BRIEF COMMUNICATION

Effect of circadian variation on neutrophil mobilization to the peripheral blood in benign constitutional neutropenia

João Tadeu Damian Souto Filho[a,b,c], Rodrigo Doyle Portugal[a], and Marcio Nucci[a]

[a]University Hospital, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; [b]Faculdade de Medicina de Campos, Campos dos Goytacazes, Rio de Janeiro, Brazil; [c]Instituto Federal de Educação, Ciência e Tecnologia Fluminense, Campos dos Gotyacazes, Rio de Janeiro, Brazil

Benign constitutional neutropenia (BCN) is an asymptomatic condition characterized by mild chronic neutropenia in patients with no history of recurrent infections. Most patients are referred for further testing, increasing health care costs. We present an alternative form of assessment of individuals with BCN based on neutrophil circadian variation. The objective of this study was to evaluate whether circadian variations of neutrophil counts would result in neutrophil values higher than neutropenia threshold in individuals with BCN. Absolute neutrophil counts (ANCs) were evaluated in 102 patients with BCN (<1500 cells/μL) and 60 age- and sex-matched controls. Paired blood counts were performed in the early morning and in the early afternoon. We observed a significant difference between morning and afternoon ANC in BCN patients (879 cells/μL, 95% CI 745−1028, $p < 0.001$), as well as in the controls (619 cells/μL, 95% CI 443−796, $p < 0.001$). The ANC increase between the two evaluations was significantly greater in BCN patients compared with controls: 83% and 27%, respectively ($p < 0.001$). The ANC increment was higher in the groups with a lower morning ANC: 105%, 63%, and 27% in the <1000, 1000−1500, and >1500 cells/μL groups, respectively ($p < 0.001$). Of all BCN patients, 73% presented with >1500 cells/μL in the afternoon and 16% improved from the <1000 cells/μL to the 1000−1500 cells/μL category. In conclusion, neutrophil circadian variation measurement allows many BCN patients to be excluded from the neutropenia threshold. We identified a simple, easy, and feasible way to assess neutrophil reserve in patients with BCN with a potential reduction in costs of the assessment. © 2018 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.

Benign constitutional neutropenia (BCN), also known as benign ethnic neutropenia, is characterized by chronic neutropenia in non-Caucasian individuals who are apparently healthy and do not have a history of recurrent infections [1−3].

The occurrence of BCN in populations of African origin is linked to polymorphisms in the Duffy Antigen

Receptor Complex (DARC) gene, recently designated Atypical Chemokine Receptor 1 (ACKR1) [4,5]. Although potentially useful, screenings using the DARC/ACKR1 gene are not currently used as diagnostic tools for BCN in clinical practice [6]. BCN diagnosis is not difficult in individuals from these ethnic groups who are healthy and do not have a history of recurrent infections. However, the diagnosis of BCN is more complex and imprecise in countries with mixed ethnicity [6,7].

The health status of individuals with BCN can be determined using diagnostic tests that evaluate the mobilization of neutrophils from reserve compartments [8]. Epinephrine, corticosteroids, endotoxins, and

etiocholanolone can mobilize neutrophils to the peripheral blood [9−12]. Likewise, vigorous exercise increases the peripheral neutrophil count [13,14].

Considering the difficulties associated with the use of external agents or stimulation via exercise to evaluate neutrophil mobilization in subjects with BCN, this study proposes an alternative form based on the circadian variation of neutrophil counts. Similar to many endogenous hormonal substances, neutrophils exhibit a daily physiological increase in healthy individuals accompanied by an increase in the serum levels of granulocyte colony stimulating factor (G-CSF) [15,16].

We hypothesized that a larger neutrophil circadian variation could be detected because patients with BCN have higher G-CSF basal levels [17] and a greater response to this cytokine [18−20]. Therefore, the objective of this study was to evaluate whether circadian variations of neutrophil counts would result in neutrophil values higher than neutropenia threshold in individuals with BCN. The confirmation of physiological increases in neutrophil levels in subjects with BCN could be of great practical interest and could minimize unnecessary tests and reduce costs.

Methods

The study population consisted of consecutive patients with asymptomatic and isolated neutropenia detected on a routine blood test and with diagnostic of BCN from 2008 to 2016 and ANC < 1500 cells/μL. An age- and sex-matched control group was composed of asymptomatic subjects with ANC > 1500 cells/μL. The exclusion criteria were: 1. patients with clinical symptoms, anemia, thrombocytopenia, fever, weight loss, adenomegaly, splenomegaly or alterations in laboratory tests, including liver, kidney, and thyroid function tests, erythrocyte sedimentation rate, C-reactive protein, serologic tests for HIV, hepatitis C and B, antinuclear antibody, and rheumatoid factor, 2. patients older than 60 years because of the higher prevalence of myelodysplasia and other concomitant diseases, and 3. patients with a history of chronic inflammatory, autoimmune, or neoplastic diseases.

Blood was collected by standard phlebotomy and leukocyte differential count was measured with a Sysmex XS-1000i analyzer (Sysmex Corporation, Kobe, Japan). A peripheral blood smear was performed and, after Wright−Giemsa staining, leukocyte differential count and morphology were reviewed. Blood collections were performed in the early morning (from 7:00 a.m. to 9:00 a.m.) and early afternoon (from 1:00 p.m. to 3:00 p.m.). Patients were instructed to sleep 8 hours the night before and not to perform physical activities between the two blood collections.

A pairwise comparison of samples from each subject was performed using Wilcoxon signed-rank test. Continuous variables were compared between groups using Kruskal−Wallis and Mann−Whitney tests. p values < 0.05 were considered statistically significant. Analyses were performed using SPSS Statistics version 21.0 (IBM Corporation, Armonk, NY, USA).

The study was approved by the Institutional Ethics Committee (CAAE 50919215.1.0000.5244) and was conducted in accordance with the principles of the Helsinki Declaration. Informed consent was obtained from all patients for being included in the study.

Results and Discussion

A total of 141 patients with BCN were evaluated and 39 were excluded for the following reasons: 23 were older than 60 years and 16 had other diseases, including: cancer (three cases), hypo- or hyperthyroidism (three cases), hepatitis C (three cases), bariatric surgery (two cases), osteoarthritis (two cases), cirrhosis (two cases), and systemic lupus erythematosus (one case). A total of 102 patients with BCN and 60 controls were included in the study (Table 1).

We observed a significant difference between morning and afternoon ANC in individuals with BCN (1038 vs. 1836, median increase of 879 cells/μL, 95% CI 745−1028, $p < 0.001$), as well as in the controls (1958 vs. 2766, median increase of 619 cells/μL, 95% CI 443−796, $p < 0.001$, Figure 1). The median difference of ANC was significantly greater in BCN patients compared with controls (879 vs. 619 cells/μL, $p = 0.012$), which represents an increase from morning ANC of 83% and 27%, respectively ($p < 0.001$). The ANC increment between the two evaluations was higher in the groups with a lower morning neutrophil count (105%, 63%, and 27% in the <1000, 1000−1500,

Table 1. Characteristics of study participants

Characteristic	BCN Patients ($n = 102$)	Controls ($n = 60$)
Male: female	36:66	23:37
Age (years), median (range)	40 (3−60)	44 (5−60)
Hemoglobin (g/dL), median (range)	13.0 (11.8−15.9)	13.4 (11.9−17.2)
Platelet count (cell × 10^3/μL), median (range)	226 (159−393)	214 (163−318)
Morning white blood count (cells/μL), median (range)	2800 (1770−4500)	3800 (2610−7800)
Morning ANC (cells/μL), median (range)	1038 (422−1490)	1958 (1511−4690)
Morning lymphocyte count (cells/μL), median (range)	1428 (627−2457)	1482 (598−3400)
Afternoon white blood count (cells/μL), median (range)	3960 (2000−7600)	5050 (2900−10500)
Afternoon ANC (cells/μL), median (range)	1836 (620−5350)	2766 (1509−7665)
Afternoon lymphocyte count (cells/μL), median (range)	1680 (510−2850)	1748 (928−3480)
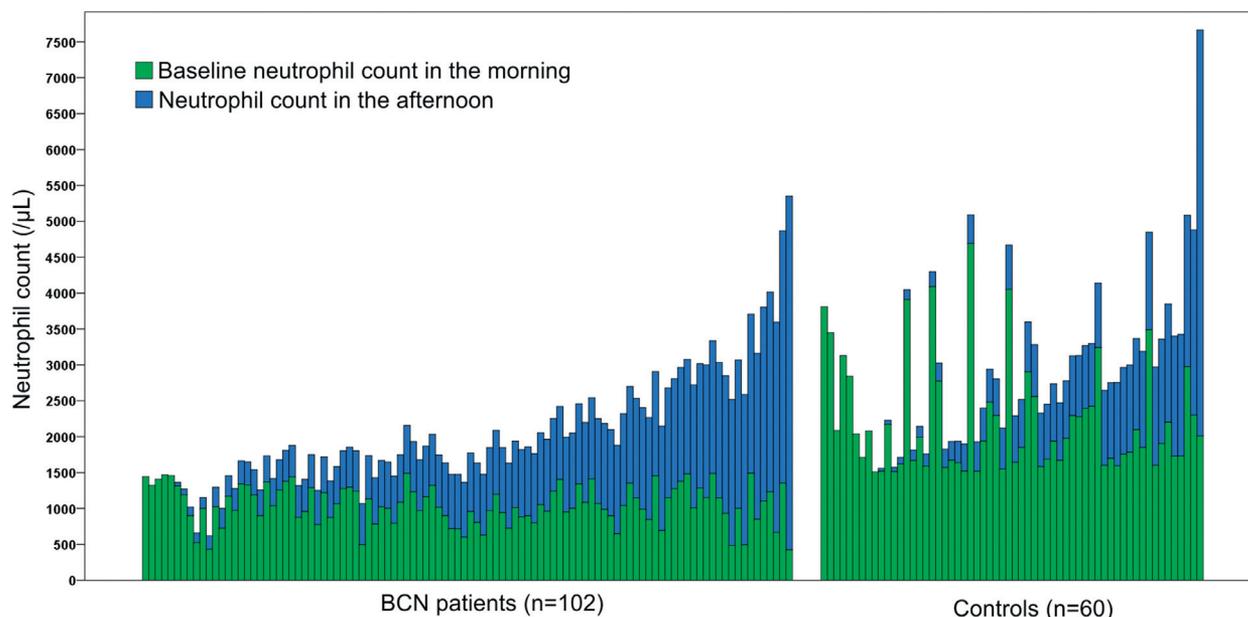
Figure 1. ANCs performed in the morning and afternoon in BCN patients ($n = 102$) and controls ($n = 60$).

and >1500 cells/μL groups, respectively; $p < 0.001$; Table 2).

Of 102 BCN patients with morning ANC < 1500 cells/μL, 74 (73%) presented with >1500 cells/μL in the afternoon and 16 (16%) improved from the <1000 cells/μL to the 1000 −1500 cells/μL category. Only 12 (12%) remained in the same neutrophil category and five of them had lower ANC in afternoon evaluation; however, this was insufficient to reduce the count to a lower category versus the morning test.

Our study showed that a neutrophil count performed in the afternoon allows many BCN patients to be excluded from the neutropenia threshold. This finding could help to determine whether individuals with BCN have an adequate number of neutrophils available when needed or if they have difficulty in mobilization in response to infections.

Possible explanations for these results are based on the circadian variation of neutrophil levels in healthy individuals, considering that neutrophil counts exhibit a trough at 8:00 a.m. and a progressive increase in the afternoon with

peak at 8:00 p.m. [15,21]. This daily increase is accompanied by an increase in serum G-CSF levels [16] and a downregulation of bone marrow expression of CXCL12, a major retention factor for cells, and its receptor CXCR4 on hematopoietic stem cells (HSCs) and neutrophils [22,23]. In addition, people of African descent have high levels of serum G-CSF versus Caucasians [17] and many studies involving donors of HSCs reported a greater proliferative and mobilization response to exogenous G-CSF among subjects with African descent than among individuals with normal baseline neutrophil counts [18−20]. These conditions can promote a greater physiological mobilization of peripheral blood neutrophils in the early afternoon in patients with BCN.

These results could also be associated with diurnal variation of endogenous cortisol and epinephrine levels [23]. Because ANC increases after exogenous glucocorticoids administration, the afternoon ANC rise could be driven by cortisol peak [10,12]. Therefore, it could resemble a positive glucocorticoid stimulation test without the glucocorticoid

Table 2. Difference between the ANCs performed in the morning and afternoon stratified by neutrophil categories

Neutrophil Category (cells/μL, n)	Median Morning ANC (cells/μL, range)	Median Afternoon ANC (cells/μL, range)	Median Neutrophil Difference (cells/μL, 95% CI)	Median Neutrophil Proportional Increase
<1000 (44)[a]	862 (422−1000)	1722 (620−5350)	903 (745−1126)[c]	105%[d]
1000−1500 (58)[a]	1250 (1001−1490)	1906 (1078−4868)	854 (662−1065)[c]	62%[d]
>1500 (60)[b]	1958 (1511−4690)	2766 (1509−7665)	619 (443−796)[c]	27%[d]

[a]BCN patients ($n = 102$).

[b]Control subjects ($n = 60$).

[c]Kruskal−Wallis test comparing three groups ($\chi^2 = 7.1$, $p = 0.029$).

[d]Kruskal−Wallis test comparing three groups ($\chi^2 = 46.5$ and $p < 0.001$). Mann−Whitney U test for post hoc pairwise comparisons: <1000 vs. 1000−1500, $p = 0.009$; all other groups, $p < 0.001$.

administration. Other possibilities for a higher afternoon ANC could be the physiological stimulation of epinephrine caused by daily activities and stress response associated with the first sample [11,13,24].

The greater neutrophil circadian variation in BCN patients could be supported by its association with polymorphism in the DARC/ACKR1 gene and its effect on leukocyte migration [4,5]. This chemokine receptor controls the levels of circulating chemokines and consequently affects neutrophil stimulation, chemotaxis, and migration [25,26]. There is substantial evidence that DARC-null neutrophils have slightly expressed gene in pathways related to HSC mobilization and leukocyte migration, leading to activated neutrophils egressing from circulation to the spleen (and possibly other organs) and causing relative neutropenia [27]. In this way, both mechanisms can undergo interference from circadian biological variations, resulting in greater afternoon ANC increase in BCN patients.

To the best of our knowledge, this is the first study to assess ANC at different times of the day for the evaluation of BCN patients. Neutrophil circadian variations also have been described in psychiatric patients with neutropenia caused by clozapine and risperidone, a situation called morning pseudoneutropenia [28]. A study of 10 patients using clozapine demonstrated a significant neutrophil increase (1130 cells/μL, $p = 0.003$) after sampling time change [29].

Our study has some limitations. It started in 2008, when the relationship between BCN and the DARC/ACRK1 gene polymorphisms was not known. Therefore, we did not perform genetic testing. Without a genetic ancestry marker, the study population was not stratified by ethnicity. Defining race is always imprecise in countries with a high frequency of inter-ethnic admixture [7]. Several studies demonstrated a week correlation between skin color or self-declared race and genetic ancestry [30]. In this way, we avoid using nongenetic ethnic stratification of case and control groups to prevent confounding results leading to erroneous conclusions [31].

In conclusion, neutrophil circadian variation measurement allows many BCN patients to be excluded from the neutropenia threshold. Our findings may affect clinical practice considering that neutrophil counts performed in the afternoon are a simple, easy, and feasible way to assess neutrophil reserve in patients with BCN and can potentially minimize unnecessary tests and reduce costs.

Acknowledgments

Conflict of interest disclosure

The authors declare no competing financial interests.

References

1. Gibson C, Berliner N. How we evaluate and treat neutropenia in adults. Blood. 2014;124:1251−1258.
2. Haddy TB, Rana SR, Castro O. Benign ethnic neutropenia: what is a normal absolute neutrophil count? J Lab Clin Med. 1999;133:15−22.
3. Thobakgale CF, Ndung'u T. Neutrophil counts in persons of African origin. Curr Opin Hematol. 2014;21:50−57.
4. Nalls MA, Wilson JG, Patterson NJ, et al. Admixture mapping of white cell count: genetic locus responsible for lower white blood cell count in the Health ABC and Jackson Heart studies. Am J Hum Genet. 2008;82:81−87.
5. Grann VR, Ziv E, Joseph CK, et al. Duffy (Fy), DARC, and neutropenia among women from the United States, Europe and the Caribbean. Br J Haematol. 2008;143:288−293.
6. Migdady Y, Olszewski AJ. Evaluation of incidental neutropenia in a multi-ethnic setting. Int J Lab Hematol. 2013;35:e6−e8.
7. Dinardo CL, Kerbauy MN, Santos TC, et al. Duffy null genotype or Fy(a-b-) phenotype are more accurate than self-declared race for diagnosing benign ethnic neutropenia in Brazilian population. Int J Lab Hematol. 2017;39:e144−e146.
8. Baldwin C, Roath S. The evaluation of neutropenia: the use of the granulocyte mobilization test. Clin Lab Haematol. 1983;5:353−360.
9. Cream JJ. Prednisolone-induced granulocytosis. Br J Haematol. 1968;15:259−267.
10. Dale DC, Fauci AS, Guerry D, IV WolffSM. Comparison of agents producing a neutrophilic leukocytosis in man: Hydrocortisone, prednisone, endotoxin, and etiocholanolone. J Clin Invest. 1975;56:808−813.
11. Joyce RA, Boggs DR, Hasiba U, Srodes CH. Marginal neutrophil pool size in normal subjects and neutropenic patients as measured by epinephrine infusion. J Lab Clin Med. 1976;88:614−620.
12. Mason BA, Lessin L, Schechter GP. Marrow granulocyte reserves in black Americans. Hydrocortisone-induced granulocytosis in the "benign" neutropenia of the black. Am J Med. 1979;67:201−205.
13. Phillips D, Rezvani K, Bain BJ. Exercise induced mobilisation of the marginated granulocyte pool in the investigation of ethnic neutropenia. J Clin Pathol. 2000;53:481−483.
14. Bain BJ, Phillips D, Thomson K, Richardson D, Gabriel I. Investigation of the effect of marathon running on leucocyte counts of subjects of different ethnic origins: relevance to the aetiology of ethnic neutropenia. Br J Haematol. 2000;108:483−487.
15. Haus E, Smolensky MH. Biologic rhythms in the immune system. Chronobiol Int. 1999;16:581−622.
16. Jilma B, Hergovich N, Stohlawetz P, Eichler HG, Bauer P, Wagner OF. Circadian variation of granulocyte colony stimulating factor levels in man. Br J Haematol. 1999;106:368−370.
17. Mayr FB, Spiel AO, Leitner JM, Firbas C, Kliegel T, Jilma B. Ethnic differences in plasma levels of interleukin-8 (IL-8) and granulocyte colony stimulating factor (G-CSF). Transl Res. 2007;149:10−14.
18. Carilli AR, Sugrue MW, Rosenau EH, Chang M, Fisk D, Medei-Hill M, et al. African American adult apheresis donors respond to granulocyte-colony-stimulating factor with neutrophil and

progenitor cell yields comparable to those of Caucasian and Hispanic donors. Transfusion. 2012;52:166–172.

19. Hsu JW, Wingard JR, Logan BR, et al. Race and ethnicity influences collection of granulocyte colony-stimulating factor-mobilized peripheral blood progenitor cells from unrelated donors, a Center for International Blood and Marrow Transplant Research analysis. Biol Blood Marrow Transplant. 2015;21:165–171.

20. Vasu S, Leitman SF, Tisdale JF, et al. Donor demographic and laboratory predictors of allogeneic peripheral blood stem cell mobilization in an ethnically diverse population. Blood. 2008;112:2092–2100.

21. Casanova-Acebes M, Pitaval C, Weiss LA, et al. Rhythmic modulation of the hematopoietic niche through neutrophil clearance. Cell. 2013;153:1025–1035.

22. Lucas D, Battista M, Shi PA, Isola L, Frenette PS. Mobilized hematopoietic stem cell yield depends on species-specific circadian timing. Cell Stem Cell. 2008;3:364–366.

23. Scheiermann C, Kunisaki Y, Frenette PS. Circadian control of the immune system. Nat Rev Immunol. 2013;13:190–198.

24. Golan K, Kumari A, Kollet O, et al. Daily onset of light and darkness differentially controls hematopoietic stem cell differentiation and maintenance. Cell Stem Cell. 2018;23:572–585.

25. Nibbs RJ, Graham GJ. Immune regulation by atypical chemokine receptors. Nat Rev Immunol. 2013;13:815–829.

26. Duchene J, Novitzky-Basso I, Thiriot A, et al. Atypical chemokine receptor 1 on nucleated erythroid cells regulates hematopoiesis. Nat Immunol. 2017;18:753–761.

27. Charles BA, Hsieh MM, Adeyemo AA, et al. Analyses of genome wide association data, cytokines, and gene expression in African-Americans with benign ethnic neutropenia. PLoS One. 2018;13:e0194400.

28. Esposito D, Chouinard G, Hardy P, Corruble E. Successful initiation of clozapine treatment despite morning pseudoneutropenia. Int J Neuropsychopharmacol. 2006;9:489–491.

29. McKee JR, Wall T, Owensby J. Impact of complete blood count sampling time change on white blood cell and absolute neutrophil count values in clozapine recipients. Clin Schizophr Relat Psychoses. 2011;5:26–32.

30. Leite TK, Fonseca RM, de Franca NM, Parra EJ, Pereira RW. Genomic ancestry, self-reported "color" and quantitative measures of skin pigmentation in Brazilian admixed siblings. PLoS One. 2011;6:e27162.

31. Manta FS, Pereira R, Caiafa A, Silva DA, Gusmao L, Carvalho EF. Analysis of genetic ancestry in the admixed Brazilian population from Rio de Janeiro using 46 autosomal ancestry-informative indel markers. Ann Hum Biol. 2013;40:94–98.