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# Flow Cytometry In The Differential Diagnosıs Of Neutropenic Patients

# Nötropenik Hastalarda Akım Sitometrinin Ayırıcı Tanıdaki Yeri

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## Abstract

**Introduction:** Although immuno-phenotyping with flow cytometry is mainly used in the diagnosis of hematological neoplasia, it also provides important contributions to revealing changes in the immune system. Flow cytometric studies are also frequently performed in neutropenic patients, as changes in neutrophil count may occur in relation to hematological neoplasms and immune system disorders.
**Objective:** Within the scope of this study, we aimed to elucidate the expressions of lymphocyte, granulocyte, and monocyte subtypes and myelomonocytic antigens on monocytes and neutrophils in neutropenia caused by different reasons and compare them with the results of healthy volunteers.
**Method:** A total of 24 patients and 13 age-and gender-matched healthy volunteers who applied to our institution have been enrolled in this study. The patient group consisted of individuals with isolated neutropenia. Examination findings, lymph node size, spleen size, and infection findings,and the laboratory parameters, CRP level, and pathological findings in the peripheral smear were recorded. Total blood obtained from the patients has been processed with flow cytometry.
**Results:** Neutrophil/lymphocyte (N/L) MFI ratio was found to be higher in the patient group than in the control group (p=0.022).A positive correlation was found between leukocyte counts and the percentage of atypical monocyte expressing CD16 (p=0.033). A negative correlation was found between platelet counts and N/L mean fluorescence intensity ratio (p=0.411) and between the platelet count and the percentage of CD11b on neutrophils (p=0.026). The percentage of neutrophils determined on leukocytes and the percentage of natural killer cells (CD30/CD56+) was statistically significantly lower in the patient group (p=0.008, p=0.001 respectively).
**Conclusion:** Regarding the results of this study one could say that clinically significant changes have been observed in lymphocyte and monocyte subtypes of patients with isolated neutropenia. Granulation was found in the ratio of N/L side scatter, mostly related to neutrophils.

**Keywords:** Neutropenia, Lymphocyte, Monocyte, Flow Cytometry, Myelomonocytic Antigens.

## Özet

**Giriş:** Akış sitometrisi ile immüno-fenotipleme esas olarak hematolojik neoplazilerin tanısında kullanılsa da immün sistemdeki değişikliklerin ortaya konmasında da önemli katkılar sağlar. Hematolojik neoplazmalar ve bağışıklık sistemi bozuklukları ile ilişkili olarak nötrofil sayısında değişiklikler meydana gelebileceğinden, nötropenik hastalarda da akım sitometri çalışmaları yapılmaktadır.
**Amaç:** Bu çalışma kapsamında, farklı nedenlerle oluşan nötropenide lenfosit, granülosit ve monosit alt tipleri ile miyelo-monositik antijenlerin monositler ve nötrofiller üzerindeki ekspresyonlarının hastalar ve sağlıklı gönüllülerin sonuçları ile karşılaştırılması amaçlandı.
**Yöntem:** Bu çalışmaya kurumumuza başvuran toplam 24 hasta ve yaş ve cinsiyet uyumlu 13 sağlıklı gönüllü alındı. Hasta grubu izole nötropenisi bulunan bireylerden oluştu. Muayene bulguları, lenf nodu boyutu, dalak boyutu ve enfeksiyon bulguları, laboratuvar parametreleri, CRP düzeyi ve periferik yaymadaki patolojik bulgular kaydedildi. Hastalardan alınan toplam kan, akım sitometrisi ile işlendi.
**Bulgular:** Nötrofil/lenfosit (N/L)ortalama floresan yoğunluk oranı hasta grubunda kontrol grubuna göre yüksek bulundu (p=0.022). Lökosit sayısı ile CD16 ifade eden atipik monosit yüzdesi arasında pozitif korelasyon bulundu (p=0.033). Trombosit sayısı ile N/L ortalama floresan yoğunluk oranı arasında (p=0.411) ve trombosit

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sayısı ile nötrofiller üzerindeki CD11b yüzdesi arasında (p=0.026) negatif bir korelasyon bulundu. Lökositlerde saptanan nötrofil yüzdesi ve doğal öldürücü hücre yüzdesi (CD30/CD56+) hasta grubunda istatistiksel olarak anlamlı derecede düşüktü (sırasıyla p=0.008, p=0.001).
**Sonuç:** Bu çalışmanın sonuçlarına bakıldığında, izole nötropenili hastaların lenfosit ve monosit alt tiplerinde klinik olarak anlamlı değişiklikler gözlendiği söylenebilir. Çoğunlukla nötrofillerle ilgili olan N/L yan saçılma oranında granülasyon bulundu.

**Anahtar Kelimeler:** Nötropeni, Lenfosit, Monosit, Akış Sitometrisi, Miyelomonositik Antijenler.

## INTRODUCTION

Neutrophils, or segmented leukocytes, develop from stem cells in the bone marrow. They are phagocytosing cells that play a role in acute inflammation and host defense against bacterial infections. Every day, 1 – 1.5 x 109/kg neutrophils are produced in the human body, and 60 – 70% of them are in the storage pool in the bone marrow while only 2 – 5% enter the circulation. Some of them settle on the vessel wall (1).

Neutropenia is the term used when the absolute neutrophil count (ANC) falls below 1.500/mm3. The ANC is calculated by multiplying the total leukocyte count by the sum of the neutrophil and rod count percentages in the blood. If the neutrophil count is below 1.000 – 1.500/mm3, it is classified as mild, below 500 – 1.000/$mm^3$ as moderate, <500/mm3 as severe, and <200/$mm^3$ as very severe (2).

Leukopenia is defined as a decrease in the absolute leukocyte count below 4.000/$mm^3$, and neutropenia as a decrease in the absolute neutrophil count below 1.500/$mm^3$. Clinical findings, blood count, peripheral smear, auto-antibodies, viral serology, histo-pathological examination of bone marrow, genetic and molecular studies and other laboratory parameters are used to determine the etiology (3,4).

Neutropenia usually occurs as a side effect of chemotherapy during the treatment of malignant disease. Neutropenia is variable in severity, sometimes enough to require delaying chemotherapy. Sometimes, it may require dose adjustment in later chemotherapy. Different pathogens can cause infectious complications in febrile neutropenia. The pathogens that are often responsible for infections in the early stages are bacteria. Today, the main factors responsible for febrile neutropenia are gram-positive coagulase-negative streptococci, S. Aureus, and enterococcus; gram-negative is E. Coli, Klebsiella spp, Enterobacter spp, and P. aeruginosa. Fungal infections such as candida and aspergillus are observed in later periods when neutropenia is prolonged. Immune neutropenia occurs in the presence of specific anti-neutrophil antibodies. These antibodies mediate splenic sequestration of opsonized cells or complement-mediated neutrophil destruction. Immune neutropenias are similar to immune thrombocytopenic purpura and immune hemolytic anemia. Isolated immune neutropenias are caused by sensitization to neutrophil-specific antigens that are not found in other hematopoietic cells. Human neutrophil-specific antigens have been defined as NA1, NA2, NB1, NC1, ND1, NE1, 9A. Human leukocyte antigens and erythrocyte antigens are also found on the surface of neutrophils. Autoantibodies against leukocyte integrins CD11/CD18 have also been identified in some neutropenic cases (3-5).

The use of Flow Cytometry in current hematological applications is increasing. The reasons behind this increase, is attributed to achieving results in a short time with an easy methodology. Flow Cytometry is highly effective for the recognition of abnormal cells and is mostly used in hematology for diagnosis, classification of diseases such as leukemia/lymphoma/myeloma, and follow-up of treatment (6).

With the Flow Cytometer, a suspended cell or particles are passed through a chamber illuminated by laser light. The signals generated during the passage of cells in front of the light are collected and analyzed. The source of the signals is the physical characteristics of the cell such as size and granularity. There may also be various fluorochromes attached to the cell. Thus, information about various properties of the cell or particle such as immunophenotype, DNA (7).

Although immuno-phenotyping with flow cytometry is mainly used in the diagnosis of hematological neoplasia, it also provides important contributions to revealing changes in the immune system. Flow cytometric studies are also frequently performed in neutropenic patients, as changes in neutrophil count may occur in relation to hematological neoplasms and immune system disorders. But because isolated neutropenias are usually associated with non-malignant causes. The diagnostic contribution of flow cytometry in isolated neutropenia is not well known, except in conditions such as large granular lymphocytosis (LGL), where CD8 T lymphocytes are clonally increased(6-8).

Within the scope of this study, we aimed to elucidate the expressions of lymphocyte, granulocyte, and monocyte subtypes and myelomonocytic antigens on monocytes and neutrophils in neutropenia caused by different reasons and compare them with the results of healthy volunteers.

## METHOD

A total of 24 patients and 13 age-and gender-matched healthy volunteers who applied to our institution have been enrolled in this study. Patient group consisted of individuals with isolated neutropenia.All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Ethics committee approval has been granted from our institutionwith protocol number 2016/86/06/03 and informed consent has been obtained from all participants.

Demographic characteristics, and clinical and laboratory parameters of the patients were recorded. In order to determine the etiology of neutropenia, infection status at the time of admission, recurrent infection history, drug use, alcohol consumption, autoimmune malignant disease history, rheumatological disease history, and family history suggestive of neutropenia were recorded.

In the physical examination findings, especially lymph node size, spleen size, and infection findings were recorded. Among the laboratory parameters, especially the CRP level, pathological findings in the peripheral smear, if any, were recorded. According to the available data and the results of the follow-up during the study, the patient was classified into a certain etiological group.

### Flow Cytometric Evaluation:

Total blood count samples to be used for flow cytometric study were placed in anticoagulant vacutainer tubes (BD Vacutainer K 3E) containing 2 ml 7.5% Ethylenediamine-Tetraacetylacid (EDTA). All blood samples were processed within 4 hours at the latest. The immuno-phenotyping panel to be analyzed and the antibody mixture to be used in the panel was put into two separate tubes.

1st tube:

CD11b-F/CD1b-PE/CD13-ECD/CD15-PC5/CD14-PB/CD45-KO

2nd tube:

TCR8-F/CD10-PE/CD19-ECD/CD4-PC5/CD56-PC7/CD3-APC/CD8-PB/CD45-KO

The amount of each antibody in the tube was determined by taking into account the previous optimization studies and 100 ml of blood sample was added to the tubes in which the antibody mixture was placed. Tubes were then vortexed and incubated in the dark for 15 minutes. After incubation, 500 ml of erythrocyte lysis solution (Optilyze C) was added to the tubes. It was again incubated for 10 minutes at room temperature and in the dark. Then, 500 µL of PBS (phosphate-buffered saline) was added to the tubes and vortexed. It was incubated at room temperature for 10 minutes in the dark and was centrifuged at 300xg for 5 minutes. Following this, 2 mL of PBS was added and vortexed, centrifuged again at 300 xg for 5 minutes, and the supernatant was discarded. Finally, 500 µL of PBS/formaldehyde was added to the tubes again and stored at +4°C until they were studied.

Samples prepared as above were analyzed with an 8-color flow cytometer (Beckman Coulter Navios, USA). The analysis of the obtained data was done with the Kaluza analysis program. Neutrophils and monocytes were gated separately using the CD45-SS (Side scatter)histogram. The expression rates and mean fluorescence intensity(MFI) of the antigens in the panel on neutrophils and monocytes were measured. CD34-SS histogram was created to determine the precursor cell ratios in peripheral blood. CD34-positive cells with low SS were detected as precursor cells. Antigen expressions of the cells and SS parameters showing their granularity were also measured. Neutrophil SS and lymphocyte SS were measured separately and compared to each other (Neutrophil SS/lymphocyte SS). Lymphocytes were selected according to CD45 expression and side scatter characteristics in the CD45-SS histogram, among the antibody combinations in the first panel, and the percentage of lymphocytes in total leukocytes was determined. The percentage of CD3-positive T lymphocytes, CD19-positive B lymphocytes, and CD3 negative/CD56-positive natural killer (NK) cells were determined by taking the gate over these lymphocytes. CD4 positive T helper and CD8 positive T suppressor lymphocyte ratio was determined on CD3 positive cells.

The CD14-positive monocyte ratio was determined by creating a CD14-SS histogram from the antibody combinations in the second panel. The CD16-expressing monocyte sub-population (non-classical monocytes) of CD14-positive monocytes was calculated from the CD14/CD16 histogram. The percentage of neutrophils in leukocytes was determined by gated the cluster of cells with moderate CD45 expression and high SS in the CD45-SS histogram. Expression intensity expressed as CD10, CD11b, CD13, CD15, and CD16 expression percentages and mean fluorescence intensity(MFI) on the separately determined monocyte and neutrophil populations was calculated by introducing these populations to the generated single-parameter histograms.

## Statistical Analysis

The data obtained in the study were analyzed with the SPSS 18.0 (Statistical Package for Social Sciences) package program. Mean and standard deviation values are given as descriptive statistics. Percentage (%) distributions were used for categorical variables. Chi-square testwas utilized for intergroup comparisons of categorical variables, and Mann Whitney U test, was used for comparisons of continuous variables.The relationship between the variables was evaluated viaSpearman Correlation analysis. The results obtained were evaluated at a 95% confidence interval($p<0.05$) significance level.

## RESULTS

A total of 24 patients with isolated neutropenia and 13 healthy volunteers have been enrolled in this research. The gender distribution of the patient group was 22 females and 2 males. When comparing the groups in terms of age and gender, the mean age of the patient group was 43.2±17.26 years, while the mean age of the control group was 36.9±10.17 years. There was no statistically significant difference between the two groups in terms of age and gender (p=0.381 and p=0.099, respectively).

The patients were divided into 6 etiological groups according to their anamnesis, clinical and laboratory findings and patients who could not be classified into a group according to these data were accepted as unclassified in a 7th group. The underlying reasons could be elaborated as:drug-induced neutropenia (n=12), autoimmune disease associated neutropenia (n=4), 2 infection associated neutropenia (n=2), cyclic neutropenia (n=1), chronic benign neutropenia (n=2), chronic idiopathic severe neutropenia (n=1), and 3 were unclassifiable.

There was no statistically significant difference between the control and patient groups in terms of hemoglobin (Hb), hematocrit (Hct), and platelet counts. As expected, total leukocyte and neutrophil counts were statistically significantly lower in the patient group than in the control group (p<0.0001) (Table 1).

**Table 1.** Comparison Of Blood Count Values Between Patient And Control Group

| | Group 1 (n=24) (Median ± SS) | Group2 (n=13) (Median ± SS) | p value |
|---|---|---|---|
| **Hb*(gr/dl)*** | 12.80±0.95 | 13.53±1.14 | 0 .058 |
| **Hct *(%)*** | 37.92±2.53 | 39.61±3.14 | 0 .070 |
| **WBC *($mm^3$)*** | 3.26±0.709 | 6.60±1.05 | ***< 0 .0001*** |
| **Neutrophil *($mm^3$)*** | 1.33±0.46 | 3.91±1 | ***< 0 .0001*** |
| **Platelets** *($mm^3$)* | 220.000±57.84 | 270.000±51.46 | ***0,022*** |

Hb: Hemoglobin, Htc: Hematocrit, Wbc: Total Leukocyte, Neu: Neutrophil**.**

The percentage of lymphocytes determined over all leukocytes by flow cytometry was found to be higher in the patient group compared to the control group (p=0.007). The percentage of natural killer (NK) cells (CD3-/CD56+) was also lower in the patient group (p=0.001). No statistically significant difference has been achieved between the patient group and the control group in terms of other lymphocyte subgroups. Neutrophil/lymphocyte (N/L) SS MFI ratio was higher in the patient group than in the control group (p=0.022) (Table 2).

The percentage of monocytes determined on all leukocytes by flow cytometry was higher in the patient group than in the control group (p=0.031). When monocyte subtypes (classical and non-classical monocytes) were evaluated between the groups, although CD16 positive non-classical monocytes were high in the patient group, this did not reach a statistical significance. The percentage of CD13 expression on monocytes was found to be lower in the patient group compared to the control group (p=0.007). The expression percentages and severity of other myelomonocytic antigens whose expression on monocytes were evaluated were not statistically significantly different between the groups.

**Table 2.** Peripheral Blood Flow Cytometry Results Of Patients According To Groups

| | Group 1 (n=24) (Median ± SS) | Group2 (n=13) (Median ± SS) | p-value |
|---|---|---|---|
| Lymphocyte ratio % | 38.3±11.9 | 29.4±6.08 | **0.007** |
| Lymphocyte CD3 % | 76.2±7.82 | 73.7±7.315 | 0.324 |
| Lymphocyte CD19 % | 9.81±4.57 | 9.89±3.43 | 0.567 |
| Lymphocyte NK % | 7.21±4.91 | 15.2 ±7.89 | **<0.001** |
| Neutrophil/Lymphocyte SS MFI | 6.10±0.66 | 5.62 ±0.46 | **0.022** |
| CD3 –CD4% | 53.7±15.4 | 59.8±11.2 | 0.131 |
| CD3 –CD8% | 38.6±9.79 | 33.8±10.55 | 0.162 |
| CD3 –TCR8D % | 9.96±12.4 | 7.2±4.39 | 0.975 |
| Monocyte ratio % | 7.78±3.1 | 5.93 ±1.35 | **0.031** |
| CD16+ monocyte ratio | 19.2±20.6 | 12.4 ±6.99 | 0.390 |
| Monocyte CD16 MFI | 11.6±7.02 | 8.33±4.08 | 0.316 |
| Monocyte CD11b% | 98.6±1.74 | 86.9±29.8 | 0.381 |
| Monocyte CD11b MFI | 15.8±6.74 | 18.5±14.1 | 0.861 |
| Monocyte CD15% | 67.2±15.9 | 59.6±20 | 0.245 |
| Monocyte CD15 MFI | 3.18±1.29 | 4.08±3.21 | 0.445 |
| Monocyte CD13% | 18.9±20.3 | 42.8±29.8 | **0.007** |
| Monocyte CD13 MFI | 4.92±3.64 | 4.99±2.47 | 0.426 |
| Neutrophil ratio % | 44.5±15.8 | 59.3±9.7 | **0.008** |
| Neutrophil CD16% | 98±1.71 | 97.4±2.9 | 0.726 |
| Neutrophil CD16 MFI | 346,5±93,2 | 420.1±149.6 | 0.127 |
| Neutrophil CD11b% | 99,5±0,34 | 99.5± 0.82 | 0.438 |
| Neutrophil CD11b MFI | 15,88±5,80 | 15.6±5.14 | 0.911 |
| Neutrophil CD15 % | 98,4±5,42 | 99.5±0.27 | 0.910 |
| Neutrophil CD15 MFI | 107,8±21,2 | 115.2±47.8 | 0.464 |
| Neutrophil CD13% | 88,6±19,7 | 93.5±4.8 | 0.656 |
| Neutrophil CD13 MFI | 14,9±23,5 | 14.3±12.8 | 0.092 |

The percentage of neutrophils determined on all leukocytes by flow cytometry was statistically significantly lower in the patient group than in the control group (p=0.008). Expression percentages and severity of myelomonocytic antigens (CD10, CD11b, CD13, CD15, CD16) on neutrophils were not statistically different between the groups.

The percentage and severity of expression of myelomonocytic markers on monocytes and granulocytes were compared between groups.

## Correlation Analysis

The relationships between the patients' blood count values, CRP and flow cytometric data were evaluated by Spearman correlation analysis. As a result of the correlation, a positive correlation has been achieved between the leukocyte count and the percentage of non-classical monocyte expressing CD16 (p=0.033).On the other hand, a positive correlation has been observed between CRP and CD16 MFI (p=0.008) and CD13 MFI (p=0.048)in monocytes. A negative correlation was found between platelet counts and N/L SS MFI ratio (p=0.411), and a negative correlation was found between the platelet count and the percentage of CD11b on neutrophils (p=0.026).Additionally,a negative correlation has been detected between CRP and CD13 percentage and expression intensity on neutrophils (p=0.03, p=0.021, respectively) (Table 3 & Table 4).

**Table 3.** Significant Correlations Between Patient Blood Count Parameters CRP And Flow Cytometric Data

| | M-CD16% | N/L SS MFI | N-CD 11b% | NK % | M-CD16 MFI | M-CD13 MFI | N-CD13 | N-CD13 MFI |
|---|---|---|---|---|---|---|---|---|
| **Leukocyte count** | r =0.436 p=0.033 | | - | - | - | - | - | - |
| **Platelet count** | - | r=-0.411 p=0.046 | *r=-0.454 p=0.026* | - | - | - | - | - |
| **CRP** | - | - | - | - | *r=0.565 p=0.008* | *r=0.436 p=0.048* | *r=-0.475 p=0.03* | *r=-0.500 p=0.021* |

**Table 4.** Percentage And İntensities Of Expression Of Lymphocytes, Monocyte Subtypes, And Myelomonocytic Antigens On Monocytes And Neutrophils İn Etiological

| | Hgb | WBC | Neu | Crp | Lenfosit oranı % | Lenfosit CD3 % | Lenfosit CD19% | Lenfosi tNK % |
|---|---|---|---|---|---|---|---|---|
| **Drug-induced neutropenia** | 12.7±0.95 | 3.36±0.75 | 1.4±0.529 | 2.31±2.5 | 34.62±10.9 | 74.7±7.2 | 11,4±5,1 | 7.21± 2.77 |
| **Autoimmune neutropenia** | 12.3±0.67 | 3.05±0.88 | 1.25±0.15 | 0.86±0.25 | 38.7±8.69 | 73.6±7.7 | 10,3± 5,22 | 7.9± 4.8 |
| **Chronic benign neutropenia** | 14.2±0.98 | 4.1 | 1.57±0.18 | 11.4±13.57 | 49.9±0.36 | 84.2±1.9 | 6,13±0,72 | 5.29± 2.23 |
| **Chronic idiopathic severe neutropenia** | 14.1 | 2.4 | 0.22 | 33 | 61.1 | 79.63 | 9,64 | 1.86 |
| **Unclassified** | 12.6±0.98 | 3.05±0.3 | 1.38±0.26 | 5±5.37 | 26.9±16.7 | 86.5±0.19 | 8,17±0,41 | 4.28± 1.2 |
| **Cyclic neutropenia** | 12.3 | 2.8 | 1.04 | 0.5 | 40.13 | 83.7 | 5,27 | 7.22 |
| **Infection** | 12.5±0.7 | 3.1±0.14 | 1.24±0.21 | 5.8±0.21 | 47.7±6.97 | 67.4±5.4 | 6.54±1.89 | 13.29± 16.27 |
| | **CD3 – CD4%** | **CD3CD8 %** | **CD3-TCR8D%** | **Monocyte Ratio %** | **Monocyte CD16%** | **Neutrophil ratio** | **G/L MFI** | |
| **Drug-induced neutropenia** | 57.8±15.2 | 37.5± 10.27 | 6.93±4.41 | 6.97±1.88 | 25.7±27.9 | 49.7±13.1 | 6.16±0.6 | |
| **Autoimmune neutropenia** | 44.1±13.2 | 47.6± 12.71 | 7.76±6.56 | 7.46±2.46 | 12.27±6.94 | 42.38±17.21 | 5.88±0.8 | |
| **Chronic neutropenia** | 64.4 ± 4 | 35.7± 3.98 | 8.83±7.74 | 6.91±0.91 | 13.8±5.65 | 31±0.38 | 5.7±0.14 | |
| **Chronic Severe neutropenia** | 48.65 | 39.6 | 11.43 | 13.7 | 6.9 | 14.6 | 7.3 | |
| **Unclassified** | 57.98±8.5 | 39.1±4.53 | 5.09±2.95 | 9.04±0.08 | 11.8±2.05 | 57.3± 21.1 | 5.49±0.16 | |
| **Cyclic neutropenia** | 44.9 | 37.2 | 19.45 | 7.88 | 13.5 | 46.5 | 6.4 | |
| **Infection-induced neutropenia** | 40.5± 32.2 | 29.8±3.09 | 33.1±42.5 | 9.9±10.2 | 16.5±1.01 | 32.4± 8.45 | 6.43±0.9 | |

## DISCUSSION

In the current study, changes in lymphocyte, monocyte and granulocyte subtypes in isolated neutropenia due to various causes were evaluated and the expression of myelomonocytic antigens on monocytes and neutrophils was measured. It was shown that natural killer cells were decreased and monocytes were increased in neutropenic patients.The N/L MFI value

obtained by dividing the neutrophil side scatter fluorescence intensityincreased in the patient group.In other words, the granularity of neutrophils was higher than normal in isolated neutropenic patients. In our study, it was also denoted that CD13 expression on monocytes was decreased compared to healthy volunteers.

Half of all cases were considered as drug-induced neutropenia. The drugs considered in the etiology were common, selective serotonin reuptake inhibitors (SSRI), serotonin nor-adrenaline reuptake inhibitors (SNRI), carbamazepine, and isotretinoin.

Although a compensatory increase in monocyte count in neutropenic patients is a common clinical observation, no research has been published on this topic in previous literature. Additionally, no study has been published showing that NK cells decrease in neutropenias. Large granular lymphocytic (LGL) leukemia is a hematological neoplasia that may present with neutropenia, a subgroup associated with NKs (9). This leukemia is a neoplasia in which cytotoxic T lymphocytes or NK cells proliferate clonally (10,11). Under normal conditions, LGLs are activated and increased significantly upon encountering antigens, then undergo apoptosis. Thesecells persist in LGL leukemia (12) but are rare lympho-proliferative disorders (2% – 6%) (10). In slow-progressing leukemias, T-cell LGL constitutes up to 85% of all LGL cases. NK-cell LGLs are divided into two subgroups in the classification of the World Health Organization as chronic NK-cell lymphoproliferative disorder (CLPD-NK) and NK-cell leukemia (13). The purpose of this distinction is to distinguish the more aggressive NK-cell leukemia from CLPD-NK, which progresses very slowly. T-LGL, slow-progressing CLPD-NK, constitutes up to 5% of all LGLs (9). Patients with LGL often present with splenomegaly, neutropenia, anemia, lymphocytosis, and some auto-immune conditions (most commonly rheumatoid arthritis). Patients presenting with these findings should definitely be evaluated for LGL leukemia (14). Although some of the patients included in our study had auto-immune diseases, there were no clinical and laboratory findings such as splenomegaly and absolute lymphocytosis suggestive of LGL. T-LGL cells with mature post-thymic immunophenotype (TCRαβ+, CD3+, CD4−, CD5dim, CD8+, CD27−, CD28−, CD45R0−, CD57+) (15).

Compared to the control group, the ratio of CD3-/CD56+ NK cells in our cases did not increase, but decreased to the full course. Again, when the cases were examined one by one, no significant increase was found in the NK cell ratio in any of the cases. In previous literature it was reported that there may be a decrease in NK cells in some hereditary immune deficiencies. In our case, which we considered as chronic idiopathic severe neutropenia, the rate of NK cells was very low (1.86%), and it was thought that there might be a hereditary immune deficiency in our cases (16).

Although CD3/TCR gamma/delta lymphocytes were not statistically different between the patient group and the control group, they were quite high in infection-related neutropenia. These lymphocytes, which make up a very small part of the lymphocytes in the peripheral blood, are mostly involved in innate immunity, are preferably distributed in peripheral tissues and have regenerative functions (17). It has been reported that these lymphocytes proliferate polyclonal in neutropenia and thrombocytopenias (18). TCR gamma/delta lymphocyte increase has been observed in some sub-etiological groups in our research, indicatingthe role of neutropenia that occurr for various reasons should be investigated.

The occurrence of the myelodysplastic syndrome as a single cytopenia other than anemia is rare, however, MDS should be considered in single cytopenias other than an anemia in adult patients. In the WHO 2016 classification, a subgroup was formed as “MDS-single series dysplasia” and there is also a subtype of MDS in this group in which only neutropenia is

present and can be confused with other isolated neutropenia (19). Although it is thought that this MDS subtype can be distinguished from other isolated neutropenia by demonstration of dysplasia and/or cytogenetic disorders, this is not always easy in clinical practice. It has been shown that flow cytometry can contribute to the diagnosis of MDS and has been suggested as an auxiliary criterion in the European Leukemia Net (ELN) 2013 MDS diagnostic guide (20).

As mentioned earlier, the N/L SS fluorescence intensity ratio, which is associated with neutrophils and their predecessor's granulation, has been suggested as an important flow cytometric parameter supporting the diagnosis of MDS (21,22). One of the 4 parameters in the scoring system created by Ogata et al. for the diagnosis of MDS was the N/L SS ratio (69). In our study, the primary purpose of measuring this parameter and myelomonocytic antigens on monocytes and granulocytes was to search for findings suggestive of MDS in our adult patients and to investigate the presence of changes specific to the cause of neutropenia.The reason of high N/L SS ratio has been attributed to an increase in granulation in neutrophils. When the etiological subgroups were examined, a statistically significant increase (in granulation) was caused by the increase in chronic idiopathic severe neutropenia, infection-related neutropenia, and drug-induced neutropenia.

When myelomonocytic antigen expressions on monocytes and neutrophils were evaluated, it was seen that the percentage of CD13 expression on monocytes was decreased compared to the control group, and the expressions of other myelomonocytic antigens in both cell groups were not different from the control group. CD13 is a membrane-bound exonuclease and has recently been shown to play a role in the phagocytosis function of monocytes and macrophages (23). Alhan et al. (2015) stated that, one of the three parameters in flow cytometric scoring to determine the prognosis of MDS was determined as CD13 expression in monocytes (24). For all these reasons, the importance of decreased CD13 expression with the increased monocyte ratio in our patients needs to be investigated further in neurotopenic patients.

Monocytes are divided into classical and non-classical subtypes according to the CD14/CD16 expression pattern. In recent years, a third group, called intermediate monocytes, has been formed among non-classical monocytes (25). Classical monocytes, which make up 90% of monocytes in the peripheral blood, express high levels of CD14 but not CD16 (CD14++/CD16-), intermediate monocytes express relatively high levels of CD14 but low levels of CD16 (CD14+/CD16+), non-classical monocytes, on the other hand, express low levels of CD14 and high levels of CD16 (CD14+/CD16++) (26).

In our study, monocytes were divided into 2 groups according to their CD14/CD16 expression pattern (monocytes with low or high expression of CD16 were considered non-classical monocytes).There was no statistically significant difference between the patient group and the control group in terms of monocyte subtypes, however, non-classical monocytes (CD16+) were higher in the patient group (mean values 19.2% in the patient group, 12.4% in the control group), especially in drug-dependent neutropenia. This difference was more pronounced (25.2%) in induced neutropenia. The main limitation of this research could be attributed to its relatively low number of sample size. Never the less, this study had illuminating insights for clinicinas and we believe that randomized controlled trials incorporating a larger cohort of patients are warranted in order to verify our results.

## CONCLUSION

Regarding the results of this study one could say that clinically significant changes have been observed in lymphocyte and monocyte subtypes of patients with isolated neutropenia. Granulation was found in the ratio of N/L side scatter, mostly related to neutrophils.

## Article Highlights

1. The percentage of lymphocytes determined from all leukocytes was found to be higher in the patient group than in the control group (p=0.007).As expected, the percentage of neutrophils determined on all leukocytes was statistically significantly lower in the patient group than in the control group (p=0.008).

2. The percentage of monocytes determined on all leukocytes by flow cytometry was higher in the patient group than in the control group (p=0.031). The percentage of natural killer (NK) cells (CD30/CD56+) was found to be lower in the patient group than in the control group (p=0.001).

3. Neutrophil/lymphocyte (N/L) MFI ratio was found to be higher in the patient group than in the control group (p=0.022).

4. A positive correlation was found between leukocyte counts and the percentage of atypical monocyte expressing CD16 (p=0.033).A negative correlation was found between platelet counts and N/L MFI ratio (p=0.411) and between the platelet count and the percentage of CD11b on neutrophils (p=0.026).

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## Ethical Declaration

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. Ethics committee approval has been granted from our institution and informed consent has been obtained from all participants.

## Abbreviations

ANC : Absolute Neutrophil Count

CLPD-NK : NK-cell lympho-Proliferative Disorder

CRP : C-Reactive Protein

EDTA : Ethylenediamine-Tetraacetylacid

ELN : European Leukemia Net

FS : Forward Scatter

LGL : Large Granular Lymphocytosis

MDS : Myelodysplastic Syndrome

MFI : Mean Fluorescence İntensity

NK : Natural Killer Cells

NSAI : Nonsteroidal Anti-İnflammatory İnhibitor

PBS : Phosphate-Buffered Saline

SLE : Systemic Lupus Erythematosus

SNRI : Serotonin Nor-Adrenaline Reuptake İnhibitor

SPSS : Statistical Package for Social Sciences

SS : Side Scatter

SSRI : Selective Serotonin Reuptake İnhibitor

WBC : Total Leukocytes

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