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Automated cell differential count in sputum is feasible and comparable to manual cell count in identifying eosinophilia

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Abstract

Introduction:

Cell differential count of induced sputum is considered the gold standard for inflammatory phenotyping of asthma but is not implemented in routine care due to its heavy time- and staff demands. Digital Cell Morphology is a technique where digital images of cells are captured and presented pre-classified as white blood cells (neutrophils, eosinophils, lymphocytes, macrophages, and unidentified) and non-white blood cells for review. With this study we wanted to assess the accuracy of an automated cell differential count in identifying the key inflammatory cells in induced sputum.

Methods:

Sputum from 50 patients with asthma was collected and processed using the standard processing protocol with one drop 20% albumin added to hinder cell smudging. Each slide was counted automatically using the CellaVision DM96 and manually by an experienced lab technician. Sputum was classified as eosinophilic or neutrophilic using 3- and 61-percent cut-offs respectively.

Results:

We found a good agreement using intraclass correlation for all target cells, despite significant differences in the cell count rate. The automated CDC had a sensitivity of 65%, a specificity of 93% and a kappa-coefficient of 0.61 for identification of sputum eosinophilia. In contrast, the automated CDC had a sensitivity of 29%, a specificity of 100% and a kappa-coefficient of 0.23 for identification of sputum neutrophilia.

Conclusion:

Automated- and manual cell counts of sputum agree with regards to the key inflammatory cells. The automated cell count had a modest sensitivity but a high specificity for the identification of both neutrophil and eosinophil asthma.

Keywords: Induced sputum, Cell differential count, Digital cell morphology, Inflammatory phenotyping, Automated Cell Counting

Introduction

Inflammation in asthma is heterogeneous and is associated with specific inflammatory cell profiles in induced sputum(1). Identification of sputum eosinophilia is important, as it is associated with increased exacerbation risk(2) and a better response to inhaled corticosteroids. Further, management guided by the level of sputum eosinophils results in improved asthma control and a reduction in asthma exacerbation rates(3–6) and sputum eosinophil count is considered a biomarker for response to IL-5 targeted therapy (7–9).

The manual differential count is a difficult and labour-intensive procedure, which might take up to 30 minutes per slide to perform(10). It is a lab procedure that requires a strong combination of experience and training in order to achieve an acceptable level of competency(10).

An important limitation of the manual cell differential count is that it relies on a subjective interpretation by the lab technician unavailable for later review.

Implementation of induced sputum as a routine measure in both clinical care and clinical trials is restricted to highly specialized centres and substantial research has been aimed towards development of other more clinically feasible biomarkers of airway inflammation.

In peripheral blood, automated cell counts have already been implemented in routine care, as it has been shown to be very effective for the differential count in peripheral blood smears(11). One technique, termed *Digital Cell Morphology*, relies on the replacement of the light microscope with a digital imaging system, which automatically locates and captures digital images of the cells from the slides and presents the images as pre-classified inflammatory cells (neutrophils, lymphocytes, eosinophils, macrophages, other and unidentified) and non-white blood cells (smudge cell and artefact).

Sputum samples are characterised by a high degree of contamination and cell damage, and while Digital Cell Morphology using the CellaVision DM96 have successfully been used to identify nucleated cells in BAL-fluid from a healthy population (11) and is routinely used in haematology it remains unknown if it is a viable method for a cell differential counts in sputum.

We hypothesized that automated cell differential counting using digital cell morphology would yield comparable amounts of eosinophil- and neutrophil granulocytes compared with manual cell differential counting and that the two methods would agree with regards to phenotype classification.

Material and methods

Sputum was collected from 56 patients referred for evaluation of asthma at the Respiratory Outpatient Clinic at Bispebjerg Hospital, Denmark as part of their participation in the BREATHE-study (Biomarker Research and E-Health Advancements To Help Everyone; Ethical approval: H-16047428) (12).

Criteria for participation were respiratory symptoms (e.g. dyspnea, cough, wheezing or chest tightness) and i) either a current positive bronchial challenge test or ii) a doctors-diagnosis of asthma and a current asthma maintenance therapy corresponding to step 4 or 5 in the 2017 GINA Asthma guidelines(13).

Sputum induction

Sputum was collected following bronchial challenge test with Mannitol. Sputum was induced using isotonic saline if FEV1 was < 70% of predicted (14).

The mannitol challenge was performed with inhalation of incremental doses of dry powder mannitol, and the patients were instructed to cough and expectorate sputum after each inhalation. Spirometry was measured 1 min after each dose, and the test was completed when a 15% decline from baseline FEV1 was recorded or when a cumulative dose of 635mg was reached (15, 16).

Sputum handling

Sputum samples were processed using the plug selection method within one hour of collection, and the plug for cell differential count (CDC) was dispersed with DTT (10). Cytospins was prepared in accordance with the ERS guidelines (17), with the exception of the addition of one drop of 20% albumin, which was added to minimize cell smudging. The slides were stained with May-Grünwald and Giemsa. Cell viability was evaluated by the trypan blue exclusion method.

The slides were collected and fixated at Bispebjerg Hospital. At Rigshospitalet, the slides were stained and automatically counted using the CellaVision DM96 (CellaVision AB, Lund, Sweden) at the Department of Clinical Biochemistry and subsequently reviewed at the Department of Pathology. Following the automated counting, the same slides were manually counted at the University of Leicester by a trained lab technician (D. Parker).

Manual sputum cell differential count:

For the manual cell counting 400 non-squamous cells were counted and the count rate (count in the percentage of the total number of counted cells) was calculated for each target cell (Eosinophils, neutrophils, epithelial cells, macrophages and lymphocytes).

Patients were classified as having eosinophilia if sputum eosinophils $\geq 3\%$ and neutrophilia if sputum neutrophils were $\geq 61\%$ (10, 18).

Automatic sputum cell differential count:

The CellaVision DM96 is an automated image analysis system developed for peripheral blood smears. Barcode-labeled, slides are placed into a magazine, containing up to 12 slides, and the machine can be loaded with up to 8 magazines. By scanning the glass slides at low power, the machine identifies potential white blood cells and then takes digital images at high magnification. The images are analyzed by an artificial neural network based on a reference database of cells and pre-classified according to white blood cell class. Reference databases exist for “blood” and “body fluids” and we chose to use the latter in this study as we considered it to be the most comparable to sputum.

We set the CellaVision DM96 software to count cells until 250 leucocytes were positively identified. The images and their suggested classification were reviewed by an experienced pathologist on a computer display for confirmation or reclassification (figure 1).

We chose a target of 250 leukocytes as it had resulted in a differential count of approximately 400 non-squamous cells after review our test-runs.

Clinical assessments:

Atopy (allergic sensitization) was defined as a specific IgE > 0.35 kU/L for a minimum of 1 out of the ten tested aeroallergens (19). Spirometry was conducted in accordance with ERS guidelines, using a MasterScreenPneumo spirometer (BD Jaeger, Würzburg, Germany (20)). Predicted values were calculated with the use of NHANES reference data (21). Fractional exhaled nitrogen oxide (FeNO) measurements were done using the Niox Vero (Circassia Limited, Oxford, UK) (22).

Blood eosinophilia was assessed by the Bispebjerg Hospital pathology laboratory using automated flow cytometry with a Sysmex XN 9000 (Sysmex Europe GmbH, Norderstedt, Germany).

Statistics

SAS Enterprise Guide version 7.1 (SAS Institute, Cary, NC, USA) was used for data analysis. For normally distributed data values are presented as mean (Standard Deviation(SD)), and for non-parametric data as median (quartile-1 (Q1); quartile-3(Q3)). The two methods were compared with regards to cell count rate (count in the percentage of the total number of counted cells) for each target cell using paired T-tests, Pearson's correlation and intraclass correlation (ICC). ICC estimates were calculated by an absolute-agreement, 2-way mixed-effects model ($k = 2$).

Subsequently, each target cell was evaluated using the Bland-Altman approach; a method specifically developed to describe the agreement between two quantitative measurements; using limits of agreement to quantify agreement (23).

We calculated the sensitivity, specificity, positive predictive value and negative predictive value for the automated method relative to the gold standard (manual CDC). Finally, we calculated the Cohen-kappa coefficient to evaluate the agreement in classification between the two methods.

Ethics:

The BREATHE-study (Biomarker Research and E-Health Advancements To Help Everyone) was approved by the local ethics committee (Ethical approval: H-16047428) and the regional data protection agency (Approval number: 2012-58-0004), and all participants signed an informed consent form.

Results

Study population

A total of 50 patients (89%) were included in the analyses, six patients were excluded due to too few cells for analysis (Figure 2). Six (12%) patients had sputum collected following induction with isotonic saline, while the remaining patients had sputum collected following a mannitol challenge test. Patient characteristics are presented in Table 1. The mean (SD) age was 43 years (16), and 54%

of the patients were female. The mean (SD) FEV1%-predicted was 87 (17) and the median (Q1, Q3) budesonide-equivalent ICS dose was 800 ug (600, 1600).

Cell differential counts

We found a statistically significant difference between the two methods in the percentage of total cells counted for each target cell, as presented in Table 2. We found a higher proportion of eosinophils ($p<0.001$) and neutrophils ($p<0.001$) using the manual cell counting, while we found a significantly larger proportion of epithelial cells ($p=0.01$), lymphocytes ($p<0.001$) and macrophages ($p<0.001$) using the automated cell count.

We found the cell count rate to be significantly correlated for all target cells; however, we only found a strong correlation for the eosinophil count ($r=0.87$, $p<0.001$) (Table 2). Figure 3, presents scatter plots, including a fitted line of the cell count rate of each target cell which for the eosinophil count supports a strong and significant correlation.

The agreement (intra-class correlation) between the two methods was good for all target cells, including both eosinophil (0.96) and neutrophil count (0.88) (24).

Using the Bland Altman approach, we plotted the difference between the two methods against the mean, as shown in figure 4. No observations were outside the limits of agreement for the eosinophil count, while 3 (6%) of observations were outside the limits of agreement for the neutrophil count which is to be expected given the definition of these limits (mean of the difference $\pm 2SD$).

Classification of inflammatory phenotypes

The automated cell differential count resulted in the classification of significantly fewer patients as having eosinophilic ($p=0.01$) and neutrophilic ($p=0.01$) airway inflammation (Table 3). Using the manual cell differential count as a reference, the automated cell differential count had a sensitivity of 65%, a specificity of 93% and a substantial agreement (kappa-coefficient of 0.61) in the identification of sputum eosinophilia (Table 4).

In contrast, the automated cell differential count had a sensitivity of 29%, a specificity of 100% and weak agreement (kappa-coefficient of 0.23) with the manual cell differential count for identification of sputum neutrophilia (table 4).

Impact of viability and contamination

Viability was significantly correlated to the absolute difference of the two methods in both eosinophil- (-0.29 , $p=0.04$) and neutrophil cell count rate (-0.333 , $p=0.02$), while contamination only was significantly correlated to the difference in neutrophil cell count rate (0.57 , $p<0.001$).

In continuation, we found the automatic sputum cell differential count to have a markedly lower sensitivity for the identification of sputum neutrophilia in samples with either high contamination (above the median ($>6.9\%$)) or low viability (equal to or below the mean (≤ 51.5)). In contrast, the sensitivity for identification of sputum eosinophilia was unchanged.

Discussion

This study shows a good agreement between an automated- and manual cell differential count for all target cells, despite significant differences in the cell count rate for each target cell. The automated

cell count had a very high specificity for identifying sputum eosinophilia and -neutrophilia, but only a modest sensitivity for the identification of eosinophilia and a poor sensitivity for identification of neutrophilia.

These findings are in line with previous analyses of the diagnostic accuracy of T2 biomarkers for sputum eosinophilia (25, 26). Therefore, we consider the current methodology – without further optimization – to be a feasible method for ruling in sputum eosinophilia and neutrophilia. Given the modest sensitivity, the absence of sputum eosinophilia should be interpreted with caution and a manual recount might be necessary. The development of a reference catalogue specifically for sputum holds potential to improve the sensitivity further. While no other studies have compared an automated cell count using the CellaVision DM96 with a manual cell count in sputum, the CellaVision DM96 has been thoroughly validated for peripheral blood smears. Correlation coefficients for neutrophil counts have been reported between 0.91-0.98, while slightly weaker correlations have been reported for eosinophil counts (0.67-0.83)(27–32). Further, strong correlations for eosinophil- (0.92 and 0.99 respectively) and neutrophil counts (0.99 and 0.99 respectively) have been reported in cerebrospinal- and abdominal fluid using the body fluids reference database. In accordance with these studies, we found a strong correlation for eosinophil counts (0.87); however, the correlation for neutrophil counts (0.54) was much weaker.

Sputum samples have in general a higher degree of contamination and cell debris compared with both BAL-fluid and blood smears (33), which we would expect to influence the detection of all cell types when using automated methods. Nonetheless, we found it to predominantly effect the identification of the neutrophils (and the neutrophilic inflammatory phenotype) and this difference in accuracy is remarkable. We believe it might be attributed to the different morphology of neutrophils in sputum compared with neutrophils in blood as we in this study have used a reference database not specifically developed for sputum.

Direct microscopy and automated cell counting have been found to be equally time-consuming for evaluation of peripheral blood smears (5.1 vs 6.4 minutes respectively); however, the CellaVision DM96 system offers several advantages over direct microscopy including the ability to re-review slides, remotely evaluate slides and perform quality control on a cell-by-cell basis. In sputum, the time consumed performing a manual differential count averages around 30 minutes in our research unit and we therefore expect the automated cell count to be more time efficient. Further, as the CellaVision DM96 utilised in routine analyses of peripheral blood it should be available at most sites with a basic laboratory facility. We believe that CellaVision DM96 has the potential to reduce the selection bias introduced with the current methodology of manual cell counting, as it enables counting of markedly more cells than the manual CDC, but future studies comparing the number of cells counted and the diagnostic accuracy are warranted.

We have in this analysis included patients with sputum collected using both mannitol and isotonic saline and we do not believe this has introduced any particular bias, as the same slide was counted using both methods. The two induction methods have shown similar results with regards to eosinophil- and macrophage rate, a trend towards a higher non-squamous cell rate has been reported in samples collected following mannitol challenge. In addition, two studies have reported a lower neutrophil rate in sputum collected following mannitol challenge compared with hypertonic saline induction(15, 16); however, this difference reached only significance in one of the studies(16).

As we have illustrated in this paper, the technique still needs further development before implementation as a routine measure for sputum cell differential count as the nature of the sputum sample is much different from samples from compartments previously assessed using the DM96 system. In the current study, we experienced a markedly better white blood cell to smudge cell ratio by improving cell dispersion with albumin, but improvements to allow for more accurate cell

identification and subsequent classification need to be pursued as illustrated by the improved diagnostic ability in the samples with the highest quality (table 4). In line with this, we believe it is necessary to develop a reference database specifically for sputum samples to account for differences as exemplified by the altered morphology of the neutrophils compared to BAL fluid and blood.

In conclusion, automated cell counting of sputum is a promising alternative to the more labourious and time-consuming manual differential count. The automated cell count offers several advantages including the ability to later re-review each counted cell. . We found a high intraclass correlation for the cell count rate for all target cells and the automated cell count to have a very high specificity for identifying sputum eosinophilia and -neutrophilia, but only a modest sensitivity for the identification of eosinophilia and a poor sensitivity for identification of neutrophilia. We believe that the development of a reference catalogue of inflammatory cells in sputum is essential to further this technique in particular to improve the sensitivity to detect eosinophilia and neutrophilia. In addition, future studies addressing the optimal number of cells to count using the CellaVision DM96 are warranted.

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Declaration of interest statement

The authors have no conflicts of interest to disclose.

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Table 1: Patient characteristics

	All
N	50
Sex; [% female]	54
Age; mean (SD)	43 (16)
Packyears; Median (Q1, Q3)	0 (0, 10)
Daily ICS dose [μ g of budesonide equivalent]; median (Q1, Q3)	800 (600, 1600)
Pre FEV1 %; mean (SD)	87 (17)
Pre FVC %; mean (SD)	100 (15)
FeNO [ppb]; median (Q1,Q3)	17 (11, 34)
Atopy*; %	60
Total IgE [$10^3 \cdot$ U/mL]; median (Q1,Q3)	126 (26, 253)
Blood eosinophils [10^9] median (Q1,Q3)	0.23 (0.10, 0.42)

*Atopy defined as a positive RAST-test for at least 1 out of 10 standard aeroallergens.

Table 2: Differential counts

	Manual CDC	Automated CDC	Difference	Agreement	Correlation	
			p	ICC	Pearsons	p
Total cells counted						
Eosinophils [%]; median (Q1, Q3)	1.6 (0.3, 6.0)	1.18 (0.0, 3.8)	0.001 †	0.96	0.87	<0.001
Neutrophils [%]; median (Q1, Q3)	69 (42, 87)	28 (10, 52)	<0.001 †	0.88	0.54	<0.001
Columnar epithelial cells [%]; (median Q1, Q3)	3.3 (1.5, 10.0)	33.7 (10.9, 57.1)	<0.001 †	0.82	0.48	<0.001
Lymphocytes [%]; (median Q1, Q3)	0.25 (0.0; 0.5)	0.5 (0.0; 1.3)	0.01 †	0.80	0.54	<0.001
Macrophages [%]; (median Q1, Q3)	13.1 (5.0; 28.3)	21.1 (12.0; 33.9)	<0.001 †	0.82	0.35	0.01
Smudge cells [smudge cells/counted cells]; median (Q1, Q3)	Na.	0.59 (0.39; 1.03)	Na.	Na.	Na.	Na.

†: paired t-test of logarithmized value.

Table 3: Classification of inflammatory phenotype

	Manual CDC	Automated CDC	P
Eosinophilic asthma (%)	40	30	0.01 ¥
Neutrophilic asthma (%)	62	18	0.01 ¥

Eosinophilic asthma: sputum eosinophils $\geq 3\%$; neutrophilic asthma: sputum neutrophils $\geq 61\%$; ¥: Chi-square test.

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Table 4: Agreement in inflammatory phenotype classification

	Sens (%)	Spec (%)	PPV	NPV	Kappa
Eosinophilic asthma	65	93	0.87	0.80	0.61
Neutrophilic asthma	30	100	1.0	0.46	0.23
High contamination (> median)					
Eosinophilic asthma	64	100	1.0	0.76	0.65
Neutrophilic asthma	7	100	1.0	0.43	0.06
Low contamination ($\leq$ median)					
Eosinophilic asthma	67	88	0.75	0.83	0.56
Neutrophilic asthma	47	100	1.0	0.50	0.38
High viability (> mean)					
Eosinophilic asthma	67	100	1.0	0.84	0.72
Neutrophilic asthma	47	100	1.0	0.37	0.3.0
Low viability ($\leq$ mean)					
Eosinophilic asthma	64	0.86	0.78	0.75	0.50
Neutrophilic asthma	0	100	0	0.48	na

Classification of inflammatory phenotype using the automated cell count relative to the classification from the manual cell differential count. Sens: sensitivity; Spec: specificity; PPV: positive predictive value; NPV: negative predictive value; Kappa: Cohen's kappa coefficient for agreement with the gold standard classification based on the CDC.

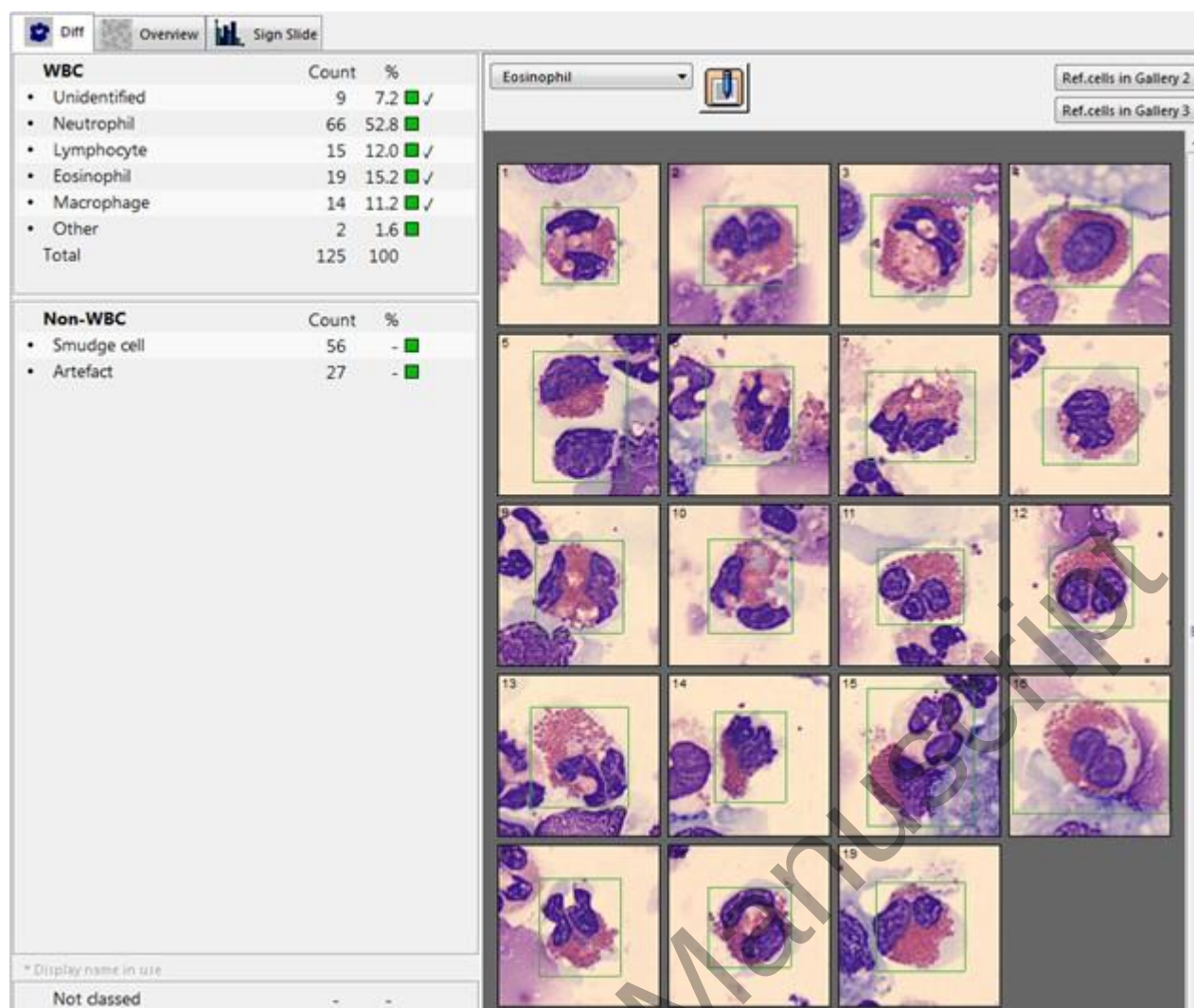


Figure 1: Classification of cells using CellaVision DM96

Pre-classified cells for review in the CellaVision Review Software.

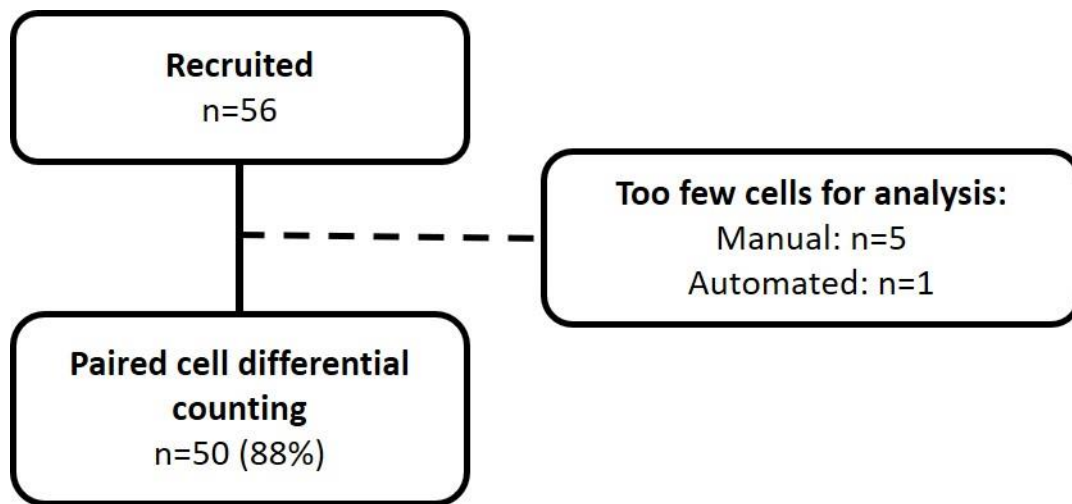


Figure 2: Study flowchart

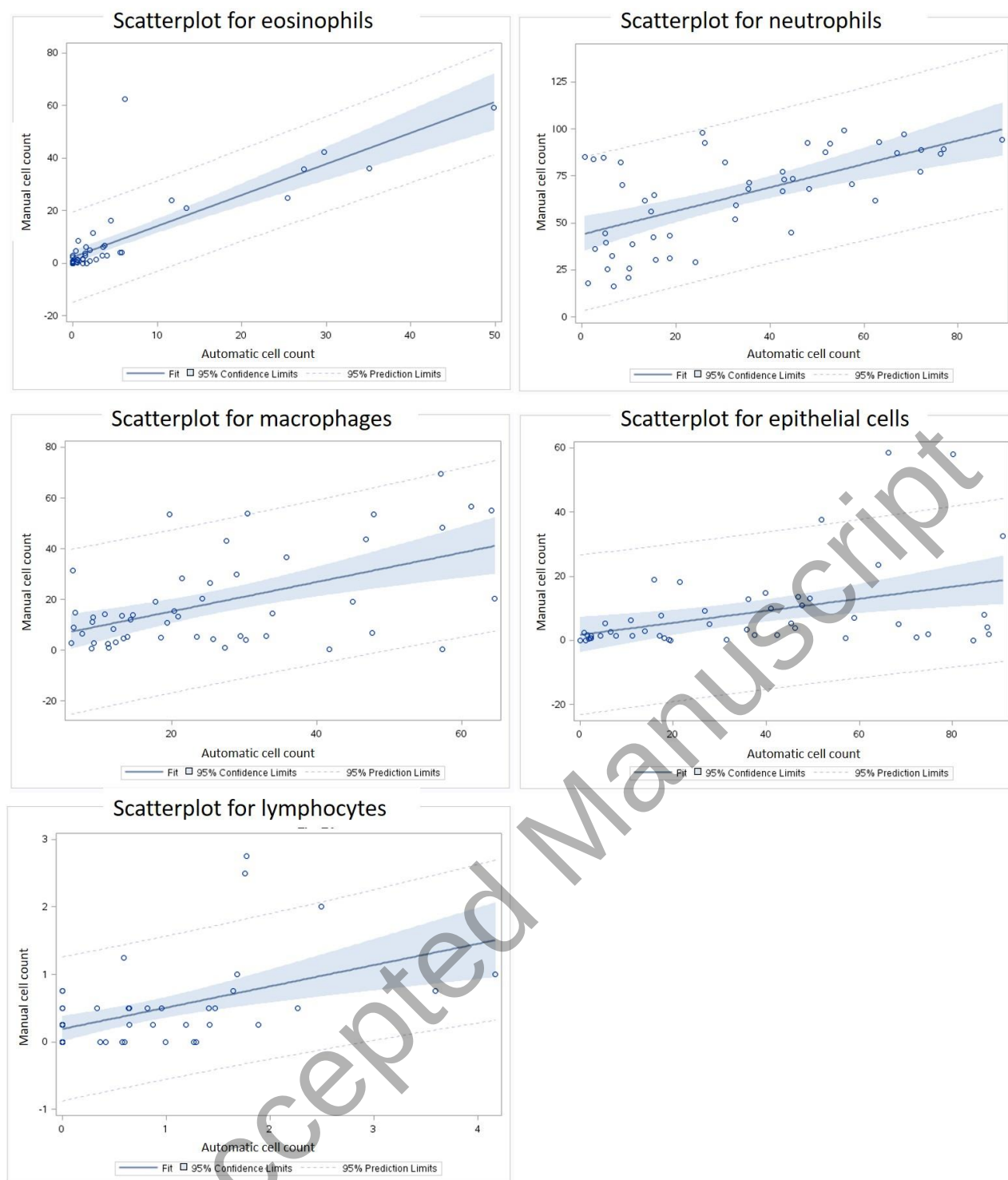


Figure 3: Scatter plots of target cells

Scatter plots of each target cell counted using each method respectively, including a fitted line and the 95%-confidence and- prediction limits.

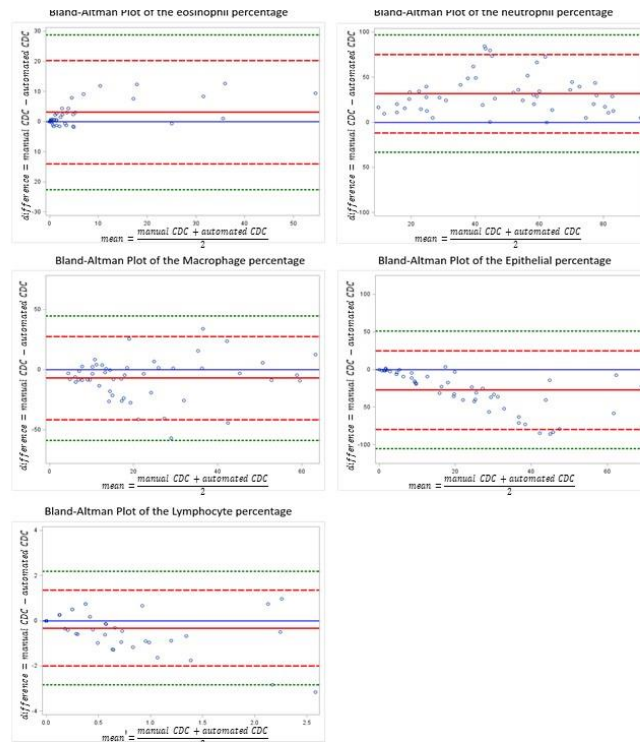


Figure 4: Bland-Altman plots of target cells.

Reference line: Blue. Mean of the difference: Solid red. Limits of agreement: Dashed red. $\pm 3SD$ of the mean of the differences: Dashed green