BACKGROUND: The treatment and prognosis of follicular lymphoma (FL) is dependant on the grade of the disease. In the World Health Organization classification of lymphoma, grading of FL into low grade (1 and 2) and high grade (3) is recommended. Grading of FL is possible in excision biopsy; histological grading is subjective and inconsistent. Grading is extremely difficult in needle core biopsies and fine needle aspirates. We attempted to grade FL using flow cytometry (FCM) and CD19/forward scatter.

MATERIALS AND METHODS: Cases of FL seen in our institution and submitted for FCM were evaluated for the percentage of cells detected beyond the 500-channel mark (on a 1024 scale) on a CD19/forward scatter dot plot. We hypothesized that these cells most likely represent centroblasts and their percentage would reflect the grade of the disease. Histological grading of the lymphoma on the open biopsies constituted the reference for FL grade.

RESULTS: Thirty-six cases of FL, including 22 males and 14 females, ranging in age from 19 to 92 years (median, 42 years), were studied. There were 17 cases of low grade (grade 1; n=10 and grade 2; n=7) and 19 cases of high grade (grade 3) FL. The percentage of cells identified beyond the 500-channel mark on CD19/forward scatter dot plot ranged from 0.12% to 12.55% (median, 4.9%) in low grade (grade 1 and 2) whereas the percentage of those cells in high grade FL ranged from 6.22% to 51.95% (median, 21%; P=0.00001).

CONCLUSION: Our findings suggest that using a CD19/forward scatter dot plot can help identify centroblasts in FL making grading possible on FCM, especially in small biopsies and fine needle aspirates.

Follicular lymphoma is one of the most common lymphomas encountered in North America, representing approximately 30% of all lymphomas seen in tertiary care pathology practice. This lymphoma is one of the few entities in the World Health Organization (WHO) classification that requires grading. Low-grade follicular lymphoma usually follows a protracted course and is, for the most part incurable. It usually requires single-agent chemotherapy or immunotherapy using monoclonal antibodies. High-grade follicular lymphoma on the other hand is an aggressive lymphoma that requires multi-agent chemotherapy and can have a higher cure rate. This suggests that grading of follicular lymphoma is one of the prerequisites for diagnosis of the disease. Grading of follicular lymphoma has, for many years, followed the grading system of Mann and Berrard. The method depends on counting the number of centroblasts per high power field. Grade 1 follicular lymphoma would have less than 5 centroblasts per high power field, grade 2 would have 5-15 centroblasts per high power field.
on forward scatter in flow cytometry, centroblasts would migrate farther than centrocytes. Centrocytes would, however, be in the same location as reactive T lymphocytes on a flow cytometric forward scatter (Figure 1). To separate centrocytes, reactive T lymphocytes and centroblasts, CD19 forward scatter would help separate the putative three categories of lymphocytes (Figure 2). In the current study we hypothesized that the percentage of cells identified at or beyond the 500-channel mark represents the percentage of centroblasts and that would in turn represent the grade of the follicular lymphoma.

Materials and Methods
All cases of follicular lymphoma diagnosed in our institution and submitted for flow cytometry formed the basis for our study. All cases had to fulfill histologic and phenotypic criteria for follicular lymphoma. Histologically the cases had to show a follicular or follicular and diffuse growth pattern with characteristic centrocytes and centroblasts. Phenotypically all cases had to express CD10, CD20, Bcl-2 and Bcl-6.

Grading of the lymphomas
All cases were independently graded by two pathologists (AT and MAO) without knowledge of the flow cytometry result. The Mann and Berrard histological grading scheme was used. Grade 1 follicular lymphoma showed less than 5 centroblasts per high power field, grade 2 lymphoma showed 5-15 centroblasts per high power field and grade 3 disease showed more than 15 centroblasts per high power field. No attempt to subdivide grade 3 follicular lymphoma into grades 3A and 3B was made for the purpose of the study. Cases were subdivided into two main categories: low-grade follicular lymphoma encompassing grades 1 and 2 disease and high-grade follicular lymphoma encompassing grades 3A and 3B follicular lymphoma.

Flow cytometry
The following monoclonal antibodies were used: T-cell markers CD1a-PE (Coulter), CD2-PE (Becton-Dickinson; BD), CD3-FITC (BD), CD4-APC (BD), CD8-PE (BD), CD7-FITC (BD), CD5-FITC (BD) whereas B-cell markers included CD10-FITC (Coulter), CD19-APC (BD), CD20-PE (BD), CD22-APC (BD), CD23 APC (BD), CD79b PE (BD), FMC-7 FITC (BD), Kappa-FITC (BD), Lambda-PE (BD), CD19 PE (Coulter), Kappa FITC (Coulter) and Lambda FITC (coul-
A monoclonal antibody against BCL-2 FITC (Pharmengin) was used. Additional antibodies used were CD45-PerCP (BD), HLA-DR-FITC (BD), CD11c-PE (BD), and 7-AAD (Coulter).

For immunophenotyping, lymph node biopsies were obtained and collected using RPMI media tube and delivered immediately to the flow cytometry section. Aliquots from single cell suspensions from the lymph node were washed twice using a PBS buffer with 1% BSA and 0.1% NaN₃. After the last wash the cells were filtered and the count adjusted to 0.5-1 × 10⁶/L of which a 100 uL was placed in each tube and incubated with the appropriate combination of monoclonal antibodies for 15–20 minutes in the dark (room temperature). Once the incubation period was finished the red cells were lysed using ammonium chloride followed by a single washing step. The cells were resuspended in 0.5 mL of 1% paraformaldehyde and stored in the refrigerator until time of analysis.

Data acquisition was performed using Cell Quest software (BD) where a minimum of 10 000 events/tube were acquired during which the normal lymphocyte elements in the sample were positioned on the 200-channel mark to act as an internal reference point. Instruments were calibrated daily using CALIBRITE beads (BD) and two levels (normal and low) of controls were run daily to ensure consistency in the functions of the instruments.

Forward scatter (FSC) vs. side scatter (SSC) to gate around the abnormal cells was performed. For identifying the percentage of abnormal cells a dot plot of FSC vs. CD19 was created, and an electronic gate was created to detect the number of cells identified at or beyond the 500-channel mark on the FSC axis and an estimation of their percentage was done.

The Wilcoxon-signed-rank test was used to test the significance of the differences between the different grade lymphomas based on the percentage of cells identified at or beyond the 500-channel mark. The analyses were performed using the S-Plus commercial program, version 6.2, 2000 (Insightful Software, Seattle, Washington, USA).

Results
There were 36 cases of follicular lymphoma identified, which included 22 male and 14 female patients. The ages ranged from 19 to 97 years (median 42 years). There were 10 cases of grade 1, 7 cases of grade 2 and 19 cases of grade 3 follicular lymphoma.

Discussion
Several prognostic indicators have been identified in follicular lymphoma. Age has been used as a prognostic indicator. Patients older than the age of 70 years had a worse prognosis according to one study. The presence of more than 6 chromosomal breaks...
has been associated with a poor outcome.\textsuperscript{14,15} Other clinical prognostic factors include serum LDH levels and performance status.\textsuperscript{16,17} The pattern of bone marrow involvement in follicular lymphoma has been shown to correlate with disease outcome, including the volume of bone marrow involvement, diffuse infiltrate and the number of lymphoma nodules.\textsuperscript{18} More recently gene expression profiling has shown that follicular lymphoma subclasses can be identified leading to the identification of disease subgroups with different biological behaviors.\textsuperscript{19,20} The technology needed to provide such gene profile at the clinical level is far from being implemented on a routine basis. Until this technology at the patient care level is available grading of follicular lymphoma has remained one of the most important aspects of predicting the behavior of the disease.\textsuperscript{21-24} Grades 1 and 2 disease usually have a persistent prolonged course with a very low probability of cure. These two grades of the disease usually require monotherapy.\textsuperscript{3,4} Grade 3 follicular lymphoma on the other hand shows a more aggressive course and shows potential for cure using multidrug chemotherapy. It thus seems that grading of follicular lymphoma is a necessity rather than an academic exercise in the management of follicular lymphoma.\textsuperscript{13-15}

The most commonly used method of grading follicular lymphoma is that of Mann and Berrard and its modification.\textsuperscript{5-8} The method has been repeatedly used to reflect the different behavioral patterns of follicular lymphoma with a certain degree of success. However, the method is for the most part, subjective and shows a great deal of inter-observer variability.\textsuperscript{7}

Additionally the method has been primarily designed to evaluate several microscopic fields. This means that the grading method is only suitable for excision biopsies. Several situations arise where excision biopsy cannot be performed for the patient.\textsuperscript{10} This means that needle core biopsies and fine needle aspirates may not be suitable for grading of follicular lymphoma. Studies have attempted to grade follicular lymphoma in fine needle aspirates with some success.\textsuperscript{25} The studies still relied on visual counts of centroblasts and/or Ki-67 proliferative indices or DNA analysis. The incorporation of histologic grade with proliferative activity has also been attempted with some degree of success.\textsuperscript{24,26}

There thus seems to be a need for a quantitative method to identify the exact percentage of centroblasts in follicular lymphoma using a reliable measuring device. We have shown that using CD19 forward scatter, three populations of lymphocytes can be identified in any given case of follicular lymphoma. The first population is that seen in the bottom left of the CD19 forward scatter plot and these are most likely T lymphocytes. The second population is that seen in the top left area of the CD19 forward scatter plot and these most likely are centrocytes. The third population is that seen in the top right portion of the CD19 forward scatter dot plot and these most likely are centroblasts. We have shown that the estimation of the percentage of these latter cells can be a reflection of the grade of the follicular lymphoma. We had a rare overlap between some of the cases. Overlap between different grades of follicular lymphoma does not necessarily mean a flaw in the flow cytometric method. Our patients should be clinically followed to see whether the flow cytometric or the histological methods are more biologically relevant. Additionally, the method can be fine-tuned to provide proper estimation of the number of centroblasts.

Although our method of estimating the grade of follicular lymphoma using CD19 forward scatter requires several studies for validation and for correlation with disease outcome, we believe that the method can be an accurate objective method of estimating the grade of follicular lymphoma and in time will replace the conventional method of grading follicular lymphoma.
Figure 5: Photomicrograph of grade 1 follicular lymphoma (H and E X400) (left); Flow cytometric dot plot histogram of CD19 forward scatter of the same case showing the percentage of cells identified beyond the 500-channel mark at 3% (right).

Figure 6: Photomicrograph of case of grade 2 follicular lymphoma (H and E X400); B1 (left); Flow cytometric dot plot histogram of CD19 forward scatter of the same case showing the percentage of cells identified beyond the 500-channel mark of 7% (right).

Figure 7: Photomicrograph of case of grade 3 follicular lymphoma (H and E X400) (left); Flow cytometric dot plot histogram of CD19 forward scatter of the same case showing the percentage of cells identified beyond the 500-channel mark at 37% (right).
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