Original Article

DNA Ploidy Analysis in Acute Leukemia by Flow Cytometry

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Abstract

Background: The detection of chromosomal abnormalities holds significant diagnostic and prognostic value in acute leukemia. While cytogenetics is the standard technique for evaluating ploidy, it is a tedious and labor-intensive process compared to flow cytometry. Flow cytometric determination of leukemic cell DNA content reflects the proliferative index of blast cells. This technique is rapid, cost-effective, sensitive, easy to perform, independent of the tumor's proliferation state, and applicable to nearly all hematological malignancies.

Materials and Methods: This study was conducted to determine DNA ploidy in all acute leukemia cases using flow cytometry over a one-year period. A total of 53 new acute leukemia cases were diagnosed based on morphology, special stains, and immunophenotyping. The DNA index was then correlated with other clinico-hematological parameters.

Results: In our study, the overall incidence of aneuploidy was 32.07%, with 24% being hyperdiploid and 8% hypoploid. The majority of the aneuploid cases were male (58.8%), while diploid cases showed a female preponderance. We observed that acute lymphoblastic leukemia (ALL) patients with hyperdiploidy demonstrated favorable prognostic factors (i.e., clinico-hematological parameters), whereas hypoploidy was associated with poorer prognostic factors. In acute myeloid leukemia (AML) patients, hyperdiploidy was linked to poor prognostic factors.

Conclusion: We recommend that each newly diagnosed case be analyzed by flow cytometry, in addition to karyotyping, for early identification of patients who may benefit from conventional induction therapy.

Keywords:

DNA ploidy, acute leukemia, flow cytometry

Introduction

DNA content is the most frequently measured entity of the cell. DNA content can be categorized as either normal (diploid) or abnormal (aneuploid). In the case of aneuploidy, the cell may gain or lose one or more chromosomes. Cells with less than a diploid chromosome content are referred to as hypoploid, whereas cells with more than a diploid but less than a tetraploid complement of chromosomes are referred to as hyperdiploid [1].

Aneuploidy can be detected with the help of traditional metaphase cytogenetics, interphase cytogenetics (fluorescent in situ hybridization (FISH), multicolor FISH, spectral karyotyping, and comparative genomic hybridization (CGH)), flow cytometry

(FCM), and image cytometry (ICM) [1]. Though cytogenetics is the standard technique to evaluate ploidy, it is a tedious, laborious, and time-consuming task and depends on the proliferative index of the sample.

Cellular DNA content measured by FCM reveals: (1) cell distribution within the major phases of the cell cycle, (2) estimates the frequency of apoptotic cells with fractional DNA content, and/or (3) discloses DNA ploidy of the measured cell population. With the advent of newer diagnostic and therapeutic modalities, FCM has become an indispensable part of the diagnosis of leukemia and also in analyzing DNA ploidy status. This technique is rapid, cost-effective, sensitive, easy to perform, not dependent on the proliferation state of the tumor, and is applicable to almost all hematological malignancies [2].

DNA ploidy is a well-known prognostic marker and has been included in the WHO 2008 classification of acute leukemia (AL) as a separate clinical and prognostically distinct entity [3]. DNA ploidy is commonly used in the risk stratification of hematolymphoid neoplasms like B-cell precursor acute lymphoblastic leukemia (BCP-ALL) and multiple myeloma (MM). In addition, its application has been expanded in the prognostication of many non-hematological cancers [4]. Hence, the present study was planned to look for frequencies of various DNA ploidy groups in acute leukemia cases using the flow cytometric method.

Materials and Methods

This prospective study was conducted in the Department of Pathology at a tertiary care institute in North India, with the aim of determining DNA ploidy in all acute leukemia (AL) cases by flow cytometry (FCM) over a period of one year. It included 53 newly diagnosed cases of AL, confirmed by morphology and immunophenotyping, which was correlated with other clinicohematological parameters. EDTA-anticoagulated peripheral blood and bone marrow aspirates were obtained for all patients. Laboratory investigations included a complete hemogram, examination of Leishman-stained peripheral blood smears and bone marrow aspiration smears, and cytochemistry (SBB, PAS). The diagnosis of AL was made based on cytomorphology and immunophenotyping.

For Immunophenotyping: Immunophenotyping was performed using an 8-color flow cytometer, BD FACS Canto II (Becton Dickinson, San Jose, CA), with a monoclonal antibody panel specific to AL for peripheral blood and bone marrow samples. Common antibodies used in the AL panel included immaturity markers (CD34, HLA-DR, terminal deoxynucleotidyl transferase [TdT], CD117), myeloid markers (cMPO, CD13, CD33), monocytic markers (CD64), B lymphoid markers (CD19, CD10, CD20, cCD79a), and T lymphoid markers (CD3, CD5, CD7, CD4, CD8). All standard protocols were followed.

For Determining DNA Ploidy: DNA ploidy was determined using the 8-color flow cytometer, BD FACS Canto II (Becton Dickinson, San Jose, CA). Peripheral blood mononuclear cells (PBMCs) served as control cells, providing a reference point for determining the DNA index of the test sample. These cells were prepared using the same reagents and procedures as the test samples, and both test and control samples were processed within an hour under identical conditions.

Mononuclear cells were prepared by density gradient centrifugation, and cell concentration was verified using an automated cell counter to ensure a concentration of 1.0 x 10^6 cells/mL. The staining procedure employed the Cycle TEST PLUS DNA reagent kit (BD Biosciences Pvt. Ltd.). Initially, trypsin was used to enzymatically disaggregate solid tissue fragments and digest cell membranes and cytoskeletons. Trypsin inhibitor and ribonuclease A were then applied to digest RNA, followed by Propidium Iodide (PI) staining at a final concentration of at least 125 µg/mL to bind stoichiometrically to DNA. List mode was acquired using Cycle TEST PLUS DNA reagents and FACS Diva software.

DNA Index Calculation: DNA index = MFI of sample population / MFI of reference diploid population (PBMC)

(MFI = mean fluorescent intensity, PBMC = peripheral blood mononuclear cell of newborn)

Interpretation: DI = 1 ± 0.05: Diploid, DI < 1.0 – 0.05: Hypoploid and DI > 1.0 + 0.05: Hyperploid

Approval from the institutional ethics committee of the University of Health Sciences was obtained. Informed consent was gathered from all participants after the study was explained to them. After hematological study and flow cytometry, biomedical waste generated during the procedures was discarded per biomedical waste (management and handling) rules [5].

The collected data were analyzed using SPSS software (version 24.0). Frequency distribution and cross-tabulation were used to create summary tables and compare items across various categories. Associations were tested using the Chi-square test, and correlation was assessed using the Spearman test. Differences between groups were considered significant only when the p-value was <0.05.

Results

A total of 53 leukemia cases were analyzed: 50.94% were Acute Lymphoblastic Leukemia (ALL), 45.28% were Acute Myeloid Leukemia (AML), and only 3.77% were Mixed Phenotypic Acute Leukemia (MPAL). Among the ALL cases, 88.8% were B-ALL (24/27), and 11.1% were T-ALL (3/27). DNA index (DI) determined by flow cytometry (FCM) in our study revealed an overall incidence of aneuploidy of 32.07%, with 24% being hyperdiploid and 8% hypoploid (Fig. 1, Fig. 2, & Fig. 3). Out of the 17 aneuploid cases, 47% (8/17) were AML, 47% (8/17) were ALL, and 5.8% (1/17) was MPAL.

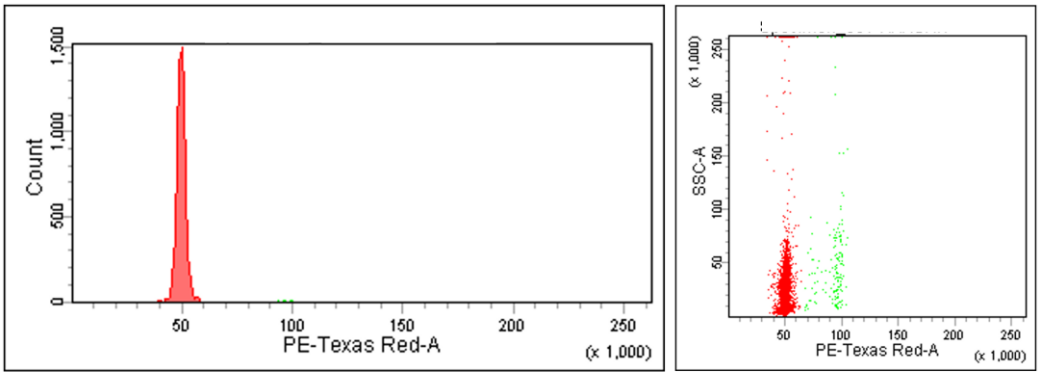

Figure 1: DNA histogram shows Diploid peak with DNA index of 1 (DI = 1)

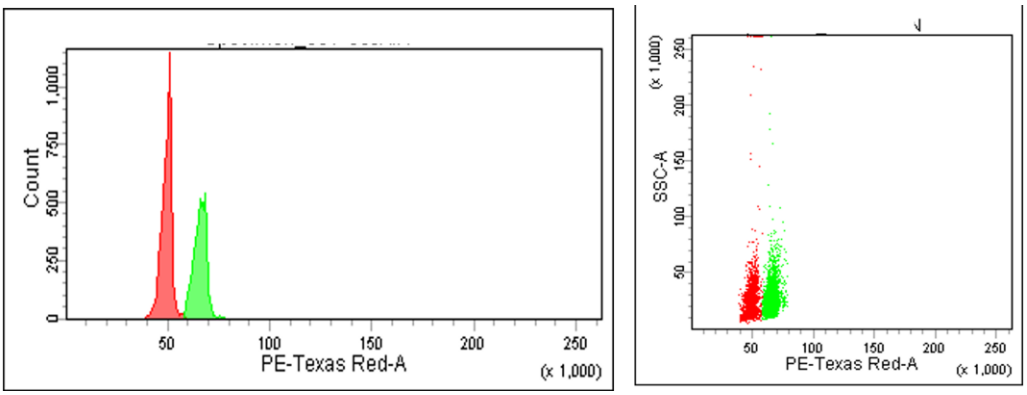

Figure 2: DNA histogram shows Hyperdiploid peak with DNA index of 1.3 (DI = 1.3)

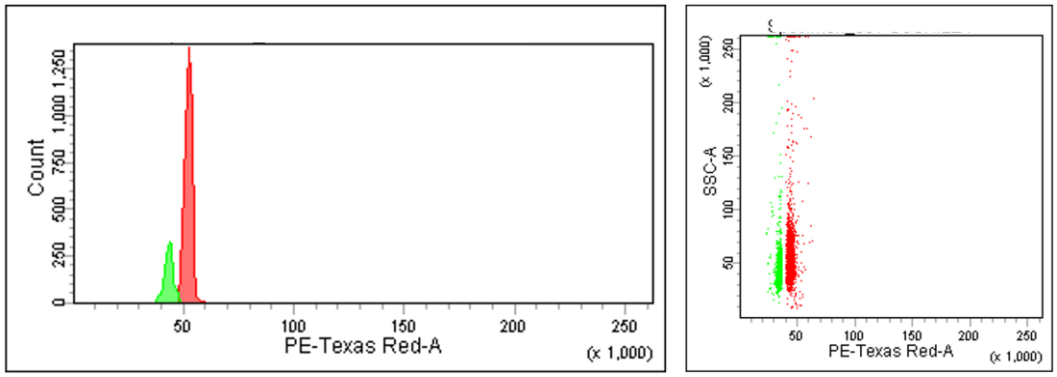

Figure 3: DNA histogram shows Hypoploid peak with DNA index of 0.83 (DI = 0.83)

There was a female predominance in diploid acute leukemia (AL) cases, constituting 72.2%, while there was a male predominance in the aneuploid population, constituting 58.8%. A statistically significant difference in the frequency of DNA aneuploidies was observed in our study.

Comparison of the mean values of hematological parameters between the diploid and aneuploid populations showed that hemoglobin (Hb) was higher in the diploid group, while total leucocyte count (TLC), platelet count, blasts% in peripheral blood film (PBF), and blasts% in FCM were higher in the aneuploid group. This difference was statistically nonsignificant (Table 1).

Table 1: DNA ploidy with hematological parameters

	Diploid (n=36)	**Aneuploid (n=17)**	**P value**
	Mean ± sd	Mean ± sd	
Hb	7.7±2.37	7.42±2.27	0.69
TLC X 10^9/L	50.0±81.4	60.7±77.8	0.653
Platelet X 10^9/L	56.4±52.3	85.7±93.4	0.15
Blasts% PBF	51.94±24.2	58.12±24.24	0.39
Blasts% FCM (SSC v/s CD45)	59.31±22.66	67.24±21.85	0.235

We also observed that patients in the diploid group were younger compared to those in the aneuploid group. However, comparison of the hematological parameters between the two groups did not yield any significant difference (Table 2 & Table 3).

Acute Lymphoblastic Leukemia: The frequency of DNA aneuploidy in ALL was 29.6%, with 18.5% being hyperdiploid and the remaining 11.1% hypoploid. The incidence of aneuploidy was 10% among children and 41.1% among adults. In our study, 27.3% of C-ALL cases had aneuploid stem lines.

Among the hyperdiploid ALL population, 4 out of 5 cases belonged to the B cell lineage. All hyperdiploid B-ALL cases had an initial TLC of <20.0 x 10^9/L, were young males, and 3 cases showed positive CD10 expression. However, no statistically significant correlation was observed between age, sex, Hb, TLC, platelet count, and ploidy ($p > 0.05$).

In the hypoploid ALL population, all cases were female adults with Hb <10.5 gm%, a platelet count <20.0 x 10^9/L, and belonged to the B cell lineage. All these cases showed positive CD10 expression. However, no statistically significant correlation was observed between age, sex, Hb, TLC, platelet count, and ploidy ($p > 0.05$).

Acute Myeloid Leukemia: The overall incidence of aneuploidy in AML was 33.3%, with 29.2% of cases being hyperdiploid and

4.1% hypoploid. There was a male preponderance among the hyperdiploid population. In 71.4% of hyperdiploid cases, Hb was <10.5 gm%, which was statistically significant (Table 3). We observed that hyperdiploidy was associated with a high TLC, an adverse prognostic factor. Increased expression of lymphoid markers such as Tdt, CD3, CD5, and CD79a was noted in aneuploid AML cases compared to the diploid population (Table 4 & Table 5). However, no statistically significant association was found among various hematological parameters between diploid and aneuploid populations ($p > 0.05$).

Table 2: Comparing various parameters among diploid, hyperdiploid and hypoploid ALL and AML population

	Parameters among diploid, hyperdiploid and hypoploid ALL									
		N	PLOIDY						Chi square	P value
			DIPLOID		HYPOPLOID		HYPERDIPLOID			
			Count	Column N %	Count	Column N %	Count	Column N %		
Sex	F	17	14	73.70%	2	66.70%	1	20.00%	4.912	0.086
	M	10	5	26.30%	1	33.30%	4	80.00%		
Age	<=10 years	10	9	47.40%	0	0.00%	1	20.00%	3.257	0.196
	>10 years	17	10	52.60%	3	100.00%	4	80.00%		
Hb (cut off 10.5 gm/dl)	<=10.5	23	16	84.20%	3	100.00%	4	80.00%	0.643	0.725
	>10.5	4	3	15.80%	0	0.00%	1	20.00%		
TLC X 10^9/L	<50.0	18	12	63.20%	2	66.70%	4	80.00%	0.505	0.777
	>50.0	9	7	36.80%	1	33.30%	1	20.00%		
Platelet X 10^9/L	<150.0	24	17	89.50%	3	100.00%	4	80.00%	0.782	0.677
	>150.0	3	2	10.50%	0	0.00%	1	20.00%		
Blasts% FCM	<20%	2	1	5.30%	0	0.00%	1	20.00%	1.523	0.467
	>20%	25	18	94.70%	3	100.00%	4	80.00%		
	Parameters among diploid, hyperdiploid and hypoploid AML									
Sex	F	14	11	68.80%	1	100.00%	2	28.60%	3.98	0.137
	M	10	5	31.20%	0	0.00%	5	71.40%		
Age	<=10 years	0	0	0.00%	0	0.00%	0	0.00%		
	>10 years	24	16	100.00%	1	100.00%	7	100.00%		
Hb (cut off 10.5 gm/dl)	<=10.5	22	16	100.00%	1	100.00%	5	71.40%	5.299	0.071
	>10.5	2	0	0.00%	0	0.00%	2	28.60%		
TLC X 10^9/L	<50.0	18	13	81.20%	0	0.00%	5	71.40%	3.381	0.184
	>50.0	6	3	18.80%	1	100.00%	2	28.60%		
Platelet X 10^9/L	<150.0	21	15	93.80%	1	100.00%	5	71.40%	2.367	0.306
	>150.0	3	1	6.20%	0	0.00%	2	28.60%		
Blasts% FCM	<20%	0	0	0.00%	0	0.00%	0	0.00%		
	>20%	24	16	100.00%	1	100.00%	7	100.00%		

Discussion

The study was conducted to determine DNA ploidy in AL using flow cytometry and to correlate DNA ploidy status with other established clinicohematological parameters of AL. The incidence of aneuploidy in 53 AL cases was 32.07% (17/53). Our findings are in concordance with Hiddeman et al. [6], who reported an incidence of 39.2%, whereas the incidence was lower in studies by Barlogie et al. [7] (26%) and Andreef et al. [8] (20%), who analyzed DNA aneuploidy in only adult populations of AL. In contrast, both pediatric and adult populations of AL were included in the present study and by Hiddeman et al. [6].

There was a statistically significant difference in male and female distribution among diploid and aneuploid populations of AL cases in our study. There was a female preponderance in diploid cases, constituting 72.2%. Among the aneuploid population, the majority were males (58.8%). In contrast, Hiddeman et al. [6] observed more aneuploidies among female ALL patients compared to male ALL patients ($p<0.05$) and found no difference in the frequencies of aneuploidies between male and female AML patients. This may have been due to the higher proportion of female AL patients in our study, small sample size, and random sampling.

Table 3: Comparing various parameters among diploid and aneuploid AML population

		N	Ploidy				Chi square	P value
			Diploid		Aneuploid			
			Count	Column N %	Count	Column N %		
Sex	F	14	11	68.80%	3	37.50%	2.143	0.143
	M	10	5	31.20%	5	62.50%		
Age	<=10 years	0	0	0.00%	0	0.00%	.	.
	>10 years	24	16	100.00%	8	100.00%		
Hb (cutoff 10.5 gm/dl)	<=10.5	22	16	100.00%	6	75.00%	4.364	**0.037**
	>10.5	2	0	0.00%	2	25.00%		
TLC X 10^9/L	<50.0	18	13	81.20%	5	62.50%	1	0.317
	>50.0	6	3	18.80%	3	37.50%		
Platelet X 10^9/L	<150.0	21	15	93.80%	6	75.00%	1.714	0.19
	>150.0	3	1	6.20%	2	25.00%		
Blasts% FCM	<20%	0	0	0.00%	0	0.00%	.	.
	>20%	24	16	100.00%	8	100.00%		

Acute Lymphoblastic Leukemia: In our study, the frequency of DNA aneuploidy in ALL was 29.6%, i.e., 8 out of 27 patients had aneuploid stem lines, which is usually associated with poor prognosis. The incidence of aneuploidy varied from 24% to 65% in the published literature. Our findings are in concordance with the studies by Sousa et al. [9] and Taleb et al. [10], while Kumar et al. [3], Almozain et al. [11], and Shahidi et al. [12] reported a higher incidence of aneuploidy.

Among these ALL cases, 70.4% were diploid, 18.5% were hyperdiploid, and the remaining 11.1% were hypoploid. A higher incidence of hyperdiploidy was reported by Taleb et al. [10] (30%), Kumar et al. [3] (65%), Shahidi et al. [12] (45%), and Khalifa et al. [13] (28.6%) compared to ours. The reason for the low frequency in our study may be due to the small number of cases in this group. However, the literature [3,9,10] shows a very low incidence of hypoploidy compared to ours. The variation in the incidence of hypodiploidy/aneuploidy could be due to the use of different DI classification systems. In our study, according to flow cytometer DNA Index (DI) results, cases were divided as diploid with a DNA index of 0.95–1.05 (DI = 1 ± 0.05), hyperdiploid with a DNA index of >1.0 + 0.05, and hypoploid with a DNA index of <1.0 – 0.05. Other studies used different DI cut-offs: Taleb et al. [10] used a DI cut-off of ≥1.16, and Kumar et al. [3] used a DI value of ≥1.10 as a cut-off for high hyperdiploidy.

The incidence of aneuploidy among children in the present study was 10%. A higher incidence of aneuploidy among the pediatric age group was reported by Kumar et al. [3] (65%), Rachieru-Sourisseau et al. [14] (60%), Shahidi et al. [12] (50%), and Hiddeman et al. [6] (40%). This could be due to the exclusive selection of pediatric B-ALL cases in most of these studies.

Among the hyperdiploid B-ALL cases, no statistically significant correlation was observed between age, sex, hemoglobin, WBC count, platelet count, and ploidy (p>0.05). Our findings align with studies by Kumar et al. [3], Taleb et al. [10], and Khalifa et al. [13], who also found no significant correlation between degree of ploidy and demographic, clinical, and hematological parameters. However, our findings contrast with Shahidi et al. [12], who observed a statistically significant correlation among hyperdiploidy, younger age, and low WBC count. Sousa et al. [9] also reported an association of hyperdiploidy with statistically significant prognostic markers, such as age between one and nine years and WBC count <20 x 10^9/L. Pui et al. [15] and Basu et al. [16] both observed a statistically significant association between ploidy and initial leukocyte count in their studies.

Among hypoploid B-ALL cases, no statistically significant correlation was observed between age, sex, hemoglobin, WBC count, platelet count, and ploidy (p>0.05).

Acute Myeloid Leukemia: AML is a heterogeneous disease with recurrent chromosomal abnormalities. Most studies on genetic abnormalities in AML use conventional cytogenetic techniques rather than flow cytometry; thus, data on aneuploidy in AML via flow cytometry is limited and underreported.

In the present study, the overall incidence of aneuploidy in AML was 33.3% (8/24). This finding is consistent with Hiddeman et al. [17] (41.5%), whereas Ameen et al. [18] reported no aneuploid population. Literature on AML aneuploidy has shown marked variability in data, ranging from 0% to 41.2%, possibly due to differences in sample size, criteria to define aneuploidy, and modalities used for analyzing ploidy [19]. We analyzed DNA ploidy using flow cytometry, which was readily available, and correlated aneuploidy status with clinical and hematological factors for prognosis.

Out of 24 AML cases, 22 (92%) had hemoglobin levels <10.5 gm%. Of the 8 aneuploid AML cases, 6 (75%) were anemic with hemoglobin <10.5 gm%. Among the aneuploid group, 71.4% (5/7) of hyperdiploid cases had hemoglobin <10.5 gm%, which was statistically significant (p=0.037, Table 4). However, due to the time-limited nature of our study, we could not follow up with patients to establish its association with prognosis. Our findings are consistent with Jahic et al. [20], who found that hemoglobin concentration <10 gm% was statistically correlated with high relapse rates. In contrast, studies by Hiddeman et al. [17], Vidriales et al. [19], and Sandahl et al. [21] found no association between hemoglobin levels and aneuploidy.

We also analyzed the aberrant expression of various antigens among diploid and aneuploid AML populations (Table 5). CD4 was the most commonly expressed aberrant marker in 62.5% of the aneuploid population. CD3, CD5, CD7, CD79a, and Tdt were also expressed in 12.5% of aneuploid AML cases. We observed an increased expression of lymphoid markers such as Tdt, CD3, CD5, and CD79a in aneuploid AML cases compared to the diploid population. Vidriales et al. [19] found a similar association between aneuploid populations and lymphoid-associated markers. Since we did not follow up with our patients, we cannot comment on the prognostic significance of aberrant lymphoid markers associated with AML aneuploidy.

There were certain limitations in our study, such as the inability to correlate with karyotyping due to the lack of conventional cytogenetics at our institute. Additionally, due to the time-limited nature of our study and lack of patient follow-up, the prognostic significance of our findings could not be assessed.

Conclusion

In our study, AL patients with hyperdiploidy revealed good prognostic factors, i.e., clinicohematological parameters, while hypodiploidy was associated with poor prognostic factors, although it did not achieve statistical significance. This study suggests that it will be beneficial to perform ploidy analysis by flow cytometry in addition to immunophenotyping at the time of diagnosis. Thus, we recommend that each newly diagnosed acute leukemia case should be analyzed by FCM in addition to karyotyping to assist with early identification and provide prognostically useful information before the initiation of induction therapy and for evaluating aneuploid leukemia following therapy.

Ethics Statement: Approval was obtained from the institutional ethics committee of the University of Health Sciences (No. IEC/18/patho09). This ethics committee is organized and operates according to the ICH-GCP guidelines and Schedule-Y requirements. Informed consent was obtained from all participants after an explanation of the study.

Code Availability: Not applicable.

Conflicts of Interest: The authors declare that they have no potential conflicts of interest.

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