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ORIGINAL ARTICLE

Using blood calprotectin as a measure of blood neutrophils

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ABSTRACT

Presently, bed-side or at home quantification of neutrophils in blood (b-neutrophils) is not practical, because cytometric methods are too expensive and technically demanding. We have explored whether calprotectin concentration in whole blood (b-calprotectin) might be a valid measure of b-neutrophils because this principle might be used in a simple and robust immunoassay device. We obtained heparin blood samples from 77 patients with possible neutropenia, most of them cancer patients treated with cytostatic drugs, and compared b-calprotectin with their b-neutrophils in a simultaneously taken EDTA-blood sample. The Spearman rank correlation coefficient between b-calprotectin and b-neutrophils was 0.986 ($p < .0001$). In a regression model of b-neutrophils as a function of age, gender, type of hematology instrument, total leukocyte count minus neutrophils, b-calprotectin, and plasma calprotectin (p-calprotectin), only b-calprotectin was a statistically significant predictor. B-neutrophils below 1×10^9/L was unlikely if b-calprotectin was above 50 mg/L. In conclusion, b-calprotectin, without adjusting for p-calprotectin, correlates closely with b-neutrophils and could be used to detect b-neutrophils below 1×10^9/L.

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Introduction

Quantification of neutrophil granulocytes (measuring b-neutrophils) are regularly done in many clinical situations, for instance in monitoring cancer patients using cytostatic medications, where neutropenia is a real danger [1]. Such measurements are easily done with modern hematology instruments [2], at least when the cells are mature and normal. However, if bed-side monitoring of b-neutrophils is wanted, just a few assays are available [3,4], and none is designed for home use. For cancer patients in home care, or for patients in remote areas where hematology instruments are lacking, a robust, bed-side assay of b-neutrophils might be useful. In the hematology laboratory, neutrophil identification and counting is usually based on detection of electrical and optical properties of the cells, while a few instruments use digital image analysis [2]. We explored a chemical method of quantifying b-neutrophils, using an immunoassay to measure the concentration of calprotectin in whole blood (b-calprotectin) [5]. This principle could be applicable to a lateral flow format [6] for bed-side testing. In healthy individuals, we previously demonstrated a firm correlation between b-neutrophils and a measure of calprotectin in neutrophils, i.e. b-calprotectin adjusted for the calprotectin concentration in plasma (p-calprotectin) [5].

However, if adjusting for the plasma concentration was unnecessary, sample centrifugation to obtain plasma could be avoided and a bed-side testing situation much relieved. If the median cell volume of neutrophils is about 300 fL [7], the median intracellular concentration of calprotectin is about 30 pg/300 fL = 100,000 mg/L, or some 100,000 times the median plasma concentration of 1.1 mg/L [5]. An individual with b-neutrophils of 4×10^9/L may have a neutrophil volume in 1 L blood of $4 \times 10^9 \times 300$ fL = 1.2 mL. So even if the plasma volume in 1 L blood is, say, 600 mL and 500 times the neutrophil volume, the concentration difference outweighs the volume difference by a factor of 200, and causes b-calprotectin to be almost exclusively dependent on b-neutrophils. Accordingly, we studied the correlation between b-neutrophils and b-calprotectin in patients with possible neutropenia, without adjusting for p-calprotectin. The results from this work may be useful for those wanting to make an assay for bed-side testing.

Methods

Population

Patients treated at St. Olavs hospital were asked to donate blood in two heparin vacuum tubes if they were treated

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with cytostatic drugs. The extra blood samples were taken simultaneously with blood samples for routine hematology testing, after written, informed consent was given. Permission was granted by the Regional Committees for Medical and Health Research Ethics (2019/580/REK sør-øst). Blood sampling was complete in 77 patients, 43 women aged 30–79 (median 59) years and 34 men aged 32–84 (median 66) years. They had various clinical diagnoses, 72 had some form of cancer, of which breast cancer was the most frequently diagnosed condition (16 (37%) of women). Consequently, most of the patients (83%) used a variety of cytostatic drugs. Routine blood samples for b-neutrophils and other hematology analyses were taken at relevant times for monitoring the cytotoxic effects and/or disease activity. We also used data from 120 healthy blood donors, part of which are previously published [5]. They were 55 women aged 20–69 (median 34) years, and 65 men aged 22–73 (median 40) years.

Laboratory methods

Blood samples for measuring b-neutrophils and other routine hematology analyses were obtained in 4 mL Vacuette K_2-EDTA tubes. Hematology analyses in patients were done using a Siemens Advia 2120i for 66 patients, Sysmex XN analyzers for 10 patients, and an Abbott CELL-DYN Sapphire analyzer for 1 patient. Sysmex XN analyzers were used for blood donors [5]. Quality control routines in the laboratory, including external quality control, ensured that there were no analytical problems with the instruments that were used to measure the blood samples, and that the neutrophil count was equally measured on the various instruments.

Blood samples for measuring b- and p-calprotectin were obtained in 4 mL Vacutainer tubes anticoagulated with lithium-heparin (Becton Dickinson, Franklin Lakes, NJ, USA). To obtain plasma, the vacuum tubes were centrifuged at 2000 × g for 10 min within 30 min after sampling. Plasma and blood were kept frozen at −80 °C and were analysed within 8 weeks after collection, mainly in batches of 10–20 samples, but the number of samples in each batch was sometimes less because of the availability of relevant patients for the project.

Calprotectin was measured by the Bühlmann fCAL® turbo test (Bühlmann Laboratories AG, Schönenbuch, Switzerland) using a Siemens Advia XPT analyzer (Siemens, Erlangen, Germany), as previously described [5]. Basically, p-calprotectin was measured in undiluted plasma samples, while b-calprotectin was measured after extraction with M-PER® Mammalian Protein Extraction Reagent (ThermoFisher, Pittsburgh, PA, USA) and B-CAL-EX from Bühlmann. For b-calprotectin the intermediate (total) coefficient of variation was 4.9% at a concentration of 50.2 mg/L, and for p-calprotectin 3.5% at a concentration of 0.517 mg/L [5].

Statistical methods

Distributions were given as range and median, and correlation between quantitative variables as the Spearman rank correlation coefficient. Wilcoxon rank-sum test was used to test the difference between the medians in two groups. Regression models of quantitative variables as a function of quantitative and categorical variables were estimated with multivariable fractional polynomials [8] to allow for non-linear associations between the dependent variable and quantitative prediction variables. p Values less than .05 were considered statistically significant. The Stata software, version 14 (StataCorp, College Station, TX, USA) was used for all statistical analyses.

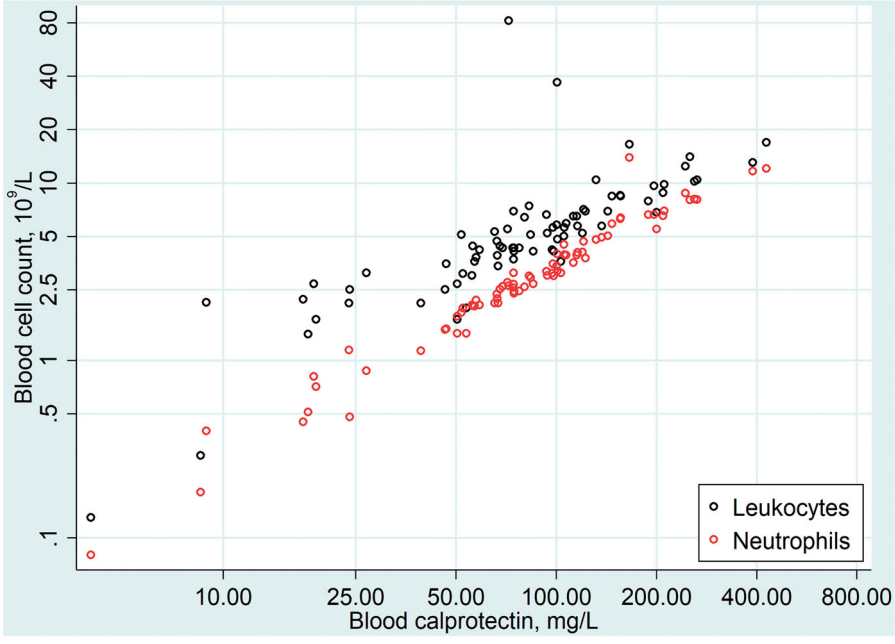

Figure 1. Cell counts of leukocytes (total) and neutrophils in blood plotted against the whole blood calprotectin concentration in 77 patients. Both axes are logarithmic.

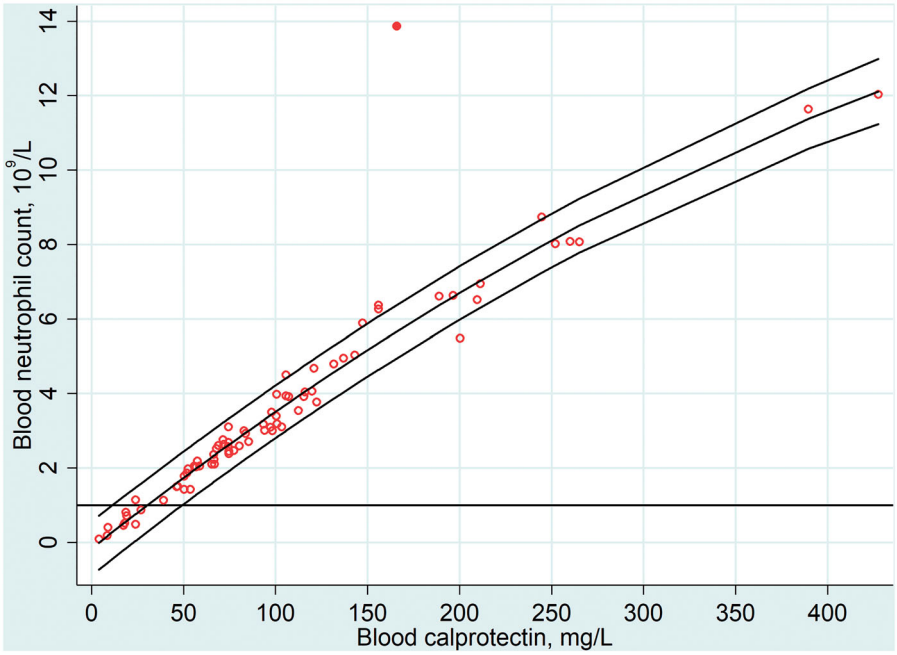

Figure 2. Cell counts of neutrophils in blood plotted against the whole blood calprotectin concentration in 77 patients. One patient with an exceptionally low whole blood calprotectin concentration per neutrophil (12 pg) is seen at the top. The neutrophil count as a function of the whole blood calprotectin concentration is shown, along with the 95% prediction interval (for new observations). The horizontal line indicates a neutrophil count of 1×10^9/L.

Results

B-neutrophils in the 77 patients were 0.08–13.9 (median 2.9) $\times$ 10^9/L, and total leukocyte counts 0.13–82.1 (median 5.1) $\times$ 10^9/L. B-calprotectin was <4.00–428 (median 82.8) mg/L. One patient with b-neutrophils of 0.08×10^9/L had b-calprotectin less than the lower limit of quantification, 4.00 mg/L, and was registered with that value. P-calprotectin was 0.338–11.9 (median 1.29) mg/L. P-calprotectin above the upper reference limit of 3.03 mg/L [5] was found in 14 (18%) of patients.

The correlation between b-calprotectin and b-neutrophils in the 77 patients is shown in Figure 1, which also indicates the total blood leukocyte count. The two patients with a total leukocyte count above 20×10^9/L were both diagnosed with chronic lymphocytic leukemia. B-calprotectin divided by b-neutrophils (b-calprotectin per neutrophil) in those patients were 25 and 27 pg, not different from the other 75 patients, where b-calprotectin per neutrophil ranged from 21 to 50 (median 30) pg, except from one patient with 12 pg b-calprotectin per neutrophil. In the 120 blood donors, the corresponding figures were 22–37 (median 29) pg, statistically not significantly different from the patients ($p = .48$). The Spearman rank correlation coefficient between b-calprotectin and b-neutrophils was 0.986 ($p < .0001$) in patients and 0.957 ($p < .0001$) in blood donors. The corresponding figures for p-calprotectin were 0.435 ($p < .0001$) in patients and 0.466 ($p < .0001$) in blood donors, respectively.

In patients, using a multivariable fractional polynomials regression model of b-neutrophils as a function of age, gender, type of hematology instrument, total leukocyte count minus neutrophils, b-calprotectin, and p-calprotectin, only b-calprotectin was a statistically significant variable. B-neutrophils as a fractional polynomials regression model of only b-calprotectin is shown in Figure 2, along with the 95% prediction interval (for new observations). The line indicating b-neutrophils of 1×10^9/L intersects with the lower limit of the prediction interval at a b-calprotectin of about 50 mg/L, indicating that patients with b-neutrophils below 1×10^9/L will have b-calprotectin below 50 mg/L. The patient with 12 pg b-calprotectin per neutrophil was visually considered to be an outlier (Figure 2), and data from that case were not included in the regression model.

Discussion

We have shown that b-calprotectin correlated closely with b-neutrophils in patients monitored for neutropenia, most of whom were cancer patients treated with cytostatic drugs. Furthermore, p-calprotectin did not provide significant information in predicting b-neutrophils. This is fortunate because it makes bedside instruments more easy to design, as no plasma measurements are needed.

Apparently, the abnormally high p-calprotectin in 18% of the patients did not override the basic ideas in the Introduction, that the relation between b-neutrophils and b-calprotectin should be virtually independent of the plasma concentration. One might be concerned that the plasma concentration still mattered in cases where b-neutrophils were very low. We had data from 9 patients with b-neutrophils below 1×10^9/L, a warning limit of significant neutropenia [1]. In those patients, the relation between b-neutrophils and b-calprotectin were the same as in the other patients (Figure 2).

The median b-calprotectin per neutrophil was not different in patients and healthy blood donors, 29–30 pg in both groups. Fagerhol et al. reported a comparable calprotectin content in neutrophils of 19–31 pg in healthy individuals;

however, those figures were adjusted for p-calprotectin and measured with a different method, and so they are not directly comparable [9]. The patient with just 12 pg b-calprotectin per neutrophil was clearly an outlier (Figure 2). We did not find an explanation for this in the clinical data, as neither diagnosis nor treatment was exceptional. Unfortunately, no measurements were repeated, so we can not exclude that these findings were due to technical errors.

The calprotectin content in other cell types was too low to be of any concern in the studied patients, as clearly shown by the two patients with a normal b-calprotectin per neutrophil despite a very high total leukocyte count due to chronic lymphocytic leukemia. However, we did not obtain blood samples from patients with a large number of myeloblasts, promyelocytes, myelocytes, and metamyelocytes in blood, so we do not know how the presence of those cells might disturb the correlation between b-calprotectin and b-neutrophils.

Another issue if b-neutrophils are to be calculated from b-calprotectin is the inter-individual variation of b-calprotectin at a certain level of b-neutrophils. Reading from Figure 2, at a level of 1×10^9/L, mean b-calprotectin is approximately 30 mg/L and the 95% prediction interval is approximately from 10 mg/L to 50 mg/L. The 95% prediction interval is equal to mean ±2 times the total (biological and analytical) standard deviation, i.e. the width of the prediction interval is about 4 times the total standard deviation. The total coefficient of variation is then $100\% \times ((50-10)/4)/30 = 33\%$. Given an analytical coefficient of variation equal to 5% for b-calprotectin (Methods), this indicates a total biological variation of $(33^2-5^2)^{0.5} = 32.6\%$. Using the present assay, patients with b-neutrophils below 1×10^9/L would be expected to have a b-calprotectin below 50 mg/L (Figure 2). In a lateral flow assay designed for bedside use, the analytical coefficient of variation is expected to be higher. For instance, increasing the analytical coefficient of variation 4 times from 5% til 20% would increase the total coefficient of variation from 33% to $(32.6^2 + 20^2)^{0.5} = 38\%$. In that case, patients with b-neutrophils below 1×10^9/L would be expected to have a b-calprotectin below 30 mg/L + $2 \times 0.38 \times 30$ mg/L = 53 mg/L. This might be acceptable and obtainable.

Ideally, sample extraction should be avoided for a bedside assay. At present, we do not see how b-calprotectin could be measured without an extraction step.

In conclusion, we have shown that b-calprotectin correlates closely with b-neutrophils in patients monitored for neutropenia, and could be used to detect b-neutrophils below 1×10^9/L.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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