The Role of Anti-Neutrophil Antibodies in the Etiologic Classification of Childhood Neutropenia: A Cross-Sectional Study in a Tertiary Center

Esin Karakilic-Ozturan, MD, Serap Karaman, MD,† Pinar Soguksu, PhD,‡ Sevim Mese, MD,‡ Ali Agacfidan, PhD,‡ Umit D. Mutlu, MD,§ Zeynep Karakas, MD,† Deniz Tugcu, MD,† Ayse Karagenc-Ozkan, MD,† and Omer Devecioglu, MD‖*

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Summary: Infections, drugs, malignancies, immunodeficiency, and autoimmunity may cause neutropenia. In primary autoimmune neutropenia, anti-neutrophil antibodies (ANeuA) bind to membrane antigens of neutrophils, which give rise to peripheral destruction of neutrophils. However, it is not always easy to detect these antibodies. This study aims to investigate the etiology of neutropenia, and at the same time to evaluate the immune mechanisms by ANeuA testing using granulocyte indirect immunofluorescence test. In our study, 310 neutropenic patients who were between 3 months and 18 years of age were evaluated. ANeuA screening tests were performed in 108 neutropenic patients (group 1), and these patients were divided into 2 subgroups as persistent neutropenia (group 1P, n = 12) and recovered neutropenia (group 1R, n = 96). Besides, a control group in the same age range was formed, consisting of 39 non-neutropenic children (group 2). ANeuA serum levels were also checked in these groups, and no statistically significant difference could be found between groups 1 and 2, or between groups 1P and 1R, regarding ANeuA levels. As a conclusion, our study was the first comprehensive research in Turkey investigating the large-scale etiology of neutropenia. Moreover, while ANeuA screening tests did not provide sufficient insight for immune neutropenia, we argue that it is not necessary for routine use and that further research in the etiology of neutropenia is required.

Key Words: childhood, neutropenia, etiology, anti-neutrophil antibody

(*J Pediatr Hematol Oncol* 2020;42:107–112)

Neutropenia is a blood disorder caused by abnormally low blood levels of neutrophils, which are important components of the immune system. By definition, neutropenia is the absolute neutrophil count (ANC) $<1000/\times10^9$/L in children who are 2 weeks to 1 year of age, and $<1500/mm^3$ in children who are of 1 year of age in whites. Yet, ethnic differences may be observed on the lower bound of ANC.[1] Neutropenia may be acute or chronic, congenital or acquired, or it may develop as a result of underproduction or increase in destruction, or as a result of intrinsic/extrinsic deficiencies affecting neutrophils and their precursors in the bone marrow.[1,2] Although its incidence varies depending on the underlying cause, the frequency of congenital neutropenia has been reported to be 1:1,000,000, while acquired neutropenia has been reported to be 1:100,000.[2] Data on the causes and frequency of neutropenia in Turkey, where this study was conducted, are limited. Yet, there is no consensus on optimal management; therefore, difficulties in the treatment and follow-up of neutropenia remain. In this cross-sectional study conducted in a tertiary center, we aimed to investigate the causes of neutropenia and the use and reliability of anti-neutrophil antibody (ANeuA) detection through the granulocyte indirect immunofluorescence test (GIIFT).

Received for publication November 25, 2018; accepted November 26, 2019.

From the Departments of *Pediatrics; †Pediatric Hematology-Oncology; ‡Microbiology; §Medical Biochemistry, Istanbul Faculty of Medicine, Istanbul University; and ‖Department of Pediatric Hematology, Memorial Sisli Hospital, Istanbul, Turkey.

Supported by Istanbul University Scientific Research Projects Unit (with project number 20409).

The authors declare no conflict of interest.

Reprints: Esin Karakilic-Ozturan, MD, Department of Pediatrics, Istanbul University, Istanbul Faculty of Medicine, Turgut Ozal Millet Ave., Fatih, Istanbul 34093, Turkey (e-mail: karakilic.esin@gmail.com).

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MATERIALS AND METHODS

Study Population

In our single-centered and cross-sectional study, which had been approved by the Ethics Committee of Istanbul University Faculty of Medicine, ~13,000 serum and blood samples collected in the Pediatric Biochemistry Laboratory between November 2015 and February 2016 were examined. The patients with an ANC $<1000/\times10^9$/L (for 3 to 12 mo of age) and $1500/\times10^9$/L (for older than 12 mo of age) had been defined neutropenic. According to neutrophil count, neutropenia is classified as *mild* (1000 to 1500×10^9/L), *moderate* (500 to 1000×10^9/L), and *severe* ($<500\times10^9$/L). In this study, 310 patients with neutropenia were enrolled, between 3 months and 18 years of age. The cause of neutropenia in 157 patients was determinate; however, we could not reach 3 of them again, and, during the reevaluation, at the beginning of the study, 42 of them had already recovered. Hence, the ANeuA screening tests were performed in the remaining 108 patients (group 1). Afterward, these patients were divided into 2 subgroups, as patients who had persistent neutropenia for 12 weeks (group 1P, n = 12), and patients who recovered from neutropenia after 3 weeks (group 1R, n = 96). Besides, a control group was formed, consisting of 39 non-neutropenic children (group 2), who had applications to the general pediatrics, social pediatrics, or adolescent health outpatient clinics for a routine health check, during the same time period. All these groups were checked for ANeuA levels. A flowchart illustrating the inclusion of patients within the study is shown as Figure 1.

Methods

Contact information and pertinent medical records on the hospital records were reviewed, and the patients were

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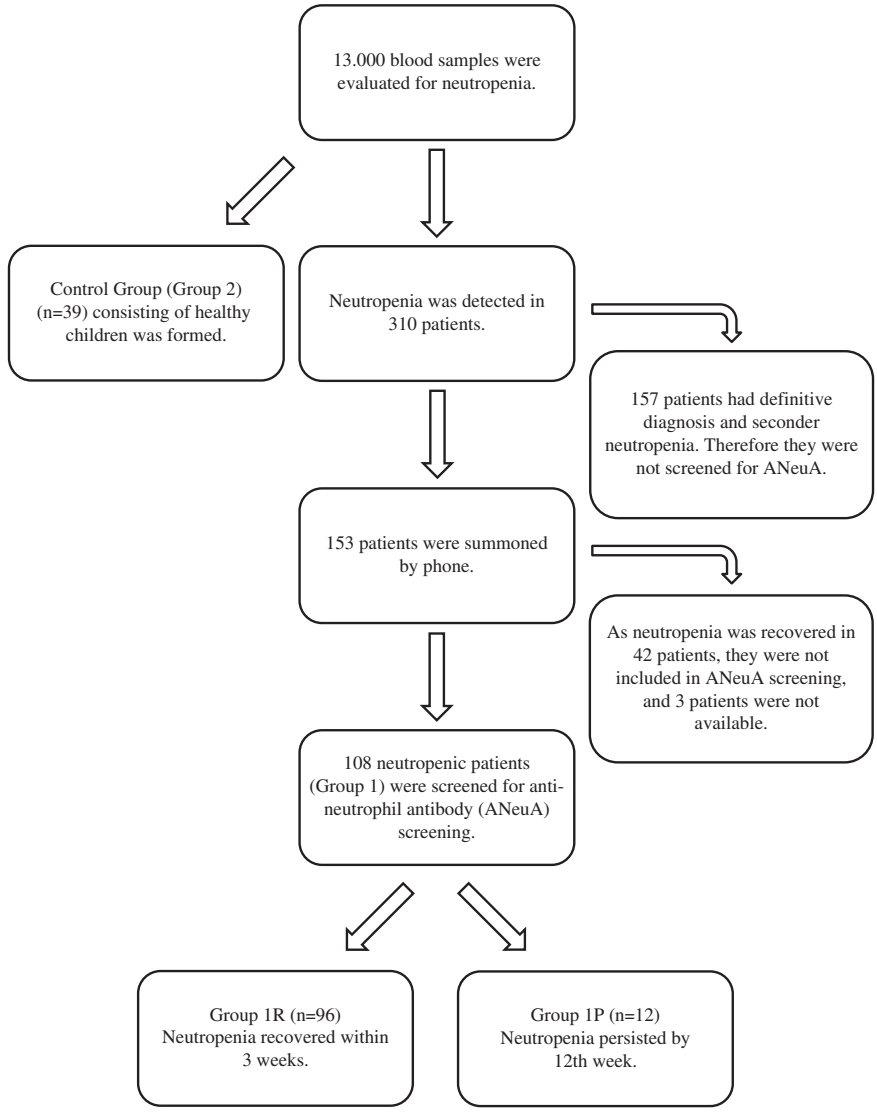


FIGURE 1. The flowchart of patient inclusion to study.

summoned by phone. The patients and their families were informed about the study, and written informed consents were obtained. The sex, age, presenting complaint, past medical history, and family history of the patients were recorded. Patients with >1 possible cause for neutropenia were added to each diagnostic category separately.

For ANeuA screening, samples were taken from all groups, and, after centrifugation, they were stored at −80°C until testing. For the group consisting of neutropenic patients, ANeuA was investigated using GIIFT in accordance with procedures. For the neutropenic group, fluorescein isothiocyanate conjugated (FITC)-labeled polyclonal anti-human IgG F(ab′)2/FITC (Dako, Glostrup, Denmark), and, for the control group, FITC-labeled negative control rabbit F(ab′)2/FITC (Dako) had been used.

To compose the neutrophil source, after an erythrocyte lysing solution (BD FACS) was added to the full blood pool, it was washed with phosphate-buffered saline, and erythrocytes were separated from the sample. In this way, donor neutrophils were obtained. Into this 50 μL neutrophil cell suspension, a 50 μL serum of the neutropenic patient and a 20 μL FITC-labeled polyclonal anti-human IgG was added. It was incubated at room temperature for 30 minutes. The same process was repeated with a negative control kit for the control group. The samples were analyzed with a flow cytometer device (BD FACS Canto; BD Bioscience). Gating the neutrophils on the FCS-SSC graph, anti-IgG values were incorporated into the count graphic. Following the incubation of donor neutrophils, patient serums and FITC-labeled anti-human IgG rabbit F(ab)2 antibody; and also donor neutrophils, negative control serums, and FITC-labeled anti-human IgG rabbit F(ab)2 antibody; histogram images in FACS Canto Analyzer were obtained (Fig. 2).

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistic Base 22.0 (SPSS Inc., Chicago, IL). Values are stated as mean ± SD and median (ranges: minimum-maximum). The frequency/prevalence analysis is indicated both as number

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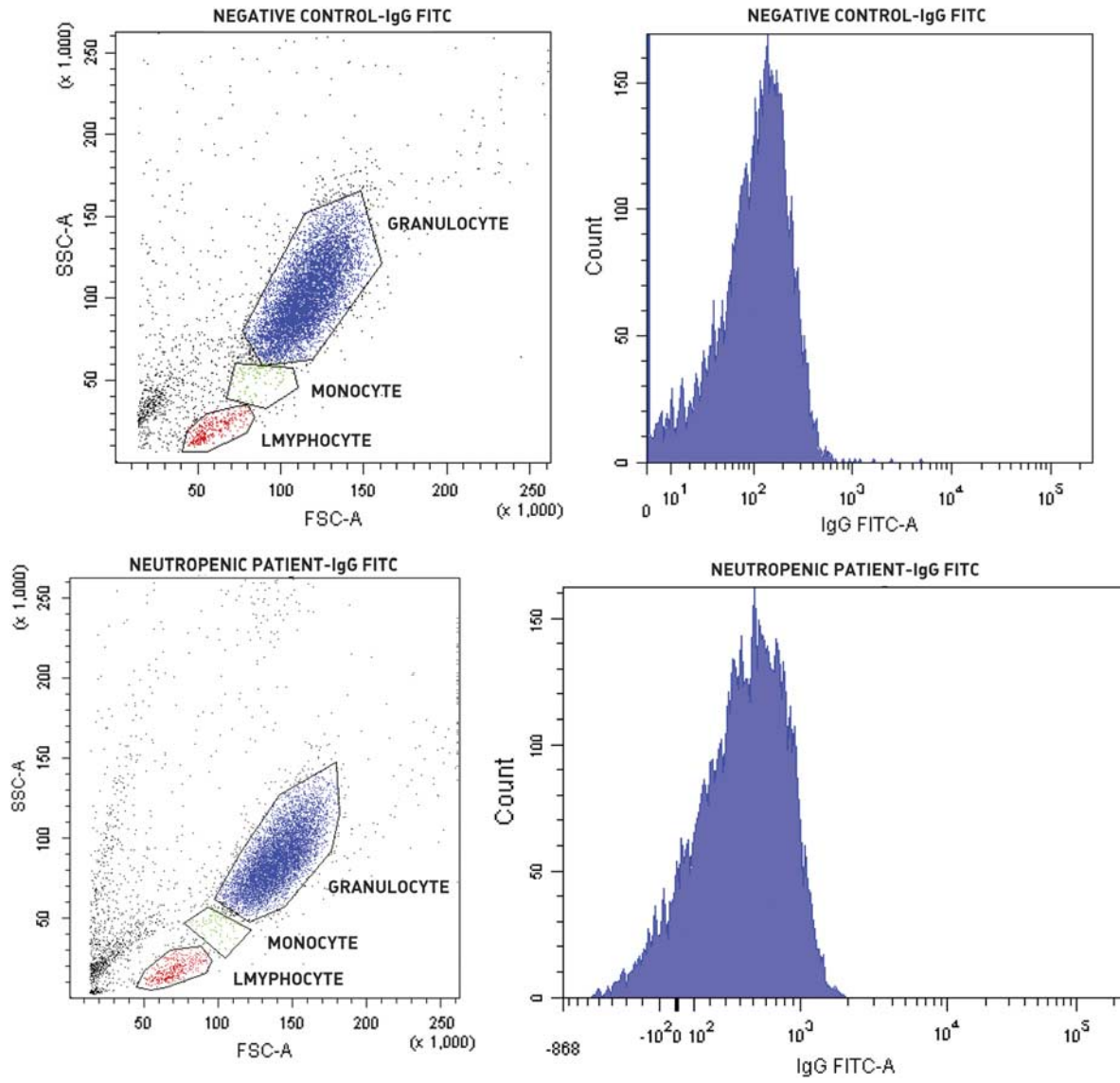


FIGURE 2. Histogram images in the FACS Canto Analyzer. full color online

and percentage. The distribution of variables was evaluated using the Kolmogorov-Smirnov test. In addition, the Mann-Whitney *U* test for the analysis of quantitative data, the Wilcoxon test for the analysis of repeated measures, and the χ^2 test for the analysis of qualitative data were applied. The significance of the *P*-value was considered to be ≤ 0.005.

RESULTS

Neutropenic patients involved in this study (n = 310) had a mean age of 5.6 ± 4.7 years (median: 4.0; minimum 0.25 to maximum 18), and the group consisted of 148 female and 162 male individuals. A total of 177 of these individuals (56.1%) had mild neutropenia, 90 of them (29%) had moderate neutropenia, and the remaining 43 (13.9%) had severe neutropenia. Mean ages of these severity groups were 6.4 ± 4.7 years (median: 5.0; minimum 0.9 to maximum 17.4), 4.5 ± 4.6 years (median: 2.6; minimum 0.25 to maximum 18), and 4.7 ± 4.4 years (median: 3.5; minimum 0.25 to maximum 17.0), respectively. While 236 of the patients (76.1%) who took part in the study presented with complaints such as fever, cough, seizures, etc., the remaining 74 (23.9%) were admitted to the hospital for routine controls without a complaint.

The etiology of all neutropenic patients and their age distribution according to the causes of neutropenia is shown in Table 1. In addition, features of groups 1, 1R, 1P, and 2 are shown and compared in Table 2. In this comparison, no statistical significance was observed with regard to sex and ANeuA levels between groups. Yet, with regard to age, group 2 was significantly older ($P > 0.018$) than group 1, but no statistical difference was observed between other groups.

DISCUSSION

Neutropenia may develop due to intrinsic or extrinsic causes in neutrophils and their precursor cells. In pediatric practice, extrinsic causes such as infection, drugs, and bone

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TABLE 1. Causes of Neutropenia and the Age Distribution of Patients

Causes of Neutropenia	Age, Mean ± SD (Median; Minimum-Maximum) (y)	Patient Number, Frequencies (%)
Infection (HIV, EBV, influenza A/B, CMV, RSV, parvovirus, coronavirus)*	3.7 ± 3.5 (2.5; 0.25-17)	140 (45.6)
Drugs	6.9 ± 5.0 (6.0; 0.4-17.4)	71 (19.8)
Immunosuppressive	7.5 ± 5.3 (6.0; 0.4-17.4)	45 (63.4)
Antiepileptic	4.4 ± 3.8 (2.5; 1.0-14)	19 (26.8)
Anti-HIV	11.5 ± 2.3 (11.5; 9.0-14.0)	4 (5.6)
Oral iron chelation/ antihypertensive	8.3 ± 4.0 (9.0; 4.0-12.0)	3 (4.2)
Malignancy	6.3 ± 4.9 (5.0; 0.4-17)	33 (9.2)
Leukemia	5.9 ± 4.5 (5.0; 1.0-16)	18 (54.5)
Solid tumor	6.8 ± 5.5 (6.0; 0.4-17)	15 (45.5)
Metabolic disease	6.7 ± 4.4 (5.0; 0.8-16)	30 (8.4)
Organic acidemia	6.0 ± 4.7 (4.0; 0.8 -15)	13 (47)
Lysosomal storage disease	7.5 ± 4.5 (8.5; 1.1-13)	8 (27)
Mitochondrial disease	6.1 ± 3.6 (4.0; 3.3-12)	5 (13)
Glycogen storage disease	8.5 ± 5.5 (7.0; 3.8-16)	4 (13)
Sequestration	10.4 ± 4.7 (9.0; 2.1-18)	26 (8.5)
Bone marrow failure	6.8 ± 5.1 (5.0; 0.4-17.4)	54 (15.1)
Congenital	8.3 ± 5.2 (9.0; 0.9-17.4)	10 (22.8)
Acquired	6.4 ± 5.0 (4.5; 0.4-17)	44 (77.2)
İmmune deficiency	7.3 ± 5.1 (6.0; 0.4-18)	61 (17.0)
Congenital	6.4 ± 5.1 (4.0; 0.9-18)	15 (21.5)
Acquired	7.6 ± 5.2 (6.0; 0.4-17)	46 (78.5)
Congenital neutropenia	5.3 ± 4.5 (3.5; 0.3-16)	15 (4.2)
Severe congenital neutropenia	5.6 ± 4.5 (4.8; 0.3-16)	14 (98.6)
Cyclic neutropenia	1.1	1 (1.4)
Chronic idiopathic neutropenia	2.9 ± 3.4 (1.6; 0.7-11)	12 (3.9)
Nutritional deficiency	9.1 ± 5.6 (10.5; 1.3-14)	4 (1.3)
Secondary autoimmune neutropenia	14 ± 2.8 (14; 12-16)	2 (0.7)
Evans syndrome	12	1 (50)
Systemic lupus erythematous	16	1 (50)

*Infection-related group for whom the microbiologic agent was determined.

CMV indicates cytomegalovirus; EBV, Epstein-Barr virus; HIV, human immunodeficiency virus; RSV, respiratory syncytial virus.

marrow infiltration are more frequent. Yet, ethnicity appears as another dimension affecting the neutrophil counts.[1,2] During our literature review, we have not encountered an extensive study on the etiology of neutropenia in Turkey. In a single-centered study by Yilmaz and Ritchey conducted between the years 2000 and 2004, 45 patients within the age range of 3 months to 17 years who had severe neutropenia were evaluated. Among them, 31 idiopathic, 6 cyclic, 3 familial, 3 congenital, and 2 autoimmune neutropenia cases were detected.[3] In a retrospective study that Lindqvist et al[4] conducted, among 71 patients, 20 infection-related, 19 chronic idiopathic, 15 autoimmune, 9 ethnic, 5 congenital, and 3 drug-related cases were reported. Another study conducted by Angelino et al[5] evaluated 104 patients with neutropenia, whereas 38 of them were acute, the rest of them were chronic. Among the acute cases, 68.4% were postinfectious, 26.3% were idiopathic, and 5.3% were drug-induced; and, among chronic cases, half were chronic idiopathic, 33.3% were autoimmune, 9% were congenital, 3% were benign familial, 3% were postinfectious, and 1.5% were drug-induced. Likewise, in our study, infection was detected as the most frequent cause in the etiology of acute neutropenia, although the incidence of chronic neutropenia was less frequent. Viral causes had usually been detected as the reasons for infection-related neutropenia.[6,7] In a study, in almost half of the 55 pediatric patients who were between 1 month and 12 years of age, the infection's cause was found to be human herpesvirus 6, enterovirus, influenza A, parvovirus, Epstein-Barr virus (EBV), and adenovirus.[6] In another study, infection was detected in almost half of the 67 neutropenic pediatric patients, whereas, in 70% of them, viral factors were also detected.[7] Furthermore, in their study, Green et al detected mild neutropenia in 10% of 58 patients hospitalized for rotavirus gastroenteritis. They noted that all of these patients were African Americans; nevertheless, the effect of ethnicity was not evaluated due to the limited number of cases.[8] In our study, there were 4 cases of human immunodeficiency virus, 2 of influenza A, and 2 of EBV, and 1 each of influenza B, coronavirus, parvovirus, respiratory syncytial virus, and cytomegalovirus; these were identified as the agents for infection among 13 patients. While 9 of the patients with neutropenia recovered, in the rest of the cases with EBV, parvovirus, coronavirus, or influenza B, the neutropenia was observed to be remaining.

As we conducted our study in a tertiary center, we consider that relatively higher rates of patients with metabolic diseases, immune deficiencies, and congenital neutropenia could have been detected.

The use of anti-inflammatory, antipsychotics, antiepileptic, antithyroid, cardiovascular system drugs and antibiotics and immunosuppressive drugs may cause drug-induced neutropenia. Although its mechanisms are indefinite, it is claimed to be dependent on toxic depression or peripheral destruction of neutrophils.[1,2,9–15] In our study, patients receiving chemotherapy were the highest group (47.9%), and patients using antiepileptic drugs (valproic acid, carbamazepine, or multiple antiepileptic drugs) were the second common group (26.8%) in the drug-related neutropenia category.

Another condition we had evaluated in classification was the development of neutropenia depending on autoimmunity. Primary autoimmune neutropenia occurs due to the destruction of neutrophils by ANeuA. Lucas and colleagues claimed that the difficulty in diagnosis is based on the difficulty in ANeuA detection, and by the insufficiency in the number of specialized laboratories testing antibodies.[16] The combination of granulocyte immunofluorescence test and granulocyte agglutination test or monoclonal antibody-specific immobilization of granulocyte antigen methods may be more sensitive and specific in antibody detection.[17] Yet, these methods are also expensive and difficult to perform. The GIIFT method may also be useful for ANeuA detection,[18–20] and, in a study conducted by Sella and colleagues, a modified GIIFT method was used, and it was declared that the positive predictive value of this test was 91.8%.[21] In this modified method, the patient's serum is incubated with normal neutrophils, and the autoantibodies are later detected using labeled secondary anti-human immunoglobulin antibodies. We also used the GIIFT method for investigating the antibodies in our study population. However, we could not find out statistically

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TABLE 2. Comparison of Sex, Age, Neutrophil Counts, Age of Neutrophil Groups, and ANeuA Levels Between Groups

	Group 1					
		Subgroups				
	All (n = 108)	Group 1R (n = 96)	Group 1P (n = 12)	*P**	Group 2 (n = 39)	*P*†
Female/male	52/56	45/51	7/5	0.547/0.329	27/12	0.058/0.055
Age (y)						
Mean ± SD	3.6 ± 3.5	3.7 ± 3.5	2.9 ± 3.4	0.302	5.3 ± 4.5	0.018
(median; minimum-maximum)	(2.2; 0.25-15)	(2.5; 0.25-15)	(1.6; 0.7-11)		(4.0; 0.5-18)	
Neutrophil counts						
Mean ± SD	977 ± 352	1038 ± 310	491 ± 293	< 0.001		< 0.001
(median; minimum-maximum)	(1000; 100-1400)	(1100; 100-1400)	(450; 200-1200)			
Neutrophil groups, n (%)						
Mild	62	61 (98.3)	1 (1.7)	< 0.001		—
Moderate	34	29 (85.3)	5 (14.7)			
Severe	12	6 (50)	6 (50)			
Age of neutrophil groups (y)						
Mean ± SD (median; minimum-maximum)						
Mild	4.6 ± 3.6 (3.5; 1-15)	4.6 ± 3.6 (3.5; 1-15)	9.0	0.179		—
Moderate	2.4 ± 3.0 (1.0; 0.25-14)	2.6 ± 3.2 (1.0; 0.25-14)	1.3 ± 0.6 (1.0; 0.9-2.0)	0.962		—
Severe	2.0 ± 3.0 (1.0; 0.25-11)	0.8 ± 0.4 (0.8; 0.25-1.3)	3.2 ± 4.0 (1.7; 0.7-11)	0.065		—
Neutrophil antibody levels (%)						
Mean ± SD (median; minimum-maximum)	6.7 ± 9.4 (3.4; 0.3-65)	7.0 ± 9.8 (3.4; 0.3-65)	3.2 ± 2.5 (2.4; 0.5-8.6)	0.120	3.7 ± 3.4 (3.0; 0.1-17)	0.088

*Comparison of sex, age, neutrophil counts, age of neutrophil groups, and ANeuA levels between groups 1R and 1P.
†Comparison of sex, age, neutrophil counts, and ANeuA levels between groups 1 and 2.
ANeuA indicates anti-neutrophil antibody.

significant differences in the comparison of AneuA levels between groups. We argue that this may be due to (i) the lack of ANeuA production in these patients, (ii) the insufficiency in determination, (iii) the insufficiency in the number of patients, and/or (iv) the probable transformation of antibodies into a negative status. By collecting serum samples several times and repeating antibody detections for 4 to 6 months or even more, the antibodies that could not be detected initially, may also be detected thereafter.[19–22] Therefore, ANeuA detection was repeated every 3 weeks until the twelfth week, but, nevertheless, as our study was cross-sectional and was limited to 3 months' duration, longer term antibody detection could not be performed.

The main limitation of our study was that it was cross-sectional, and it had been time-consuming to reach the patients and their records on the basis of laboratory data. Because of the multicausality of neutropenia's development, we had difficulty in forming homogenous groups. ANeuA detection is a test, which is expensive and of a highly variable nature, depending on numerous factors. Therefore, false-negative and positive results may cause confusion in the diagnosis. Besides, as the control group was formed among the children referred to our healthy and adolescent outpatient clinics during this cross-sectional period, who also agreed to participate in the study, a slight difference occurred between the mean ages of both groups.

To conclude, there may be many causes of neutropenia, and autoimmune mechanisms may be responsible for the development of neutropenia, even though ANeuA may not always be detected. In contrast, the ANeuA detection in neutropenic patients provided insufficient insight into the etiology of neutropenia and clinical progress in our study. We argue that it might not be necessary for routine use, but yet the need for extensive research in the field of etiology remains.

ACKNOWLEDGMENT

The authors thank Professor Dr Firdevs Bas for her valuable contribution to the statistical analysis carried out in this study.

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