DOI: 10.4149/BLL_2023_049

*Bratisl Med J 2023; 124 (4)*

320 – 329

EXPERIMENTAL STUDY

# The effects of metamizole on hematopoietic progenitor cells: Suppression of hematopoiesis stimulation *in vitro*

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**ABSTRACT**

BACKGRAUND: There is evidence that the adverse effects of metamizole occur due to the effect of the drug on the hematopoietic stem/progenitor cells, and therefore, the disruption of hematopoiesis. Therefore, our study aimed to evaluate the effects of metamizole on hematopoietic stem/progenitor cells using cell culture techniques.

MATERIAL AND METHODS: In our study, samples were taken from stem cell products of healthy allogeneic stem cell transplant donors. The colony-forming unit (CFU) assay was used for the cells obtained from these samples. In addition, the drug effects on cell proliferation were evaluated with the MTT. Furthermore, the cell colonies were labelled with immunofluorescent antibodies and the effects of metamizole on cell types formed in culture were evaluated.

RESULTS: We determined that metamizole negatively affects the proliferation of cells, especially starting from 10 μM. As a result of the evaluation of colonization, we saw that the number of colonies decreased with increasing concentrations. Granulocyte-macrophage colonies were more affected at increasing concentrations than other colonies. As a result of the evaluations of our in vitro study, it was also shown as an important finding that the individual effects of the drug were highly variable.

CONCLUSION: CFU method can be used as a suitable method to investigate the effects of drugs and toxic substances on hematopoiesis. We also think it may be suitable for pre-analysing hematopoietic side effects in new drug research. In addition, using stem cell samples in studies may contribute more easily to the in vitro simulation of hematopoietic differentiations *(Fig. 7, Ref. 29)*. Text in PDF *www.elis.sk*

KEY WORDS: metamizole, hematopoietic progenitor cells, hematopoiesis, CFU assay, adverse effect.

## Introduction

Metamizole (dipyrone) is an antipyretic and analgesic drug used to treat fever and different types of pain (1). Although the mechanism of metamizole has not been fully elucidated, its effect is mainly related to the inhibition of cyclooxygenases. Although classified as a non-steroidal anti-inflammatory drug, metamizole has weak anti-inflammatory properties (2). Despite a well-known good gastrointestinal safety profile, the reports of spontaneous adverse drug reactions indicate the presence of metamizole-induced blood dyscrasias, including metamizole-induced neutropenia and agranulocytosis, a more severe form (1). Risk estimates for patients exposed to the drug vary from 1/2.000 to 1/1.000.000 (3). Although metamizole is still widely used in many countries as an effective analgesic, it has not been approved or been withdrawn from the market in some countries, including the USA, France and Australia, due to concerns about its safety, especially the risk of agranulocytosis (4). Epidemiological studies show that the risk of agranulocytosis associated with short-term use of metamizole is low; however, the risks associated with long-term use are still controversial (5, 6).

Moreover, in large-scale studies, it has been reported that the case rates of agranulocytosis and aplastic anaemia are exaggerated and that the drug is safer than previously thought (7). Similarly, another review has indicated that negative reports for the adverse effects of the drug were somewhat biased since these studies were conducted with small sample sizes and the actual incidence was not very high; therefore, the evaluation of patients should be based on their pre-drug clinical characteristics (8). The undesirable effects of metamizole, the frequently used drug due

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**Acknowledgements:** This study was supported by Alanya Alaaddin Keykubat University Scientific Research Projects Unit. Project ID: 2018-04-02-MAP01.

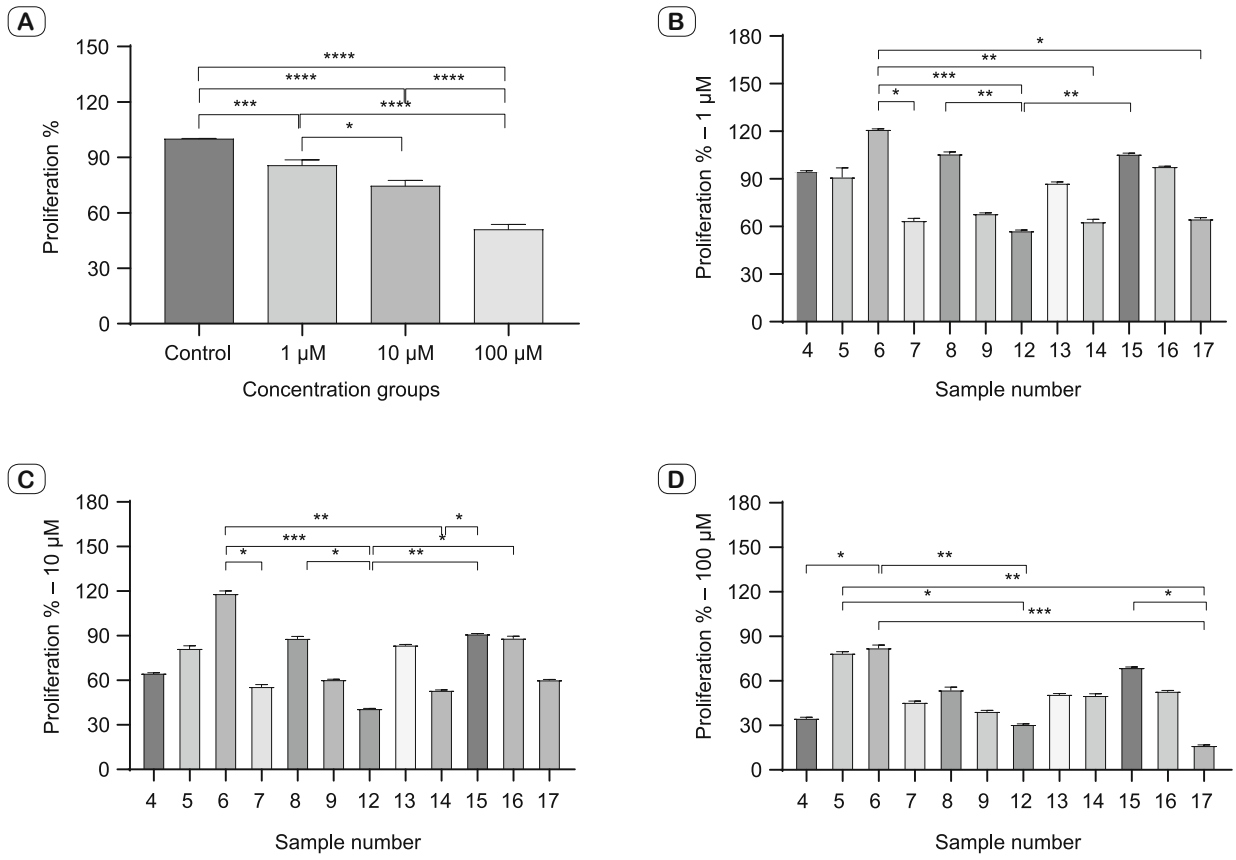


**Fig. 1. The findings of MTT assay that evaluated the proliferative/cytotoxic effects of metamizole. A: Comparison of % proliferation values between groups. Control (100 ± 0.22 %) 1 µM (85.7 ± 2.95 %), 10 µM (74.6 ± 3.02 %) and 100 µM (51.02 ± 2.7 %). Inter-individual differences in the effects of metamizole on proliferation at 1 µM (B), 10 µM (C) and 100 µM (D) concentrations. Data shown as mean ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.**

to its potent analgesic effect, on the hematopoietic system are generally based on case studies. There are no experimental studies investigating the mechanisms of action of metamizole on the hematopoietic system.

Human hematopoietic stem/progenitor cells are usually derived from bone marrow, cord blood, or peripheral blood and are commonly used to study hematopoiesis. The colony-forming unit (CFU) method, which evaluates their colony-forming abilities in a semi-solid medium, is used to study the proliferation and differentiation patterns of hematopoietic progenitors. In this *in vitro* method, the cells isolated from the stem cell material are suspended at a certain concentration and cultured in a semi-solid medium containing growth factors (9).

In this study, we used peripheral blood stem cell products obtained from healthy allogeneic stem cell transplant donors to examine the effects of metamizole on hematopoietic stem cells. Mononuclear cells (MNCs), including hematopoietic stem/progenitor cells, were isolated, and hematopoiesis was mimicked *in vitro* up to the precursors of terminal cells. It was aimed to show, for the first time, the effects of metamizole on the proliferation and colony-forming capacity of MNCs obtained from stem cell-rich transplant materials using an *in vitro* method.

## Materıals and methods

*Hematopoietic stem cell collection*

The study has been approved by Alanya Alaaddin Keykubat University, Faculty of Medicine, Clinical Ethics Committee, with the date 13/04/2018 and the decision number 2018/1, according to the ethical standards of the Declaration of Helsinki. In addition, this study was financially supported by Alanya Alaaddin Keykubat University Scientific Research Projects Unit. (Project ID: 2018-04-02-MAP01).

Peripheral blood stem cell materials were collected from healthy allogeneic stem cell donors at Başkent University, Adana Dr. Turgut Noyan Application and Research Center, Adana Adult Bone Marrow Transplantation and Cellular Therapy Center. The samples taken to analyze the materials before transplantation and regarded as waste products were used for the study. All patients

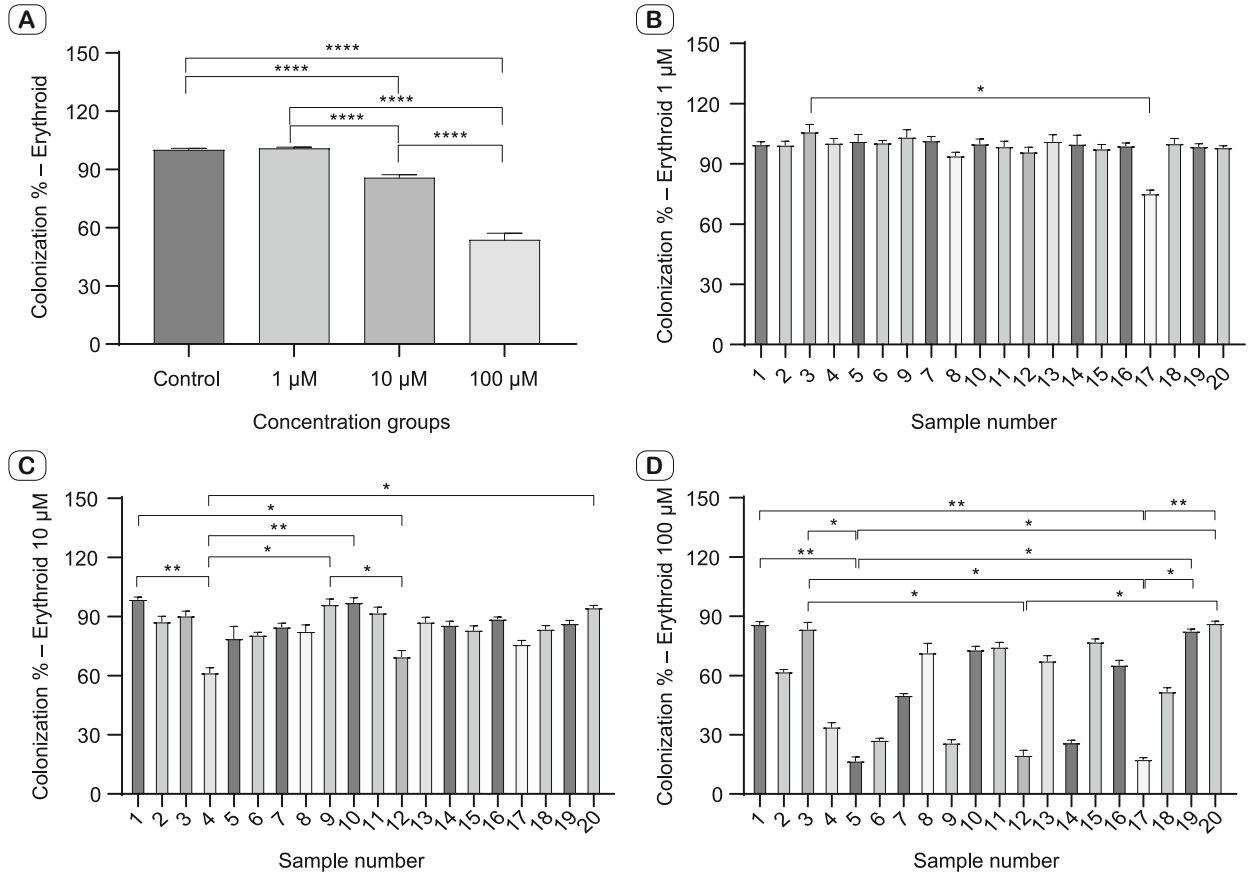


**Fig. 2. The effects of metamizole on CFU-E and BFU-E. A: There was a significant decrease in colony-forming capacity between groups with increasing concentration of metamizole (Control: 100 ± 0.84 %, 1 µM: 100.7 ± 0.76 %, 10 µM: 85.7 ± 1.62 %, 100 µM: 53.58 ± 3.46 %). Inter-individual differences in the effects of metamizole on erythroid colony forming capacity at 1 µM (B), 10 µM (C) and 100 µM (D) concentrations. Data shown as mean ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.**

provided written informed consent. *In vitro* studies were conducted at Alanya Alaaddin Keykubat University, Faculty of Medicine.

*Mononuclear cell isolation*

MNCs were isolated using a density gradient centrifugation using Histopaq1077 (Sigma-Aldrich Corporation, United Kingdom) solution. In brief, stem cell material diluted to 6 ml with phosphate-buffered saline (PBS) was carefully layered onto 3 ml Histopaque-1077. The mononuclear cells were collected after centrifugation at 400 g for 30 min. Carefully aspirated MNCs from the histopaque-plasma interface were then washed with PBS and centrifuged at 400 g for 5 min. The supernatant was discarded, and the cells were resuspended at $1x10^6$ cells per ml of cell culture medium prepared with Iscove's Modified Dulbecco's Medium (IMDM, StemCell Technologies, Canada).

*MTT cell proliferation assay*

The MTT [3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay was used to measure the short-term (48 h) cytotoxic/proliferative effect of metamizole on the hematopoietic cells.

MNCs isolated from 12 healthy donors were seeded in 96-well plates ($1x10^4$ per well) and treated with 1, 10 and 100 µM metamizole in RPMI-1640 supplemented with phytohemagglutinin, fetal bovine serum (FBS) and antibiotics. Plates were incubated for 48 h at 37 °C in 5 % $CO_2$ and 95 % relative humidity. At the end of the period, the cells were incubated with MTT (Sigma-Aldrich Corporation, United Kingdom) solution at 37 °C for 4 h. Subsequently, the supernatant was removed, and the formed formazan crystals were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich Corporation, United Kingdom). The optical density (OD) value at 570 and 630 nm was measured using a microplate spectrophotometer (BioTek Synergy H1, USA). The absorbance values of each well were then converted to % proliferation values to calculate cell viability.

*Colony-forming unit (CFU) assay*

The colony-forming unit-mix (CFU-mix) assay was performed to reveal the long-term effects of metamizole on the mixed-colony formation. MNCs isolated from 20 peripheral blood stem cell materials by density gradient centrifugation were cultured in a

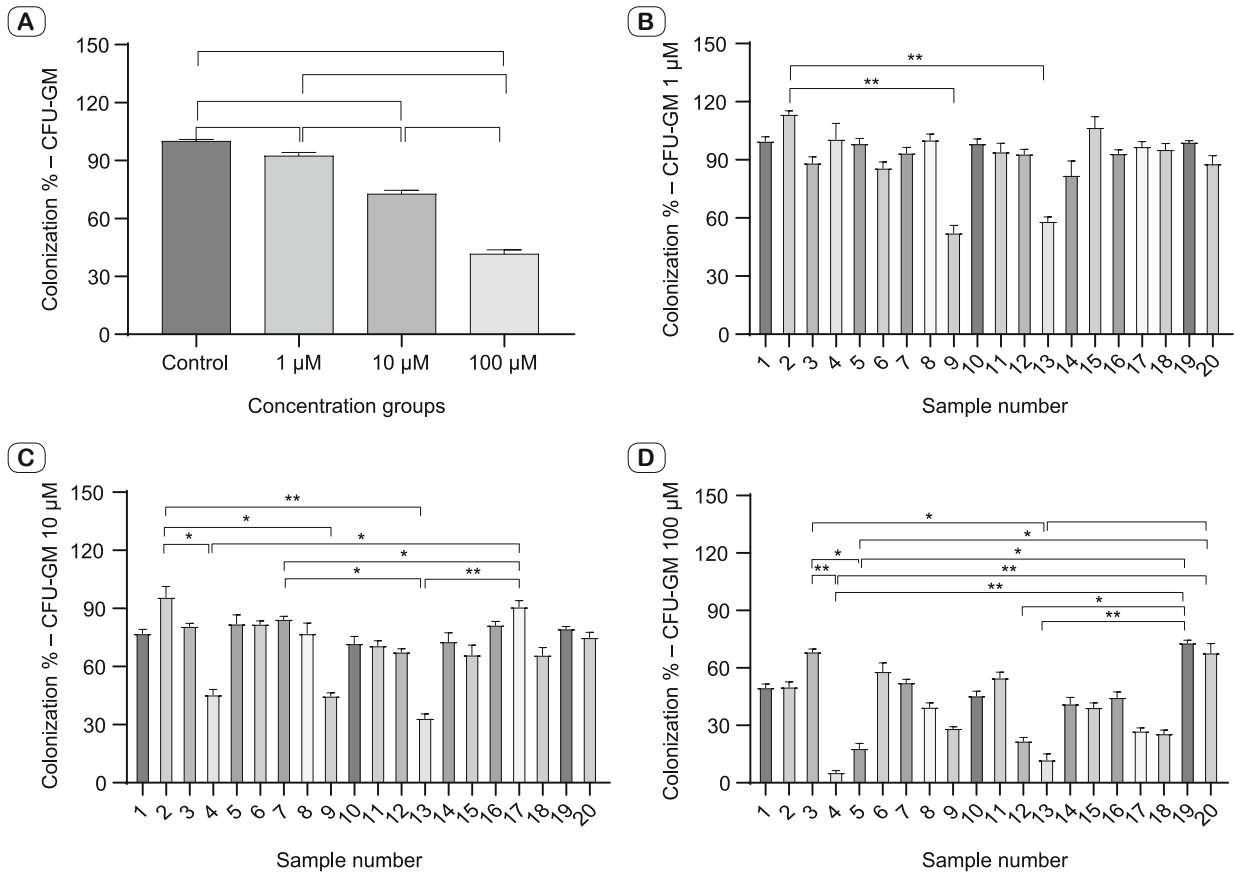


**Fig. 3. The effects of metamizole on CFU-GM. A: There was a significant decrease in colony-forming capacity between groups with increasing concentration of metamizole (Control: 100 ± 0.88 %, 1 µM: 92.44 ± 1.77 %, 10 µM: 72.73 ± 1.83 %, 100 µM: 41.64 ± 2.16 %). Inter-individual differences in the effects of metamizole on CFU-GM colony forming capacity at 1 µM (B), 10 µM (C) and 100 µM (D) concentrations. Data shown as mean ± SEM. * p < 0.05), ** p < 0.01, *** p < 0.001, **** p < 0.0001.**

semi-solid methylcellulose medium (MethoCult4034, Stem Cell Technologies Inc., Canada). The cells of control and metamizole (1, 10 and 100 µM) groups were then transferred to 35-mm Petri dishes in duplicate at a density of $1x10^5$ cells/ml and incubated at 37 °C in a 5 % $CO_2$ incubator for 14–16 days. Morphological analyses and colony count were performed manually using an inverted microscope (Zeis, Axio Vert.A1, Germany). Colonies were classified based on their morphology: Erythroid (colony-forming unit-erythroid, CFU-E and burst forming unit-erythroid, BFU-E), granulocyte-macrophage (colony forming unit-granulocyte-macrophage, CFU-GM), granulocyte, erythroid, macrophage, megakaryocyte (colony-forming unit- granulocyte, erythroid, macrophage, megakaryocyte, CFU-GEMM) (10). Colony numbers of the control group were matched to 100% capacity, and the colony-forming capacities of the other groups were calculated accordingly.

*Immunofluorescent marking*

Immunofluorescent marking was designed to determine how increasing concentrations of metamizole affect the stem cells and the development of monocyte/macrophage, granulocyte, and megakaryocyte precursors. Cells from pooled colonies were spread on slide glass. Marking was performed using primary antibodies (Miltenyibiotec, Germany) specific to hematopoietic cells and examined with an inverted fluorescent microscope. Samples were marked with anti-human-CD14-FITC and CD68-FITC for monocyte/macrophage progenitors, CD61-FITC for megakaryocyte progenitors, CD66b-FITC for granulocyte progenitors and CD34-FITC for hematopoietic stem cells. The nucleus was marked with DAPI (Sigma-Aldrich, USA) to detect the cells under the microscope clearly. The number of positive cells to the total number of cells was calculated.

*Statistical analysis*

Data from the MTT and CFU assays were calculated as mean ± the standard error of the mean (SEM). Shapiro-Wilk test was used for testing normality. For the data sets which followed the normal distribution, differences between groups were analysed using one-way ANOVA followed by Bonferroni's post hoc test. Since the individual analyses of each concentration in each sample did not follow normal distribution, individual data were analysed by the

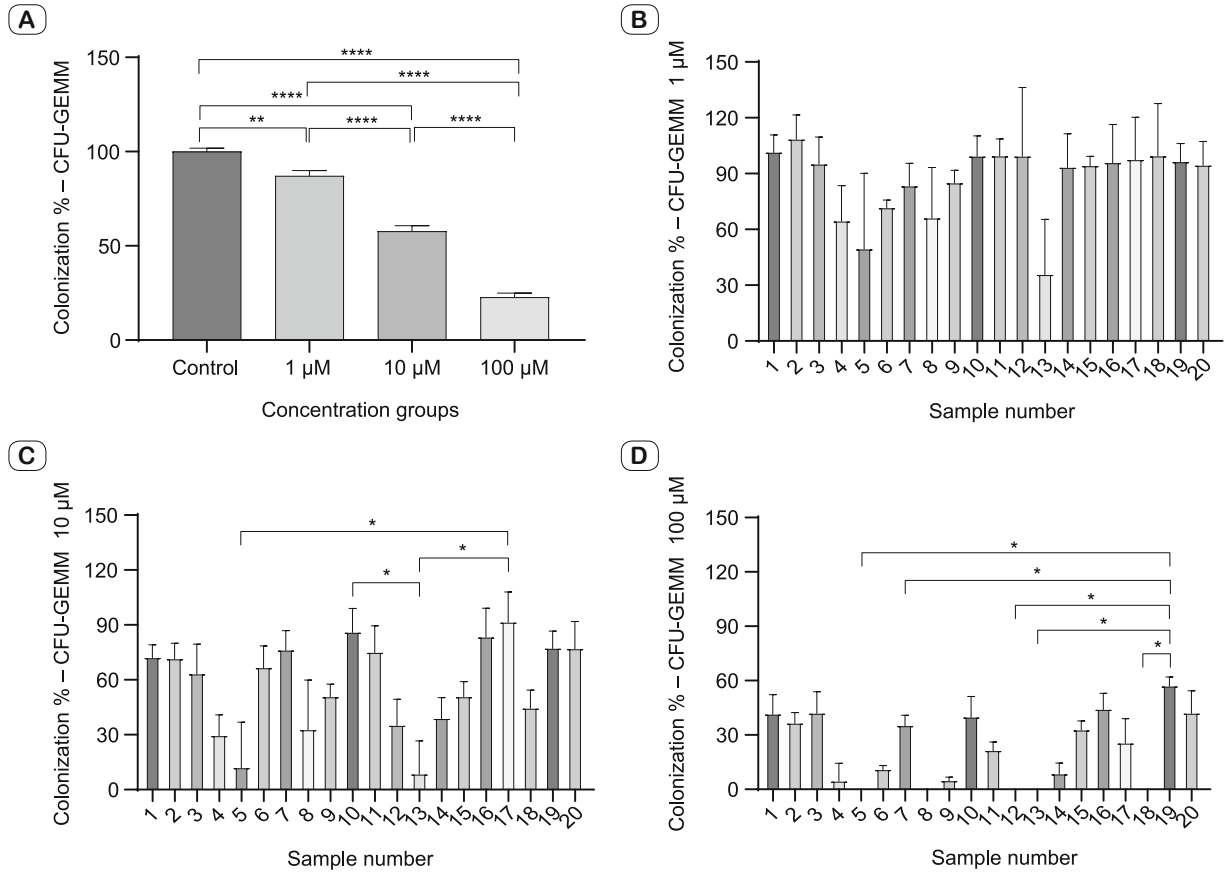


**Fig. 4. The effects of metamizole on CFU-GEMM. A: There was a significant decrease in colony-forming capacity between groups with increasing concentration of metamizole (Control: 100 ± 1.83 %, 1 μM: 87.08 ± 0.89 %, 10 μM: 57.67 ± 3.06 %, 100 μM: 22.73 ± 2.25 %). Inter-individual differences in the effects of metamizole on CFU-GEMM colony forming capacity at 1 μM (B), 10 μM (C) and 100 μM (D) concentrations. Data shown as mean ± SEM. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001.**

non-parametric Kruskal-Wallis test. All statistical analyses were performed using GraphPad Prism 9.0 software (SanDiego, CA, USA). $p \leq 0.05$ were considered statistically significant.

## Results

*Cell viability and proliferation*

There was a significant difference between the control and metamizole (1–10–100 μM) groups showing that increasing concentration of metamizole has negative effects on cell proliferation (Fig. 1A). However, significant inter-individual differences in metamizole effects were found among different concentration groups. The highest proliferation capacity in the 1 μM and 10 μM groups was detected in sample (s) 6 (121.8 ± 0.22 % and 119 ± 0.95 %, respectively), the lowest one was detected in s12 (57.93 ± 0.36 % and 41.64 ± 0.12 %, respectively) (Figs 1B–C). In the 100 μM group, the high concentration group, s6 (82.87 ± 1.1 %) showed the highest proliferation capacity, while the lowest capacity was found in s17 (17.05 ± 0.29 %) (Fig. 1D).

***CFU assay results***

*Erythroid colonies*

Total erythroid colonies (CFU-E+BFU-E) were counted. There was a significant difference in colony-forming capacity between the control and metamizole (10–100 μM) groups (Fig. 2A). No significant difference was detected between the control and metamizole 1 μM groups. Inter-individual analyses revealed that only the s3 (106.5 ± 3.72 %) and s17 (75.69 ± 1.84 %) were significantly different in their colony-forming capacity at 1 μM concentration (Fig. 2B). As in the MTT test, individual differences increased primarily in the 10 and 100 μM concentration groups of metamizole. The highest proliferation capacity in the 10 μM group, the intermediate concentration group, was detected in s1 (99.31 ± 1.27 %), and the lowest one was detected in s4 (62 ± 2.67 %) (Fig. 2C). Colony numbers generally decreased at the high concentration of 100 μM, while the highest capacity was detected in s21 (86.9 ± 122 %) and the lowest in s5 (17.27 ± 2.12 %) (Fig. 2D). These results show that metamizole has a negative effect on erythroid colony formation at increasing concentrations. Particu-

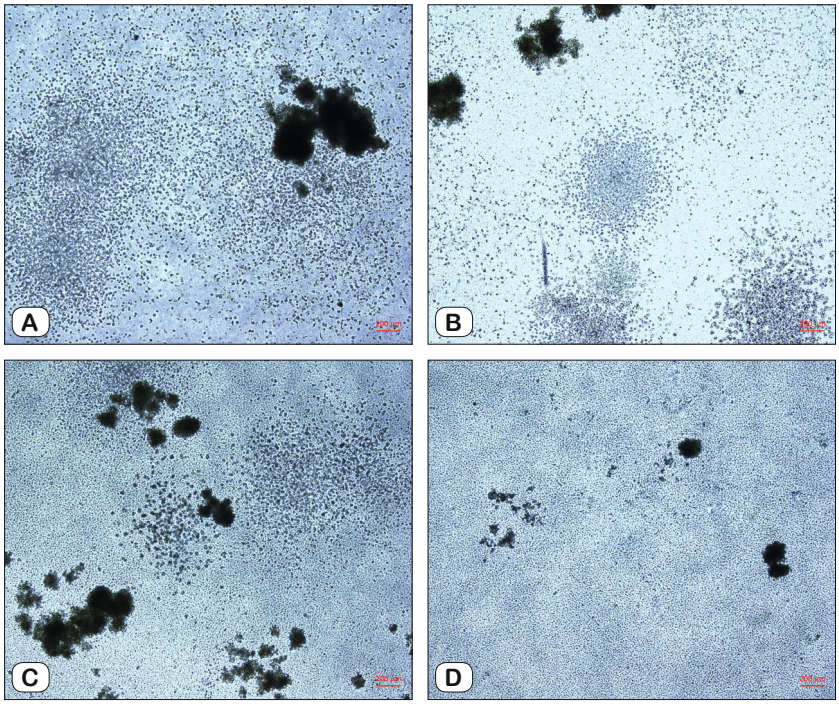


**Fig. 5. The effects of metamizole on colony-formation capacity at increasing concentration. A: Control, B: Metamizole 1 µM, C: Metamizole 10 µM, and D: Metamizole 100 µM.**

larly at higher concentrations, inter-individual differences between individual results also increase.

*Granulocyte-macrophage colonies (CFU-GM)*

There was a significant decrease in the number of CFU-GM colonies between the control and metamizole (1- 10–100 µM) groups (Fig. 3A). Inter-individual analyses showed that the highest and lowest proliferation capacities were detected in s2 (114 ± 1.95 %) and s9 (52.85 ± 4.02 %) for 1 µM group, in s2 (96.29 ± 5.7 %) and s13 (33.75 ± 2.4 %) for 10 µM group and in s19 (73.58 ± 1.52 %) and s4 (5.88 ± 1.17 %) for 100 µM group, respectively. Individual differences in the effects of colony formation were raised with increasing concentrations of metamizole (Figs 3B–D).

*Granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) colonies*

There was a significant decrease in the number of CFU-GEMM colonies between the control and metamizole (1–10–100 µM) groups (Fig. 4A). Individual differences were detected in CFU-GEMM colony-forming capacities for each concentration group. The highest and lowest proliferation capacities were detected in s2 (109 ± 6.6 %) and s13 (36.6 ± 14.85 %) for 1 µM group, in s17 (92 ± 8.33 %) and s13 (9.09 ± 9.09 %) for 10 µM group, respectively (Figs 4B–C). For the 100 µM group, the highest proliferation capacity was detected in s19 (57.58 ± 2.54 %), while no CFU-GEMM colonies were detected in s5, 8, 12, 13 and 18 (Fig. 4D).

In the morphological examinations, the colony numbers obtained from each sample were individually different. However, all colony formations decreased microscopically with increasing concentration (Fig. 5).

*Immunofluorescent marking findings*

Although the colony numbers of erythroid, CFU-GM and CFU-GEMM decreased with increasing concentrations of metamizole, no significant difference was detected with the immunofluorescent marking (Fig. 6). Although the total number of cells decreased, these cell ratios were preserved. On the other hand, a significant decrease at 100 µM concentration was found in granulocyte progenitors (Fig. 7).

## Discussion

This study is the first *in vitro* evidence of the effects of metamizole on the proliferation and colony formation ability of MNCs isolated from hematopoietic stem cells. Our findings showed that metamizole negatively affects the proliferation of cells at increasing concentrations starting from 10 µM, decreases the number of erythroid colonies at increasing concentrations starting from 10 µM, and the number of CFU-GM and CFU-GEMM colonies at increasing concentrations starting from 1 µM. In addition, the determination of inter-individual differences in the colony formation for each concentration was another important finding of the study.

In a study previously conducted in our country, children admitted to the hospital who used metamizole were evaluated for 12 months. During this period, granulocytopenia and agranulocytosis were detected in 13 children. The study results revealed that the incidence of metamizole-related agranulocytosis in pediatric patients admitted to the hospital was 1/10,000, and it was suggested that clinicians should not consider metamizole as the first-line therapy in the paediatric population (11). Agranulocytosis was detected in one of the two cases with metamizole-related adverse effects in Antalya, Turkey. This patient was treated and discharged from the hospital with normal blood count values on the 14th day (12).

In a retrospective case-control study by Blaser et al, 57 patients with metamizole-associated leukopenia were classified and compared based on their clinical characteristics before using metamizole. According to the data obtained, it has been reported that allergy and hepatitis C are the risk factors for metamizole-associated leukopenia (13). In our study, the cell products of healthy individuals were only used.

The incidence of metamizole-induced agranulocytosis was found 0.56 per million in the study of Ibáñez et al., which included 17 haematology laboratories in Barcelona, Spain, between 1980 and 2004. Patients who developed agranulocytosis in the study

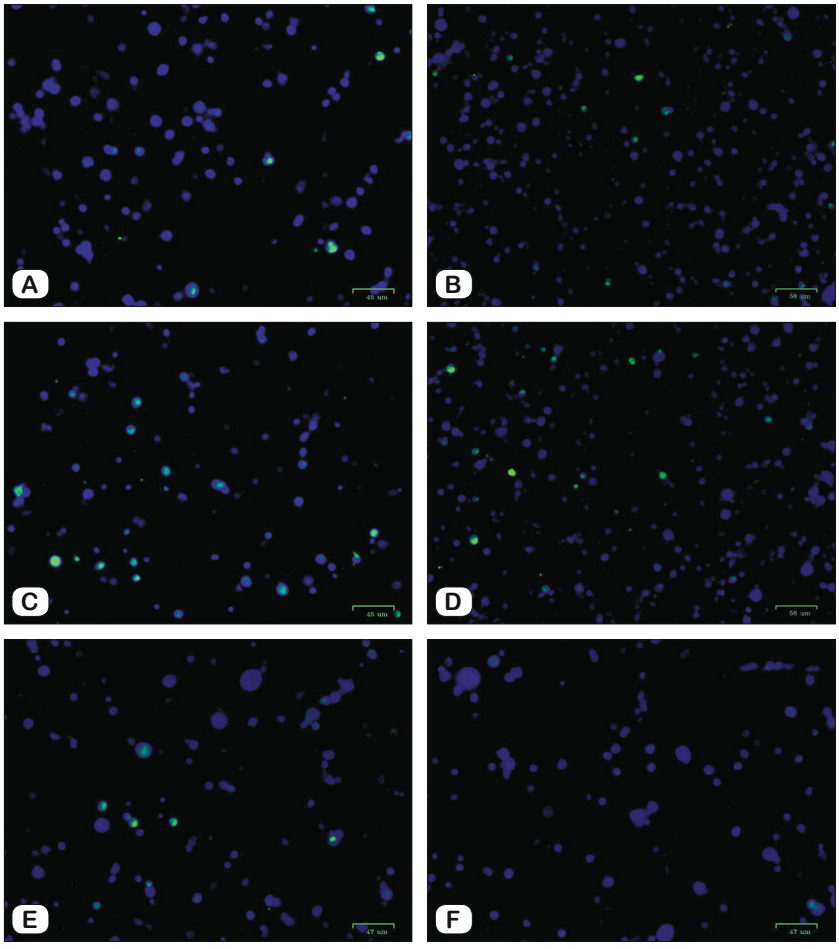


**Fig. 6. Immunofluorescence marking of (A-B) monocyte/macrophage progenitors (CD14-FITC and CD68-FITC, respectively), (C–D) granulocyte progenitors (CD66b-FITC), (E) megakaryocyte progenitors (CD61-FITC) and (F) hematopoietic stem cells (CD34-FITC). Green colour refers positive. Cell nuclei were marked with DAPI (blue).**

used metamizole longer than the others. Nevertheless, researchers reported low incidence, but there is a risk of agranulocytosis associated with metamizole (14).

In a study involving 51 hospitals in Germany, metamizole-induced agranulocytosis was detected in 26 of 88 patients with validated agranulocytosis and who used metamizole. The incidence rate of metamizole-induced agranulocytosis was determined as 0.96 per million. The researchers reported that the drug should not be sold without a prescription due to its adverse effects. They also emphasized that informing patients about this adverse effect is critical, and the increasing use of metamizole should be considered (15).

The mechanisms underlying metamizole-induced agranulocytosis have not been fully elucidated. Current data indicate the direct toxic effect on the progenitor cells in the bone marrow (16) or an immunological process (17). Garcia-Martinez et al. showed that *in vivo* concentrations of metamizole and its active metabolite 4-methylaminoantipyrine did not affect granulocytic differentiation and did not induce the related apoptosis of terminally differentiated granulocytes. Therefore, they concluded that the mechanism of agranulocytosis caused by metamizole is of immune-allergic origin (3). The genetic factors may also be involve in the process (6).

Bone marrow biopsies of patients in whom metamizole had adverse effects showed inhibition of granulocyte maturation at the promyelocyte and myelocyte stages (18). Therefore, the effect of metamizole on granulocytes might occur in the bone marrow, not in the peripheral circulation (19).

CFU method based on the ability of cells to colonize in a culture medium was used to determine the mechanisms of haematopoiesis after the initiation of bone marrow transplantation (20). With the increase in bone marrow transplantation, it has also been used to determine the quality of allogeneic and autologous transplantation materials, but it has lost its value in this field with flow cytometry. However, it is currently used in the qualification and validation of stem cell harvesting, especially in accredited transplant centres. CFU assay is also used to contribute to the differential diagnosis of changes in platelet and erythrocyte counts in the blood (21, 22), as well as hematopoietic malignancies (23). Although it is a proper model, this method has not made ground in research on drugs affecting haematopoiesis. In recent years, studies have reported that this method can be used as a model for investigating the toxic effects of drugs on haematopoiesis and describing these models (24, 25).

Apart from toxic substances, a study evaluates the effects of used drugs on haematopoiesis with this method. Samalidou et al examined the in vitro effects of liposomal amphotericin B, voriconazole and caspofungin, which are used to treat fungal infections in stem cell transplant recipients, on haematopoiesis, utilizing this model together with an *in vivo* experiment in mice. These drugs may cause adverse effects like metamizole but with different incidences, in the form of decreased number of leukocytes. Samalidou et al determined that particularly amphotericin B caused a decrease in CFU-GM colonies in the increasing concentration. No toxic effect was observed with other antifungals (26). Our findings showed that metamizole decreased CFU-GM colonies at the increasing concentration, similar to the effect of antifungals. Moreover, it was determined that there was a decrease in erythroid and CFU-GEMM colonies.

In the only *in vitro* study evaluating the effects of metamizole on myeloid cells, the effects of diclofenac and acetylsalicylic acid were compared with the effects of metamizole. It has been shown

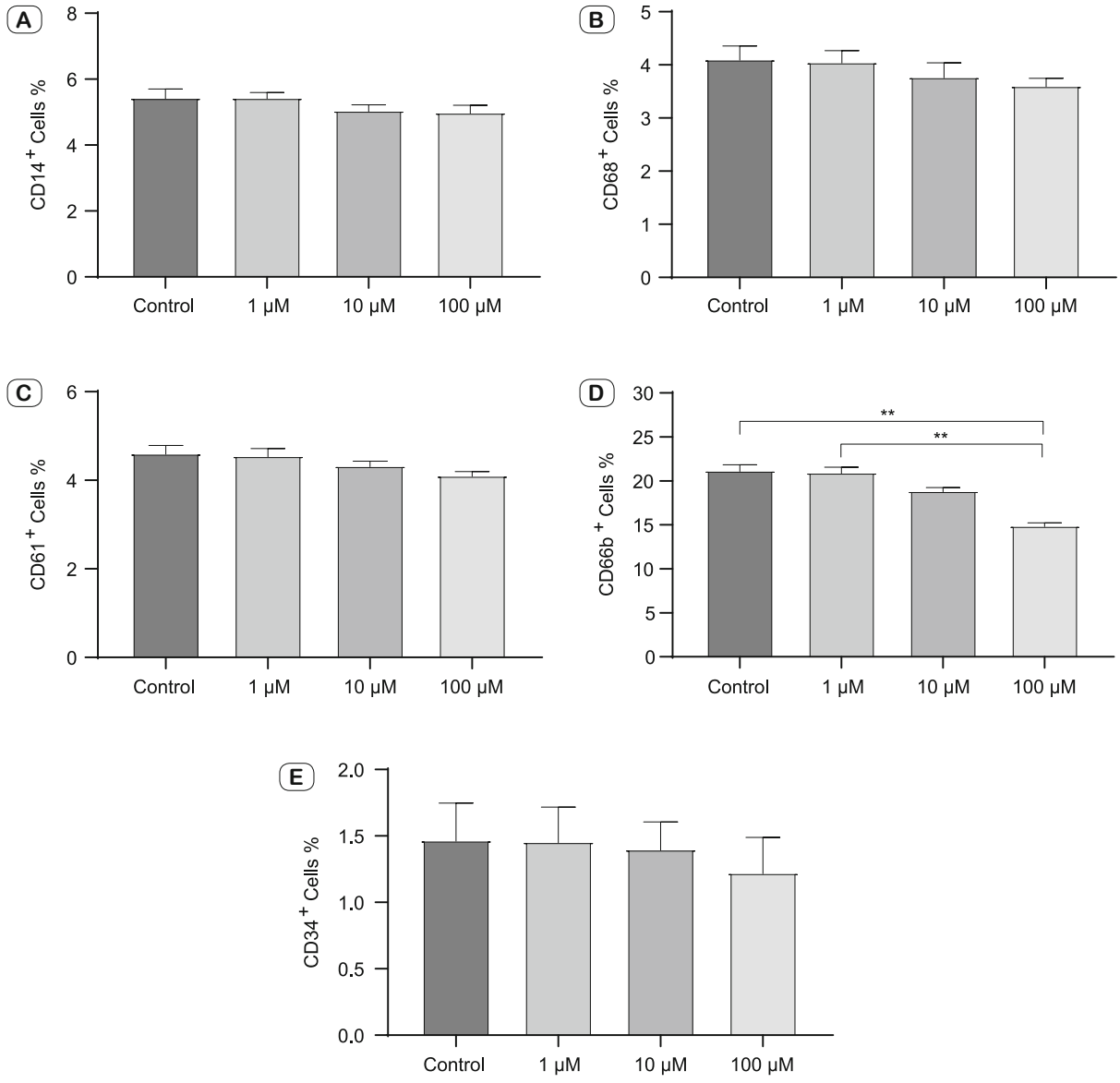


**Fig. 7. The ratio of (A-B) monocyte/macrophage progenitors (CD14-FITC and CD68-FITC, respectively), (C) granulocyte progenitors (CD66b-FITC), (D) megakaryocyte progenitors (CD61-FITC) and (E) hematopoietic stem cells (CD34-FITC). Although a decrease in colony forming capacity was detected with increasing concentration, only granulocyte progenitors were significantly lower than control (21.22 ± 0.75 %), metamizole 1 µM (21 ± 0.68 %) and metamizole 100 µM (14.94 ± 0.41 %) groups. Data shown as mean ± SEM. ** p < 0.01.**

that metamizole does not have higher myelotoxicity than these active ingredients. The HL60 cell line (acute promyelocytic leukaemia) and differentiated granulocytes from these cells were used in the study. Furthermore, the apoptotic analysis of these drugs was performed on isolated healthy granulocytes. It was found that apoptosis induced by high concentrations of metamizole affected 30% of HL60 promyelocytes, but the apoptotic effect was slightly less in differentiated granulocytic cells. This study determined that the effects of metamizole in plasma concentrations were minor (3). It should be noted that the HL60 cells are immortal cancer cells proliferating unlimited and uncontrolled. These cells can also develop drug resistance. Furthermore, the use of terminal granulocytes in the apoptosis test limited the described mechanisms since the current data indicate that the drug probably affects granulocyte maturation in the bone marrow (18). In our study, all the steps to the precursors of terminal cells in haematopoiesis were simulated *in vitro* using bone marrow-derived progenitor cells. As in the study mentioned above, our study also suggests that metamizole affects promyelocyte differentiation. However, the severity of this effect varies by individual.

There are also limited *in vitro* studies showing that metamizole affects cancer cells (27–29). The basis of these studies is the effects of metamizole on the cell production process such as agranulocytosis. In two studies with the osteosarcoma cell line MG-63, it has been shown that the drug has apoptotic effects on cells starting from 10 μM concentration. Moreover, the drug has also been effective in the differentiation of cells (27, 28). A study with A549 and HeLa cell lines reported that metamizole reduced cell proliferation and viability (29).

Adverse effects of metamizole on hematopoietic cells are based on case reports or their meta-analyses. It has been reported that immunological mechanisms (3) or genetic factors may play a role in developing of these adverse effects (3, 6). Our study indicates that there may be individual variability in the effect of metamizole on hematopoietic cells. As seen in the graphics above, there are significant differences in colonization between individuals at increasing concentrations under the effect of metamizole. These findings suggest that genetic differences, perhaps yet undetermined polymorphisms may be responsible for the serious adverse effects associated with haematopoiesis. This behavioural difference between individuals is therefore important and may lead to further studies on this topic.

## Conclusion

The effects of metamizole, which has apoptotic and antiproliferative effects on cancer cell lines, on haematopoiesis should be investigated experimentally, taking into account the individual differences related to possible mechanisms. Additionally, studies considering genetic-individual differences in affected patients should be further designed. The development of in vitro methods is also important in investigating the effects of drug on haematopoiesis. We wanted to give an example of this with our study. Especially in these studies, the use of samples with dense stem cells may also be advantageous.

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Received November 8, 2022.
Accepted November 14, 2022.