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# Flow Cytometry Analysis of Recurrent or Persistent Lymphadenopathy in Patients with Nodular Lymphocyte-Predominant Hodgkin Lymphoma

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ABSTRACT

**Objectives:** We recently examined the utility of flow cytometric analysis in the diagnosis of nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) by examining reactive T-cell features. This study aims to compare these features in sequential biopsies of persistent or recurrent lymphadenopathy in patients with NLPHL.

**Methods:** We reanalysed the histopathology and flow cytometry findings of 9 patients with multiple biopsies for persistent or recurrent lymphadenopathy and either initial or recurrent NLPHL. A flow cytometry signature was considered suggestive of NLPHL if ≥12% of T-cells expressed CD57 or ≥3% of T-cells co-expressed CD4 and CD8.

**Results:** A flow cytometry signature considered suggestive of NLPHL was seen in 18 of 20 specimens. Based on histopathology, 11 were diagnosed as NLPHL, 3 were initially underdiagnosed as atypical lymphoid proliferation, and 4 were initially incorrectly diagnosed as negative or progressive transformation of germinal centers. Flow cytometry showed similar expression patterns of CD57 and CD4/CD8 in T-cells between initial and subsequent biopsies. The remaining 2 specimens lacked the flow cytometry signature suggestive of NLPHL and were histopathologically diagnosed as reactive hyperplasia.

**Conclusion:** Flow cytometry analysis based on our criteria is highly sensitive in detecting NLPHL. Correlation with the cytospin cytology may increase the diagnostic specificity. A negative flow essentially ruled out the possibility of NHLPHL.



## Background

Nodular lymphocyte-predominant Hodgkin lymphoma (NLPHL) is a rare, indolent B-cell lymphoma. It accounts for approximately 5% of all Hodgkin lymphomas and predominantly affects men ages 30-50 years. It is important to accurately identify NLPHL as its prognosis and therapeutic modalities are unique. The diagnosis of NLPHL is currently based on its morphologic and immunohistochemical features; this diagnostic approach usually requires an excisional lymph node biopsy. Morphologic findings include a nodular or a nodular and diffuse proliferation of scattered, large neoplastic cells known as lymphocyte-predominant (LP) cells, or popcorn cells, which are situated among small, non-neoplastic lymphocytes and histiocytes [1]. LP cells are typically positive for CD20, CD45, CD79a, Bcl-6, Pax-5, OCT2, and BOB1 and negative for CD15 [2-4]. This immunophenotypic profile based on immunohistochemistry is neither sensitive nor specific. It can be difficult to distinguish NLPHL from other lymphoid neoplasms with similar architectural characteristics, such as lymphocyte-rich classic Hodgkin Lymphoma (LRCHL), T-cell/histiocyte rich large B-cell lymphoma (TCRLBCL), follicular lymphoma, and follicular hyperplasia with progressive transformation of germinal centers (PTGC) [1, 4, 5]. In certain cases, morphologic examination of these disease entities may

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demonstrate strikingly similar patterns of atypical large lymphocytes present in a background of smaller lymphocytes.

In previous studies, researchers examined the clinical utility of flow cytometric analysis in the diagnosis of NLPHL by examining features of the reactive T-cells [6-8]. They concluded that there is an increased number of CD57+ T-cells and/or double-positive (CD4+/CD8+) T-cells among the cases previously diagnosed as NLPHL. In our current study, we examined cases of persistent or recurrent lymphadenopathy in patients with an established diagnosis of NLPHL. We found that the T-cell flow cytometry signature is highly predictive of histopathological diagnosis. It may help to improve the diagnosis when the specimen is a needle core biopsy or histopathology features are atypical.

## Materials and Methods

### I Case Selection

Cases of NLPHL at Beaumont Health were retrospectively identified. Initial diagnoses were based on morphology and immunohistochemistry as outlined in the 2017 World Health Organization classification [9]. The NLPHL cases were selected for further analysis if it was found in the clinical history that the patient experienced persistent or recurrent lymphadenopathy either before or after the diagnosis was established. A total of 9 cases were identified, each with 2 to 3 specimens, yielding a total of 20 specimens.

These cases were divided into two groups: i) initial work-ups that were inconclusive but later diagnosed as NLPHL and ii) work-ups initially diagnosed as NLPHL with subsequent recurrent lymphadenopathy. The H&E sections and immunohistochemical sections of all cases were reviewed. Several cases were signed out as reactive hyperplasia, reactive hyperplasia with progressive transformation of germinal centers, or atypical reactive hyperplasia. The original diagnoses from pathology reports are displayed in (Table 1). Recommended revision of the diagnosis is noted in the table based on comprehensive review in this study. The cytospin slides of single cell suspensions and touch imprints of biopsies were also examined if they were available. This study was approved by the Beaumont Health System Institutional Review Board.

### II Flow Cytometry

The protocol and antibodies used in this study were previously described [7]. The original flow cytometry listmode data were reanalysed with Kaluza software (Beckman Coulter) to assess the percentage of helper T-cells (CD4+/CD8), suppressor T-cells (CD4-/CD8+), double positive T-cells (CD4+/ CD8+), CD4:CD8 ratio, and the percentage of T-cells expressing CD57. The antibodies that were used during this time period and relevant to this study are as follows: CD3 (clone SK7; BD Biosciences, San Jose, CA), CD4 (clone SK3; BD Biosciences), CD8 (clone SK1; BD Biosciences), CD57 (clone HNK-1; BD Biosciences; used from 2002-2004), and CD57 (clone NC1; Beckman Coulter; used from 2005-2013).

The cell parameters analysed included proportion of T cells that were double positive for CD4 and CD8, CD4/CD8 ratios, and proportion of T cells positive for CD57 expression. Specifically, double-positive (CD4 + CD8+) T cells were defined as those T cells having a CD4 + and CD8 + staining intensity in excess of internal B cells. CD57 + T cells were defined as those T cells having the CD57 staining intensity in excess of internal B cells. Flow cytometry was considered positive if ≥ 12% of T-cells expressed CD57 or ≥ 3% of T-cells co-expressed CD4 and CD8 in the absence of features of non-Hodgkin lymphoma (absence of abnormal T-cells, B-cells and NK-cells). These cut-offs were established based on receiver operating characteristic (ROC) curve analysis published previously [8]. The expression patterns of CD4, CD8, and CD57 were examined. Cytospin preparations of single cell suspensions were also examined for LP cells, if available.

## Results

### I Initial Workup for Persistent Lymphadenopathy which was Later Diagnosed as NLPHL: Cases 1, 3, 4, and 8

All 9 cases are summarized in (Table 1). Needle core biopsies were performed as an initial diagnostic approach in 3 cases (cases 1, 3 and 4). Flow cytometric studies showed that B-cells were polytypic. T-cells showed no evidence of aberrant antigen expression but did exhibit features suggestive of NLPHL. The diagnoses of atypical lymphoid infiltrates were rendered for the initial biopsies of cases 1 and 3 (specimens 1A and 3A) based on histopathology evaluation alone and the diagnoses of NLPHL were made for the subsequent excisional biopsies (specimens 1B and 3B). Upon review, we found that the same flow cytometry signatures of NLPHL were present in the initial needle core biopsies as well as in the subsequent excisional biopsies; case 1 is shown in (Figure 1).

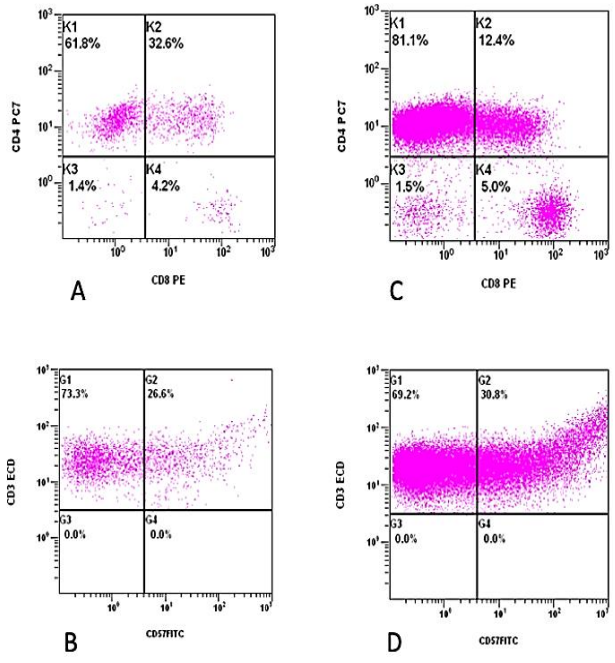


**Figure 1:** Expression patterns of CD4, CD8 and CD57 in T-cells in case 1. Plots A and B represent specimen 1A and plots C and D represent specimen 1B. Displayed are dot plots of all analysed T-cells.

CD57+ T-cells and double positive T-cells were increased and the expression patterns of CD57, CD4 and CD8 in T-cells were classic as

reported previously. When specimen 4A was signed out, the pathologist made a diagnosis consistent with NLPHL by considering the flow cytometric features, the presence of LP cells on cytospin preparation, and the histopathological features of the core biopsy. Although excisional biopsy was recommended for confirmation, the clinician and patient decided to perform another needle core biopsy (specimen 4B). Both flow cytometry and histopathology studies showed nearly identical findings between specimens 4A and 4B.

Case 8 was a challenge during the initial diagnostic workup. The initial excisional biopsy (specimen 8A) showed histopathological features interpreted as reactive hyperplasia with progressive transformation of germinal centers (RH/PTGC). However, lymphadenopathy was persistent. Three months later, a second excisional biopsy was performed, and it was again interpreted as RH/PTGC based on histopathological features (specimen 8B). However, lymphadenopathy was persistent and slowly progressive. Three years later, the third excisional biopsy (specimen 8C) was performed with histopathological features interpreted as NLPHL. Because unique T-cell signatures in NLPHL were unknown previously, the flow cytometric studies were only reported as negative for NHL (non-Hodgkin lymphoma). The flow cytometry listmode data were reanalysed retrospectively and it was found that there was indeed a unique T-cell signature suggestive of NLPHL present in all three specimens, as shown in (Figure 2). LP cells were identified on retrospective review of the cytospin preparation.

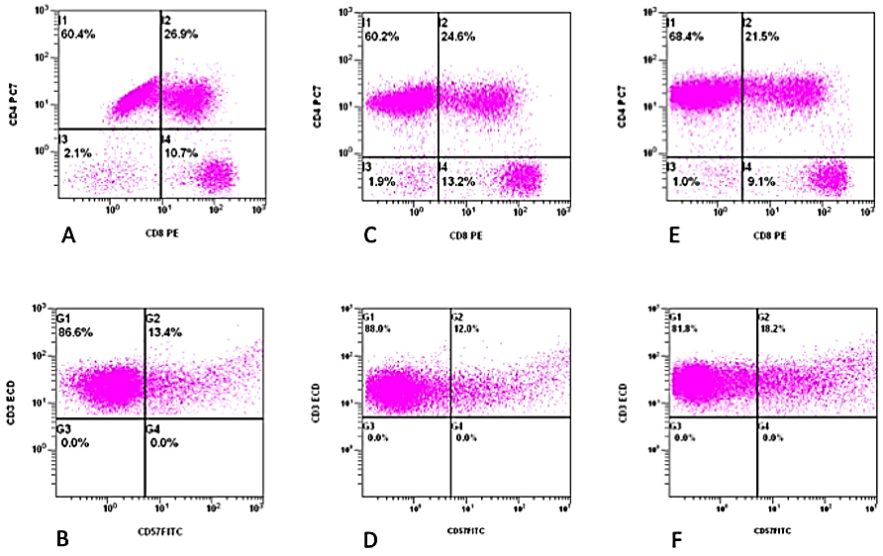


**Figure 2:** Expression patterns of CD4, CD8 and CD57 in T-cells in case 8. Plots A and B represent specimen 8A, plots C and D represent specimen 8B and plots E and F represent specimen 8C. Displayed are dot plots of all analysed T-cells.

**Table 1:** Summary of all 20 specimens of 9 NLPHL patients.

| Case | Date | Specimen | Histopathology | FCI | CD57+ (%) | CD4+CD8+ (%) |
|---|---|---|---|---|---|---|
| 1-A | 03/08 | Core | Atypical | Positive | 27 | 33 |
| 1-B | 05/08 | Excision | NLPHL | Positive | 30 | 14 |
| 2-A | 06/03 | Excision | NLPHL | Positive | 48 | 21 |
| 2-B | 05/08 | Excision | RH | Negative | 7 | 1 |
| 3-A | 01/10 | Core | Atypical | Positive | 40 | 10 |
| 3-B | 02/10 | Excision | NLPHL | Positive | 47 | 3 |
| 4-A | 05/10 | Core | NLPHL | Positive | 25 | 20 |
| 4-B | 06/10 | Core | NLPHL | Positive | 25 | 21 |
| 5-A | 06/08 | Excision | NLPHL | Positive | 19 | 12 |
| 5-B | 12/11 | Excision | RH | Negative | 4 | 2 |
| 6-A | 02/07 | Excision | NLPHL | Positive | 24 | 21 |
| 6-B | 11/12 | Excision | NLPHL | Positive | 13 | 9 |
| 7-A | 04/12 | Excision | NLPHL | Positive | 15 | 19 |
| 7-B | 06/12 | Core | Atypical | Positive | 13 | 6 |
| 8-A | 06/07 | Excision | RH/PTGC | Positive | 13 | 27 |
| 8-B | 10/07 | Excision | RH/PTGC | Positive | 15 | 23 |
| 8-C | 01/11 | Excision | NLPHL | Positive | 19 | 22 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 9-A | 03/03 | Excision | NLPHL | Positive | 22 | 1 |
| 9-B | 04/08 | Excision | RH | Positive | 6 | 6 |
| 9-C | 09/09 | Excision | RH/PTGC | Positive | 9 | 8 |

FCI: Flow Cytometric Immunophenotyping; NLPHL: Nodular Lymphocyte-Predominant Hodgkin Lymphoma; RH: Reactive Hyperplasia, RH/PTGC: Reactive Hyperplasia with Progressive Transformation of Germinal Centers.

### II Recurrent Lymphadenopathy in Patients with Known History of NLPHL: Cases 2, 5, 6, 7, and 9

As seen in (Table 1), five cases (2, 5, 6, 7 and 9) were of patients with a history of NLPHL who developed recurrent lymphadenopathy. Their concurrent flow cytometric studies were reanalysed. Flow cytometric immunophenotype (FCI) positivity for NLPHL was present in specimens 2A, 5A, but absent in specimen 2B and 5B. The FCI are concordant with the diagnosis based on histopathology in these two cases. LP cells were present in the cytospin slide of specimen 2A, 5A but absent in specimen 2B and 5B. For illustration, FCI of T cells of case 5 is shown in (Figure 3).

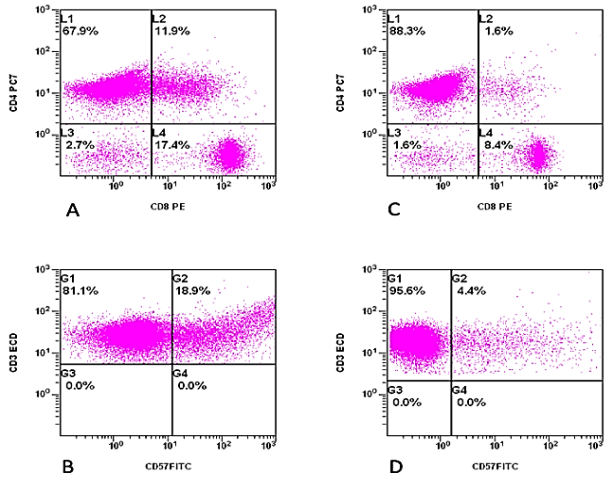


**Figure 3:** Expression patterns of CD4, CD8 and CD57 in T-cells in case 5. Plots A and B represent specimen 5A and plots C and D represent specimen 5B. Displayed are dot plots of all analysed T-cells.

Case 6 showed recurrent lymphadenopathy due to NLPHL based on histopathology evaluation. FCI were positive for NLPHL in specimen 6A and 6B. LP cells were present in cytospin slides of specimens 6A and 6B. The recurrent lymphadenopathy of case 7 was evaluated by needle core biopsy and interpreted as atypical lymphoid proliferation based on histopathology. When concurrent flow cytometry listmode data were reanalysed, features suggestive of NLPHL were seen in specimen 7B, similar to specimen 7A. Residual or recurrent NLPHL was very likely based on these findings. The cytospin preparation indeed demonstrated atypical cells with features consistent with LP cells. Unfortunately, further follow-up data were not available.

Case 9 is of a patient who had developed lymphadenopathy five years after the initial diagnosis of NLPHL. Excisional biopsy was performed twice and was interpreted as reactive hyperplasia (specimen 9B) and RH/PTGC (specimen 9C) based on histopathology evaluation. The cytogenetic study of specimen 9C was reported to show an abnormal karyotype with trisomy 12 in 2 of 20 metaphase cells, which was confirmed by fluorescence in-situ hybridization (FISH), arguing against the diagnosis of RH/PTGC. Retrospective review of the original flow cytometry listmode data persistently showed a T-cell signature suggestive of NLPHL (Figure 4). LP cells were seen in the cytospin preparations of all specimens.

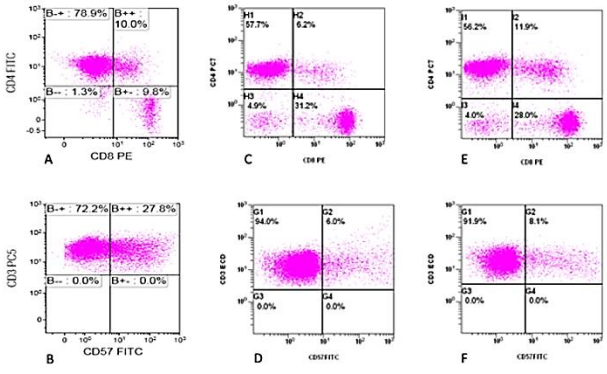


**Figure 4:** Expression patterns of CD4, CD8 and CD57 in T-cells in case 9. Plots A and B represent specimen 9A, plots C and D represent specimen 9B, and plots E and F represent specimen 9C. Displayed are dot plots of all analysed T-cells.

### Discussion

The diagnosis of NLPHL can be difficult when based solely on the histopathologic evaluation of an excisional biopsy. It is even more difficult when material is obtained by needle aspiration or core biopsy. This case series confirms the utility of flow cytometry in the initial diagnostic work up and post-therapeutic monitoring of NLPHL. There are two possible criteria for a case to be classified as NLPHL by flow cytometric study of the T cells: at least 3% of T-cells co-expressing CD4 and CD8 or 12% T-cells expressing CD57 based on previous studies [8]. Of 20 specimens reported in this study, both criteria were concordant in 16 (80%) specimens. Other specimens showed discordant results and they happened to be from the same individual (case 9), with an initial diagnosis of NLPHL showing high CD57+ T-cells (22%) but low CD4+CD8+ T-cells (1%) in specimen 9A. The subsequent lymphadenopathy showed low CD57+ T cells (<12%) and high CD4+CD8+ T cells (>3%) in both specimen 9B and 9C. This switching is of great interest and its significance is unclear. Specimens 9B and 9C were originally diagnosed as RH (9B) or RH/PTGC (9C) based on histopathologic evaluation, but this was not supported by cytogenetic studies showing trisomy 12. Further studies with more cases are necessary to further explore the biological and diagnostic significance of CD57+ T cells and CD4+CD8+ T cells in NLPHL.

Flow cytometry interpretation appears to be more sensitive than histopathologic evaluation of NLPHL in 7 of 20 specimens. However, the possibility of false positive flow cytometry interpretation cannot be ruled out. Of interest, 3 specimens were diagnosed as PTGC based on histopathology, whereas the flow cytometry results were suggestive of NLPHL. It has been well described that PTGC has been frequently

described in association with NLPHL, though the majority of patients with PTGC never develop NLPHL. A much smaller proportion of patients with NLPHL have synchronous, antecedent, or subsequent PTGC [10]. Since PTGC and early or residual NLPHL may show overlapping pathological features, we believe these 3 specimens likely represent misinterpretation of early or residual NLPHL as PTGC. At least in case 9, the concurrent cytogenetic studies showed structural or numerical abnormalities that argued against PTGC. Further studies with a larger group of cases are needed to determine if flow cytometry is useful in differentiating PTGC with risk of transforming to NLPHL from those without.

Making a diagnosis of NLPHL based on histopathologic evaluation of needle core biopsy poses is a great challenge to many pathologists. This may explain why "atypical lymphoid proliferation" is often a preferred choice of diagnostic label in this situation (specimens 1-A, 3-A, 7-B in Table 1). Considering positive FCI in such context, the pathologist may be able to upgrade their diagnosis from atypical to suspicious for or consistent with NLPHL when nodal architecture evaluation is very limited. In patients with a history of NLPHL, it appears to be reasonable to consider fine needle aspiration or needle core biopsy as the initial approach to evaluate for recurrence if a representative portion of the specimen is submitted for concurrent flow cytometry studies. In this setting, the flow cytometry panel should include a CD57 antibody in addition to the routine antibody panel for a NHL workup including CD3, CD4 and CD8. When B-cells are polytypic and the T-cells show normal antigen expression with no signature suggestive of NLPHL, reactive hyperplasia is very likely. The absence of LP cells or other atypical cells on cytological preparation may offer additional assurance. This approach may reduce the need for an invasive excisional biopsy.

Traditionally, flow cytometry of lymphadenopathy has been focused on the evaluation for NHL by identification of immunophenotypically monotypic or aberrant lymphocytes. Since reactive T-cells may have unique immunophenotypic features suggestive of NLPHL, we strongly recommend considering NLPHL in the differential diagnosis when such flow cytometry features are detected. To improve the diagnosis of NLPHL, we would like to propose the following algorithm, shown in (Figure 5). On difficult cases, cytogenetic studies would be diagnostically useful because LP cells often have complex cytogenetic abnormalities [11, 12].

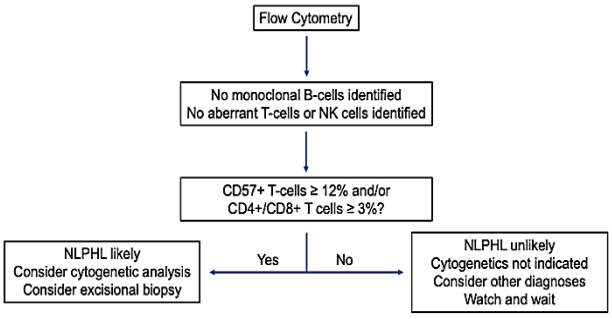


**Figure 5:** Diagnostic algorithm in the flow cytometric evaluation for NLPHL in patients with persistent or recurrent lymphadenopathy.

In summary, we have shown that this approach is highly sensitive in diagnosing NLPHL. However, its specificity remains to be further elucidated. Cytological evaluation for LP cells (Figure 6) may reduce the possibility of a false positive interpretation; thus, careful correlation of the flow cytometry and cytological features is highly recommended.

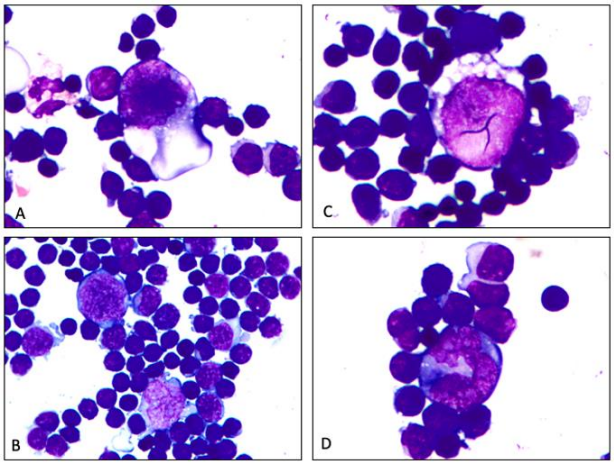


**Figure 6:** Representative LP cells on cytospin slides (Wright and Giemsa stains, magnification 1000x). LP cells are large, atypical cells which are scattered among small lymphocytes. Their cytoplasm is variable; from **A)** very abundant or **B, C,** and **D)** minimal. The cell may have **B)** visible nucleoli or **D)** an embryonic-shaped nucleus.

**Funding**

None.

**Conflicts of Interest**

None.

**Competing Interests**

None.

**Ethics Approval (Include Appropriate Approvals or Waivers)**

Approved by IRB.

**Consent to Participate (Include Appropriate Statements)**

Not applicable.

**Consent for Publication (Include Appropriate Statements)**

Not applicable.

**Availability of Data and Material (Data Transparency)**

All original data archived.

**Code Availability (Software Application or Custom Code)**

Not applicable.

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