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# Influence of repeated subcutaneous G-CSF injections on selected blood parameters relevant for monitoring programmes in sports drug testing

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**The use of growth factors in sports is restricted under the terms of the World Anti-Doping Code (WADC). While the beneficial effects of erythropoietin (EPO) on erythropoiesis and therefore its performance-enhancing properties have been well documented and established for decades, the aim of this study was to elucidate the relevance of the cytokine G-CSF in a doping control context, particularly concerning its influence on selected blood parameters representing central aspects of the Athlete Biological Passport. For that purpose, the effect of repeated subcutaneous granulocyte colony-stimulating factor (G-CSF) injections in therapeutic dosages (10 μg/kg/d) on white blood cells, erythrocytes, hemoglobin, hematocrit and percent reticulocytes was analyzed by using commonly employed fluorescence flow cytometry-based approaches. A total of 20 people were tested (14 male, 6 female) and both white blood cell count and reticulocyte percentages were found to significantly increase following a 5-day treatment with G-CSF. Simultaneously, all other volume-dependent parameters (red blood cell count, hemoglobin, hematocrit) slightly but significantly decreased. Due to the relevance of these measurands for the validity of blood tests for doping controls and the anecdotal evidence of G-CSF being potentially misused by elite athletes, G-CSF analyses might be indicated in case of unusually altered blood profiles. Copyright © 2012 John Wiley & Sons, Ltd.**

**Keywords:** G-CSF; blood parameters; reticulocytes; white blood cells; Athlete Biological Passport

## Introduction

Hematopoietic growth factors such as erythropoietin (EPO) or granulocyte colony-stimulating factor (G-CSF) are a family of cytokines regulating the proliferation and differentiation of hematopoietic progenitor cells.[1] In general, the use of such growth factors is restricted under the terms of the World Anti-Doping Agency (WADA) and the annually issued Prohibited List.[2,3] The performance-enhancing properties of EPO are well documented and other hematopoietic growth factors also hold a considerable potential for misuse in sports.

G-CSF is a hematopoietic growth factor used for the mobilization of CD34+ hematopoietic progenitor cells from the bone marrow to the blood.[4–6] Moreover, G-CSF increases the survival, proliferation, differentiation, and functional activation of cells of the neutrophil lineage, thus, among other aspects, regulating granulopoiesis.[1,5,7] Human G-CSF consists of 174 amino acids arranged in four anti-parallel α-helices and has a molecular weight of approximately 20 kDa.[6–8] A variety of cell types including monocytes/macrophages, fibroblasts and endothelial cells are able to produce and secrete G-CSF in response to an appropriate stimulus.[9,10] Such stimulatory agents are for example lipopolysaccharides (LPS), tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1). For this reason, the plasma levels of G-CSF in healthy humans are generally very low (< 78 pg/ml) increasing only in case of bacterial infections and other stress stimuli.[7,9–12]

Currently, three pharmaceutical products of G-CSF have received clinical approval.[6] Filgrastim (Amgen, Thousand Oaks, CA, USA) is a recombinant, non-glycosylated protein expressed in *Escherichia coli*, which bears an additional methionine-residue at its N-terminus. A stabilized, glycosylated form of recombinant G-CSF named Lenograstim (Chugai Pharmaceuticals, Tokyo, Japan) is produced in Chinese hamster ovary cells.[6,8] Additionally, Amgen has developed a PEGylated variant of Filgrastim which features an increased plasma half-life.[6,13] Despite the different persistence *in vivo*, all recombinant forms of G-CSF have a similar biological activity and bioavailability following subcutaneous or intravenous administration.[6,8] Clinical applications for these drugs are, for example, the treatment of neutropenia caused by chemotherapy or hematopoietic stem cell transplantation and the G-CSF mediated mobilization of CD34+ peripheral

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blood stem cells.[6,8,13] Moreover, the influence of G-CSF on the number and activity of mature neutrophils predestines this drug for the treatment of bacterial infections.[8,9]

In the present study, the influence of repeated subcutaneous G-CSF injections on different blood parameters including hematocrit, hemoglobin, red blood cell count, white blood cell count and percentage of reticulocytes was analyzed by means of fluorescence flow cytometry. Since these parameters are essential for sports drug testing monitoring programmes, the relevance of G-CSF as potential performance-enhancing or masking agent in sports was to be elucidated.

## Materials and methods

### G-CSF administration and sample collection

In the course of regular peripheral blood stem cell donations, 20 healthy volunteers (14 male, 6 female, aged 21–57 years) performed subcutaneous self-injections of 10 micrograms of Lenograstim (Granocyte®, Chugai Pharma, Frankfurt a. Main, Germany) per kilogram body weight for a total of five consecutive days. Written informed consent was obtained from each donor and the study protocol was conformed to the ethical guidelines of the Declaration of Helsinki as reflected in an approval by the institutional ethical review committee. From each volunteer, two blood samples were collected in EDTA Vacutainer® (BD Biosciences, Heidelberg, Germany) tubes before and after G-CSF administration and analyzed within 36 h. Until analysis, samples were stored at 4°C in order to prevent cellular damage.

### Measurement of blood parameters

Prior to hematological analysis, blood samples were homogenized on a roller mixer at room temperature (RT) for at least 15 min. For blood analysis, a Sysmex (Norderstedt, Germany) XT-2000i fluorescence flow cytometer was used and the performance of the instrument checked by measuring quality controls provided by the manufacturer. A total of five blood parameters comprising white blood cells ($10^3$/μl), red blood cells ($10^6$/μl), hemoglobin (g/dl), hematocrit and percent reticulocytes were determined according to standard procedures. Paired Student's *t*-test was used to perform a statistical evaluation of the recorded data and to determine the significance of the observed alterations. Additionally, R software (version 2.8.1) was used to create box plots (logarithmic and non-logarithmic scale) of the data.

### Determination of G-CSF plasma concentrations

Following hematological analysis, plasma, buffy coat and red blood cells were separated by centrifugation for 2 min at 1000 x *g* and RT. The plasma supernatant was transferred to a fresh tube and stored at −20 °C until further analysis.

Preliminary tests with urine samples collected after G-CSF administration and literature research[14] have shown that only minute amounts of intact G-CSF (≤ 67 pg/ml) are renally eliminated. Consequently, plasma specimens were chosen for the determination of G-CSF. The plasmatic G-CSF concentrations before and after repeated administration of Lenograstim were determined by means of a commercial G-CSF ELISA kit (R&D Systems, Wiesbaden, Germany). The assay was performed according to the manufacturer's instructions without dilution and all samples were measured in duplicate.

### Enumeration of CD34+ cells

In order to evaluate the mobilization of hematopoietic progenitor cells from the bone marrow to the peripheral blood, the number of circulating CD34+ cells before and after G-CSF administration was determined by using the two-platform ISHAGE (International Society of Hemotherapy and Graft Engineering) protocol.[15] Thereby, the absolute number of leukocytes is determined by using an automatic cell counter (CellDyn, Abbott Diagnostics, Wiesbaden, Germany). Simultaneously, the number of CD34+ events expressed as percentage of CD45+ events is measured with an Epics XL flow cytometer (Beckman Coulter, Krefeld, Germany) equipped with a 488-nm argon-ion laser. By multiplying the percentage of CD34+ cells with the total number of WBCs, the absolute number of CD34+ cells (/μl) in the sample can be calculated.

For flow cytometric analysis, a total of 40 μl of peripheral blood was incubated with 10 μl each of anti-CD45-FITC antibody (J33) and anti-CD34-PE antibody (clone 581) (Pharmingen/Becton Dickinson, San José, CA, USA) in duplicate. Flow cytometric measurements were performed within 1 hour and until analysis, all samples were stored at 4 °C and in the dark.

## Results and discussion

### Determination of G-CSF plasma concentrations

A total of 40 plasma samples were analyzed, collected before and after repeated subcutaneous injection of recombinant human G-CSF. As shown in Table 1, only 5 of the 20 samples collected prior to the G-CSF administration contained detectable amounts of G-CSF and all measured values were below the lowest standard of 39 pg/ml (mean: 2.6 pg/ml ± 7.2). Following five consecutive days of subcutaneous self-injections of 10 μg of Lenograstim per kg of body weight, G-CSF plasma concentrations were found to range between 16 and more than 2500 pg/ml (mean: 1603.4 pg/ml ± 687.9).

Generally, the plasma levels of G-CSF in healthy humans are low (less than 78 pg/ml) and increase only in response to different stress stimuli such as bacterial infections.[7,9–11] Thus, only small amounts of G-CSF were expected in the plasma samples collected before G-CSF administration. After five days of medication, the plasma levels increased to an average value of 1603.4 pg/ml with a relatively high standard deviation of 688 pg/ml. Depending on the dosage, Lenograstim (Granocyte®) has a bioavailability between 26 and 61% and the serum elimination half-life was found to range between 3 and 4 h.[14,16] As the volunteers were all treated with the same dosage of the cytokine (10 μg/kg/day), the widespread range of G-CSF plasma levels observed in this study could indicate an inaccurate subcutaneous application as well as a varying clearance of the cytokine. Despite this considerable variability in plasma G-CSF concentration, atypically elevated plasma levels could serve as an indicator for an administration of the growth factor to athletes. However, as the physiological G-CSF amount in the blood can increase in case of bacterial infections to more than 2000 pg/ml,[12] the evaluation of additional inflammatory parameters might be necessary to distinguish between G-CSF doping and an inflammatory response.

### Enumeration of CD34+ cells

Besides the treatment of neutropenia, the main clinical use of G-CSF is the mobilization of CD34+ hematopoietic progenitor

**Table 1.** G-CSF plasma concentrations and its influence on selected blood parameters: CD34+ count (# CD34+), white blood cell count (# WBC), red blood cell count (# RBC), hemoglobin (HGB), hematocrit (HCT) and percent reticulocytes (%Ret)

| Volunteer Number | Gender | G-CSF [pg/mL] | | # CD34+ [/μL] | | # WBC [$10^3$/μL] | | # RBC [$10^6$/μL] | | HGB [g/dL] | | HCT [%] | | %Ret | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Before | After | Before | After | Before | After | Before | After | Before | After | Before | After | Before | After |
| 1 | male | 0.0 | 16.5 | 1.3 | 89.0 | 7.17 | 44.97 | 4.99 | 4.89 | 14.3 | 14.2 | 43.6 | 44.6 | 0.86 | 1.93 |
| 2 | male | 31.6 | 908.8 | 4.3 | 44.0 | 8.71 | 36.13 | 5.12 | 4.96 | 16.0 | 15.4 | 48.5 | 47.1 | 1.00 | 1.35 |
| 3 | male | 10.3 | 2597.8 | 0.8 | 20.0 | 6.02 | 33.36 | 4.79 | 4.71 | 15.6 | 15.3 | 44.9 | 45.3 | 1.84 | 2.30 |
| 4 | male | 0.0 | 1243.5 | 1.1 | 53.0 | 4.52 | 48.72 | 4.88 | 4.36 | 14.9 | 13.3 | 44.4 | 40.4 | 1.50 | 2.40 |
| 5 | male | 0.0 | 126.7 | 2.9 | 101.0 | 6.55 | 64.54 | 5.77 | 5.50 | 16.5 | 15.6 | 51.4 | 49.5 | 0.81 | 1.36 |
| 6 | male | 0.0 | 2590.1 | 1.8 | 31.0 | 2.90 | 22.87 | 4.96 | 4.29 | 14.9 | 13.2 | 45.8 | 40.7 | 0.45 | 1.22 |
| 7 | male | 0.0 | 1731.5 | 1.0 | 38.0 | 5.21 | 35.68 | 5.01 | 4.93 | 14.7 | 14.7 | 43.4 | 44.5 | 0.61 | 1.59 |
| 8 | male | 0.0 | 1644.6 | 1.1 | 126.0 | 6.10 | 55.12 | 5.53 | 5.08 | 15.7 | 14.7 | 47.4 | 44.5 | 1.88 | 2.47 |
| 9 | male | 6.8 | 1663.0 | 2.8 | 83.0 | 4.85 | 36.29 | 5.24 | 4.73 | 16.0 | 14.9 | 48.9 | 45.8 | 1.15 | 1.71 |
| 10 | male | 0.0 | 1694.0 | 3.4 | 45.0 | 3.65 | 28.89 | 5.32 | 4.83 | 15.4 | 14.2 | 46.3 | 43.7 | 1.05 | 1.86 |
| 11 | male | 0.0 | 1106.1 | 2.5 | 65.0 | 5.73 | 40.54 | 4.71 | 4.82 | 14.9 | 15.3 | 42.9 | 45.2 | 0.81 | 1.74 |
| 12 | male | 0.0 | 1515.4 | 2.9 | 255.0 | 6.53 | 80.49 | 5.39 | 4.96 | 16.3 | 14.9 | 48.9 | 46.6 | 1.61 | 2.09 |
| 13 | male | 0.0 | 1670.9 | 2.1 | 87.0 | 5.89 | 45.24 | 5.10 | 5.10 | 15.8 | 15.6 | 45.3 | 46.6 | 1.35 | 2.03 |
| 14 | male | 0.0 | 1677.6 | 3.2 | 152.0 | 7.94 | 46.13 | 5.53 | 5.13 | 16.7 | 15.5 | 49.2 | 47.9 | 1.02 | 1.68 |
| 15 | female | 0.0 | 2634.0 | 2.3 | 77.0 | 6.73 | 34.51 | 4.59 | 4.12 | 14.2 | 13.1 | 43.8 | 40.2 | 0.85 | 2.15 |
| 16 | female | 0.0 | 2499.9 | 4.6 | 35.0 | 3.00 | 26.43 | 4.66 | 4.40 | 13.2 | 12.7 | 41.0 | 40.4 | 0.51 | 2.26 |
| 17 | female | 2.7 | 1713.4 | 2.4 | 98.0 | 9.03 | 51.48 | 4.46 | 4.34 | 13.9 | 13.8 | 42.0 | 42.1 | 1.39 | 2.43 |
| 18 | female | 0.4 | 1672.3 | 3.9 | 50.0 | 5.72 | 48.19 | 5.69 | 5.23 | 13.5 | 12.9 | 42.1 | 40.5 | 0.87 | 1.37 |
| 19 | female | 0.0 | 1695.1 | 2.1 | 68.0 | 6.06 | 48.44 | 4.86 | 4.61 | 14.4 | 13.6 | 43.5 | 43.5 | 1.00 | 1.70 |
| 20 | female | 0.0 | 1667.6 | 3.3 | 51.0 | 8.25 | 32.69 | 4.28 | 4.31 | 12.8 | 13.1 | 40.3 | 40.9 | 1.01 | 2.21 |
| **Mean ♂** | | 3.5 | 1441.9 | 2.2 | 84.9 | 5.84 | 44.21 | 5.17 | 4.88 | 15.55 | 14.77 | 46.49 | 45.17 | 1.14 | 1.84 |
| **SD ♂** | | 8.36 | 717.30 | 1.04 | 59.40 | 1.51 | 14.38 | 0.30 | 0.30 | 0.70 | 0.76 | 2.50 | 2.40 | 0.42 | 0.38 |
| **Student's T-Test p-value ♂** | | 6.68E-06 | | 0.00023 | | 1.78E-07 | | 0.00052 | | 0.00061 | | 0.04448 | | 1.93E-08 | |
| **Mean ♀** | | 0.5 | 1980.4 | 3.1 | 63.2 | 6.47 | 40.29 | 4.76 | 4.50 | 13.67 | 13.20 | 42.12 | 41.27 | 0.94 | 2.02 |
| **SD ♀** | | 1.00 | 416.81 | 0.92 | 20.59 | 1.94 | 9.46 | 0.45 | 0.36 | 0.56 | 0.38 | 1.25 | 1.18 | 0.26 | 0.37 |
| **Student's T-Test p-value ♀** | | 0.00013 | | 0.00150 | | 0.00033 | | 0.02338 | | 0.07126 | | 0.23528 | | 0.00193 | |
| **Mean** | | 2.6 | 1603.4 | 2.5 | 78.4 | 6.03 | 43.04 | 5.04 | 4.77 | 14.99 | 14.30 | 45.18 | 44.00 | 1.08 | 1.89 |
| **SD** | | 7.15 | 687.88 | 1.08 | 51.93 | 1.68 | 13.23 | 0.40 | 0.36 | 1.09 | 0.99 | 2.98 | 2.76 | 0.39 | 0.38 |
| **Student's T-Test p-value** | | 4.26E-09 | | 4.09E-06 | | 9.66E-11 | | 1.84E-05 | | 8.03E-05 | | 0.01686 | | 1.88E-09 | |

cells from the bone marrow into the peripheral blood for subsequent stem cell collection and transplantation.[12] The efficiency of this process can be evaluated by means of fluorescence flow cytometry. Within this study, the number of circulating CD34+ cells was found to increase from 2.5 (± 1.1) to 78.4 (± 52.0) cells per µl of blood (Table 1 and Figure 1). Therefore, the repeated subcutaneous injections of Lenograstim successfully led to an enhanced release of hematopoietic progenitor cells from the bone marrow. The high standard deviation probably indicates individual responses to the treatment with the cytokine, which may vary depending on different parameters such as the age of the stem cell donor.[17]

### Influence of repeated subcutaneous G-CSF injections on blood parameters

In order to elucidate the relevance of G-CSF in a doping control context, the influence of repeated subcutaneous G-CSF injections on different blood parameters was investigated. Leukocytes ($10^3$/µl), red blood cells ($10^6$/µl), hemoglobin (g/dl), hematocrit and percent reticulocytes were measured before and after G-CSF administration (Table 1 and Figure 1). The white blood cell content of all 20 samples increased significantly due to the G-CSF administration from 6.0 x $10^3$ (± 1.7 x $10^3$) to 43.0 x $10^3$ (± 13.2 x $10^3$) cells per µL blood ($p < 0.001$). As the stimulation of granulopoiesis is one of the main functions of G-CSF,[1,5,7] the elevated number of circulating white blood cells (leukocytosis) is the logical consequence of G-CSF administrations. In addition, the percentage of circulating reticulocytes considerably increased from 1.1 % (± 0.4 %) to 1.9 % (± 0.4 %) ($p < 0.001$). As G-CSF generally stimulates the release of hematopoietic progenitor cells from the bone marrow into the peripheral blood, an elevated number of circulating reticulocytes due to repeated G-CSF administrations seems to be conclusive. Additionally, earlier studies have reported positive (side) effects of G-CSF on erythropoiesis and the cytokine was found to induce the release of erythropoietic progenitor cells from the bone marrow into the peripheral blood and their migration to the spleen.[18–20]

By contrast, slight but statistically significant decreases of red blood cell count ($p < 0.001$), hematocrit ($p < 0.05$) and hemoglobin ($p < 0.001$) were observed. The number of circulating erythrocytes declined from 5.0 x $10^6$ (± 0.4 x $10^6$) to 4.8 x $10^6$ (± 0.4 x $10^6$) cells per µl of blood, the amount of hemoglobin from 15.0 g/dl (± 1.1 g/dl) to 14.3 g/dl (± 1.0 g/dl) and the hematocrit from 45.2 % (± 3.0 %) to 44.0 % (± 2.8 %). The lowered values in hematocrit and other volume-dependent parameters might indicate a slight imbalance between hydrostatic and colloid osmotic intravascular pressure due to the elevated number of circulating leukocytes (which contribute with their globular cell structure to the total blood volume in average with approximately 60 ml/L) without inflammatory response or attendance for extravasation. Moreover, as detailed in Table 1, the statistical evaluation of the measured blood parameters might indicate a gender-dependent difference in response to G-CSF administration as the observed decrease in hemoglobin concentration is significant only for male ($p = 0.00061$) and not for female subjects ($p = 0.07126$); however, due to the rather limited number of individuals particularly in the female group, the observed bias will require further studies to verify or falsify the tendency.

Overall, one of the most distinct effects of the repeated G-CSF injections is the massively increased number of circulating leukocytes. This parameter is recorded routinely along with those considered within the Athlete Biological Passport haematological module[21] but its potential relevance for doping control purposes is yet undetermined. The incidence of a leukocytosis might result from severe pathological conditions such as infections or leukemia but also physical exertion can lead to a so-called

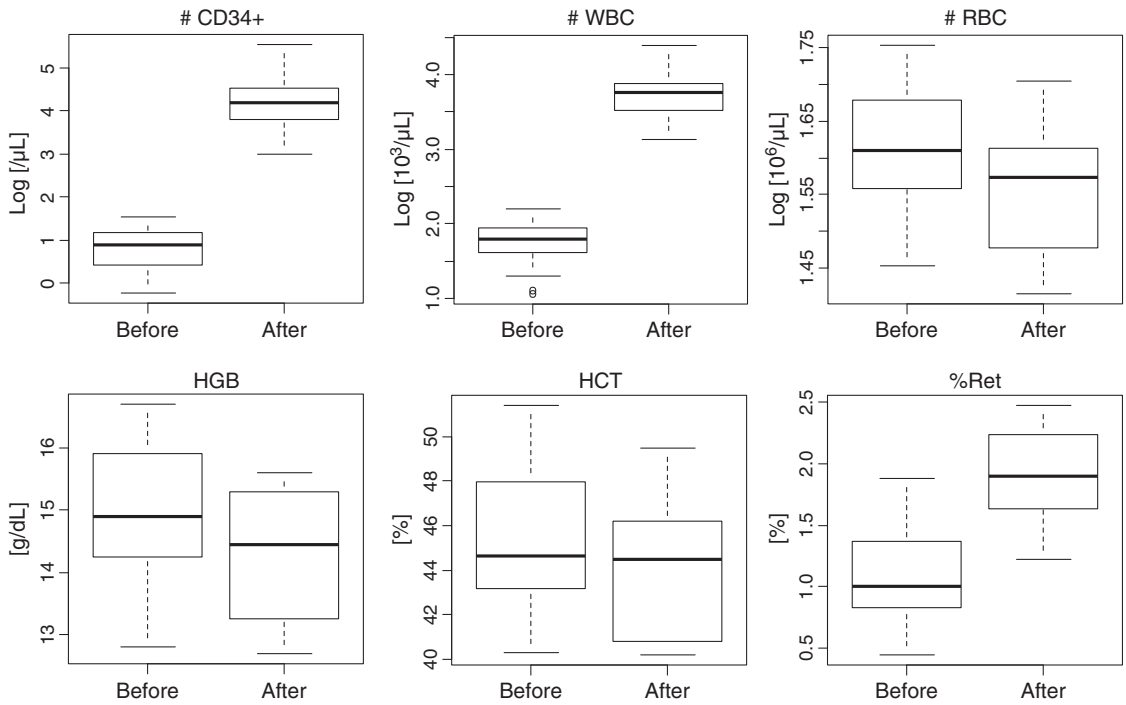


**Figure 1.** Log-transformed box-plot presentation of parameters monitored before and after G-CSF intervention with 20 volunteers.

'stress leukocytosis' with occasionally extremely elevated WBC counts.[22,23] As such, the WBC count might become an interesting measurand to be monitored by anti-doping authorities given the fact that athletes with significantly elevated WBCs due to serious infection or hematologic diseases would not be able to compete or exercise; hence, differential diagnosis of acute leukocytosis in athletes in training or competition would mostly be limited to stress-induced leukocytosis or the use of G-CSF. Consequently, testing athletes with leukocytosis concerning elevated plasma G-CSF levels might be warranted. Since stress leukocytosis resolves within hours, the frequent occurrence of significantly increased WBC counts in- as well as out-of-competition would raise concerns of doping.

## Concluding remarks

In the past, several growth factors such as EPO or human growth hormone (hGH) have gained much attention as performance-enhancing agents.[2,3] Circumstantial evidence suggests that the cytokine G-CSF is misused by athletes and can potentially affect blood parameters relevant for monitoring programs in sports drug testing. Therefore, the aim of this study was to elucidate the relevance of G-CSF in a doping control context by analysing its influence on selected blood parameters included in the Athlete Biological Passport. The analysis of 40 samples collected before and after medication yielded a significant increase of both white blood cell count and percent reticulocytes as well as a simultaneous decrease of red blood cell count, hematocrit and hemoglobin. As these parameters are highly relevant for the validity of blood tests for doping controls and G-CSF was found to have an obliterating effect on blood profiles, its analysis might be indicated in case of unusual findings in blood passport analyses. Such unusual (or atypical) findings concerning blood parameters can also include pathologically elevated WBC counts, which might be relevant from a doping control as well as medical perspective since the origin of a leukocytosis can be manifold and, thus, the information potentially relevant concerning the athlete's health status. In summary, further studies investigating the biological effects of G-CSF and its influence on variables measured within the Athlete Biological Passport as well as the relevance of WBC in a doping control setting might be necessary.

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