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Unique characteristics of leukocyte volume, conductivity and scatter in chronic myeloid leukemia

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ABSTRACT

Background: Modern automated hematology analyzers provide quantitative data on leukocyte size and structure that may be useful to distinguish reactive from neoplastic cellular proliferations. We compared leukocyte volume, conductivity and scatter (VCS) characteristics of chronic myeloid leukemia (CML), bcr-abl1-positive patients with those of non-neoplastic neutrophilia.

Materials and methods: Complete blood counts and VCS data (LH750 hematology analyzers, Beckman Coulter) from 38 newly-diagnosed CML patients, 65 CML on imatinib mesylate therapy, 58 patients with elevated age-specific neutrophil counts due to varied causes, 100 pregnant women and 99 healthy controls were collated and compared. Receiver-operating-characteristic curves, logistic regression models and classification trees were studied for their abilities to distinguish various groups.

Results: Untreated CML had higher mean neutrophil volume and mean monocyte volume (MNV and MMV), mean lymphocyte scatter (MLS) and higher standard deviations of the mean neutrophil volume and conductivity (MNV-SD and MNC-SD) over all other groups ($p < 0.0001$ for all). MNV, MNC-SD and MLS distinguished CML from reactive neutrophilia + pregnancy groups (sensitivities 89.5%, 94.7%, 94.7% and specificities 90.6%, 95.6% and 94.0% respectively). Combination of MNV>163.0 AND MNC-SD>12.69 was 89.5% sensitive and 100% specific for CML. Two algorithmic classification-tree approaches using VCS parameters alone (i.e. without the aid of blood count parameters) correctly separated 100% cases of untreated CML from all others.

Conclusion: Successful distinction of untreated but not post-imatinib CML patients from subjects who were either normal, pregnant or had reactive neutrophilia by automated analyzer-derived cell-population data opens possibilities for their applications in diagnosing and understanding the pathogenesis of CML.

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At a glance commentary

Scientific background on the subject

Automated hematology analyzers generate quantitative data on the volume, conductivity and scatter (VCS) parameters of circulating leukocytes. These have been shown to be of diagnostic value in sepsis, bacterial, parasitic and viral infections, lymphocytic proliferations and myelodysplastic syndromes. These indices are available rapidly and at a low cost.

What this study adds to the field

We found significant differences in the VCS characteristics of leukocytes from chronic myeloid leukemia (CML) patients versus those with reactive neutrophilia. These changes were observed in multiple cellular lineages and reverted to near-normal after successful therapy. This opens possibilities for the application of our results in diagnosis and monitoring of CML.

Modern automated hematology analyzers, in addition to accurately and rapidly enumerating and classifying blood cells, also yield several quantitative leukocyte parameters. These have been shown to be valuable in myriad clinical settings. The volume, conductivity and scatter parameters (VCS, Beckman Coulter Inc., Table 1), for instance, have been applied to the study of sepsis in neonates [1] and the elderly [2], bacterial infections [3], malaria and dengue [4] etc. Analyzers have also been studied for their ability to diagnose various causes of lymphocytosis [5] and to detect myelodysplasia [6]. However, no systematic study has previously attempted to evaluate leukocytic data for their ability, if any, to differentiate reactive neutrophilic states from chronic myeloid leukemia (CML).

One reason for this lacuna is that in the vast majority of CML, the total and differential leukocyte counts, supplemented by the leukocyte alkaline phosphatase (LAP) score are sufficient to make a low-cost presumptive diagnosis and guide further work-up. However, we postulated that assessing the analyzer's leukocyte cell population data might help glean insights into basic differences between cells in different conditions. Thus, we studied VCS indices in CML patients before and after imatinib therapy and compared them to reactive neutrophilic states including the physiological neutrophilia of pregnancy. The aim was to identify differences, if any, and to possibly develop software-based flagging algorithms to distinguish benign from CML-associated neutrophilia.

Materials and methods

This prospective cohort study was conducted from January to September 2015 in the Hematology Department of a tertiary-level, state-funded, teaching hospital. The following study groups were enrolled from among persons undergoing testing in various departmental laboratories:

Group 1: Newly-diagnosed CML patients (n = 38). The diagnosis of CML was suspected based on clinical findings, blood counts, blood film morphology and the cytochemical LAP score. It was confirmed in all patients by conventional cytogenetics for the Philadelphia chromosome and reverse-transcriptase polymerase chain reaction (RT-PCR) for the *bcr-abl1* fusion transcripts as described previously [7]. Pediatric CML (age <16 years) and patients who had been previously treated were excluded. Patients were enrolled regardless of the phase of CML (chronic phase as well as those who initially presented in acceleration or blast crisis).

Group 2: CML patients on imatinib therapy (n = 65). This group included previously diagnosed CML patients who were being followed-up at intervals of least every three months while on treatment with imatinib mesylate.

Group 3: Patients with reactive neutrophilia (n = 58). Adult patients presenting to the medical or surgical emergency, with an absolute neutrophil count $\geq 8.5 \times 109/L$ and without any evidence of a hematological malignancy on hemogram including differential leukocyte counts were enrolled.

Group 4: Pregnant women (n = 100). Pregnant women undergoing complete blood counts as part of the routine antenatal laboratory work-up were included. Those with febrile illnesses or any known co-morbidities were excluded.

Group 5: Healthy controls (n = 99). Baseline hemogram data from self-reported healthy individuals with normal range blood counts were recruited as controls.

Clinical and demographic details of all patients (i.e. groups 1 to 4) were accessed from clinical charts, laboratory records, and the hospital information system. All 360 blood samples were anticoagulated with dipotassium Ethylenediamine tetra acetato (EDTA) (1.5 mg per ml of blood) and were subjected to Complete blood count with differential leukocyte count mode on automated analyzer (CBC + DIFF) mode analysis (complete blood count with differential leukocyte count, DLC) on one of two LH750™ automated analyzers (Beckman Coulter Inc, Miami, FL, USA) within 6 h of collection. Twenty Complete blood count (CBC) parameters, warnings, flags and the complete VCS indices were recorded. The 18 VCS parameters we studied are listed in Table 1 and the derivation of their nomenclature explained. Rigorous quality control was exercised on both instruments including VCS optimization, regular Latron™ controls and periodic recalibrations.

Statistical analysis

The non-parametric Mann–Whitney U and Levene tests were used to compare parameters between the groups due to the non-Gaussian, wide spreads of the VCS parameters, with several showing standard deviations in excess of their means. Receiver-operating-characteristic (ROC) curves and areas-under-curve were evaluated to compare diagnostic efficiencies. Logistic regression models yielding dichotomous

Table 1 An explanation of the 18 VCS parameters analyzed in this study.

	Neutrophils		Lymphocytes		Monocytes	
Volume	MNV	MNV-SD	MLV	MLV-SD	MMV	MMV-SD
Conductivity	MNC	MNC-SD	MLC	MLC-SD	MMC	MMC-SD
Scatter	MNS	MNS-SD	MLS	MLS-SD	MMS	MMS-SD

The first alphabet M in each parameter refers to "Mean", the second alphabet (N/L/M) refers to the cell type (neutrophil, lymphocyte or monocyte respectively), the third alphabet (V/C/S) refers to the cell property measured by the analyzer (volume, conductivity or scatter respectively). Each parameter has the mean value, as well as a standard deviation (a measure of its spread).

outcomes using CBC and VCS parameters were computed and their diagnostic efficacies compared, also using ROC curves. And finally, classification trees constructed using VCS parameters alone were assessed for their abilities to separate untreated CML cases from benign controls (groups 3, 4 and 5). All analyses were done using MedCalc™ software (v.12.7.0, Ostend, Belgium).

Results

The demographic and salient CBC findings of the newly diagnosed-versus-treated CML patients are given in Table 2.

Analysis of VCS parameters was approached in the following three steps. First, we compared parameters in group 1 (untreated CML) against groups 3, 4 and 5 (all benign controls) to detect differences between neoplastic and non-neoplastic leukocytes. CML cases displayed significantly greater mean neutrophil and monocyte volumes (MNV and MMV) and higher mean lymphocyte scatter (MLS) along with increased variability (i.e. standard deviations) of mean neutrophil volume and conductivity (MNV-SD and MNC-SD) and lymphocyte conductivity (MLC-SD) ($p < 0.001$ for all) (Table 3). Additionally, within the controls, the means of the MNV, MNC-SD, MLS and MLC-SD in pregnancy (group 4) were the highest among all controls, being statistically significantly higher than group 5 of normal controls ($p < 0.05$ for all, Students t-test).

Secondly, we compared group 1 versus group 3, to assess the ability of leukocyte VCS parameters only, without the aid of Total leukocyte count (TLC), to distinguish malignant from benign reactive neutrophilia. Parameters most successful in this analysis were MNV (Area under the receiver-operating-characteristic curve, AUC 0.956, sensitivity 89.5%, specificity 90.6%), MNC-SD (AUC 0.989, sensitivity 94.7%, specificity 95.6%) and MLS (AUC 0.983, sensitivity 94.7%, specificity 94.0%) (Table 4 and Fig. 1).

A combination of MNV>163.0 + MNC-SD>12.69 showed 89.5% sensitivity and 100% specificity for CML versus reactive neutrophilia. In addition, on comparing group 2 (treated CML) versus groups 4 + 5 (pregnancy plus normals), both the neutrophil parameters (MNV and MNC-SD) declined post-therapy in group 2 to reach normal ranges. Overall post-therapy, means of nine of the 20 CBC parameters normalized (i.e only the TLC, platelet count, the percentages of lymphocytes, eosinophils, and basophils, absolute counts for these 3 cell types, and the nRBC% were different between group 2 vs. group 4 + 5, while the hemoglobin, RBC count, MCV, MCH, MCHC, Red cell distribution width (RDW) and the neutrophil and monocyte percentages and absolute counts did not show a significant difference between the groups; $p > 0.05$, Levene test of variances). Similarly, 10 out of the 18 VCS parameters normalized (i.e. MNV, MNC, MNC-SD, MNS-SD, MLV, MLV-SD, MLS, MMS, MMS-SD, MMC-SD normalized while the MNV-SD, MNS, MLS-SD, MLC, MLC-SD, MMV, MMV-SD and MMC did not normalize; $p > 0.05$, Levene test of variances).

In order to identify biological differences, if any, between CML and non-CML leukocytes, we compared groups 1 + 2 versus groups 3 + 4 + 5. On multivariate analysis of all CML cases, whether treated or untreated, versus all non-CML cases, the following statistical model (logistic regression equation) with 11 parameters was generated by the statistical software (MedCalc™ v.12.7.0, Ostend, Belgium) after evaluating all input parameters:

CML (untreated or treated), if −60.4311 + 0.063252*[TLC] + 0.098075*[MCV] − 0.051514*[MMS] − 0.1878*[MLS-SD] + 0.15707*[RDW-CV] + 0.0043441*[Platelet count] − 0.092057*[Neutrophil percentage in automated differential count] + 0.083528*[MNV] + 0.16667*[MNS] + 0.57967*[MNS-SD] + 0.21165*[MLV] was greater than −1.0452.

This model's 84.6% sensitivity and 83.7% specificity indicated that the VCS data, when combined with CBC results, approached the accuracy of a well-trained haematologist (using the total and differential leukocyte counts) at predicting malignant leukocytes.

And finally, two algorithmic approaches (classification trees, shown in Figs. 2 and 3) that were constructed using VCS parameters alone could correctly separate 100% of the cases into untreated CML (group 1) versus all non-neoplastic subjects (groups 3 + 4 + 5).

Discussion

Automated hematology analyzers have a well-established role in providing informative data on red cells' size and its variations. In contrast, automated quantitative leukocyte analysis has, for the most part, been limited to total and differential WBC counts. Only recently has research into these parameters begun to make the transition into clinical laboratory practice. Accelerating this research are the various original equipment manufacturing firms with their varied technologies. For instance, instruments from Sysmex International (XE-2100) [8], Abbott Diagnostics (CELL-DYN Sapphire) [9], Horiba Inc. (Pentra MS CRP) [10] and Beckman Coulter (LH780) [11] have all been recently applied for the automated diagnosis of myelodysplasia. The basic premise underlying all these efforts is that electronic flags generated by using either cut-offs for individual parameters or by using algorithms incorporated into laboratory information systems can be used to initiate further specific testing.

Table 2 Demographic and salient CBC findings of the five groups.

	Untreated CML (n = 38)	CML on Imatinib (n = 65)	Reactive neutrophilia (n = 58)	Antenatal women (n = 100)	Normal controls (n = 99)
Age (years)	42.3 ± 13.2 (16–82)	42.2 ± 12.2 (18–65)	44.2 ± 18.2 (14–82)	30.1 ± 6.7 (19–45)	34.1 ± 10.3 (19–65)
Gender; M:F	20:18	40:25	41:17	0:100	76:23
Hemoglobin (gram/dL)	9.3 ± 1.8 (6–13)	11.2 ± 2.4 (4.1–16.4)	10.6–2.3 (5.6–16.1)	10.6 ± 1.9 (4.6–13.9)	13.4 ± 1.4 (11.5–16.9)
Total leukocyte count (× 10^9/L)	162.6 ± 93.6 (45.3–376.5)	6.9 ± 8.07 (0.6–42.3)	16.9 ± 6.0 (10.4–40.5)	9.9 ± 3.5 (5.6–28.7)	7.5 ± 2.1 (0.5–13.6)
Platelet count (× 10^9/L)	353.5 ± 325.9 (83–1461)	220.0 ± 174.1 (4.0–891)	205.2 ± 110.5 (3–619)	197.5 ± 87.2 (13.1–468.6)	213.8 ± 68.5 (110.5–407.3)
Absolute neutrophil count (× 10^9/L)	133.8 ± 83.4 (8.5–335.1)	3.6 ± 3.5 (0.3–32.3)	13.8 ± 4.9 (8.7–29.1)	7.2 ± 3.7 (3.1–26.6)	5.5 ± 1.5 (3.07 –9.12)
Absolute lymphocyte count (× 10^9/L)	9.9 ± 8.4 (2.6–39.2)	2.4 ± 2.5 (0.1–19.5)	1.7 ± 1.7 (0.2–13.4)	1.8 ± 0.6 (0.4–3.5)	2.3 ± 0.6 (1.1–4.3)
Absolute monocyte count (× 10^9/L)	5.7 ± 13.6 (0.0–65.5)	0.6 ± 0.5 (0.1–3.8)	2.4 ± 10.4 (0.1–81.0)	0.6 ± 0.6 (0.03–2.81)	0.6 ± 0.2 (0.2–1.5)
Absolute eosinophil count (× 10^9/L)	5.8 ± 10.6 (0.01–57.10)	0.2 ± 0.2 (0.0–1.0)	0.19 ± 0.3 (0.0–1.3)	0.16 ± 0.21 (0.00–1.870	0.24 ± 0.14 (0.04–0.62)
Absolute basophil count (× 10^9/L)	0.7 ± 1.7 (0.1–9.9)	0.08 ± 0.2 (0.0–1.2)	0.04 ± 0.1 (0.0–0.7)	0.04 ± 0.04 (0.0–0.3)	0.04 ± 0.02 (0.01–0.09)

Note: All values apart from gender are given as mean ± SD (range). Values are automated results derived from the LH780 analyzers, not manual differential counts. The basophil count was typically underestimated by the analyzers.

The VCS indices that were found for the first time in our study to distinguish CML from reactive neutrophilia with moderate accuracy are rapidly generated by analyzers at no extra cost during routine WBC analysis, without any requirement for an additional blood sample. It must however be documented that most cases of CML can be easily distinguished by blood counts and smear evaluations alone from most reactive neutrophilia cases, without any need for the VCS indices. The potential advantage of VCS technology lies in that it may be able to tell if the patient has CML or not, even before a smear is prepared, without reliance on the clinical background or a cytochemical stain.

In our study, we turned our attention next to whether the significant differences in the VCS indices could help provide biological insights into CML leukocytes that differentiate them from non-CML-related neutrophilia. The reversion of many of the indices to normal after therapy, as well as the fact that pregnant women show higher values of certain VCS parameters compared to other controls, suggests that these indices might represent an accurate measure of increased neutrophil turnover and activity that is driven by intrinsic and extrinsic factors. The current study lacks the power and design to prove or disprove any pathogenetic assumptions, but it does serve as a proof-of-concept of the power of qualitative leukocyte analysis. Future research may shed more light on leukocyte structure and biology in CML and other illnesses using these parameters.

The control group of pregnant women was included since pregnancy represents a special physiological state known to show neutrophilic leukocytosis and mild maturational shift to the left [12] and we wanted to ensure that the observed changes in CML weren't simply ones of neutrophilia. One of the criticisms of prior studies on VCS has been that a sufficient variety of normal and diseased controls were not included, and hence the specificity of the results remained in doubt.

The complex logistic regression equation generated by our study to separate CML from non-CML cases incorporates

Table 3 VCS parameters that were highly significantly different between group 1-versus-groups 3 + 4 + 5 (p-values were less than 0.001 for all).

Groups → CPD parameters ↓	Untreated CML (n = 38)	CML on Imatinib (n = 65)	Reactive neutrophilia (n = 58)	Antenatal women (n = 100)	Normal controls (n = 99)
MNV	187.7 ± 20.3	149.9 ± 14.3	147.5 ± 10.2	152.2 ± 10.7	143.0 ± 7.8
MNV-SD	48.8 ± 5.8	23.3 ± 6.2	24 ± 5.1	19.4 ± 55.1	19.6 ± 1.9
MNC-SD	23.3 ± 8.2	7.3 ± 3.3	6.6 ± 1.3	8.7 ± 3.0	5.7 ± 1.0
MLS	98.4 ± 15.4	68.7 ± 9.6	64.5 ± 6.2	71.0 ± 7.0	64.6 ± 8.0
MLC-SD	18.2 ± 7.1	10.8 ± 7.7	11 ± 2.6	11.8 ± 4.9	9.2 ± 2.3
MMV	177.6 ± 32.7	169.0 ± 12.0	164.1 ± 23.5	164.2 ± 9.8	162.6 ± 8.6

Abbreviations: MNV: mean neutrophil volume; MNV-SD: standard deviation of MNV; MNC-SD: standard deviation of the mean neutrophil conductivity; MLS: mean lymphocyte scatter; MLC-SD: standard deviation of mean lymphocyte conductivity; MMV: mean monocytes volume.

Table 4 Performances of the parameters most successful in distinguishing untreated CML (group 1) from reactive neutrophilia (group 3).

	MNV	MNC-SD	MLS
AUC	0.956	0.989	0.983
p-value	<0.0001	<0.0001	<0.0001
Criterion	>163.046	>12.69	>81.827
Sensitivity	89.47	94.74	94.74
Specificity	90.57	95.6	94.03

Abbreviations: AUC: Area under the receiver-operating-characteristic curve; MNV: mean neutrophil volume; MNC-SD: standard deviation of the mean neutrophil conductivity; MLS: mean lymphocyte scatter.

several parameters. These need validation before being employed in clinical laboratories. Ultimately the selected models would need to be fed into the automated analyzer's software, and all calculations would be done by the computer to generate flags. Inclusion of the platelet count in the models appears reasonable since the myeloproliferative neoplasms often have thrombocytosis, while platelet counts are normal to low in many non-neoplastic emergency conditions with reactive neutrophilia. The inclusion of MCV and RDW-CV may be due to the fact that the elevated numbers of leukocytes in CML were analyzed together with RBCs in the red cell + platelet analysis channel of the analyzers where cells are not lysed [13].

The presence of lymphocyte number, percentage as well as scatter parameters as significantly predictive of CML both before and after therapy are more difficult to explain. However, these apparent lymphocytic changes are likely to be influenced by the absolute basophilia present in untreated CML. Basophils localize in the upper right-hand quadrant of the lymphocyte box in a plot of discriminant function 1 (mainly light scatter) versus size in Beckman Coulter instruments. They need to be distinguished from lymphocytes and monocytes by gating out neutrophils and eosinophils in a plot of size versus discriminant function 3, thereby resulting in unreliable basophil counts, a problem that is present in instruments from other manufacturers as well [13].

It is interesting to reflect on the possible causes of such distinctive VCS features between the diseased and non-diseased groups, even though CML neutrophils are morphologically indistinguishable from their normal counterparts [14]. In CML, the granulocytic expansion is attributable chiefly to the markedly increased cell numbers in the terminal (band and segmented neutrophils) as well as middle (myelocytes and metamyelocytes) stages of maturation [13]. The LH750™ analyzers can flag immature precursors, but not separate them from mature neutrophils, thereby resulting in a "neutrophil" VCS dataset that is invariably "contaminated" by the VCS indices of the preceding cells in the series [13]. Since cell volume decreases steadily from promyelcytes to neutrophil stages, this explains why untreated CML had markedly higher MNV values than any control group (Table 3). The inclusion of cellular stages with variable volumes would also explain the anisocytosis seen in CML, as exemplified by the markedly increased MNV-SD (Table 3).

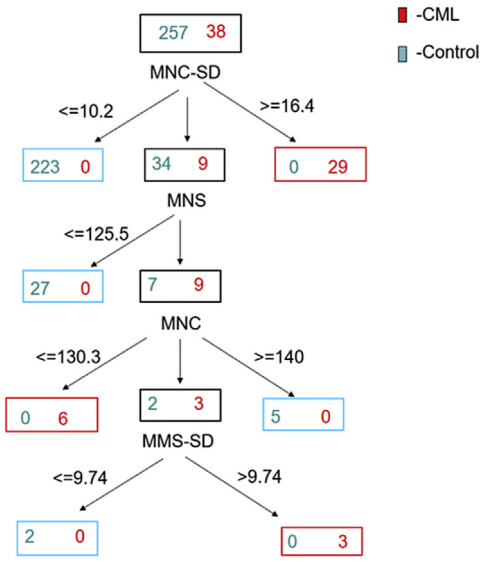

Fig. 2 An algorithmic approach (classification tree) using only the VCS parameters MNC-SD, MNS, MNC and MMS-SD could correctly separate 100% of the cases into untreated CML (group 1) versus all non-neoplastic subjects (groups 3 + 4 + 5).

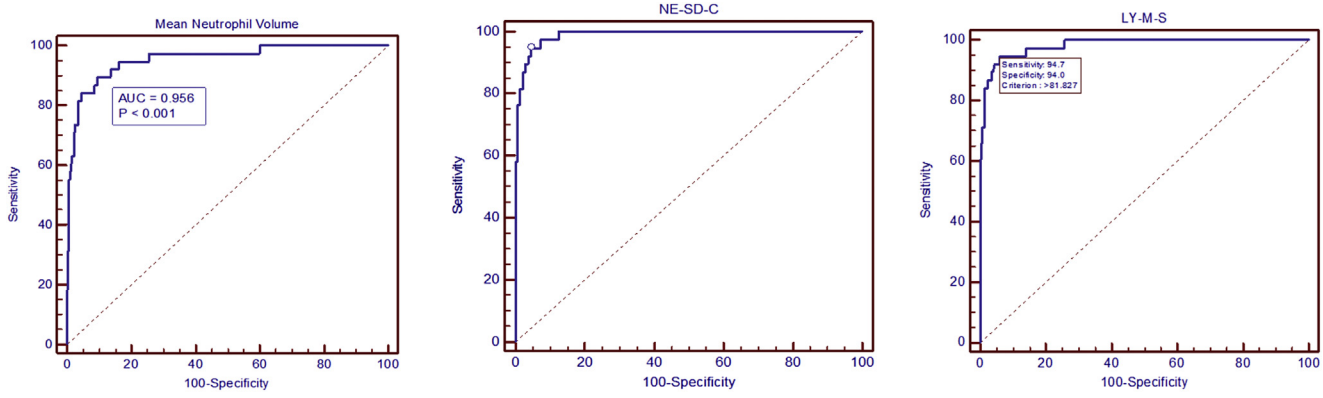

Fig. 1 Receiver-Operating-Characteristic curves for the three parameters most successful in distinguishing untreated CML (group 1) from reactive neutrophilia (group 3), i.e. MNV, MNC-SD and MLS (depicted left to right respectively).

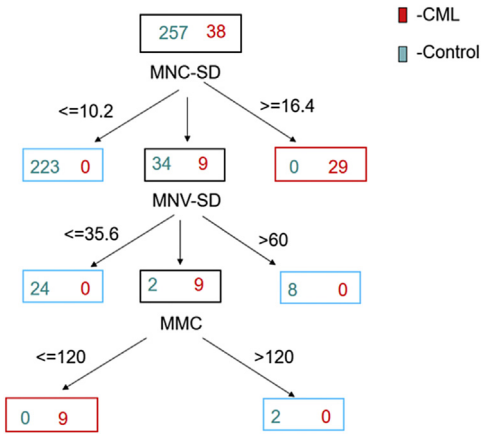

Fig. 3 Another classification tree using the neutrophil and monocyte VCS parameters MNC-SD, MNV-SD and MMC correctly classifies 100% of the cases into untreated CML (group 1) versus all non-neoplastic subjects (groups 3 + 4 + 5).

The markedly increased variation in neutrophil conductivity (MNC-SD) and to a lesser extent, in lymphocyte conductivity (MLC-SD), in untreated CML (Table 3) is more difficult to explain. The latter is almost certainly due to basophils getting included along with the lymphocytes (supported by high lymphocyte scatter seen in CML cases, Table 3) since the analyzer cannot distinguish them accurately in most specimens [13,14]. The variation in neutrophil conductivity occurs possibly because the alternating radiofrequency range current differentially short-circuits the bipolar lipid layer of neutrophils versus precursor cells' membranes, allowing the energy to penetrate the cell and generate a signal dependent on their size, internal structure, chemical composition and nuclear volume.

This study has certain shortcomings. It is possible that the differences in VCS parameters might simply be the results of discrepant characteristics within groups such as age, gender, total white count, etc. Some differences in age and gender were inevitable when comparing pregnant women versus a specific malignancy versus unselected patients with neutrophilia. Another problem is that we used the automated leukocyte percentages and absolute counts in our statistical analysis. Autoanalyzers yield erroneous counts in several situations [13,14]. However, our aim was to test the automated counter's outputs for their diagnostic and biological informativeness, since expert pathologists/hematologists and experienced technologists would usually not require the instrument's data anyway to differentiate CML from reactive neutrophilia. An ideal study would compare CML with those leukemoid reactions where the TLC is very high (for e.g. >50 × 109/L), but such cases would take a long time to accrue.

The decline of VCS indices after therapy would be interesting to evaluate further as a tool to monitor CML patients at low cost. CML patients on therapy could be followed-up at predetermined timepoints to assess if the VCS parameters correlate with molecular/cytogenetic/hematological monitoring results.

Conflicts of interest

The authors declare no conflicts of interest.

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