CHRONIC LYMPHOCITIC LEUKEMIA

• The most common leukemia in western world

• Characterized by the accumulation of mature monoclonal B cells in the blood, secondary lymphatic tissues and bone marrow

• Characterized by a heterogeneous clinical course
• For a more accurate *diagnostic definition*

• For a biologically-based *prognostic stratification* of patients

• For an optimal *therapeutic* (or non-therapeutic) *algorithm*
INTEGRATED APPROACHES UTILIZED FOR A MODERN CHARACTERIZATION OF CHRONIC LYMPHOCYTIC LEUKEMIA

- MORPHOLOGY
- IMMUNOPHENOTYPE
- CYTOGENETICS
- MOLECULAR GENETICS
- P53 SEQUENCE
- IG GENE REARRANGEMENTS
- GENOMIC PROFILING
- WHOLE GENOME SEQUENCING
CHRONIC LYMPHOCYTIC LEUKEMIA

DIAGNOSIS

CELL COUNT

MORPHOLOGY

IMMUNOPHENOTYPE
For the diagnosis of CLL two data are necessary and sufficient.

The number of clonal B-lymphocytes: more than 4,000-5,000/ul

The peculiar immunophenotype
CLL patient at the time of the diagnosis
CLL patient at the time of the diagnosis
Typical CLL
CLL / PL Morphology
Smudge cells on peripheral-blood smear of patient with chronic lymphocytic leukemia. The arrows show examples of smudge cells. The smudge cell percentage is estimated by counting 200 lymphocytes and/or smudge cells; the smudge cell number is then divided by total number of cells counted (smudge cells intact lymphocytes) and multiplied by 100%.

Nowakowski et al, J Clin Oncol 2009
Smudge cells on peripheral blood smears of one patient with CLL. The arrow shows an example of a smudge cell. The smudge cell percentage was determined by counting lymphocytes and/or smudge cells following the method described by Nowakowski et al.
Treatment-free and overall survival curves of 100 patients with CLL according to smudge cell percentages. Kaplan–Meier curves depict the cumulative proportion of untreated patients with CLL according to the time interval since diagnosis and OS in the same cohort. Median times to first treatment were 40 months vs. 116 months. Median OS was 161 months vs. 244 months. Statistical analysis was performed using the log-rank test. The number of patients in each group is shown in brackets.
CLL

Slg ±
CD5+
CD22−/+
FMC-7−/+
CD23++
CD19+
CD20 ±
B-CELL CHRONIC LYMPHOPROLIFERATIVE DISEASES
IG LIGHT CHAIN EXPRESSION

CLONALITY

Expression of kappa chain

or

lambda chain
MIF OF IG-LIGHT CHAIN EXPRESSION IN B-CELL CHRONIC LYMPHOPROLIFERATIVE DISEASES

Norm B  CLL  PLL  HCL  MCL  FL
Smlg  Bright  Dim  Bright  Bright  Bright  Bright

MIF: Dim <1000 Bright >1200
**B-CHRONIC LYMPHOPROLIFERATIVE DISEASES IMMUNOPHENOTYPE (TdT-)**

<table>
<thead>
<tr>
<th></th>
<th>CLL</th>
<th>PLL</th>
<th>HCL</th>
<th>MCL</th>
<th>FL</th>
<th>SLVL</th>
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<tbody>
<tr>
<td>SmIg</td>
<td>±</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD19</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>CD5</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CD20</td>
<td>±</td>
<td>+</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
</tr>
<tr>
<td>CD23</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>±</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>FMC7</td>
<td>±</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>CD25</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>DBA.44</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>CD103</td>
<td>-</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
LEUKEMIC MCL

CD5+ CD23 -

CD5+ CD23 +

CLL
CD200 ANTIGEN
Flow Cytometric Evaluation

Differential Diagnosis
between
Chronic Lymphocytic Leukemia = positive
Mantle Cell Lymphoma = negative
CD200 expression in CD5+ lymphoproliferative disorders
### Lymphocyte sub-populations: reference values

<table>
<thead>
<tr>
<th></th>
<th>Donors with age &gt;18 years</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Mean ± SD</strong></td>
<td><strong>Median (range)</strong></td>
<td></td>
</tr>
<tr>
<td>WBC (ul)</td>
<td>6629 ± 1540</td>
<td>6660 (4410-9940)</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>34 ± 7</td>
<td>34 (20-43)</td>
<td></td>
</tr>
<tr>
<td>Lymphocytes (ul)</td>
<td>2198 ± 533</td>
<td>1970 (1260-3060)</td>
<td></td>
</tr>
<tr>
<td><strong>Lymphocyte sub-populations</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD19 (%)</td>
<td>10 ± 3</td>
<td>9 (6-18)</td>
<td></td>
</tr>
<tr>
<td>CD19 (ul)</td>
<td>218 ± 108</td>
<td>179 (113-524)</td>
<td></td>
</tr>
<tr>
<td>CD3 (%)</td>
<td>72 ± 6</td>
<td>73 (68-84)</td>
<td></td>
</tr>
<tr>
<td>CD3 (ul)</td>
<td>1595 ± 448</td>
<td>1556 (857-2570)</td>
<td></td>
</tr>
<tr>
<td>CD3+/CD4+ (%)</td>
<td>44 ± 7</td>
<td>931 (642-1498)</td>
<td></td>
</tr>
<tr>
<td>CD3+/CD4+ (ul)</td>
<td>945 ± 225</td>
<td>931 (643-1498)</td>
<td></td>
</tr>
<tr>
<td>CD3+/CD8+ (%)</td>
<td>25 ± 7</td>
<td>27 (11-39)</td>
<td></td>
</tr>
<tr>
<td>CD3+/CD8+ (ul)</td>
<td>572 ± 255</td>
<td>630 (158-1193)</td>
<td></td>
</tr>
<tr>
<td>CD56+/CD16+/CD3- (%)</td>
<td>13 ± 6</td>
<td>11 (4-27)</td>
<td></td>
</tr>
<tr>
<td>CD56+/CD16+/CD3- (ul)</td>
<td>275 ± 133</td>
<td>247 (74-566)</td>
<td></td>
</tr>
</tbody>
</table>
B-Cell Monoclonal Diseases

Rawstron AC, et al

Monoclonal B-cell lymphocytosis and chronic lymphocytic leukemia
<table>
<thead>
<tr>
<th>Feature at Presentation</th>
<th>Progressive Lymphocytosis (N=51)</th>
<th>Death (N=62)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cutoff Point or Category (no. of subjects)†</td>
<td>Univariate (Log-Rank P Value)</td>
</tr>
<tr>
<td>Age — yr</td>
<td>68 (72/113)</td>
<td>0.59</td>
</tr>
<tr>
<td>Sex</td>
<td>M/F (89/96)</td>
<td>0.40</td>
</tr>
<tr>
<td>Hemoglobin — g/dl</td>
<td>12.5 (38/147)</td>
<td>0.25</td>
</tr>
<tr>
<td>Platelet count — per mm³</td>
<td>295,000 (144/41)</td>
<td>0.35</td>
</tr>
<tr>
<td>Lymphocyte count — per mm³</td>
<td>6800 (143/42)</td>
<td>0.008</td>
</tr>
<tr>
<td>B-cell count — per mm³</td>
<td>1900/4000 (31/108/46)</td>
<td>0.005</td>
</tr>
<tr>
<td>T-cell count — per mm³</td>
<td>1700 (131/54)</td>
<td>0.40</td>
</tr>
<tr>
<td>B cells expressing CD38 — %‡</td>
<td>2 (58/116)</td>
<td>0.54</td>
</tr>
</tbody>
</table>

* The 5-year risk of progressive lymphocytosis was 26% (95% confidence interval [CI], 19 to 34) and of death 26% (95% CI, 20 to 33). Optimal cutoff points for outcome prediction were defined according to Youden’s J value. These cutoff points were used for variables entered into the Cox proportional-hazards analysis of the defined groups.

† With the exception of sex and B-cell count among subjects with progressive lymphocytosis, the numbers in parentheses are the number of subjects with a value at or above the cutoff point and the number of subjects with a value below the cutoff point, respectively. For sex, the numbers in parentheses are the number of men and the number of women, respectively. Two cutoff points for B-cell count were identified for the risk of progressive lymphocytosis, since the J value peaked multiple times between the counts of 1900 and 4000 per cubic millimeter. The numbers in parentheses are the numbers of subjects with B-cell counts below 1900, between 1900 and 4000, and above 4000, respectively.

‡ Hazard ratios were calculated for having a value at or above the cutoff point as compared with a value below the cutoff point, except for sex, for which the hazard ratios were calculated for being male as compared with being female, and except for progressive lymphocytosis according to B-cell count, for which the hazard ratios were calculated for having a count at or below 4000 as compared with a value above 4000.

§ Data for B-cell expression of CD38 were not available for all subjects.
IMMUNOPHENOTYPE

- Flow cytometer: FacsCalibur/FacsCanto, Becton Dickinson
- Antigens: CD45, CD5, CD19, CD20, CD22, CD23, CD200, CD79b, FMC7, kappa, lambda, CD38, **CD43, CD81**, ZAP-70, CD14, CD3, CD4, CD8, CD16, CD56
- Analysis: CellQuest/PaintGate Becton Dickinson
### Eight color monoclonal antibody combinations (FACSCanto II cytometer)

<table>
<thead>
<tr>
<th>FITC</th>
<th>PE</th>
<th>PerCP</th>
<th>PE-Cy7</th>
<th>APC</th>
<th>APC-Cy7</th>
<th>HV-450</th>
<th>HV-500</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ig lambda</td>
<td>Ig kappa</td>
<td>CD20</td>
<td>CD19</td>
<td>CD5</td>
<td>CD3</td>
<td>CD38</td>
<td>CD45</td>
</tr>
<tr>
<td>CD20</td>
<td>CD23</td>
<td>CD19</td>
<td>HLA-DR</td>
<td>CD5</td>
<td>CD3</td>
<td>CD38</td>
<td>CD45</td>
</tr>
<tr>
<td>CD81</td>
<td>CD5</td>
<td>CD20</td>
<td>CD19</td>
<td>CD43</td>
<td>CD45</td>
<td>CD3</td>
<td>HLA-DR</td>
</tr>
<tr>
<td>CD20</td>
<td>CD49-d</td>
<td>CD19</td>
<td>CD5</td>
<td>CD3</td>
<td>CD45</td>
<td>CD38</td>
<td>HLA-DR</td>
</tr>
<tr>
<td>CD20</td>
<td>CD200</td>
<td>CD19</td>
<td>CD5</td>
<td>CD3</td>
<td>CD45</td>
<td>CD38</td>
<td>HLA-DR</td>
</tr>
</tbody>
</table>
An example of CLL patient

CD20+/CD19+/CD5+/CD23+/Ig kappa+/CD200+/CD49d+/CD38-/CD43+/CD81-
MRD+ (0.084%) of CLL patient (Pb)
MRD+ (1.59%) of CLL patient (BM)
Case 7: MRD- of CLL patient (Pb)
Case 7: MRD+ (0.018%) of CLL patient (BM)
IMMUNOPHENOTYPE

VALUE OF QUANTIFICATION EXPRESSION OF ANTIGENS
QUANTIFICATION OF ANTIGEN EXPRESSION BY FLOW CYTOMETRY

MIF = Mean Intensity Fluorescence

ABC = Antibodies Bound per Cell
EXPRESSION OF CD20 ANTIGEN
EXPRESSION OF CD22 ANTIGEN
EXPRESSION OF CD19 ANTIGEN

[Graphs showing the expression of CD19 antigen for different samples labeled as LLC, LNH E, and LAL B. The graphs display histograms with count on the y-axis and CD19 APC on the x-axis.]
EXPRESSION OF CD52 ANTIGEN

CLL cells

T-lymphocytes
IMMUNOPHENOTYPIC PROGNOSTIC FACTORS

- CD38: type II transmembrane glycoprotein acting both as ectoenzyme and receptor for CD31 antigen
- ZAP-70: signal transduction molecule
- CD49d: adhesion molecule, member of the integrin superfamily
CD38 EXPRESSION
EVALUATED BY FLOW CYTOMETRY

FacsCalibur, Becton Dickinson
CD19/CD38/CD3/CD5
FITC/PE/PerCP/APC

FACS/Canto, Becton Dickinson,
CD45/CD38/CD3/CD19/CD5/CD20
FITC/PE/PerCP/PE–cy7/APC/APC-cy7
## CD38 EXPRESSION OF CLL CELLS

<table>
<thead>
<tr>
<th>Patients</th>
<th>CD38+ &gt;10%</th>
<th>CD38+ &gt;20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>CLL (n=110)</td>
<td>19 (17.2%)</td>
<td>15 (13.6%)</td>
</tr>
<tr>
<td>CD5+ NHL (n=46)</td>
<td>25 (54%)</td>
<td>19 (41.3%)</td>
</tr>
<tr>
<td>NHL (CD5-) (n=51)</td>
<td>8 (15.6%)</td>
<td>6 (11.7%)</td>
</tr>
</tbody>
</table>

GENTILE ET AL BJH 2005
Treatment-free survival according to the 7% CD38+ cut-off point
Gentile et al, BJH 2005
ZAP-70 Expression and IgVH Mutational Status

Mutation $\text{IgV}_H$  Unmutated $\text{IgV}_H$

Survival Probability According to ZAP-70 Expression (Stage A)

$<20\%$ ZAP-70

$\geq 20\%$ ZAP-70

P = 0.01

Years after Diagnosis
Zap-70 negative

Zap-70 positive

26%

0.3%
ZAP-70 expression which method for the analysis

ISO-Method

T-Method

T/B Ratio-Method

Caso negativo

Caso positivo

ZAP-70 Alexa Fluor 488 Clone 1E7.2 (Caltag)
Correlation of Flow Cytometrically Determined Expression of Zap-70 Using the SBZAP Antibody with IgVH Mutation Status and Cytogenetics in 1,229 Patients with Chronic Lymphocytic Leukemia

Wolfgang Kern,* Frank Dicker, Susanne Schnittger, Claudia Haferlach, and Torsten Haferlach,
Cytometry B Clin Cytom 2009

T-Method

T/B Ratio-Method
Cut-off = 4.5
**Method**

**T/B Ratio**

**Method**

**T/B Ratio**

**Positive case**

**T/B Ratio**

**Negative case**

**T/B Ratio**

**Ratio T/B = 1.2**

**Ratio T/B = 5.6**

**SBZAP-70 PE (Beckman Coulter)**
Caso 1: CD49-d+/CD38+/ZAP-70+/IgVH germline

Caso 2: CD49-d-/CD38+/ZAP-70+/IgVH germline

Caso 3: CD49-d+/CD38-/ZAP-70+

CLL: CD49-d/CD38/ZAP-70
CLL: CD49-d/CD38/ZAP-70

Caso 4: CD49-d+/CD38-/ZAP-70-

Caso 5: CD49-d-/CD38-/ZAP-70-
GENETIC / MOLECULAR PROGNOSTIC FACTORS

- FISH
- CARYOTYPE
- *IGHV* STATUS
- *P53* GENE SEQUENCE
- *ATM* GENE SEQUENCE
- NEW GENES: NOTCH1, SFB3F1, BIRC3
- GENE PROFILE
- MiRNA
- CGH
- SNIP
Frequency of Chromosome Lesions in CLL (comparison of CC and FISH)

<table>
<thead>
<tr>
<th>Aberration</th>
<th>Cytogenetics (*)</th>
<th>FISH (**)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clonal</td>
<td>30-50%</td>
<td>82%</td>
</tr>
<tr>
<td>Normal</td>
<td>50-70%</td>
<td>18%</td>
</tr>
<tr>
<td>del 13q14 single</td>
<td>7-11%</td>
<td>36-45%</td>
</tr>
<tr>
<td>12q trisomy</td>
<td>13-19%</td>
<td>11-14%</td>
</tr>
<tr>
<td>del 11q22-23/ATM</td>
<td>6-13%</td>
<td>8-25%</td>
</tr>
<tr>
<td>del 17p/p53</td>
<td>1-5%</td>
<td>3-7%</td>
</tr>
<tr>
<td>del 6q21</td>
<td>1%</td>
<td>2%</td>
</tr>
</tbody>
</table>

(*) % total abnormal
(**) Dohner et al, 2001 EHA
SURVIVAL AND SPECIFIC CHROMOSOME ABERRATIONS
DOHNER ET AL, NEJM (2000)
<table>
<thead>
<tr>
<th>GENES</th>
<th>CHROMOSOME</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light chain (\lambda)</td>
<td>22</td>
</tr>
<tr>
<td>Light chain (\kappa)</td>
<td>2</td>
</tr>
<tr>
<td>Heavy chain</td>
<td>14</td>
</tr>
</tbody>
</table>

The primary structure of the antigen-binding site of B-lymphocytes’ BCR is determined by the DNA sequence of its variable (V) domains. These are comprised of recombined \(V_H\), D and \(J_H\) segments in the heavy (H) chains and \(V_L\) and \(J_L\) segments in the light (L) chains.

Additional diversity is provided by the insertion or deletion of nucleotides at the joints of recombination.
Il Ficoll è specifico per l’isolamento di cellule mononucleate. Dopo centrifugazione a 2000 rpm per 20 min si stratificano:

- Gli eritrociti
- I polimorfonucleati;
- La soluzione di Ficoll;
- Cellule mononucleate (linfociti e monociti);
- Plasme e piastrine.
ONE STEP PCR

Si utilizza un set di primers specifici per il riarrangiamento VDJ della catena pesante delle Ig in particolare:

Un primer forward (Leader o FR1) ed un primer reverse degenerato (JH).
Risultato di una PCR effettuata utilizzando come Primer 5’ VH3 Leader e come Primer 3’ JH

Da questa PCR possiamo evincere che 2 pazienti affetti da LLC presentano un riarrangiamento delle Ig che coinvolge il segmento genico VH3. Come controllo positivo è stato utilizzato un paziente affetto da LLC che presenta un riarrangiamento delle Ig che coinvolge il segmento genico VH3, confermato da precedenti analisi della sequenza.
SURVIVAL by IGHV STATUS

Hamblin et al. 1999

Damle et al., Blood 1999
PERCENT OF IGHV MUTATED AND UNMUTATED CLL CASES STUDIED AT OUR INSTITUTION AT DIAGNOSIS

30.6% % mutated
69.4% % unmutated
If we assume that these recombination events are random, the likelihood that specific $V_H$, $D$, and $J_H$ genes would be used in the same $V_HDJ_H$ rearrangement is $1/6600$ ($1/44 \times 1/25 \times 1/6$), for a specific $V_L$ and $J_L$ gene is $1/200$ ($1/40 \times 1/5$) or $1/124$ ($1/31 \times 1/4$) for a $\kappa$ versus a $\lambda$ rearrangement, respectively.

Only $1/1320000$ B-cells would be predicted to randomly express the same $V_H$, $D$, $J_H$, $V_\kappa$, and $J_\kappa$ segments in its BCR.

GERMINAL CENTER AND LYMPHOMAGENESIS


Naive B-cell

IgV hypermutation
Ig isotype switch

Apoptosis

CLL
Memory B-cells
Plasma cells

IgV mutations
CLL - MCL - FL + BL + DLCL + MM +

BCL-6

Syndecan
GeneChip® Probe Array

Human Genome U95A v2
- 8,000 genes with known function
- 4,500 genes without known function
- 100 controls
Mouse Genome
Rat Genome
Yeast Genome
E. Coli Genome
GeneChip® Probe Arrays

GeneChip Probe Array

Hybridized Probe Cell
- Single stranded, labeled RNA target
- Oligonucleotide probe

Image of Hybridized Probe Array
- 1.28 cm
- 20 μm

- Millions of copies of a specific oligonucleotide probe
- >400,000 different complementary probes
Affymetrix® Instrument System

Platform for GeneChip® Probe Arrays
CLL is a unique disease with two different features:


Klein et al (J exp med 2001)
I fattori trascrizionali interagiscono con le sequenze regolatrici di molti geni aumentandone l’espressione
ZAP-70 Expression and IgVH Mutational Status

Survival Probability According to ZAP70 Expression (A Stage)

Crespo et al, NEJM 2003
TP53 Function
TP53 GENE

Transactivation (1-42; 43-62)
Proline-rich (63-97)
DNA binding (102-292)
Oligomerisation (323-356)
Regulation (363-393)

Phosphorylation site
Acetylation site

N-            C-

I  II  III  IV  V

I
II
III
IV
V

-Phosphorylation site
-Acetylation site
# TP53 MUTATIONS

<table>
<thead>
<tr>
<th>Cod</th>
<th>Res</th>
<th>Mut</th>
<th>Effects on the protein structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>175</td>
<td>Arg</td>
<td>His</td>
<td>Breaks crucial H-bond bridging loops L2 and L3</td>
</tr>
<tr>
<td>248</td>
<td>Arg</td>
<td>Gln</td>
<td>Breaks main contact with DNA in minor groove</td>
</tr>
<tr>
<td>273</td>
<td>Arg</td>
<td>His</td>
<td>Breaks main contact with DNA in major groove</td>
</tr>
<tr>
<td>248</td>
<td>Arg</td>
<td>Trp</td>
<td>Breaks main contact with DNA in minor groove</td>
</tr>
<tr>
<td>273</td>
<td>Arg</td>
<td>Cys</td>
<td>Breaks main contact with DNA in major groove</td>
</tr>
<tr>
<td>282</td>
<td>Arg</td>
<td>Trp</td>
<td>Destabilizes DNA binding in the major groove</td>
</tr>
</tbody>
</table>
Frequency of TP53 mutation gene in different phases of the disease

- Diagnosi: 97%
- Progressione: 91%
- Resistenza: 86.7%

Frequenza delle mutazioni in TP53 nelle varie fasi della malattia
Distribution of normal function of p53 protein in different phases of CLL

- LLC esordio: 84.62%
- LLC progressione: 77.27%
- LLC resistenti: 52.94%
NORMAL FUNCTION OF P53 PROTEIN
Frequency of Function Alterations of p53 protein in different phases of CLL

- LLC esordio: 15.38%
- LLC progressione: 22.73%
- LLC resistenti: 47.06%
Disfunctions of P53 protein
Alterazioni nel pathway di p53

DNA damage

ATM

MDM2

p53

p21

miR-34a

Puma, Bax

CDKs, CCND1, BCL2, E2F, MYCN, SIRT1, etc

Cell cycle arrest

Apoptosis

11q- / ATM mutation

17p- / TP53 mutation

miR-34a ↓
Mechanisms of ATM structural alterations in CLL.
## ATM gene mutations in CLL patients (8/57).

<table>
<thead>
<tr>
<th>ID N°</th>
<th>Patient(s)</th>
<th>Nucleotide change</th>
<th>Protein change</th>
<th>Type</th>
<th>G/S</th>
<th>Allelic Status</th>
</tr>
</thead>
<tbody>
<tr>
<td>3664</td>
<td>M.R.</td>
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<td>D479T</td>
<td>Missense</td>
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<td>5948</td>
<td>I.A.</td>
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<td>D479T</td>
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<td>12</td>
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<td>D479T</td>
<td>Missense</td>
<td>12</td>
<td>U</td>
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<td>3988</td>
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<td>I826L</td>
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<td>4046</td>
<td>V.A.</td>
<td>2502insA</td>
<td>–</td>
<td>Frameshift (T)</td>
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<td>U</td>
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<td>5116</td>
<td>C.F.</td>
<td>IVS29+5G&gt;A</td>
<td>–</td>
<td>Splicing (T)</td>
<td>29</td>
<td>S</td>
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<td>3706</td>
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<td>57</td>
<td>G</td>
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</table>
Statistical analysis of treatment-free survival.

Comparison between ATM-mutated and non-mutated CLL patients.

Comparison between MLPA+ and MLPA− CLL patients.

Model of the ATM protein.

PERCENT OF IgVH MUTATED AND UNMUTATED CLL CASES STUDIED AT OUR INSTITUTION (72 CASES)

69.4% mutated
30.6% unmutated
microRNA

- I microRNA sono piccole sequenze di circa 22nt, non codificanti che regolano a livello post-trascrizionale l’espressione dei geni targets.
- Ad oggi sono stati identificati, nell’uomo, circa 450 microRNAs.
- I microRNAs svolgono un ruolo fondamentale nello sviluppo cellulare, nel differenziamento, nel ciclo cellulare e nell’apoptosi.
- I microRNAs sono coinvolti nell’oncogenesi:
  - mediante repressione di geni oncosoppressori
  - mediante mancata repressione di un oncogene
Biogenesi dei microRNA

MicroRNA nell’emopoiesi

- miR-181, 142 e 223 sono regolati e regolano la differenziazione emopoietica.

- miR-155 è iperespresso nei linfomi e nei topi induce disordini linfoproliferativi B.

- miR-223 regola la differenziazione granulocitica.

- miR-221 e 222 svolgono un ruolo nell’eritropiesi e nella crescita leucemica.

- miR-15 e mirR-16 sono fortemente downregolati in un sottogruppo di LLC, mentre mir-21, 150 e 155 sono maggiormente espressi nelle LLC rispetto ai linfociti B periferici.
Cloning shows that a set of miRs is de-regulated in CLL.
RT-qPCR corroborates the up-regulation of miR-21, miR-150 and miR-155, and shows down-regulation of miR-92 in CLL patients.
miR-223, miR-150 and miR-29b are differentially expressed between IgVH mutated and germline cases

These miRs may have prognostic significance; in line with these findings, miR-150 is consistently down-modulated in ZAP-70 negative patients
WES
(Whole Exome Sequencing)

• Con il progredire delle tecnologia, è oggi possibile sequenziare l’esoma umano

• Fine ultimo: identificazioni di mutazioni non precedentemente identificate, che potrebbero essere patogenetiche e possano rappresentare nuovi bersagli terapeutici per lo sviluppo di terapie mirate
**WES: Metodologia**

- **Estrazione DNA**
  - Image

- **Controllo Qualità DNA**
  - Agilent Bioanalyzer
  - Nanodrop spectrophotometer

- **Preparazione library**

- **Analisi dei Dati**
  - Image

- **Exome sequencing**
High Confidence Diffs

In coding regions

Novel (not in dbSNP130)

Depth of coverage > 8 (variable)

Non Synonymous

Genes involved
WES: Validazione dei dati

- Identificazione di una mutazione mediante WES
- Validazione della stessa e dei geni coinvolti in un determinato pathway mediante sequencing
- Valutazione di casi addizionali per lo studio della mutazione (e del suo pathway) in una casistica allargata
**A**

A diagram showing the gene structure with markers for EGF repeats (1-36), LNR, HD, PRAM, and Ankyrin. The markers are also labeled for TAD (Topologically Associated Domain) and PEST (Proline-Enriched Segments).

**B**

A gel electrophoresis image labeled "CLL samples," showing various bands at 300bp and 200bp.

**C**

A Kaplan-Meier survival curve comparing ARMS negative (ARMS neg) and ARMS positive (ARMS pos) groups. The curve shows a significant difference (p<.001) in survival probability. The table below the curve provides the number at risk, events, total, median, and 95% CI for each group.

<table>
<thead>
<tr>
<th>Category</th>
<th>ARMS neg</th>
<th>ARMS pos</th>
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<tr>
<td>Events</td>
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<td>12</td>
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<tr>
<td>Total</td>
<td>283</td>
<td>26</td>
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<tr>
<td>Median</td>
<td>13.0</td>
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<tr>
<td>95% CI</td>
<td>10.1-15.9</td>
<td>0.8-14.7</td>
</tr>
</tbody>
</table>
A

B

\[ \begin{array}{cccccc}
\text{No. at Risk} & \text{GL} & \text{M} \\
\text{NOTCH1} & 275 & 34 & 39 & 13 & 2 & 1 & 0 \\
\text{NOTCH1} & 133 & 8 & 0 & 0 & 0 & 0 & 0 \\
\end{array} \]

\[ \begin{array}{cccccc}
\text{Events} & \text{Total} & \text{Median} & 95\% \text{ CI} \\
\text{NOTCH1} & 62 & 275 & 13.9 & 10.5-17.3 \\
\text{NOTCH1} & 16 & 34 & 3.5 & 0.7-7.3 \\
\end{array} \]

\[ \begin{array}{cccccc}
\text{No. at Risk} & \text{GL} & \text{M} \\
\text{NOTCH1} & 204 & 26 & 44 & 16 & 7 & 1 & 1 & 0 & 0 & 0 \\
\text{NOTCH1} & 123 & 16 & 16 & 1 & 0 & 0 & 0 & 0 & 0 & 0 \\
\end{array} \]

\[ \begin{array}{cccccc}
\text{Events} & \text{Total} & \text{Median} & 95\% \text{ CI} \\
\text{NOTCH1} & 52 & 204 & 16.2 & 12.4-19.9 \\
\text{NOTCH1} & 15 & 26 & 8.5 & 4.9-12.2 \\
\end{array} \]

p<.001

p=.002
A

NOTCH1 GL
NOTCH1 M

p<.001

B

NOTCH1 GL
NOTCH1 M

p=.026

Events Total Median 95% CI
NOTCH1 GL 111 275 8.8 6.9-10.6
NOTCH1 M 24 34 1.9 0.6-3.2

Events Total 5-year risk 95% CI
NOTCH1 GL 14 275 6.8% 3.3-10.3
NOTCH1 M 5 34 18.3% 1.7-34.9
**BIRC3 disruption in CLL.**

(A) Prevalence of BIRC3 disruption in clinical MBL, in CLL at diagnosis, in fludarabine-refractory CLL, in fludarabine-sensitive CLL, and in RS. Numbers on top indicate the actual number of mutated samples over the total number analyzed. (B) Schematic diagram of the BIRC3 protein with its key functional domains. Color-coded symbols indicate the type and position of the mutations in BIRC3. (C) Graphic representation of segmentation data from 4 CLL patients carrying BIRC3 deletion. Deletions start from a centromeric break that truncates BIRC3 and removes its terminal exons, including exon 9, which encodes the RING domain. Sample 09-361 harbors a focal loss of 411 kb on 11q22 involving BIRC3 and its homolog, BIRC2. Each track represents one sample; white denotes a normal (diploid) copy number and blue indicates region of a copy number loss (Integrative Genomics Viewer software; http://www.broadinstitute.org/igv; assembly NCBI36/hg18). Individual genes in the region are aligned in the bottom panel. Dual-color FISH validates the occurrence of 11q22 deletion involving BIRC3 but sparing ATM in sample 09-361 (RP11-17708-BIRC3 specific probe in green and LSIATM probe in orange). (D) Mutual relationship of the BIRC3 disruption with other genetic lesions in CLL at diagnosis and in fludarabine-refractory CLL. In the heat map, rows correspond to identical genes and columns represent individual patients color-coded based on the gene status (white indicates wild-type and red, mutations and/or deletion of TP53, mutations and/or deletion of BIRC3, mutations of SF3B1, and mutations of NOTCH1).
The NF-κB pathway is activated in CLL patients harboring BIRC3 disruption.


©2012 by American Society of Hematology
Kaplan-Meier estimates of OS according to BIRC3 disruption.

SF3B1 mutations in CLL and RS.

Prevalence, mutual relationship with other genetic lesions, and clinical impact of SF3B1 mutations in CLL.

A currently more appealing model is that multiple antigens select out B-cells for leukemic transformation. This selection could cull out B-cells from the entire available BCR repertoire or from a BCR repertoire that is limited to a distinct subset of normal B-lymphocytes.

Keating M.J. et al., Hematology 2003
Model of the Development and Evolution of CLL Cells

B-cell GC reaction and proposed cellular derivation of B-cell lymphomas.

- Antigen-activated naïve B cells differentiate into centroblasts which introduce somatic hypermutations into the IgV gene during the clonal expansion in the dark zone. Centroblasts then differentiate into centrocytes and move to the light zone, where B cells with improved antigen-binding are selected. A subset of centrocytes finally differentiates into memory B cells or plasma cells. The GC reaction involves the risk of oncogenic transformation at several steps of differentiation, resulting in the development of different lymphoma subtypes. The proposed cellular derivation of the various B-cell lymphomas and multiple myeloma is indicated. Most lymphoma subtypes are thought to originate from the oncogenic transformation of a GC-derived B cell. Some lymphoma subtypes, including GC-type DLBCL, show similarities with hypermutating GC centroblasts; a subgroup of lymphomas including ABC-type DLBCL may originate from a centrocyte/plasmablast; for the proposed cellular derivation of CLL and hairy cell leukemia, see text. GC, germinal center; ABC, activated B cell.

_U. Klein, R. Dalla-Favera / Seminars in Cancer Biology_
A model for the cellular derivation of CLL.

- Naïve B cells may be driven into either T-dependent (IgV hypermutation in the GC) or T-independent (no IgV hypermutation) immune responses. Upon completion of these responses, the cells differentiate into antigen-experienced memory/marginal zone B cells. These cells may then be continuously activated through persisting antigen and acquire genetic alterations that could lead to the outgrowth of clones with aMBL phenotype and ultimately to oncogenic transformation.
miR-15a/16-1 down regulate the expression of several proliferation associated proteins that have important roles in the G0/G1-S phase transition. The absence of miR-15a/16-1 causes a faster or stronger activation of cyclin D2 and D3 as well as cyclin-dependent kinases CDK4 and CDK6 which are critical cell cycle check points, and also cyclin E, thus leading to faster phosphorylation of retinoblastoma (Rb) and hence accelerated entry into cell cycle by activating E2F. Likewise, the E2F-regulated cell cycle-associated proteins CHK1 and MCM5 may contribute to the pro-proliferative phenotype of miR-15a/16-1-deficient cells. miR-15a/16-1 targets are indicated by gray boxes.
A proposed model of CLL multistep pathogenesis and of its clinical implications.

**Initiating lesion(s)**
- miRNA15/16 deletion
- other (?)

**Microenvironmental interactions**
- BCR / antigen
- CD38 / CD31
- CD49d / VCAM1

**Accumulation of additional pathogenetic lesions**
- ATM mutation / deletion
- TP53 mutation / deletion
- NOTCH1 mutations
- SF3B1 mutations
- other (?)

**Predisposition**
**Initiation**
**Clonal expansion**
**Chemorefractoriness**
Richter transformation

Rossi D, Gaidano G Haematologica 2012;97:5-8
CLL at presentation
- del13q14 (50%)
- +12 (15%)
- ATM (15%)
- TP53 (5-10%)
- NOTCH1 (10%)
- SF3B1 (5-10%)
- BIRC3 (4%)
- MYD88 (3-5%)

Chemorefractoriness
- TP53 (40%)
- BIRC3 (25%)
- SF3B1 (25%)
- ATM (25%)
- NOTCH1 (25%)
- del13q14 (50%)

RS
- TP53 (60%)
- NOTCH1 (30%)
- MYC (28%)
- del13q14 (20%)
- +12 (15%)
- ATM (12%)
- SF3B1 (5%)
- BIRC3 (0%)
CLL-derived BCRs possess autonomous signalling capacity.
CLL BCRs induce cell-autonomous signalling.
Autonomous signalling of CLL-derived BCRs is mediated by the recognition of a BCR-intrinsic epitope.
Cell-autonomous Ca$^{2+}$ signalling is elevated in primary CLL B cells.
Espressione di antigeni nella linea B

± CD5
CD19
CD20
CD22
CD52

Stem cell → Pre-B → Early B → Intermediate B → Mature B → Plasmacytoid B → Plasma

ALL ↔ CLL, PLL ↔ Burkitt’s, FL, DLCL, HCL ↔ WM ↔ MM
Meccanismi citotossici dei MoAbs

- Cellule effettrici/Complemento
- Apoptosi
- Radionuclide
- Tossina
L’antigene CD20 bersaglio razionale per l’immunoterapia dei LNH-B con anticorpi monoclonali

- L’antigene CD20
  - Vitale per proliferazione e sopravvivenza cellulare
  - Non presente in circolo
  - Non internalizza o modula al contatto con l’anticorpo
  - Espresso solo da cellule B

Non presente su cellule staminali emopoietiche e plasmacellule
Radioimmunoterapia dei LNH

**90Y Zevalin**

- Ibritumomab (murine antibody parent of Rituximab)
- Tiuxetan (MX-DTPA) conjugated to antibody forming strong urea-type bond
  - Stable retention of 90Y

**Yttrium-90**

- $T_{1/2} = 64$ hours
- Outpatient administration
- Beta emission $\chi_{90} = 5$ mm
Alemtuzumab (anti-CD52)

Anticorpo IgG1 umanizzato:
Bassa immunogenicità

- Antigene CD52:
  - Intensamente espresso su:
    - Tutti i linfociti
    - Monociti e macrofagi
    - Spermatozoi
    - Eosinofili
  - Non espresso sulle cellule staminali emopoietiche
  - Non modula /non mascherato
  - Espresso anche sulla maggior parte dei linfociti maligni
CAMPATH-1H (α-CD52)

Mechanisms of Action

Activated complement

ADCC

Apoptosis
Interazione tra p53 e MDM2

Genotoxic stress

ATM/ATR
Chk2
CK2

p53

mdm2

degradazione P53

Proteasome

p53

Target gene

p53

mdm2

Interazione tra p53 e MDM2
Receptor signaling in CLL. (A) The BCR is composed of membrane immunoglobulin bound to CD79a/CD79b.

Integrin signaling in CLL. (A) Inside-out integrin signaling.

Kinase inhibitors in CLL. (A) Chemical structures of signal kinase inhibitors.