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# **Integration of Immature Granulocytes quantification with the version 2.0 UniCel DxH 800 in the HematoFlow strategy**

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**Running Title:** Validation of the immature granulocyte count by DXH 800

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**Keywords:** Immature Granulocytes, HematoFlow, DxH 800, EGC, Productivity, Slide Review Rates, WBC Differentials

## **Abstract**

### **Objectives:**

Our aim was to define whether the Early Granulocyte Cell marker (EGC%\_DxH) parameter might replace immature granulocytes counts obtained by HematoFlow (IG%\_HF) and/or manual differential count (IG%\_manual).

### **Methods:**

We conducted a study over a ten day period in Feb 2014 whereby 402 samples were analyzed for the IG flag. We correlated the EGC%\_DxH versus IG%\_HF and IG%\_manual, identified any discrepant results and finally looked at the impact on our workflow by incorporation of the EGC% into our WBC Differential algorithm.

### **Results:**

On an initial training set a ROC curve analysis showed a threshold of 0.9% for EGC%\_DxH (sensitivity of 91.7%, specificity of 93.5% and an area under the curve of 0.965). Further analysis of the dataset (259 samples) found a correlation of the EGC%\_DxH to all our IG% counting methods ( $r=0.963$ ). Incorporation of the EGC%\_DxH into the WBC HematoFlow Differential resulted in a 36% reduction of samples requiring HematoFlow and/or slide review.

### **Conclusions:**

The EGC% generated by the DxH 800 can be easily incorporated into existing HematoFlow and slide review algorithms.

## Introduction

Immature granulocytes (IG) are the precursors of neutrophils and include promyelocytes, myelocytes and metamyelocytes. IG are normally found in the bone marrow. With the exception of newborns, they are rarely observed in the peripheral blood of normal, healthy individuals. Therefore, the presence of IG in a peripheral blood sample is considered abnormal and indicates increased myeloid cell production, which can be the result of infection (especially of bacterial origin), as a response to severe inflammation, hematological myeloid diseases such as myelodysplastic or myeloproliferative syndroms or, finally, as a result of metastatic bone marrow infiltration by tumors. Accordingly, enumerating IG can be helpful, not only for detecting the aforementioned diseases, but also in monitoring treatment and progression of these diseases.<sup>1</sup>

To date, the reference method for their enumeration is still the microscopic slide review.<sup>2</sup> Unfortunately, manual counts show a large inter-observer variation and poor reproducibility, especially when IG are present in low numbers in the peripheral blood.<sup>3</sup>

The UniCel DxH 800 (Beckman Coulter, Brea, CA) is an automated analyzer capable of performing complete blood counts (CBC) and the leukocyte differential.<sup>4</sup> In addition, IG are classified using the expanded VCSn (Volume, Conductivity, Scatter with multiple angles) technologies on the DxH 800 and are called Early Granulocyte Cells (EGC%\_DxH).

In our laboratory the differential workflow and slide review incorporates the DxH 800 connected to the HematoFlow (HF) (Beckman Coulter) immunodifferential flow cytometry process.<sup>5</sup> HematoFlow is an innovative technique that uses a cocktail of six monoclonal antibodies for immunophenotyping and production of an accurate

extended WBC differential including an IG count.<sup>5</sup> The 2.0 version of the gating software has been recently released and is implemented in our routine lab.<sup>6</sup> With this workflow, qualitative flags generated by the DxH 800 (i.e. Blast, Atypical lymphocyte and IG) trigger a HematoFlow immunodifferential with or without slides review. IG is the most frequent flag generated by Haematology Analyzers in our experience and this finding has been confirmed by other reports in the literature.<sup>7</sup> Therefore accurate, automatic and rapid quantification of IG could improve productivity, avoid unnecessary analyses and improve the time of reporting for clinicians to take appropriate action.

The primary objective of our study therefore was to evaluate the performances of the new EGC%\_DxH count and compare this with the reference method used routinely in our laboratory. The secondary objective was to determine the reduction in the number of HF and/or slide reviews by the integration of the EGC%\_DxH parameter into the algorithm of our routine workflow.

## Material and Methods

### Patient samples and study design

During a 10 day period (from January 28<sup>th</sup> to February 8<sup>th</sup> 2014), consecutive blood samples exhibiting an IG flag after CBC and leukocyte differential on DxH 800 were included in this study. CBC and leukocyte differential were performed on K2-EDTA anticoagulated blood samples from the clinical departments of University Hospital of Rennes, as recommended by manufacturer instructions. This non-interventional study did not require patient consent according to French law. In total 6,913 CBC samples were routinely processed during this evaluation period. As defined in our laboratory procedures, the IG flag does not allow automatic validation and generates a HF differential for verification. Briefly, after red blood cell lysis and immunostaining with CytoDiff Reagent on a FP1000 sample preparation system (Beckman Coulter), the HematoFlow leukocyte differential is performed as previously described with a FC500 flow cytometer (Beckman Coulter).<sup>5</sup> ~~The CytoDiff CXP software version 2.0 was employed for automatic gating of the population of interest.<sup>6</sup> Accordingly to previous internal evaluation comparing Hematoflow and manual slide review, the threshold for IG obtained by Hematoflow (IG%\_HF) is at 1.4% for a cut off at 1% on manual review (unpublished data). As such, in our routine procedure IG%\_HF <1.4% is reported as zero on the CBC diff report. value of IG obtained by HematoFlow (IG%\_HF) is considered as negative when the percentage is lower than 1.4% (unpublished data). As immature granulocytes are considered as pathological condition, in these cases, the IG% is reported as zero on the CBC diff report.~~

The HF algorithm is able to detect the 5 regular populations (neutrophils, eosinophils, basophils, lymphocytes, monocytes) as well as 2 abnormal populations (immature granulocytes and blasts). After exclusion of B-cells (Side scatter (SS<sup>low</sup>/CD19<sup>pos</sup>),

monocytes ( $CD36^{pos}/CRTH2^{neg}$  and  $CD2^{neg}$ ) and mature neutrophils ( $SS^{high}/CD16^{pos}$ ), IG are defined as cells with high granularity on SS axis with lower expression of CD45 and CRTH2 than eosinophils on a biparametric dot-plot  $CD2-CRTH2/CD45$ .<sup>5,8</sup> The IG phenotype is confirmed by a lower expression of CD16 and CD45 than mature neutrophils on  $SS/CD16$  and  $SS/CD45$  plots respectively which is used in the process of the validation. In our workflow, when IG are higher than 15% without previous results in the seven preceding days or if leukocytes are missidentified by HematoFlow, a systematic manual slide review is required to confirm the amount of IG.

### **Statistical analysis**

For consecutive samples presenting with the IG flag on DxH 800, we recorded the automatic flags, absolute leucocyte count,  $EGC\%_{DxH}$ ,  $IG\%_{HF}$  and a 200 cells manual differential count ( $IG\%_{manual}$ ). Differences between these values were calculated. Demographic data (age and clinical departments) were also collected. Discordant cases were defined either as a difference higher than 5% between the two techniques or by qualitative discrepancies ( $EGC\%_{DxH}^{neg}$  and  $IG\%_{HF}^{pos}$ ). For these cases, microscopic leucocyte differential were performed and each slide was retained for further review. Standard statistical methods including Spearman correlation, linear regression and Bland-Altman test were used for data analysis using GraphPad 5.0 (Prism Software).

An initial training set enabled to compare  $EGC\%_{DXH}$  with the two reference methods  $IG\%_{HF}$  and  $IG\%_{manual}$  and define an  $EGC\%_{DxH}$  threshold with receiver operating characteristic (ROC) curve using the MedCalc statistics program (MedCalc Software, Mariakerke, Belgium). A validation set was used to evaluate the

integration of EGC%\_DxH in our routine HematoFlow workflow. A second sample set evaluated a validation reflex algorithm to integrate EGC%\_DxH parameter in our laboratory workflow.

## **Results**

### **Samples**

During the period from January 28<sup>th</sup> to February 8<sup>th</sup> 2014, 6,913 samples were tested in our laboratory for a CBC and leukocytes differential profile. For 470 out of these 6,913 samples (6.8%), an IG flag was generated thus not allowing automatic validation and triggering a HF differential. After exclusion of error flagged samples (R-flag) for EGC% parameter, 402 (85.5%) patients were available for analysis in this study. Both HF and slide review were performed for the training set on 143 samples whereas in the validation set of 259 patients, a HF panel with or without slide review was performed according to our routine workflow.

The median age of these patients was 60.5 years (Interquartile range 36.5-74.5 years). The source of the samples included 46.3% from General Medical Departments, 15.3% from Emergency and Intensive Care Departments, 9.5% from Surgical Departments, 6.2% from Pediatric Non Hematology-Oncology Departments and 22.3% from Hematology-Oncology departments (17.6% adult and 4.5% pediatric).

To validate the repeatability of the IG count on the DxH 800, 3 random samples with low, intermediate and high count of IG (mean at 2.13, 5.26 and 20.86, respectively) were re-run 10 times. Coefficients of variation were calculated at 9.1%, 3.9% and 4.7% respectively. Then, we assessed the longitudinal reliability of EGC%\_DxH by

testing two samples with low or high level of IG% at the beginning and at the end of each series (every 8 hours in our institution). The coefficient of variation obtained with this method on two consecutive series, on the three DxH 800 with three different operators was of 7.63% and 5.70% for low (3% of IG) and high level (10% of IG) respectively (data not shown).

### **Threshold calculation on a training set**

A first training set of 143 patients was evaluated in order to define the best threshold for EGC%\_DxH compared to our reference methods: Flow cytometry (Hematoflow) and manual 200 cells differential count (Figure 1). The median EGC%\_DxH was at 1.4% ranged from 0.0 to 17.3%. The median value of IG%\_HF was at 2.1% (from 0.0 to 35.0%). EGC%\_DxH and IG%\_HF were correlated ( $r=0.873$ ) (Figure 1A). No significant bias was evidenced by Bland-Altman analysis (Figure 1B). Forty-six samples (32.2%) were considered as negative and 97 (68.8%) positive with IG%\_HF (threshold at 1.4%). ROC curve analysis showed a positive threshold of EGC%\_DxH at 0.9% with a sensitivity of 91.8% and a specificity of 93.5% and an area under the ROC curve of 0.965 (Figure 1C). The concordance rate between the two methods was at 92.3%.

After slide review, IG%\_manual showed a median value of 2.0% (from 0.0% to 35.0%) and exhibit a good correlation with EGC%\_DxH with ( $r=0.899$ ) (Figure 1D). No significant bias was evidence with Bland-Altman analysis (Figure 1E). Forty-two samples (29.4%) were considered as negative and 101 (70.6%) exhibited an IG%\_manual higher than 1.0%. The concordance rate was 91.8%. ROC curve analysis showed that the same threshold of 0.9% for EGC%\_DxH exhibited a sensitivity of 90.1% and a specificity of 97.6% with an area under ROC curve of

0.979.

The good correlation observed between EGC%\_DxH and both IG%\_HF and IG%\_manual enabled to evaluate this new parameter in our workflow with a threshold at 0.9%.

### **Comparison of IG% on the DxH 800 and with HF strategy**

In the validation set of 259 patients, we compared the performance of DxH 800 and HF for IG% quantification with the thresholds previously defined (Figure 2). The mean EGC%\_DxH was 1.8% (range from 0.0% to 77.9%) and a mean IG%\_HF of 1.9% (range from 0.0% to 44.0%). The linear regression was comparable with the initial training set data ( $y=1.188x+1,086$  with a Spearman correlation at  $r=0.775$ ). Considering the thresholds previously calculated, results from DxH and HematoFlow were concordant in 226 samples (87.3%) of cases with 123 DxH<sup>neg</sup>/HF<sup>neg</sup> and 103 DxH<sup>pos</sup>/HF<sup>pos</sup> (Figure 2A).

For 33 samples (12.7%) discrepant results was found. Six samples were considered false negative (DxH<sup>neg</sup>/HF<sup>pos</sup>) and 27 false positive (DxH<sup>pos</sup>/HF<sup>neg</sup>). Under these conditions, EGC%\_DxH exhibited a sensitivity of 79,2%, a specificity of 95.3%, a negative predictive value of 94.5% and a positive predictive value of 82.0%. Not surprisingly the source of samples (clinical department site) influenced the rate of discrepant samples. The percentage of discrepant samples was much higher from the Hematology-Oncology departments (44.4%) compared to those from non-Hematology-Oncology departments (22.3%) and was statistically significant ( $p=0.02$ ). Major discrepancies defined as a difference higher than 5% between IG%\_HF and EGC%\_DxH were found in 16 samples. These are denoted and plotted in red in figure 2C and in Table 1 (Range from 5.6 to 77.9%). Further analysis of these 16

samples showed seven had a “Blast” flag requiring HF independently of IG% status. Eight samples presented with others cytopenias (thrombocytopenia or neutropenia). Eleven of these samples emanated from the Hematology-Oncology departments. The biggest difference was found in a case of myeloblastic leukemia with 76% of myeloblasts identified both by HF and slide review. These myeloblasts were assessed as EGC% by the DxH (Figure 3A).

We proposed a validation algorithm integrating the EGC%\_DxH for patients presenting with “Imm Gran” flag (Figure 2C). Quantitative flags “Blasts” or “Atypical lymphocytes” require HF analysis whatever the differential count or EGC%\_DxH. Then, in the absence of EGC (EGC%\_DxH ≤ 0.9), the blood differential count can not be validated and requires HF or slide review in case of i) lymphocytosis, monocytosis or cytopenia (thrombocytopenia or neutropenia) if no concordant results in the previous 7 days ii) previous results without IG in the last 7 days iii) first hematology consultation. In all other cases without additional flags for WBC review the results for EGC%\_DxH < 0.9 can be validated and reported as 0.0% on CBC-diff report. In presence of EGC%\_DxH > 0.9, HF is still necessary in case of i) unknown cytopenia or lymphocytosis or monocytosis ii) major IG% discrepancies (delta > 5%) in the last 7 days or absence of previous results iii) first hematology consultation. In other situations the EGC% results can be reported as part of WBC differential without performing IG count with HF analysis. Using this algorithm, 92 out of the 259 patients (36%) could be validated (37 EGC%\_DxH<sup>neg</sup> and 55 EGC%\_DxH<sup>pos</sup>) directly after DxH analysis (Figure 2B). This procedure efficiently selected samples requiring HF. Indeed, 9 out of the 33 discrepant samples were validated with EGC%\_DxH values: 7 were DxH<sup>neg</sup>/HF<sup>pos</sup> whereas 2 were DxH<sup>pos</sup>/HF<sup>neg</sup>. Interestingly, seven corresponded to IG% amount lower than 2%. The last 2 samples EGC%\_DxH<sup>neg</sup>

were quantified at 4% and 26% with HF. For these samples, HF count was overestimated, due to eosinophils being incorrectly classified as IG (Figure 3B). Therefore our results showed real discrepancies only concerned in very low counts of IG% where the clinical impact is negligible. Moreover, EGC%\_DxH validated results showed excellent correlation with results obtained with our actual workflow (HF and eventual slide review) with a spearman correlation coefficient of  $r=0.953$  and a linear regression of  $y=1.339x+0,689$  (Figure 2B).

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## Discussion

The algorithm within the DxH 800 Hematology Analyzer System identifies, extracts and enumerates the immature granulocyte subpopulation (called early granulocyte cells) of white cells within the granulocyte population using mathematical transformation and/or combinations of the VCSn measurements which the granularity, the size and the opacity of each cell. “Immature Gran” (IG) flag is the most frequent DxH 800 flag that requires HematoFlow differential validation and possible eventual slide review in our actual routine workflow. The sensitivity of the IG flagging enables us to validate the leukocyte differential without IG for unflagged samples.<sup>9</sup> For IG flagged samples the HematoFlow procedure and especially slide review is time consuming and might require an excellent level of expertise in morphology. **Noteworthy, our routine workflow has been implemented with the CytoDiff CXP version 2.0.<sup>6</sup> This new version benefit of improvements notably useful in defining IG that were frequently misidentified with the version 1.0; for instance IG were sometime miscounted as eosinophils or CD16<sup>dim</sup> polynuclear neutrophils incorrectly counted as IG, leading to high variability in IG count.<sup>6</sup> The version 1.0 was employed in recent papers and to our knowledge our work is the first one using the new version.<sup>10</sup> We showed that the EGC%\_DxH correlated well with HF with a positive cut-off of 0.9% in an IG flagged population. In our ten day study of 6,913 samples we were able to demonstrate that incorporation of the EGC% from the DxH 800 into our routine slide review algorithm had a positive impact.**

EGC%\_DxH showed a very good overall correlation with our reference methods, HF and/or slide review ( $r=0.953$ ). Linear regression and correlation obtained for EGC is comparable with the other classical leukocyte subpopulations which are present usually in smaller numbers - particularly monocytes and eosinophils which present

with similar range of values in the peripheral blood.<sup>5,7,8</sup> The EGC%\_DxH correlation as per our study showed similar performance to the quantification of immature granulocytes as seen with those generated by the Sysmex XE analyser platform.<sup>11</sup> Based on our study incorporation of the EGC% into our routine algorithm results in a reduction of 92/259 (36%) of samples that requires either HematoFlow differential and/or microscopical slide review.

In those samples where the EGC% was discrepant with HF we found some samples where eosinophils were misidentified as IG. This mis-classification can be identified by the operator, either by the difference in eosinophils count between DxH 800 and HematoFlow, or by the observation of the SS/CD45 and SS/CD16 plots (eosinophils are CD45high whereas IG are CD45low) (Figure 3B). Another possible reasons for IG mis-classification include exceptions when mature neutrophils can lose CD16 in pathologic conditions like severe sepsis<sup>12</sup> and paroxysmal nocturne hemoglobinuria<sup>13</sup> leading to false IG identification. These conditions can be easily suspected on HF histograms. Indeed, IG presents a gradual gain of CD16 during their maturation. Therefore, they exhibit a continuum of CD16 expression and as such a specific pattern on SS/CD16 histogram. In case of hematologic malignancy (PNH) or myelodysplastic syndromes, the pathologic cells exhibit a low but homogenous CD16 expression leading to a distinct cluster on SS/CD16 histogram (Figure 3C). Otherwise, myeloblastic blasts can be identified as immature granulocytes by DxH but accurate flagging avoid automatic validation. HF histogram reviewing enable to suspect blasts with their lower CD45 expression and SS intensity, leading to a systematic slide review for each new diagnosis. Indeed, HF never substitutes a manually performed differential count for the initial diagnosis of hematologic malignancies. In all these malignant conditions where there is a potential risk of

autovalidation of an incorrect EGC%, the incorporation in our algorithm of other criteria (flags, unknown cytopenia and samples from hematology departments) results in these samples being accurately identified and processed for HF and/or slide review (see Figure 2C). As reported by et Allou et al, the workflow can also be improved by an automated flagging strategy on HematoFlow reviewing that enables an automatic validation of HF leukocyte differential<sup>14</sup>.

Therefore in conclusion DHX800 accurately quantifies the presence of immature granulocytes through the new EGC%\_DxH parameter and can be safely integrated into the WBC differential workflow and the HematoFlow process. Thus we propose an improved validation algorithm, which integrates the EGC%\_DxH from the DxH 800 Haematology Analyzer with our current HematoFlow strategy to further streamline and improve our slide review process. The EGC%\_DxH parameter is automatically generated by the DxH 800 so therefore is available at no additional cost. It allows us to further reduce our slide review rate in IG flagged samples by 36% and also allows for improved turn-around time for reporting to clinicians in the various departments we service. Only minor discrepancies in low IG% may be missed however as our study showed these have little if any clinical relevance.

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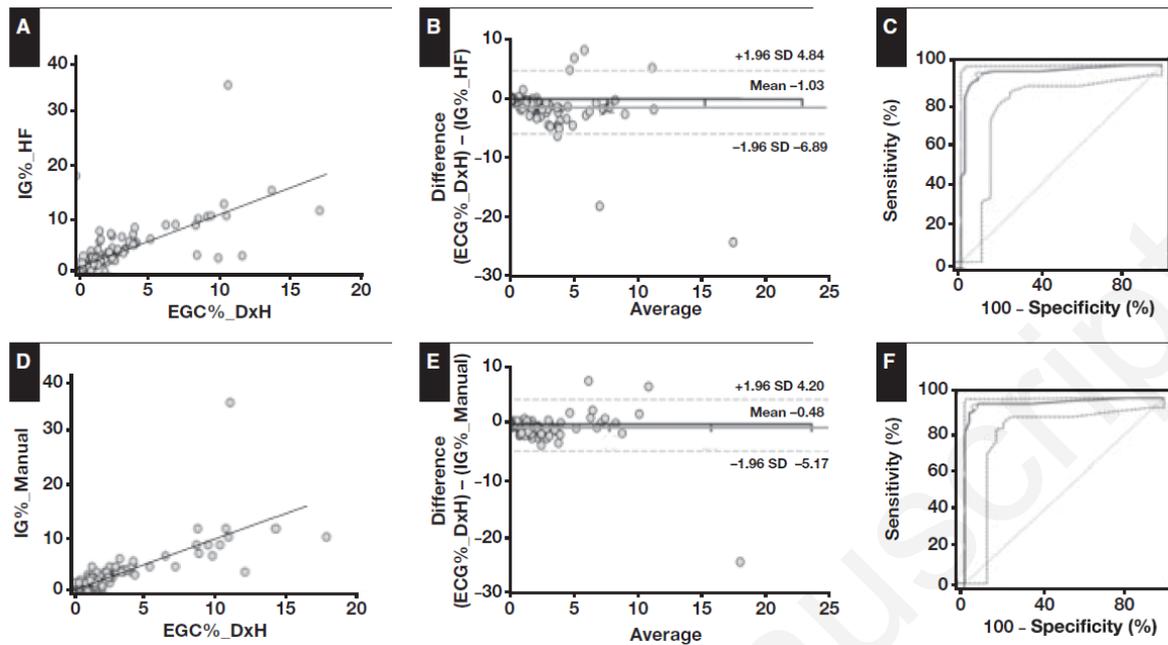
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**Table 1: Major discrepancies between EGC%\_DxH and IG%\_HF in validation set**

	Sex/age	Department	WBC (G/L)	Flag	EGC_DxH (%)	Imm Gran_HF (%)	Slide review	Validation
EGC% DxH <sup>pos</sup> /HF <sup>neg</sup>	F/36	<b>Hematology</b>	24.8	<b>Blast</b>	<b>77.9</b>	<b>0.0</b>	0% (76% blast) Fig 3A	No
	F/42	Medicine	2.1	Neutropenia	<b>0.0</b>	<b>7.4</b>	0 % (7% eosinophils) Fig 3B	No
	F/40	Medicine	3.4	Neutropenia	<b>0.0</b>	<b>11.4</b>	11%	No
EGC% DxH <sup>neg</sup> /HF <sup>pos</sup>	F/64	<b>Hematology</b>	0.8	Neutropenia	<b>0.0</b>	<b>12.1</b>	0% (13% eosinophils)	No
	F/74	Surgery	18.9	No	<b>0.0</b>	<b>26.1</b>	1% (19% eosinophils)	<b>Yes</b>
	F/31	<b>Hematology</b>	3.4	Neutropenia	<b>0.0</b>	<b>34.7</b>	0% (45% of CD16-negative neutrophils in HPN) Fig 3C	<b>Yes</b>
	M/8	<b>Hematology</b>	6.0	Thrombocytopenia	<b>8.9</b>	<b>14.6</b>	15%	<b>Yes</b>
	M/8	<b>Hematology</b>	8.0	Thrombocytopenia	<b>5.6</b>	<b>11.8</b>	12%	<b>Yes</b>
	M/8	<b>Hematology</b>	4.9	Thrombocytopenia	<b>9.2</b>	<b>15.7</b>	10%	<b>Yes</b>
	F/15	<b>Hematology</b>	9.1	<b>Blast</b>	<b>8.6</b>	<b>15.8</b>	12%	No
EGC% DxH <sup>pos</sup> /HF <sup>pos</sup>	M/8	<b>Hematology</b>	6.3	Thrombocytopenia	<b>7.7</b>	<b>15.0</b>	9%	<b>Yes</b>
	F/64	<b>Hematology</b>	85.0	<b>Blast</b>	<b>18.5</b>	<b>10.6</b>	11%	No
	M/66	ICU	8.0	<b>Blast</b>	<b>1.0</b>	<b>9.3</b>	9%	No
	M/82	Medicine	45.8	<b>Blast</b>	<b>2.0</b>	<b>10.8</b>	11%	No
	F/15	<b>Hematology</b>	8.5	<b>Blast</b>	<b>9.9</b>	<b>22.4</b>	13%	No
	M/7	<b>Hematology</b>	10.6	<b>Blast</b>	<b>6.0</b>	<b>26.0</b>	10%	No

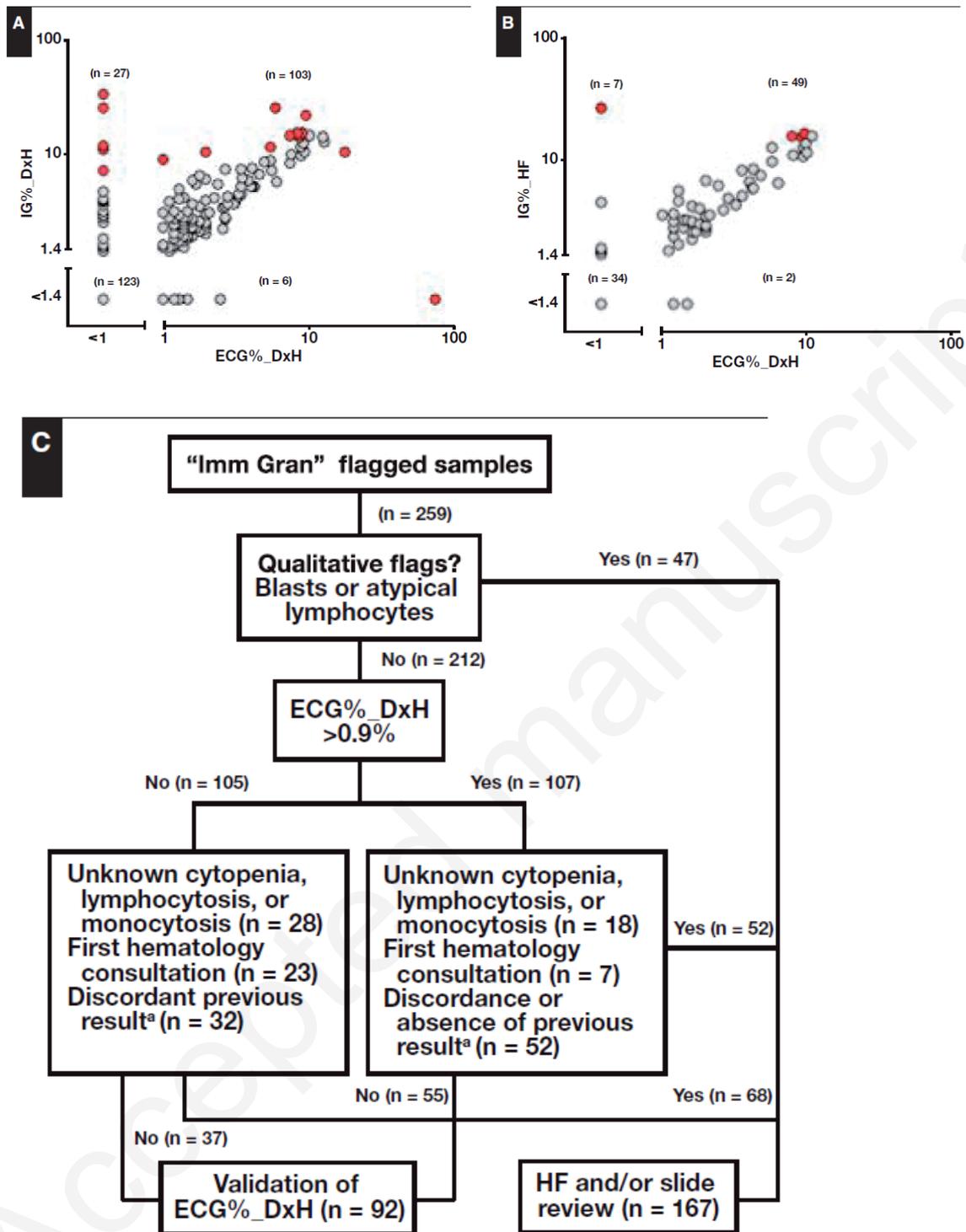
ICU : Intensive Care Unit ; PNH: Paroxysmal Nocturnal Hemoglobinuria

## Figures legends



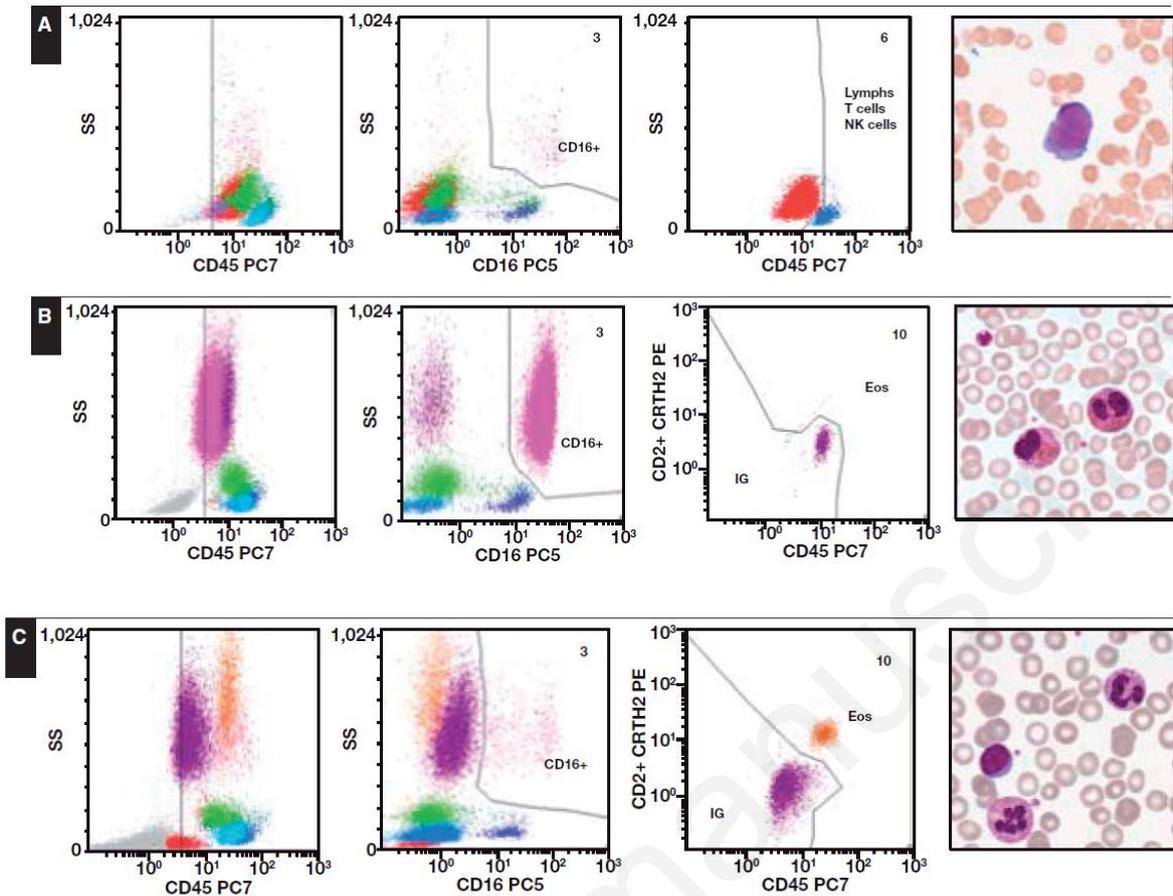
**Figure 1: Threshold definition of ECG%\_DxH**

**A-** Comparison between ECG%\_DxH and IG%\_HF in the training set (N=143). **B-** Bland-Altman analysis between ECG%\_DxH and IG%\_HF. **C-** Receiver operating characteristic curve identified a threshold of 0.9% for ECG%\_DxH (AUC: area under ROC curve). **D-** Comparison between ECG%\_DxH and IG%\_Manual. **E-** Bland-Altman analysis between ECG%\_DxH and IG%\_manual. **F-** ROC curve identified a threshold of ECG%\_DxH at 0.9%.



**Figure 2: Integration of EGC%\_DxH in HematoFlow strategy**

**A-** Comparison of EGC%\_DxH and IG%\_HF in validation set (N=259): linear regression  $y=1.188x+1,086$  with a Spearman  $r=0.775$ . Logarithmic representation of results after thresholds application. **B-** Logarithmic representation of the 92 validated EGC%\_DxH following the algorithm of validation detailed on figure C. **C-** Reflex algorithm for EGC%\_DxH validation. N represents the number of samples in the subgroup. <sup>a</sup>Discordant results are defined by IG% delta above 5% or qualitative discrepancies with previous results in the last 7 days.



### Figure 3: Example of major discrepancies

**A-** Representative HematoFlow (HF) dotplots identifying blasts Xm (red dots) defined as  $CD45^{low}/SSC^{low}/CD16^{neg}$  cells.  $EGC\%_{DxH}$  was quantified at 77.9% and slide review confirmed the presence of 76% of myeloblasts (right picture) without immature granulocytes (IG). **B-** HF dotplots identified 5% of IG whereas  $EGC\%_{DxH}$  was  $<0.9\%$ . Slide review exhibited 4% of eosinophils and no IGs. Note the high CD45 expression, CRTH2 positivity and CD16 negativity of purple dots suggesting a false identification of IGs. **C-**  $CD16^{neg}$  neutrophils identified as IG by HematoFlow in the context of a confirmed paroxysmal nocturne hemoglobinuria. Note the absence of continuum in CD16 expression in purple cells. Slide review did not evidenced IG but only neutrophils, according to  $EGC\%_{DxH}$ .