)	18 (30.5)	1.00	1.00	1.00	1.00
Yes	61 (14.4)	43 (22.3)	13 (22.0)	1.21 (0.73–2.00)	1.01 (0.57–1.77)	1.22 (0.56–2.66)	1.39 (0.59–3.27)
Missing	4 (0.9)	1 (0.5)	0 (0.0)				
During pregnancy							
≤11 mo.							
No	205 (48.4)	62 (32.1)	24 (40.7)	1.00	1.00	1.00	1.00
Yes	50 (11.8)	26 (13.5)	4 (6.8)	1.72 (0.99–2.99)	1.49 (0.77–2.89)	0.68 (0.23–2.06)	0.96 (0.30–3.10)
Missing	0 (0.0)	1 (0.5)	0 (0.0)				
12–23 mo.							
No	129 (30.5)	83 (43.0)	25 (42.4)	1.00	1.00	1.00	1.00
Yes	37 (8.8)	21 (10.9)	6 (10.1)	0.89 (0.49–1.63)	0.83 (0.40–1.68)	0.84 (0.32–2.19)	1.09 (0.38–3.11)
Missing	2 (0.5)	0 (0.0)	0 (0.0)				

[a]Adjusted odds ratio by use of oral contraceptives during pregnancy, maternal age at birth, maternal education, birth weight, and infant skin color; [b]three months before pregnancy; ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; *n* = number of cases; Adj. OR, adjusted odds ratio; CI, confidence interval; mo. = months.

[b]Preconception (legend: three months before pregnancy).

Table 4: Maternal smoking and alcohol consumption interaction risk for early age leukemia, Brazil, 1999–2007.

Type of analysis/smoke burden[b]	Alcohol consumption[a]					
	ALL			AML		
	Abstainers	Occasional drinkers	Frequent drinkers	Abstainers	Occasional drinkers	Frequent drinkers
Periconception						
Crude OR (95% CI)						
Nonsmokers	1.00	1.67 (0.96–2.94)	1.14 (0.63–2.06)	1.00	2.01 (0.92–4.36)	0.97 (0.38–2.47)
Moderate smokers	0.64 (0.36–1.12)	0.00	2.10 (0.77–5.78)	0.66 (0.28–1.58)	1.56 (0.18–7.60)	0.45 (0.04–4.67)
Heavy smokers	2.10 (0.69–6.44)	1.78 (0.51–6.17)	1.36 (0.20–9.53)	0.99 (0.19–8.34)	0.00	0.00
Adj.[c] OR (95% CI)						
Nonsmokers	1.00	0.88 (0.39–1.98)	1.22 (0.59–2.53)	1.00	2.01 (0.81–4.96)	1.19 (0.43–3.27)
Moderate smokers	1.00 (0.51–1.94)	0.00	1.46 (0.48–4.43)	0.64 (0.25–1.66)	1.28 (0.16–10.2)	0.38 (0.03–4.29)
Heavy smokers	1.89 (0.28–12.69)	0.97 (0.22–4.29)	1.41 (0.16–12.3)	1.16 (0.13–10.5)	0.00	0.00
During pregnancy						
Crude OR (95% CI)						
Nonsmokers	1.00	1.24 (0.63–2.45)	1.19 (0.62–2.30)	1.00	1.63 (0.67–3.98)	0.21 (0.03–1.58)
Moderate smokers	1.00 (0.55–1.81)	1.69 (0.44–6.45)	0.91 (0.23–3.55)	0.44 (0.13–1.48)	0.00	0.00
Heavy smokers	2.39 (0.47–12.0)	0.00	1.68 (0.09–32.3)	2.10 (0.21–20.6)	0.00	0.00
Adj.[c] OR (95% CI)						
Nonsmokers	1.00	0.88 (0.39–1.98)	1.22 (0.59–2.53)	1.00	1.78 (0.66–4.80)	0.22 (0.03–1.71)
Moderate smokers	1.00 (0.51–1.94)	1.26 (0.26–6.16)	1.00 (0.23–4.44)	0.35 (0.10–1.31)	0.00	0.00
Heavy smokers	1.89 (0.28–12.69)	0.00	1.61 (0.05–51.0)	3.47 (0.32–37.0)	0.00	0.00

[a]OR, odds ratios, and 95% CI (confidence intervals) for leukemia according to categories of joint tobacco and alcohol exposures comparing models that assuming independence of effects and effect modification; level of pregnancy alcohol consumption: abstainers (0 glasses/week); occasional drinkers (≤1 glass/week); frequent drinkers (≥1 glass/week); [b]level of pregnancy smoke consumption: nonsmokers; moderate smokers (1–≤20 cigarettes/day); heavy smokers (≥20 cigarettes/day); ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; *n* = number of cases; Adj. OR, adjusted odds ratio; CI, confidence interval; [c]adjusted OR by use of oral contraceptives during pregnancy, maternal age at birth, maternal education, birth weight, and infant skin color.

consequently associated with several health hazards, including miscarriages, fetal distress, prematurity, malformations, fetal growth retardation, infections, and neurological and respiratory sequels, in addition to intellectual disabilities [35, 36]. This study was not able to determine the strength or direction of any association with maternal alcohol intake. Beer was the beverage most commonly consumed by mothers in this study. Other than beer, few mothers reported high intake levels of spirits and wines. A slight increase in ALL risk following the amount of beer consumption at preconception period was observed, adj. OR = 1.30 (95% CI 0.71–2.36) for weekly intakes of ≤1 glass and adj. OR = 1.47 (95% CI 0.88–2.46) for >1 cup, P trend < 0.03. Data from Brazilian Antidrug Secretary Survey indicated that 9–12% of women between 18 and 44 years of age reported alcohol consumption on a regular basis and 38–44% of women in the reproductive age were abstainers. According to this survey, beer is the most popular beverage choice, pointed by 58% of Brazilian women, followed by wine (34%) and spirits (14%) [37]. Of note, the overall frequencies of abstainers mothers in this epidemiological study were 59.8%, and similar to those reported in population-based surveys [37], beer was by far the preference. The different patterns and types of alcohol drinks consumed according to distinct socioeconomic status in Brazil should be considered; maternal wine consumption is relatively low compared to other studies that provided this information [22, 23].

This null association between maternal alcohol consumption and EAL risk regardless of all the period of exposure during pregnancy is consistent with the literature data, similar to overall childhood risks, which pointed out an inverse association especially according to wine intake, OR = 0.7 (95% CI 0.5–0.9) [22, 38, 39]. Slater et al. reported statistically significant inversed association between maternal alcohol use during pregnancy and IL [34]. In the Canadian, French, and Australian studies, wines are the beverage most consumed. Milne et al. also observed U-shaped associations with paternal alcohol consumption in the year before pregnancy, by reduced risk at moderate levels of wine and beer consumption and increased risk associated with high levels

of beer intake [23]. Wines contain antioxidant compounds, such as polyphenols, that possibly accomplish the protective effect of DNA damaging of ethanol and acetaldehyde [23]. Nonetheless this speculation is in opposite direction to the causative effect of polyphenols in IL proposed [3]. IL is strongly associated with *MLL-r*, mainly in ALL cases diagnosed before the first year of life [31]. The confirmation of the same *MLL-r* by retrospective analyses of neonatal blood has led to the proposal that transplacental exposure to topoisomerase-II inhibitors during pregnancy would be one of the causation factors of IL [3, 32, 40]. Based on experiments that demonstrated block of topoisomerase-II function by some substances, including phenols, which inhibit the resealing of broken DNA-strand ends, the formation of *MLL* translocations would be associated with exposures to such substances [41, 42].

Alcohol drinking is a behavior that often accompanies tobacco smoking, and its role in childhood cancer causality is scarce [16, 20]. The interaction between of maternal alcohol consumption and tobacco smoking as addictive effect in the risk of EAL was tested. The risk magnitudes for a model assuming effect modification were compared with the baseline model assuming independence of effects, but no effect modification was observed in strength of the associations.

This analysis has some limitations as consequence of case-control study in such rare settings. The hospital-based case-control study design may introduce selection bias depending on the chosen comparison groups [43]. Therefore, we recruited controls with a variety of indications for hospitalization and enrolled controls from general hospitals in the same cities, though not necessarily the same hospitals, in which the cases were diagnosed. As in the majority of the case-control approach, the analysed data of variables were dependent on level of perception of maternal report (more accurate in mothers' cases) for variables such as drug and/or tobacco use (that might cause their child leukemia) causing recall bias. Some possible explanation for the imprecise exposure estimates could be that some exposures were possibly being underreported. Sample size was limited mainly to infant AML and for stratified analysis based on *MLL* status. The reduced numbers of infants with low frequency of maternal exposures report make OR unstable, thus resulting in imprecise estimates of association. Another weakness of our study are the data on paternal smoking before and during pregnancy were not collected during the interviews.

On the other hand, the study has strengths regarding the large series of IL cases, given they are rare, compared with other studies that have tested childhood leukemia in older children.

Thereby, data from the use of tobacco smoking in this study did not show evidence of a modification effect by concomitant maternal use of alcohol and tobacco during pregnancy. In this regard, recently, our group demonstrated the increased risk association between maternal smoking and EAL with *MLL-r* modulated by genetic susceptibility [44]. The significant associations found could guide the design of other observational studies in childhood leukaemia, emphasizing the genetic susceptibility in the mechanistic pathway leading to leukaemia in early childhood.

5. Conclusion

This study does not support the hypothesis of an increased risk of EAL associated with maternal alcohol consumption during pregnancy. Additionally, no effect modification was observed in strength of the associations with maternal tobacco and smoking. Nevertheless, parents should be advised to limit alcohol intake when planning a pregnancy due to the premise that alcohol metabolites cause DNA damage in gametes and fetal cells according to experimental models.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Clinical Study

Role of Genetic Polymorphisms of Deoxycytidine Kinase and Cytidine Deaminase to Predict Risk of Death in Children with Acute Myeloid Leukemia

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Cytarabine is one of the most effective antineoplastic agents among those used for the treatment of acute myeloid leukemia. However, some patients develop resistance and/or severe side effects to the drug, which may interfere with the efficacy of the treatment. The polymorphisms of some Ara-C metabolizing enzymes seem to affect outcome and toxicity in AML patients receiving cytarabine. We conducted this study in a cohort of Mexican pediatric patients with AML to investigate whether the polymorphisms of the deoxycytidine kinase and cytidine deaminase enzymes are implicated in clinical response and toxicity. Bone marrow and/or peripheral blood samples obtained at diagnosis from 27 previously untreated pediatric patients with *de novo* AML were processed for genotyping and *in vitro* chemosensitivity assay, and we analyzed the impact of genotypes and *in vitro* sensitivity on disease outcome and toxicity. In the multivariate Cox regression analysis, we found that age at diagnosis, wild-type genotype of the CDA A79C polymorphism, and wild-type genotype of the dCK C360G polymorphism were the most significant prognostic factors for predicting the risk of death.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of leukemias that result from the clonal transformation of a primitive stem/progenitor cell by more than one genetic aberration. It accounts for 20–25% of all childhood acute leukemias and is responsible for more than one-half of the leukemia deaths.

Current pediatric AML protocols result in 85%–90% complete remission rates [1], with long-term survival rates for patients who achieve remission in the range of 60%–70% and event-free survival rates between 45% and 55% [2–6].

This has made possible thanks to the intensification of chemotherapy, better risk group stratification, increased use of allogeneic hematopoietic stem-cell transplantation, and improvements in supportive care. However, resistance to chemotherapy remains a major cause of treatment failure among pediatric patients with AML, with adverse side effects contributing to morbidity and mortality.

Cytarabine (1-β-D-arabinofuranosylcytosine, Ara-C) is a structural analog of deoxycytidine (dCyd) and has been the mainstay of treatment for AML for more than four decades [7]. It is a hydrophilic molecule that requires facilitated diffusion via nucleoside-specific membrane transport carriers [8]. To enter the cell, Ara-C binds to the human equilibrative nucleoside transporter (hENT1), but, when administered at high doses, the drug can be taken up by passive diffusion [9]. Inside the cell, Ara-C is phosphorylated to monophosphate (Ara-CMP) by deoxycytidine kinase (dCK); afterwards, two further phosphorylations are catalyzed by pyrimidine kinases

to convert Ara-CMP into the active metabolite ara-cytidine-5′-triphosphate (Ara-CTP) [10]. Ara-CTP competes with dCTP for DNA polymerases α, β, and δ; its incorporation into DNA results in chain termination, with consequent inhibition of DNA and RNA synthesis, causing leukemic cell death. Thus, the intracellular concentration of Ara-CTP is one of the determinants of the antileukemic effect of Ara-C. [11]. The catabolism of Ara-C results from rapid irreversible deamination by cytidine deaminase (CDA) to the noncytotoxic metabolite arabinoside uridine (Ara-U), while the enzyme 5′-nucleotidase (NT5C2) dephosphorylates Ara-CMP, thereby stopping the production of Ara-CTP [12]. Ara-CMP can also be converted into Ara-U by the enzyme deoxycytidylate deaminase (DCTD).

Resistance to Ara-C has been associated with conditions that reduce the intracellular concentration of Ara-CTP, such as inefficient cellular uptake of Ara-C, reduced Ara-C activation, or increased Ara-C inactivation, mainly due to variations in the activity or expression of hENT1 or of the enzymes that participate in the Ara-C metabolic pathway [13]. Malignant cells with a larger intracellular pool of cytosine deoxynucleotides can also be more resistant to Ara-C as a result of a competitive inhibition of dCK activity by dCTP [14].

Several studies have demonstrated genetic variation in key genes of the pharmacokinetic pathway of Ara-C with a consequent variation in the intracellular concentrations of Ara-CTP that can result in differences in toxicity and in the clinical response to AML treatment. These findings provide the molecular basis for explaining interpatient variability to cytidine analogues [15–17]. However, few studies have focused on the association of these genetic polymorphisms with clinical outcome and toxicity in patients with AML treated with cytarabine-based chemotherapy [18–20], and no study has been done on a Mexican population.

We conducted this study in a cohort of Mexican pediatric patients with AML to investigate whether the CDA Single Nucleotide Polymorphism (SNP) A79C and the dCK SNPs C360G and C201T are implicated in clinical response and toxicity of this leukemia.

2. Patients and Methods

2.1. Patient Samples. Bone marrow and/or peripheral blood samples were obtained at diagnosis from 27 previously untreated pediatric patients with *de novo* AML. We excluded patients with Down Syndrome, promyelocytic leukemia, treatment-related AML, those who abandoned therapy before remission could be documented, and cases without sufficient biomaterial for *in vitro* cytotoxicity assay and genetic analyses.

The diagnosis of AML was based on bone marrow examination, according to the FAB and WHO classification criteria; immunophenotype was determined by flow cytometry, and molecular analyses were performed by real time PCR.

2.2. Treatment Protocol. The patients were treated according to a modified NOPHO-AML 93 protocol [3]. All received a first course of Ara-C, etoposide, doxorubicin, and 6-mercaptopurine (6-MP) (ATEDox); since there is no 6-tioguanine available in Mexico, it was substituted by 6-MP. Bone marrow was assessed by morphology on day 16. Good responders received a second ATEDox course, while poor responders received an Ara-C plus mitoxantrone (AM) course. Patients with more than 5% blasts in the bone marrow after the AM course were given a course of high dose of Ara-C plus etoposide (HA2E). If remission was not achieved, the child was classified as a nonresponder. All patients in remission were treated with courses of high-dose cytarabine-based consolidation. The total number of courses was six, with the exception of the patients who required three induction courses to achieve remission. No patient underwent stem-cell transplantation in the first remission.

The patients were stratified according to their response to the first induction course. Good risk patients were those with less than 5% blasts after the first cycle.

Toxicities were assessed using National Cancer Institute common terminology criteria (version 3.0).

2.3. Chemicals. Ara-C was obtained from Pfizer Laboratories (Cytosar U), MTT [3-(4,5-dimethylthiazol)-2,5-diphenyltetrazolium bromide] from SIGMA-Aldrich (St. Louis, USA). Tissue culture reagents and supplies were purchased from assorted vendors. PCR primers were obtained from Operon, Inc. (The Woodlands, TX, USA). Taq polymerase and restriction enzymes were purchased from New England Biolabs (Beverly, MA, USA).

2.4. Cell Culture. Mononuclear cells were isolated by sedimentation using Ficoll-Paque Plus (GE Healthcare, Sweden) density gradient centrifugation and cryopreserved at −80°C until use. Cell pellets were resuspended in 0.5 mL of RPMI-1640 medium (Thermo Scientific HyClone), plus 10% fetal calf serum, 100 U/mL of penicillin, 100 U/mL of streptomycin, 0.5 μg/mL of amphotericin B, and pH 7.0. To eliminate granulocytes, the cell pellets were frozen at −70°C and then thawed. The cells were incubated at 37°C under 5% CO_2 and were cryopreserved in 10% DMSO.

Before performing the experiments, we prepared smears of cell suspensions using double staining with Wright and myeloperoxidase stains to confirm, by morphology, that leukemic blasts were more than 95%.

2.5. In Vitro Chemosensitivity Assay. The *in vitro* Ara-C chemosensitivity of myeloblasts was assessed with the MTT assay using a 3-day cell culture assay.

Cryopreserved cells were thawed and cultured in 96-well plates with 10,000 cells per well in triplicate. Cytarabine was added at different concentrations: 0, 0.001, 0.01, 0.1, 1.0, 10, 100, and 1000 μg/mL. The control wells (Ara-C concentration 0) contained myeloblasts with culture medium, without cytarabine, and blank wells contained only medium. After incubating the cells for 72 hours at 37°C in a humidified atmosphere containing 5% CO_2, 100 μL of 10% MTT was added to each well, and the plates were incubated for additional 4 hours. Formazan crystals were dissolved by adding 100 μL

Table 1: Primer sequences used for amplification of the *dCK* and *CDA* genes.

Gene	Sequence	Product (bp)
dCK A	5′ GCC TTC TCC CCA GAT GAG TT 3′	583 bp
dCK B	3′ CAC TGG CGG GCC TGC GGG 5′	
CDA A	5′ GAC ACA CCC AAG GGG AGG A 3′	205 bp
CDA B	3′ GAC TGT AGG GGC AGT AGG CTG AAT 5′	

of isopropanol, and the optical density (OD) was measured spectrophotometrically at 570 nm. Results were considered evaluable only when the control wells contained more than 70% myeloblasts after 3 days of culture.

For every patient, cell viability was calculated for each drug concentration using the following equation (mean OD treated wells/mean OD control wells) × 100%, after correction for the background OD of the blank wells. To determine the IC_{50} value, we created *XY* data tables where *X* is log (Ara-C concentration) and *Y* is cell viability and performed a nonlinear regression analysis using the GraphPad 5 software.

2.6. DNA Extraction and Genotyping. Diagnostic peripheral blood samples were collected in EDTA-containing tubes. The blood was treated with ammonium chloride 1X, centrifuged at 2500 rpm for 15 minutes, washed with PBS, and centrifuged again. The cell pellet was then resuspended in 2 mL of PBS. DNA was extracted using the QIAamp DNA Blood Midi Kit (Qiagen, Düsseldorf, Germany) and stored at −20°C.

The polymorphisms C201T and C360G of the *dCK* gene and the polymorphism A719G of the *CDA* gene were identified by the PCR-RFLP (restriction fragment length polymorphism) method. Genomic DNA was amplified using a Mastercycler gradient thermocycler (Eppendorf, Germany), with oligonucleotides specific for each gene (Table 1) [21, 22].

2.7. Detection of the A79C Polymorphism of the CDA Gene. Genomic DNA was amplified using oligonucleotides specific for the A79C polymorphism of the *CDA* gene, 2.5 mM dNTPs, 3 mM $MgCl_2$, 1X buffer (20 mMTris-HCl and 2 mM $MgSO_4$), and 0.5 U DNA Taq polymerase. The amplification conditions were as follows: initial denaturation at 95°C for 3 min followed by 40 cycles at 95°C for 40 sec, 63°C for 40 sec, and 72°C for 30 sec, with a final extension at 72°C for 1 min. A 205-bp PCR product was generated. After the amplification reaction was performed, 10 μL of the PCR product was digested with 2 U of the restriction enzyme *Eco RI* at 37°C for 2 h. In the absence of the A79C polymorphism, the enzyme cuts the DNA at position 79, generating one fragment at 180 bp. When a polymorphism is present, the cutting site is eliminated and the amplified product remains the original size (205 bp) (Figure 1(a)).

2.8. Detection of the C201T Polymorphism of the dCK Gene. Genomic DNA was amplified using oligonucleotides specific for the C201T polymorphism of the *dCK* gene, 2.5 mM dNTPs, 3 mM $MgCl_2$, 1X buffer (20 mM Tris-HCl and 2 mM $MgSO_4$), and 0.5 U DNA Taq polymerase. The amplification conditions were as follows: initial denaturation at 95°C for 3 min followed by 40 cycles at 94°C for 60 sec, 65°C for 38 sec, and 72°C for 60 sec, with a final extension at 70°C for 5 min. A 583-bp PCR product was generated. After the amplification reaction was performed, 10 μL of the PCR product was digested with 5 U of the restriction enzyme *Bgl I* at 37°C for 4 h. In the absence of the C201T polymorphism, the enzyme cuts the DNA at position 201, generating two fragments at 379 bp and 204 bp. When a polymorphism is present, the cutting site is eliminated and the amplified product remains the original size (583 bp) (Figure 1(b)).

2.9. Detection of the C360G Polymorphism of the dCK Gene. Genomic DNA was amplified using oligonucleotides specific for the *C360G* polymorphism of the *dCK* gene, 2.5 mM dNTPs, 3 mM $MgCl_2$, 1X buffer (20 mMTris-HCl and 2 mM $MgSO_4$), and 0.5 U DNA Taq polymerase. The amplification conditions and primers were the same as for the C201T polymorphism. A 583-bp PCR product was generated. After the amplification reaction was performed, 10 μL of the PCR product was digested with 5 U of the restriction enzyme *Kas I* at 37°C for 4 h. In the absence of the C360G polymorphism, the enzyme cuts the DNA at position 360, thereby generating three fragments at 276 bp, 214 bp, and 93 bp. When a polymorphism is present, one cutting site is eliminated, thereby yielding two products of 490 bp and 93 bp (Figure 1(b)).

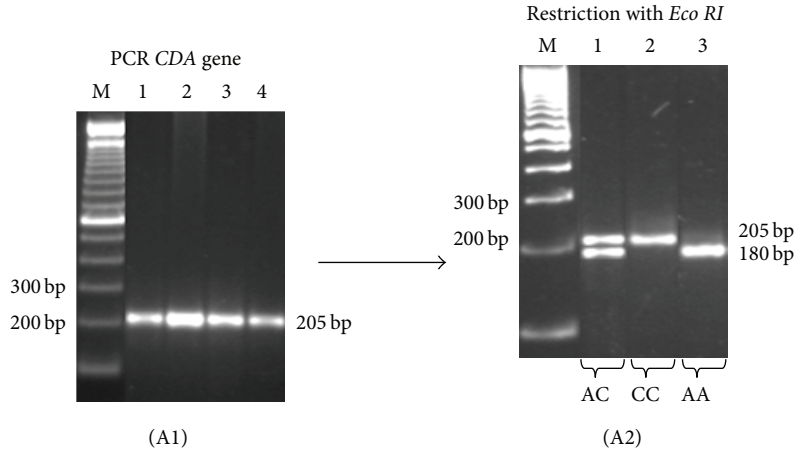
All restrictions and PCR products were visualized using UV light on 1.5% agarose gel stained with ethidium bromide.

2.10. Statistical Analysis. The categorical variables and *in vitro* Ara-C chemosensitivity were compared across genotypes using Fisher's exact test.

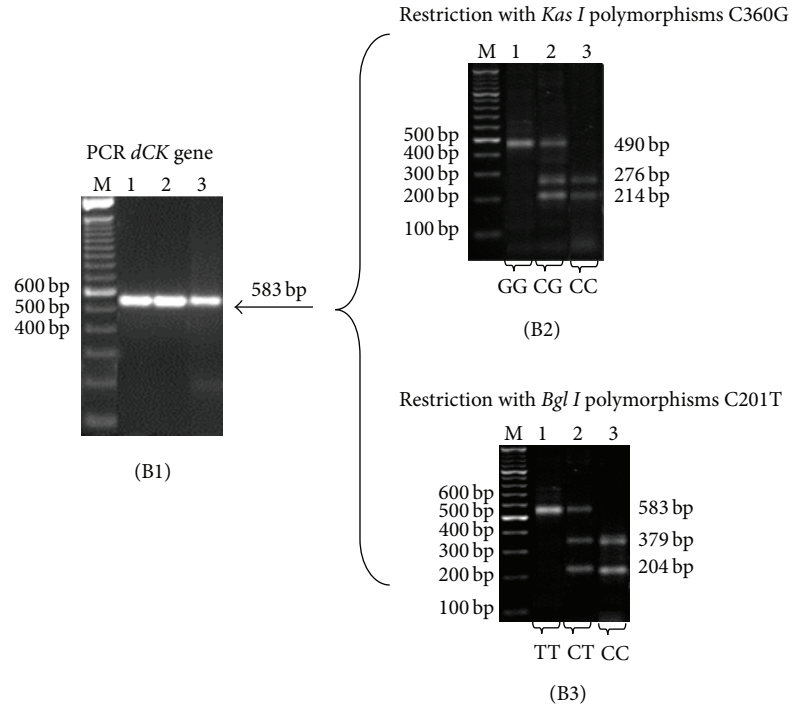
Due to the skewness of the distributions, a logarithmic transformation was performed on the chemosensitivity values. According to their chemosensitivity, the patients AML cells were classified as sensitive (IC_{50} < 2.5 μg/mL), intermediate (IC_{50} 2.5–5 μg/mL), and resistant (IC_{50} > 5 μg/mL) [23].

Event-free survival (EFS) was calculated from the date of diagnosis until relapse or death. Overall survival (OS) was calculated from the date of diagnosis until death by any cause or last follow-up using the Kaplan-Meier method, and differences between groups were estimated by the log rank test.

The Cox model was used to identify independent prognostic variables affecting overall survival. All *P* values were considered significant when ≤0.05.



(a) Detection of the CDA A79C polymorphism



(b) Detection of the dCK C360G and C201T polymorphisms

Figure 1: Identification of the genotypes of *CDA* and *dCK* genes. (a) Polymorphism A79C of *CDA* gene. (A1) *CDA* gene PCR. M 100 bp DNA ladder; lines 1–4: PCR product prior to digestion 205 bp. (A2) Restriction with *Eco RI*. M 100 bp DNA ladder; line 1: heterozygous (AC); line 2: variant homozygous (CC); line 3: wild-type homozygous (AA). (b) Polymorphism C360G and C201T of *dCK* gene. (B1) *dCK* gene PCR. M 100 bp DNA ladder; lines 1–3: PCR product prior to digestion 583 bp. (B2) Restriction with *Kas I*. M 100 bp DNA ladder; line 1: variant homozygous (GG); line 2: heterozygous (CG); line 3: wild-type homozygous (CC). (B3) Restriction with *Bgl I*. M 100 bp DNA ladder; line 1: variant homozygous (TT); line 2: heterozygous (CT); line 3: wild-type homozygous (CC).

The statistical analysis was carried out with the SPSS program (version 20) and the Stata software (version 12) was used for Cox regression analysis.

2.11. Definitions. Complete remission was defined as a bone marrow blast count of less than 5% on morphologic examination, with absolute neutrophil count >1,000/μL, platelets >80/μL, and no evidence of extramedullary disease.

Nonresponders were defined as all those patients that did not achieve complete remission after three induction courses and survived the first 6 weeks of treatment.

Deaths that occurred after induction failure or relapse were classified as leukemia-related, and the deaths that were not preceded by these events were considered as treatment-related.

Early deaths were defined as the deaths that occurred within the first 6 weeks of treatment, regardless of the cause.

The IC_{50} was defined as the concentration of drug necessary for growth inhibition of 50% compared with control cells cultured in the absence of Ara-C.

3. Results

3.1. Patient Characteristics. From January 2008 to January 2013, 51 consecutive AML pediatric patients were treated in our institution; of these, 10 had PML, 5 had treatment-related AML, 3 had Down Syndrome, in 4 cases there was insufficient biomaterial for the analyses, and 2 patients refused chemotherapy. A total of 27 patients were eligible.

The median age at diagnosis was 5.91 years (ranging from 18 days to 16.5 years). Eleven (40.7%) were male. The morphological subtypes were M1 9 cases (33.3%), M2 7 (25.9), M4 8 (29.6%), M5 1 (3.7%), and M6 2 patients (7.4%).

Initial characteristics and clinical outcomes of these 27 patients are summarized in Table 2.

3.2. Genotype Information. The genotype frequencies of the *dCK* C201T polymorphism were C/C 9 (33.3%), C/T 11 (40.7%) and T/T 7 (25.9%) of the *dCK* C360G polymorphism were C/C 10 (37%), C/G 14 (51.9%), and G/G 3 (11.1%), and of the *CDA* A79C polymorphism were A/A 15 (55.6%), A/C 6 (22.2%) and C/C 6 (22.2%) (Table 3).

3.3. Therapy Response and Disease Outcomes. At the time of analysis the median follow-up was 21 months. Twenty patients (74.1%) achieved remission after the first induction course, 1 (3.7%) entered remission after the second course, 3 (11.1%) were nonresponders, and 3 (11.1%) died before remission could be assessed.

Among the 21 patients who achieved complete remission, there were 5 relapses. Two patients underwent peripheral stem-cell transplantation after relapse, one is still alive, and the other died from relapse after transplant. One patient abandoned treatment before transplant while being still in second remission, one patient developed refractory leukemia, and one was treated with chemotherapy alone; he is still alive and disease-free at 28 months after relapse.

Thirteen patients died; 8 (61.5%) were treatment-related and 5 (38.5) were leukemia-related deaths. Of them, 5 were early deaths, 4 were due to infectious complications, and one was as a consequence of pulmonary hemorrhage. The nonresponder patients died of infections while being treated with intensive chemotherapy, 3 patients died in remission during consolidation therapy, and 2 died after relapse.

3.4. Toxicity Analysis. Chemotherapy toxicity was recorded for a total of 115 courses of chemotherapy in 27 patients.

The 115 cycles were complicated by fever requiring intravenous antibiotic or antifungal therapy. In 24 of these events (20.8%), signs of septic shock were present and 12 patients died from septic shock.

The most common grade ≥3 toxicities observed were neutropenia in 85.2% of the cycles, thrombocytopenia in 25.2%, febrile neutropenia in 25.2%, neutropenic enterocolitis in 6%, and mucositis in 5.2%.

Table 2: Initial characteristics and clinical outcomes of 27 pediatric patients with AML.

Number of patients	27
Age, median (yr), range	5.9 (0.05–16.5)
Sex	
Male	11 (40.74)
Female	16 (59.26)
Total blood counts	
Leucocytes, median (range)/uL	23,000 (3,100–254,000)
Platelets, median (range)/uL	36,000 (3,000–152,000)
Hemoglobin, median (range) g/dL	6.8 (2.8–13.0)
WBC, >100,000/uL, *n* (%)	8 (29.6)
CNS positive, *n* (%)	2 (7.4)
FAB, *n* (%)	
M1	9 (33.3)
M2	7 (25.9)
M4	8 (29.6)
M5	1 (3.7)
M6	2 (7.4)
M7	0
Remission	
After the first induction course *n* (%)	20 (74.1)
After the second induction course *n* (%)	1 (3.7)
Nonresponders *n* (%)	3 (11.1)
Not assessed	3 (11.1)
Relapses *n* (%)	5 (18.5)
Deaths *n* (%)	13 (48.1)
Early deaths	5/13 (38.4)
Treatment-related	8 (61.5)
Leukemia-related	5 (38.5)

We found significant association between the 201 CC genotype of the *dCK* gene and hematological toxicity grades ≥3 ($P = 0.038$), and a trend towards association was found between the wild-type genotype 79AA of the *CDA* gene and hematological toxicity grades ≥3 ($P = 0.057$). No association was found between the C360G genotypes of the *dCK* gene and severe toxicity.

3.5. In Vitro Chemosensitivity. The median Ara-C IC_{50} value was 5.27 μg/mL, 3 patients exhibited a sensitive pattern, 10 had intermediate sensitivity, and 14 were resistant ($IC_{50} > 5\,\mu$g/mL).

3.6. Survival Analysis. Three out of the 5 patients who relapsed were Ara-C resistant and 2 had intermediate chemosensitivity, but no significant association could be demonstrated between in *vitro* sensitivity to Ara-C and the risk of relapse ($P > 0.1$).

We found a trend toward association between the wild-type genotype 360 CC of the *dCK* gene and *in vitro* resistance to Ara-C (76.9% of the wild types were resistant versus 35% of the variants) ($P = 0.056$). And the same was found with the wild-type genotype 201 CC (81.8% of the wild types were

Table 3: Frequencies of C201T and C360G polymorphisms of the *dCK* gene and A79C of the *CDA* gene in 27 Mexican pediatric patients with acute myeloid leukaemia.

CDA gene		*dCK* gene			
Polymorphism A79C		Polymorphism C360G		Polymorphism C201T	
Genotype	Frequency % ($n = 27$)	Genotype	Frequency % ($n = 27$)	Genotype	Frequency % ($n = 27$)
AA	15 (55.6%),	CC	10 (37%)	CC	9 (33.3%)
AC	6 (22.2%)	CG	14 (51.9%)	CT	11 (40.7%)
CC	6 (22.2%).	GG	3 (11.1%)	TT	7 (25.9%)

resistant versus 36.4% of the variants) ($P = 0.064$). There was no association between the A79C polymorphisms and *in vitro* Ara-C chemosensitivity.

The survival analysis using the Kaplan-Meier method revealed that, in the C360G polymorphisms of the *dCK* gene, the variant genotype significantly increased OS (50.3% versus 20%) ($P = 0.006$) and EFS (37.8% versus 20.0%) ($P = 0.028$). For the C201T polymorphism of the *dCK* gene, the outcome was significantly worse for the patients carrying the wild-type genotype OS (53% versus 22.2%) ($P = 0.001$) and EFS (44.7 versus 11.1%) ($P = 0.001$). For the A79C genotypes of the *CDA* gene, we observed a significant difference in OS (75% versus 38.1%) ($P = 0.041$) and a marginal difference in EFS among patients who carried the variants 79AC and 79CC compared to those with the wild type (Figure 2). We also found a significant difference between the *in vitro* Ara-C sensitivity pattern and survival (Figure 3). The patients who exhibited a resistant pattern ($n = 14$) had a significantly worse OS (19%) compared to the patients that had an intermediate sensitivity (30%) ($P = 0.036$) and to those who were sensitive to Ara-C (100%) ($P = 0.031$), while age, gender, subtype, and leukocyte counts at diagnosis did not have an impact on survival.

3.7. Cox Regression Analysis. In the multivariable Cox regression analysis, we included age, gender, FAB subtype, leukocyte count at diagnosis, response to the first induction course, *in vitro* chemosensitivity, C360G SNPs and C201T SNPs of the *dCK* gene, and A79C SNPs of the *CDA* gene. We found that the most significant prognostic factors were age at diagnosis, wild-type genotype of the A79C polymorphism, and wild-type genotype of the C360G polymorphism (Table 4). Gender, FAB subtype, leukocyte counts at diagnosis, response to the first induction course, and Ara-C *in vitro* chemosensitivity did not show significant effect on the risk of death.

4. Discussion

Treatment of acute myeloid leukemia remains a challenge worldwide. It is a fact that lower-income countries have not achieved the same survival rates as high-income countries, and their results are not easily reproducible despite the use of the same protocols [24].

In low-income countries, treatment-related mortality remains a significant cause of treatment failure, while in high-income countries relapse is the leading cause of death. Possible explanations for this difference include variations in supportive care interventions, sociocultural aspects, nutritional status, and genetic factors.

Cytarabine is the essential drug in AML induction and consolidation chemotherapy. The response to and the toxicity of Ara-C treatment are characterized by significant interindividual differences, and the development of Ara-C resistance is still a major problem, raising the possibility that the pharmacogenetics of this agent may play a role in the difference of outcomes. Many single nucleotide polymorphisms (SNPs) and haplotypes of *dCK* and *CDA*, which contribute to susceptibility to Ara-C, have been identified in Africans, Europeans, and Chinese [24–26]; however, there are few reports about the relation between dCK and CDA polymorphisms and AML outcome [18–20].

We analyzed the impact of the polymorphisms of genes encoding cytidine deaminase (CDA A79C) and deoxycytidine kinase (dCK C360G, C201CT) and of *in vitro* sensitivity to Ara-C on disease outcome and toxicity in 27 *de novo* pediatric AML patients.

In this study, treatment-related mortality represented the main cause of failure (61.5% of the deaths), similar to the mortality pattern reported in other Latin American countries [24].

We found that children with the 79CC genotype of the *CDA* gene and the 360CC genotype of the *dCK* gene had significantly higher risk of dying, and the 201CC genotype was also associated with higher incidence of hematological toxicity grades ≥3, as well as less probability of survival.

In a previous study, Children's Oncology Group demonstrated that children with a low-activity 79CC genotype were at an increased risk of treatment-related mortality [19]. Some *in vitro* studies have shown that increased CDA enzyme activity has a role in the development of resistance to cytarabine [27–29]. The A79C polymorphism has also been associated with Ara-C toxicity [30], while the dCK-360G allele was found to increase the risk of mucositis after exposure to low-dose cytarabine in childhood ALL therapy [31].

Shi et al. reported an association between genotypes 360CC and 201CC of the *dCK* gene and poor clinical outcome in AML patients treated with Ara-C-based chemotherapy [25]. These authors demonstrated that the transcriptional activity of the 5′ flanking region of the homozygous variants of the *dCK* gene was eightfold greater than that of the wild type, suggesting that variant genotypes may provide

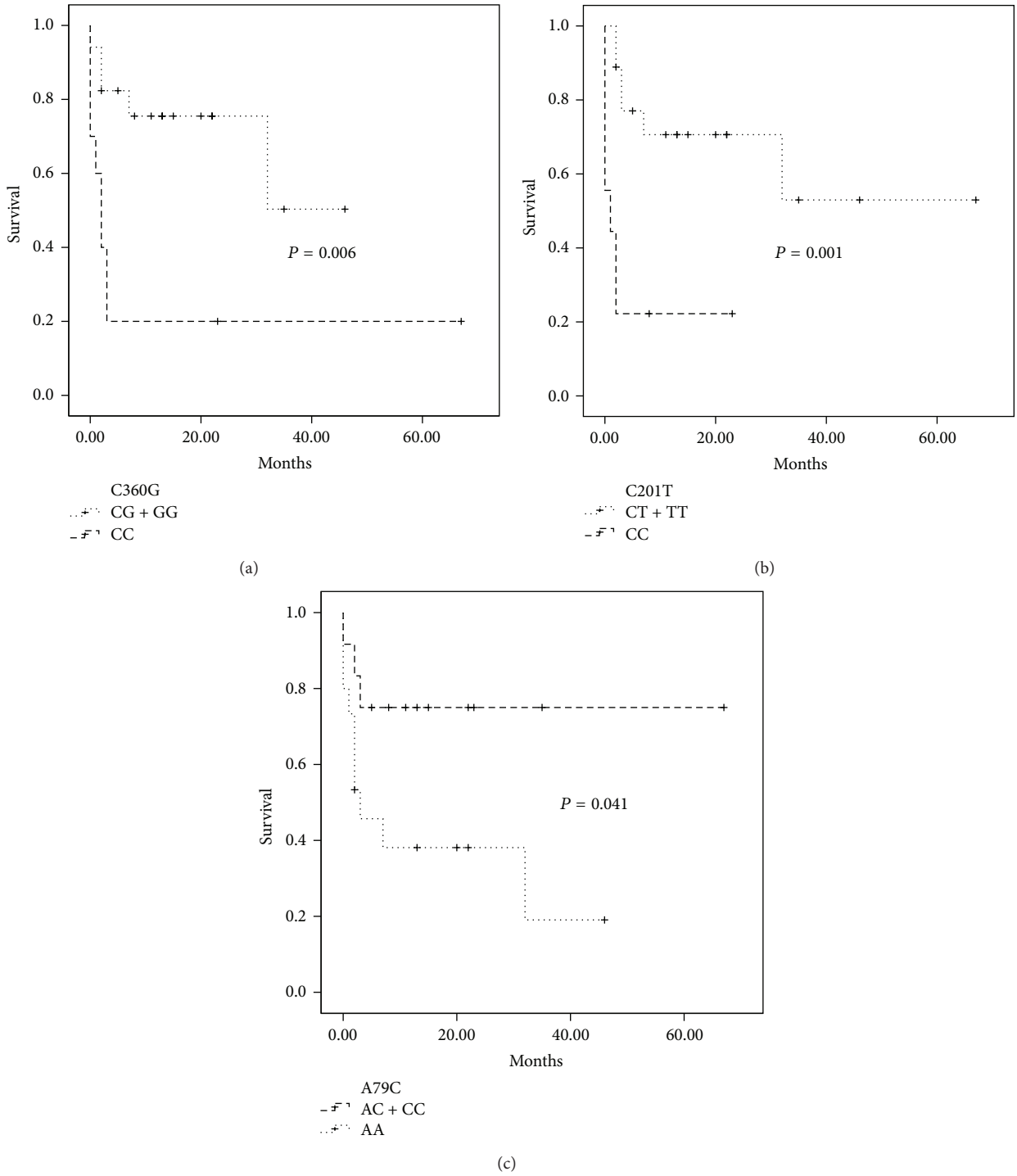


Figure 2: Kaplan-Meier curves of overall survival by genotypes. (a) *dCK* C360G SNPs, (b) *dCK* C201T SNPs, and (c) *CDA* A79C SNPs.

acellular transcriptional machinery with more efficient promoter/enhancement elements.

Age was one of the most significant prognostic factors, as has been found by previous pediatric studies [32, 33]. The Nordic Society for Pediatric Hematology and Oncology reported that children of 10 years of age or older had an inferior prognosis compared to younger children [3].

In our study, *in vitro* resistance to Ara-C seemed to be associated with low probability of survival, although this did not achieve statistical significance in multivariate analysis, possibly due to the limited sample size.

We were not able to demonstrate an association between response to the first induction course and disease outcome, as has been previously described by the NOPHO and other

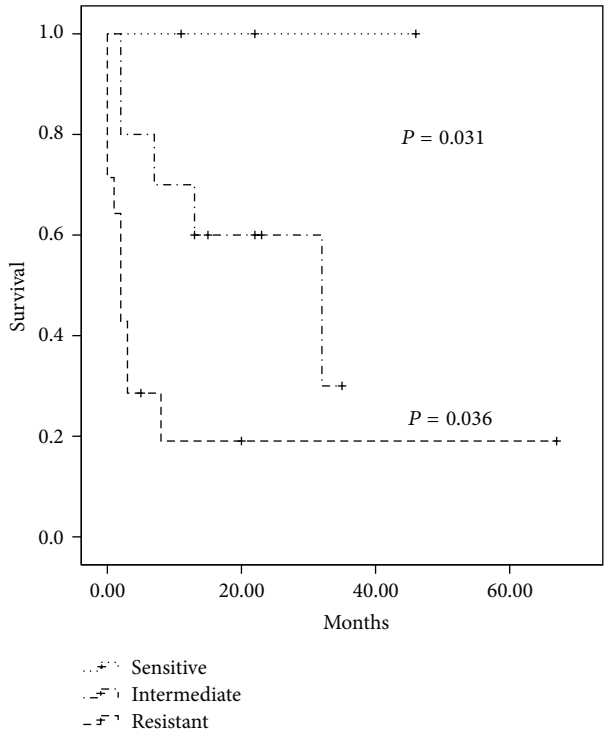


Figure 3: Kaplan-Meier curve of overall survival stratified according to the *in vitro* Ara-C sensitivity.

groups. This is probably related to the fact that most of the patients died relatively early from causes related to treatment and not as a result of resistance.

Early identification of patients at risk of severe toxicity allows for early intervention to reduce morbidity and mortality; equally important is the identification of those cases at risk of developing chemoresistance. Regarding the pharmacogenomics of cytarabine, clinical studies have identified a biomarker (Ara-CTP levels) and a main candidate gene (*dCK*), while cell-based models have identified candidate SNPs associated with these phenotypes.

We tested the hypothesis that the analysis of drug targets for polymorphism can help to establish gene-based information for the treatment of AML patients; we investigated functional SNPs in the *CDA* and *dCK* genes and found that some polymorphisms have a role in predicting survival and toxicity.

In a hospital like ours, where intensive care support, transfusion therapy, and appropriate treatment of infections are not a major problem, such a high rate of treatment-related mortality would not be expected. Thus, we believe that genetic factors are contributing to a higher frequency of serious adverse events leading to lower survival rates.

The frequency of homozygous wild-type genotype 79 AA of the *CDA* gene observed in this study was higher than that found in Caucasian populations [19, 34–36], who report the highest rates of survival in AML; however, it was lower than that of Asian populations [18, 37], suggesting that this genotype might be more common in Mexican than in Caucasian populations. Regarding the frequency of the wild-type genotype 360CC of the *dCK* gene, we found a lower frequency than that described in Chinese and French populations [25, 34], although there are few reports about the frequencies of these polymorphisms. These results must be interpreted with caution because they are based on a small number of patients and, although we found that both genotypes represent a risk factor, there are several enzymes involved in the metabolic pathway of Ara-C and various polymorphisms can be present in each. Further prospective studies on the clinical impact of these SNPs are warranted in large cohorts of Mexican/Hispanic ad in other populations to verify these results.

Table 4: Multivariate analysis (Cox proportional hazards model) of overall survival in patients with AML treated with Ara-C ($n = 27$).

Regressors	Hazard ratio	[95% Conf. Interval]
Age at diagnosis	0.817	0.683–0.979
Wild-type 79AA	8.314	1.798–38.436
Wild-type 360CC	11.064	1.588–77.056
Wild-type 201CC	0.769	0.140–4.231

Pharmacogenetics has become a major concern in personalized medicine; some markers such as TPMT, DPYD, or UGT1A1 are routinely used in clinical practice. The next challenge will be to incorporate new genotype-phenotype correlations into clinically useful diagnostics and prognostic stratification.

5. Conclusion

Despite the limited sample size, some of our results are statistically significant and consistent with previous reports. We consider that these findings add evidence to the role that polymorphisms C360T and C201T of the *dCK* gene and A79C of the *CDA* gene have to anticipate toxicities and predict treatment outcome and toxicity in patients with AML treated with cytarabine-based chemotherapy.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Bone Marrow Cells in Acute Lymphoblastic Leukemia Create a Proinflammatory Microenvironment Influencing Normal Hematopoietic Differentiation Fates

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B-cell acute lymphoblastic leukemia (B-ALL) is a serious public health problem in the pediatric population worldwide, contributing to 85% of deaths from childhood cancers. Understanding the biology of the disease is crucial for its clinical management and the development of therapeutic strategies. In line with that observed in other malignancies, chronic inflammation may contribute to a tumor microenvironment resulting in the damage of normal processes, concomitant to development and maintenance of neoplastic cells. We report here that hematopoietic cells from bone marrow B-ALL have the ability to produce proinflammatory and growth factors, including TNFα, IL-1β, IL-12, and GM-CSF that stimulate proliferation and differentiation of normal stem and progenitor cells. Our findings suggest an apparently distinct $CD13^+CD33^+$ population of leukemic cells contributing to a proinflammatory microenvironment that may be detrimental to long-term normal hematopoiesis within B-ALL bone marrow.

1. Introduction

Inflammatory cells and their products are important regulators of the tumor microenvironment. Based on experimental models and epidemiological data, increasing evidence has suggested the close connection between chronic inflammation and carcinogenesis, particularly in colorectal cancer, gastric cancer, and liver and lung neoplastic diseases [1–3]. Moreover, it has been estimated that about 25% of tumors are associated with chronic inflammatory processes caused by infections or unknown causes [1]. Inflammation may associate with cancer through two potentially connected pathways: the extrinsic, which results from external factors promoting latent inflammatory responses, and the intrinsic, conducted by oncogenes or tumor suppressor genes that activate the expression of inflammation-related programs [4]. For both of them, the cellular and cytochemical components within the tumor microenvironment are crucial. Macrophages are the most widely described inflammatory cells related to cancer [5, 6], while tumor-associated fibroblasts have been identified as good producers of proinflammatory cytokines and growth factors in skin, breast, and pancreas malignancies [7]. The participation of cells of the immune response in the initiation, growth, and tumor progression, as well as in the response to antineoplastic therapy, is apparently mediated by a variety of proinflammatory cytokines, including IL-1β,

IL-1α, TNFα, IL-6, IL-12, and IFNγ, and chemokines as CCL2 and CXCL12 [8, 9]. They play a role in tumorigenesis and tumor-associated inflammation has made them potential targets for adjuvant therapy in cancer. Of special interest, transcription factors such as NFκB and STAT3 may cooperate to promote the development and progression of cancer, by controlling the expression of antiapoptotic and proliferative genes, in addition to intervening in the regulation of angiogenesis and invasion [1, 8, 10]. Accordingly, the TLR-MyD88 signaling pathway has been shown to promote tumorigenesis in primary carcinogen models. Thus, together, cytokines, chemokines, and their receptors are keys to the binomial cancer-inflammation entity, compromising sometimes the physiological functions and promoting normal cell senescence, concomitant to proliferation and survival of tumor cells with invasive and metastatic properties [9, 11].

Despite the increasing evidence of anomalies in the bone marrow microenvironment of hematologic malignancies, which may govern stem cell activity and lead to disease (reviewed in [12, 13]), knowledge about the role of inflammation in leukemogenesis is scarce. At present, B-cell acute lymphoblastic leukemia is the most common cause of cancer in children worldwide. It is characterized by uncontrolled production of hematopoietic B-precursor cells within bone marrow (BM), with inexorable damage to their differentiation and functional properties. Although a number of genetic aberrations potentially contribute to leukemogenesis, the cellular origin of B-ALL is a fundamental issue under research [12, 14, 15]. Both, cell culture systems and animal models of human-mouse xenotransplantation and leukemic reconstitution suggest that leukemia-initiating cells have characteristics of primitive progenitors [16] and are sensitive to instability lineage triggered by extrinsic stimuli [13, 17]. According to the original hypothesis of leukemogenesis of Greaves, the occurrence of multiple consecutive lesions in hematopoietic cells can trigger malignant transformation, opening the possibility that not only the oncogenic damage inherent in the early stages of development, but abnormal microenvironmental factors could contribute directly or indirectly to generation or maintenance of leukemic precursors [18]. The implication of specific genetic aberrations in the triggering of ALL-inflammation setting will be highly relevant to investigate. A role of the fusion gene ETV/RUNX1 TEL/AML1 in the TGF signaling constitutes a milestone entailing this immune regulatory pathway to leukemogenesis, by compromising the normal cell responses to its inhibitory function. Accordingly, the favoring of reactive oxygen species by ETV6/RUNX1 has been recently reported that apparently origins in the B-cell progenitor compartment [19–21]. Furthermore, the competition of leukemic cells with normal hematopoietic cells by the normal marrow niches and their apparent contribution to formation of abnormal inhibitory microenvironments have suggested the exhaustion or migration of normal precursors, resulting in almost total BM replacement by malignant cells [22–24]. Remarkably, B-ALL primitive cells before and upon cultures have been shown to produce high levels of TNFα and IL-6, suggesting that an inflammatory microenvironment prevails in this BM disorder ([25, 26] and our unpublished observations). More recently, the studies from Guzman et al. indicate that leukemic stem cells in acute myeloid leukemia have constitutively activated the transcription factor NFκB [27, 28], a fact that may, in turn, harm the normal biology of hematopoietic niches. To determine whether lymphoblastic leukemia precursors contribute to an inflammatory BM microenvironment that may impact the earliest hematopoietic development from the onset of the disease, we have now investigated and confirmed the production of soluble factors, including cytokines, chemokines, and growth factors by mononuclear cells from B-ALL BM. By using *in vitro* proliferation assays and controlled culture systems, the impact of ALL-derived proinflammatory factors on normal hematopoietic differentiation potentials was examined, suggesting that within ALL marrow, normal primitive cells are driven into cycle and prompted to produce both lymphoid and innate lineage cells.

2. Materials and Methods

2.1. Patient Characteristics and Sample Collection. Fifty-four children referred to the "Federico Gomez" Children's Hospital (Mexico City, Mexico) and diagnosed with B-cell precursor acute lymphoblastic leukemia were included in the study. Among them, 34 patients fulfilled the criteria for high-risk disease by blood cell count, age, T-cell phenotype, or Ph+ chromosome, whereas 17 for fulfilled the criteria standard-risk. Within the high-risk group, 35% of the patients were female and 65% were male, while the standard risk group included 24% female and 76% male patients. The median age values were 7.8 year old (2 mo–18 yr) and 4.8 (2 yr–9 yr) for the high-risk and standard-risk group, respectively. BM specimens were collected by aspiration before any treatment, respecting international and institutional guidelines. Control BM specimens were obtained from healthy children undergoing minor orthopedic surgery. All procedures were approved by the Ethics, Research and Biosafety Committee of the "Federico Gómez" Children's Hospital (Registry HIM/018/2013) in Mexico City. Umbilical cord blood (UCB) samples were obtained from normal full-term neonates. All samples were collected after informed consent from the parents.

2.2. ALL Phenotyping and Pattern Definition. Patients fulfilling morphological criteria of ALL according to the French-American-British group (FAB) were stratified in line with clinical risk of relapse and phenotypic expression of CD10, CD19, CD20, CD22, CD79α, Kappa, Lambda, anti TdT, CD3, CD5, CD7, CD13, CD14, CD15, CD33, myeloperoxidase, CD45, and CD34. Patient groups were further defined in accordance with aberrant expression of myeloid markers, with 25% of the cells expressing the myeloid antigen being considered as positive. Cytokine production based on the expression of two or more antigens was assessed in ALL.

2.3. Cell Isolation and Supernatant Collection. Mononuclear cells (MNC) from B-cell precursor ALL patients were prepared by Ficoll-Paque Plus (GE Healthcare Bioscience) gradient separation and placed in culture with Alpha MEM medium (Gibco by Life Technologies) (200,000 MNC per

200 μL medium). Supernatants were collected at 24 h and evaluated for cytokine concentration (experimental time = 24 h). Additional 200 μL of culture medium was added to the same cells and their supernatants were reevaluated 24 h later (experimental time = 48 h).

2.4. Cytokine Detection. Supernatants were collected and stored at −20°C until analysis. The cytokine, chemokine, and growth factor content in supernatants were measured by Multiplex Milliplex Map Immunoassay (Merck Millipore), following the manufacturer's recommended protocols. The assay included SDF-1, BCA-1, LIF, TRAIL, SCF, TSLP, FGF-2, Flt-3L, G-CSF, GM-CSF, IFNα, IFNγ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-10, IL-12(p40), IL-12(p70), IL-15, IP-10, TNFα, and VEGF.

2.5. Immunofluorescence Microscopy. ALL and control MNC were fixed using 4% paraformaldehyde and hydrated with PBS. Permeabilization was performed with 0.01% Triton X-100 (Bio-Rad) and blocking with 2% BSA for 30 minutes at 37°C. Anti-pNFκB p65 (serine 536) and anti-pSTAT3 (tyrosine 705) antibodies (Cell Signaling) were incubated for 1 hour at 37°C followed by incubation with goat anti-rabbit Alexa 488 (Invitrogen) antibody. Slides were mounted with Vectashield with propidium iodide (Vector). Overlay of images from the different fluorescent channels was performed using ImageJ software (NHI) and Image Pro Plus software.

2.6. Purification of Precursor Cells. Mononuclear cells (MNC) from umbilical cord blood (UCB) were prepared by Ficoll-Paque Plus (GE Healthcare Bioscience) gradient separation. $CD34^+$ cells containing hematopoietic stem and progenitor cells were enriched from the MNC fraction using the Human CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec) according to manufacturer instructions, with final purity frequencies between 85 and 95%. Cells were enumerated before analyses, and the purity confirmed by flow cytometry.

2.7. CFSE Proliferation Assay. Carboxyfluorescein diacetate succinimidyl ester (CFSE) was used for *in vitro* labeling of cells to trace multiple generations using dye dilution by flow cytometry (Molecular Probes). $CD34^+$ CB cells were incubated with 10 mM CFSE and then exposed in a 120-hour culture to supernatants collected from control BM MNC (Control SN), noninflammatory BM MNC (Non Infl SN), or inflammatory BM MNC (Infl SN). After the 5 days, harvested cells were analyzed by flow cytometry for their phenotype and the number of cell divisions. Dilution of fluorescence intensity to monitoring up to 8 cell divisions was estimated using the application for cell proliferation within the FlowJo 7.6.1 software.

2.8. Stromal Cell Co-Cultures. UCB precursor cells were placed on MS-5 stromal cell monolayers and cocultured with them for 3 weeks in the presence of ALL MNC supernatants and with lymphoid conditions, according to a modified previous report [26]. The α-modified essential medium (α-MEM) was supplemented with 10% fetal bovine serum, 1 ng/mL Flt3-L (FL), 2 ng/mL SCF, 5 ng/mL IL-7, and 10 ng/mL IL-15 (Preprotech) and contained 100 U/mL penicillin and 100 mg/mL streptomycin. Supernatants collected from control BM MNC (Control SN), noninflammatory BM MNC (Non Infl SN), or inflammatory BM MNC (Infl SN) were used in a 3 : 1 medium : supernatant volume ratio. Coculture systems were incubated at 37°C in a humidified atmosphere of 5% CO_2. Media and supernatants were weekly replaced. This controlled system promotes the efficient differentiation of hematopoietic stem, progenitor, and precursor cells towards B cells, NK cells, lymphoid-related dendritic cells, and some myeloid cells. Cell frequencies and final yield per input values were calculated on the basis of specific lineage production within each condition.

2.9. Proliferation and Differentiation Analyses by Flow Cytometry. Phenotyping of UCB hematopoietic cells from lymphoid cocultures was performed by six-color flow cytometry on a FACSCanto flow cytometer (BD Biosciences). Cells were enumerated after culture to calculate cell frequencies and yields per input progenitor prior to staining with directly conjugated fluorescent antibodies (Invitrogen and BD Pharmingen). For the CFSE proliferation assay, fluorochrome-conjugated antibodies anti-lineage markers (CD3, CD8, TCR, CD56, CD14, CD11b, CD20, CD19, and CD235a) and anti-CD34 were used to identify the Lin^-CD34^+, Lin^-CD34^-, and Lin^+CD34^- populations. On the other hand, at the end of the differentiation cultures, harvested cells were stained with antibodies anti-CD34, -CD56, -CD11c, -CD19, -CD11b, and -CD14, and newly produced cells were identified by their phenotype as follows: stem and progenitor cell fraction as $CD34^+$; NK precursor cells as $CD34^+CD56^+$ cells; NK cells as $CD34^-CD56^+$ cells; dendritic cells as $CD11c^+$, and B cells as $CD19^+$, whereas myeloid cells were $CD11b^+CD14^-$ or $CD11b^+CD14^+$ cells. Analysis of flow cytometry data was performed using the FlowJo 7.6.1 software, and final yield per input values was calculated on the basis of specific lineage cell frequencies within each condition.

2.10. Statistics. The Prism (version 5.01, GraphPad) and SPSS software were used for statistical analysis. Comparisons between groups were performed with the unpaired t-test. P values were two-tailed and were considered significant if less than 0.05. Additionally, for the aberrant expression of myeloid markers, a distributional analysis of the data was made and found no normal distribution. Thus, comparison groups were performed with the nonparametric test U Mann-Whitney, comparing the medians and taking α of 5% to define statistical significance.

3. Results and Discussion

3.1. Two Groups of B-ALL Patients according to BM Hematopoietic Cell Cytokine Production. The hematopoietic microenvironment within bone marrow (BM) is constituted by a cellular network and its products (including extracellular matrix, cytokines, chemokines, and growth factors), which

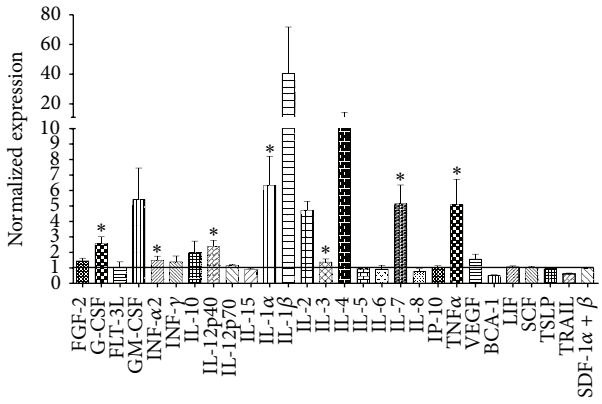

Figure 1: Bone marrow cells from childhood acute lymphoblastic leukemia produce proinflammatory cytokines and hematopoietic growth factors. ALL mononuclear cells from BM aspirates were cultured for 24 h, and supernatants were further collected and assayed for 28 cytokines, chemokines, and growth factors. Normalized production relative to normal bone marrow cells was tabulated.

form a highly organized three-dimensional structure to support hematopoiesis [12, 29]. Under normal conditions, the current model of hematopoietic microenvironment includes at least two specific cell niches, according to which stem cells require interaction with osteoblasts and endothelial cells, whereas the earliest progenitors are dependent on the contact with stromal cells expressing CXCL12/SDF1, and downstream lineage committed precursors of B cells require IL-7. The recent discovery of regulation of the hematopoietic developmental pathways by pathogen and/or danger recognition by primitive cells suggests that Toll-like receptors (TLR) are involved in the early cell fate decisions and contribute to the emergent replenishment of innate hematopoietic cells in the context of inflammatory settings [13, 30–38]. Moreover, the production of proinflammatory cytokines and growth factors, including TNFα, IL-12, IL-3, IL-6, IL-8, GM-CSF, and MCP-1, as a result of TLR signaling in normal progenitor cells, has been documented [37, 39–41]. In response to these inflammatory signals, hematopoietic stem and early progenitors can, in turn, produce cytokines that promote their mobilization and expedite differentiation to innate cells [42, 43].

Derived from the tumor microenvironment, leukemic cells have been shown to harm normal biology of hematopoietic cells [22]. Therefore, cytokines and growth factors were now quantified in supernatants derived from B-ALL MNC. Remarkably, the proinflammatory factors IL-1α, IL-1β, and TNFα were highly overproduced when compared to production levels by their normal counterpart, with up to 40-time increases for IL-1β (Figure 1). Moreover, some cytokines, interferons, and growth factors participating in inflammatory responses, including G-CSF, GM-CSF, IFNα, and IL-12 were substantially elevated in B-ALL. The same was true for early lymphoid-related growth factors, like IL-7 (Figure 1). Thus, for the first time, hematopoietic precursors in childhood B-cell acute lymphoblastic leukemia have been shown to produce proinflammatory and hematopoietic growth soluble factors. Modest increments were observed in IFNγ, IL-10, and VEGF, while no changes were registered in Flt3L, IL-15, IL-5, IL-6, IL-8, SCF, and SDF-1. Accordingly, although the evaluation of cytokines and growth factors in patients with acute lymphoblastic leukemia has been achieved mostly in serum and plasma specimens, a couple of relevant reports indicate their production by leukemic blasts in the bone marrow [44–47], supporting the notion that malignant cells directly contribute to the pathogenesis of ALL by creating an inflammatory microenvironment.

It remains to be seen whether the cytokine production in B-ALL results from a response to TLR ligation by pathogen-associated or damage-associated molecular patterns (PAMPs or DAMPs, resp.) [13, 37] or from a genetic aberration leading to a constitutive activation of inflammatory pathways [28]. As we have additionally found a significant production of inflammatory cytokines plus IL-6 by BM mesenchymal stromal cells (MSC) from B-ALL (unpublished data), we assume that some extrinsic stimuli may contribute to a bystander release of hematopoietic cytokines through activation of MSC [48].

Despite the heterogeneity in the abundance of the produced cytokines among patients, analyses of data dispersion indicated the existence of two groups, defined according to concentration of the produced cytokines upon 24 h culture (Figure 2(a)). Strikingly, a distinct and minor group behaved differently from most patients and from the normal controls, as they produced the highest amount of growth factors, interferons, and proinflammatory factors. Supernatants derived from this minor group and containing proinflammatory factors were designated as "inflammatory supernatants," whereas supernatants containing low or normal amounts of proinflammatory factors were considered "noninflammatory supernatants." Normal supernatants were derived from BM mononuclear cells from nonhematological individuals.

To explore the possibility of a local and transient cell preactivation, cell cultures were continued for additional 24 h upon the former supernatant collection. Concentration graphs in Figure 2(b) suggest a long-term activation of inflammatory pathways, independent on the continuous stromal cell contact within BM.

In order to investigate the pathways involved in the triggering of proinflammatory microenvironment, the activation status of p65 NFκB and STAT3 was examined by indirect immunofluorescence (Figure 3). Among patients, the heterogeneity in activation levels of these transcription factors was a constant. However, samples showing a proinflammatory profile were mostly positive for phosphorylated NFκB and STAT3, suggesting that, in this group of patients, the proinflammatory property is apparently mediated by the activation of NFκB and STAT3 pathways (Figure 3, top panel). In contrast, BM samples identified as noninflammatory showed much less activation of both NFκB and STAT3, and their normal counterpart shows scant basal activation. Interestingly, these transcription factors cooperate to promote the development and progression of cancer, and inhibitors of NFκB and STAT3 have been proposed as therapeutically useful in switching the inflammatory nature of tumor environments. After activating their signaling pathways,

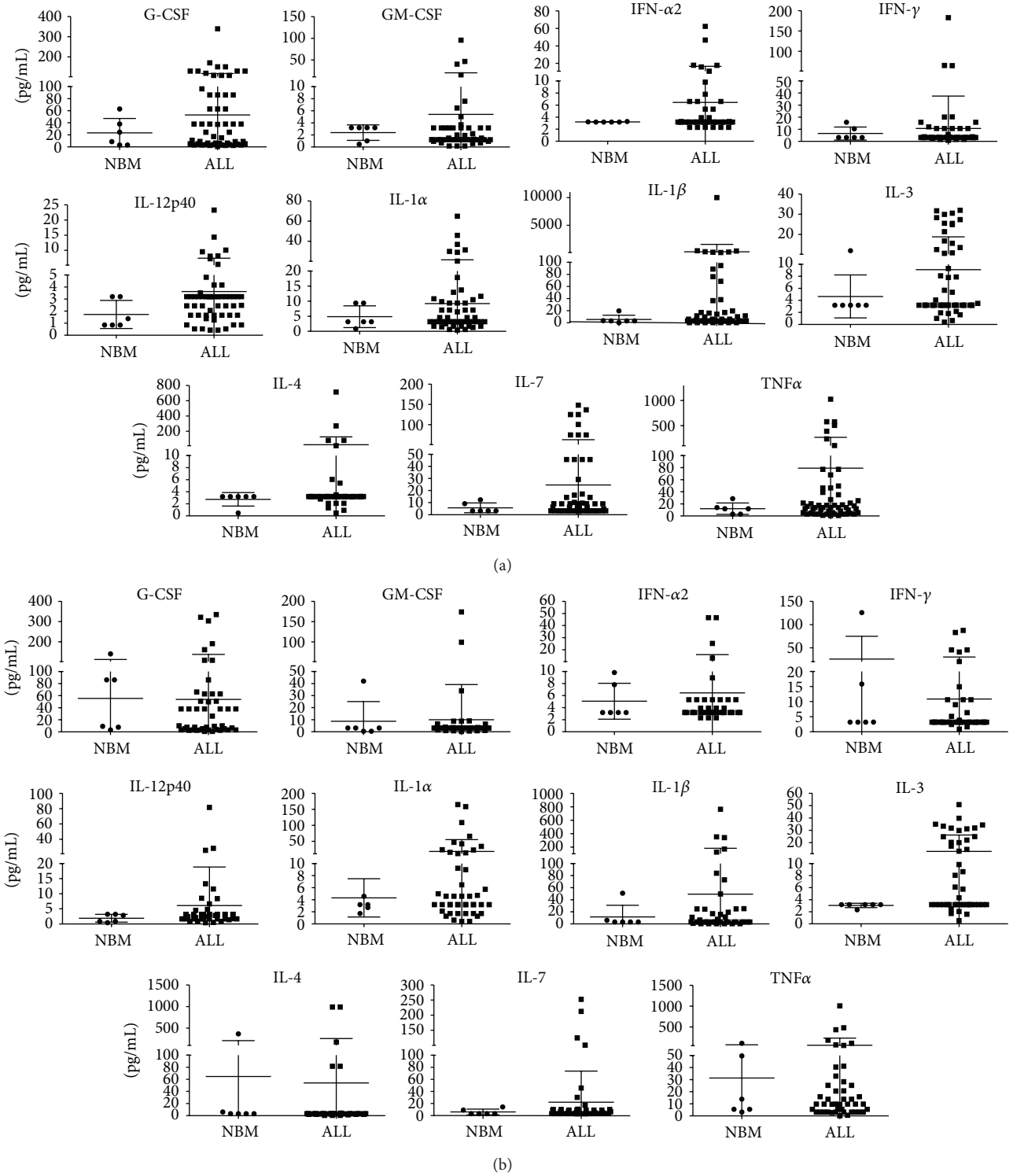

Figure 2: A distinct group of patients is highly producer of proinflammatory cytokines and hematopoietic growth factors. ALL or normal BM (NBM) mononuclear cells were cultured for 24 h (a) and supernatants immediately collected. Cells were provided with additional culture medium and supernatants collected 24 h later (b). The distribution of highly produced cytokines in NBM and ALL patients is shown.

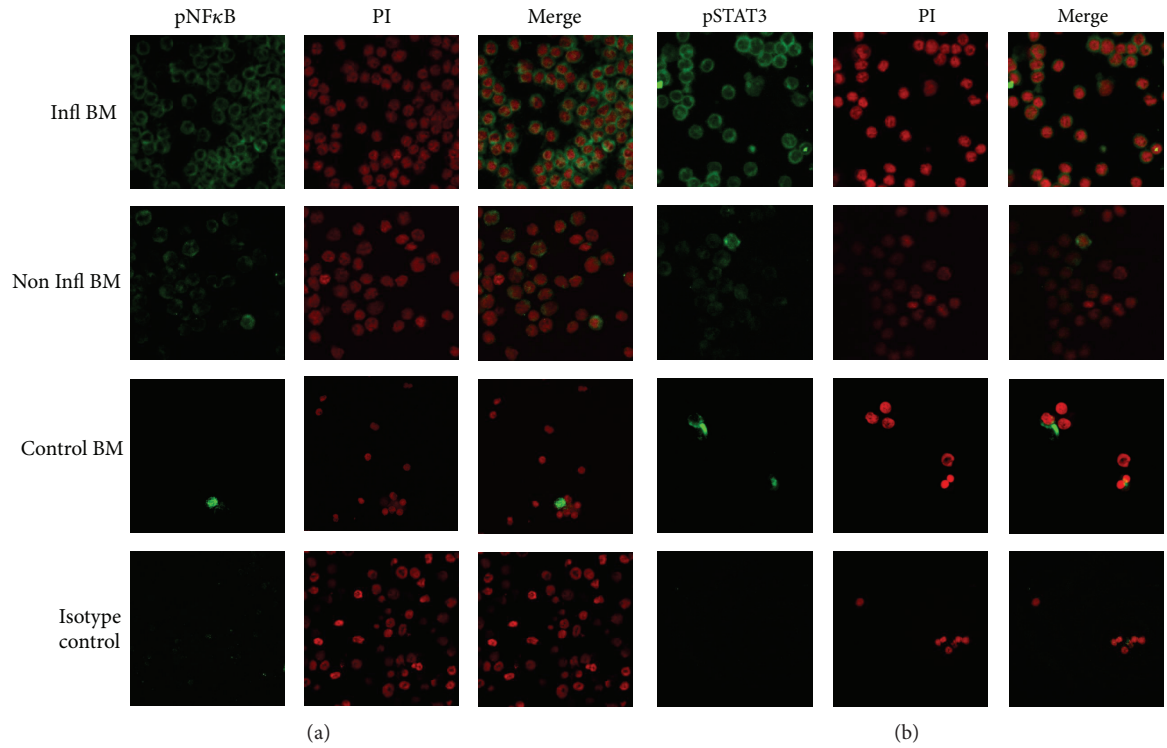

Figure 3: A proinflammatory profile is associated with activation of NFκB and STAT3. Immunofluorescence microscopy was used to demonstrate pNFκB p65 and pSTAT3 transcription factors within MNC fractions from ALL patients showing a proinflammatory profile (Infl BM) and a noninflammatory profile (Non Infl) or from normal individuals (Control BM). Propidium iodide was used for nuclear staining. A representative figure of four independent experiments is shown.

the expression of antiapoptotic and proproliferative genes is under their control in both neoplastic and normal tissues [1, 49]. Moreover, studies indicate that leukemic stem cells in acute myelogenous leukemia have constitutively activated NFκB [28], while STAT3 participates in the maintenance and self-renewal of HSC in TEL-AML1 t(12 : 21) ALL [50] and helps in the rebounding of the hematopoietic system through the control of myeloid progenitors differentiation [51].

3.2. The Inflammatory Profile Is Related to B-ALL BM Coexpressing Myeloid Markers. Among the 54 ALL patients included in this study, 34 fulfilled the criteria for high-risk disease by blood cell count, age, or Ph+ chromosome, whereas 17 fulfilled the criteria for standard-risk (Table 1). The median age values were 7.8 year old (2 mo–18 yr) and 4.8 (2 yr–9 yr) for the high-risk and standard-risk group, respectively. Independent of the conventional risk stratification, 20 patients showed aberrant coexpression of one or more myeloid markers, including CD13 and CD33.

Correlation tests were conducted by the Pearson method (not shown), showing weak or absent associations between risk stratification groups (Table 1) and the production of proinflammatory cytokines. No associations were found neither between single lymphoid markers (CD10, CD19, or CD20) or coexpressed single myeloid markers (CD13, CD14, CD15, or CD33) with production of particular cytokines. However, when the studied population was classified according to concomitant coexpression of 3 lymphoid markers (CD10, CD19, and CD20) or the aberrant expression of the myeloid markers CD13 and CD33, four groups were identified: a CD10+CD19+CD20+ positive group, which coexpressed these 3 B-lymphoid markers; a group lacking concomitant expression of CD10, CD19, and CD20, but expressing less than 3 of these B-lymphoid markers (CD10+/−CD19+/−CD20+/−) (Table 2); a CD13+CD33+ positive group; and a group expressing less than these 2 myeloid markers (Table 3). First, Mann-Whitney test was applied after the Kolmogorov-Smirnov analysis to discard normal distribution, and significant differences between the lymphoid positive (CD10+CD19+CD20+) and negative (CD10+/−CD19+/−CD20+/−) groups were found for IL-1β, TNF-α, G-CSF, and IFN-α2 with $P \leq 0.05$ (Table 2). On the other hand, patients with aberrant coexpression of CD13 and CD33 in more than 25% of BM MNC were considered as a positive group in Table 3. Strikingly, *U* Mann-Whitney test showed significant differences between the two myeloid groups for IL-1α, IL-1β, IL-12p40, TNF-α, and GM-CSF (Table 3). Thus, aberrant expressions of myeloid markers within B-ALL BM apparently condition the abnormal production of proinflammatory factors. It remains to be investigated whether cells expressing myeloid markers constitute the origin of these factors, or they function as target and respond to proinflammatory factors by upregulating myeloid-related molecules. Alternatively, they might be newly differentiated under the emergency settings of ALL [42, 43, 48].

Table 1: Patient characteristics.

Patient	Age (yr)	Sex	WBC/mm^2	Phenotype	Risk stratification Risk factor	Aberrant expression of myeloid markers	Concomitant expression of CD10/CD19/CD20
1	4	F	33700	B-ALL	SR	NEG	POS
2	4	F	7600	B-ALL	SR	CD15	POS
3	11	F	65000	B-ALL	HR Leukocytosis	NEG	POS
4	4	M	26400	B-ALL	SR	CD13/CD33	NEG
5	11	M	36600	B-ALL	HR Age	CD13/CD33	NEG
6	5	M	23000	B-ALL	HR PhCr+	CD13	POS
7	5	M	8900	T-ALL	HR T-ALL	NEG	POS
8	2	F	91500	B-ALL	HR Leukocytosis PhCr+	NEG	POS
9	1	M	40000	B-ALL	HR Age	NEG	NEG
10	10	F	1500	B-ALL	HR Age	NEG	POS
11	12	F	1300	B-ALL	HR Age	CD13/CD15	NEG
12	2	M	39000	B-ALL	SR	NEG	POS
13	5	M	11400	B-ALL	SR	NEG	NEG
14	13	M	3400	B-ALL	HR Age	NEG	NEG
15	5	M	1100	B-ALL	SR	CD13	NEG
16	2	M	5300	B-ALL	SR	NEG	NEG
17	3	F	260000	B-ALL	HR PhCr+	CD13	NEG
18	3	M	85700	B-ALL	HR Leukocytosis	CD13	NEG
19	4	M	161500	B-ALL	HR	NEG	POS
20	13	M	1600	M-ALL	HR	CD13/MPO	NEG
21	13	M	2600	B-ALL	HR Leukocytosis	NEG	NEG
22	3	M	400500	T-ALL	HR T-ALL	NEG	NEG
23	3	M	52500	B-ALL	HR Leukocytosis	CD-13	NEG
24	3	F	35100	B-ALL	SR	CD15	NEG
25	10	M	3500	B-ALL	SR	NEG	NEG
26	10	F	1200	B-ALL	HR Age	CD13/CD15	NEG
27	6	M	115000	B-ALL	HR Leukocytosis	CD13	NEG
28	2	M	—	B-ALL	—	NEG	POS
29	6	M	131000	B-ALL	HR Age	CD13	NEG
30	6	M	83500	B-ALL	HR Leukocytosis	NEG	POS
31	3	F	2700	B-ALL	SR	NEG	POS
32	6	M	13100	B-ALL	SR	NEG	POS

Table 1: Continued.

Patient	Age (yr)	Sex	WBC/mm^2	Phenotype	Risk stratification Risk factor	Aberrant expression of myeloid markers	Concomitant expression of CD10/CD19/CD20
33	6	M	12600	B-ALL	SR	NEG	NEG
34	3	M	15600	B-ALL	SR	NEG	POS
35	4	F	—	B-ALL	—	NEG	NEG
36	6	M	1000	B-ALL	SR	NEG	NEG
37	14	F	600	B-ALL	HR PhCr+	NEG	NEG
38	14	M	163000	B-ALL	HR Leukocytosis	NEG	POS
39	10	M	5900	B-ALL	HR Age	CD13	POS
40	4	M	16100	B-ALL	HR PhCr+	NEG	NEG
41	15	M	113000	B-ALL	HR Age Leukocytosis	NEG	NEG
42	6	M	42100	B-ALL	SR	NEG	NEG
43	15	M	47400	B-ALL	HR Age	NEG	NEG
44	9	M	185000	B-ALL	HR Leukocytosis	NEG	POS
45	7	M	4000	B-ALL	SR	CD13	NEG
46	17	M	—	B-ALL	HR Age	NEG	NEG
47	0.9	F	334700	B-ALL	HR Age	CD33	POS
48	1	F	87900	B-ALL	HR Leukocytosis	NEG	POS
49	7	M	10500	B-ALL	SR	CD13/CD15	NEG
50	8	M	—	B-ALL	—	CD15	NEG
51	9	M	1900	B-ALL	HR	NEG	NEG
52	0.4	F	41600	B-ALL	HR Age	CD13	NEG
53	8	F	52500	B-ALL	HR Leukocytosis	NEG	NEG
54	8	F	350000	B-ALL	HR Leukocytosis	NEG	POS

yr: years old; WBC: white blood cell count; M: male; F: female; B-ALL: B-cell precursor acute lymphoblastic leukemia; T-ALL: T-cell acute lymphoblastic leukemia; M-ALL: mixed acute leukemia; HR: high risk; SR: standard risk.

3.3. Early Progenitor Cells Are Driven into Cycle by Inflammatory Factors in B-ALL. CFSE dilution assays were performed to test progenitor cell responses to inflammatory factors produced by the subset of cases of ALL expressing the myeloid antigens CD13 and CD33 within BM (Figure 4). Moderate acceleration of lineage commitment from Lin^-CD34^+ to Lin^+CD34^- stages was recorded when multiparametric flow cytometry was used for phenotyping of the newly produced cells upon 5-day culture in lymphoid conditions (Figure 4(a)). In contrast, the induction to cell proliferation states by noninflammatory and inflammatory supernatants was clear (Figures 4(b) and 4(c)). While there was no apparent influence on the earliest cell compartment by noninflammatory factors, the differentiating Lin^-CD34^- stage was selectively prompt to proliferate. Of special interest, inflammatory conditions clearly showed the awakening of primitive cells (Figure 4(c), left panel), as well as an intense activity on more differentiated Lin^-CD34^- and Lin^+CD34^- cells. The net positive effect was most appreciated when total absolute cell numbers of each particular stage were analyzed with respect to cell divisions (Figure 4(d)). Progenitor cells were driven into cycle by inflammatory conditions, as seen in the transition from cell generation 0 (G0) to generation 1 (G1) and downstream to generation 2 (G2). The promotion effect was also observed in differentiating Lin^-CD34^- cells, where most cell generations from G1 to G6 contained mainly cells from the inflammatory condition (Figure 4, middle panel). In contrast, developing mature cells were restrictively promoted to proliferate up to G2 and in a similar extent as under noninflammatory conditions. According to these observations, previous reports indicate that during chronic inflammation, awakening of stem and progenitor cells leads to exhaustion of the pool [32, 34, 52]. Deciphering if the same is true for normal hematopoietic cells within ALL BM will

Table 2: BM-expressing less than 3 B-ALL lymphoid markers produce high levels of proinflammatory factors.

Cytokine	Expression of 3 lymphoid markers (CD10+ CD19+ CD20+) Median (pg/mL) IR	Less than 3 positive lymphoid markers (CD10+/− CD19+/− CD20+/−) Median (pg/mL) IR	P
IL-1α	3.20 4.07	4.58 9.20	0.202
IL-1β	3.20 6.90	10.00 8.80	0.004**
IL-12p40	2.44 2.20	3.00 2.83	0.167
TNFα	9.85 13.72	19.00 54.73	0.032*
G-CSF	9.00 45.92	37.93 102.38	0.049*
GM-CSF	3.00 0.45	3.20 1.22	0.08
IL-7	5.00 6.39	9.18 42.51	0.104
IFN-α2	0.700 0.700	2.20 3.80	0.008**

* $P = 0.05$; ** $P = 0.001$.
Concomitant expression of CD10, CD19, and CD20. U-Mann Whitney test; IR: interquartile range; CMN: mononuclear cells.

Table 3: Aberrant expression of myeloid markers in B-ALL and production of proinflammatory factors.

Cytokine	Expression of the myeloid markers CD13 and CD33 Median (pg/mL) IR	Expression of less than two myeloid markers (CD13 or CD33) Median (pg/mL) IR	P
IL-1α	46.00 52.38	3.20 96.39	0.009**
IL-1β	429.00 9911.00	3.20 13.96	0.008**
IL-12p40	8.00 6.80	3.00 1.53	0.051*
TNFα	237.00 342.49	13.92 22.14	0.022*
G-CSF	37.00 104.17	24.32 81.19	0.558
GM-CSF	7.00 1.69	3.20 0.20	0.015*
IL-7	17.00 36.71	6.00 12.80	0.222
INF α2	8.00 12.80	3.20 3.80	0.122

* $P = 0.05$; ** $P = 0.001$.
Concomitant expression of CD13 and CD33. U-Mann Whitney test; IR: interquartile range.

be highly relevant and may help to predict risk or protective scenarios.

3.4. A Proinflammatory Environment of ALL BM Prompts Normal Hematopoietic Differentiation. To determine whether the ALL-inflammatory setting could influence early hematopoietic progenitor cell fate decisions, $Lin^{-}CD34^{+}$ cells from UCB were purified, exposed to normal or ALL supernatants, and cultured during 3 weeks under lymphoid conditions. Further cell harvesting revealed that CD34, NK, B, DC, and myeloid cell production were substantially increased by ALL-derived inflammatory supernatants when compared to normal controls. At the end of coculture, CB lymphoid progenitors showed at least twice yield per input progenitor upon incubation with ALL-factors (Figure 5). Of particular interest was the overproduction of innate immune NK and $CD14^{+}$ myeloid cells, which resemble the stem and progenitor cell behavior under stress conditions or threatening infections [13, 32, 37, 43].

The function of some soluble factors is fundamental within the tumor microenvironment: TNFα potentiates growth of malignant skin, ovarian, and pancreatic tumors and

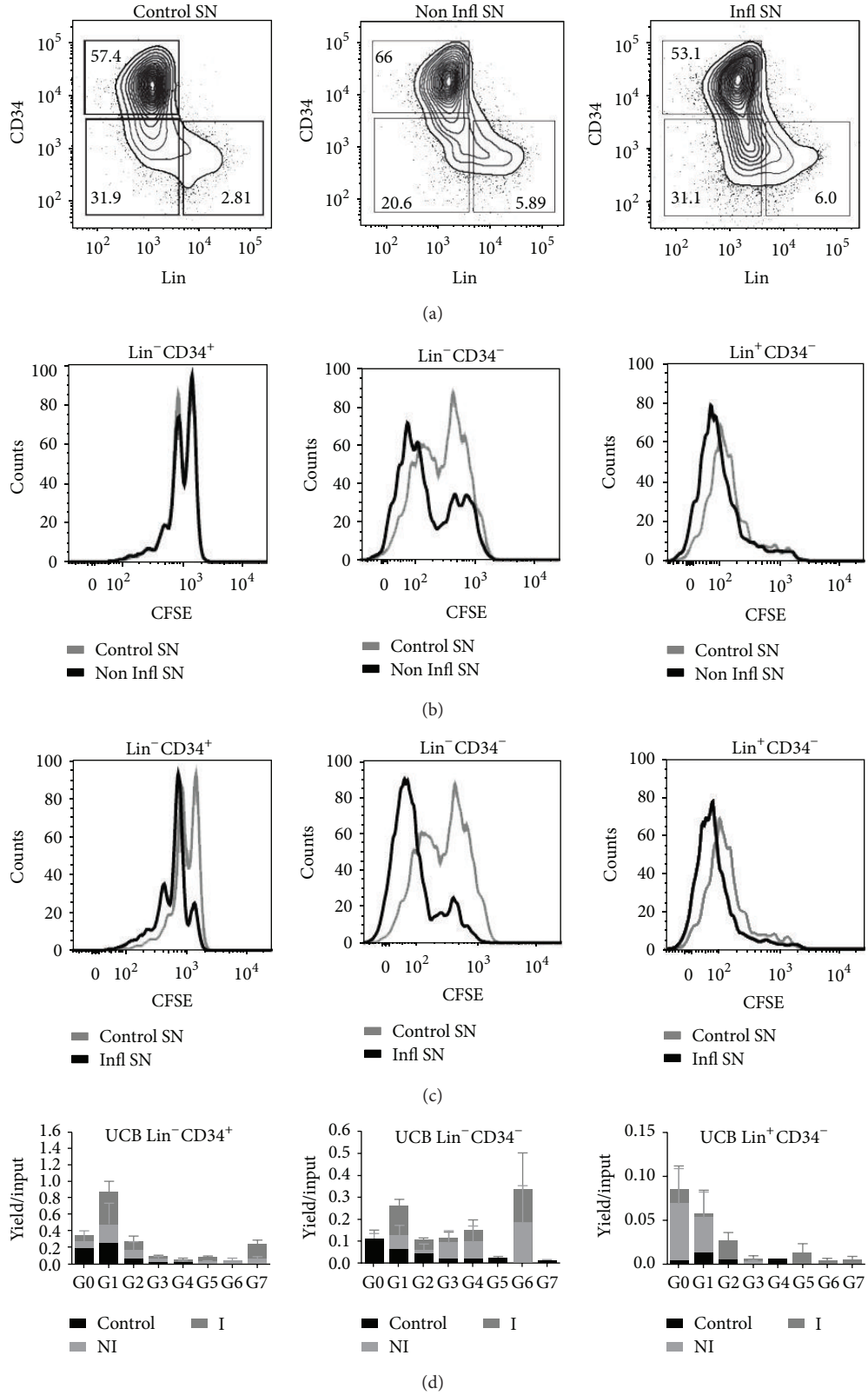

Figure 4: Proinflammatory factors produced by hematopoietic ALL BM cells promote the proliferation of normal primitive cells. Umbilical cord blood $CD34^+$ cells were purified by magnetic separation and analyzed for their proliferation capabilities after 120 h exposure to control, noninflammatory, and inflammatory supernatants. The indicated gates were used to determine cell frequencies within the culture for $Lineage^-CD34^+$ primitive cells, $Lineage^-CD34^-$ differentiating precursor cells, and $Lineage^+CD34^-$ maturing cells (a) and to examine the number of cell divisions within each population. Exposure of cells to noninflammatory supernatants is shown in (b), whereas the inflammatory culture conditions are shown in (c). Total cell numbers within each cell generation and from each treatment condition were calculated and expressed as yields per input progenitor (d). Analyses of cell divisions (expressed as number of cell generations) were performed using the FlowJo software. Representative data of two independent experiments.

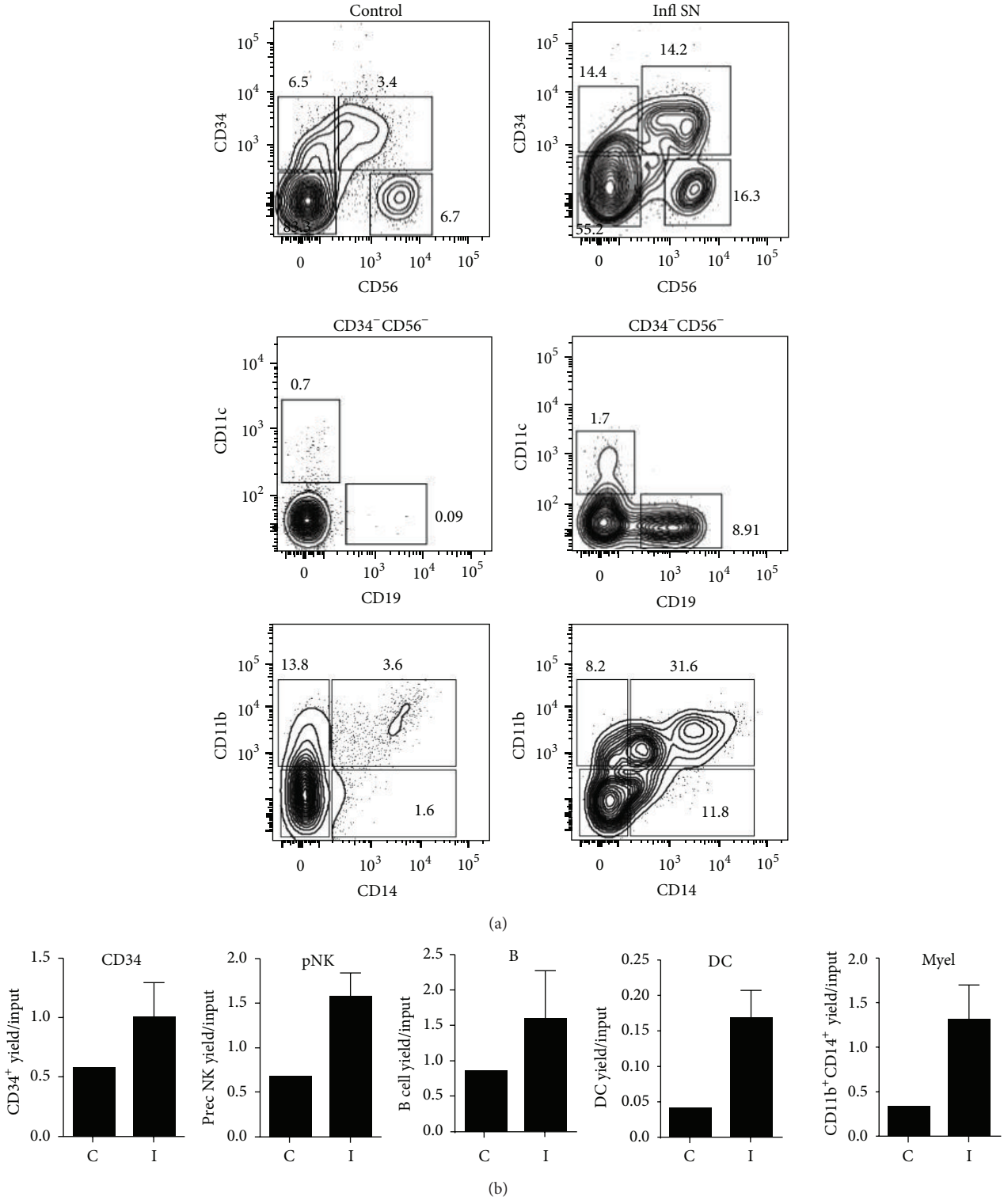

Figure 5: Early lymphoid- and myeloid-cell differentiation is substantially accelerated by inflammatory factors within ALL BM. $CD34^+$ cells were purified from UCB and stimulated with control or inflammatory supernatants collected from normal or ALL BM, followed by a 3-week-stromal cell coculture. Newly produced cells were further identified and enumerated by multiparametric flow cytometry (a). The indicated gates were used to determine cell frequencies within the culture for $CD34^+$ cells, $CD34^+CD56^+$ differentiating NK precursor cells, and $CD56^+$ NK cells (shown in (a) upper panel). Further fractionation of the $CD34^-CD56^-$ compartment allows the identification of $CD11c^+$ DC and $CD19^+$ B cells ((a) middle panel). Myeloid cells appeared as $CD11b^+$ or more mature $CD11b^+CD14^+$ cells ((a) lower panel). Total cell numbers from each treatment condition were calculated and expressed as yields per input progenitor (b). Control SN or C: control supernatant; Infl SN or I: inflammatory supernatant. Data are representative of three independent experiments. Supernatant from different individuals was used for each experiment.

is part of a network of proinflammatory molecules including CXCL12 and CCL2 chemokines, IL-6, macrophage inhibitory factor (MIF), and vascular endothelial growth factor (VEGF), among others [11]. For hematological neoplasias, there are experimental observations indicating the use of autocrine TNFα by leukemic initiating cells for their survival and proliferation, in a NFκB-dependent manner [53]. On the other hand, the participation of IL-1β in the pathogenesis of acute myeloid leukemia has been documented, indicating that its signals in leukemic stem cells disrupt the dormant state and induce its proliferation potential [54]. Moreover, regulation of the NLRP3 inflammasome may explain the activity of IL1β in promyelocytic leukemia, as described in other models [55, 56]. The contribution of a particular inflammasome in the activation of inflammatory processes in B-ALL remains to be determined, as well as the putative activator of the oligomer inside the ALL cells.

To our knowledge, this is the first investigation on the influence of an inflammatory microenvironment in early hematopoietic development during lymphoid leukemia settings. The findings suggest that an ALL subset expressing myeloid antigens is associated with *in vitro* secretion of a number of cytokines, predominantly Th1-type, which, in turn, regulate proliferation and differentiation of primitive normal cells. Our data entails the NFκB and STAT3 signaling in ALL cells with the creation of abnormal hematopoietic niches that may disrupt effective long-term blood cell formation processes. The mechanisms involved may include induction of growth factor receptors [37]. Of special interest will be the implication of specific genetic aberrations in the triggering of ALL-inflammation setting. Although a role for NFκB in leukemogenesis is possible, the etiology and maintenance of ALL remain unknown in most cases [57]. The included patients in this study were not clinically identified as infected, arguing in favor of a "sterile" local inflammation. Besides, a conspicuous population of individuals behaved as "non-inflammatory," suggesting that the abnormal environment appears as a consequence of the activity of leukemic cells.

4. Concluding Remarks

There are great expectations for the biomedical research to unravel fundamental aspects of ALL pathogenesis from the earliest events of normal and neoplastic differentiation within bone marrow. Although the stability of the hematopoietic system has long been recognized, it has become clear that plasticity of stem and progenitor cells allows the crucial regulation of primitive cell compartments during inflammation. Furthermore, recent studies have highlighted the importance of chronic inflammation in development and maintenance of some malignancies. Our findings suggest the manipulation of the environment by a special type of leukemic cells that might disrupt the normal HSC-niche communication at the time tumor progression is promoted. The relevance of a rigorous multiparametric flow cytometry phenotyping and purification of the suggested leukemic cell populations producing proinflammatory cytokines is high for current and future investigations.

Decoding its possible relationship to the biology and prognosis of childhood acute leukemias is crucial for the design of therapeutic strategies based on manipulation of the tumor microenvironment.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

EBV, HCMV, HHV6, and HHV7 Screening in Bone Marrow Samples from Children with Acute Lymphoblastic Leukemia

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Acute lymphoblastic leukemia (ALL) is the most common cancer in childhood worldwide and Mexico has reported one of the highest incidence rates. An infectious etiology has been suggested and supported by epidemiological evidences; however, the identity of the involved agent(s) is not known. We considered that early transmitted lymphotropic herpes viruses were good candidates, since transforming mechanisms have been described for them and some are already associated with human cancers.

In this study we interrogated the direct role of EBV, HCMV, HHV6, and HHV7 human herpes viruses in childhood ALL. Viral genomes were screened in 70 bone marrow samples from ALL patients through standard and a more sensitive nested PCR. Positive samples were detected only by nested PCR indicating a low level of infection. Our result argues that viral genomes were not present in all leukemic cells, and, hence, infection most likely was not part of the initial genetic lesions leading to ALL. The high statistical power of the study suggested that these agents are not involved in the genesis of ALL in Mexican children. Additional analysis showed that detected infections or coinfections were not associated with prognosis.

1. Introduction

Acute lymphoblastic leukemia (ALL) is the most common childhood cancer worldwide and Mexico has reported one of the highest ALL incidence rates [1, 2]. While new therapies have notably improved ALL outcome in recent years [3], pathogenic events leading to disease development remain largely unknown [4]. An infectious etiology has been suggested by different hypothesis favoring either direct or indirect mechanisms of transformation [5–7]. For infectious agents, direct oncogenic mechanisms refer to expression of viral oncogenes together with deregulation of cellular oncogenes and/or tumor suppressor genes. Indirect mechanisms are mainly triggered by an inflammatory milieu with production of mutagenic molecules or immunosuppression with loss of the cancer immune surveillance mechanisms [8]. The former mode of transformation implies that the infectious agent acts from within the cell and thus, after the cancer clonal expansion, it is carried in all tumor cells, as it has been documented for tumor herpes viruses [9, 10]; while indirectly acting infectious carcinogenic agents do not necessarily infect the cell forming the tumor.

The *Delayed infection* hypothesis by Greaves proposes that exposure to infectious agents very early in life protects from ALL as it modulates maturation of the immune system. However, late exposures lead to an aberrant immune response promoting leukemogenesis by an indirect mechanism [5]. On the opposite side, Smith proposes that oncogenic viruses transmitted during intrauterine life or the first year of life are able to infect immature lymphocytes and promote leukemia through a direct transformation mechanism [7]. Epidemiological, clinical, and molecular evidence have been searched to support either direct or indirect mechanisms with variable and even opposite results (reviewed in [11]). We considered that the high incidence of childhood ALL in Mexico City was more closely correlated with high incidence of early infection of a developing country [1]. In agreement, we recently showed that serious infections requiring hospitalization in the first year of life were associated with increased risk of ALL in children with Down syndrome [12], better supporting the notion that early infections can promote pediatric leukemia as proposed by the Smith's hypothesis.

In this study, we explored the idea that viruses from which oncogenic capacities have been documented may promote leukemogenesis through a direct transforming mechanism. We selected members of the herpesviridae family, Epstein Barr virus (EBV), human cytomegalovirus (HCMV), and human herpes virus 6 (HHV6A and B) and 7 (HHV7) because they are lymphotropic viruses often transmitted in the first months of life. EBV is a potent transforming agent and has been consistently associated with several human malignancies including pediatric lymphomas [10]. Although, HCMV and HHV6 are not considered carcinogenic, their transforming ability has been shown in *in vitro* studies [13–16]. Additionally, HCMV has been defined as oncomodulator because of its ability to infect tumor cells and alter proliferation, survival, angiogenesis, and invasiveness increasing the tumor aggressiveness [17, 18]. HHV6 has previously been associated with several hematological malignances, including childhood acute leukemia, through serological case-control studies, although with heterogeneous results [19, 20]. An HHV7 transforming role has not been shown; however, there are proposals about its role as cofactor in T-cell and B-cell lymphomas [21, 22]. Moreover, HHV7 may potentiate the pathogenic role of other herpes viruses [23, 24]. We assessed whether EBV, HCMV, HHV6, and HHV7 were involved in the genesis of childhood B-cell and T-cell ALL through a direct transformation mechanism. ALL bone marrow samples were tested by two PCRs with different detection limits. Considering that viruses acting through direct transforming mechanisms behave like driver genetic lesions that are preserved throughout tumor development, we designed a PCR test to equate the number of infected cells with the number of tumor cells and an even more sensitive PCR to detect evidence of infection. We found that less than 20% of the samples were positive by at least one of the viruses tested. Because positive samples showed low infection levels, these data do not support a direct role for EBV, HCMV, HHV6, and HHV7 in the genesis of pediatric ALL from Mexican children. Further analysis of the positives samples showed no association between detected infections or coinfections and prognosis.

2. Materials and Methods

2.1. Ethics Statement. This study was approved by the ethical and scientific review boards of the Mexican Institute of Social Security (IMSS): the National Commission of Scientific Research and the Ethics Committee on Research. Prior to sample collection, parents of enrolled patients were informed on the nature of the study and those who were willing to participate signed a letter of consent; children older than 10 years also signed a letter of assent. All patients enrolled were treated according to the ethical guidelines of our institution.

2.2. Patients and Biological Samples. The cases recruited in this study belong to the Mexican Inter-institutional Group for the Identification of the Causes of Childhood Leukaemia (MIGICCL; Mexico City, Mexico), a member of the Childhood Leukemia International Consortium (CLIC) since 2012. During the period of the study (January 1, 2010, to August 30,

2012) there were 553 patients diagnosed with B- or T-cell ALL; however, there were sufficient bone marrow samples from only 70 pretreatment patients (66 from B-cell ALL and 4 from T-cell ALL) to include in the present study (Table 1). Two mL of bone marrow were collected in 0.1 M sodium citrate solution (TEKNOVA, Hollister CA, USA) from the included patients and mononuclear cells were isolated by a density gradient centrifugation on Histopaque-1077 (Sigma-Aldrich, St. Luis, NO).

2.3. Control Cell Lines and Plasmid DNA. For EBV detection, Raji (ATCC, CCL-86) and Ramos (ATCC, CRL-1596) cell lines were used as positive and negative controls, respectively. Both cell lines were cultured in advanced RPMI medium supplemented with 4% of fetal bovine serum and 1X hepes (all from Gibco, Carlsbad, CA) and maintained at 37°C in 5% of CO_2. Control plasmids were constructed for HCMV, HHV6, and HHV7 detection. Purified HCMV viral genome was kindly provided by Dr. M. Ruiz-Tachiquin (Pediatric Hospital at the CMN SXXI, IMSS, Mexico City, Mexico). HHV6 DNA was obtained from MOLT3 cell line (ATCC CRL-1552) infected with viral particles of HHV6 Z29 strain (ATCC VR-1467). HHV7 DNA was purified from infected lymphocytes kindly provided by Dr. E. Sevilla (National Institute of Respiratory Diseases, Mexico City, Mexico). A fragment of HCMV UL83, HHV6 U94, and HHV7 U42 genes were PCR amplified using primers described in Table 2 (standard PCR) and cloned into T-easy pGEM plasmid (Promega, Madison, WI) according to manufacturer's instructions. The identity of cloned products was confirmed by sequencing.

2.4. DNA Purification. DNA was purified from bone marrow mononuclear cells or control cell lines using QIAamp DNA extraction kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Plasmid DNA was purified using PureLink Quick Plasmid Miniprep Kit (Invitrogen, Carlsbad, CA) following the manufacturer's instruction. Purified DNA was quantified using a spectrophotometer NanoDrop 1000 (Thermo Fisher Scientific, Waltham, MA). The DNA quality and integrity were evaluated through electrophoretic landslide, optical density (260/280 ratio), and amplification of β-actin endogenous gene (genomic DNA).

2.5. Standard and Nested PCRs. Standard and nested PCR mix contained 1x Taq Polymerase buffer, 1.5 to 2.5 mM $MgCl_2$ ($MgCl_2$ concentration was optimized for every PCR), 200 μM deoxynucleotide triphosphate (dNTP), 2.5 U of Taq Polymerase (all from Thermo Fisher Scientific, Waltham, MA), and 200 nM of each primer (IDT Technologies). For standard PCR, 100 ng of DNA from samples or control cell lines or 5.5×10^{-5} ng from control plasmid (equivalent to the number of moles contained in 100 ng of genomic DNA) were used. Plasmids were linearized with restriction enzyme NdeI (New England BioLabs) before use and mixed with DNA from Daudi cell line in order to run the amplification reaction under identical mass/volume DNA concentration for both samples and controls. For the nested PCR, 0.5 or 0.05 μL (1 : 100 or 1 : 1000 dilution resp.) of product of first round PCR was used as template. All PCR reactions were carried out in a final volume of 50 μL. All primer sequences and cycling conditions used are detailed in Table 2. PCR products were analyzed by electrophoresis in 1.8% agarose gels stained with ethidium bromide and photographed under ultraviolet light using the Quantum ST4 System (VilberLourmat, Torcy, Marne-la-Vallée, FR). The viral identity of PCR amplified fragments was confirmed by sequencing.

Table 1: Demographic and clinic characteristics of the patients $N = 70$.

Age at diagnosis (years)	Median (range)
	7.6 (0.8–15.7)
Gender	N (%)
Male	33 (47)
Female	37 (53)
Birthplace	N (%)
Mexico City	63 (91)
Other Southern Mexican States	7 (9)
Percentage of blasts in bone marrow	Average (range)
	87% (25%–100%)
Immunophenotype*	N (%)
B-cell precursor	66 (94.3)
T-cell	4 (5.7)
FAB classification	N (%)
L1	43 (62)
L2	26 (36)
L3	1 (2)
Genetic rearrangements**	N (%)
ETV6-RUNX1	6 (12)
E2A-PBX1	2 (4)
No rearrangement	42 (84)

*Immunophenotype was determined according to international parameters [54].
**Molecular diagnosis of ETV6-RUNX1, E2A-PBX1, MLL-AF4 and major and minor BCR-ABL genetic rearrangements were performed only in 50 samples. Percentages shown were calculated using 50 as denominator.

2.6. Nucleotide Sequencing. The cloned viral gene fragments and PCR amplified fragments were excised from 1% agarose gels and purified using QIAquick gel extraction kit (Qiagen Hilden, Germany), following the manufacturer's instructions. Direct automated sequencing of both forward and reverse strands was carried out using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA). About 20 ng of template DNA was added for each reaction; the program included 25 cycles as described: denaturation at 95°C for 30 sec, annealing at 50°C for 15 sec, and extension at 60°C for 4 min. Samples were analyzed in an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA). The obtained sequences were examined using the Nucleotide BLAST program [25].

2.7. Statistical Analysis. The statistical power of the study was calculated considering the sample size ($N = 70$)

Table 2: PCR cycling conditions and primers sequences.

Virus/endogenous gene	Type of PCR	Cycling conditions[a]	Primers sequences (5′ → 3′)
EBV	Standard	95°C/40 sec, 57°C/1 min, 72°C/1.5 min (30X)	**F** CCATGTAAGCTTGCCTCGAG **R** GCCTTAGATCTGGCTCTTTG [55][c]
EBV	Nested	95°C/20 sec, 57°C/30 sec, 72°C/45 sec (15X)	**F** CTTTGTCCAGATGTCAGGGG **R** GCCTGAGCCTCTACTTTTGG[b]
CMV	Standard	95°C/1 min, 55°C/1 min, 72°C/1 min (35X)	**F** AAGATGCGGTAGATGTCGTT **R** CTGCGCTCTTCTTTTTCGAT[b]
CMV	Nested	95°C/45 sec, 55°C/45 sec, 72°C/45 sec (15X)	**F** TTCTGACCCTGAACCGTAG **R** CGACGAAGAACTCGTAACC[b]
HHV6[d]	Standard	95°C/1 min, 50°C/1 min, 72°C/1 min (30X)	**F** GTGCGCTATAAAATCGATAGC **R** TGATTTCCGTTGTGTGTTTTCC[b]
HHV6[d]	Nested	95°C/30 sec, 56°C/30 sec, 72°C/30 sec (15X)	**F** GTCTCTTCGTATCCACGCG **R** CGTTCCCGTCGAAGAAATC[b]
HHV7	Standard	95°C/45 sec, 53°C/45 sec, 72°C/45 sec (30X)	**F** TTTTTACATTTGGCTTGCTTTTTG **R** ATATTTCTGTACCTATCTTCCCAA [56][c]
HHV7	Nested	95°C/30 sec, 55°C/30 sec, 72°C/30 sec (15X)	**F** GAACGGTTTGCTTAGATTGC **R** GCAGACCAAACTCCACAAATTC[b]
β-actin	Standard	95°C/1 min, 60°C/1 min, 72°C/1.5 min (30X)	**F** CCTAAGGCCAACCGTGAAAAG **R** TCTTCATGGTGCTAGGAGCCA [57][c]

[a] All amplification runs included an initial denaturation step at 95°C for 5 minutes and a final extension step at 72°C for 10 minutes. Annealing temperatures were optimized for every reaction.
[b] These primers were designed in our laboratory using the Primer-BLAST program [25].
[c] The specificity of the previously reported primers was corroborated using Primer-BLAST program [25].
[d] HHV6 primers recognize both HHV6A and HHV6B subtypes.

and the probability to detect viral genomes supporting a direct transformation mechanism in at least one patient. The hypothetical frequencies of infection were 10% and 5%. The statistical package used for this analysis was Epi Info version 7.1.4 (Centers for Disease Control and Prevention, http://wwwn.cdc.gov/epiinfo/). Odds ratios with 95% confidence intervals were calculated to identify if the detected infections were associated with high-risk leukemia, relapse, or death. Mann-Whitney U test was used to compare age in months and leukocyte count with infection. The statistical package used for these analyses was Epidat version 3.0.

3. Results

3.1. Study Population. The study included 70 pretreatment children diagnosed with ALL, 66 were B-cell ALL and 4 were T-cell ALL. The diagnosis of ALL fulfilled the morphological and phenotypic criteria, including that at least 25% of the cells from the bone marrow sample were leukemic blasts. Demographic and clinic characteristics of the patients were extracted from medical records and are presented in Table 1.

3.2. PCRs Limit of Detection. To know the limit of detection of the standard PCRs, we first carried out amplification reactions using serial dilutions of DNA from control cell lines and plasmids (Figure 1). For EBV detection, we employed Raji cell line, which derives from an EBV-associated lymphoma and carries about 50 viral episomes per cell [26]. The amplification target was the BamHI W fragment, which is eleven times repeated in the EBV genome. The lower limit of detection of our reaction was of 7.5×10^3 viral genomes (Figure 1(a)). We decided to use 9.5×10^3 cells from bone marrow samples for EBV detection because we speculated that ALL samples might have similar frequencies to EBV episomes than those found in EBV-associated lymphomas. Still, if the number of viral genomes in leukemic cells were as low as the minimum reported for an EBV-associated cancer, namely, 7 viral genomes per cell in EBV-associated nasopharyngeal carcinoma [27], our test would be able to detect positive samples in as low as 25% of blasts (Figure 1(a)). For HCMV, HHV6, and HHV7 detection, we used plasmid DNA as control (see Material and Methods section). The limit of detection was about 0.97×10^3 plasmid copies for every reaction (Figures 1(b), 1(c), and 1(d)). As we did not know the number of HCMV, HHV6, and HHV7 genomes that could be present in each leukemic blast, DNA from control plasmid and samples were used at equal molar concentration. We determined that using 15.5×10^3 cells from samples (and 15.5×10^3 plasmid copies mixed with DNA from 15.5×10^3 negative cells as control) would be sufficient to detect up to one viral genome per cell even in samples with only 25% of leukemic blasts (Figures 1(b), 1(c), and 1(d)). We have previously used these PCRs in which the frequency of infected cells matches the frequency of cancer cells to address direct viral oncogenesis [28–30].

3.3. Viral Screening. Once the limit of detection was established for every reaction, we screened the presence of viral genomes in the samples. We do not observe any positive sample for any herpes virus tested by the first round PCR (Figures 2(a), 3(a), 4(a), and 5(a)). EBV, HCMV, and HHV6 positive samples were only detected through nested PCR in

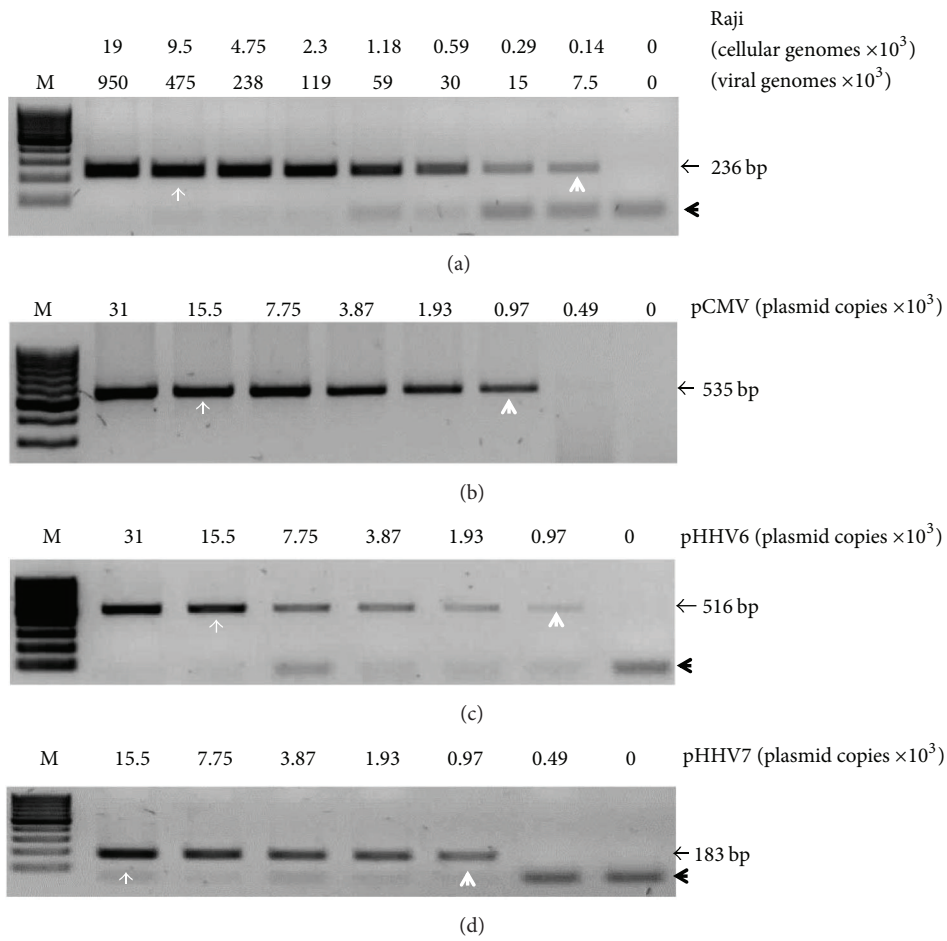

Figure 1: The PCRs limit of detection. Serial dilutions of DNA from control cell line and plasmid were PCR amplified in order to know the lower limit of detection of our screening test. For EBV (a), the limit of detection is expressed in number of cellular and viral genomes (from Raji cell line). For HCMV (b), HHV6 (c), and HHV7 (d) the limits of detection are expressed as plasmid copies. Lower limits of detection are indicated with a white arrowhead. White arrows point to the number of cells or plasmid copies that were used for viral detection in ALL samples. The sizes of PCR products are indicated. Black arrowheads point to residual primers.

the following frequencies: 14%, 19%, and 9%, respectively, while HHV7 was not detected by this method (Figures 2(b), 3(b), 4(b), and 5(b)). Considering that direct carcinogenic viruses contribute to oncogenesis acting like driver genetic lesions, we would consider that if viral infection had a role in the transformation of the cell subjected to oncogenic clonal expansion, the progeny of that cell should all carry the viral genome. In that scenario, the viral genome should have been detected by the first PCR in agreement with the previously established limit of detection. All samples showed high DNA integrity (not shown) and the amplification of *β-actin* endogenous gene was positive (Figures 2(c), 3(c), 4(c), and 5(c)).

3.4. Power of the Study. We carried out a post hoc power analysis to calculate the probability that our results were true based in the obtained sample size and the frequency of the infection. The frequency of the infection was hypothetical because up to today none of the viruses studied have been shown to cause ALL. The statistical power test indicated that the probability to detect at least one positive subject (in the first PCR, supporting a direct transforming) from our study population ($N = 70$) was 99.99% and 95.00% from hypothetical frequencies of infection of 10% and 5%, respectively. Thus, our results suggest that EBV, HCMV, HHV6, and HHV7 are not involved in the genesis of childhood ALL in Mexican children.

3.5. Analysis of Infection and Prognosis. A possible role of the detected infections in prognosis was explored. Considering a previously documented probable interaction between the herpes viruses tested [31–35] we calculated the association between every infection or coinfection and the risk for high-risk leukemia, relapse, or death (Table 3). Coinfections included only double-infections: EBV+/HCMV+, EBV+/HHV6+, and HCMV+/HHV6+; triple infections were not detected. We did not find any significant association with this analysis (Table 3). We also compared the age (in months) at the moment of diagnosis and the leukocyte count between infected and noninfected children finding

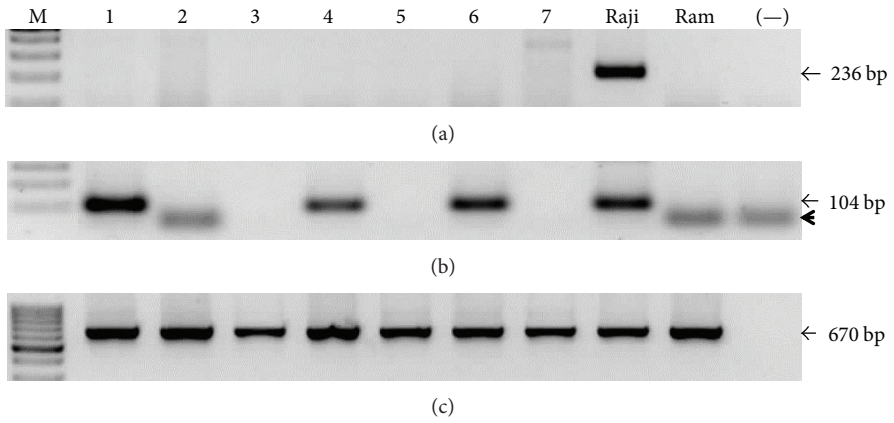

Figure 2: EBV screening. Image of seven representative samples showing that all of them were EBV negative by first round PCR (a) and positivity was only detected by nested PCR in 10 (14%; showing three) samples (b). Raji and Ramos (Ram) cell lines were used as positive and negative controls, respectively. A reaction without DNA was routinely run (−). (c) Amplification of *β-actin* endogenous gene. The sizes of PCR products are indicated. The black arrowhead points to residual primers.

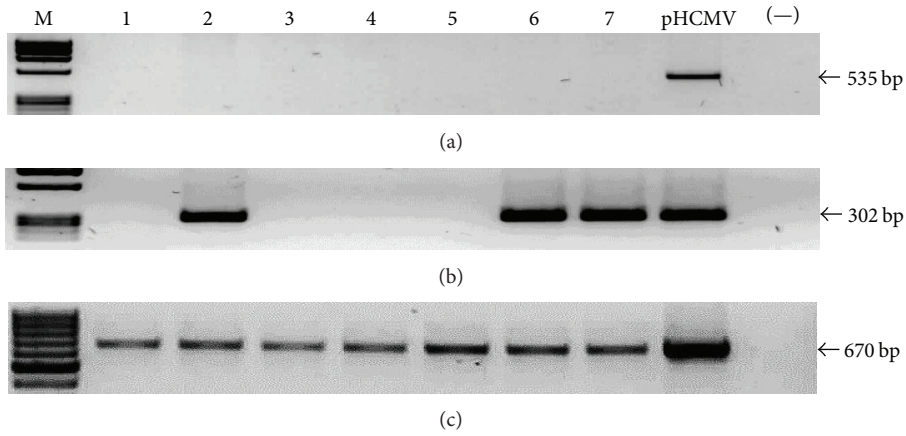

Figure 3: HCMV screening. Image of seven representative samples showing that all of them were HCMV negative by first round PCR (a) and positivity was only detected by nested PCR in 13 (19%, showing three) samples (b). Plasmid DNA was used as positive control (pHCMV). A reaction without DNA was routinely run (−). (c) Amplification of *β-actin* endogenous gene. The sizes of PCR products are indicated.

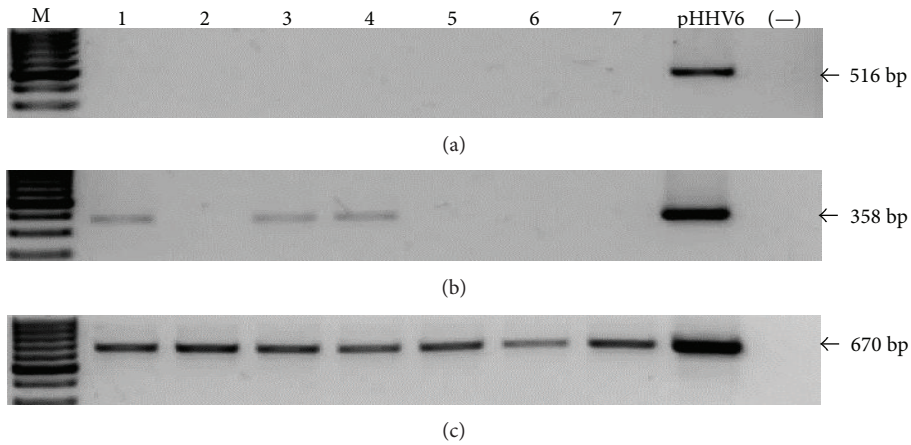

Figure 4: HHV6 screening. Image of seven representative samples showing that all of them were HHV6 negative by first round PCR (a) and positivity was only detected by nested PCR in 6 (9%, showing three) samples (b). Plasmid DNA was used as positive control (pHHV6). A reaction without DNA was routinely run (−). (c) Amplification of *β-actin* endogenous gene. The sizes of PCR products are indicated.

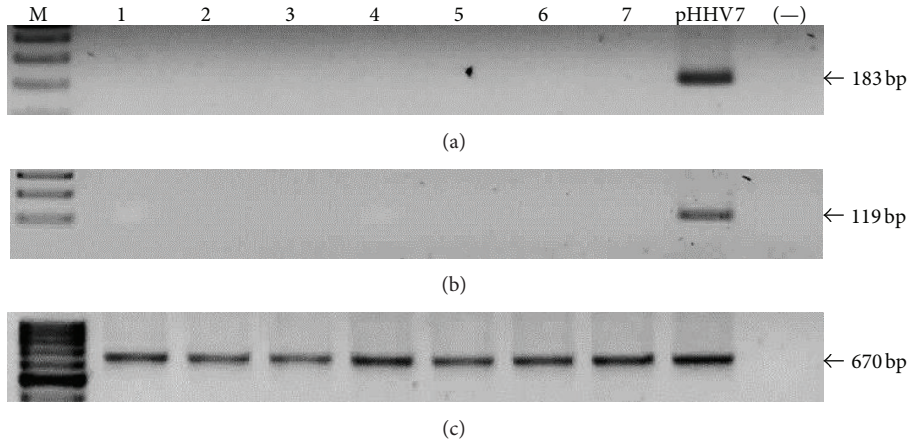

Figure 5: HHV7 screening. Image of seven representative samples showing that all of them were HHV7 negative by both first round PCR (a) and nested PCR (b). Plasmid DNA was used as positive control (pHHV7). A reaction without DNA was routinely run (−). (c) Amplification of *β-actin* endogenous gene. The sizes of PCR products are indicated.

Table 3: Infection and prognostic factors.

Virus	EBV+	HCMV+	HHV6+	Any coinfection
N (%)	10 (14%)	13 (19%)	6 (9%)	6 (9%)
High-risk leukemia				
OR (95% IC)	0.43 (0.12–1.57)	0.72 (0.22–2.32)	2.29 (0.4–15.32)	1.12 (0.24–5.2)
P	0.29	0.75	0.4	1
Relapse				
OR (95% IC)	1.19 (0.27–5.36)	0.88 (0.20–3.82)	1.10 (0.15–6.63)	0.81 (0.12–4.67)
P	1	1	1	1
Death				
OR (95% IC)	0.17 (0.02–1.14)	0.16 (0.02–1.08)	0.04 (0.04–2.81)	0.37 (0.05–2.03)
P	0.89	0.08	0.65	0.41

OR: odds ratio; CI: confidence interval. $P < 0.05$ was considered significant.

no significant differences (not shown). These results argued against a possible indirect role of these infections in the onset and the course of disease.

4. Discussion

Epidemiological data suggest the involvement of infections in the etiology of childhood ALL; however, the identity of the potentially implicated agent(s) has not been identified. Based on the original Smith's proposal about a direct causative role of viral agents in ALL development [7], we screened the presence of EBV, HCMV, HHV6, and HHV7 viral genomes in B-cell and T-cell ALL samples by a standard PCR aiming to match the number of infected cells with the number of leukemic blasts. The infectious etiology of childhood leukemia has been mainly proposed for common B-cell ALL [5, 7]. However, in this work, the viral screening in T-cell ALL was performed as an exploratory analysis based in the known T-cell tropism of the studied herpes viruses. Also, EBV is the causative agent of T-cell lymphomas [36, 37] and a role of EBV in the etiology of T-cell leukemia has been suggested [38].

We found no evidence of the participation of EBV, HCMV, HHV6, and HHV7 in the B-cell and T-cell ALL development. Positive samples were detected only through a more sensitive nested PCR, most likely reflecting normal levels of infection according to the age of the tested population. Positivity only by nested PCR does not support a relationship between infection and leukemogenesis. Still, the nested PCR was implemented as an additional control to globally validate our detection system. Based on the knowledge that the herpes viruses under study are ubiquitously acquired early in life and maintain a reservoir population in bone marrow, we expected to obtain positive samples by a sensitive detection, reflecting the common infection occurring in the pediatric population.

Previous studies looked for the presence of DNA viruses in bone marrow or peripheral blood from childhood ALL samples, namely, JVC, BKV, and SV40 polyomaviruses [39, 40]; TT anellovirus [41] and EBV and HHV-6, -7, and -8 herpes viruses [42]. Nevertheless all of them have failed to find evidence of their participation in the disease. Although, there is a previous work analyzing the participation of herpes viruses in leukemogenesis with similar negative results, based on the epidemiological differences between our country and

developed countries related to the increased prevalence of infections and childhood leukemias in Mexico [1], we hypothesized that a mechanism of direct leukemogenesis could explain some cases of leukemia in our population. An advantage of our work over previous studies is the number of samples analyzed, $N = 70$ in our study versus $N = 15–47$ in others; which conferred it a higher statistical power. Also, other works screened the presence of viral genomes of EBV and HHV6 herpes viruses [43], JVC and BKV polyomaviruses [44], and parvovirus B19 [45] in archived neonatal blood spots collected at birth from children who developed leukemia, however, these works lost the window of infections happening from birth to the presentation of the disease. Additionally, this is the first work reporting no association between HCMV and childhood ALL. We have also previously reported that human T-cell lymphotropic virus type 1 (HTLV1) and mouse mammary tumor virus - like virus (MMTV-LV) were not involved in the genesis of childhood ALL [29].

Indirect mechanisms of leukemogenesis are also possible but addressing those mechanisms was beyond the aim of this study. Still, we did not find any evident association between nested PCR positive patients and clinical data. Previous studies have supported a role for EBV in genetic instability and EBV-associated Burkitt's lymphoma is characterized by a *Myc* genetic translocation [46–48]. However, the low number of patients with genetic translocations in our study ($N = 8$) precluded an analysis of EBV infection and ALL-associated genetic translocations with significant statistical power. It is proposed that herpes viruses often act in concert potentiating their pathological effects in cases of chronic fatigue syndrome, infectious mononucleosis, and/or posttransplant disease [31–35]. Although, we had a sizable number of infection positive patients, most of them were single infected, with only 6 (9%) showing evidence of infection by more than one herpes virus (see Table 3). Therefore, we do not have any evidence of a possible interaction by the tested viruses.

It is possible that the lymphoproliferation that characterizes ALL results from continuous immune stimulation triggered by repetitive infections. However, almost any infectious agent would be responsible for this effect and only tumor viruses with documented or possible direct transforming properties were tested here. There exist some examples of chronic antigenic stimulation leading to lymphomas, for instance, MALT lymphoma after *Helicobacter pylori* persistent infection and hepatitis C virus associated lymphoma [49–52]. Since those are adult neoplasias arising after decades of antigenic stimulation it is unlikely that pediatric ALL could result from a similar mechanism and arise as the immune system responds to a single infection. In any case, it is more likely that multiple antigenic challenges combined with maturing lymphocytes with a genetic lesion, for example, an intrauterine translocation, were involved in the genesis of ALL. In lymphomas of mature cells, the identity of the target of the antigen receptor (VDJ recombination clone) gives light to the origin of the lymphoproliferation. Since ALL occurs in progenitor cells in which more often the leukemic blasts have not completed the rearrangements of the antigen receptor, this is a scenario more difficult to corroborate.

An important limitation of all studies searching for infectious agents in pediatric ALL is that all have been performed individually, testing samples through standard techniques of molecular biology (PCR and/or Southern blot). Such strategies are restricted to agents whose genomic sequences have been at least partially described. Future investigations should use next-generation sequencing technologies and bioinformatic analysis that are more able to detect the full spectrum of viruses including those still not described, as it has been recently shown for Merkel cell polyomavirus [53]. Besides tumor viruses, indirect mechanisms of leukemogenesis also need to be addressed in order to better understand the role that a history of infection has in the development of pediatric ALL.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

J. M. Mejía-Aranguré and E. M. Fuentes-Pananá contributed equally to this work.

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Research Article

Clinical Utility of Ammonia Concentration as a Diagnostic Test in Monitoring of the Treatment with L-Asparaginase in Children with Acute Lymphoblastic Leukemia

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L-asparaginase (ASP) is an enzyme used as one of the basic regimens in the acute lymphoblastic leukemia (ALL) therapy. Because of the possibility of the enzyme inactivation by antibodies, monitoring of ASP activity is essential. The aim of the study was to examine if plasma concentration of ammonia, a direct product of the reaction catalyzed by ASP, can be used in the assessment of ASP activity. A group of 87 patients with acute lymphoblastic leukemia treated in the Department of Pediatric Oncology and Hematology in Krakow was enrolled to the study. ASP activity and ammonia concentration were measured after ASP administrations during induction. A positive correlation was found between the ammonia concentration and ASP activity ($R = 0.44$; $P < 0.0001$) and between the medium values of ammonia concentration and ASP activity ($R = 0.56$; $P < 0.0001$). The analysis of ROC curves revealed the moderate accuracy of the ammonia concentration values in the ASP activity assessment. It was also found that the medium value of ammonia concentrations can be useful in identification of the patients with low (<100 IU/L) and undetectable (<30 IU/L) ASP activity. The plasma ammonia concentration may reflect ASP activity and can be useful when a direct measurement of the activity is unavailable.

1. Introduction

L-asparaginase (ASP), an enzyme catalyzing hydrolysis of asparagine to aspartic acid and ammonia, is one of the basic regimens used in the treatment of acute lymphoblastic leukemia (ALL). Neoplastic blasts have reduced expression of asparagine synthetase and thus need asparagine from the circulating blood [1]. ASP causes asparagine depletion from plasma, leading to inhibition of protein biosynthesis in blast cells, cell cycle arrest, and finally cellular death [2]. The treatment efficacy is related to the duration and grade of the reduction of the asparagine concentration in plasma and cerebrospinal fluid, which depends on the ASP activity. It is considered that the therapeutic ASP activity is above 100 IU/L [3–6] but a complete asparagine depletion was observed in some patients with lower enzyme activity [3, 5, 6].

ASP can be derived from *Escherichia coli* (*E. coli*) or *Erwinia chryzantemi* (*Erwinia*) [7]. There are native and pegylated (PEG-ASP) preparations available, which differ in pharmacokinetics. Distinct treatment schedules are used for each preparation to assure the optimal efficacy in most of the patients.

ASP, as a nonhuman protein, can cause antibodies development. Hypersensitivity can be clinically visible or "silent," when the drug activity decreases without clinical symptoms [8–12]. The reported frequency of antiasparaginase antibodies is variable and reaches up to 70% [7, 12]. The frequency of hypersensitivity reactions ranges from 0% to 45% [7, 12].

Table 1: The protocols of the ALL IC 2002 program with L-ASPA administration.

Protocol I	5000 IU/m^2/day—days: 12, 15, 18, 21, 24, 27, 30, and 33
Protocol II	10000 IU/m^2/day—days: 8, 11, 15, and 18
Protocol III	10000 IU/m^2/day—days: 1, 4, 8, and 11
HR blocks 1–3	25000 IU/m^2/day—days: 6 and 11

Both situations are an indication for switching to another ASP preparation (pegylated or from another bacterial source) to assure the efficacy of the treatment. Therapy monitoring with a systematic ASP activity measurement seems to be crucial to recognize silent inactivation and react to it properly.

Ammonia is a direct product of the reaction catalyzed by ASP. There are few studies concerning the ammonia concentration during a treatment with ASP [13–17]. A significant increase of ammonia levels after an intravenous ASP administration was described [13, 14, 16]. In some patients, symptomatic hyperammonemia occurs, especially after PEG-ASP, probably due to its prolonged half-life [13].

The ammonia measurement, as an easily available, fast, and cheap test, may be useful as a surrogate parameter of ASP activity. The aim of the study was to assess the clinical relevance of ammonia concentration during the ASP therapy.

2. Patients and Methods

Between June 2005 and October 2008, 97 children with newly diagnosed ALL started treatment with the international program ALLIC 2002 in the Department of Pediatric Oncology and Hematology of the Children's University Hospital in Krakow, Poland. Eighty-seven patients had blood samples available for the ASP activity and ammonia concentration monitoring.

There were 45 boys (52%) and 42 girls (48%), aged from 1.2 to 17 (median: 6, mean: 7; standard deviation/SD/: 4.3 years). 71 patients (81.6%) were diagnosed with common B-ALL (cALL), 3 (3.4%) with pro-B ALL, 2 (2.3%) with transitional ALL, and 11 (12.7%) with T-ALL. Among 71 patients with cALL, 25 were in the standard risk group (SRG), 31 in the intermediate risk group (IRG), and 15 in the high risk group (HRG). One child with proB-ALL and 2 with transitional ALL were in SRG, 2 patients with proB-ALL and 7 with T-ALL in IRG, and 4 with T-ALL in HRG.

All patients received native *E. coli* ASP (Asparaginase Medac, Medac Gesellschaft für klinische Spezialpräparate mbH, Hamburg, Germany) during induction (Protocol I), reinduction (Protocol II and III), and HR blocks (Table 1).

The blood samples for ASP activity and ammonia concentration were collected before each ASP administration during induction (3 days after the preceding administration). Serum for ASP activity evaluation was frozen at −80°C until the analysis was carried out. ASP activity was evaluated with MAAT test (Medac Asparaginase-Aktivitäts-Test, Medac Gesellschaft für klinische Spezialpräparate mbH, Hamburg, Germany). Blood for ammonia evaluation was taken before and 24 hours after ASP administrations to heparinized capillary tubes (lithium heparin) and transported on ice to the laboratory where immediately tested to eliminate the effect of ASP activity in vitro. In all patients, the activity of transaminases was monitored to recognize hepatotoxicity. All analyses were performed in the Department of Clinical Biochemistry in the Polish-American Institute of Pediatrics (Jagiellonian University Medical College, Krakow, Poland).

Statistical analyses were performed with the STATISTICA 8 software. Correlations were assessed with Spearman correlation analysis. Mann-Whitney U test was used to compare the differences between the two groups. The prognostic capacity of ammonia tests was evaluated in terms of the receiver-operating characteristic (ROC) curve. It was assumed that a test with an area under curve (AUC) of more than 0.9 has high accuracy, AUC from 0.7 to 0.9 indicates moderate accuracy, from 0.5 to 0.7, low accuracy, and below 0.5, a chance result [18].

3. Results

The activity of ASP was analyzed in 374 samples. The mean ASP activity was 257 (standard deviation (SD): 168) and median was 248 (range: 0–2063) IU/L. Activity below 100 IU/L was noted in 59 (16%) samples in 19 (21%) patients. In 15 (4%) samples, activity was undetectable (<30 IU/L). It was noted in 7 (8%) children.

The ammonia concentration was assessed in 535 samples taken before the administration of ASP (3 days after the preceding dose) and in 536 samples taken 24 hours after ASP. The mean ammonia concentration before ASP administration was 38 μmol/L (SD: 28 μmol/L) and median was 32 μmol/L (range: 1.4–208 μmol/L). One day after ASP administration, the mean ammonia concentration was 103 μmol/L (SD: 64 μmol/L) and median was 88 μmol/L (range: 5–491 μmol/L). The median increase of the ammonia concentration 24 hours after ASP administration with reference to the concentration before that ASP dose was 51 μmol/L. We did not observe symptoms of hyperammonemia.

There was a positive correlation between the ammonia concentration 24 hours and 3 days after ASP administration and the activity of the drug 3 days after the administration (R Spearman: 0.34 and 0.44, resp.; $P < 0.0001$).

For each patient, the mean value of ammonia concentrations (separately for the measurements performed 24 hours and 3 days after the drug administration) and the mean value of ASP activities were calculated. A significant correlation between the mean values of the ammonia concentration and the mean value of ASP activity was found (R Spearman 0.41; $P < 0.0001$ and 0.56 $P < 0.0001$ for ammonia measurements performed 24 hours and 3 days after ASP administration, resp.). The mean ammonia concentration was lower in patients with decreased ASP activity (<100 IU/L) in at least one measurement than in children with the therapeutic activity of the enzyme in all tested samples (ammonia 24 hours after ASP: median 70 μmol/L and 106 μmol/L, resp.; ammonia 3 days after ASP: median 29 μmol/L and 42 μmol/L, resp.; $P < 0.00001$).

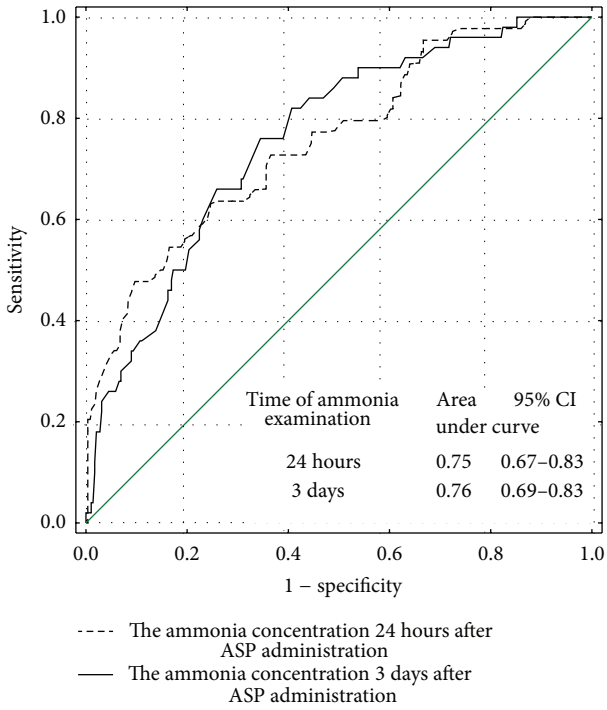

Figure 1: The usefulness of the ammonia concentration measured 24 hours and 3 days after ASP administration in detection of low (<100 IU/L) ASP activity—ROC curve.

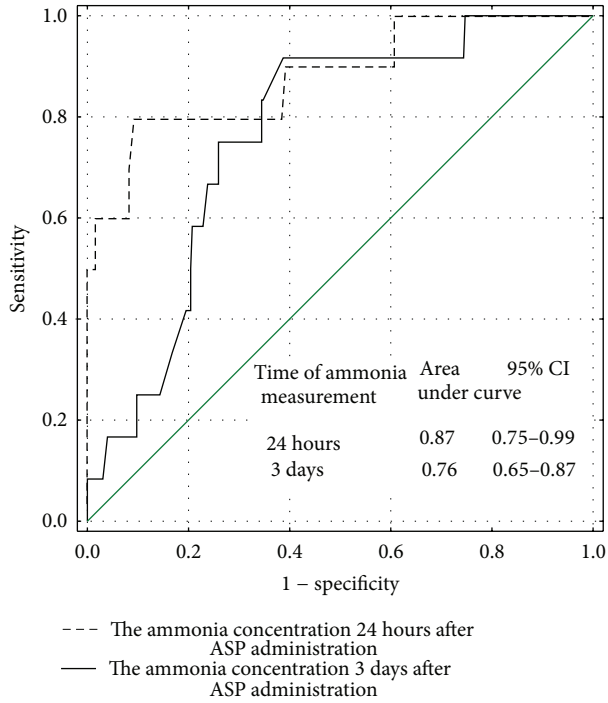

Figure 2: The usefulness of the ammonia concentration measured 24 hours and 3 days after ASP administration in detection of undetectable (<30 IU/L) ASP activity—ROC curve.

On the basis of a ROC curves analysis, the usefulness of ammonia concentration in detection of low and undetectable ASP activity was stated (AUC for ammonia concentration 24 hours and 3 days after ASP administration: 0.75 and 0.76, resp.; for low ASP activity; 0.87 and 0.76, resp.; for undetectable ASP activity—Figures 1 and 2).

The optimal cut-off point for detection of low ASP activity was 64 μmol/L and 29 μmol/L for the ammonia concentration 24 hours and 3 days after ASP administration, respectively. For undetectable ASP activity, the ammonia cutoff was 38 μmol/L (24 hours after ASP) and 24 μmol/L (3 days after ASP). The parameters assessing the value of the tests recognizing low and undetectable ASP activity for different cut-off points of ammonia concentration are shown in Tables 2 and 3.

The mean value of ammonia concentration during induction was found to be useful to distinguish between the patients with the therapeutic ASP activity in all measurements during induction and the patients with low or undetectable ASP activity in at least one determination during induction (AUC for low and undetectable ASP activity: 0.85 and 0.84, resp.) (Figures 3 and 4).

To identify the patients with low and undetectable ASP activity, the optimal cutoff points for the mean ammonia concentration determined 24 hours after ASP administration were 91 μmol/L and 90.5 μmol/L, respectively, and for the mean ammonia concentration determined 3 days after ASP administration, 36 μmol/L and 29 μmol/L, respectively. The parameters assessing the ability of the tests to identify the patients with low and undetectable ASP activity for different cut-off points of the mean ammonia concentration are shown in Tables 4 and 5.

Table 2: The parameters assessing the ability of the ammonia concentration to recognize low (<100 IU/L) ASP activity (95% CI in brackets).

	The ammonia concentration after ASP administration	
	24 hours ≤ 64 μmol/L	3 days ≤ 29 μmol/L
Sensitivity	0.64 (0.51–0.76)	0.76 (0.62–0.87)
Specificity	0.75 (0.72–0.76)	0.65 (0.59–0.71)
Positive predictive value	0.30 (0.24–0.35)	0.27 (0.22–0.30)
Negative predictive value	0.93 (0.90–0.95)	0.94 (0.91–0.96)

Hepatotoxicity grade III and IV WHO was found in 5 patients. The ammonia concentration in this group of patients did not differ significantly from the ammonia concentration in the patients without severe hepatotoxicity. No correlation between the ammonia concentration and transaminases activity was found.

4. Discussion

ASP is among the most important agents in the therapy of ALL. Treatment schedules for each preparation assure clinical efficacy in the majority of patients. Taking into

Table 3: The parameters assessing the ability of ammonia concentration to recognize undetectable (<30 IU/L) ASP activity (95% CI in brackets).

	The ammonia concentration after ASP administration	
	24 hours ≤ 38 μmol/L	3 days ≤ 24 μmol/L
Sensitivity	0.80 (0.50–0.94)	0.75 (0.47–0.91)
Specificity	0.90 (0.89-0.90)	0.74 (0.73–0.75)
Positive predictive value	0.21 (0.13–0.25)	0.09 (0.06–0.12)
Negative predictive value	0.99 (0.98–1.0)	0.99 (0.97–1.0)

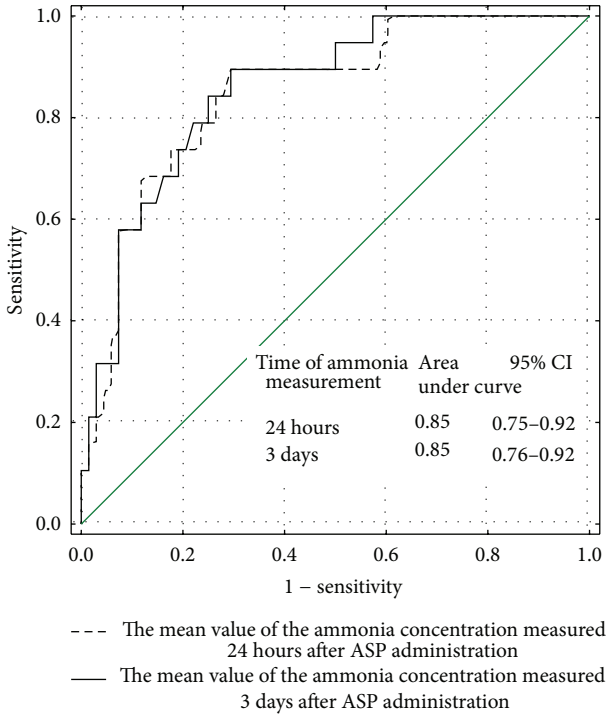

Figure 3: The usefulness of the mean value of the ammonia concentration measured 24 hours and 3 days after ASP administration in identifying the patients with low (<100 IU/L) ASP activity in at least one examination—ROC curve.

account the risk of ASP inactivation by antibodies, the therapy monitoring with ASP activity measurements is very important [5, 6, 8].

Ammonia is a direct product of the reaction catalyzed by ASP and its plasma concentration can be easily measured as a routine, inexpensive laboratory test. Hyperammonemia is a well-known complication of ASP administration [13–15, 19]. Steiner et al. described ammonia fluctuations in 10 patients treated with ASP, up to sevenfold above the normal values 1 day after ASP administration and slow returning to the normal level within 2 consecutive days, but the authors did not monitor ASP activity [16]. Watanabe et al. found that ex vivo ammonia production well correlated with ASP activity, but only 5 patients were enrolled to the study and 23 samples

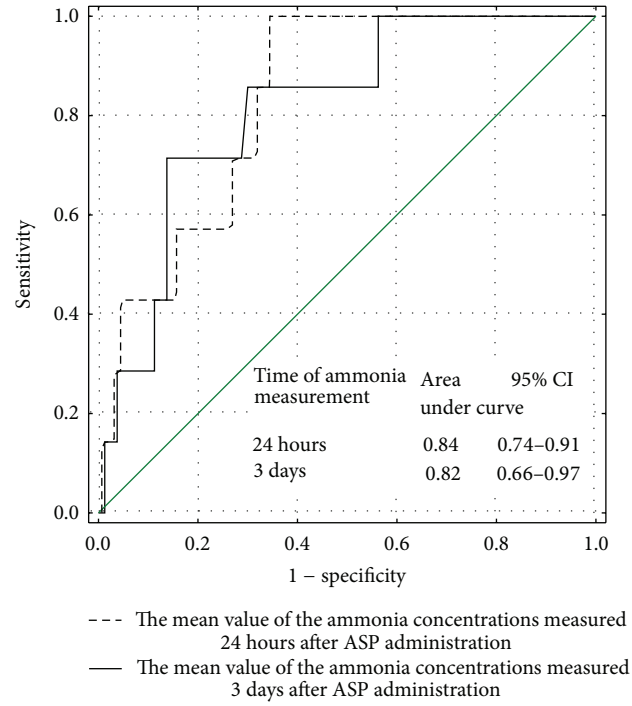

Figure 4: The usefulness of the mean value of the ammonia concentration measured 24 hours and 3 days after ASP administration in identifying the patients with undetectable (<30 IU/L) ASP activity in at least one examination—ROC curve.

were tested [17]. To the authors' knowledge, till now, there has been no publication describing a correlation between plasma ammonia level and ASP activity in larger cohort.

Ammonia produced by ASP reaction is removed in the liver by incorporation into the urea cycle, so the ammonia level may be influenced by a hepatic dysfunction. Therefore, we monitored the liver function in the studied patients. There were 5 patients with WHO grade III and IV hepatotoxicity, but their ammonia concentrations were not significantly higher than those of other patients. We did not find any correlation between the ammonia concentration and transaminase activity in our patients.

We analyzed the ammonia concentration 24 hours and 3 days after ASP administration during induction. The ammonia concentration correlated with ASP activity. The utility of ammonia measurements in recognizing low and undetectable ASP activity was assessed. On the basis of an ROC curves analysis, we found the moderate accuracy of the test. The optimal ammonia concentration thresholds to identify low and undetectable ASP activity were determined. The sensitivity and specificity of these tests were acceptable especially for undetectable activity of the enzyme, but the positive predictive values (PPV) were very low, which means a high percentage of false positive results.

A better test accuracy was found for the mean value of ammonia concentrations. An analysis of the ROC curve revealed that the mean value of ammonia concentrations can be useful to identify patients with low and undetectable

Table 4: The parameters assessing the ability of the mean value of the ammonia concentrations to recognize patients with low ASP activity in at least one examination (2 cut-off points, 95% CI in brackets).

	The mean value of the ammonia concentration			
	24 hours after ASP administration		3 days after ASP administration	
	≤46 μmol/L	≤91 μmol/L	≤20 μmol/L	≤36 μmol/L
Sensitivity	0.17 (0.04–0.41)	0.89 (0.67–0.99)	0.22 (0.06–0.48)	0.89 (0.67–0.99)
Specificity	0.99 (0.92–1.0)	0.70 (0.58–0.81)	0.99 (0.92–1.0)	0.70 (0.58–0.81)
Positive predictive value	0.75 (0.19–0.99)	0.46 (0.28–0.63)	0.80 (0.28–0.99)	0.46 (0.30–0.60)
Negative predictive value	0.82 (0.72–0.89)	0.96 (0.86–0.99)	0.83 (0.73–0.90)	0.96 (0.89–0.99)

Table 5: The parameters assessing the ability of the mean value of the ammonia concentrations to recognize patients with undetectable ASP activity in at least one examination (2 cut-off points, 95% CI in brackets).

	The mean value of the ammonia concentration			
	24 hours after ASP administration		24 hours after ASP administration	
	≤53 μmol/L	≤90.5 μmol/L	≤14 μmol/L	≤29 μmol/L
Sensitivity	0.43 (0.10–0.82)	1.0 (0.59–1.0)	0.14 (0.01–0.58)	0.71 (0.29–0.96)
Specificity	0.96 (0.89–0.99)	0.66 (0.55–0.76)	0.99 (0.93–1.0)	0.86 (0.77–0.93)
Positive predictive value	0.50 (0.12–0.88)	0.21 (0.08–0.38)	0.50 (0.13–0.99)	0.31 (0.11–0.59)
Negative predictive value	0.95 (0.88–0.99)	1.0 (0.93–1.0)	0.93 (0.85–0.97)	0.97 (0.90–1.0)

ASP activity in at least one determination during induction. Using ammonia measurements, 89% of patients with low ASP activity and 70% of patients with the therapeutic ASP activity could be correctly recognized and among the patients with positive results (the mean ammonia level below the threshold), 46% would be those with low ASP activity. Taking into account the fact that low ASP activity occurred in 21% of the examined patients, false positive results would amount to 23% of all results. That would result in recognizing silent inactivation in the patient with the therapeutic enzyme activity and unwarranted switching to another ASP preparation. As all ASP preparations have similar efficacy and safety profile while used at proper doses [5, 8, 11], it would have mainly financial consequences, because of higher costs of the pegylated preparations or those derived from *Erwinia*.

A reduction of false positive results could be achieved by decreasing the threshold of ammonia level. An increase of false negative results is synonymous with more patients with unrecognized silent inactivation, at risk of an ineffective treatment and a symptomatic allergic reaction.

5. Conclusions

The ammonia concentration correlates with ASP activity. The lower the ammonia concentration is, the higher the probability of low ASP activity is. Although the ammonia concentration measurement is not accurate enough to replace a direct ASP activity assay, it can be helpful when a direct assay is not available. Identification of the patients with low and especially undetectable ASP activity "silent inactivation" with such an easily available measurement could help in making decisions about switching to another ASP preparation to ensure the efficacy of the treatment.

Conflict of Interests

The authors declare that there is no actual or potential conflict of interests in relation to this paper.

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Research Article

Prevalence of Gene Rearrangements in Mexican Children with Acute Lymphoblastic Leukemia: A Population Study—Report from the Mexican Interinstitutional Group for the Identification of the Causes of Childhood Leukemia

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Mexico has one of the highest incidences of childhood leukemia worldwide and significantly higher mortality rates for this disease compared with other countries. One possible cause is the high prevalence of gene rearrangements associated with the etiology or with a poor prognosis of childhood acute lymphoblastic leukemia (ALL). The aims of this multicenter study were to determine the prevalence of the four most common gene rearrangements [*ETV6-RUNX1, TCF3-PBX1, BCR-ABL1*, and *MLL* rearrangements] and to explore their relationship with mortality rates during the first year of treatment in ALL children from Mexico City. Patients were recruited from eight public hospitals during 2010–2012. A total of 282 bone marrow samples were obtained at each child's diagnosis for screening by conventional and multiplex reverse transcription polymerase chain reaction to determine the gene rearrangements. Gene rearrangements were detected in 50 (17.7%) patients. *ETV6-RUNX1* was detected in 21 (7.4%) patients, *TCF3-PBX1* in 20 (7.1%) patients, *BCR-ABL1* in 5 (1.8%) patients, and *MLL* rearrangements in 4 (1.4%) patients. The earliest deaths occurred at months 1, 2, and 3 after diagnosis in patients with *MLL, ETV6-RUNX1*, and *BCR-ABL1* gene rearrangements, respectively. Gene rearrangements could be related to the aggressiveness of leukemia observed in Mexican children.

1. Introduction

Leukemia is the most common cancer in children worldwide, and acute lymphoblastic leukemia (ALL) is the most common subtype, accounting for 80% of all cases [1]. Mexico has two major problems in relation to childhood leukemia: it has one of the highest incidences of childhood leukemia in the world [2], and it has significantly higher mortality rates for this disease compared with other countries [3].

The related factors for these two problems in Mexico are not completely understood. However, it has been suggested that one factor could be the high prevalence of gene rearrangements associated with the etiology or with a poor prognosis of children with ALL [4, 5].

In Mexico City, three studies have reported the frequencies of gene rearrangements in children with ALL. They were single hospital studies based on a small number of cases [4–6]. Pérez-Vera et al. [4] reported a low frequency of the *ETV6-RUNX1* gene rearrangement in 57 Mexican patients with leukemia from the Instituto Nacional de Pediatría. In another study by Jiménez-Morales et al. [5], a high proportion of *TCF3-PBX1* cases was reported in 53 ALL patients. Finally, Daniel-Cravioto et al. [6], in one of the hospitals at the Instituto Mexicano del Seguro Social (IMSS), reported a high frequency of the *MLL-AF4* gene rearrangement, which has been associated with leukemia of a poor prognosis.

When a child is diagnosed with leukemia in Mexico City, the detection of gene rearrangements is not routinely performed. Therefore, the prognostic stratification and the choice of chemotherapy treatment are based on clinical characteristics, laboratory tests, and the immunophenotype [7].

There is no available population level information on the prevalence of gene rearrangements in Mexican patients with childhood ALL.

The aims of this multicenter study were to determine the prevalence of the four most common gene rearrangements [*ETV6-RUNX1*, *TCF3-PBX1*, *BCR-ABL1*, and *MLL* rearrangements] and to explore their relationship with mortality rates during the first year of treatment in ALL children from Mexico City.

2. Materials and Methods

2.1. Patients. The Mexican Interinstitutional Group for the Identification of the Causes of Childhood Leukemia (MIGICCL) conducted a prospective study of newly diagnosed ALL patients below the age of 19 years between January 1, 2010, and December 31, 2012, in eight public hospitals in Mexico City. The diagnosis of ALL was based on bone marrow morphology and immunophenotyping. Patients were treated with existing local treatment protocols, which differ from one hospital to another. The present study was approved by the National Ethics and Scientific Committees with the following number: 2009-785-001. Informed consent was obtained from the children's parents in accordance with the Declaration of Helsinki.

2.2. Hospitals. It has been estimated that the majority (97.5%) of children with leukemia are treated in nine public hospitals of Mexico City (Figure 1). The remaining cases are treated at private institutions [2]. The Instituto Nacional de Pediatría (INP) did not participate in this study because approval from the INP Institutional Review Board was not granted.

Participating hospitals represent four different Mexican Health Institutions: the Hospital de Pediatría, Centro Médico Nacional (CMN) "Siglo XXI," the Hospital General "Gaudencio González Garza," CMN "La Raza" and the Hospital General Regional "Carlos McGregor Sánchez Navarro" from the Instituto Mexicano del Seguro Social (IMSS), the Hospital Infantil de México Federico Gómez, the Hospital General de México and the Hospital Juárez de México from the Secretaria de Salud (SSa), the Hospital Pediátrico de Moctezuma from the Secretaría de Salud del Distrito Federal (SSDF), and the Hospital CMN "20 de Noviembre" from the Instituto de Seguridad Social al Servicio de los Trabajadores del Estado (ISSSTE).

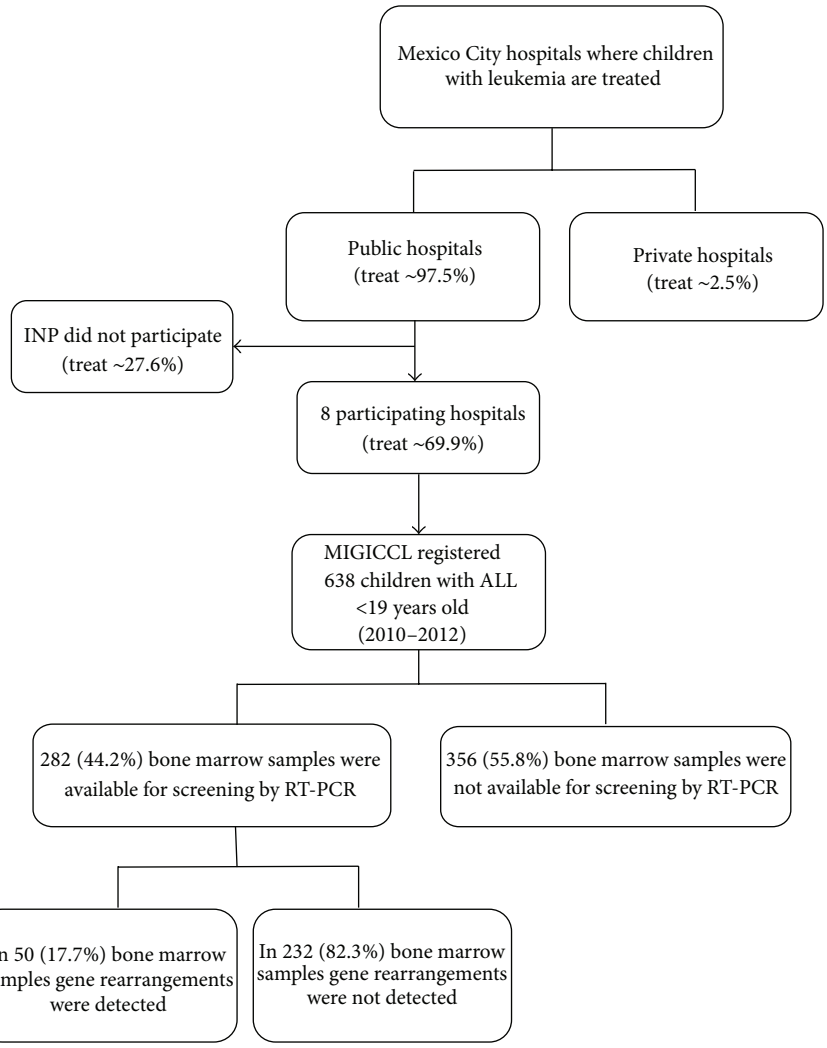

Figure 1: Flow chart of the selection process. Mexican Interinstitutional Group for the Identification of the Causes of Childhood Leukemia (MIGICCL) study of newly diagnosed ALL patients below the age of 19 years between January 1, 2010, and December 31, 2012, in eight public hospitals in Mexico City.

2.3. Clinical Data and Definitions. The following clinical data were collected from patient records: information regarding sex, age at diagnosis, immunophenotype classification, white blood cell (WBC) count at diagnosis, and date of the patient's last visit to the hospital or the date of the patient's death as of one year after ALL diagnosis.

According to the National Cancer Institute (NCI) risk classification, patients were classified as having a standard risk [with ages ranging from 1 to 9.99 years and initial white blood cell count (WBC) $< 50 \times 10^9$/L)] or a high risk [age <1 or ≥10 years and/or initial WBC $\geq 50 \times 10^9$/L] [8].

Early mortality was defined as a patient's death within the first year from the time of ALL diagnosis.

2.4. Detection of Gene Rearrangements. The identification of gene rearrangements was carried out at the Unidad de Investigación Médica en Inmunología e Infectología, Hospital de Infectología "Dr. Daniel Méndez Hernández," "La Raza" from the IMSS.

2.4.1. Cell Lines. In this study, cell lines were obtained from the American Type Culture Collection (ATCC) and used as positive controls for RT-PCR: the t(12; 21) positive cell line Reh (ATCC CRL-8286) (having the *ETV6/RUNX1* fusion gene); SUP-B15 (ATCC CRL-1929) (having t(9;22)(q34;q11), t(4;14) (p11;q24), der(4)t(1;4) (p11;q33), t(9;22) (q34;q11), der(10)t(3;10) (q25;q26), and (16); Philadelphia chromosome is present); RS4;11 (ATCC CRL-1873) (with t(4;11)(q21;q23); K-562 (ATCC CCL-243); $p210^{Bcr-Abl}$ $p185^{Bcr-Abl}$ positive cell line for *BCR-ABL major and minor*, respectively. B1H1 (ATCC 59171) HL-60 promyelocytic leukemia was used as a negative control. The t(1;19) (q23;p13) positive cell line 697 was obtained from DSMZ-German collection of microorganisms and cell cultures (Braunschweig, Germany). All cell lines were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum and antibiotics (Invitrogen, Carlsbad, CA) and maintained according to the manufacturer's instructions. The total RNA was extracted from cell lines and used as internal controls (positive and negative).

2.4.2. RNA Isolation and Synthesis of Complementary DNA (cDNA). Total RNA was extracted from leukemic and normal cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quality of RNA was confirmed by the presence of intact ribosomal RNA (28s and 18s bands) by denaturing agarose gel electrophoresis and visualized by UV illumination. A total of 3 μg of total RNA was reverse-transcribed using the Superscript one-step RT-PCR with Platinum Taq Kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The cDNA was incubated at 94°C for 10 min to inactivate the reverse transcriptase. Finally, the synthesized cDNA was stored at −20°C until use.

2.4.3. Detection of Gene Rearrangements by Polymerase Chain Reaction (PCR). Gene rearrangements were detected with a conventional RT-PCR assay as described in Daniel-Cravioto et al. [6] in a previous study. In addition, a commercial Multiplex RT-PCR kit was used according to the manufacturer's instructions (Hemavision, DNA-Technology A/S, Aarhus, Denmark). This screening assay covering 28 different fusion transcripts was used to test for the presence of more than 80 fusion transcript variants. After cDNA synthesis, the PCR amplification was performed in two steps: first, a master PCR amplification followed by nested PCR to screen for the presence of fusion transcripts and second a split-out PCR amplification followed by nested PCR to identify specific fusion transcripts [9, 10]. We obtained one hundred percent concordance between our in-house procedure and the commercially available kit. Because we were only interested in the four rearrangements, we decided, once validated, to use the conventional RT-PCR assay as described in Daniel-Cravioto et al. [6].

3. Statistical Analysis

Statistical analyses were performed using SPSS IBM (Statistical Package for the Social Sciences, Inc., Version 21, Chicago, IL, USA) and relative frequencies regarding the four most frequent gene rearrangements were obtained.

4. Results

From 2010 to 2012, the MIGICCL registered 638 pediatric patients newly diagnosed with ALL from 8 public hospitals of Mexico City of which 334 (52.4%) were male. The entire population median age was 6.2 years (75 months; range from 2 to 222 months). According to the NCI risk classification, 356 patients (55.8%) were classified as standard risk and 282 (44.2%) as high risk. The median WBC at diagnosis was 10.07×10^9/L (range from 0.46 to 970×10^9/L). According to their immunophenotype, 543 (85.1%) patients were classified as having B-cell precursor ALL, 69 (10.8%) patients as having the T-cell immunophenotype, and 26 (4.1%) patients as having biphenotypic leukemia. Early mortality occurred in 86 (13.5%) patients in our cohort study.

The analysis to detect the most frequent gene rearrangements was carried out on 282 (44.2%) available samples (Figure 1), and gene rearrangements were detected in 50 (17.7%) from these patients. Patient characteristics according to molecular subgroups are displayed in Table 1.

ETV6-RUNX1 was detected in 21 patients (7.4%), of whom 14 (66.7%) patients were female. The *ETV6-RUNX1* gene rearrangement was observed in 18 (85.7%) children under the age of 10 and B-cell precursor was the predominant immunophenotype (Table 1). Moreover, the majority of patients (66.7%) had a standard risk and early mortality occurred in 1 (4.8%) patient with this fusion gene.

The *TCF3-PBX1* translocation was present in 20 (7.1%) patients. The B-cell precursor immunophenotype was observed in 19 (95%) patients and 1 patient (5%) had biphenotypic leukemia. In addition, ten patients (50%) with this rearrangement were classified as high risk and 1 (5%) patient died.

The frequency of patients with the *MLL* rearrangements was 1.4% ($n = 4$) of which 3 (75%) were female. Three patients presented the *MLL-AF4* rearrangement, and one case presented the *MLL-AF17* rearrangement. The patients' age ranged between 3 and 137 months with a median of 43 months (3.5 years). These patients had a median WBC of 210.7×10^9/L (range from 17.4 to 970×10^9/L) and 75% ($n = 3$) of *MLL* cases were classified as high risk patients. One of the four patients (25%) presented early mortality (Table 1).

The *BCR-ABL1* gene rearrangement was detected in five (1.8%) patients, four (80%) were male. The median age at diagnosis was 102 months (range from 15 to 185). Four (80%) patients had a B-cell precursor immunophenotype, and in this molecular subgroup early death occurred in two patients (40%).

Interestingly, the earliest deaths occurred at months 1, 2, and 3 after diagnosis in patients with the *MLL*, *ETV6-RUNX1,* and *BCR-ABL1* gene rearrangements, respectively.

5. Discussion

This is the first report regarding the prevalence of the four most frequent gene rearrangements in pediatric patients with ALL from eight public hospitals where ~69.9% of the all Mexico City's children with leukemia are treated [2].

5.1. ETV6-RUNX1 Gene Rearrangement. The frequency of *ETV6-RUNX1* in the present study (7.4%) is consistent with reports from developing countries. In a previous study from 26 pediatric cases from the Hospital La Raza in Mexico City, the frequency was 3.8% [6]. In India [14] and Brazil [15], reported frequencies were 7% and 11.3%, respectively (Table 2). However, we observed a lower frequency of the *ETV6-RUNX1* rearrangement in comparison to reports from developed countries [11–13, 20]. Interestingly, the disparities regarding gene rearrangement prevalence among countries could be explained by environmental factors as playing an important role in the development of childhood leukemia, as would be the case of Mexico [1, 6].

Interestingly, *ETV6-RUNX1* patients with a B-cell precursor immunophenotype were predominantly female. This finding is consistent with previous reports [21, 22]. Notably, in our study, the majority of *ETV6-RUNX1* cases were

Table 1: Patient characteristics in molecular subgroups of childhood acute lymphoblastic leukemia (ALL). Results from the Mexican Interinstitutional Group for the Identification of the Causes of Childhood Leukemia (MIGICCL).

Patient characteristics	Gene rearrangement					
	ETV6-RUNX1 ($n = 21$) n (%)	*TCF3-PBX1* ($n = 20$) n (%)	*BCR-ABL1* ($n = 5$) n (%)	*MLL* rearrangements ($n = 4$) n (%)	Undetected ($n = 232$) n (%)	Samples not available for screening ($n = 356$) n (%)
Gender						
Male	7 (23.3)	10 (50)	4 (80)	1 (25)	115 (49.5)	197 (55.3)
Female	14 (66.7)	10 (50)	1 (20)	3 (75)	117 (50.4)	159 (44.7)
Age (in months)						
Range	31–144	9–187	15–185	3–137	5–222	2–203
Median age	75	95.5	102	43	84.5	68.5
Age groups						
<1 year	—	1 (5)	—	2 (50)	4 (1.7)	14 (3.9)
1–9 years	18 (85.7)	12 (60)	3 (60)	1 (25)	158 (68.1)	237 (66.6)
10–14 years	3 (14.3)	5 (25)	1 (20)	1 (25)	51 (22)	78 (21.9)
≥15 years	—	2 (10)	1 (20)	—	19 (8.2)	27 (7.6)
WBC count $\times 10^9$/L						
Range	2.2–306	0.9–137.6	4.6–149.5	17.4–970	0.4–910	0.6–697.8
Median	15.8	21.2	31.2	210.7	10.8	9.0
Immunophenotype						
B-cell precursor	20 (95.2)	19 (95)	4 (80)	3 (75)	205 (88.4)	292 (82)
T-cell	—	—	—	1 (25)	24 (10.3)	44 (12.4)
Biphenotypic	1 (4.8)	1 (5)	1 (20)	—	3 (1.3)	20 (5.6)
NCI risk classification						
Standard	14 (66.7)	10 (50)	3 (60)	1 (25)	121 (52.2)	207 (58.1)
High	7 (33.3)	10 (50)	2 (40)	3 (75)	111 (47.8)	149 (41.9)
Early death (1st year)						
Yes	1 (4.8)	1 (5)	2 (40)	1 (25)	31 (13.4)	50 (14)
No	20 (95.2)	19 (95)	3 (60)	3 (75)	201 (86.6)	306 (86)

Table 2: Proportions of the four molecular subgroups reported in the present study, other Mexican studies, and studies from other countries.

Author, year of publication [Reference]	Country	Number of patients	*ETV6-RUNX1* *n*(%)	*TCF3-PBX1* *n* (%)	*MLL* rearrangements *n* (%)	*BCR-ABL1* *n* (%)
MIGICCL study, 2014 [present study]	Mexico City	287	24 (7.4)	20 (7.1)	4 (1.4)	5 (1.8)
Other Mexican studies						
Pérez-Vera et al., 2008 [4]	Mexico City	59	5 (8.7)	—	5 (8.7)	1 (1.7)
Jiménez-Morales et al., 2008 [5]	Mexico City	53	7 (13.5)	6 (11.5)	—	2 (3.8)
Daniel-Cravioto et al., 2009 [6]	Mexico City	26	1 (3.8)	—	17 (65.4)	1 (3.8)
Studies from other countries						
Amor et al., 1998 [11]	Australia	66	22 (33.0)	—	—	—
Zuna et al., 1999 [12]	Czech Republic	190	41 (21.6)	—	—	—
Codrington et al., 2000 [13]	England	56	22 (39.0)	—	—	—
Siraj et al., 2003 [14]	India	259	18 (7.0)	18 (7.0)	2 (4)*	14 (5.0)
Mesquita et al., 2003 [15]	Brazil	88	10 (11.4)	5 (5.7)	1 (1.1)	1 (1.1)
Pui et al., 2003 [16]						
White children	USA	338	64 (18.9)	10 (3.0)	10 (3.0)	8 (2.4)
Black children	USA	68	9 (13.2)	8 (11.8)	1 (1.5)	4 (5.9)
Aldrich et al., 2006 [17]						
Non-Hispanic White	USA	140	34 (24.3)	6 (4.3)	4 (2.9)	1 (0.7)
Hispanic	USA	151	19 (12.6)	7 (4.6)	3 (2.0)	2 (1.3)
Lazic et al., 2010 [18]	Serbia	70	12 (17.1)	6 (8.6)	0	7 (10.0)
Alonso et al., 2012 [19]	Argentina	380	49 (12.9)	19 (5.0)	40 (10.5)	6 (1.6)

MIGICCL: Mexican Interinstitutional Group for the Identification of the Causes of Childhood Leukemia.
— Not screened.
* Other *MLL* rearrangements were detected in two (4%) of 50 samples analyzed by Southern blot.

under the age of 10. It has been reported that *ETV6-RUNX1* translocation is most commonly observed in children with leukemia under this age [23].

5.2. TCF3-PBX1 Gene Rearrangement. The proportion of positive patients for the *TCF3-PBX1* gene rearrangement represents one of the highest reported worldwide [14–19, 24] (Table 2).Our findings agree with results reported by Jiménez-Morales et al. [5] who found that the frequency of *TCF3-PBX1* was 11.5% in children treated at a single institution in Mexico City. In our cohort, the proportion of *TCF3-PBX1* is intermediate with respect to those reported for white children (3.0%) and black children (11.8%) [16]. In addition, it has been reported that the *TCF3-PBX1* fusion gene is associated with a poor outcome in ALL patients [25, 26]. Therefore, the high frequency of *TCF3-PBX1* could possibly explain the high mortality rates observed in Mexican children with ALL.

5.3. MLL Rearrangements. With regards to the translocations involving the chromosome 11q23 gene *MLL*, a lower frequency was observed (1.4%) compared to that reported by Daniel-Cravioto et al. [6] at the Hospital La Raza of Mexico City (65.4%). However, our findings are consistent with the reported in developed countries (Table 2). The results reported in the work by Daniel-Cravioto et al. [6] regarding the frequency of this gene rearrangement could be explained by an event called random variability, which is very probable when frequencies of extremely rare diseases are reported. For example, in 1996, the incidence of childhood leukemia corresponded to 80 cases per million in Mexico City, and this value was an exceptional and astonishing observation that has not registered again [27]. On the other hand, the median age of the *MLL* rearrangements subgroup was 42 months, and two patients were less than 1 year of age. These findings are consistent with the literature surmising that this type of gene rearrangement is associated with leukemia in early life [28].

5.4. BCR-ABL1 Gene Rearrangement. We observed a relatively low frequency (1.8%) of the *BCR-ABL1* gene rearrangement in comparison to previously reported studies carried out in Mexico City (Table 2). In addition, our findings are similar to those reported in Brazil (1.1%) [15] and Argentina (1.6%) [19]. Interestingly, two *BCR-ABL1* cases died during the first year of treatment. This gene rearrangement confers a poor prognosis in ALL patients [24] unless treated with tyrosine kinase inhibitors [TKIs] or hematopoietic stem cell transplant [HSCT]) [26].

5.5. Early Mortality during the First Year of Treatment. In our cohort ($n = 638$), the mortality rate during the first year of treatment was high (13.5%) considering that 5-year survival

rates in developed countries, currently, are higher than 90% [29]. Early mortality was observed in patients with positive gene rearrangements too. In a study conducted by Curado et al. [3] they reported that Mexico is among the few countries worldwide that has failed to reduce the mortality caused by childhood leukemia. The authors also mentioned that in recent years there has been a significant increase in mortality rates for childhood leukemia in this country [3]. Children who are classified as having a lower risk are treated with less intensive chemotherapy, while those classified as having a higher risk might be given a higher dose of chemotherapy than required leading to a higher rate of toxicity and higher mortality rates [26]. The inclusion of gene rearrangement detection in risk-adapted therapy has significantly contributed to improve the survival rate of pediatric patients with ALL in developed countries [25, 30]. However, in the majority of Mexico City public hospitals, current risk allocation schemes do not include the detection of gene rearrangements [7]. This might explain why Mexican children with ALL have higher mortality rates compared to developed countries where molecular techniques are routinely carried out in all patients with leukemia [24]. However, to draw solid conclusions about the impact of gene rearrangements on the survival of Mexican children with ALL, a long-term followup is necessary.

6. Conclusions

We present results from a multicenter study carried out by the MIGICCL on the frequency of the four most common gene rearrangements in Mexican pediatric ALL patients. We found a particularly high prevalence of the *TCF3-PBX1* gene rearrangement (associated with a poor prognosis) and a low prevalence of the *ETV6-RUNX1* fusion gene (associated with a better prognosis) in comparison to developed countries [25, 31]. Studies on the frequency of gene rearrangements in children with ALL are very heterogeneous; this is most likely due to ethnic and/or environmental factors. Moreover, this is a significant finding, as it may help to explain the high mortality rates observed in Mexican children with ALL; however, further research is needed to elucidate this hypothesis. We recommend the routine detection of gene rearrangements at ALL diagnosis in Mexican children to provide a better prognostic stratification and help to decide the most appropriate treatment, as already implemented in developed countries.

Conflict of Interests

The authors declare that they have no conflict of interests.

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