

## SILVER STAINED NUCLEOLAR ORGANIZING REGIONS (AGNOR) IN CHRONIC LYMPHOID LEUKEMIAS

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### CHRONIC LYMPHOID LEUKEMIAS

#### Definition

Are a complex group of disorders characterized by clonal neoplastic proliferation of cytologically and immunophenotypically mature lymphocytes (B, T and NK) with predominant blood and bone marrow manifestations.

#### *Classification of chronic lymphoid leukemias*

- 1- The French – American – British group proposal for the classification of chronic lymphoid leukemias (1989)
- 2- The REAL classification (1994)
- 3- The proposed WHO classification of lymphoid neoplasms (1997)

### CHRONIC LYMPHOCYTIC LEUKEMIA (CLL)

#### Diagnosis

The International Workshop on CLL, IWCLL (1981, updated in 1989) proposed the following criteria for the diagnosis of CLL:

- 1- A sustained peripheral blood lymphocyte count greater than  $10 \times 10^9/L$  with most of the cells being mature-appearing lymphocytes.
- 2- Bone marrow aspirate showing greater than 30% lymphocytes.
- 3- Peripheral blood lymphocytes identified as monoclonal B cells.

The diagnosis of CLL is confirmed if criteria 1 plus 2 or 3 are present. If the peripheral blood lymphocyte count is less than  $10 \times 10^9/L$  then both criteria 2 and 3 must be present.

## Prognosis

### 1. Clinical Staging Systems

- I. Rai staging system (1975). II. Binet staging system (1977), (1981). III. The IWCLL system (1981).*
- 2. The absolute lymphocyte count (ALC).*
- 3. The total tumor mass (TTM).*
- 4. The lymphocyte doubling time (LDT).*
- 5. Lymphocyte morphology.*
- 6. BM aspirate.*
- 7. BM histology.*
- 8. Immunotype.*
- 9. Cytogenetics.*
- 10. Molecular genetics.*
- 11. Age, sex, and response to therapy.*
- 12. Transformations: (1) Prolymphocytoid transformation. (2) Immunoblastic transformation (Richter's syndrome). (3) Acute lymphoblastic leukemia.*

## PROLYMPHOCYTIC LEUKEMIA (PLL)

- The diagnosis of PLL requires that > 55% of the circulating leukemic lymphocytes are prolymphocytes.
- The typical immunophenotype of B-PLL includes: SmIg+, FMC7+, CD20+, CD22+ and CD79b+, CD5 (low). Most T - PLL cases are CD2, CD3, CD5, CD7 and CD4 positive while CD8 negative.

## PLASMA CELL LEUKEMIA (PCL)

- PCL may occur de novo (primary PCL) or may develop in patients with multiple myeloma, MM (secondary PCL).
- PCL is diagnosed when the number of identifiable plasma cells in the peripheral blood is greater than  $2 \times 10^9/L$ .
- **Three morphological appearances can be encountered in PCL**  
*(1) Lymphoplasmacytic (20% of cases) (2) Plasmacytic (75% of cases) (3) Plasmablastic (5% of cases)*
- The typical combination of positive markers in PCL is CD38+ and CyIg+ (kappa or lambda).

SmIg -, CD19-, 20-, 21-.

### **The nucleolus organizer regions**

#### **Definition**

Nucleolus organizer regions (NORs) are specific regions on certain metaphase chromosomes, which cause the formation of nucleoli in interphase.

#### **Metaphase NORs**

In human karyotype, NORs are located in the secondary constriction areas of the short arms of each of the five acrocentric chromosomes 13, 14, 15, 21 and 22.

#### **Each NOR is composed of**

1. rDNA.
2. Histone proteins.
3. AgNOR proteins: include the following proteins:
  - a. Nucleolin:
  - b. Nucleolar protein B23,
  - c. RNA polymerase I (RPI) with its various subunits.
  - d. The upstream binding factor (UBF).

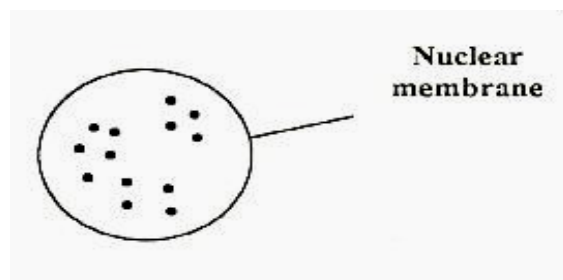
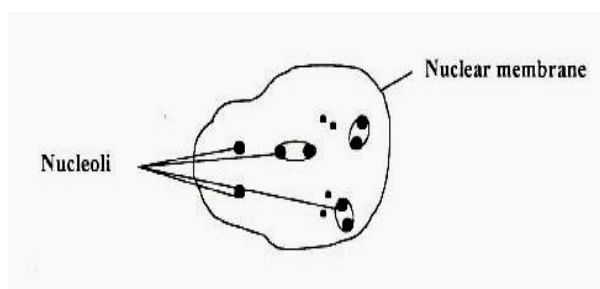
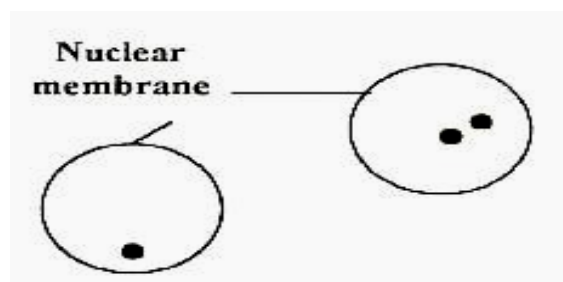
#### **Analysis of AgNOR**

##### *A. Quantitative analysis:*

1. Counting method:
  - i. Mean AgNOR dots (mAgNOR) technique:
  - ii. Another AgNOR parameter is the mean number of AgNOR clusters.
2. Morphometric method (image cytometry, ICM).
3. Quantification of AgNOR by flow cytometry (FCM)

##### *B. Qualitative analysis of AgNOR*

1. The percentage of cells with one or two compact nucleoli.
2. The percentage of cells with clusters.
3. The percentage of cells with several scattered dots

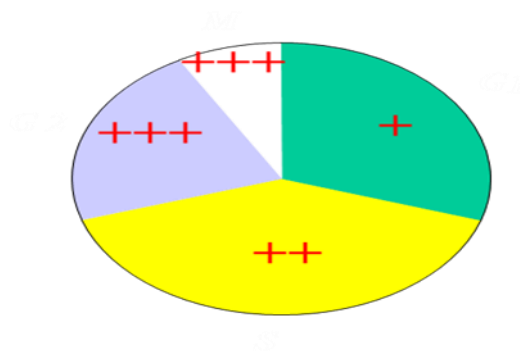


### The cellular pathophysiologic conditions that affect AgNOR

1. Metabolic activity.
2. Cell ploidy.
3. Cell maturation.
4. Cell proliferation.

### AgNOR and proliferation

- AgNOR amount in relation to cell cycle



*G1 green, S yellow, G2 blue, M white*

**AgNOR & proliferation****Interphase AgNOR quantity  $\propto$** 

- TLI(thymidine labeling index)
- BLI(bromodeoxyuridine labeling index)
- the percentage of S-phase cells determined by flow cytometry
- the proportion of Ki67 positive cells

**Interphase AgNOR quantity  $1/\propto$** 

- cell doubling time.

in vitro.

in vivo

**AgNOR and CLL****Lymphocytes with one AgNOR cluster(%)**

$1/\propto$

- *age*
- *hemoglobin concentration*
- Lymphocytes with one AgNOR cluster(%)

$\propto$

- ***Total tumor mass(TTM)***
- ***Lymphocyte doubling time (LDT)***
- ***(Binet stage C, bulky LNs, massive splenomegaly and ALC > 100 x 10<sup>9</sup>)***

**Predicts The duration of stable phase or treatment free period of CLL****AIMS OF THE STUDY**

- ❖ To analyze nuclear organizer region (Ag-NOR) parameters of lymphocytes in chronic lymphoid leukemias.
- ❖ To find whether AgNOR parameters can differentiate between benign and neoplastic lymphocytosis.
- ❖ To compare PB vs. BM AgNOR parameters.
- ❖ To find whether AgNOR parameters correlate with prognostically significant clinico-hematological parameters in CLL.

**CASES AND METHODS*****Normal Group***

11 healthy persons aged 8 - 70 years PB AgNOR studies

***Reactive Group***

10 cases aged 1 mo - 9 years PB AgNOR studies.

***CLL Group 43 cases (27 New, 16 retrospective) aged 35 - 76 years***

- Clinical studies
- Hematological studies
- PB & BM AgNOR studies

***PLL & PCL Group 1 PLL 2 secondary PCL 1 primary PCL***

- Clinical studies
- Hematological studies
- PB AgNOR studies

**Clinical studies**

- Age, sex, LAP, hepatomegaly, splenomegaly and the number of LN areas involved.

**Specimens**

- *Patients diagnosed during the period of study:*
- *PB and BM samples.*
- *Cases diagnosed before starting the study:*
- *Leishman stained PB and BM smears were reviewed.*

**Hematological studies**

- *\*PB studies:*
- *Blood counts:* Hb, PCV, WBC count and platelet count. *WBC differential count:* LC%, ALC, PLC%, APLC
- *Cytological analysis*
- **Differential count of cytological features of PB lymphocytes:**
- lymphocyte size, N/C ratio, chromatin pattern, cells with nucleoli, cells with cleaved lymphocytes, cells with azurophilic granules, cells with plasmacytoid features.

- Cytological classification of PB lymphocytes:
- Six morphologically different lymphocytes were observed:

### **Morphological classification of CLL**

- According to the FAB group, T. Vallespi and D. Oscier proposals:
- CLL with atypical lymphocytes morphology
- <10 %prolymphocytes
- <10 %large lymphocytes
- <15 %lymphocytes with cleaved nuclei
- <15 %lymphocytes with azurophilic granules
- <15 %lymphocytes with plasmacytoid features
- CLL with typical lymphocyte morphology
- \* *BM studies:*
- *BM smears*
- Differential count of marrow cellular elements.
- *BM biopsy* ( 20 cases)
- Diffuse, nodular, interstitial or mixed nodular and interstitial.

### **Diagnostic criteria**

#### ***The diagnosis of CLL was based on:***

1. PB lmyphocytosis of  $> 10 \times 10^9/l$
2. BM lymphocytic infiltration of  $> 40\%$
3. Immunophenotyping (31 cases)

CD3-, CD19+, CD21+ and CD10-. Five cases were CD5- while 26 cases were CD5+.

#### ***The diagnosis of PLL was based on:***

$> 55\%$  prolymphocytes in the PB.

#### ***The diagnosis of PCL was based on:***

1.  $> 2 \times 10^9/l$  plasma cells in the PB negative results on 2. Immunophenotyping using the above mentioned markers was negative.

### **Staging**

Binet clinical staging system was applied.

AgNOR studies.

### *Specimens*

AgNOR staining was applied to PB and BM smears. For the CLL cases diagnosed before starting the study, Leishman's stained BM smears were obtained and AgNOR staining was done after 24 hrs. destaining in absolute methanol.

### *Equipments*

A sensitive balance. An incubator.

### *Solutions*

1. Aqueous silver nitrate solution (50%)
2. colloidal developer solution (2% gelatin, 1% formic acid)
3. Sodium thiosulphate (5%)

### *Procedure*

The silver staining protocol proposed by the International Committee for AgNOR quantification that was submitted during the 1st workshop held in Berlin in 1993, was followed.

- I. Fixation
- II. Impregnation and development step
- III. Treatment with sodium thiosulphate.
- IV. Mounting in Canada balsam.

### *Analysis of AgNOR stained slides*

**Eight AgNOR configurations (patterns) have been identified in AgNOR stained smears for cases of chronic lymphoid leukemia:**

1. Cells with one large brown-black dot.
2. Cells with two large brown-black dots.
3. Cells with three small brown-black dots.
4. Cells with four small brown-black dots.
5. Cells with scattered small dots ( $\geq 5$  dots).
6. Cells with a single cluster ( $\geq 2$  black-brown small dots within a light brown nucleolar matrix).
7. Cells with more than one cluster.
8. Cells with cluster(s) and dots.



The percentage of each AgNOR pattern was obtained by differential counting of these patterns from 200 consecutive lymphocytes and division by 200.

The mean AgNOR dot per cell (nucleus) i.e. mAgNOR was obtained by counting all silver – stained dots in the 200 consecutive lymphocytes whether these dots were individually distributed or clustered and dividing the number of these dots by 200.

AgNOR parameters included both the percentage of each AgNOR pattern and the mean AgNOR (mAgNOR).

### Statistical analysis

SPSS ver. 10 software was used for statistical analysis. The level of  $< 0.05$  was considered significant.

### A. Description of the results

1. The normal group.
2. The reactive group.

Peripheral blood AgNOR parameters among the normal and the reactive groups.

Parameter	Range	Median	Mean	SD
<b>Normal group</b>				
Nuclei with 1 or 2 dots %	77 – 100	97	95.1	6.98
Nuclei with 3 dots %	0 – 23	3	4.9	6.98
mAgNOR	1.1 – 1.79	1.2	1.29	0.21
<b>Reactive group</b>				
Nuclei with 1 or 2 dots %	76 – 97	91	89.7	7.66
Nuclei with 3 dots %	0 – 14	2.5	4.2	4.54
Nuclei with 4 dots %	0 – 2	0	0.6	0.84
Nuclei with $\geq 5$ dots %	0 – 2	0	0.3	0.67
Nuclei with one cluster %	0 – 22	3	5.2	0.46
mAgNOR	1.21 – 1.74	1.39	1.4	0.31

### 3. The CLL cases:

#### I. Clinical parameters:

##### i. Age:

35 years - 76 years (median 60 years).

*ii. Sex:*

The M:F ratio was 1.5: 1.

*iii. Organomegaly:*

LAP (80%)

Splenomegaly (58%).

**II. Hematological parameters:****Haematological parameters among CLL patients**

Parameter	Range	Median	Mean	SD
Hb (g/L)	44 – 160	108	105	24
Platelet count $\times 10^9/L$	3 – 550	140	165	110
WBC count $\times 10^9/L$	14 – 600	63	125	169
ALC $\times 10^9/L$	10.3 – 600	59	105	133
PLC %	0 – 10	0	1	2
Absolute PLC $\times 10^9/L$	0 – 12	0	1.3	3.4
ANC $\times 10^9/L$	0.67 – 12.3	3.9	4.6	2.7
B.M lymphocyte %	45 – 100	83	83	15.5

**Cytological parameters among CLL patients**

Parameter	Range	Median	Mean	SD
Lymphocyte size				
Small cell %	43 – 100	100	93	14.8
Large cell %	0 – 57	0	7	14.8
Lymphocyte N/C ratio				
High %	27 – 100	100	88.2	20.1
Low %	0 – 73	0	11.8	20.1
Lymphocyte with clumped nuclear chromatin	73 – 100	100	95.4	14.8
Lymphocyte with nucleoli %	0 – 25	0	2.3	5.9
Lymphocyte with cleaved nuclei %	0 – 70	2	7.3	13.5
Lymphocyte with azurophilic granules %	0 – 13	0	0.76	2.4
Plasmacytoid lymphocyte %	0 – 5	0	0.15	0.79

**Morphological subtypes of CLL**

Typical CLL 62.7%

CLL with large lymphocyte 21%

CLL with cleaved nuclei 11.6%

CLL with large lymphocytes and cleaved nuclei 4.7%

**BM histology:** Diffuse 55%, mixed 25%, interstitial 20%

**III. Staging:** A 30%, B 33%, C 37%

**IV. AgNOR parameters among CLL patients**

Parameter	Range	Median	Mean	SD
<b>Peripheral Blood</b>				
Nuclei with 1 or 2 dots %	35 – 97	88	81.73	15.29
Nuclei with 3 dots %	0 – 18	3.5	4.91	4.39
Nuclei with 4 dots %	0 – 10	0	1.31	2.43
Nuclei with $\geq 5$ dots %	0 – 36	0	1.77	7.04
Nuclei with one cluster %	1 – 35	6	10.19	9.64
mAgNOR	1.18 – 5.38	1.48	1.7	0.8
<b>Bone marrow</b>				
Nuclei with 1 or 2 dots %	43 – 96	82	82	12.7
Nuclei with 3 dots %	0 – 28	3	4.24	5.15
Nuclei with 4 dots %	0 – 8	0	0.57	1.57
Nuclei with $\geq 5$ dots %	0 – 4	0	0.29	0.94
Nuclei with one cluster %	2 – 32	12	12.17	8.26
mAgNOR	1.21 – 2.45	1.53	1.54	0.23

**Clinical and hematological parameters among PLL and PCL patients**

Parameter	PLL	Secondary PCL case no.1	Secondary PCL case no.2	Primary PCL
Age (yrs)	70	48	57	36
Sex (M/F)	M	M	F	M
LN (+/-)	-	+	+	+
Splenomegaly (+/-)	+	+	+	+
Hb g/L	100	62	50	55
Platelet count $\times 10^9/L$	200	20	54	14
Total WBC $\times 10^9/L$	179	25	20	15
ALC $\times 10^9/L$	172	17.5	16	9.5
Differential WBC count % [ $\times 10^9/L$ ]	L: 35 [62] PLC: 61 [109]	L: 21 [3.7] PC: 16 [2.8] LPC: 50 [8.8] P: 13 [2.3]	L: 44 [7] PC: 6 [1] LPC: 50 [8]	L: 63 [6] PC: 2 [0.2] LPC: 35 [3.3]

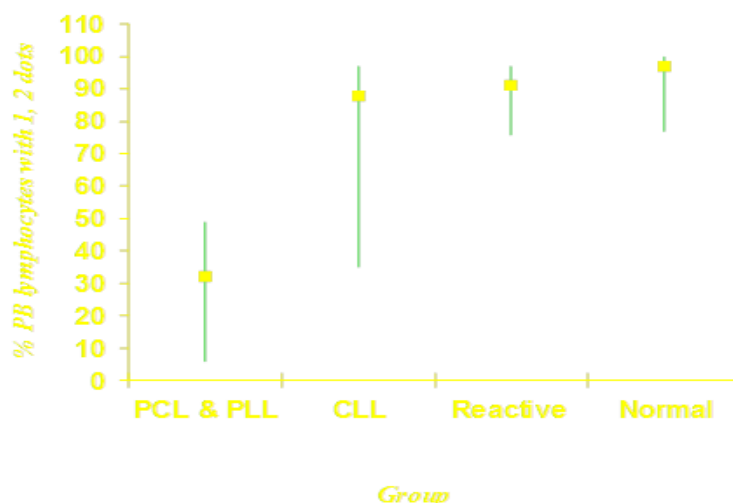
*L: lymphocyte, PLC: prolymphocyte, PC: plasma cell, LPC: lymphoplasmacyte, P: plasmablast*

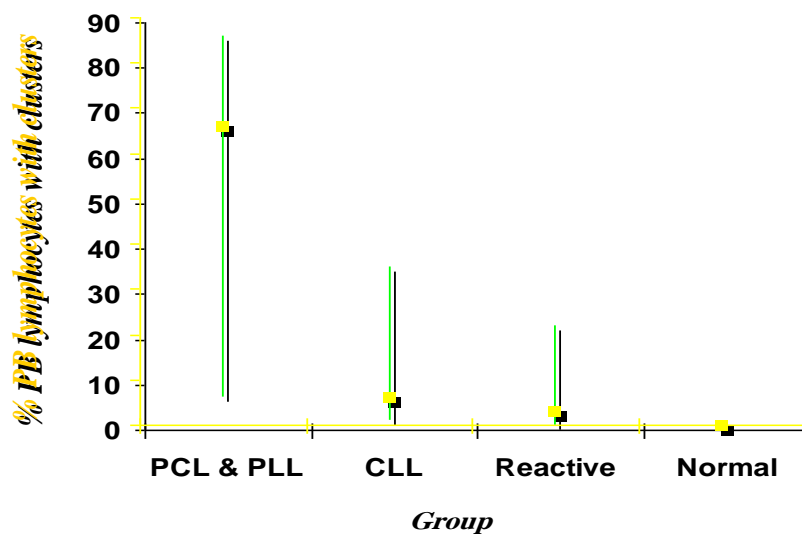
*Peripheral blood AgNOR parameters among PLL and PCL patients*

Parameter	PLL	1°PCL Case No.1	1°PCL Case No. 2	1°PCL
Nuclei with 1 dot %	16	4	35	26
Nuclei with 2 dots %	2	2	10	23
Nuclei with 3 dots %	0	0	1	15
Nuclei with 4 dots %	1	0	4	15
Nuclei with ≥ 5 dots %	0	8	0	15
Nuclei with one cluster %	75	30	50	6
Nuclei with more than one cluster %	6	50	0	0
Nuclei with cluster(s) and dots %	0	6	0	0
mAgNOR	2.82	6.31	2.82	3.27

#### B. Statistical analysis of the results

*Comparison between the normal group, the reactive group, the CLL group and the PLL & PCL group regarding AgNOR parameters.*

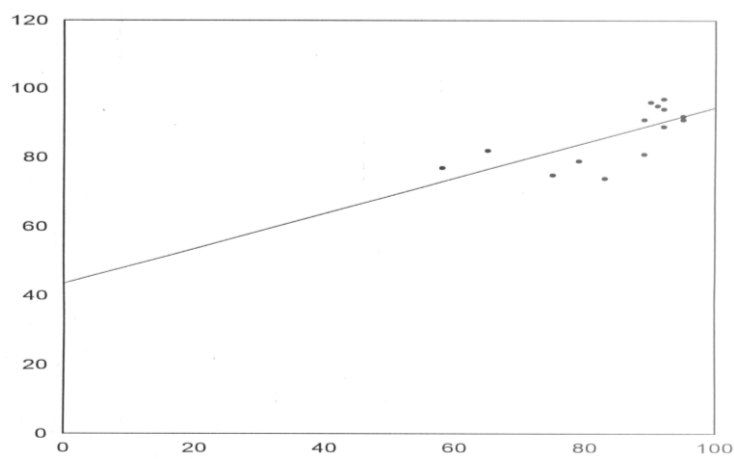




## II. CLL group

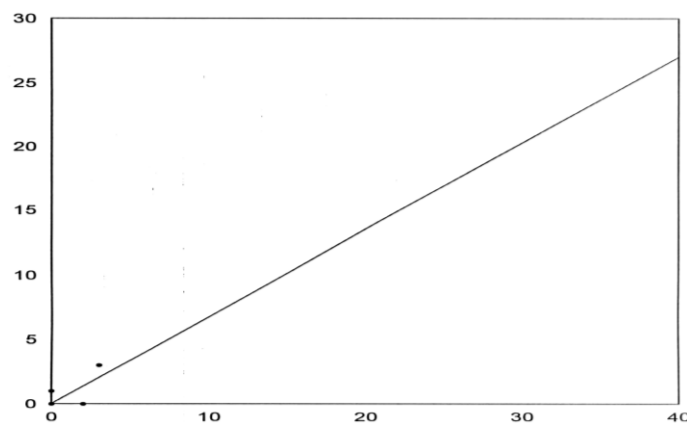
### 1. PB vs. BM AgNOR

BM Lymphocytes with 1 or 2



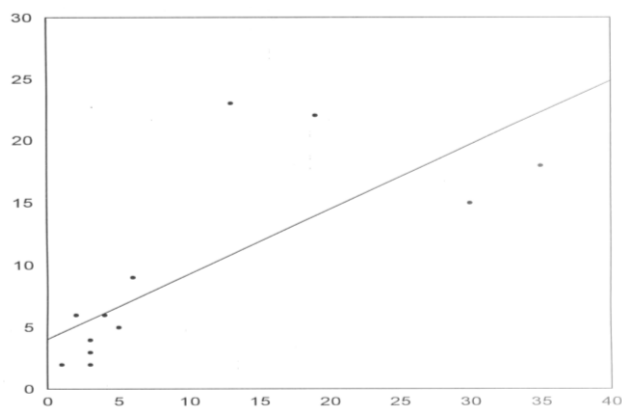
PB Lymphocytes with 1 or 2 dots (%)

BM lymphocytes with  $\geq 5$  dots (%)



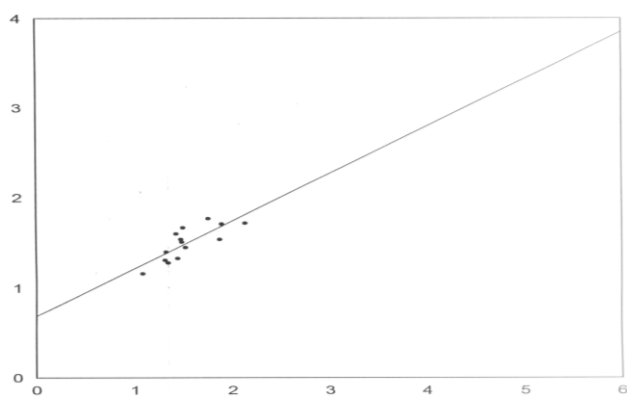
**PB lymphocytes with  $\geq 5$  dots (%)**

**BM lymphocytes with clustered NORs (%).**



**PB lymphocytes with clustered NORs (%).**

**The mAgNOR of BM lymphocytes**



**The mAgNOR of PB lymphocytes**

## **2. AgNOR parameters in relation to each clinicohematological parameter**

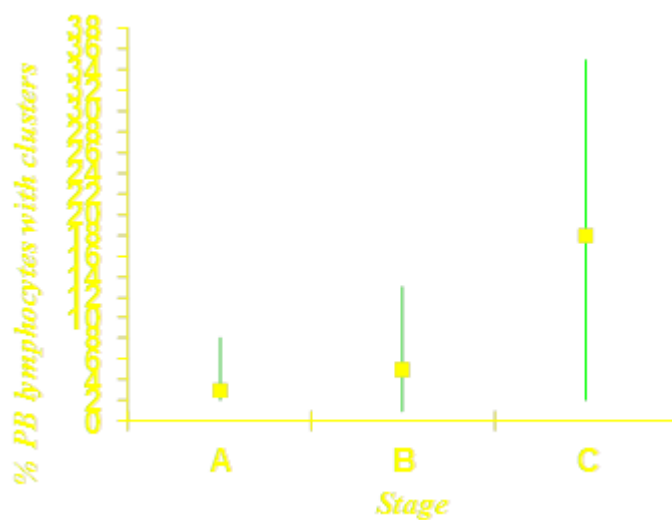
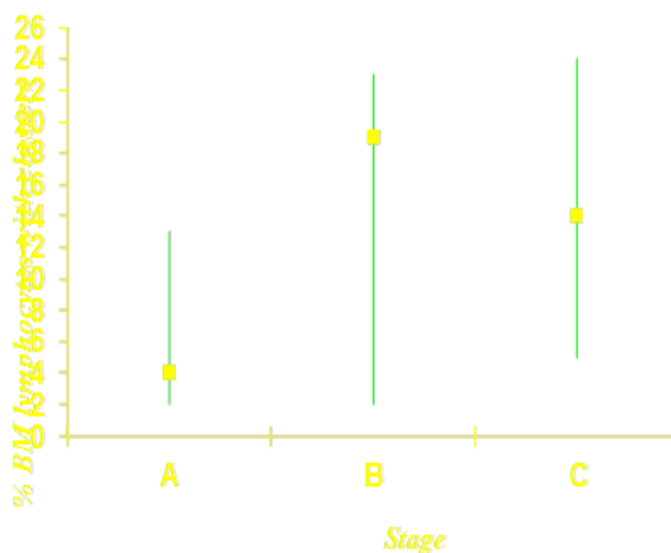
### ***i. Age.***

Correlation of the proportion of BM lymphocytes with a single cluster with age was on the borderline of being statistically significant ( $p=0.05$ ).

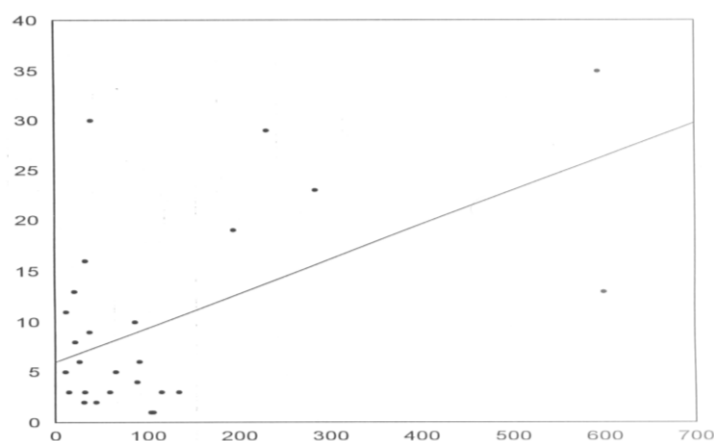
### ***ii. Sex.***

No significant difference was found between male and female regarding AgNOR parameters.

### ***iii. The Binet staging system.***

**PB lymphocytes with clusters % in relation to stage****BM lymphocytes with clusters % in relation to stage****iv. ALC**

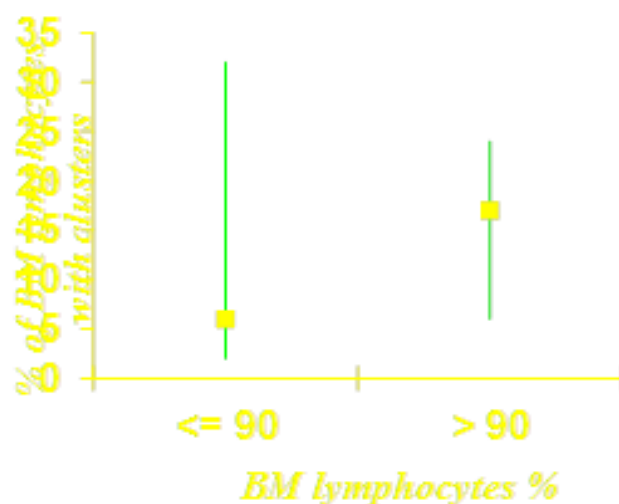
There was strong positive correlation between the ALC and the proportion of PB lymphocytes with a single cluster ( $p < 0.005$ ).

**PB lymphocytes with clustered NORs (%)****The absolute lymphocyte count (ALC,  $\times 10^9$ )**

v. AgNOR in relation to morphological subtypes of CLL: NO SIGNIFICANT DIFFERENCE

**vi. BM infiltration patterns**

No significant difference regarding AgNOR parameters was found between CLL with diffuse infiltration pattern and CLL with non-diffuse infiltration pattern (i.e. interstitial and mixed patterns).

**vii. BM lymphocyte percentage of all nucleated cells****viii. CD5- CLL**

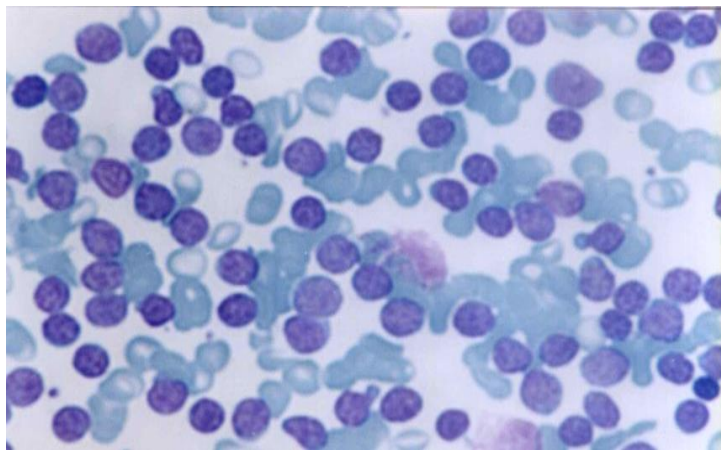
AgNOR parameters did not differentiate between CD5+ and CD5- CLL.



**CASES**

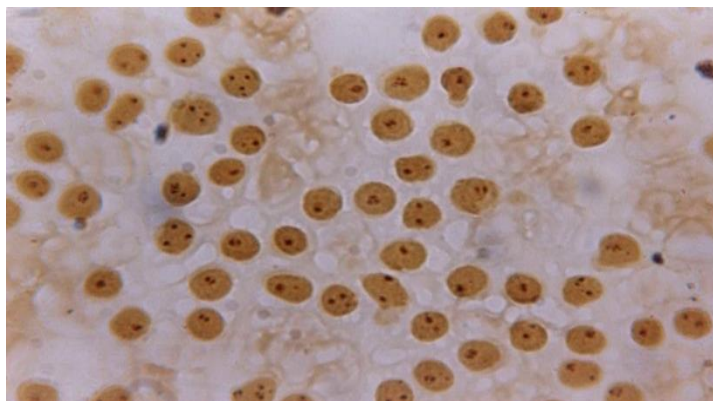
1-CLL with ALC of  $600 \times 10^9/l$ . Leishman stain (x1000).

Cells are small with high N/C ratio and clumped chromatin



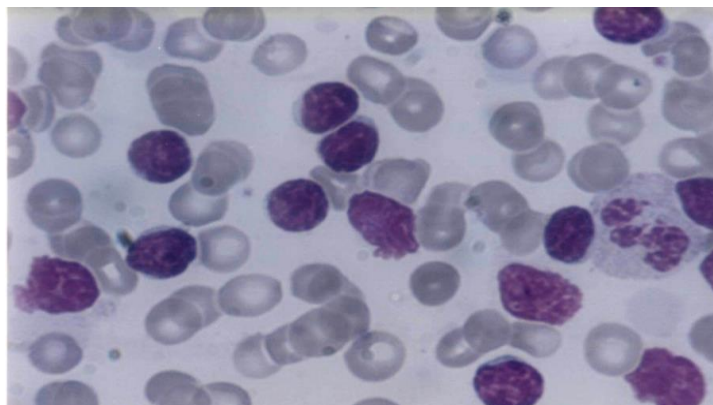
The same case stained with AgNOR technique (x1000).

AgNOR patterns include cells with 1 dot, 2 dots, 3 dots and 4 dots with many cells showing clusters of 2 or more dots

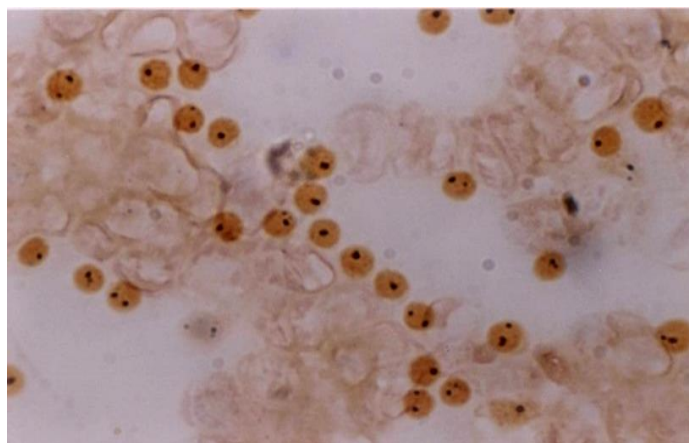


2-CLL with ALC of  $100 \times 10^9/l$ . Leishman stain (x1000).

Cells are small with high N/C ratio and clumped chromatin

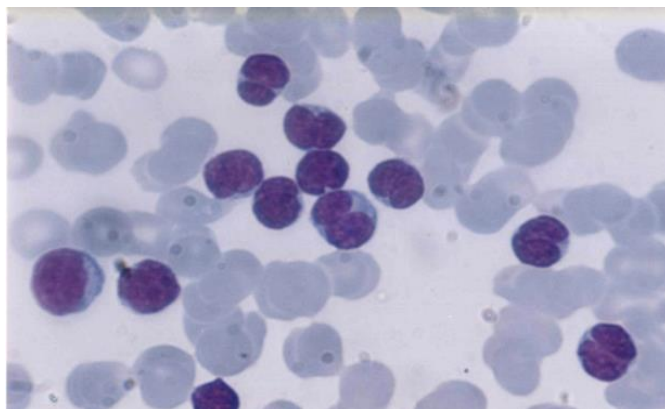


The same case stained with AgNOR technique (x 1000). Most lymphocytes have 1 or 2 dots



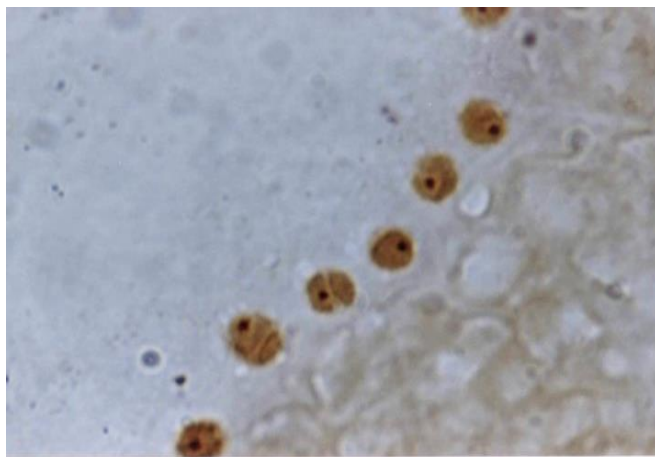
3-CLL with 70% lymphocytes with cleaved nuclei (CD5-ve, CD10-ve\*). Leishman stain (x1000)

The clefts are deep, dividing the nucleus into 2 lobes

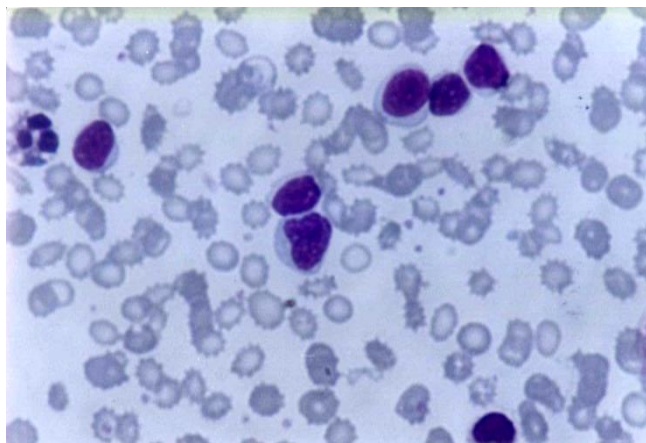


The same case stained with AgNOR technique (x1000).

Most cells have 1 AgNOR dot

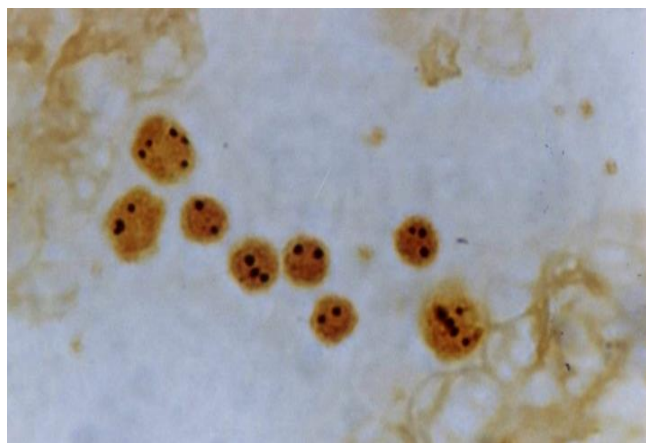


**4-CLL with large lymphocytes (CD5+, CD19+). Leishman stain x1000. Cells are > 2 RBCs with low N/C ratio and condensed chromatin**

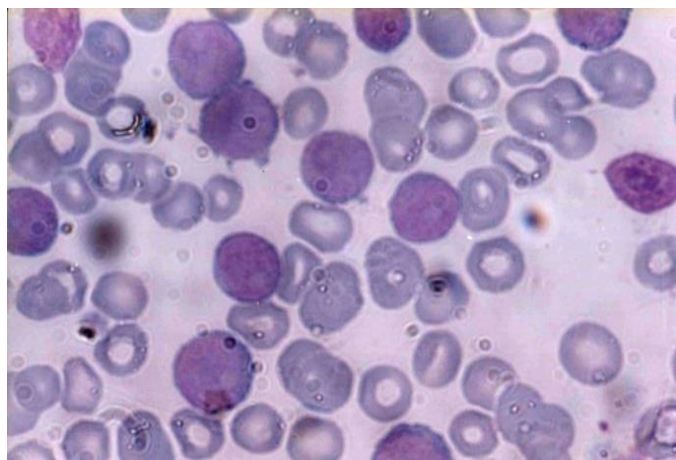


**The same case stained with AgNOR technique (x1000).**

**Cells with 2 dots, 3 dots and 5 dots are seen together with a cell with a cluster**



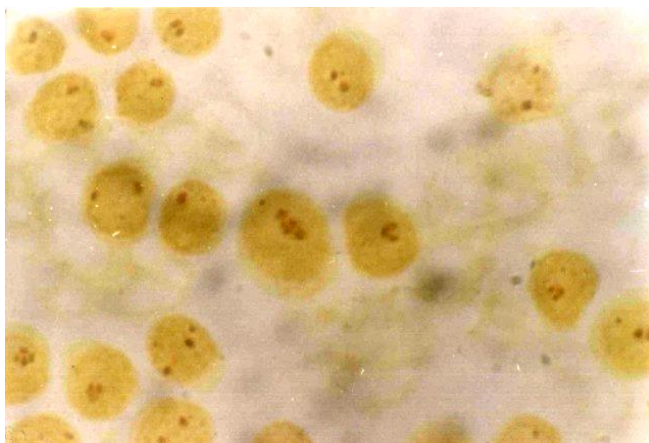
**5-PLL with 70% prolymphocytes. Leishman stain (x1000)**



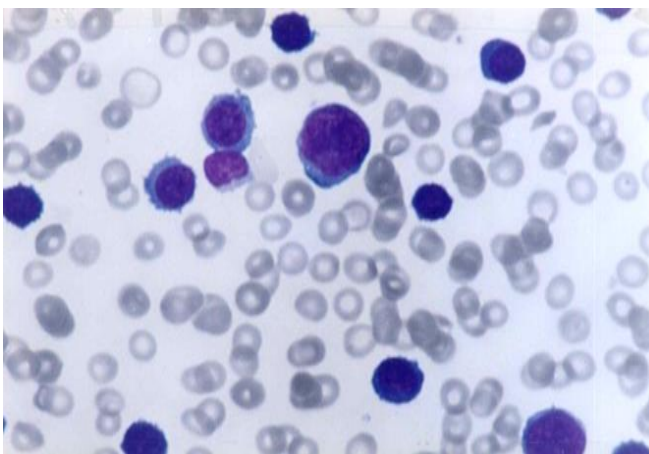


The same case stained with AgNOR technique (x1000).

Most cells show a single cluster of AgNORs

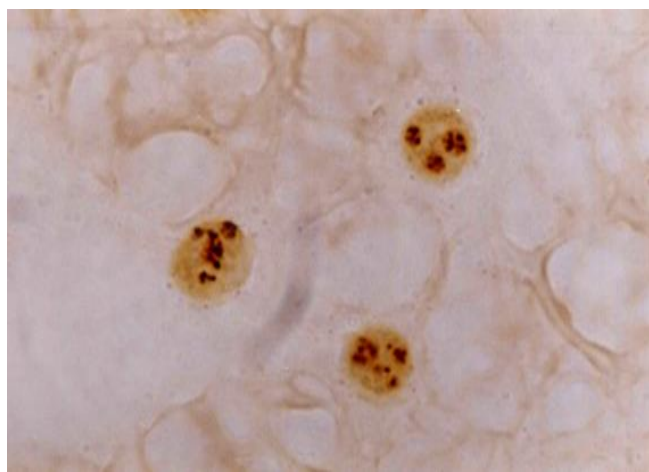


6-The case with secondary plasma cell leukemia. Leishman stain (x1000). plasmacytoid lymphocytes and plasmablasts are seen



The same case stained with AgNOR technique (x1000).

Cells with more than one cluster are seen



## CONCLUSIONS

1. Normal peripheral blood lymphocytes show one NOR, two NORs and less commonly three NORs.
2. Clustered NORs are found in the PB lymphocytic cells in cases with reactive and neoplastic lymphocytosis but not in normal individuals.
3. The predominant AgNOR pattern observed in CLL was cells with 1 or 2 NORs in the majority of cases while cells with clustered NORs formed a minor but highly variable proportion. On the contrary, clustered NORs was the pattern found in the nuclei of a major proportion of lymphocytic cells in PLL and secondary PCL. AgNOR was of scattered pattern in the nuclei of lymphocytic cells in 10PCL.
4. Cells with more than one cluster, or with clusters and dots were only found in PLL and 20PCL with plasmablasts and they formed a major proportion of cells in the latter.
5. The proportion of cells with 1 or 2 dots, the proportion of cells with clusters and the mAgNOR were the patterns that differentiated significantly between CLL vs normal lymphocytes and between PLL & PCL vs CLL, reactive and normal groups.
6. The proportion of cells with clustered NORs was the AgNOR parameter that correlated with significant prognostic parameters in CLL.
7. AgNOR parameters are not helpful in differentiating CLL from reactive lymphocytosis.
8. Both PB and BM are useful for AgNOR analysis.
9. The percentage of PB lymphocytes with a single cluster differentiated significantly between stage C vs. each of stage A and B and it correlated significantly with ALC while the percentage of BM lymphocytes with a single cluster differentiated significantly between each of stage B and stage C vs. stage A and it is correlated with BM lymphocytes percentage. Correlation with age was on the borderline of being statistically significant.
10. Atypical CLL didn't show significant difference from typical CLL regarding AgNOR.
11. CD5 -ve CLL and CLL with PLCs < 10%, plasmacytoid lymphocytes < 15% and lymphocytes with azurophilic granules < 15% showed AgNOR parameters similar to those found in typical CLL.
12. Recommendations
13. Evaluation of AgNOR at diagnosis as a prognostic factor in CLL cases requires clinical follow up studies to establish whether or not there is a correlation between a high proliferative rate (as determined by high proportion of lymphocytes with clusters) and survival.

14. Investigating the changes of AgNOR before and after treatment may provide informations about response to treatment, treatment selection and overall survival.
15. Analysis of AgNOR using image or flow cytometry can provide reproducible AgNOR data that may show more significant correlations.