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ORIGINAL RESEARCH

IL-6 −572C>G and CARD8 304T>A Genetic Polymorphisms are Associated with the Absolute Neutrophil Count in Patients with Hematological Malignancies Under Chemotherapy: An Application of Multilevel Models to a Preliminary Pharmacogenetic Study

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Purpose: Neutropenia is a common event in patients undergoing cytotoxic chemotherapy for the treatment of a hematological malignancy. Some polymorphisms, as *IL-6* −572C>G (rs1800796), *IL-1β* −31 G>A (rs1143627), and *CARD8* 304T>A (rs2043211), in genes related to the inflammatory process, could affect the level of absolute neutrophil count (ANC) after chemotherapy. Since an efficient inflammatory process enhances neutrophil survival, we hypothesize that these polymorphisms are associated with ANC.

Patients and Methods: We carried out a prospective cohort study in two hospitals in Santiago, Chile. The patients included were adults diagnosed with acute myeloblastic leukemia, acute lymphoblastic leukemia, or non-Hodgkin's lymphoma, undergoing cytotoxic chemotherapy. We use a multilevel linear regression model to test our hypothesis. The best model was selected using the Akaike's information criterion (AIC).

Results: We analyzed 1726 hemograms and ANCs from 172 hospitalizations from 32 patients. The results show that CC and CG genotypes of *IL-6* −572 C>G polymorphism are associated with higher ANCs compared with the GG genotype (Ln (ANC) ~ 0.81 IC95% 0.02–1.55). Similarly, TT and AT genotypes of *CARD8* 304T>A polymorphism were related to higher ANCs compared with AA (Ln (ANC) ~ 0.95 IC95% 0.02–1.82). IL-1β genetic polymorphism had no statistically significant association with ANC.

Conclusion: *IL-6* rs1800796 −572C>G and *CARD8* rs2043211 304T>A polymorphisms are associated with the absolute neutrophil count in patients undergoing cytotoxic chemotherapy for treatment of hematological malignancies. Our findings might be useful to improve the safety of chemotherapy through predictive ANC models.

Keywords: pharmacogenetics, neutropenia, hematological neoplasms, leukemia, lymphoma, interleukin-6, CARD8 protein

Introduction

The primary treatment of hematological malignancies (HM) is cytotoxic chemotherapy, whose goal is to suppress the bone marrow to make the cancer cells disappear or to avoid the formation of new ones. Severe neutropenia, defined as an absolute neutrophil count (ANC) lower than 500 cells/mm^3,[1] is common in these

patients (30% prevalence) and makes them more susceptible to life-threatening infections (11% of mortality).[2,3] The earlier neutropenia starts, and the longer it lasts, the higher the risk.[1,4,5] Currently, it is not possible to predict the value of ANC, so we cannot know the start or length of neutropenia.

Chemotherapy involves inflammation due to cell destruction; neutrophils maturation, release, and survival depend on an efficient response.[6,7] Some proteins involved in the inflammatory response, as Interleukin 6 (IL-6), Interleukin 1β (IL-1β), and caspase recruitment domain family, member 8 (CARD8), had polymorphic variants that can explain the interindividual variability of chemotherapy.

IL-6 participates in the regulation of hematopoiesis, inflammation, and immune response,[8,9] and stimulates the production and mobilization of neutrophils in the bone marrow.[10,11] Moreover, CARD8 is a crucial component in the regulation of nuclear signaling and the activation of IL-1β[12] The reduced expression of IL-1β and IL-1R and the structural alteration of CARD attenuate the immune system through poor neutrophil recruitment.[13]

An essential problem in pharmacogenetic studies, particularly in developing countries, is to reach an appropriate sample size for robust results. However, there are statistical methods that allow making preliminary and well-controlled analyses, whether enough information from every patient is available. Multilevel models (MM) allow us to analyze repeated measures and grouped data in a reduced sample of patients.

We hypothesize that genetic polymorphisms on *IL-6*, *IL-1β*, and *CARD8* are associated with the ANC value in patients with HM receiving cytotoxic drugs using an MM to control by correlation and confounding variables.

Patients and Methods

We carried out a prospective cohort study from October 2017 to November 2018 at the Oncologic Hospital "Fundación Arturo Lopez Pérez" (FALP) and the Clinical Hospital of the University of Chile (HCUCH) in Santiago, Chile.

We included adult patients diagnosed with acute myeloblastic leukemia, acute lymphoblastic leukemia or non-Hodgkin's lymphoma, and had neutropenia after cytotoxic chemotherapy. They were enrolled before the first chemotherapy cycle and followed up during everyone. We excluded patients regularly taking immunosuppressive medication, pregnant women, and patients with a diagnosis of immunodeficiency.

ANC was treated as a continuous variable, and it was obtained from hemograms directly when the laboratory provided it. When not provided, it was calculated using the following formula:[14]

$$ANC = White\ blood\ cells\ count * \left(\frac{(\%polymorphonuclear\ neutrophils + \%bands)}{100}\right)$$

All patients signed a written informed consent and an agreement to participate in this study. The study was carried out following the strict ethical procedures recommended by the Ethics Committee of the Clinical Hospital of the University of Chile (approval received on July 18th, 2018) and the Eastern Metropolitan Health Service (approval received on July 4th, 2017), following the procedures suggested in the Declaration of Helsinki, with Chilean Laws 20.120, 20.584 and 19.628 and with the guidelines of the Good Clinical Practices.

Genotyping Analysis

DNA was isolated from peripheral blood samples using the High Pure PCR Template Preparation Kit (Catalog Number, 11,796,828,001; Roche Diagnostics GmbH, Mannheim, Germany).

IL-6 rs1800796, *IL-1β* rs1143627, and *CARD8* rs2043211 polymorphism were analyzed using TaqMan® SNP Genotyping Assay (Catalog number, 4,362,691; Thermo Fisher Scientific, Waltham, MA, United States) in a Stratagene Mx30d00p real-time PCR system (Agilent Technologies, Santa Clara, CA, United States). Every sample was analyzed in triplicate to ensure reliability (Laboratory Protocol dx.doi.org/10.17504/protocols.io.bfuujnww).

Statistical Analysis

We use a multilevel linear regression model, which allowed us to account for intraindividual variability regarding the chemotherapy scheme and cycle and to use correlated outcome data. The unit of observation was the ANC, not the patient.

Our MM had four levels[15] (Table 1), 1) "Patient," the upper level, where genetic variables were assessed; 2) "Scheme" with eight possibilities, depending on the treatment; 3) "cycle" level and 4) "time" level, where the daily ANC and the administration of Granulocyte Colony-Stimulating Factor (G-CSF) were measured. Moreover,

Table 1 Multilevel Diagram

Name of Level	Subscript to Index the Level	Member Categories in Each Level	Variables Measured on Each Member of the Level
Patient	i = 1, …, 32	ID (1–32)	$Diagnosis_i$, *IL-6* $Genotype_i$, *IL-1β* $Genotype_i$, *CARD8* $Genotype_i$, $Hospital_i$
Scheme	j = 1, …, 9	• Hyper CVAD phase I[a], • Hyper CVAD phase II[b], • 15–30 induction[c], • 15–30 consolidation[d], • 3 + 7 (induction)[e], • Cytarabine (consolidation), • R BAC[f], • FLAG – Ida[g], • ARCHIMBAUD[h]	No variables measured at this level
Cycle	k = 1, …, 6	Cycle (1–6)	No variables measured at this level
Time	t = 0, …, 50	Day (0–50)	ANC_{ijkt}, $G\text{-}CSF_{ijkt}$, $G\text{-}CSFxDay_{ijkt}$, $G\text{-}CSFxHospital_{ijkt}$

Notes: The Multilevel diagram shows the organization of data, the lower level is contained or nested in the upper level, ie, every patient could have different schemes, every scheme could have different cycles, and in every cycle, there are several time measurements. The multilevel diagram also indicates what explanatory variables are measured at each level. [a]Hyper CVAD phase I: cyclophosphamide, vincristine, doxorubicin, dexamethasone. [b]Hyper CVAD phase II: methotrexate, cytarabine. [c]15–30 induction: dexamethasone, vincristine, daunorubicin, L-asparaginase. [d]15–30 consolidation: 6- mercaptopurine, cyclophosphamide, cytarabine, vincristine, L-asparaginase. [e]3 + 7 (induction): cytarabine, daunorubicin. [f]R BAC: rituximab, bendamustine, cytarabine. [g]FLAG – Ida: fludarabine, Ara-C (cytarabine), G-CSF, idarubicin. [h]ARCHIMBAUD: mitoxantrone, cytarabine, etoposide. G-CSFxDay: Interaction between the administration of G-CSF and the time. G-CSFxHospital: Interaction between the administration of G-CSF and the hospital where it was administrated.

Abbreviations: IL6, Interleukin 6; CARD8, caspase recruitment domain family, member 8; ANC, absolute neutrophil count; G-CSF, granulocyte-colony stimulating factor.

Table 2 Absolute Neutrophil Count by Genotype and Therapeutic/Morbid Characteristics

	Total	IL6 GG	IL6 CC/CG	p – value	CARD8 AA	CARD8 TT/TA	p-value
ANC Mean ± SD ANC Median (IQR)	1726.1 ± 90.6 326 (0–1980)	1299.3 ± 175.7 207 (0–1420)	1798.2 + 108.1 407 (0–2184)	0.038[I]*	1424.3 ± 93.1 146.5 (0–1980)	2059.8 ± 155.3 418 (0–1997)	0.001[I]*
Diagnosis, ANC Mean ± SD							
• ALL	1784.5 ± 3431.2	1530.0 ± 355.1	1842.4 ± 154.9	0.404[II]	2207.2 ± 354.4	1660.0 ± 151.8	0.107[II]
• AML	1042.0 ± 2078.9	1139.5 ± 205.4	1015.0 ± 75.2	0.487[II]	889.9 ± 75.1	1270.1 ± 145.1	0.012[II]*
• NHL	3183.1 ± 6112.9	2171.4 ± 459.1	3463.7 ± 396.2	0.103[II]	2382.4 ± 210.1	3749.4 ± 534.8	0.039[II]*
Scheme, ANC Mean ± SD							
• Hyper CVAD phase I[a]	3348.9 ± 7001.1	2497.4 ± 1146.0	3434.4 ± 461.2	0.533[II]	2041.3 ± 502.9	3743.8 ± 538.7	0.096[II]
• Hyper CVAD phase II[b]	2177.7 ± 3887.4	2888.4 ± 647.4	2035.1 ± 199.7	0.109[II]	2358.1 ± 396.7	2114.8 ± 229.7	0.5932[II]
• 15 – 30 induction[c]	450.1 ± 589.0	292.0 ± 64.1	586.0 ± 95.8	0.016[II]*	445.1 ± 146.9	451.7 ± 65.9	0.963[II]
• 15 – 30 consolidation[d]	1436.3 ± 1886.8						
• 3 + 7 (induction)[e]	1095.8 ± 2778.1	1655.5 ± 500.0	894.1 ± 167.6	0.064[II]	633.9 ± 143.3	1796.1 ± 392.3	0.002[II]*
• Cytarabine (consolidation)[f]	1225.9 ± 1852.2	1019.0 ± 192.2	1275.8 ± 102.5	0.263[II]	1216.0 ± 111.8	1239.6 ± 151.4	0.898[II]
• R BAC[g]	2602.3 ± 2255.2	1340.6 ± 202.3	2857.4 ± 243.7	0.007[II]*	2857.4 ± 243.7	1340.6 ± 202.3	0.007[II]*
• FLAG – Ida[h]	585.4 ± 1545.9	372.7 ± 160.4	645.2 ± 166.3	0.404[II]	296.4 ± 116.7	715.5 ± 186.8	0.150[II]

Notes: *p-value <0.05. [I]Tested using the Mann–Whitney test [II]Tested using an independent samples *t*-test. ANC: absolute neutrophil count. ALL: acute lymphoblastic leukemia. AML: acute myeloblastic leukemia. NHL: non-Hodgkin's lymphoma. [a]Hyper CVAD phase I: cyclophosphamide, vincristine, doxorubicin, dexamethasone. [b]Hyper CVAD phase II: methotrexate, cytarabine. [c]15 – 30 induction: dexamethasone, vincristine, daunorubicin, L-asparaginase. [d]15 – 30 consolidation: 6- mercaptopurine, cyclophosphamide, cytarabine, vincristine, L-asparaginase. [e]3 + 7 (induction): cytarabine, daunorubicin. [f]R BAC: rituximab, bendamustine, cytarabine. [g]FLAG – Ida: fludarabine, Ara-C, G-CSF, idarubicin. [h]ARCHIMBAUD: mitoxantrone, cytarabine, etoposide.

Abbreviations: SD, standard deviation; IQR, interquartile range; IL6, interleukin 6; CARD8, caspase recruitment domain family, member 8.

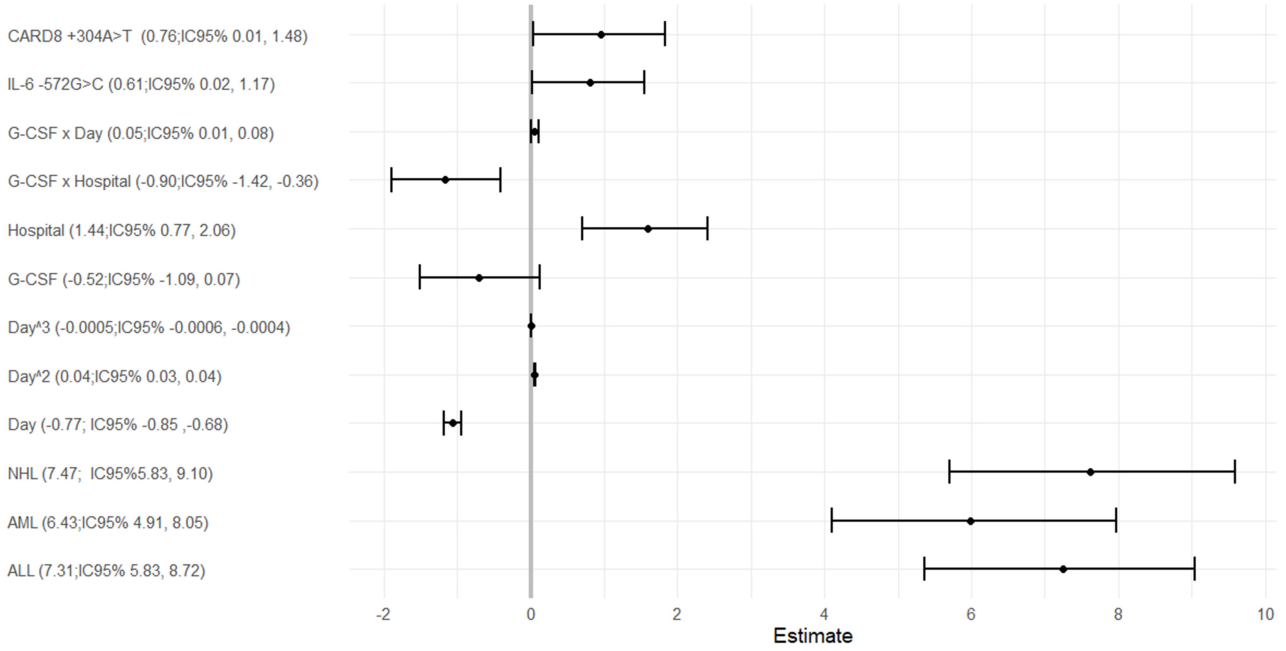

Figure 1 Factors associated with Absolute Neutrophil Count (ANC).
Abbreviations: CARD8, caspase recruitment domain family member 8; IL-6, interleukin 6; G-CSF, granulocyte-colony stimulating factor; ANC, absolute neutrophil count; ALL, acute lymphoblastic leukemia; AML, acute myeloblastic leukemia; NHL, non-Hodgkin's lymphoma; G-CSFxDay, interaction between the administration of G-CSF and the time; G-CSFxHospital, interaction between the administration of G-CSF and the hospital where it was administrated.

we included two interactions: 1) G-CSF/time and 2) administration of G-CSF/hospital.

To improve the fit, we made a logarithmic transformation of the dependent variable. The "0" ANC values were replaced by "1" due to mathematical incompatibility. As this variable has a polynomic behavior, we added the variables Day^2 and Day^3 to the model (other models in Supplementary Table 1). The base model to add other explanatory variables is:

$$\ln(ANC)_{ijkt} = \beta_0 + \beta_{0i} + \beta_{0ij} + \beta_{0ijk} + {}_1Day + {}_2Day^2 + {}_3Day^3 + e_{ijkt}$$

where $\beta_{0i}, \beta_{0ij} and \beta_{0ijk}$ are random intercept effects for "patient," "scheme within patient," and "cycle within scheme within patient," respectively.

The best model was selected using Akaike's Information Criterion (AIC), and variables were added to the model depending on their statistical significance in univariate analysis. The diagnosis was analyzed using the "cell means model." Other categorical variables were analyzed using a reference category.

Analyses were performed using the software R (v3.6.1) and RStudio (v2.1.5001) with the package "lme4"[16]

Results

We analyzed 1726 ANCs from 32 patients. The most common scheme was "Hyper CVAD" (38% of patients in Phase I, and 50% of patients in Phase II). Table 2 shows the ANC values in the sample. Allele frequencies of the studied polymorphisms are shown in Supplementary Table 2.

Figure 1 shows genetic and non-genetic associated factors to ANC (univariate analysis in Supplementary Table 3). For *IL6* −572C>G polymorphism, genotypes CC and CG are associated with higher ANCs compared with the GG genotype (Ln(ANC)~0.81 IC95% 0.02–1.55). Similarly, for *CARD8* 304T>A polymorphism, genotypes TT and AT were related to higher ANCs compared with AA (Ln(ANC)~0.95 IC95% 0.02–1.82). *IL-1β* had no statistically significant association with ANC.

About non-genetic associated factors, we found that, besides time, the HCUCH was related to higher levels of ANC after chemotherapy compared to FALP, but when the patient received G-CSF at HCUCH had lower ANC than FALP. The interaction between time and the use of G-CSF was associated positively with the ANC.

The Ln(ANC) values correlation overtime was strongly determined by the scheme and rather than by the cycle,

with an Intraclass Correlation Coefficient (ICC) for the scheme of 0.4 and not much more due to cycle (see Supplementary Table 1 which shows ICCs).

Discussion

ANC monitoring could improve anticancer treatment by predicting toxicity. Previous predictive models have not included genetic factors, but drug-specific variables.[17] Previous studies have included some drug-related genes and the use of oral mucosal neutrophil count as a way to predict the onset and resolution of neutropenia;[18] in this study, we use drug-independent polymorphisms and non-genetic factors as possible explanations for ANC variability in patients undergoing chemotherapy for HM.

We found that patients carrying C allele of *IL-6* −572 C>G polymorphism had higher ANC levels during chemotherapy, according to other studies,[19,20] supporting the idea that G allele causes a change in the regulatory region that implies a lower expression of the gene and poor neutrophils' survival.[19] Also, this polymorphism was previously related to a lower level of plasmatic cytokines, supporting our results.[19]

CARD8 304T>A polymorphism causes cysteine to change to a stop codon (p.C10X).[21] Thus, the formed protein is not fully functional, which decreases the activation of IL-1β; Figure 2 shows the relationship between the proteins and the polymorphisms.[22] Therefore, the lower activity of pro-inflammatory proteins caused by the AA genotype in this study could lead to early neutrophil apoptosis and, therefore, to lower levels of ANC after chemotherapy. Nevertheless, the association with plasmatic level cytokines has not been described.

The time is one of the most important explanatory variables on the behavior of ANC; linear time (Day) has a negative coefficient, which means, in the first days after chemotherapy starts, the ANC decreases. Then, with the passing of days, quadratic time (Day^2) becomes more critical, making the ANC rise again (positive coefficient); cubic time (Day^3) finally makes the ANC decrease to

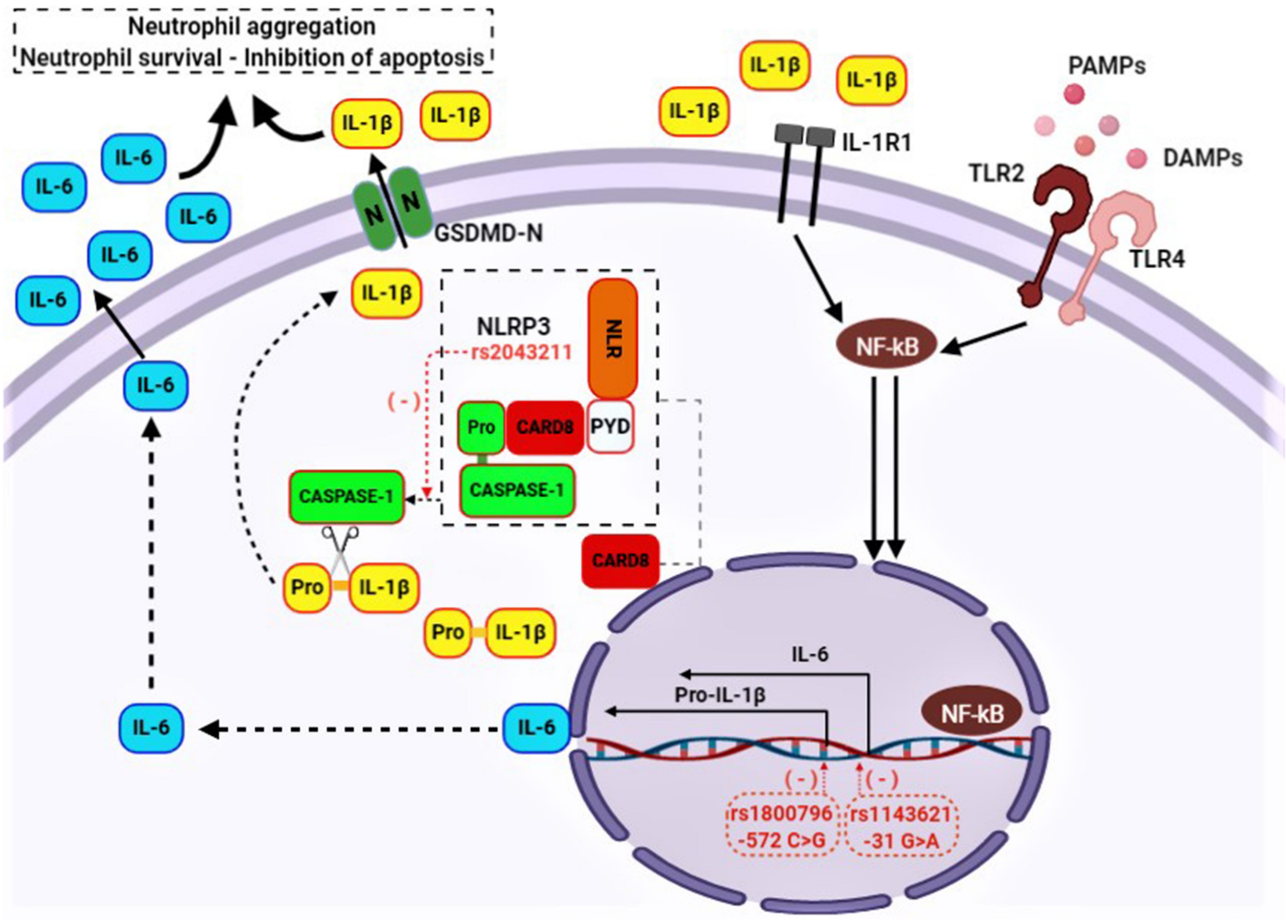

Figure 2 Relationship between protein function, studied polymorphic variants, and the effect in neutrophils survival.
Abbreviations: CARD8, caspase recruitment domain family member 8; IL-6, interleukin 6; G-CSF, granulocyte-colony stimulating factor; ANC, absolute neutrophil count; TLR2, toll-like receptor 2; NF-κB, nuclear factor kappa light chain enhancer of activated B cells.

normal levels. This polynomial fit is the best model to explain the curve of neutrophils after chemotherapy administration (Supplementary Table 1).

The interaction of time with the G-CSF administration had a positive association, indicating that, while time increases, the medication's effect is more significant due to the effect reaches its maximum some days after the first dose.[23,24] Besides, the negative association between hospitals and G-CSF administration may be explained because, at HCUCH, G-CSF was used at the beginning of the neutropenia period, whereas at FALP, toward the end of the period; hence, it was more common to have lower levels of ANC during medication administration at HCUCH than at FALP.[25]

Multilevel modeling allows us to work with correlated data and meets the assumption of linear regression, generating a proper way to obtain more reliable results. However, despite our analysis, the main limitation of this study is the small sample size, because it does not allow carry out combinatorial analyses; the low number of patients examined could mask potential associations, especially for low-frequency polymorphisms. Thus, our results should be used only to help to demonstrate the association between ANC and polymorphisms, not the effect in the outcome. Moreover, future studies should consider the effect of other actors in the inflammatory response and also focus on a specific chemotherapy scheme or diagnosis.

In the future, our results could be used to generate or improve predictive models to enhance the safety of chemotherapy for HM. Prediction of the ANC lower than 500 cells/mm^3 could help to improve antimicrobial prophylaxis, preventive isolation, and early discharge, decreasing the morbidity of patients and improving their quality of life.

Conclusion

In conclusion, *IL-6* −572 C>G polymorphism was associated with a lower neutrophil count, similarly to *CARD8* 304T>A polymorphism. Non-genetic factors associated were the administration of G-CSF, the hospital, and the time. The multilevel analysis allows us to manage correlated data and to have more reliable results.

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Disclosure

The authors report no conflicts of interest in this work.

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