Age-Dependent Reference Ranges for Automated Assessment of Immature Granulocytes and Clinical Significance in an Outpatient Setting

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- Context.—New generations of hematology analyzers have made the routine automated quantification of immature granulocytes (IGs) in peripheral blood samples accessible as a powerful clinical parameter.

- Objective.—The use of IGs has previously been studied mostly in hospitalized patients with sepsis. We investigated the use of IGs in the outpatient setting. Establishment of precise normal outpatient IG reference ranges is a prerequisite for clinically meaningful interpretation of the parameter.

- Design.—We analyzed a large outpatient population comprising more than 2400 samples to determine age-stratified normal reference ranges for IGs.

- Results.—Using nonparametric statistical approaches, we show that 1-tailed 95th percentile estimates for relative and absolute IG concentrations up to the age of 10 years are 0.30% and 30.0 $\mu$L$^{-1}$, respectively. For individuals above the age of 10 years, the respective 95th percentile estimates are approximately twice as large at 0.74% and 60.0 $\mu$L$^{-1}$. No differences were seen between male and female reference ranges. Taking nonparametric 90% confidence intervals for each estimate into account, we recommend the following IG upper reference range limits for routine outpatient use: 0.30%/40.0 $\mu$L$^{-1}$ (≤10 years) and 0.90%/70.0 $\mu$L$^{-1}$ (>10 years). Up to the age of 10 years, the most common pathologies associated with elevated IG counts in outpatients were infections, in particular, otitis media, upper and lower respiratory infections, and gastroenteritis. By contrast, above the age of 10 years, the most common causes were hematologic malignancies, drug therapy (glucocorticoids, chemotherapy), severe infections, and pregnancy (young females).

- Conclusions.—The use of appropriate reference ranges makes IGs a powerful hematologic parameter for outpatient care that is associated with differential diagnoses that are distinctly characteristic of that setting.

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Modern automated hematology analyzers are capable of measuring additional parameters beyond the traditional complete blood cell count with 5-part white cell differential. Among these, the immature granulocyte (IG) parameter in peripheral blood has recently gained considerable interest. Immature granulocytes are defined as the collection of maturing granulocytic myeloid cells that have differentiated beyond the myeloblast stage, but have not yet reached the stages of band form neutrophils, eosinophils, or basophils.1 Immature granulocytes thus comprise promyelocytes, myelocytes, and metamyelocytes. Because of their low abundance under normal conditions (≪1%), IGs are generally difficult to quantify precisely by manual smear examination. However, flow cytometry–based analyzers routinely count many thousands of cells per sample and are thus capable of precisely enumerating even very rare cells in peripheral blood.2 Previous work has focused almost exclusively on IGs in hospitalized patients and their potential role for predicting sepsis.3,4 Similarly, even “normal” reference ranges for IGs have largely been derived from and validated against hospitalized patients.3,4 Furthermore, reference ranges and clinical associations of IGs in pediatric populations have not been studied comprehensively.

In this article, we use a large data set of more than 2400 samples from nonhospitalized outpatients to derive a variety of IG reference ranges stratified by age and sex. One great advantage is that, because of the size of the data set, we can make exclusive use of nonparametric statistical methods.5 These are considered ideal and are the gold standard because a priori statistical assumptions with regard to sample distribution are not needed.5 We then apply these reference ranges to assemble lists of differential diagnoses frequently observed in outpatients with significantly elevated IG counts.

MATERIALS AND METHODS

Sample Collection and Analysis

East Boston Neighborhood Health Center (Boston, Massachusetts) is a comprehensive outpatient-only medical center covering both primary care and extensive specialty care, including large pediatric, obstetrics/gynecology, and geriatric services. All peripheral venous blood samples were collected in K$_2$EDTA anticoagulant. All hematologic measurements were performed...
on the same Sysmex XT-1800i instrument (Sysmex, Kobe, Japan). All samples were analyzed within 4 hours of collection and those with markedly abnormal results were routinely repeated. Three levels of commercial quality control specimens (Sysmex c-Check) were run at least daily. Quality was further monitored via participation in a hematology proficiency testing program of the College of American Pathologists.

The XT-1800i performs a differential count based on a combination of light scatter and fluorescence emission. An aliquot of blood is diluted after lysis of red blood cells and incubated with a polymethine-based RNA- and DNA-binding fluorescent dye. Neutrophils, eosinophils, monocytes, and lymphocytes are differentiated on the basis of their light scatter and fluorescence expression characteristics using electronic cluster analysis protocols. The XT-1800i electronically determines the cluster of IGs from the granulocyte cluster in the differential histogram. IGs are recognized by their increased fluorescence.

### Data Analysis and Statistics

It is preferable to use quantile estimates that do not depend on the underlying distribution of analytic values. Such distribution-free estimators are based on the order statistics from the sample. The order statistics are the n sample values sorted into ascending order as follows:

\[ X_{(1)} \leq X_{(2)} \leq \ldots \leq X_{(n)} \]

A nonparametric estimator of the pth quantile, \( \hat{F}^{-1}(p) \), can be calculated as follows:

\[
\hat{F}^{-1}(p) = \begin{cases} 
X_{(k)} & \text{if } p \leq \frac{k}{n+1}, \\
X_{(k)} + \left( h - [h] \right) \left( X_{(k+1)} - X_{(k)} \right) & \text{if } \frac{k}{n+1} \leq p < \frac{k+1}{n+1},
\end{cases}
\]

where \( h = (n + 1)p \) and \([h]\) is the largest integer not greater than \( h \). This expression corresponds to a linear interpolation of the expectations for the order statistics for the uniform distribution on [0,1] (see also Clinical and Laboratory Standards Institute standard C28-A3).13

The nonparametric \( \gamma \times 100\% \) confidence interval of \( \hat{F}^{-1}(p) \) consists of the interval defined by the 2 order statistics \( (X_{(0)}, X_{(\gamma)}) \), where

\[
\sum_{i=1}^{r-1} \left( \frac{n}{i} \right) p^{(1-p)^{i-1}} \geq \gamma,
\]

while minimizing \((r - l)\) under the appropriate constraint: \(1 \leq l \leq r \leq n \) if \( p < 1/(n + 1) \), \( 1 \leq l \leq r = n \) if \( p \geq n/(n + 1) \), \( 1 \leq l \leq \lfloor h \rfloor \) if \( r = n \) if \( 1/(n + 1) \leq p < n/(n + 1) \) and \( h = \lfloor h \rfloor \), or \( 1 \leq l \leq \lfloor h \rfloor + 1 \leq r \leq n \) if \( 1/(n + 1) \leq p < n/(n + 1) \) and \( h - \lfloor h \rfloor > 0 \).12

### RESULTS

#### Age-Dependent IG Reference Ranges

To determine IG reference ranges, we started with a total data set comprising 2571 samples (Table 1). Because this total data set contained samples from patients with abnormal WBC counts, we eliminated samples that fell outside age-dependent WBC reference ranges to generate a reference range data set comprising 2443 samples (Table 1). Age and sex characteristics of the 2 data sets were very similar, with the exception that the number of elderly females was lower in the reference range data set because of higher prevalence of abnormal samples in this patient group.

Figure 1, A and B, shows patient age distribution histograms for the reference sample data set. In Figure 1, A, the distribution is shown by age decade for the range 0–100 years, and in Figure 1, B, the first decade is shown in further detail. Each histogram bar indicates the number of contributing samples from male and female patients as black and gray areas, respectively. It is apparent that the number of samples from patients up to the age of 10 years (1426) was larger than the sum of samples from all other age groups (1017). In particular, the pediatric population up to the age of 7 years was well represented (Figure 1, B).

The overall distribution of IG% concentrations in the reference range data set is depicted in Figure 2. The mode (ie, most frequent value of IG%) was 0.0%, which means that, in normal individuals, the number of IGs in peripheral blood fell most frequently below the instrument’s detection...
limit. Thus, the left limit of any IG# and IG% normal reference range will correspond to 0.0 m
L
2
1
and 0.0%, respectively. Conversely, clinically useful abnormal IG concentrations will only correspond to elevated values. By convention, clinical reference ranges capture the variability of 95% of normal individuals. To define a normal IG reference range, we were therefore, only concerned with determining the upper (ie, 1-tailed) limit of normal corresponding to the 95th percentile, with abnormal values falling into the right-sided tail beyond the 95th percentile. The large number of samples included in this study and their broad age distribution permitted the use of nonparametric sample statistics for estimating percentiles. Nonparametric methods are ideal because no assumptions with regard to underlying statistical distributions need to be made and no model-based data fitting needs to be performed.

We asked whether different age groups may require distinct IG reference ranges. The mathematical approach outlined in “Materials and Methods” was applied to estimate 95th percentiles and associated 90% CIs for both IG# and IG% concentrations (Table 2; Figure 3, A and B). We discovered that the results naturally grouped patients into those 10 years or younger and those older than 10 years, respectively. Interestingly, 95th percentile estimates in the latter group were approximately twice as large as in the former.

Focusing on the 2 age groups (≤10 years and >10 years), we found that IG upper limits of normal were essentially the same for both males and females, that is, independent of patient sex (Table 3), while again displaying comparable quantitative differences between the 2 age groups (Figure 4, A and B). We are not aware of currently developed nonparametric statistical quantile test procedures that allow testing of whether the 95th percentile estimates are significantly different between the 2 age groups. However, we have begun theoretical work toward such a quantile test. Nevertheless, it is important to note that none of the respective 90% CIs overlap (Figure 4, A and B).

Clinical Significance of Elevated IG Concentrations in an Outpatient Setting

Automated evaluation of IGs has been studied almost exclusively in hospitalized patients, in particular limited to the setting of sepsis and severely ill individuals. We were thus especially interested in examining the clinical associations of abnormal IG counts in nonhospitalized outpatients that may represent a very different spectrum of conditions and disorders. Although peripheral blood IG counts are readily available in an outpatient clinic setting from an automated analyzer such as the Sysmex XT-1800i, clinical impact, usefulness, and associated differential diagnoses have remained undefined for this large patient population. Figure 5, A and B, depicts the overall distributions of IG% concentrations for the total data set, separated by age groups (≤10 years and >10 years). The number of samples with elevated IG% was, as expected, greater than in the reference range data set (Figure 2) because patients with abnormal WBC counts were now included. In particular, some patients above the age of 10 years had highly increased values (>10%).

Scatterplots of the relationships between total WBC count and either IG% or IG# are instructive (Figures 6 and 7). It is apparent that the total WBC count does not generally correlate with elevated IG% or IG# concentrations. Using the data shown in Figures 6 and 7, we identified patients with significantly elevated relative and/or absolute IG concentrations and investigated the clinical scenarios that were most commonly associated (Table 4; Figure 8, A and B). Up to the age of 10 years, the
Alternative approaches need to make as-
sumptions about the underlying distribution of a parameter. For example, they may assume a normal distribution or that after some numerical (e.g., logarithmic) transformation of the primary data near normality is achieved. Although one potential advantage is that such parametric methods do not require data sets of the magnitude available to us in this study, we feel that the advantages of nonparametrically derived reference ranges far outweigh their disadvantages. Furthermore, recent statistical work has shown that so-called robust nonparametric strategies may be applied to even relatively small data sets and frequently outperform parametric approaches.

Through careful analysis of age- and sex-stratified reference range estimates (Tables 2 and 3), we found that IG concentrations for patients aged 10 years or younger and for those older than 10 years require distinct normal reference ranges with nonoverlapping 90% CIs, whereas values for males and females are similar (Figures 3 and 4).

Based on our data and accounting for the nonparametric 90% CI for each 95th percentile estimate, we recommend the following IG%/IG# upper limits of normal for routine clinical use with particular emphasis on outpatients (Table 3): 0.30%/40.0 µL⁻¹ (≤10 years) and 0.90%/70.0 µL⁻¹ (>10 years). Based on available method comparison data, we believe that these cutoffs should be very comparable and readily transferable to other methods of measuring IGs, including dedicated flow cytometry using cell-specific surface markers, other hematology analyzers, or manual counting.

We applied these cutoff criteria to an unselected cohort of 2571 samples to identify patients with significantly elevated IG counts in both age groups (Figures 5 through 7). We then asked which clinical scenarios were most often associated with this hematologic abnormality (Table 4; Figure 8, A and B). Both groups shared infections (such as respiratory infections) and drug therapy (glucocorticoids, chemotherapy), severe infections, and pregnancy (young females). The most common pathologies associated with elevated IG counts in outpatients were infections, in particular otitis media, upper and lower respiratory infections, and gastroenteritis. By contrast, above the age of 10 years, the most common causes were hematologic malignancies, drug therapy (glucocorticoids, chemotherapy), severe infections, and pregnancy (young females).

### Table 2. Age-Stratified Nonparametric Estimates of Upper Limits of Normal (95th Percentiles) for both IG# and IG% and Associated 90% CIs

<table>
<thead>
<tr>
<th>Group</th>
<th>Samples</th>
<th>Outliers</th>
<th>IG# UL (µL⁻¹)</th>
<th>IG# 90% CI (µL⁻¹)</th>
<th>IG% UL (%)</th>
<th>IG% 90% CI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤1 y</td>
<td>171</td>
<td>3</td>
<td>40.0</td>
<td>30.0–50.0</td>
<td>0.30</td>
<td>0.20–0.40</td>
</tr>
<tr>
<td>1–2 y</td>
<td>243</td>
<td>0</td>
<td>40.0</td>
<td>30.0–40.0</td>
<td>0.30</td>
<td>0.30–0.40</td>
</tr>
<tr>
<td>2–5 y</td>
<td>699</td>
<td>0</td>
<td>30.0</td>
<td>30.0–40.0</td>
<td>0.30</td>
<td>0.30–0.40</td>
</tr>
<tr>
<td>5–10 y</td>
<td>313</td>
<td>1</td>
<td>30.0</td>
<td>30.0–40.0</td>
<td>0.30</td>
<td>0.30–0.40</td>
</tr>
<tr>
<td>10–20 y</td>
<td>120</td>
<td>0</td>
<td>65.9</td>
<td>40.0–130.0</td>
<td>0.70</td>
<td>0.40–1.10</td>
</tr>
<tr>
<td>&gt;20 y</td>
<td>641</td>
<td>1</td>
<td>60.0</td>
<td>50.0–60.0</td>
<td>0.80</td>
<td>0.70–0.90</td>
</tr>
<tr>
<td>Total</td>
<td>2443</td>
<td>10</td>
<td>40.0</td>
<td>40.0–50.0</td>
<td>0.50</td>
<td>0.50–0.50</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; IG#, absolute IG concentration; IG%, relative IG concentration; UL, upper limit.

### Table 3. Nonparametric Estimates of Upper Limits of Normal (95th Percentiles) for Both IG# and IG% and Associated 90% CIs

<table>
<thead>
<tr>
<th>Group</th>
<th>Samples</th>
<th>Outliers</th>
<th>IG# UL (µL⁻¹)</th>
<th>IG# 90% CI (µL⁻¹)</th>
<th>IG% UL (%)</th>
<th>IG% 90% CI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>2443</td>
<td>10</td>
<td>40.0</td>
<td>40.0–50.0</td>
<td>0.50</td>
<td>0.50–0.50</td>
</tr>
<tr>
<td>≤10 y</td>
<td>1426</td>
<td>4</td>
<td>30.0</td>
<td>30.0–40.0</td>
<td>0.30</td>
<td>0.30–0.30</td>
</tr>
<tr>
<td>&gt;10 y</td>
<td>1017</td>
<td>6</td>
<td>60.0</td>
<td>50.0–70.0</td>
<td>0.74</td>
<td>0.70–0.90</td>
</tr>
</tbody>
</table>

Abbreviations: CI, confidence interval; IG#, absolute IG concentration; IG%, relative IG concentration; UL, upper limit.

* Bolded numbers correspond to cutoffs recommended for clinical use in an outpatient setting. See also Figure 3.

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Figure 3. Graphic comparison of age-stratified immature granulocyte (IG) upper limits of normal (95th percentiles) for (A) relative and (B) absolute IG concentrations with corresponding 90% confidence intervals (CIs; bars). Note the differences, including nonoverlapping CIs, between age groups up to 10 years and age groups above 10 years. Age intervals are left-exclusive and right-inclusive, respectively. See also Table 2.

Figure 4. Pairwise comparison of gender- and age group-stratified immature granulocyte (IG) upper limits of normal (95th percentiles) for (A) relative and (B) absolute IG concentrations with corresponding 90% confidence intervals (CIs, bars). Values for males (M) and females (F) are shown as blue and red dots, respectively. Black dots show values when samples from both sexes are combined. Age intervals are left-exclusive and right-inclusive, respectively. See also Table 3.

Figure 5. Histograms depicting the relative immature granulocyte (IG) concentration distribution for the total data set (n = 2571), including both reference range samples and abnormal samples. A. Samples from patients 10 years or younger (n = 1449). B. Samples from patients older than 10 years (n = 1122). Intervals are left-inclusive and right-exclusive, respectively. Note the markedly skewed distribution of values.
Figure 6. Scatterplots using patients from the total data set aged 10 years or younger (n = 1449) illustrate the relationship between total white blood cell (WBC) concentrations and (A) relative or (B) absolute IG concentrations. The dashed red line indicates separation between normal values (below: less than or equal to the upper limit of the 90% confidence interval [CI] of the 95th percentile estimate) and abnormal values (above: greater than the upper limit of the 95% CI of the 95th percentile estimate). Note that quantization of IG concentrations along the ordinates is due to smallest instrument-reported absolute value differences of 0.1% and 10.0μL⁻¹ for relative and absolute IG values, respectively (see “Materials and Methods”).
Table 4. Examples of Clinical Scenarios in Which Elevated IG% and IG# Were Observed in the Total Data Set$^a$

<table>
<thead>
<tr>
<th>IG% (%)</th>
<th>IG# (µL–1)</th>
<th>WBC (10^11µL–1)</th>
<th>Age, y</th>
<th>Clinical Setting</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤10 y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.3</td>
<td>180</td>
<td>13.4</td>
<td>5.3</td>
<td>PNA</td>
</tr>
<tr>
<td>1.1</td>
<td>130</td>
<td>11.7</td>
<td>5.6</td>
<td>URI</td>
</tr>
<tr>
<td>1.1</td>
<td>60</td>
<td>5.4</td>
<td>4.3</td>
<td>UTI</td>
</tr>
<tr>
<td>0.8</td>
<td>130</td>
<td>15.7</td>
<td>3.2</td>
<td>OM</td>
</tr>
<tr>
<td>0.8</td>
<td>140</td>
<td>17.8</td>
<td>3.9</td>
<td>Glucocorticoid therapy</td>
</tr>
<tr>
<td>0.7</td>
<td>90</td>
<td>12.1</td>
<td>2.0</td>
<td>URI, OM</td>
</tr>
<tr>
<td>&gt;10 y</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>39.9</td>
<td>225 530</td>
<td>565.2</td>
<td>47.5</td>
<td>CML</td>
</tr>
<tr>
<td>11.7</td>
<td>320</td>
<td>2.7</td>
<td>78.1</td>
<td>Chemotherapy (R-CHOP) for DLBCL</td>
</tr>
<tr>
<td>7.3</td>
<td>1042</td>
<td>14.2</td>
<td>83.1</td>
<td>UTI</td>
</tr>
<tr>
<td>6.2</td>
<td>1050</td>
<td>17.0</td>
<td>77.6</td>
<td>PNA</td>
</tr>
<tr>
<td>4.2</td>
<td>670</td>
<td>15.9</td>
<td>73.5</td>
<td>Pregnancy</td>
</tr>
<tr>
<td>3.9</td>
<td>450</td>
<td>11.7</td>
<td>65.0</td>
<td>Chemotherapy (azacitidine) for MDS</td>
</tr>
<tr>
<td>2.7</td>
<td>200</td>
<td>7.4</td>
<td>70.9</td>
<td>CML</td>
</tr>
<tr>
<td>2.5</td>
<td>400</td>
<td>15.8</td>
<td>27.9</td>
<td>Pregnancy</td>
</tr>
</tbody>
</table>

Abbreviations: CML, chronic myelogenous leukemia; CMML, chronic myelomonocytic leukemia; DLBCL, diffuse large B-cell lymphoma; IG, immature granulocyte; IG#, absolute IG concentration; IG%, relative IG concentration; MDS, myelodysplastic syndrome; OM, otitis media; PNA, pneumonia; R-CHOP, rituximab, cyclophosphamide, hydroxydaunorubicin (doxorubicin), Oncovin (vincristine), and prednisone/prednisolone; URI, upper respiratory infection; UTI, urinary tract infection; WBC, white blood cells.

$^a$ See also Figure 8.

Analogous to the observed difference in IG reference range cutoffs (≤10 years, lower; >10 years, higher), we found that even the most abnormal IG counts in the younger age group were quite low compared with abnormal IG counts in the older age group (compare Figure 6, A and B, with Figure 7, A and B). In fact, using only one simple cutoff for all age groups would have missed many small but clinically significant IG elevations in young children with clearly associated disease (examples in Table 4).

Our results significantly expand the use of IGs beyond the current realm of monitoring hospitalized patients with sepsis. The combination of carefully derived age-stratified IG reference ranges and upper cutoff values with distinct lists of associated differential diagnoses encountered in an outpatient setting, establishes IGs as a powerful and clinically significant hematologic parameter.

The authors wish to thank Ian Giles, MD, Sysmex America (Mundelein, Illinois), for advice on retrieving data from the XT-1800i. We are indebted to Narayanawamy Balakrishnan, PhD, Department of Mathematics and Statistics, McMaster University (Hamilton, Ontario, Canada), for discussions about the current lack of nonparametric statistical tests for quantile estimates. We have begun to work jointly to close this gap. Dr Roehrl acknowledges research grant support from the American Cancer Society, Atlanta, Georgia (grant IRG-72-001-35-IRG). Dr Roehrl is a Faculty Fellow of the Karin Grunenbaum Cancer Research Foundation, Cambridge, Massachusetts.

References

Figure 7. Scatterplots using patients from the total data set older than 10 years (n = 1122) illustrate the relationship between total white blood cell (WBC) concentrations and (A) relative or (B) absolute immature granulocyte (IG) concentrations. The dashed red line indicates separation between normal values (below: less than or equal to the upper limit of the 90% confidence interval [CI] of the 95th percentile estimate) and abnormal values (above: greater than the upper limit of the 90% CI of the 95th percentile estimate). Note that quantization of IG concentrations along the ordinate is not apparent in this plot because of different ordinate scales compared to Figure 6.

Figure 8. Pie charts depicting relative distribution of the most common clinical causes of abnormally elevated immature granulocyte (IG) concentrations derived from the total data set (n = 2571). A, Pie chart derived from patients aged 10 years or younger. B, Pie chart derived from patients older than 10 years. The following criteria were applied for inclusion in these statistics (IG%, relative concentration of IGs [percentage of total white blood cells]; IG#, absolute concentration of IGs per microliter): IG% ≥ 0.7% and/or IG# ≥ 90 µL–1 (≤10 years old); IG% ≥ 2.0% and/or IG# ≥ 200 µL–1 (>10 years old). These criteria are stricter than the individual 95th percentile cutoffs for IG% and IG# because, if we consider a patient abnormal if at least one of the 2 IG parameters is above cutoff, individual cutoffs need to be increased to ~98th percentiles (0.95^2 = 0.975), in order to maintain an overall ≤5% false-positive rate (multiple testing phenomenon). See also Table 4.