Immunophenotypic Patterns of Lymphomas in a Tertiary Hospital, Lagos, Nigeria.

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Introduction
Lymphomas are lymphoid cell neoplasms that commonly affect the lymph nodes but can also affect any tissue of the body. They are broadly divided into Hodgkin lymphoma (HL) and Non-Hodgkin lymphoma (NHL). The latter is a heterogeneous group of clinico-pathological diseases, each with its distinctive response to treatment and prognosis.1,2 Lymphomas are estimated to account for about 3-4 % of cancers worldwide.3 Reviewed literatures from major tertiary hospitals revealed that lymphomas, mostly NHL, ranked among the leading cancers in Nigeria.4,5,7,8 Ghana,9 Uganda10 and South Africa11 Epidemiologic studies have shown

Abstract
Aims and Objectives: This study aims to classify the various types of lymphomas seen in our center by morphology and immunophenotyping using basic antibody panel.
Materials and Methods: Using 3-5 member antibody panels in sequential batches as primary, secondary and tertiary antibodies selected from the list of available antibodies, immunohistochemical staining were done on 110 cases of formalin-fixed paraffin-embedded lymphoma tissue blocks from the archive. A prior microscopic assessment of the hematoxylin-eosin-stained sections of each tissue block was done. All cases were diagnosed and sub typed by their morphology and pattern of antigen immunoreactivity.

Results: of the 110 cases of lymphoma, non Hodgkin lymphoma (NHL) constituted 84.5 % (93 of 110 cases), while Hodgkin lymphoma (HL) was 15.5 % (17 of 110 cases). The B cell NHL was 65.5 % (71 of 110 cases) and T cell NHL was only 20 % (22 of 110 cases).

Conclusion: This study further underscored the importance of immunohistochemistry in diagnostic haematolymphoid oncology. Starting with the availability of at least 10 antibodies (CD20, CD 79a, CD5, CD10, BCL6, CYCLIN D1, CD3, BCL2) and good diagnostic skill, most of the tertiary hospitals in developing countries will be able to perform, to a large extent, meaningful diagnosis of most of the common lymphomas. The list can be expanded with time as demanded. More detailed studies will be necessary to find out the potential infective aetiological factors in the development of high grade B-cell lymphomas in the environment.

Key note: Lymphoma; Immunohistochemistry: Subtyping.
remarkable differences in the distribution of lymphoma subtypes among different populations\textsuperscript{12} (Africans,\textsuperscript{13} Caucasians\textsuperscript{3} and in the Asians \textsuperscript{14,15})

The contemporary management of these neoplasms requires that they are accurately classified and subtyped according to the WHO system of classification. This classification encompasses clinical, morphological, immunological, cytogenetic and molecular attributes of the neoplastic cells, and provides guidelines in their treatment.\textsuperscript{14} The impact of this has been reflected in the resource-rich settings where treatment of these malignancies are increasingly being associated with unprecedented rates of long-term cure and control unlike what obtains in sub-Saharan Africa where lymphoma diagnosis is still by the morphologic appearances of the H and E stained tissue.\textsuperscript{15} While H and E histomorphology is the first step and a gold standard in surgical pathology, it is highly of limited diagnostic and prognostic value especially as regards lymphoma. Only a few cases of lymphomas with typical diagnostic features could be identified when they are presented with the usual conventional morphology.\textsuperscript{18,19}

Despite the acclaimed increase in the incidence of some subtypes of lymphomas in association with the significant increase in HIV prevalence in sub-Saharan Africa, very little progress has been made to improve on its diagnostic challenge. Unlike other cancers, the multiple parametric investigations (histology, cytology, immunophenotyping, cytogenetics, imaging and clinical) ensure appropriate disease diagnosis and classification but this constitutes a major challenge as the facilities are lacking in most tertiary centers in this region.\textsuperscript{18}

Immunophenotyping permits different lymphocyte subpopulations to be defined by their expression of a particular constellation of cell surface antigens, and the use of an ever – expanding supply of well defined monoclonal antibodies (MAbs) permits precise immunophenotyping of lymphocyte subpopulations. The lack of facilities for immuno-subtyping hampers accurate diagnosis and classification as well as research into the lymphoid malignancies in developing countries. This partly accounts for poor treatment outcome for patients with lymphomas because of incorrect or incomplete diagnosis\textsuperscript{17}. With the use of immunophenotyping, more than 95% of lymphomas could be identified for therapeutic and prognostic purposes.

This study aims to classify the various types of lymphomas seen in our center by immunophenotyping using basic antibody panel that could lead to identification of B and T cell types, immature and mature lymphomas as well as sorting the differentials of low and high grade lymphomas.

Materials and Methods

A search of database identified all patients diagnosed with lymphoma at the department of Anatomic and Molecular Pathology, Lagos University Teaching Hospital, Lagos, South West Nigeria was searched to identify patients with diagnosis of lymphoma, excluding myeloid and plasma cell neoplasms. This study excluded cases with inadequate material and those diagnosed from bone marrow alone. From January 2007 to December 2012, a total number of 104 cases with definitive diagnoses of lymphomas were included. Clinical information including demographics (age, gender) and site involvement were extracted from the laboratory request forms submitted with the pathological samples.

Hematoxylin and Eosin stained sections of the formalin-fixed, paraffin embedded blocks were retrieved from the archive. These were reviewed with the assistance of the haematopathologists at Haematopathology Oncology unit, Department of Pathology, Tata Memorial Hospital, India and Department of Pathology, Dr Ram Manohar Lohia Institute of Medical Sciences, Lucknow, India. Depending on the morphologic impression of the H and E stained slides, antibody panels were selected for the immunohistochemical staining. Members of each panel were selected from the list of available antibodies in the Department of pathology, Dr Ram Manohar Lohia Institute of Medical Sciences, Lucknow, where the procedure was done manually using the departmental protocol manual. The list of available antibodies were CD3, CD5, CD4, CD7, CD8, CD10, CD15, CD20, CD21, CD23, CD30, CD34, CD43, CD45RO, CD38, CD138, CD79, CD99, CD 25, CD 56, CD 57 EMA (epithelial membrane antigen), cyclinD1, BCL-2, BCL-6, ki-67, TdT, PD1 and pancytokeratin.

For the immunohistochemistry, 2-3 micron sections were cut from each block for each antibody marker. The sections were made on charged slides, deparaffinized with xylene and then rehydrated for 2-3 minutes. The slides were then incubated in water bath for 20 min at 97°C. To reduce nonspecific back ground staining due to endogeneous peroxidase, the sections were incubated in Hydrogen peroxide for 10 minutes. Each of these steps was followed by citrate buffer wash. To block background staining, protein block was applied and the sections were incubated for 5 minutes. Primary antibodies were added followed by incubation for 1 hour and the wash in buffer solution. Sequential addition of secondary and tertiary antibodies was done with each step followed by incubation for 10 minutes (slides in the dark) were done. The slides were then washed in buffer, distilled water and then buffer again. DAB was then added to the slides which were then incubated for 5 minutes and washed with distilled water. Finally the slides were counter-stained and cover-slipped.

The antibodies, hydrogen peroxide, DAB and blocking agents for reagents were from Thermo scientific while others were prepared in the laboratory. These antibodies were used in combinational panels in batches (primary, secondary and tertiary antibody panels). The members of each panel were selected on the background of the morphologic appearances.
of the tumour cells. Primary panel consisted of B-cell (CD20, CD79a) and T-cell (CD3) antigens. CD45, CD15, and CD30, EBV, MUM1 and pan-cytokeratin were added to the primary panel if large atypical cells are seen while CD38/CD138 were added in cases with numerous plasma cells. In cases with alteration of B-cell areas and presence of small /intermediate lymphoid cell, antibodies against CD5, CD10, CD23, cyclin D1, CD43, BCL-2 and BCL-6, Ki67 were used for the diagnosis and subtyping of low grade lymphomas (SLL, FL, MCL, MZL).

Ki67, Tdt and CD23 were used to determine the mitotic activity, immature lymphomas and presence of follicular dendritic cells respectively while pan-cytokeratin and S100 were used to identify non lymphoid malignancies. For cases with expansion of interfollicular area where T-cell lymphoma was a consideration, secondary and even tertiary antibody panels were selected from the following antibodies; CD4, CD5, CD7, CD8, CD10, PD1, CD25, CD56, CD57, CD30, ALK-1 for further characterization. All cases were diagnosed and sub typed in accordance with the criteria of WHO classification of Tumors of Haematopoietic and lymphoid Tissue (2008). The immunnostains were as well reviewed in conjunction with the haematopathologists Approval for the study was obtained from The Research and Ethical Committee of Lagos University Teaching Hospital (LUTH).

Results
Of the 110 cases of lymphomas, the overall male: female ratio was 1.4:1 while the mean age for adult and childhood NHL were 46 and 9 years respectively. NHL constituted 84.5 % (93 of 110 cases), while HL was 15.5 % (17 of 110 cases). The B cell NHL was 65.5 % (71 of 110 cases) and T cell NHL was only 20 % (22 of 110 cases).

Of the B cell NHL, DLBL constituted 45.1 % (32 of 71 cases), while SLL and FL were 21.1 % (15 of 71) and 10.0 % (7 of 71) respectively. Burkitt lymphoma was only 7.0 % (5 of 71); while 5 (7.0 %) and 3 (4.2 %) cases of MCL and MZL were seen respectively. Only 3 (4.2 %) and 1 (1.4 %) cases of MALTOMA and precursor B cell lymphoma were seen, respectively.

T / NK–cell lymphomas constituted 20% (22/110) of all the lymphomas and 23.6 % of NHL in this study. 21 extranodal lymphomas consisting of 8 T-cell lymphomas and 13 B cell lymphomas were seen. Of the B cell extranodal lymphomas, 3 types (primary central nervous system lymphoma, primary effusion lymphoma and plasmablastic lymphoma) with clinical evidence of AIDS were seen.

PTCL constituted 45.5% (10 of 22) of the T cell lymphoma in this study. Precursor T cell lymphoma constituted 22.7 % (5 of 22), while 18.2% (4 of 22) were cases of MF and only 13.6% (3 of 22) were cases of ALCL.

Table 1 indicates the age distribution of patients with the various types of non Hodgkin lymphomas while Figures 1
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Figure 3: Frequency distribution of B cell NHL.

All the 17 cases of HL were nodal and most commonly involved the cervical region. The male to female ratio was 1:0.7. The age range was 10 to 79 years while the mean age was 43 years. Of all the HL, 10 (58.8%) were seen in the younger age group (16-45 years) while 3 each (17.6%) were seen in the middle age (46-60) and elderly (> 60 years). Only one case was seen in the childhood age group (0-15 years). Mixed cellularity and nodular sclerosis were 35.3% (6 of 17) and 29.4% (5 of 17), respectively while 3 cases (17.6%) each were lymphocyte rich and lymphocyte depleted. No case of nodular lymphocyte predominant type was seen.

Figure 1: Hand E and Immunohistochemistry Staining Patterns of B Cell Small Lymphocytic Lymphoma

Loss of the follicular architecture, population of monomorphic lymphoid cells positive for CD20, CD79a, CD5, CD23, BCL2.

Figure 2: Hand E and Immunohistochemistry Staining Patterns of Follicular Lymphoma

Lymph node with nodular architecture, lymphoid cell in the nodules are strongly positive for CD20, CD10, BCL2, BCL6, CD21 but negative for CYCLIN D1. CD3 and CD5 stain the T cells in the parafollicular region.

Figure 3: Hand E and Immunohistochemistry Staining Patterns of Mantle Cell Lymphoma

Lymph node tissue with loss of follicular architecture. It show presence of monotonous lymphoid cells positive for CD20, CD5 but negative for CD10, BCL6 and CD23. CD3, CD5 marks T cells.
Discussion
The study showed that NHL predominates over HL and that among the NHL, B cell lymphoma is more commonly seen than T cell type. It also showed that DLBCL, followed by SLL is the most common type of lymphoma in our center. These findings are consistent with findings in literature from African and Asian countries but are different from reports from developed countries in Europe and America where the incidence of follicular lymphoma is high next to that of DLBCL.5,12,13

The study showed that with the use of antibody panel consisting of CD20, CD79a, CD5, CD23, CD10, CyclinD1, BCL2, BCL6, Ki67, most mature B cell lymphoma subtypes
can be easily identified and classified. Depending on the experience of the pathologist, the selection can be in batches (primary, secondary and tertiary) of 3-5 antibodies before a final conclusion is made. However, the diagnosis of DLBL could be more challenging due to its heterogenous morphology and resemblance of the tumour cells to the cells of other lymphomas with large cells and the possibility of loss of any of the pan B cell markers like CD19, CD20, CD22, and CD79a. The categorization of DLBL into Post germinal center B cell- like and activated B cell-like types, using the variation in the reactivity of the tumour cells to BCL6, MUM1, and CD10 could not be done in this study as MUM1 was not used in this study. Studies have shown that this classification does not correlate with the genetic classification and has not been proved to be of significant therapeutic / clinical importance. One case of T cell / Histiocytic rich DLBL, a rare variant of DLBL was seen in this study. This variant is usually of a major diagnostic challenge. Without immunohistochemistry (IHC) and good interpretive skill, it may be difficult to differentiate it from other lymphomas with abundant inflammatory background and some cases of benign lymphoid proliferations with reactive immunoblasts. Other DLBL sub-types have bearing to the site of origin and are usually diagnosed with the use of IHC. Two of these involving the CNS and skin were seen.

Burkitt Lymphoma occupied the 4th position among the B non-Hodgkin lymphomas seen in this study. This lymphoma was endemic in the African continent and over recent years, its incidence increased in endemic areas of Africa, overlapping with the epidemic of HIV and malaria in the region.21,22 This study supported the low incidence of BL previously reported among childhood lymphomas and adult lymphomas in Lagos23 as well as among all recent cases of lymphomas seen at Ibadan.6 However, this is in contrast to the Northern parts of Nigeria4 and some other sub-Saharan African countries like Uganda10 andTanzania,12 where it still remains the most common lymphoma among children and HIV infected patients. Before the HIV/ AIDS endemcity in Africa, reviewed literatures showed that the incidence rate of BL in Nigeria, Mali, the Gambia and Congo were lower than those reported in Uganda but substantially higher than those observed in the western countries.22 This variation in the incidence of BL might be due to country differences in reporting practices or actual country based differences due to changes in life style and HIV epidemic as well as variation in the standard of living, malaria incidence and other related environmental risk factors. All the cases of BL were seen in children with the usual jaw involvement (age range of 2 to 8 years and mean age of 4 years). Only one case of BL was seen in an adult involving the ovary. The cases show the usual morphology and IHC pattern of pan B cell markers and CD10, BCL6 positivity and very high mitotic index with Ki67 and Negative reactivity for BCL2 and CD5.

The low grade lymphomas constituted about 17 % of the NHL and showed male: female ratio of 2:1. Among these, SLL ranked highest, followed by FL and MCL. SLL/ CLL is seen as the most common adult leukaemia in the Western World and are most common among men than women as seen in this study.24 However, in our study, middle age individuals (46-60 years) with the median age of 53 years were more affected compared to elderly age recorded in literature from the developed countries. Follicular lymphoma represented a lower frequency (8%) among the NHL and 6.7% of all lymphomas seen in this study. This finding is consistent with findings in literature where its incidence is much lower in developing countries including Asia and Eastern Europe.25,26 The few cases of FL, MCL and other lymphomas with small cell morphology precludes adequate inferences about the diseases. With the use of CD5, CD23, CD10, BCL6 and Cyclin D1, they can be differentiated from one another. The identification of MCL is of therapeutic importance as this is managed as a high grade lymphoma.27

T cell lymphoma is noted to be of low incidence in African and Western countries and this has been further confirmed in this study. The frequency (20.0%) of T cell Lymphoma in this study is slightly higher than reported in the English literature (12%) of the NHL.24,26 Reviewed literature showed little or no information on T cell lymphomas in Africa. Sub Saharan Africa, with the exception of South Africa, was not represented in the report of an International study that evaluated lymphoma from US, Europe and Asia.24 However, this study support the findings of international study of lymphoma as well as a few others that peripheral T cell lymphomas are more common in the Asians countries but less frequent in the western and African countries.24,26 This variation, although might have a genetic basis, could be due to environmental factors, most especially, the high prevalence of viral infections like HTLV-1 and EBV which are associated with T cell lymphomas.28,29 PTCL and MF followed by ALCL were the most common mature T cell lymphomas among the well defined entities of mature nodal T cell lymphomas seen in this study.

This study showed that HL (15.5%) is less common than NHL and is more predominant in males. It also showed the bimodal peak age of involvement at young adult and mid adult ages. These findings are in keeping with findings in literature on HL.6,12,30,31 However, there was no bimodal age distribution in the report of Qun-Pei et al from China.15 The predominance of mixed cellularity HL and the rarity of nodular lymphocyte predominant HL in this study is in agreement with documented reports from Africa and Asia, but contrast those from Ibadan and western countries.
which showed nodular sclerosis as the most common variant.\textsuperscript{12,32}

The diagnosis of HL could be very straightforward with the presence of typical Reed-Sternberg / lymphohistocytic (RS/LH) cells or variants of these cells against an inflammatory background and varying degree of fibrosis. The use of IHC could be of significant value in controversial cases in which there is resemblance to other lymphomas with large cells.\textsuperscript{34} With the pattern of expression of the large cells with CD30, CD15, CD45, CD20, CD3, EMA, a more definitive diagnosis could be made. Seldomly, the use of second panel of markers like Oct2, BOB1, EBV, PAX5, MUM1 which were not available and hence not used in this study, might be required. The differentiation of nodular lymphocyte predominant Hodgkin lymphoma (NLPHL) from other HL and T cell / Histiocytic cell rich DLBCL is of both therapeutic and prognostic value.\textsuperscript{33}

The precursor lymphomas in this study were mainly T cell, of equal sex distribution and nodal in origin. The mean age of occurrence was 23 years. These findings are different from findings in the literature with the mean age of occurrence below 15 years in about 64\% of reported cases and it’s predominance in males.\textsuperscript{34,35,36}

Of the 21 extranodal lymphomas seen in this study, none were of extranodal HL. This findings as well as the low occurrence of lymphocyte depleted HL and a few or none of the HIV related lymphomas at Ibadan\textsuperscript{a} and Zaria\textsuperscript{a} could corroborate the believe that there is no substantial proof that there has been an impact of HIV epidemicity in the country on lymphoma incidence. However, the predominance of DLBCL and BL among the extranodal cases as well as the clinical association of HIV/AIDS with the types of lymphomas that are commonly associated with HIV (primary lymphoma of central nervous system, pleural effusion lymphoma and plasmablastic lymphoma) among 3 out of the 7 patients with DLBCL extra nodal lymphoma, is significant and worth mentioning. This proportion could be higher, in this and any other similar studies in the country, if all the high grade lymphomas and HL were subjected to HIV antigenic markers by IHC / molecular studies as done in other parts of Africa and western world where significant impart has been recorded.

All the major lymphoma categories show higher male: female ratio (figure 2) as documented in the literature but the overall mean age is significantly lower than what is obtained in developed countries.

From this study we observed that a panel of 8 to 10 antibodies consisting of CD45, CD20, CD79a, CD3, CD15, CD30, CD34/TdT, Ki67, BCL2, pancytokeratin (Ae1/Ae3), desmin, should be able to distinguish a reactive follicular lesion from follicular lymphoma, identify non lymphoid malignancies, determine the cell-type of the lymphoma (either as B or T). One should also be able to identify most cases of classical Hodgkins lymphomas and determine whether the lymphoma is of high grade / low grade or of immature / mature type (lymphoblastic lymphomas) as well as suitability for anti CD 20 drug (Rituximab).

However, for a more comprehensive diagnosis and subtyping of lymphomas, a larger list of antibodies made up of CD45, CD20, CD79a, CD1a, CD3, CD4, CD5, CD8, CD7, CD23, CD25, CD10, CD43, CyclinD1, CD38, CD138, BCL2, BCL6, Ki67, Alk-1, CD15, CD30, CD34, CD56, CD68, Tdt, MUM1, PAX5, Bob-1 Oct-2, PD-1 pancytokeratin, desmin, SI00, LMP-1 (EBV), HLV-1, HIV, HHV-8 are required. From the list, 3 to 5 antibodies are selected to form panels of varying combinations that can be used in batches as required.

For a non-equivocal case of lymphoma on H and E morphology assessment, the beginning antibody panel will include antibodies against B-cell (CD20, CD79a) and T-cell (CD3, CD5) antigens. CD45, CD15, and CD30, EBV, MUM 1 and pancytokeratin should be added if large atypical cells are seen as in Hodgkin lymphoma, anaplastic large cell lymphoma, poorly differentiated carcinomas while CD 38 / CD 138 and monoclonal light chain immunoglobulin (kappa / lambda) should be considered in cases with numerous plasma cells for the diagnosis of plasma cell neoplasm. Based on morphology, if there is alteration of B-cell areas and the lymphoid cell are small to intermediate in size, antibodies against CD5, CD10, CD23, cyclin D 1, Ki 67, B-cell lymphoma 2 protein (BCL-2), and B-cell lymphoma 6 protein (BCL-6; the prototype of the B-cell transcription factors) should be used to subtype the low grade lymphomas (SLL, FL, MCL, MZL) for further characterization. Differentiation of low grade from high grade lymphomas and diffuse large B cell lymphoma from Burkitt lymphoma requires inclusion of Ki67 while EBV, BCL2 and Ki67 are important to differentiate DLBCL from BL. Tdt and CD 34 will mark immature cells.

Selection of antibodies for T NK cell lymphoma is complex than that of B cell lymphomas. If there is expansion of interfollicular areas and a T-cell lymphoma is being considered, selection of secondary and even tertiary antibody panels from the following antibodies; CD2, CD4, CD5, CD7, CD8, CD56, CD57, PD 1, CD 25, CD 30, ALK-1 as well as staining for the expression of cytoplasmic cytotoxic granules like granzyme B, perforin, T cell intracytoplasmic antigen (TIA) and presence of viral antigens (HLV-1, HIV, HHV-8) for further characterization would be necessary.\textsuperscript{37}

PAX-5, MUM-1 should be used to differentiate B cell and HL from T cell lineage in difficult cases as well as
distinguishing lymphomas of activated B cells from those of post germinal center B cell NHL.

When confronted with the challenges of distinguishing a benign from a malignant lymphoproliferative lesion, expression of aberrant antigens like BCL2, CD43, CD56, ALK-1 by the tumour cells as well as loss of pan T cell markers (CD3, CD5, CD7) or co-expression of or loss of both CD4 and CD8, could be of diagnostic importance.

The interpretations of immunohistochemical staining patterns in lymphoproliferative lesions could be highly demanding. Inexperienced individuals should not shy away from asking for assistance when needed. It is important to note that despite the number of antibodies used and the experience of the pathologist, there remain varying numbers of few cases of lymphomas that will require molecular techniques to demonstrate immunoglobulin or T cell receptor monoclonality before they could be diagnosed.

**Conclusion.**
This study showed that lymphoma immunophenotyping is required for lymphoma diagnosis and subtyping. It revealed no significant difference in the relative frequency distribution of the various subtypes of lymphomas, except for the low and high relative frequencies of Burkitt and small lymphocytic lymphomas, respectively.

Our study showed that most lymphoma subtypes can be identified, classified and differentiated from benign and nonlymphoid neoplasias with a panel of about 5 to 8 antibodies. The panels found useful in this study were CD20 or CD79a, CD5, CD23, CYCLIN D1, CD10, BCL6, and Ki-67 for mature B cell lymphoma, including DLBCL, CD 15, CD30, CD45, pancytokeratin, EMA, ALK 1 for cases with large cell morphology and CD 3, CD7, CD4, CD8, PD1, CD25, CD10, and CD23 for mature T cell lymphomas. A consideration for Acute lymphoma / leukemia will require in addition to B and T cell markers (CD3,CD79a), CD34 and Tdt. To differentiate between reactive and malignant lymphoproliferative lesions, BCL 2, CD 43, CD56, ALK -1 are required.

**Acknowledgement**
We owe our gratitude to the management of The Punch Newspapers Nigeria Ltd for the donation of lymphoma research grant to College of Medicine, University of Lagos, in memory of late Mr. Olu Aboderin.

We express our gratitude to Professor AAF Banjo of The Department of Anatomic and Molecular Pathology, College of Medicine, University of Lagos, the management of The Specialist Laboratory and Dr U Igbokwe of The department of Cellular Pathology, Queen’s Hospital Rom Valley, United Kingdom for their consultancy services.

We also acknowledge the contributions of Dr Sumeet Gujral (Associate professor) of the Haematopathology Oncology unit, Department of Pathology, Tata Memorial Hospital, India as well as Dr Nusat Husain (Professor and Head of Department) and Dr Pradyumn Singh (Additional professor) of The department of pathology, Dr Ram Manohar Lohia Institute, of Medical Sciences, Lucknow, India.

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