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# Prediagnostic transcriptomic markers of Chronic lymphocytic leukemia reveal perturbations 10 years before diagnosis

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**Background:** B-cell lymphomas are a diverse group of hematological neoplasms with differential etiology and clinical trajectories. Increased insights in the etiology and the discovery of prediagnostic markers have the potential to improve the clinical course of these neoplasms.

**Methods:** We investigated in a prospective study global gene expression in peripheral blood mononuclear cells of 263 incident B-cell lymphoma cases, diagnosed between 1 and 17 years after blood sample collection, and 439 controls, nested within two European cohorts.

**Results:** Our analyses identified only transcriptomic markers for specific lymphoma subtypes; few markers of multiple myeloma ($N = 3$), and 745 differentially expressed genes in relation to future risk of chronic lymphocytic leukemia (CLL). The strongest of these associations were consistently found in both cohorts and were related to (B-) cell signaling networks and immune system regulation pathways. CLL markers exhibited very high predictive abilities of disease onset even in cases diagnosed more than 10 years after blood collection.

**Conclusions:** This is the first investigation on blood cell global gene expression and future risk of B-cell lymphomas. We mainly identified genes in relation to future risk of CLL that are involved in biological pathways, which appear to be mechanistically involved in CLL pathogenesis**.** Many but not all of the top hits we identified have been reported previously in studies based on tumor tissues, therefore suggesting that a mixture of preclinical and early disease markers can be detected several years before CLL clinical diagnosis.

**Key words:** epidemiology, lymphoma, chronic lymphocytic leukemia, mRNA analyses, prospective cohort

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## introduction

Non-Hodgkin lymphomas (NHLs) are a heterogeneous collection of lymphoproliferative B- and T-cell malignancies [1], among which B-cell lymphomas [follicular lymphoma (FM), diffuse large B-cell lymphoma (DLBCL), chronic lymphocytic leukemia (CLL), and multiple myeloma (MM)] are the most common [2]. Relatively little is known about NHL etiology [3] and limited prediagnostic markers are currently known. Global gene expression investigation of various chronic conditions, including cancers [4–7], has led to the discovery of molecular signatures predictive of future risk, survival, or response to treatment. To our knowledge to date, no genome-wide gene expression studies have been published using peripheral blood mononuclear cells (PBMCs) to assess the future risk of lymphoma and potentially identify transcriptomic profiles reflecting both the early stages of the disease as well as the influence of risk factors on lymphomagenesis [8]. Further-more, exploration of the transcriptomic profiles as a function of time to diagnosis (TtD) could potentially provide information on gene trajectories involved in disease development and result in predictive sets of disease (progression) markers [9].

Within the EnviroGenomarkers project (www.envirogenomarkers.net), we conducted a global gene expression study using PBMCs from B-cell lymphoma cases collected 1–17 years before disease diagnosis, and healthy controls nested within two large European prospective cohorts.

## materials and methods

### envirogenomarkers data

The EnviroGenomarkers study was approved by the committees on research ethics at the relevant institutions. It includes participants from the Italian component of the European Prospective Investigation into Cancer and Nutrition [10] (EPIC-Italy, $N = 47\,749$ volunteers aged 35–70) and the Northern Sweden Health and Disease Study (NSHDS, $N = 95\,000$ healthy individuals aged 40–60) [11]. Anthropometric measurements, lifestyle factors, and a blood sample were collected at recruitment (EPIC-Italy 1993–1998; NSHDS 1990–2006). Incident B-cell lymphoma cases were identified through local Cancer Registries (loss to follow-up <2%) and occurred between 1 and 17 years after recruitment. Cases were classified into subtypes according to the SEER ICD-0-3 morphology [12].

### biosamples and genome-wide expression profiles

We recently demonstrated that high-quality RNA can be obtained from stored PBMC samples from the EPIC-Italy and NSHDS cohorts [13]. We also showed that samples not cold-stored within 2 h after blood collection had significantly different expression profiles than fresh samples, and therefore only PBMC samples that had been placed in cold storage within 2 h after blood collection were included in the current study. Gene expression profiles were acquired using the Agilent $4 \times 44$K human whole genome microarray platform.

We analyzed a total of 281 B-cell lymphoma cases and 281 controls matched on sex, age (±2.5 years), center, fasting status, and date of blood collection (±6 months) in two analytical phases. In addition to the lymphoma study, the EnviroGenoMarkers project also comprises 100 breast cancer (BC) case–control pairs (corresponding to 87 and 93 successfully analyzed cases and controls, respectively), which were added in our B-cell lymphoma analyses as unmatched controls in order to maximize statistical power. The final numbers of successfully analyzed samples were 263 B-cell lymphoma cases and 439 controls (supplementary Table S1, available at *Annals of Oncology* online). Technical performance and quality of the microarrays was assessed according to a protocol described previously [13].

### statistical analysis

As proposed before [14], we developed a linear-mixed model controlling for potential technically induced noise (nuisance variation) and investigated the relationship between the expression level of each probe and the disease outcome (see supplementary Section S1, available at *Annals of Oncology* online). The general formulation of our mixed model for a given probe defines its expression level observed in participant $i$ ($Y^i$) as follows:

$$Y^i \sim \alpha + \beta_1 X^i + \beta_2 FE^i + u^{A^i} + \in^i,$$

where $\alpha$ is the intercept of the model, $\varepsilon^i$ is the residual error, and $X^i$ is the outcome of interest, a binary variable indicating if individual $i$ is a B-cell lymphoma case or not. The resulting regression estimate $\beta_1$ can be expressed as the fold-change ($f$) by $f = 2^{\beta}$. $FE^i$ is a vector of fixed effect observations for individual $i$ and corresponding regression coefficients are compiled in the vector $\beta_2$. Fixed effect covariates included the matching criteria (age, gender and country), the experimental phase (1 or 2), a set of a priori potential confounders as observed in previous analyses of lymphoma within the EPIC cohort [15, 16]: body mass index (BMI, continuous), education (5 classes), physical activity (4 classes), smoking at enrollment (3 classes), and alcohol consumption at enrollment (continuous), and a binary variable indicating if the participant was a BC case or not. Nuisance variation was modeled through a random intercept model where $u^{A^i}$ represents the shift associated to $A^i$, the value of the random effect variable(s) $A$ observed for individual $i$. The dates of the three main steps of sample processing were used as random effect variables: RNA isolation, hybridization, and dye labeling. Model was fitted, using the R-statistical package *lme*4, on all 29 662 probes separately, and we accounted for multiple testing using a stringent Bonferroni correction, setting the family-wise error rate (FWER) to 5%. Analyses were (i) carried out on the full population and (ii) stratified by major histological subtypes, and a series of sensitivity analyses were performed.

Transcripts identified by the genome-wide screen were further investigated through gene-enrichment analyses (see supplementary Section S2, available at *Annals of Oncology* online), and gene trajectories linking expression level and TtD were investigated using both linear and generalized additive models.

## results

In supplementary Table S2, available at *Annals of Oncology* online, the characteristics of the study population with respect to the main demographic covariates are summarized. Among the study participants, cases (number of successfully analyzed samples) included CLL ($n = 39$), DLBCL ($n = 41$), FL ($n = 38$), MM ($n = 72$), other B-cell lymphomas ($n = 69$), and four unspecified B-cell lymphoma. The distribution of B-cell lymphoma cases by histological subtype, cohort, and TtD is summarized in supplementary Table S3, available at *Annals of Oncology* online.

### genome-wide transcriptomic profiles

The linear-mixed model fitted to all B-cell lymphoma cases and controls revealed nine significant associations at a Bonferroni 5%

**Table 1.** Strongest associations between expression level and NHL

| Agilent ID | Full population | | Lymphoma subtype | | | | | | | | Symbol |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | CLL ($N = 39$) | | DLBL ($N = 41$) | | FL ($N = 38$) | | MM ($N = 72$) | | |
| | $f$[a] | $P$-value | $f$[a] | $P$-value | $f$[a] | $P$-value | $f$[a] | $P$-value | $f$[a] | $P$-value | |
| A_23_P26854 | 1.79 | 2.65E−10 | 24.25 | 6.06E−60 | 1.15 | 2.92E−01 | 1.19 | 1.95E−01 | 1.06 | 5.46E−01 | ARHGAP44 |
| A_23_P500400 | 1.59 | 6.39E−10 | 16.45 | 3.71E−81 | 0.97 | 7.40E−01 | 1.26 | 1.64E−02 | 0.96 | 5.02E−01 | ABCA6 |
| A_23_P210581 | 0.76 | 2.94E−08 | 0.71 | 7.29E−04 | 0.91 | 2.97E−01 | 0.84 | 4.67E−02 | 0.68 | 8.06E−08 | KCNG1 |
| A_23_P145889 | 1.25 | 3.24E−07 | 3.27 | 5.55E−36 | 1.01 | 1.00E−01 | 1.12 | 9.73E−02 | 0.97 | 5.84E−01 | CDK14 |
| A_24_P29733 | 1.27 | 7.06E−07 | 3.89 | 3.74E−41 | 1.01 | 8.45E−01 | 1.20 | 1.90E−02 | 0.95 | 1.00E+00 | CDK14 |
| A_23_P130158 | 1.53 | 1.02E−06 | 14.32 | 1.17E−46 | 1.00 | 6.00E−01 | 1.19 | 1.76E−01 | 0.93 | 3.79E−01 | WNT3 |
| A_23_P384127 | 1.22 | 1.08E−06 | 2.20 | 3.11E−18 | 1.04 | 5.51E−01 | 1.11 | 1.61E−01 | 1.06 | 3.17E−01 | – |
| A_32_P44394 | 1.24 | 1.20E−06 | 2.66 | 2.26E−25 | 1.12 | 1.63E−01 | 1.16 | 5.62E−02 | 1.05 | 4.08E−01 | AIM2 |
| A_23_P419213 | 1.21 | 1.44E−06 | 2.28 | 8.45E−24 | 1.13 | 4.89E−02 | 1.19 | 3.40E−03 | 0.99 | 9.25E−01 | KIAA1407 |

Probes declared significant and listed in the table were identified using a Bonferroni-corrected per-test significance level ensuring a FWER control at 5%. Corresponding $P$-values and effect size estimates obtained for subtype-specific analyses, only considering cases of a single subtype at a time and keeping all controls (regardless of the subtype of their matched case) are also given.

[a]Fold-change ($f$) is derived from the regression coefficient estimate ($\beta$) by the mixed model: $f = 2^{\beta}$.

FWER level (Table 1). Analyses by B-cell lymphoma subtype showed that eight of the nine probes show highly significant $P$-values exclusively in the CLL-specific analysis, while the remaining probe seems to be mainly driven by MM. Consistently, when CLL cases are excluded from the population, the $P$-values for these nine probes dramatically increase (supplementary Table S4, available at *Annals of Oncology* online) while, as expected, the MM-driven candidate remains (but more weakly) associated to disease status. Our data do not support the presence of a common signal associated with all B-cell lymphoma subtypes.

Subtype-specific analyses showed numerous associations for CLL ($N = 745$ at Bonferroni FWER 5%) and the 60 strongest signals are reported in Table 2, a. Other subtypes did not provide any realistic candidate signals, with the exception of MM for which we found a few ($N = 3$) weaker candidates with moderate effect sizes (Table 2, b). Subsequent analyses were therefore limited to CLL.

## CLL-specific transcriptomic signals

High levels of correlation and strong clustering were observed among the 745 CLL-specific probes (supplementary Figure S3A, available at *Annals of Oncology* online). Consistently, the scree plot from the principal component analysis shows that only 25 components are necessary to explain 80% of the variance within the data (supplementary Figure S3B, available at *Annals of Oncology* online). While the two first principal components only explained <45% of the variance, they are able to clearly discriminate more than 65% of the CLL cases from controls (supplementary Figure S3C, available at *Annals of Oncology* online).

As illustrated in supplementary Figure S4, available at *Annals of Oncology* online, the vast majority of the CLL-specific signals show gene upregulation in cases, with the 20 strongest associations showing up to 25-fold upregulation. A few signals show downregulation in cases, but their association ($P$-values) tends to be weaker.

The 745 CLL-specific probes are spread across all chromosomes (Figure. 1A), and show a consistent overexpression pattern in cases regardless of the chromosome they relate to (Figure 1B). However, a cluster of three very strong signals ($P < 10^{-40}$) emerges in chromosome 17 with large effect size (fold-change >13.9) (Figure 1C).

The predictive ability of the CLL-specific signals was assessed by running a stepwise logistic regression procedure described in supplementary Section S2, available at *Annals of Oncology* online. Results (Figure 2) show excellent predictive performances of the model, even when a single probe is used to predict disease status (the maximum AUC found for a univariate model was based on probe A_23_P500400—gene ABCA6—and was over 90%). As expected, predictive ability improves with the number of probes included in the model and ranges between 89% and 96% for models including 20 probes. Potential for overfitting was assessed and ruled out from a cross-validation procedure (supplementary Section S2, available at *Annals of Oncology* online).

Additional robustness analyses showed that the inclusion over BC cases and controls yielded increased power without introducing a bias, and showed that the strongest findings were detected in both cohorts (supplementary Section S3, available at *Annals of Oncology* online).

## biological interpretation of the findings: gene-enrichment analysis

Based on the consistency of the findings across cohorts, insights into the underlying biological process were sought by running gene-enrichment analyses on the 745 CLL-specific markers from the full population (supplementary Section S4, available at *Annals of Oncology* online). The results are summarized in Table 3 and show over 30 significantly enriched pathways and gene ontology terms. The identified pathways all relate to proliferation, differentiation, activation, and regulation of B cells, the

**Table 2.** Summary of the subtype-specific analyses

| Rank[a] | Agilent ID | $f$[b] | *P*-value | Gene | Rank[a] | Agilent ID | $f$[b] | *P*-value | Gene | Rank[a] | Agilent ID | $f$[b] | *P*-value | Gene |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| (a) CLL-specific analysis | | | | | | | | | | | | | | |
| 1 | A_23_P500400 | 16.42 | 3.7E−81 | ABCA6 | 21 | A_23_P201211 | 5.41 | 3.0E−33 | FCRL5 | 41 | A_23_P147578 | 2.29 | 3.5E−26 | – |
| 2 | A_32_P53234 | 5.26 | 4.1E−60 | – | 22 | A_24_P376848 | 4.11 | 1.4E−32 | FCRL5 | 42 | A_32_P116989 | 2.20 | 3.6E−26 | ZCCHC18 |
| 3 | A_23_P26854 | 24.26 | 6.1E−60 | ARHGAP44 | 23 | A_23_P310931 | 2.29 | 2.1E−32 | CNR2 | 43 | A_23_P76402 | 2.10 | 4.8E−26 | TCTN1 |
| 4 | A_23_P130158 | 14.37 | 1.2E−46 | WNT3 | 24 | A_23_P46039 | 3.40 | 4.4E−32 | FCRLA | 44 | A_23_P39067 | 2.36 | 4.9E−26 | SPIB |
| 5 | A_23_P27332 | 3.82 | 3.0E−44 | TCF4 | 25 | A_23_P164773 | 3.39 | 8.1E−32 | FCER2 | 45 | A_23_P116533 | 2.13 | 7.1E−26 | SWAP70 |
| 6 | A_23_P131024 | 5.28 | 1.0E−43 | ZBTB32 | 26 | A_23_P160751 | 3.89 | 3.2E−31 | FCRL2 | 46 | A_23_P253321 | 2.35 | 7.5E−26 | PNOC |
| 7 | A_24_P691826 | 5.60 | 2.3E−43 | – | 27 | A_24_P402588 | 2.20 | 3.5E−31 | BCL11A | 47 | A_23_P85269 | 2.98 | 8.5E−26 | TTN |
| 8 | A_24_P29733 | 3.89 | 3.7E−41 | CDK14 | 28 | A_23_P163697 | 2.71 | 4.6E−31 | SYT17 | 48 | A_23_P259393 | 1.85 | 1.3E−25 | SFMBT1 |
| 9 | A_23_P67529 | 3.18 | 7.7E−41 | KCNN4 | 29 | A_23_P132378 | 3.35 | 2.0E−30 | CELSR1 | 49 | A_32_P44394 | 2.65 | 2.3E−25 | AIM2 |
| 10 | A_24_P931428 | 3.69 | 4.0E−37 | TCF4 | 30 | A_23_P17269 | 2.08 | 3.3E−30 | CCDC88A | 50 | A_23_P342131 | 1.81 | 3.5E−25 | CYBASC3 |
| 11 | A_32_P108156 | 3.68 | 9.7E−37 | MIR155HG | 31 | A_23_P45786 | 2.30 | 5.7E−30 | COL9A2 | 51 | A_24_P662636 | 3.70 | 8.3E−25 | – |
| 12 | A_23_P145889 | 3.26 | 5.6E−36 | CDK14 | 32 | A_23_P102113 | 2.10 | 7.5E−30 | WNT10A | 52 | A_23_P8961 | 2.39 | 9.7E−25 | IL7 |
| 13 | A_23_P20427 | 2.42 | 6.5E−36 | RHOBTB2 | 33 | A_23_P370830 | 3.92 | 6.3E−28 | KLHL14 | 53 | A_23_P113572 | 2.77 | 1.2E−24 | CD19 |
| 14 | A_32_P48054 | 2.86 | 9.2E−36 | CNR2 | 34 | A_23_P21758 | 2.81 | 1.1E−27 | ADAM28 | 54 | A_32_P107029 | 2.14 | 2.5E−24 | NAPSA |
| 15 | A_23_P85250 | 3.01 | 1.5E−35 | CD24 | 35 | A_24_P184803 | 3.81 | 1.3E−27 | COCH | 55 | A_23_P4551 | 2.10 | 6.1E−24 | SETBP1 |
| 16 | A_23_P156907 | 4.38 | 5.6E−35 | SOBP | 36 | A_24_P54390 | 2.81 | 3.1E−27 | RASGRP3 | 56 | A_23_P419213 | 2.28 | 8.4E−24 | KIAA1407 |
| 17 | A_24_P319647 | 3.37 | 2.4E−34 | FCRL2 | 37 | A_23_P31725 | 3.09 | 4.4E−27 | BLK | 57 | A_32_P73507 | 2.85 | 1.3E−23 | CHDH |
| 18 | A_32_P49854 | 2.08 | 2.4E−34 | – | 38 | A_24_P410605 | 2.96 | 1.4E−26 | ROR1 | 58 | A_23_P7185 | 2.54 | 4.2E−23 | STAP1 |
| 19 | A_23_P56553 | 2.59 | 3.1E−34 | METTL8 | 39 | A_23_P40108 | 3.76 | 2.3E−26 | COL9A3 | 59 | A_32_P72067 | 2.81 | 4.4E−23 | – |
| 20 | A_23_P124335 | 2.90 | 8.2E−34 | – | 40 | A_23_P30736 | 2.13 | 3.4E−26 | HLA-DOB | 60 | A_23_P91764 | 2.17 | 5.6E−23 | TNFRSF13C |
| (b) MM-specific analysis | | | | | | | | | | | | | | |
| 1 | A_24_P139620 | 0.99 | 2.9E−11 | USP21 | | | | | | | | | | |
| 2 | A_23_P210581 | 0.68 | 8.1E−08 | KCNG1 | | | | | | | | | | |
| 3 | A_32_P8813 | 0.67 | 4.22E−07 | LOC283663 | | | | | | | | | | |

(a) lists the 60 strongest associations found for the CLL-specific analysis and (b) the three significant associations found for MM-specific analyses.

[a]Rank: Probes are ordered with respect to their estimated strength of association with the disease status.

[b]Fold-change ($f$) is derived from the regression coefficient estimate ($\beta$) by the mixed model: $f = 2^{\beta}$.

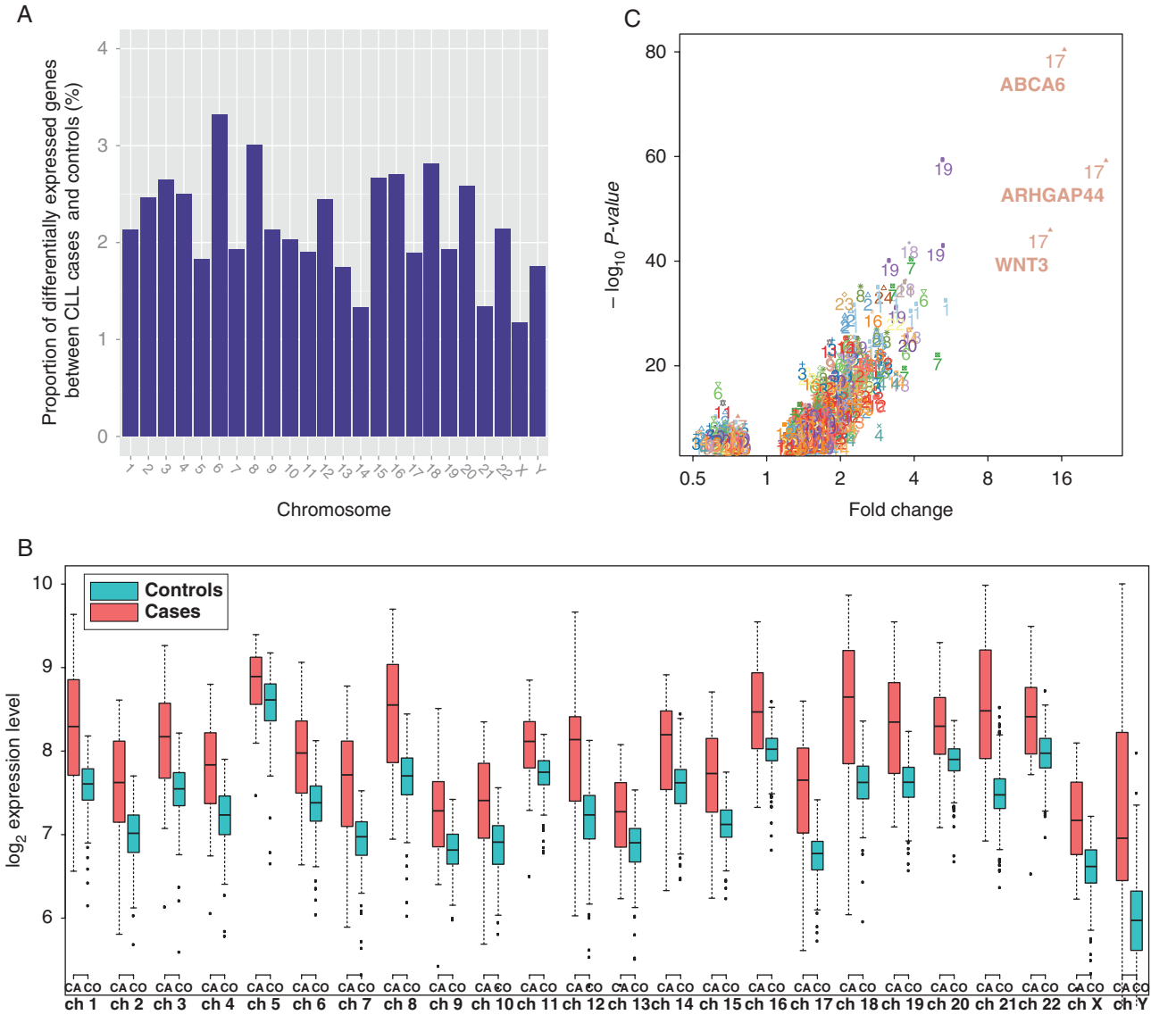


**Figure 1.** Physical repartition of the genes whose expression is measured by the 745 CLL-specific candidates. The per-chromosome proportion of significant probes (Figure 2A) is calculated from the 739 probes whose chromosome is annotated over the total number of probes assayed per chromosome. Figure 2B summarizes the expression levels in cases and controls for the probes relating to each of the 24 chromosomes (total 739 in which the chromosome is annotated). Figure 2C displays for each probe (labeled and colored accordingly to the chromosome they belong to) the *P*-value measuring the association with the disease status as a function of their effect size estimate (fold-change).

Pleckstrin homology domain (intracellular cell signaling) and immune system regulation.

## relationship between CLL-specific transcriptomic markers and time to diagnosis

The results presented above suggest the existence of gene expression signals strongly related to future risk of CLL and present in blood several years before diagnosis. In order to evaluate prediagnostic/preclinical nature of these signals, we ran our CLL-specific analyses on cases enrolled less or more than 6 years before disease onset. Supplementary Figure S7, available at *Annals of Oncology* online clearly shows a large overlap between candidates significant in both TtD strata and in the pooled analysis ($n = 245$). Additional stratification of TtD shows that based only on the six CLL cases diagnosed more than 10 years after enrollment, 47 of the 50 strongest and 68 of the 100 strongest associations found in the full population are still observed.

We also investigated the temporal evolution of expression of the main signals observed among CLL cases only. For the 10 strongest transcriptomic signals, we observed a consistent upregulation while approaching diagnosis (supplementary Figure S8, available at *Annals of Oncology* online). Furthermore, we observed stronger effect sizes (absolute values of the slope with TtD) for the strongest signals with both TtD (supplementary Figure S9A, available at *Annals of Oncology* online) and CLL

**Table 3.** Summary of the results of the gene-enrichment analyses

| Database | Term | Count | *P*-value | Fold enrichment | Bonferroni 5% |
|---|---|---|---|---|---|
| GOTERM_BP_FAT | GO:0051249~regulation of lymphocyte activation | 24 | 1.6E−11 | 5.85 | 3.6E−08 |
| GOTERM_BP_FAT | GO:0002694~regulation of leukocyte activation | 25 | 2.9E−11 | 5.43 | 6.2E−08 |
| GOTERM_BP_FAT | GO:0046649~lymphocyte activation | 27 | 4.3E−11 | 4.89 | 9.4E−08 |
| GOTERM_BP_FAT | GO:0050865~regulation of cell activation | 25 | 8.9E−11 | 5.15 | 1.9E−07 |
| GOTERM_BP_FAT | GO:0045321~leukocyte activation | 28 | 6.9E−10 | 4.17 | 1.5E−06 |
| GOTERM_BP_FAT | GO:0050670~regulation of lymphocyte proliferation | 17 | 8.1E−10 | 7.39 | 1.8E−06 |
| GOTERM_BP_FAT | GO:0070663~regulation of leukocyte proliferation | 17 | 9.7E−10 | 7.30 | 2.1E−06 |
| GOTERM_BP_FAT | GO:0032944~regulation of mononuclear cell proliferation | 17 | 9.7E−10 | 7.30 | 2.1E−06 |
| GOTERM_BP_FAT | GO:0050671~positive regulation of lymphocyte proliferation | 14 | 2.3E−09 | 9.18 | 4.9E−06 |
| GOTERM_BP_FAT | GO:0032946~positive regulation of mononuclear cell proliferation | 14 | 2.9E−09 | 9.02 | 6.3E−06 |
| GOTERM_BP_FAT | GO:0070665~positive regulation of leukocyte proliferation | 14 | 2.9E−09 | 9.02 | 6.3E−06 |
| GOTERM_BP_FAT | GO:0030098~lymphocyte differentiation | 18 | 2.9E−09 | 6.30 | 6.4E−06 |
| GOTERM_BP_FAT | GO:0050863~regulation of T cell activation | 19 | 3.2E−09 | 5.86 | 7.0E−06 |
| GOTERM_BP_FAT | GO:0001775~cell activation | 29 | 7.0E−09 | 3.65 | 1.5E−05 |
| GOTERM_BP_FAT | GO:0051251~positive regulation of lymphocyte activation | 17 | 8.7E−09 | 6.32 | 1.9E−05 |
| SP_PIR_KEYWORDS | B-cell | 8 | 1.2E−08 | 23.53 | 4.6E−06 |
| GOTERM_BP_FAT | GO:0002521~leukocyte differentiation | 19 | 2.0E−08 | 5.23 | 4.4E−05 |
| GOTERM_BP_FAT | GO:0002696~positive regulation of leukocyte activation | 17 | 3.2E−08 | 5.79 | 7.0E−05 |
| GOTERM_BP_FAT | GO:0030888~regulation of B cell proliferation | 10 | 3.7E−08 | 12.88 | 8.1E−05 |
| GOTERM_BP_FAT | GO:0002684~positive regulation of immune system process | 25 | 4.5E−08 | 3.79 | 9.9E−05 |
| GOTERM_BP_FAT | GO:0050867~positive regulation of cell activation | 17 | 6.3E−08 | 5.52 | 1.4E−04 |
| GOTERM_BP_FAT | GO:0050864~regulation of B cell activation | 12 | 1.1E−07 | 8.49 | 2.4E−04 |
| GOTERM_BP_FAT | GO:0050870~positive regulation of T cell activation | 14 | 1.4E−07 | 6.65 | 3.1E−04 |
| GOTERM_BP_FAT | GO:0002520~immune system development | 26 | 1.9E−07 | 3.40 | 4.2E−04 |
| INTERPRO | IPR011993:Pleckstrin homology-type | 27 | 2.1E−07 | 3.30 | 1.7E−04 |
| GOTERM_BP_FAT | GO:0048534~hemopoietic or lymphoid organ development | 25 | 2.4E−07 | 3.47 | 5.3E−04 |
| GOTERM_BP_FAT | GO:0042110~T cell activation | 17 | 3.8E−07 | 4.87 | 8.2E−04 |
| INTERPRO | IPR001849:Pleckstrin homology | 25 | 5.3E−07 | 3.34 | 4.1E−04 |
| KEGG_PATHWAY | hsa05340:Primary immunodeficiency | 9 | 4.0E−06 | 9.08 | 4.6E−04 |
| KEGG_PATHWAY | hsa04662:B cell receptor signaling pathway | 12 | 6.2E−06 | 5.65 | 7.1E−04 |
| GOTERM_BP_FAT | GO:0051249~regulation of lymphocyte activation | 24 | 1.6E−11 | 5.85 | 3.6E−08 |
| GOTERM_BP_FAT | GO:0002694~regulation of leukocyte activation | 25 | 2.9E−11 | 5.43 | 6.2E−08 |

Pathways found significantly enriched are reported on the basis of their Bonferroni 5% adjusted *P*-values. Gene-enrichment analyses are based on the 745 CLL-specific candidates found for the full population.

status (supplementary Figure S9B, available at *Annals of Oncology* online). This suggests an overall tendency for an increase in intensity of CLL-specific signals closer to diagnosis.

## discussion

As expected from the biological heterogeneity of B-cell lymphomas [17], our results do not support the existence of genes whose change in expression is common to the pathogenesis of all or multiple histological subtypes of NHL. Instead, and despite the limited number of CLL cases available, our analyses led to the identification of several strong signals associated with prospective CLL risk (more than 10 years before diagnosis). These include ABCA6, ARHGAP44, Wnt3, TCF4, ZBTB32, CDK14, KCNN4, and TCF4, which showed (except for Wnt3), consistency across both cohorts studied. While variation in the proportion of different subtypes of normal leukocytes may have contributed to these transcriptomic signals, it is unlikely to have been differential by disease status, and by histological subtypes. The substantial overexpression (up to 25 fold) in cases and the trend toward increased expression while approaching diagnosis suggest that the CLL-related signals reflect, at least partly, markers of disease progression arising from subpopulations of cells in which disease initiation has occurred long before diagnosis. This is further supported by the fact that some of the strongest associations we found (e.g. ARHGAP44, ABCA6, and WNT3) are strongly upregulated in CLL malignant cells [18, 19].

Most cases of CLL are believed to be preceded by monoclonal B-cell lymphocytosis, a hematological condition commonly found in normal subjects, increases with age and which evolves to CLL at a low rate (1%–2% per year) [20, 21], raising the possibility that the CLL-related profile we have observed may arise, at least to some extent, in CLL-like MBL cells. Some support for this possibility comes from the inclusion in the latter profile of a number of genes related to Wnt signaling (e.g. Wnt3, Wnt10A,

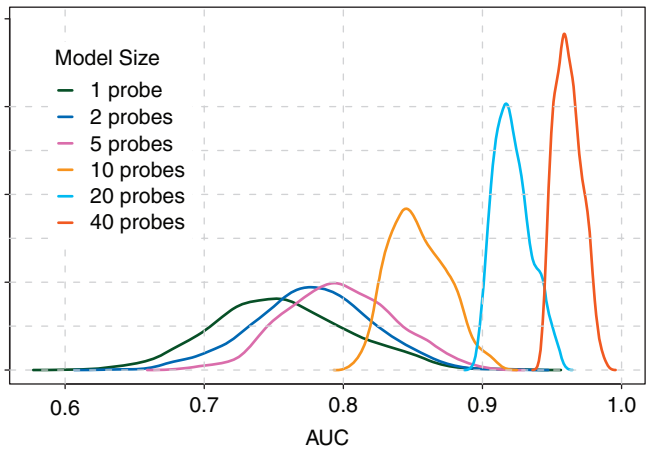


**Figure 2.** Quantitative assessment of the predictive abilities of the CLL candidate probes. Combinations of probes were selected based on a stepwise procedure including one probe at a time in a logistic model. At each step of the iterative procedure, an additional probe was added to the model such that it maximized the gain in AUC for the resulting ROC curve compared with the probe combination retained at the previous step. The plot presents the density estimate of the AUC at different steps of the algorithm (models containing 1, 5, 10, 20, and 40 probes).

ARHGAP44, TCF4, CDK14, and ZBTB32) which has been recently reported to be activated in MBL [22]. Furthermore, of 20 genes reported as being differentially expressed in CLL-like MBL [22], 4 (PRKCB, PAG1, TCL1A, ROR1) fall among the CLL-related genes identified in the current study. On the other hand, the MAPKinase and protein kinase A pathways, reported to be activated in MBL cells, were not among those indicated by our CLL-related profile. As such, the identified profile seems to be only in part drive by MBL. This is strengthened by the observation that the identified CLL-transcriptomic profile predicts more than 80% of the cases, whereas only ~5%–10% of subjects of the MBL phenotype would be expected to progress to CLL over the 6-year average follow-up period of our study. Taken together, these observations are compatible with the possibility that the CLL-related differential expression profile detected is due to clones of malignant or premalignant cells, including MBL cells, present at low concentrations in our blood samples several years before clinical onset, and which evolve toward CLL via specific transcriptomic signals. This may not be surprising as, for most patients, CLL is indolent and progresses slowly, and it may take years for clinical symptoms to arise.

The most common chromosomal abnormalities in CLL, using conventional and molecular cytogenetics, are trisomy 12, del(13)(q14), del(11)(q22–23), del(17)(p13), and del(6)(q21) [23]. We did not find any strong evidence of chromosome specificity for our signals, except possibly for chromosomes 17, 18, and 19.

Due to the heterogeneity of NHL pathologies, and despite its reasonable size, our study was not sufficiently large to enable the in-depth investigation of signals associated with histological types other than CLL. The strongest associations we have identified were almost exclusively associated with CLL, but we cannot exclude the possibility that, with greater statistical power, transcripts specific for other subtypes would be identified.

In conclusion, from our agnostic search, several transcriptomics signals have been found to be associated with CLL risk in preclinical blood samples taken many years before actual diagnosis. The identified transcripts point toward an important contribution of B-cell signaling, and B-cell activation and proliferation in the etiology of CLL.

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## disclosure

The authors have declared no conflicts of interest.

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## appendix

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