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LETTER TO THE EDITOR

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Adequate scattergram interpretation increases the reliability of automated polymorphonuclear (pmn) cell counts from ascitic fluid of patients with liver cirrhosis

Dear editors,

Accurate and fast characterization of the cellular components of body fluids is crucial for the diagnosis of several potentially life-threatening diseases. The number and composition of white blood cells (WBC), for example, may point to an infectious origin and guide early treatment, even if results of microbiological work up are still pending. Microscopic differentiation from cytospin preparations is still the gold standard to quantify leukocyte subpopulations in body

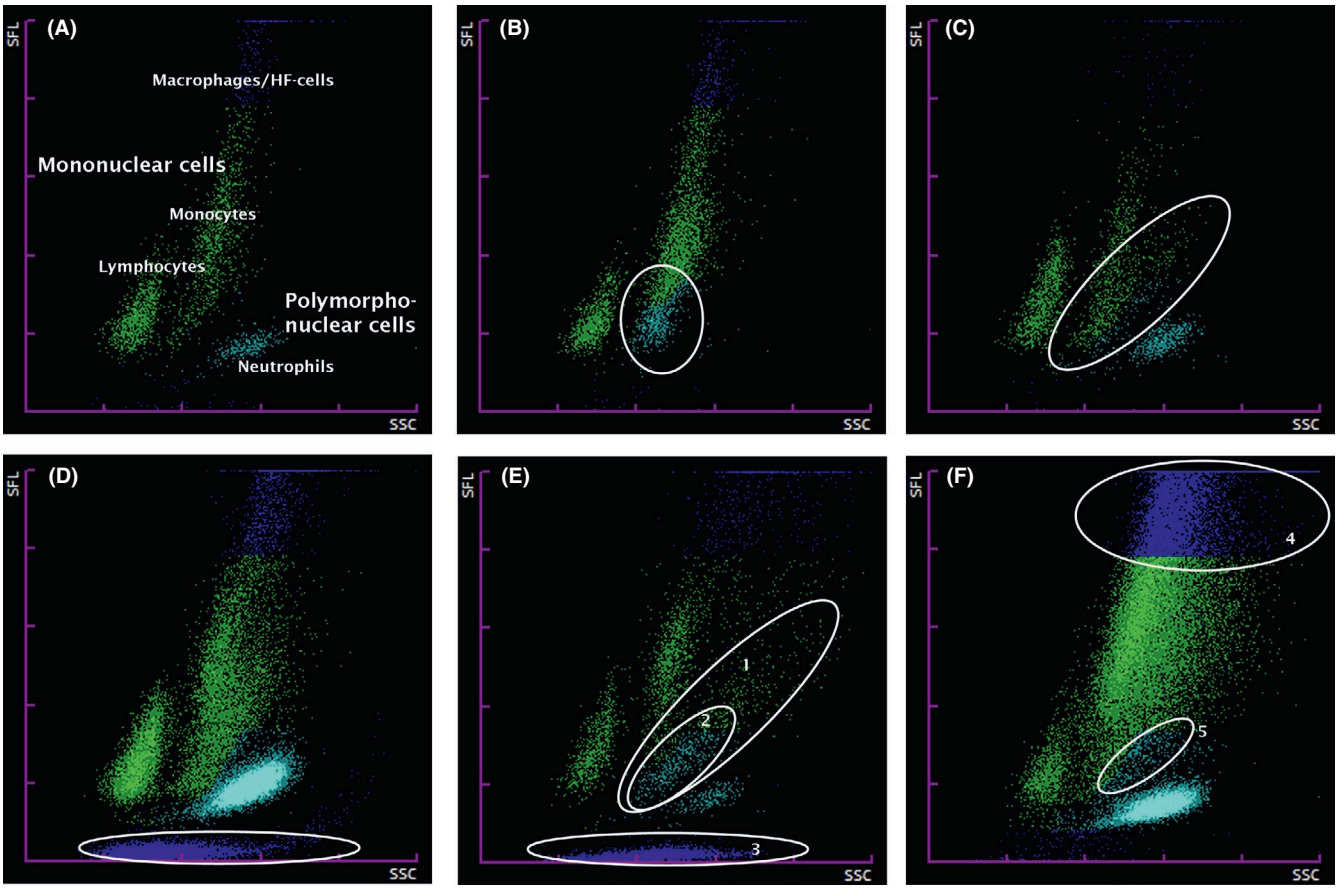

FIGURE 1 Examples of scattergram features (indicated by white circles) that were analyzed for their association with deviating results of microscopic and automated PMN counts. A) Inconspicuous scattergram with marked cell populations, B) misclassification (eg, MN obviously classified as PMN), C) diagonal population between monocytes and PMN designated as MN, D) increased debris area, E) misclassification (2) within a diagonal cell population (1) (classified for both, MN and PMN) and increased debris area (3), and F) increased frequency of High Fluorescent (HF-)cells (4) and discrete misclassification (5). Green spots denote cellular events designated as MN; light blue spots denote cellular events designated as PMN; strong blue spots denote events designated as debris (D, E) or HF (F); SFL... side fluorescent light; SSC... side-scattered light

fluids. Aside from being time- and labor-consuming, this procedure is statistically imprecise and associated with inter-operator variability. Moreover, at many medical facilities, it is not available on a 24-hour basis.[1] The use of automated hematology analyzers to characterize the cellular components of a body fluid is considered an appropriate alternative to microscopic differentiation. It offers shorter turnaround times without the need for specific sample preparation.[2] Reports on the accuracy of automated differential counts are ambivalent, especially for body fluids with low cell counts.[2-6]

Spontaneous bacterial peritonitis (SBP) is a life-threatening complication of liver cirrhosis. Current guidelines define the threshold for diagnosis of SBP at an ascitic polymorphonuclear cell (PMN) count above 0.250 10^9/L.[7] Moreover, patients that do not fulfill the criteria of SBP at presentation are at higher risk to subsequently develop SBP, if the PMN count in the ascitic fluid exceeds 0.100 10^9/L.[8] Although it is consensus that PMN counts should be provided within a short time frame to ensure prompt initiation of antimicrobial treatment, definite recommendations on the method of quantification do not exist. In the present study, we compare PMN counts obtained from microscopic examination with automated PMN counts performed in parallel by a Sysmex XN-2000 hematology analyzer. Moreover, we aimed to identify visual features of the analyzer's WBC scattergram that could indicate the need for a microscopic recount.

In our laboratory, the diagnostic work up of ascitic fluid includes measurement of total nucleated cell (TNC) and WBC counts on the automated hematology analyzer XN-2000 (software version 00-18B; Sysmex, Kobe, Japan), followed by a microscopic differential count, if the TNC count exceeds 0.100 10^9/L. Microscopic 100-cell differential counts are performed by experienced biomedical technicians and/or laboratory physicians from cytospin preparations stained according to a modified Wright protocol (Hema-Tek®, Siemens Healthineers, Erlangen, Germany). Microscopic PMN comprise neutrophils, eosinophils, and basophils. The present evaluation retrospectively compares the routinely reported microscopic PMN counts with the corresponding automated PMN counts of 118 consecutive ascites samples that had been sent to our laboratory for diagnostic work up between January 2016 and April 2018. The automated PMN counts had been measured on the XN-2000 in parallel with TNC and WBC counts and stored in the database of our laboratory data management system without being further considered for the patient report. The study complies with the World Medical Association Declaration of Helsinki and was approved by the Ethics Committee of the Medical University of Vienna (No. 1869/2017).

The hematology system Sysmex XN-2000 is equipped with a specific body fluid mode. Cells are differentiated by fluorescence flow cytometry. This method is capable of classifying cells according to their size, structure, and content by staining them with a proprietary fluorescence dye that binds cellular nucleic acids. Within the flow cell, a semiconductor laser beam irradiates the leucocytes and generates both, scattering of the light into different directions and fluorescence. For a differential count, laterally scattered light (side-scatter, SSC), which rises with increasing internal complexity (eg, granulation) of the cells, is plotted against side-fluorescence light, a surrogate of the cell's amount of nucleic acids. Thereby, granulated cells with low amounts of accessible nucleic acids (PMN: neutrophils/basophils, eosinophils) can be distinguished from mostly nongranulated cells with varying contents of nucleic acids (mononuclear cells, MN: lymphocytes, monocytes) (Figure 1A). In addition, high fluorescence (HF) cells, which are not included in the WBC but in the TNC count, are recorded (Figure 1A). This population primarily indicates the presence of tumor cells; however, mesothelial cells and macrophages may also fall into this category. A technical warning flag (WBC abnormal scattergram) may be created if the analyzer's software is unable to discriminate WBC from ghosts.

WBC scattergrams of the study samples were extracted from the database of the laboratory data management system and retrospectively reviewed by two expert laboratory physicians and a technical expert. In particular, images and associated data were screened for the presence of events that might have potentially interfered with the correct classification of automated PMN, including i) misclassification or overlap of populations (eg, MN obviously classified as PMN or vice versa; debris classified as cell population), ii) an atypically (diagonal between PMN and monocytes) located population designated as MN, iii) a prominent debris area, and iv) an increased proportion of HF cells (Figure 1). The presence of abnormal morphological features was graded 0 = not present, 1 = present to a low/intermediate degree, and 2 = present to a high degree. For each feature, the final grade of scattergram abnormality was defined as the maximum score assigned by any of the evaluating experts (eg, the grade for misclassification was "2," if any of the experts scored the scattergram as "2"). HF cell rates were calculated as *(total cell count – WBC count) / WBC count*. Rates between 20% and 40% were graded as "1," rates ≥ 40% were classified as "2."

Median automated TNC and WBC counts of the 118 study cases were 0.269 10^9/L (0.185 - 0.521 10^9/L) and 0.236 10^9/L (0.163 - 0.453 10^9/L), respectively. Among the microscopic counts, the median percentage of PMN was 14% (6%-32%) and the median absolute number of PMN was 0.031 10^9/L (0.013-0.079 10^9/L). The median neutrophil count was 0.029 10^9/L (0.011-0.079 10^9/L), and the median counts of eosinophils and basophils were 0 10^9/L (0-0 10^9/L), each. In samples exhibiting >=1% of eosinophils (n = 20) or basophils (n = 18), the median counts were 0.003 10^9/L (0.002-0.010) and 0.003 10^9/L (0.002-0.006), respectively.

Among the automated counts, the median percentage of PMN was 15.4% (9.6 - 29.6%) and the median absolute number of PMN was 0.034 10^9/L (0.019 - 0.079 10^9/L). 21 cases (18%) exhibited PMN counts above 0.250 10^9/L (median 1.360 10^9/L [0.374 - 3.292 10^9/L]). None of the cases was flagged by the WBC abnormal scattergram flag.

A comparison between relative PMN counts of the Sysmex XN-2000 and the microscopically determined percentage of PMN displayed a strong correlation ($\rho = 0.829$, $P < .0001$). The Passing-Bablok regression line, which did not significantly deviate from

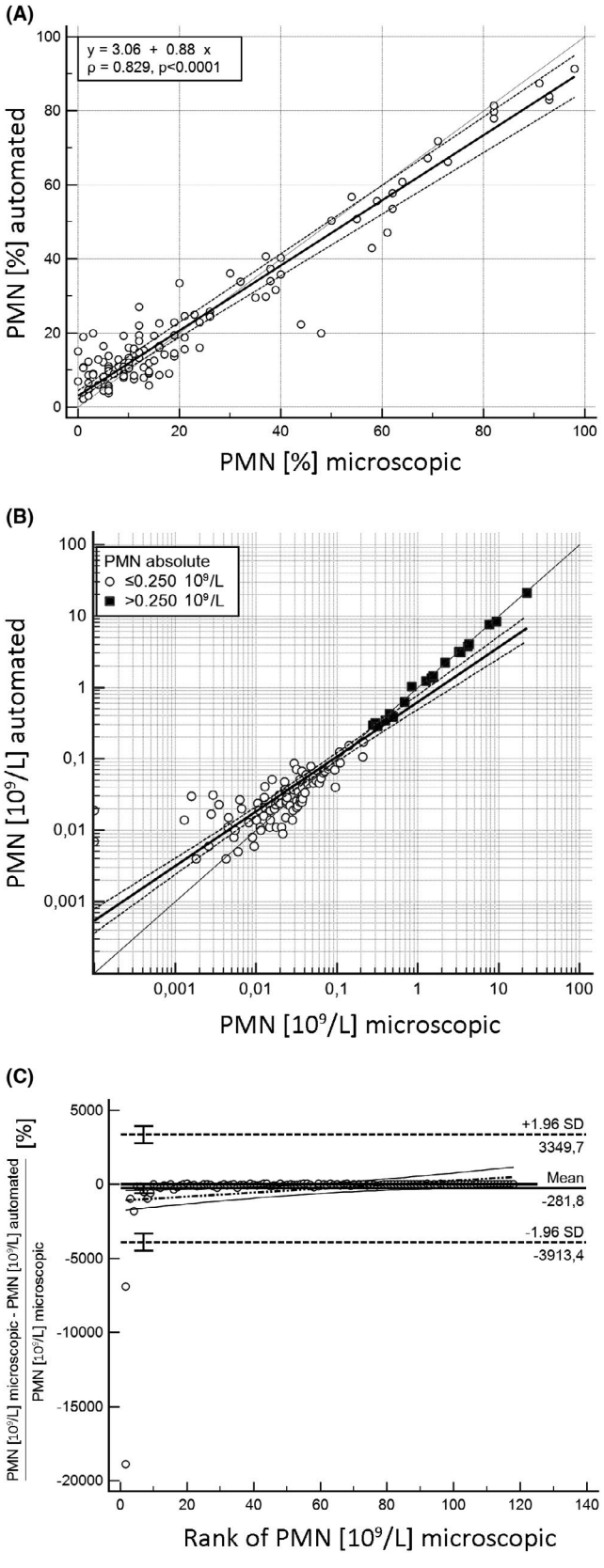

FIGURE 2 Comparison of microscopic and automated polymorphonuclear cell (PMN) counts. Passing-Bablok regression lines for relative (A) and absolute (B) counts (B: open circles denote microscopic PMN counts ≤ 0.250 10^9/L, closed squares denote microscopic PMN counts > 0.250 10^9/L); C) Bland-Altman plot with ranks of absolute PMN counts (from 0 to 118) given on the x-axis to enhance readability. To enable the calculation of % differences (y-axis), microscopic PMN counts = 0 were changed to 0.0005 10^9/L

linearity (P =.79), exhibited small systematic and proportional differences (Figure 2A): $Y = 3.06 + 0,88X$. Absolute PMN counts correlated with a Spearman's ρ of 0.894 (P <.0001). In the Passing-Bablok regression analysis, the intercept was 0.00 and the slope was 0.93 (Y = 0.00 + 0.93 X) (Figure 2B). When comparing Spearman's correlation coefficients of relative and absolute counts, the association between automated and microscopic absolute PMN counts was more pronounced than for relative counts ($p_{one\text{-}sided}$ for difference of correlation coefficients = 0.026).

Although both methods appeared to be well comparable, differences in relative PMN counts ranged from 0.1% to 28.0% (3.9% [1.9 - 6.7%]). The degree of the difference was independent of the total WBC count (ρ=−0.043, P =.647), although relative and absolute PMN counts tended to diverge more distinctly at lower PMN count levels (ie, below 0.100 10^9/L) (Figure 2A,B). As further illustrated in the Bland-Altman plot, automated PMN counts were overestimated among the samples with the lowest microscopic counts (Figure 2C).

Importantly, all samples with a microscopic PMN count of > 0.250 10^9/L also exhibited > 0.250 10^9 /L PMN on the XN-2000 (Figure 2B).

The extent of the difference between microscopic and automated PMN counts was linked to scattergram features indicating misclassification of cellular events, an atypically (diagonal between PMN and monocytes) located population designated as MN, a prominent debris area and/or an increased proportion of HF cells (Figure 1). Misclassification of cellular events was the only scattergram feature positively associated with the magnitude of the difference between counting methods (ρ = 0.20, P =.027, Figure 3A). More specifically, misclassification of grade 2 was significantly correlated with a higher difference between PMN counts (grade "2" vs. grade "0": Mann-Whitney U = 185, P =.024), whereas misclassification of grade 1 was not associated with a higher difference between counting methods (P =.176, Figure 3E).

The above reported findings underline the good agreement between microscopic and automated PMN counts of ascitic fluid samples[9,10] Our findings also emphasize the importance of visual validation of the WBC scattergram to check the plausibility of automated PMN counts in ascitic samples.[2,11,12] It must be mentioned that the results presented here are valid for the BF mode of the Sysmex XN-2000 and cannot be transferred to other devices without further evaluation. Nevertheless, it can be assumed that obvious misclassification of cell populations may distort the count result of any type of analyzer.

In conclusion, this study recommends automated PMN counting as a reliable method to guide clinical decision-making in patients with liver cirrhosis that may improve the permanent availability of PMN count measurements in clinical practice. Consideration of the WBC scattergram is essential to identify samples that should be subjected to a microscopic recount.

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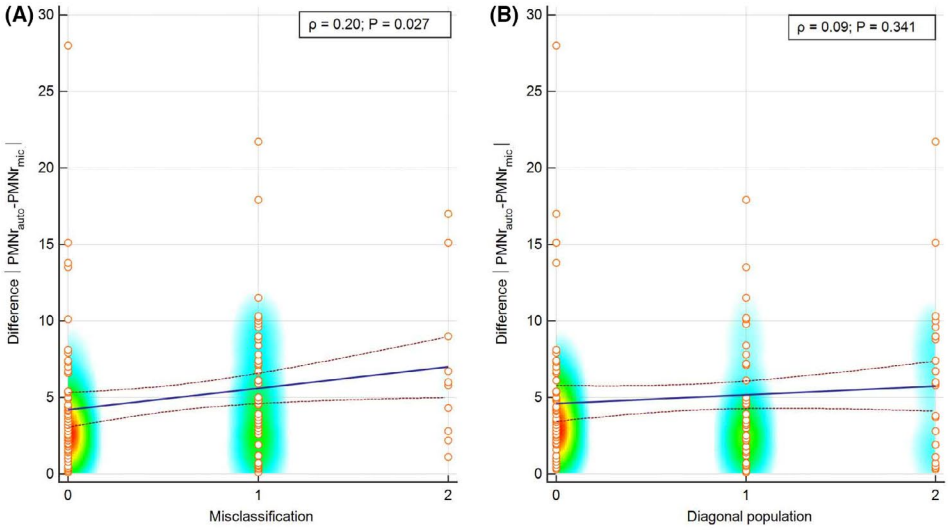

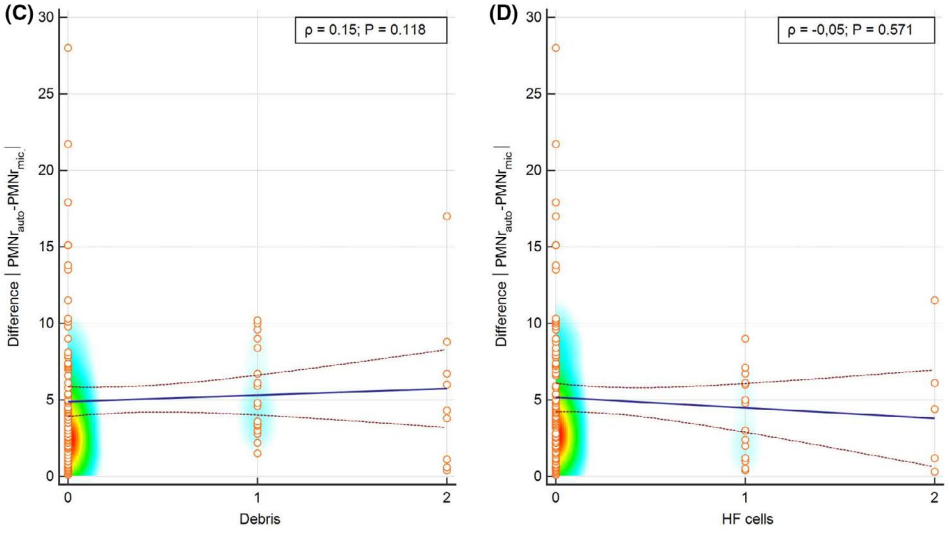

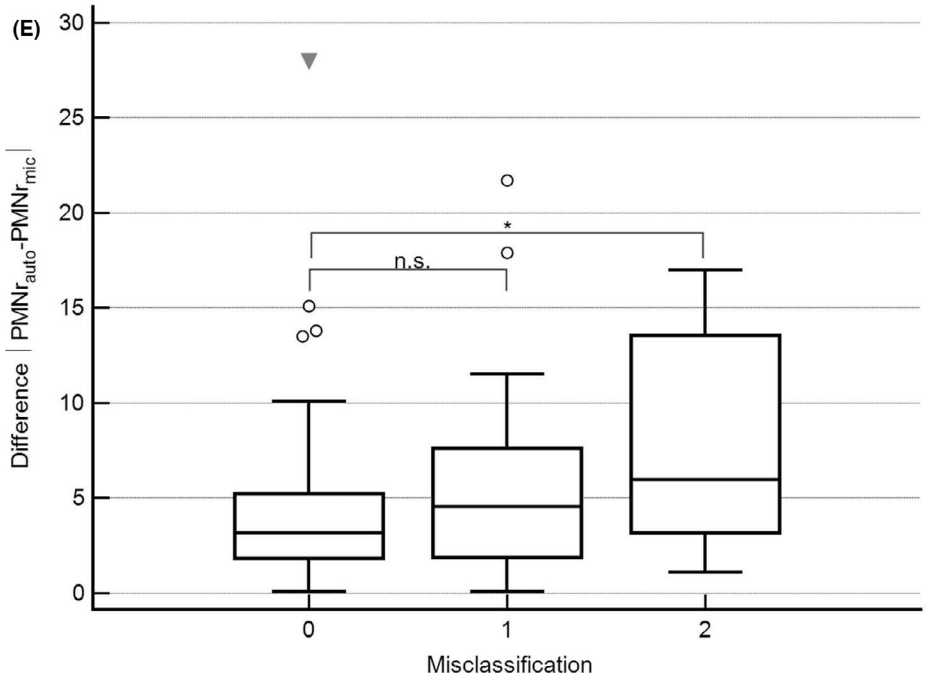

FIGURE 3 A-D) Differences in relative automated and microscopic PMN counts among samples with scattergrams not showing the particular feature (0), or showing it to a low/intermediate degree (1), or to a high degree (2). The heat map indicates the frequency of occurrence for the measurement points located in this area. E) Differences in relative automated and microscopic PMN counts between samples with scattergrams showing no (0), a low/intermediate (1), or a high (2) degree of misclassification. n.s.... not significant, *... $P < .05$, $PMNr_{auto}$... automated PMN counts [%], $PMNr_{mic...}$ microscopic PMN counts [%]

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CONFLICT OF INTEREST

RR is affiliated with Sysmex Austria GmbH. All other authors declare that they have no conflicts of interest.

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