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# Association Between Bruton's Tyrosine Kinase Gene Overexpression and Risk of Lymphoma in Primary Sjögren's Syndrome

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**Objective.** We undertook this study to analyze whole blood gene expression and to investigate the role of B cell genes in primary Sjögren's syndrome–related non-Hodgkin lymphoma (primary SS-NHL).

**Methods.** Peripheral whole blood samples were collected from 345 well-phenotyped patients with primary SS enrolled in the prospective Assessment of Systemic Signs and Evolution in Sjögren's Syndrome (ASSESS) cohort. Transcriptomic analysis was performed using human Clariom S Arrays (Affymetrix). In our primary analysis, we considered patients with incident lymphoma (i-primary SS-NHL) as the case group and all patients without lymphoma as the comparison group. In our sensitivity analyses, we considered all patients with primary SS-NHL, including those with a history of lymphoma (h-primary SS-NHL), as the case group and primary SS patients without lymphoma, stratified on their risk factors of lymphoma, as the comparison group.

**Results.** Twenty-one patients with primary SS-NHL (including 8 with i-primary SS-NHL and 13 h-primary SS-NHL) were eligible for transcriptomic analysis; we compared these patients to 324 primary SS controls without lymphoma, including 110 with moderate to severe disease activity and 61 with no risk factor of lymphoma. Functional clustering analyses revealed an enrichment of genes related to innate and adaptive immunity, including B cell–related genes. Bruton's tyrosine kinase (BTK) and a proliferation-inducing ligand (APRIL) genes were overexpressed before the occurrence of lymphoma in patients with incident lymphoma compared with patients without lymphoma. In sensitivity analyses, BTK was consistently up-regulated across all comparisons performed. BTK expression was associated with risk of lymphoma on multivariate analyses, which considered 9 validated predictors of lymphoma in primary SS.

**Conclusion.** BTK and APRIL were overexpressed in the peripheral blood of primary SS patients prior to lymphoma. The association between BTK, APRIL, and primary SS-NHL requires confirmation in other prospective cohorts.

## INTRODUCTION

Primary Sjögren's syndrome (primary SS) is a B cell–driven autoimmune disorder and represents the second most frequent

systemic autoimmune disease, predominantly affecting middle-aged women (1). Among systemic autoimmune diseases, primary SS is associated with the highest risk of lymphoma (2–4) and therefore represents a relevant model of lymphomagenesis

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related to autoimmunity. This immune-mediated inflammatory disease is clinically characterized by the disabling triad of pain, dryness, and fatigue and is immunologically associated with anti-nuclear antibodies directed to ribonucleoproteins SS antigen A (SSA), called anti-Ro/SSA, and SS antigen B (SSB), called La/SSB; circulating IgM autoantibodies specific for IgG-Fc, called rheumatoid factors (RFs); and the presence in up to one-third of patients of ectopic lymphoid structures infiltrating the target organs of the disease, mainly the salivary and lacrimal glands. Systemic manifestations can occur in 30% of patients.

The most severe complication of primary SS is lymphoma, occurring in 5–10% of patients, which is associated with increased mortality (5). Lymphoma can occur before, concomitantly, or after the diagnosis of primary SS. Primary SS-related lymphomas are most frequently associated with long-lasting disease with systemic complications (6). The subtypes of lymphoma observed in primary SS patients are non-Hodgkin malignant B cell lymphoma (NHL) with a predominance of marginal-zone B cell lymphoma (MZL) arising from the target organs of the autoimmune disease (e.g., salivary and lacrimal glands), which explains the frequency of the subtype mucosa-associated lymphoid tissue-MZL (MALT-MZL) (7–10). The second most frequent primary SS-related lymphoma subtype is diffuse large B cell lymphoma (DLBCL), an aggressive B cell lymphoma that can occur de novo and predominantly upon transformation of a preexisting indolent MZL (9), as demonstrated by the clonal relationship between MZL and DLBCL reported in primary SS (11). Similar to that shown in patients without autoimmune diseases but with chronic viral infections, such as hepatitis C complicated by cryoglobulinemia (12,13), chronic antigen-induced B cell stimulation is thought to be the pivotal mechanism leading to lymphomagenesis in primary SS (14–16).

In primary SS, there is a continuum in the lymphomagenesis process, from polyclonal autoreactive B cell activation to monoclonal malignant proliferation (14). However, molecular actors involved in the transition between autoimmunity and lymphoma are incompletely characterized, and the identification of new biomarkers of primary SS-related lymphoma is needed. A recent study showed that, unlike other extranodal MZLs, primary SS-related MALT-MZLs are genomically stable (low mutational burden, few copy number alterations, lack of lymphoma-related mutation profile) (17). The occurrence of lymphoma is intimately linked with chronic B cell activation occurring in secondary and tertiary lymphoid organs, specifically within ectopic lymphoid

structures organizing in the target tissues of the disease. Given the role of cytokines promoting B cell stimulation and growth, such as a proliferation-inducing ligand (APRIL) and B cell-activating factor/B lymphocyte stimulator (BAFF/BLyS) in ectopic lymphoid structure organization (18) and primary SS pathogenesis, the latter could also be involved in lymphomagenesis. Of note, BAFF and its 3 receptors, BCMA, TACI, and BAFF receptor (BAFFR), contribute to autoreactive B cell survival and growth and have been found to be associated with primary SS systemic disease activity and primary SS-related lymphomas (19–22).

Thus, investigating B cell activation-related genes and pathways driving survival and growth of autoreactive B cells may provide clues for a better understanding of lymphomagenesis complicating primary SS. In addition, lymphomas complicating primary SS are likely to emerge following a multiple hit process involving clonal selection of autoreactive B cells expressing cell surface B cell receptor (BCR) with RF activity (RF-BCR) (23–25), able to engage immune complexes, and activating NF- $\kappa$ B pathways through TLR7–TLR9 and BCR downstream signaling by recruiting Bruton's tyrosine kinase (BTK), thus leading to enhanced proliferative and survival signals on clonal B cells. The promising results of targeted therapeutic strategies using BTK inhibitors in DLBCLs (26) and MZLs (27) highlight the importance of BTK within the BCR signaling pathways in B cell NHLs outside autoimmune diseases but currently unexplored in primary SS-related lymphomas.

On the basis of previous gene expression profiling studies, a type 1 interferon signature (28,29), as well as a signature associated with fatigue, was detected in primary SS patients (30), therefore emphasizing the transcriptional approach as a powerful tool to uncover novel pathways implicated in the pathogenesis of primary SS. In this peripheral whole blood transcriptomic study, we aimed to analyze the gene expression signature and the role of B cell activation-related genes with regard to lymphoma in a cohort of patients with primary SS.

## PATIENTS AND METHODS

**Patient recruitment.** The Assessment of Systemic Signs and Evolution in Sjögren's Syndrome (ASSESS) cohort is a French nationwide multicenter prospective cohort of patients with primary SS that was established in 2006 with a 10-year follow-up

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to clarify disease evolution, identify predictive factors, and investigate underlying mechanisms of primary SS-associated lymphomas (6,31) (see Appendix A). In addition to collection of demographic and clinical data at enrollment and every year during follow-up, serum samples and peripheral whole blood RNA and DNA samples were collected from patients at enrollment for further biologic and molecular assessments. All patients who enrolled in the cohort fulfilled the American–European Consensus Group criteria for primary SS (32). The diagnosis of NHL was proven by biopsy, and the NHL subtype was assessed according to the World Health Organization Classification of Tumours of Haematopoietic and Lymphoid Tissues (33).

**Study design.** We analyzed patients with primary SS and incident NHL during follow-up (i-primary SS-NHL group) and patients with primary SS and a history of NHL (h-primary SS-NHL group) separately and also in an aggregate group (all-primary SS-NHL group). This distinction was applied to account for heterogeneity in patients with primary SS-NHL, especially for potential differences between the i-primary SS-NHL and h-primary SS-NHL groups. Patients who received rituximab and/or cyclophosphamide in the 6 months preceding enrollment were excluded, to take into account the predictable therapeutic impact on gene expression profiling, particularly that of B cell-related genes, given the usual B cell depletion effects of such immunomodulatory treatments (34–36).

Patients without lymphoma were assigned to 3 control populations according to their risk factors of lymphoma and disease activity: 1) all primary SS patients without lymphoma (all-primary SS control group), regardless of their disease activity and lymphoma predictors; 2) primary SS patients without lymphoma and with no risk factors (low-risk primary SS control group) among 9 validated predictors of lymphoma in primary SS, which included moderate to severe systemic disease activity (evaluated by the EULAR Sjögren's Syndrome Disease Activity Index [ESSDAI]  $\geq 5$ ) (37), previous parotid swelling, purpura, lymphocytopenia, CD4/CD8 T cell ratio  $\leq 0.8$ , RF positivity, cryoglobulinemia, monoclonal component, and low C4 level (2,6,38) (this comparator limits the risk of including patients who might be at risk of lymphoma occurring throughout and after the 10 years of follow-up); and 3) primary SS patients without lymphoma but with moderate to severe systemic disease activity at enrollment (ESSDAI  $\geq 5$  control group) (this comparator allows for identifying relevant predictors in an at-risk population because the ESSDAI score is associated with increased risk of lymphoma) (6).

In this case-control discovery transcriptomic study, nested within the prospective ASSESS cohort, a primary analysis and 8 separate sensitivity analyses were planned to compare differential gene expression profiles between the subgroups of primary SS-NHL and primary SS without lymphoma. The primary analysis considered incident lymphomas (i-primary SS-NHL) as the case

group and all patients without lymphoma (all-primary SS controls) as the comparison group, to identify potential predictive biomarkers of primary SS-NHL. In some sensitivity analyses, we analyzed patients with incident and prevalent lymphoma together to investigate the pathogenesis of previous or subsequent lymphoma. Nevertheless, to acknowledge potential differences in gene expression profiles between patients with prevalent lymphoma and those with incident lymphoma, these groups were also analyzed separately. Sensitivity analyses 1 and 2 compared i-primary SS-NHL patients versus low-risk primary SS and ESSDAI  $\geq 5$  controls. Six separate sensitivity analyses explored gene expression profiles of h-primary SS-NHL patients and all-primary SS-NHL patients (including patients with i-primary SS-NHL and h-primary SS-NHL) with those of each of the previously described control populations: low-risk primary SS controls, ESSDAI  $\geq 5$  controls, and all-primary SS controls without lymphoma.

**Ethics approval.** All patients included in the ASSESS cohort gave their consent for participation. The ASSESS cohort, promoted by the Assistance Publique - Hôpitaux de Paris, was approved by the institutional review board and the ethics committee of Hôpital Bichat and the “Commission Nationale Informatique et Libertés.”

**Laboratory techniques and microarray data generation.** Total mRNA was extracted from patients' peripheral whole blood samples using the PAXgene Blood mRNA kit (PreAnalytix GmbH).

**Microarray data generation.** The quality of samples was assessed with Bioanalyzer 2100, with an Agilent RNA6000 nano-chip kit. After validation of RNA quality, 75 ng of total RNA was reverse transcribed using the GeneChip WT Plus Reagent Kit (Affymetrix). Briefly, the resulting double-stranded complementary DNA (cDNA) was used for in vitro transcription with T7 RNA polymerase (all steps are included in the wild-type cDNA synthesis and amplification Affymetrix kit).

After purification according to the Affymetrix protocol, 5.5  $\mu\text{g}$  Sens Target DNA was fragmented and labeled with biotin. Control of fragmentation involved using Bioanalyzer 2100, and cDNA was then hybridized to GeneChip human Clariom S (Affymetrix) at 45°C for 17 hours.

After overnight hybridization, chips were washed on the fluidic station FS450 in accordance with Affymetrix-specific protocols and scanned using the GCS3000 7G. We analyzed the scanned images using Expression Console software (Affymetrix) to obtain raw data (CEL files) and metrics for quality control. Data were deposited in GEO (accession no. GSE140161; <https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE140161>).

**Microarray data pre-processing and analysis.** Microarray computational analyses were performed with R 3.5.0.

Briefly, raw data were loaded and normalized using the Robust Multi Array algorithm. The quality of data normalization was verified by visual inspection of intensity distribution across arrays and by principal component analysis (PCA) (from FactoMineR package), with all genes used as the input. No outlier was detected by either approach. Microarray probes were annotated with use of the `clariomshumanhttranscriptcluster.db` package. If several probes were mapped to the same gene, the probe with the highest coefficient of variation was selected to represent the expression of the corresponding gene. All differentially expressed genes (DEGs) were included for PCA. PCA was used to identify separate clusters between primary SS-NHL and primary SS control groups.

We performed differential gene expression analysis using the `limma` package (based on linear models and empirical Bayesian method). The DEGs with  $P \leq 0.05$  were considered target genes and were further analyzed. A focused analysis on B cell activation-related genes was planned. These genes included tumor necrosis factor (TNF) super family 13 (*TNFSF13*) coding for APRIL, *TNFSF13B* coding for BAFF, tumor necrosis factor receptor super family 17 (*TNFRSF17*) coding for its receptor BCMA, *TNFSF10* coding for TNF-related apoptosis-inducing ligand (TRAIL), C-X-C motif chemokine ligand 13 (*CXCL13*), cluster of differentiation 180 (*CD180*), myeloid differentiation 88 (*MYD88*), and *BTK*, which were selected a priori for particular importance in promoting B cell stimulation and growth in the pathogenesis of primary SS and their potential role in association with BCR downstream signaling in lymphomagenesis.

**Laboratory assessments.** Laboratory assessments of patients in the ASSESS cohort was performed as previously described (31). This included CD4/CD8 T-cell ratio and B cell-associated serum biomarkers collected at enrollment, such as serum levels of total  $\gamma$ -globulins (g/L), IgG (g/L),  $\lambda$  and  $\kappa$  free light chains of Ig levels (mg/L), RF titers (IU/mL),  $\beta_2$ -microglobulin (mg/L), and BAFF (pg/mL).

#### Statistical analyses of clinical data and microarrays.

Demographic, clinical, and laboratory quantitative variable results are expressed as the mean  $\pm$  SD or the median (range), depending on the Gaussian nature of the distribution. Normality was assessed with the Shapiro-Wilk's test. For unsupervised analyses, gene expression data were filtered by the resulting  $P$  values at 5%, and the Benjamini and Hochberg method was used to estimate the false discovery rate. For the planned analysis of B cell-related genes and for pathway analyses, the false discovery rate was not used, given the exploratory nature of these analyses based on the whole blood in a limited sample size. We performed gene set enrichment analysis for DEGs with significant fold changes in expression of  $P$  values less than or equal to 0.05 using DAVID web-based software (National Institutes of Health) for functional annotation clustering (39).

**A priori specified set of criteria for replication of B cell-related gene results.** B cell-related genes that were consistently up-regulated across the primary and all sensitivity analyses were selected for replication using real-time quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and for inclusion in multivariate analyses.

#### Real-time qRT-PCR assessment of BTK expression.

The up-regulation of *BTK* observed in microarrays was confirmed by qRT-PCR with the same RNA samples (using peripheral whole blood) in 12 of 13 patients with history of lymphoma (RNA was exhausted for 1 patient) and in all 8 patients with incident lymphoma compared to 21 randomly selected patients without lymphoma and no risk factor of lymphoma and compared to 21 randomly selected patients without lymphoma and with ESS-DAI  $\geq 5$ . Total RNA (75 ng) was reverse transcribed to cDNA using Maxima Reverse Transcriptase Kit (Thermo Fisher Scientific). qRT-PCR was performed with the LC480 Instrument (Roche Diagnostics) according to the manufacturer's recommended protocol. Reverse-transcribed RNA was amplified using the ProbeMaster kit (Roche Diagnostics) and TaqMan gene expression assay ID HS00975865\_m1 for *BTK* (Thermo Fisher Scientific).

Data were normalized with 2 reference genes (peptidylpropyl isomerase B [*PPIB*] and succinate dehydrogenase complex flavoprotein subunit A [*SDHA*]), obtained from a pilot study of housekeeping genes with the GeNORM algorithm. Housekeeping genes were measured with the SYBR Green I Master kit (Roche Diagnostics). Sequences of PCR primers of housekeeping genes were 5'-AGACTGCGGCAAGATCGAGGTG-3' (forward) and 5'-GGGCGCTGCACAGACGGTCA-3' (reverse) for *PPIB* and 5'-GCATTCTACGACACCG-3' (forward) and 5'-GTGCAATA GCGAGTGG-3' (reverse) for *SDHA*. The technician was blinded to patient status when performing RT-PCR and used anonymized samples. Each reaction was run in triplicate.

For each triplicate reaction, the standard deviation of the quantification cycle was calculated. Triplicate reactions with SD  $< 0.3$  validated the measure, and the mean of the quantification cycle was calculated. For triplicate reactions with SD  $> 0.3$ , the outlier result was excluded and the mean of the remaining duplicates was calculated. To estimate the reproducibility of triplicates across reactions, the intraclass correlation coefficient was calculated. The comparative threshold cycle method was used to calculate the amplification fold, as specified by the manufacturer.

All laboratory techniques were performed at the Institut Cochin (Paris, France). Validation data for *BTK* gene expression obtained by qRT-PCR were compared between groups with the Mann-Whitney test, with  $P$  values less than or equal to 0.05 considered significant.

**Multivariate analyses.** To explore the association between lymphoma and candidate gene expression, multivariate logistic regression analyses were performed, with adjustment for 9 validated

predictors of lymphoma in primary SS (systemic complications [ESS-DAI  $\geq 5$ ], previous parotid swelling, purpura, lymphocytopenia, CD4/CD8 ratio  $\leq 0.8$ , RF positivity, cryoglobulinemia, monoclonal component, and low C4 level), selected as predetermined covariates. Odds ratios (ORs) and 95% confidence intervals (95% CIs) were estimated. Adjusted ORs were estimated for each of the 9 predetermined covariates in bivariate analyses. The 9 predetermined covariates were included in a multivariate model. We considered *P* values less than or equal to 0.05 as significant for bivariate and multivariate analyses. The bivariate and multivariate analyses were performed with logistic regression models in the R 3.5.3 environment.

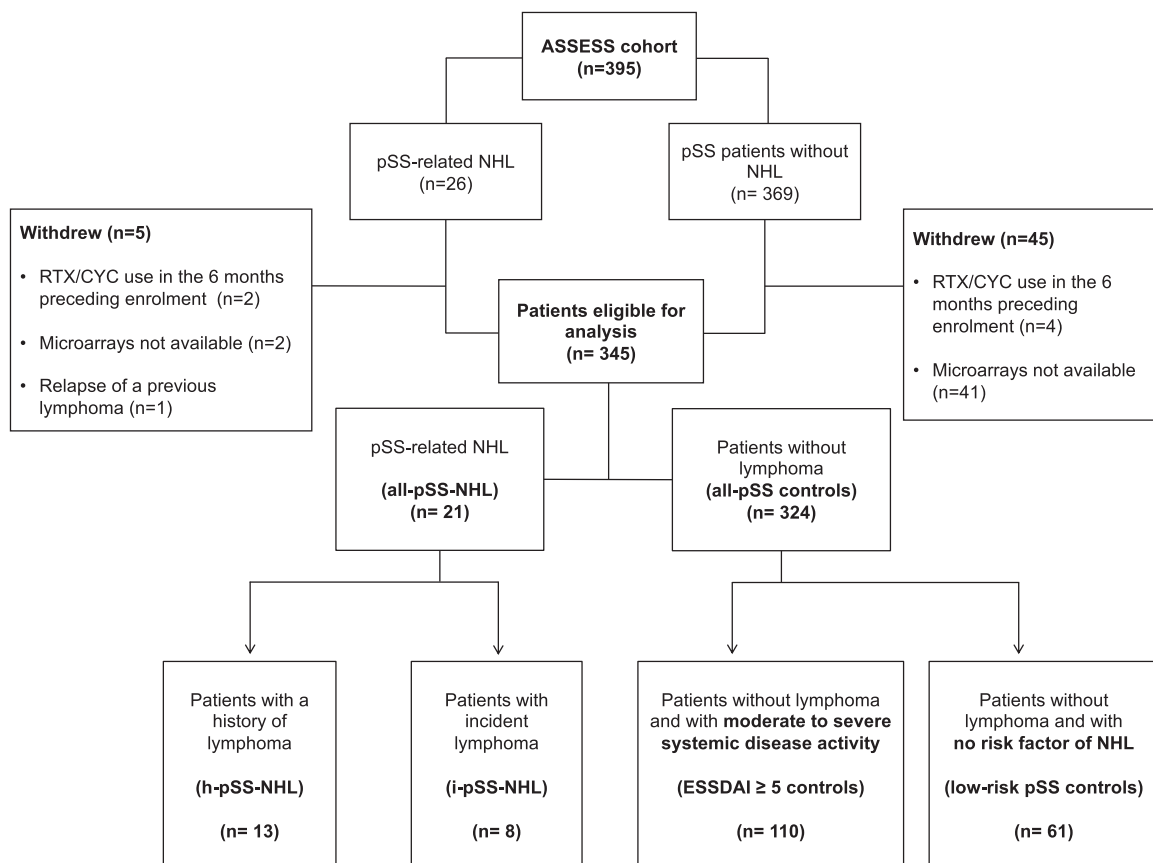
## RESULTS

**Patient characteristics.** At enrollment, 18 patients with primary SS had a history of lymphoma. During the 10-year follow-up, incident lymphoma developed in 8 patients and 1 patient with a history of lymphoma had a relapse. At last follow-up, 17 primary SS patients had a history of lymphoma without relapse, 1 patient had a relapse, and 8 patients with no previous lymphoma had incident lymphoma. Two patients with a history of lymphoma were excluded because of cyclophosphamide or rituximab use in the 6 months

before enrollment. A patient with relapse of previous NHL was entirely excluded from the analysis because of lack of consensus in classifying the particular situation of reoccurrence of a previous lymphoma in the i-primary SS-NHL or the h-primary SS-NHL subgroup. Microarray data were not available for 2 other patients with a history of lymphoma. Overall, 21 primary SS-NHL patients were eligible and available for analysis, including 13 with h-primary SS-NHL with no relapse and 8 with i-primary SS-NHL (Figure 1).

Among primary SS patients with available microarray data and who did not receive rituximab or cyclophosphamide in the 6 months before enrollment, 324 patients had no lymphoma, including 110 with moderate to severe systemic disease activity (ESSDAI  $\geq 5$ ) and 61 with no risk factor of lymphoma (Figure 1). Table 1 summarizes the demographic and immunologic features of patients with primary SS-NHL and the 3 control populations analyzed in the present study.

**Characteristics of lymphomas.** MALT-MZL was the dominant histologic subtype, representing 52% of all primary SS-NHL cases ( $n = 21$ ). MALT-MZL affected mostly the salivary glands in 10 patients (48%) and the lung in 1 patient (4.8%). MALT-MZL was followed by nodal and splenic MZL in 2 patients



**Figure 1.** Study flow diagram. ASSESS = Assessment of Systemic Signs and Evolution in Sjögren's Syndrome; pSS = primary Sjögren's syndrome; NHL = non-Hodgkin lymphoma; RTX = rituximab; CYC = cyclophosphamide; ESSDAI = EULAR Sjögren's Syndrome Disease Activity Index.

**Table 1.** Characteristics of patients with primary Sjögren's syndrome–related non-Hodgkin lymphoma and patients with primary Sjögren's syndrome without lymphoma at enrollment in the ASSESS cohort\*

|   | Primary SS-NHL patients†    |                           |                          | Primary SS controls‡                  |                              |                                   |
|---|-----------------------------|---------------------------|--------------------------|---------------------------------------|------------------------------|-----------------------------------|
|   | All-primary SS-NHL (n = 21) | h-primary SS-NHL (n = 13) | i-primary SS-NHL (n = 8) | Low-risk primary SS controls (n = 61) | ESSDAI ≥5 controls (n = 110) | All primary SS controls (n = 324) |
| Women   | 19 (90)                     | 12 (92)                   | 7 (88)                   | 60 (98)                               | 104 (95)                     | 308 (95)                          |
| Age at first symptoms of primary SS, years                          | 49.1 ± 13.9                 | 50.8 ± 15.3               | 46.5 ± 11.8              | 48.8 ± 12.8                           | 45.2 ± 15.6                  | 46.4 ± 13                         |
| Age at primary SS diagnosis, years                                  | 52.1 ± 11.8                 | 52.7 ± 14.1               | 53.5 ± 7.3               | 52.5 ± 12.6                           | 52.2 ± 13.5                  | 51.4 ± 12.5                       |
| Age at lymphoma diagnosis, years                                    | 59.3 ± 12.3                 | 56.1 ± 13.8               | 64.4 ± 7.4               | NA                                    | NA                           | NA                                |
| Time from primary SS diagnosis to ASSESS enrollment, years          | 7 (1–19)                    | 7 (1–13)                  | 6.5 (2–19)               | 5 (0–30)                              | 5.5 (0–30)                   | 5 (0–30)                          |
| Time from first symptoms of primary SS to lymphoma diagnosis, years | 11 (–2 to 45)               | 4 (–2 to 14)              | 14 (10–45)               | NA                                    | NA                           | NA                                |
| Time from history of lymphoma to enrollment, years                  | –                           | 5 (1–11)                  | –                        | NA                                    | NA                           | NA                                |
| Time from enrollment to occurrence of lymphoma, years               | –                           | –                         | 5 (1–9)                  | NA                                    | NA                           | NA                                |
| Follow-up duration, years   | 8.9 (1.2–10.3)              | 9.3 (1.2–10.3)            | 8 (4–9.6)                | 8.9 (0–11)                            | 8.9 (0–10.6)                 | 8.8 (0–11)                        |
| Deaths from all causes  | 4 (19)                      | 3 (23)                    | 1 (12.5)                 | 3 (5)                                 | 6 (5)                        | 14 (4)                            |
| Deaths induced by lymphoma  | 2 (9.5)                     | 1 (7.7)                   | 1 (12.5)                 | NA                                    | NA                           | NA                                |
| Clinical predictive factors   |                             |                           |                          |                                       |                              |                                   |
| Glandular domain of the ESSDAI score                                | 5 (24)                      | 3 (23)                    | 2 (25)                   | 0                                     | 18 (16)                      | 33 (10)                           |
| Purpura   | 6 (29)                      | 5 (38)                    | 1 (12.5)                 | 0                                     | 18 (16)                      | 33 (10)                           |
| Immunologic predictive factors                                      |                             |                           |                          |                                       |                              |                                   |
| SSA antibody positivity   | 18 (86)                     | 10 (77)                   | 8 (100)                  | 36 (59)                               | 83 (75)                      | 242 (75)                          |
| SSB antibody positivity   | 16 (76)                     | 9 (69)                    | 7 (88)                   | 34 (56)                               | 78 (71)                      | 218 (67)                          |
| RF positivity   | 10 (48)                     | 5 (38)                    | 5 (75)                   | 0                                     | 44 (40)                      | 123 (38)                          |
| Cryoglobulinemia  | 5 (24)                      | 3 (23)                    | 2 (25)                   | 0                                     | 16 (15)                      | 45 (14)                           |
| Monoclonal component  | 5 (24)                      | 4 (31)                    | 1 (12.5)                 | 0                                     | 19 (17)                      | 35 (11)                           |
| Lymphopenia <1,000/mm <sup>3</sup>                                  | 7 (33)                      | 3 (23)                    | 4 (50)                   | 0                                     | 27 (25)                      | 54 (17)                           |
| Low C4 level  | 13 (67)                     | 8 (62)                    | 5 (75)                   | 0                                     | 54 (50)                      | 120 (37)                          |
| CD4/CD8 ratio ≤0.8  | 6 (29)                      | 4 (31)                    | 2 (25)                   | 0                                     | 5 (5)                        | 10 (3)                            |
| Lymphoid infiltrates in minor salivary gland biopsies               | 21 (100)                    | 13 (100)                  | 8 (100)                  | 51 (80)                               | 93 (85)                      | 275 (85)                          |
| Disease activity  |                             |                           |                          |                                       |                              |                                   |
| ESSDAI  | 4 (0–18)                    | 7 (0–18)                  | 4 (1–11)                 | 0 (0–4)                               | 9.5 (5–24)                   | 2 (0–24)                          |
| High disease activity (ESSDAI ≥5)                                   | 10 (48)                     | 7 (54)                    | 3 (37.5)                 | 0                                     | 110 (100)                    | 110 (34)                          |
| Predictor of primary SS-NHL   | 3 (1–7)                     | 3 (1–7)                   | 3 (1–6)                  | 0                                     | 3 (1–7)                      | 2 (0–7)                           |
| Histologic type of lymphoma   |                             |                           |                          |                                       |                              |                                   |
| Salivary gland MALT marginal zone lymphoma                          | 10 (48)                     | 6 (46.2)                  | 4 (50)                   | NA                                    | NA                           | NA                                |
| Lung MALT-MZL   | 1 (4.8)                     | 1 (7.7)                   | 0 (0)                    | NA                                    | NA                           | NA                                |
| Nodal MZL   | 2 (9.5)                     | 1 (7.7)                   | 1 (12.5)                 | NA                                    | NA                           | NA                                |
| Splenic MZL   | 1 (4.8)                     | 1 (7.7)                   | 0 (0)                    | NA                                    | NA                           | NA                                |
| DLBCL   | 4 (19)                      | 2 (15.4)                  | 2 (25)                   | NA                                    | NA                           | NA                                |
| Follicular lymphoma   | 1 (4.8)                     | 0 (0)                     | 1 (12.5)                 | NA                                    | NA                           | NA                                |
| EBV-induced lymphoproliferation                                     | 1 (4.8)                     | 1 (7.7)                   | 0 (0)                    | NA                                    | NA                           | NA                                |
| T cell lymphoma, mycosis fungoid                                    | 1 (4.8)                     | 1 (7.7)                   | 0 (0)                    | NA                                    | NA                           | NA                                |
| Treatment with delay of >6 months before enrollment                 |                             |                           |                          |                                       |                              |                                   |
| Rituximab   | 6 (28.6)                    | 6 (46)                    | 0 (0)                    | 0 (0)                                 | 5 (4.5)                      | 7 (2.2)                           |
| Cyclophosphamide  | 2 (9.5)                     | 2 (15)                    | 0 (0)                    | 0 (0)                                 | 5 (4.5)                      | 7 (2.2)                           |
| Rituximab + cyclophosphamide  | 1 (4.8)                     | 1 (7.7)                   | 0 (0)                    | 0 (0)                                 | 1 (0.9)                      | 1 (0.3)                           |

\* Values are the number (%) of patients, the mean ± SD, or the median (range). Primary SS = primary Sjögren's syndrome; NHL = non-Hodgkin lymphoma; ESSDAI = EULAR Sjögren's Syndrome Disease Activity Index; NA = not applicable; SSA/B = SS antigen A/B; RF = rheumatoid factor; MALT = mucosa-associated lymphoid tissue; MZL = marginal-zone B cell lymphoma; DLBCL = diffuse large B cell lymphoma; EBV = Epstein-Barr virus.

† Primary SS-NHL groups included those with incident lymphoma (i-primary SS-NHL) and those with history of lymphoma (h-primary SS-NHL).  
‡ Primary SS groups without lymphoma included patients regardless of their disease activity and lymphoma predictors (all-primary SS controls), those with moderate to severe systemic disease activity (ESSDAI ≥5 controls), and those with no risk factor of NHL (low-risk primary SS controls). No patient from primary SS-NHL or primary SS groups without lymphoma received rituximab or cyclophosphamide in the 6 months before enrollment.

(9.5%) and 1 patient (4.8%), respectively. The DLBCL subtype was diagnosed in 4 patients (19%). Three other NHL histologic subtypes were identified in the ASSESS cohort, including 1 follicular lymphoma, 1 Epstein-Barr virus-induced lymphoproliferation, and 1 T cell epidermotropic cutaneous lymphoma (mycosis fungoides) (4.8%) (Table 1).

**Differential gene expression profile of primary SS patients with or without lymphoma.** Among the 18,625 analyzable genes and according to  $P \leq 0.05$  threshold of significance, we showed that 870 genes were differentially expressed in the primary analysis, which compared patients with i-primary SS-NHL ( $n = 8$ ) versus all patients without lymphoma (all-primary SS controls;  $n = 324$ ); 422 genes were up-regulated, and 448 were down-regulated. The total number of DEGs in all secondary analyses is shown in Supplementary Table 1, available on the *Arthritis & Rheumatology* website at <https://onlinelibrary.wiley.com/doi/10.1002/art.42550>. Unsupervised analyses by PCA (data not shown) did not discriminate between patients with lymphoma and any of the 3 control populations.

In the primary analysis, we used functional annotation clustering to analyze the 422 significantly up-regulated genes obtained after comparing i-primary SS-NHL to all-primary SS controls (Supplementary Table 2, available on the *Arthritis & Rheumatology* website at <https://onlinelibrary.wiley.com/doi/10.1002/art.42550>). Among the top 20 pathways identified in this analysis, the first 7 pathways were involved in nonspecific cellular functions, including cell membrane, lysosomal, and endosomal functions, DNA binding, oxidation–reduction, kinases, and vesicle trafficking–related/autophagy genes, followed by immune-related genes (25/422 genes;  $P = 5.2 \times 10^{-2}$ ) (Supplementary Table 2).

Functional annotation clustering of the 448 down-regulated genes in the primary analysis revealed enrichment of genes involved in nuclear function (163/448 genes;  $P = 3.6 \times 10^{-5}$ ), transcription regulation (93/448;  $P = 7.2 \times 10^{-7}$ ), and cancer-associated pathways (24/448;  $P = 3.6 \times 10^{-3}$ ), including apoptotic genes (18/448;  $P = 1.4 \times 10^{-1}$ ), T cell costimulation genes (6/448;  $P = 1.6 \times 10^{-3}$ ), and DNA damage response and repair process–associated genes (10/448;  $P = 4.4 \times 10^{-1}$ ), suggesting impairment of several antitumoral processes (Supplementary Table 3, available on the *Arthritis & Rheumatology* website at <https://onlinelibrary.wiley.com/doi/10.1002/art.42550>).

Higher numbers of DEGs were observed in the sensitivity analysis that compared all-primary SS-NHL (i-primary SS-NHL and h-primary SS-NHL) with 61 patients without lymphoma and with no risk factor of lymphoma (low-risk primary SS controls) (Supplementary Table 1, available at <https://onlinelibrary.wiley.com/doi/10.1002/art.42550>). The list of 1,177 genes that were up-regulated in the combined group of primary SS-NHL patients compared to the primary SS controls at low risk for NHL was submitted for functional annotation clustering analysis. The analysis identified a predominant enrichment of immunity-related

pathways ( $P = 1.6 \times 10^{-18}$ ), including adaptive immunity and BCR signaling ( $P = 8.3 \times 10^{-2}$ ), innate immunity ( $P = 7.4 \times 10^{-24}$ ), and particularly interferon signaling pathways ( $P = 3.0 \times 10^{-2}$ ), among the top up-regulated functional pathways (Supplementary Table 4, available on the *Arthritis & Rheumatology* website at <https://onlinelibrary.wiley.com/doi/10.1002/art.42550>).

In accordance with the results of the primary analysis, genes down-regulated in the sensitivity analysis of the all-primary SS-NHL group versus low-risk controls were related to nuclear function (541/1,290;  $P = 1.6 \times 10^{-34}$ ), transcription regulation (281/1,290;  $P = 1.2 \times 10^{-22}$ ), apoptosis (43/1,290;  $P = 2.7 \times 10^{-1}$ ), T cell costimulation (9/1,290;  $P = 2.8 \times 10^{-3}$ ), and DNA damage response and repair process–associated genes (33/1,290;  $P = 9.3 \times 10^{-4}$ ), including base excision repair, nucleotide excision repair, and mismatch repair–associated genes (Supplementary Table 5, available on the *Arthritis & Rheumatology* website at <https://onlinelibrary.wiley.com/doi/10.1002/art.42550>). We performed separate gene clustering analyses for all patients with lymphoma (including patients with a history of NHL and those with incident lymphoma), which we compared to the 2 other control populations (ESSDAI  $\geq 5$  and all-primary SS controls). In these analyses, similar enriched pathways were found (data not shown).

**Planned analysis of genes involved in B cell activation.** In the primary analysis, in which we compared i-primary SS-NHL patients to all-primary SS controls, *APRIL* and *BTK*, but not *BAFF*, *BCMA*, or *TRAIL*, were significantly up-regulated (Table 2; Figure 2B), as observed in a sensitivity analysis that compared i-primary SS-NHL patients to primary SS patients with ESSDAI  $\geq 5$  (Table 2). Conversely, *BTK*, *APRIL*, and *BAFF* showed significant up-regulation in a sensitivity analysis comparing i-primary SS-NHL patients to low-risk primary SS controls.

In sensitivity analyses comparing h-primary SS-NHL patients to all-primary SS controls and to ESSDAI  $\geq 5$  controls, only *BTK* was significantly up-regulated (Table 2). In the analysis comparing h-primary SS-NHL patients versus low-risk primary SS controls, *BAFF*, *BCMA*, *TRAIL*, and *BTK*, but not *APRIL*, were up-regulated (Table 2). Furthermore, in a sensitivity analysis comparing all-primary SS-NHL to all-primary SS controls, *BCMA* and *BTK* were up-regulated, but not *BAFF*, *APRIL*, or *TRAIL* (Table 2). In the comparison of all-primary SS-NHL patients with ESSDAI  $\geq 5$  controls, only *BTK* showed significant overexpression (Table 2). Finally, in the comparison of all-primary SS-NHL patients with low-risk primary SS controls, *BTK*, *BAFF*, *APRIL*, *BCMA*, and *TRAIL* were up-regulated (Table 2 and Figure 2B). The other B cell–associated genes analyzed, *CXCL13*, *MYD88*, and *CD180*, were not significantly expressed in the primary analysis or in any of the sensitivity analyses performed.



**Table 2.** Results of primary and sensitivity analyses for B cell activation-associated genes\*

| Official gene symbol   | i-primary SS-NHL versus all-primary SS controls (primary analysis)       |                | i-primary SS-NHL versus low-risk primary SS controls (sensitivity analysis)   |                | i-primary SS-NHL versus ESSDAI $\geq$ 5 controls (sensitivity analysis)   |                |
|------------------------|--|----------------|---|----------------|---|----------------|
|                        | Fold change  | <i>P</i> value | Fold change   | <i>P</i> value | Fold change   | <i>P</i> value |
| <i>BTK</i>             | 1.2  | 0.01           | 1.2   | 0.004          | 1.17  | 0.03           |
| <i>BAFF (TNFSF13B)</i> | 1.15   | 0.3            | 1.36  | 0.02           | 1.15  | 0.4            |
| <i>BCMA (TNFRSF17)</i> | 1.11   | 0.2            | 1.17  | 0.08           | 1.1   | 0.4            |
| <i>TRAIL (TNFSF10)</i> | 1.09   | 0.4            | 1.18  | 0.08           | 1.04  | 0.7            |
| <i>APRIL (TNFSF13)</i> | 1.3  | 0.003          | 1.3   | 0.001          | 1.3   | 0.006          |
| <i>CXCL13</i>          | 0.99   | 0.9            | 0.99  | 0.9            | 0.99  | 0.8            |
| <i>MYD88</i>           | 1.073  | 0.4            | 1.1   | 0.1            | 1.04  | 0.7            |
| <i>CD180</i>           | 0.8  | 0.2            | 0.8   | 0.2            | 0.83  | 0.3            |
|                        | h-primary SS-NHL versus all-primary SS controls (sensitivity analysis)   |                | h-primary SS-NHL versus low-risk primary SS controls (sensitivity analysis)   |                | h-primary SS-NHL versus ESSDAI $\geq$ 5 controls (sensitivity analysis)   |                |
|                        | Fold change  | <i>P</i> value | Fold change   | <i>P</i> value | Fold change   | <i>P</i> value |
| <i>BTK</i>             | 1.2  | 0.007          | 1.2   | 0.003          | 1.16  | 0.02           |
| <i>BAFF (TNFSF13B)</i> | 1.2  | 0.2            | 1.3   | 0.01           | 1.12  | 0.4            |
| <i>BCMA (TNFRSF17)</i> | 1.13   | 0.09           | 1.2   | 0.03           | 1.11  | 0.2            |
| <i>TRAIL (TNFSF10)</i> | 1.1  | 0.3            | 1.2   | 0.04           | 1.04  | 0.7            |
| <i>APRIL (TNFSF13)</i> | 1.02   | 0.9            | 1.05  | 0.6            | 1.002   | 0.9            |
| <i>CXCL13</i>          | 1.03   | 0.4            | 1.02  | 0.4            | 1.02  | 0.4            |
| <i>MYD88</i>           | 0.99   | 0.9            | 1.02  | 0.7            | 0.96  | 0.5            |
| <i>CD180</i>           | 1.1  | 0.6            | 1.04  | 0.8            | 0.91  | 0.5            |
|                        | All-primary SS-NHL versus all-primary SS controls (sensitivity analysis) |                | All-primary SS-NHL versus low-risk primary SS controls (sensitivity analysis) |                | All-primary SS-NHL versus ESSDAI $\geq$ 5 controls (sensitivity analysis) |                |
|                        | Fold change  | <i>P</i> value | Fold change   | <i>P</i> value | Fold change   | <i>P</i> value |
| <i>BTK</i>             | 1.2  | 0.0003         | 1.2   | 0.0002         | 1.17  | 0.003          |
| <i>BAFF (TNFSF13B)</i> | 1.17   | 0.1            | 1.3   | 0.002          | 1.13  | 0.2            |
| <i>BCMA (TNFRSF17)</i> | 1.13   | 0.04           | 1.2   | 0.01           | 1.11  | 0.2            |
| <i>TRAIL (TNFSF10)</i> | 1.09   | 0.2            | 1.3   | 0.02           | 1.04  | 0.6            |
| <i>APRIL (TNFSF13)</i> | 1.12   | 0.06           | 1.15  | 0.05           | 1.1   | 0.1            |
| <i>CXCL13</i>          | 1.02   | 0.5            | 1.02  | 0.6            | 1.02  | 0.6            |
| <i>MYD88</i>           | 1.02   | 0.6            | 1.06  | 0.3            | 0.99  | 0.8            |
| <i>CD180</i>           | 0.97   | 0.8            | 0.94  | 0.6            | 0.99  | 0.9            |

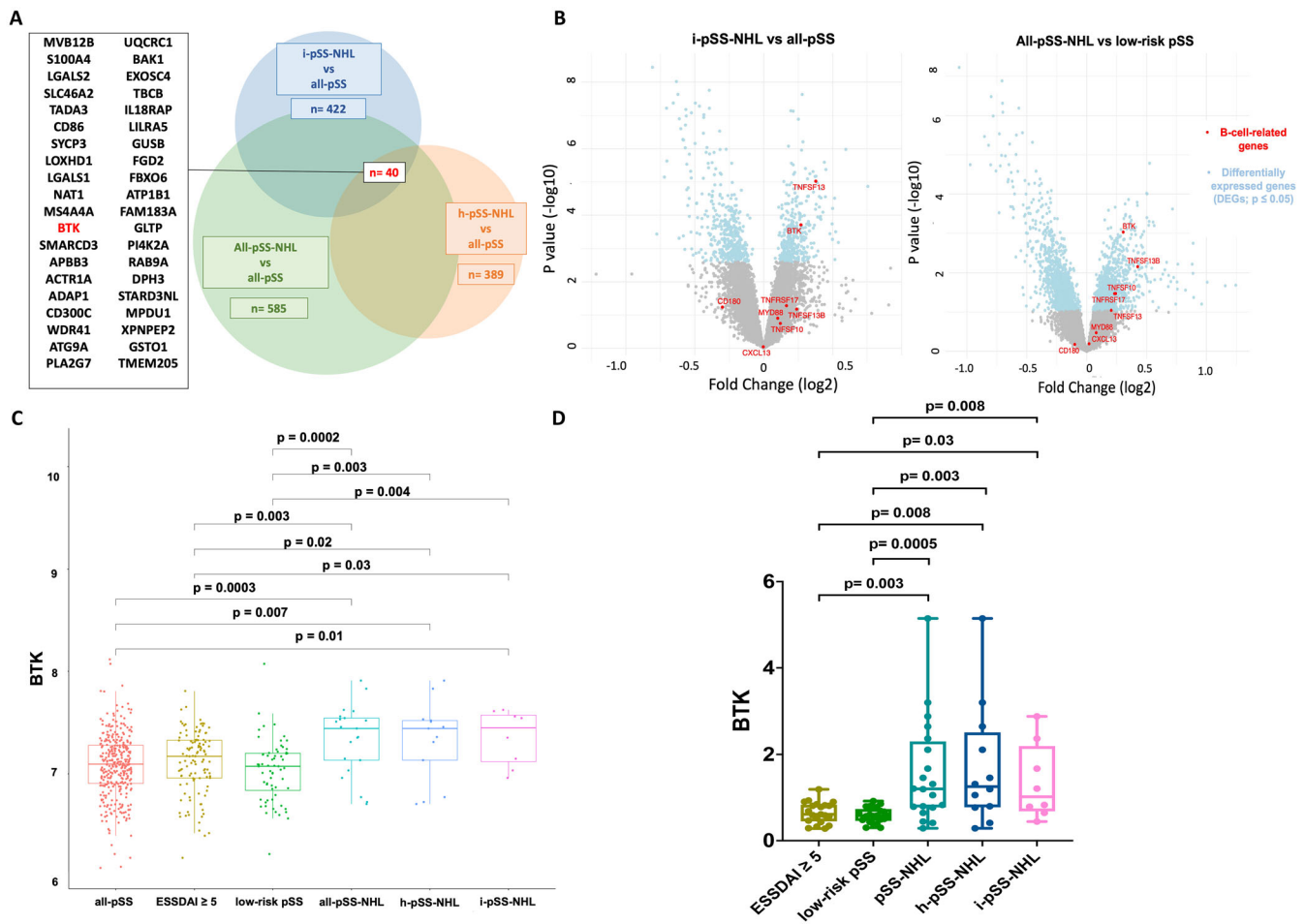
\* Statistical analyses were not adjusted for multiple comparisons. *BTK* = Bruton's tyrosine kinase; *TNFSF* = tumor necrosis factor superfamily; *TNFRSF* = tumor necrosis factor receptor superfamily; *TRAIL* = tumor necrosis factor-related apoptosis-inducing ligand. See Table 1 for other definitions.

**Conservation of *BTK* gene up-regulation across all comparisons.** To test which of the B cell activation genes was conserved in all groups, we used Venn diagrams to indicate the overlapping genes between primary SS-NHL subgroups and the 3 control groups (Figure 2A and Figure 3). Among the 40 overlapping up-regulated genes (see Supplementary Table 8, available at <https://onlinelibrary.wiley.com/doi/10.1002/art.42550>), in comparing i-primary SS-NHL, h-primary SS-NHL, and all-primary SS-NHL patients versus all-primary SS controls, and among the 261 overlapping up-regulated genes, in comparing all-primary SS-NHL patients and the 3 control populations, the only B cell activation-related gene consistently identified across comparisons was the gene coding for *BTK* (Figure 2A and Figure 3).

Among the top up-regulated genes involved in B cell activation, *BTK* showed a significant differential expression profile in all

comparisons performed and notably versus the low-risk primary SS controls (Figure 2B). Of note, *BTK* was significantly up-regulated in the primary analysis between i-primary SS-NHL and all-primary SS controls and in every comparison between patients with lymphoma (i-primary SS-NHL, h-primary SS-NHL, all-primary SS-NHL) and the 3 control populations without lymphoma (low-risk primary SS controls, primary SS with ESSDAI  $\geq$ 5 controls, all-primary SS controls) (Figure 2C). The up-regulation of *BTK* observed in microarrays was confirmed by qRT-PCR (Figure 2D; Supplementary Table 6, available on the *Arthritis & Rheumatology* website at <https://onlinelibrary.wiley.com/doi/10.1002/art.42550>).

**Correlation of *BTK* with B cell serum biomarker levels.** To examine the association between *BTK* expression, B cell-associated serum biomarkers, CD4/CD8 T cell ratio, and



**Figure 2.** Differential expression of Bruton's tyrosine kinase (*BTK*) gene in patients with primary Sjögren's syndrome (SS)-related NHL and in patients without lymphoma. **A**, Venn diagram showing the number of up-regulated overlapping genes among patients with incident primary SS-related NHL (i-pSS-NHL), patients with primary SS and a history of lymphoma (h-pSS-NHL), and all patients with primary SS-NHL versus all primary SS patients without lymphoma (all-pSS controls). The *BTK* gene was the only B cell-associated gene among the 40 overlapping genes in these 3 comparisons. **B**, Volcano plots showing the distribution of differentially expressed genes (DEGs) in the i-pSS-NHL group versus the all-pSS control group (primary analysis) and the all-pSS-NHL group versus the low-risk pSS control group (sensitivity analysis). B cell-related genes (*TNFSF13/APRIL*, *TNFSF13B/BAFF*, *TNFRSF17/BCMA*, *TNFSF10/TRAIL*, *BTK*, *CD180*, *CXCL13*, *MYD88*) are shown in red, with right showing up-regulated DEGs. DEGs with  $P \leq 0.05$  are shown in blue. **C**, *BTK* gene expression level in patients with lymphoma (all-pSS-NHL, i-pSS-NHL, and h-pSS-NHL groups) compared to patients without lymphoma (low-risk pSS control, ESSDAI  $\geq 5$  control, and all-pSS control groups). **D**, real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis of *BTK* gene expression in peripheral blood of patients in the pSS-NHL ( $n = 20$ ), i-pSS-NHL ( $n = 8$ ), and h-pSS-NHL ( $n = 12$ ) groups at enrollment versus patients without lymphoma and with no risk factor of lymphoma (low-risk pSS controls;  $n = 21$ ) and with moderate to severe systemic disease activity (ESSDAI  $\geq 5$  controls;  $n = 21$ ). Intraclass correlation coefficient for qRT-PCR triplicates was 0.8 (95% CI 0.8–0.86). Validation data for *BTK* gene expression obtained by qRT-PCR were compared by Mann-Whitney test, with  $P \leq 0.05$  considered significant. See Figure 1 for other definitions.

disease activity at enrollment, we performed correlation analyses. *BTK* expression was significantly associated with total  $\gamma$ -globulins, IgG,  $\lambda$  and  $\kappa$  free light chains of Ig levels, RF titers, and serum levels of  $\beta_2$ -microglobulin and BAFF, all surrogate markers of B cell activation. *BTK* was also correlated with disease activity (ESSDAI) but not with the T cell surrogate marker CD4/CD8 T cell ratio (Supplementary Figure 1, available on the *Arthritis & Rheumatology* website at <https://onlinelibrary.wiley.com/doi/10.1002/art.42550>).

**Association between *BTK* expression level and lymphoma on multivariate analyses.** To address the

association between *BTK* expression at enrollment and lymphoma development, we performed bivariate and multivariate analyses, first between all patients with and without lymphoma and second between patients with lymphoma and patients without lymphoma and moderate to severe disease activity (ESSDAI  $\geq 5$  controls). On univariate analyses, *BTK* level was significantly associated with lymphoma when we compared patients with and without lymphoma (OR 16.3, 95% CI 3.5–77.5;  $P = 0.0004$ , OR expressed for an increase of 1 in the numerical value of *BTK*, when *BTK* values fluctuated from 6.08 to 8.1) and when we compared patients with lymphoma



**Figure 3.** The *BTK* gene was the only B cell-associated gene among the overlapping genes across all comparisons. **A–D**, Venn diagrams showing the number of up-regulated overlapping genes between all groups with lymphoma (i-pSS-NHL, h-pSS-NHL, and all-pSS-NHL) compared with groups without lymphoma (all-pSS controls [A and D], low-risk pSS controls [B and D], and pSS with ESSDAI  $\geq 5$  controls [C and D]). See Figure 1 for other definitions.

and patients without lymphoma and with ESSDAI  $\geq 5$  (OR 13.9, 95% CI 2.2–89.4;  $P = 0.006$ ). We observed the same associations with *BTK* and lymphoma in bivariate models adjusted for each of the following validated risk factors of lymphoma in primary SS: previous parotid swelling, purpura, lymphocytopenia, CD4/CD8 ratio  $\leq 0.8$ , RF positivity, cryoglobulinemia, monoclonal component, and low C4 level for the comparison with ESSDAI  $\geq 5$  controls and the same factors and ESSDAI  $\geq 5$  for the comparison with all-primary SS controls (Supplementary Table 7, available on the *Arthritis & Rheumatology* website at <https://onlinelibrary.wiley.com/doi/10.1002/art.42550>).

On multivariate analysis of all patients with and without lymphoma, taking into account these 9 potential confounding factors, *BTK* level at enrollment remained associated with lymphoma (adjusted OR 47.6, 95% CI 5.5–411.9;  $P = 0.0005$ ), as did CD4/CD8 T cell ratio  $\leq 0.8$  (adjusted OR 7.4, 95% CI 1.6–34.3;  $P = 0.01$ ) (Table 3). In the comparison of all patients with lymphoma and those without lymphoma and moderate to

severe systemic disease activity at enrollment (ESSDAI  $\geq 5$ ), *BTK* level was also associated with lymphoma in a multivariate model that included 8 validated risk factors of lymphoma (the same factors as above, but excluding ESSDAI) (adjusted OR 33.6, 95% CI 3.0–375.5;  $P = 0.004$ ) and CD4/CD8 ratio  $\leq 0.8$  (adjusted OR 9.5, 95% CI 2.0–45.8;  $P = 0.005$ ) (Table 3).

## DISCUSSION

The present study is, to our knowledge, the first report of a peripheral whole blood gene expression profiling study with a specific focus on B cell-related genes in primary SS-NHL. Lymphoma subtypes in our study included mostly MALT-MZLs over nodal/splenic MZLs and DLBCLs, which is consistent with previous studies addressing epidemiologic and histologic features of NHL complicating primary SS (8,10).

The primary analysis was exploratory and aimed to compare the gene expression profiles of patients with incident lymphoma

**Table 3.** Multivariate analyses estimating the risk of lymphoma at enrollment in the ASSESS cohort in patients with lymphoma versus patients without lymphoma\*

| Variable                 | Model 1 all-primary<br>SS-NHL versus all-primary SS controls |         | Model 2 all-primary<br>SS-NHL versus ESSDAI $\geq 5$ controls |         |
|--------------------------|--|---------|---|---------|
|                          | OR (95% CI)  | P value | OR (95% CI)   | P value |
| BTK                      | 47.6 (5.5–411.9)   | 0.0005  | 33.6 (3.0–375.5)  | 0.004   |
| CD4/CD8 ratio $\leq 0.8$ | 7.4 (1.6–34.3)   | 0.01    | 9.5 (2.0–45.8)  | 0.005   |
| Lymphopenia              | 2.0 (0.6–6.8)  | 0.3     | 1.12 (0.3–3.9)  | 0.9     |
| Low C4                   | 2.6 (0.8–8.9)  | 0.1     | 1.9 (0.6–6.8)   | 0.3     |
| RF                       | 1.1 (0.3–3.7)  | 0.9     | 1.2 (0.4–4.4)   | 0.7     |
| Cryoglobulinemia         | 1.6 (0.4–6.3)  | 0.5     | 2.4 (0.6–9.5)   | 0.2     |
| Monoclonal component     | 1.9 (0.4–8.6)  | 0.4     | 0.9 (0.2–4.3)   | 0.9     |
| ESSDAI $\geq 5$          | 1.1 (0.4–3.6)  | 0.8     | –   | –       |
| Purpura                  | 1.6 (0.4–7.0)  | 0.5     | 1.5 (0.3–6.5)   | 0.6     |
| Parotid swelling         | 2.5 (0.7–9.7)  | 0.2     | 1.7 (0.4–7.0)   | 0.5     |

\* Model 1 included the 9 predictors of primary SS-NHL (systemic complications [ESSDAI  $\geq 5$ ], previous parotid swelling, purpura, lymphocytopenia, CD4/CD8 ratio  $\leq 0.8$ , RF positivity, cryoglobulinemia, monoclonal component, and low C4 level) to compare patients with versus without lymphoma. Model 2 included 8 predictors (same factors as for model 1, excluding ESSDAI) to compare patients with lymphoma versus patients without lymphoma and ESSDAI  $\geq 5$ . Odds ratios (ORs) and 95% confidence intervals (95% CIs) are expressed for an increase in the numerical value of 1 in the original scale of each covariate. BTK = Bruton's tyrosine kinase; see Table 1 for other definitions.

versus the profiles of all-primary SS patients without lymphoma, to uncover a signature and potential biomarkers associated with lymphoma development in primary SS. We detected no specific signature associated with lymphoma between patients with incident lymphoma and those without lymphoma. Functional clustering analyses, in the primary analysis and in all sensitivity analyses, indicated enrichment of numerous pathways, including notably innate and adaptive immunity, nuclear factors, and transcription regulators. In the sensitivity analysis in which we compared all-primary SS-NHL patients to low-risk primary SS controls, we identified these pathways ranking first. The planned analysis of B cell activation genes allowed for detecting *APRIL* and *BTK* up-regulation in the primary analysis. Of note, the planned analysis of genes involved in B cell activation revealed that *BTK* was the only such gene to be consistently up-regulated, in both the primary analysis and all sensitivity analyses, which was confirmed by qRT-PCR.

B cell hyperactivation is a key feature of primary SS pathogenesis and lymphomagenesis (15). In the primary analysis, in which we compared i-primary SS-NHL patients to all-primary SS controls, we found significant up-regulation of *BTK* and *APRIL* but not *BAFF* and *BCMA*, the shared receptor for BAFF and APRIL. In contrast, *BTK*, *APRIL*, *BAFF*, and *BCMA* were up-regulated in all patients with primary SS-NHL compared with patients with low risk of NHL. BAFF and APRIL are pivotal cytokines involved in B cell proliferation, survival, and isotypic switch. Increased serum levels of BAFF are correlated with autoantibody titers and disease activity and are more frequent in primary SS-related lymphoma. Elevated APRIL serum levels have been associated with B cell lymphoproliferations, such as chronic lymphocytic leukemia (40) and DLBCL in systemic lupus erythematosus and rheumatoid arthritis (41).

A role for APRIL in autoimmunity and development of lymphoid infiltrates in primary SS has also been reported (18). *APRIL*

was found to be up-regulated in patients with i-primary SS-NHL versus all control populations and in all-primary SS-NHL versus patients with low risk of NHL. This could suggest an association between *APRIL* up-regulation and lymphoma development. This finding illuminates *APRIL* as a potential biomarker associated with incident lymphoma in primary SS, which should be replicated in a larger study sample.

We then focused on DEGs involved in B cell activation that were consistently up-regulated in every subgroup of patients with lymphoma compared with the 3 control populations. Among the overlapping up-regulated genes within these comparisons, the only B cell activation-related gene was *BTK*. *BTK* was significantly and consistently up-regulated at enrollment in all subgroups of primary SS-NHL analyzed, including patients who developed incident lymphoma during the 10-year follow-up. Therefore, *BTK*, a pivotal BCR transducer, was overexpressed in the peripheral blood of patients before the occurrence of lymphoma.

*BTK* plays a pivotal role in B cell activation and proliferation through BCR signaling (42) and therefore might represent a key pathogenic player in the transition between B cell chronic polyclonal activation and malignant lymphoproliferation in primary SS. Given the pathogenesis of primary SS, a lymphoid source of *BTK* is likely, but one cannot exclude that myeloid cells are also involved, which should be evaluated on sorted cells. Likewise, *BTK* was associated with B cell-related serum biomarkers such as total  $\gamma$ -globulins, IgGs, RFs,  $\lambda$  and  $\kappa$  free light chains,  $\beta_2$ -microglobulin, and BAFF serum levels.

Our present results suggest that *BTK* overexpression in peripheral blood might be detected before the occurrence of lymphoma, which is of particular interest when investigating predictive biomarkers of primary SS-NHL. Systemic disease activity is associated with risk of lymphoma in primary SS (6). Likewise, other clinical or biologic predictors of lymphoma in primary SS are routinely tested, such as parotid swelling, purpura,

lymphocytopenia, CD4/CD8 T cell ratio  $\leq 0.8$ , RF positivity, cryoglobulinemia, monoclonal component, and low C4 level. Therefore, the predictive value of *BTK* with regard to all of these validated predictors needed investigation. In our study, *BTK* remained overexpressed in patients with lymphoma compared with patients with moderate to severe disease activity (ESSDAI  $\geq 5$  controls) at enrollment. In addition, multivariate analyses showed the association between *BTK* expression and lymphoma, even when compared to “at-risk” patients with moderate to severe systemic disease activity (ESSDAI  $\geq 5$  controls). Of note, with the limited sample size and associated statistical power, wide CIs and risk of substantial residual confounding in the logistic regression models indicate that these findings from the multivariate analysis should be interpreted with caution and must be replicated in larger cohorts.

The extent of lymphocytic infiltration in salivary glands and, in one study, the presence of tertiary lymphoid organs within salivary glands were found to be associated with risk of lymphoma in primary SS (43,44). These parameters were not available in our study; however, high expression of BTK in parotid glands of primary SS patients was previously shown to be associated with the development of well-organized tertiary lymphoid organs displaying germinal centers (45).

Our present results are in accordance with 2 recent studies that showed increased expression of both BTK mRNA (46) and protein in B cells of patients with primary SS (45), which suggests a role for the BCR signaling pathway in primary SS pathogenesis. Downstream BCR signaling pathways, including BTK, have been recently investigated in autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, and primary SS, indicating a role for BTK in the activation of autoreactive B cells and production of antinuclear autoantibodies and RFs (45,47). Moreover, preclinical studies conducted on animal models of systemic autoimmune diseases, such as rheumatoid arthritis (48) and systemic lupus erythematosus (49), showed a strong *in vivo* effect of BTK inhibition, supporting its implication in autoimmunity. A second-generation BTK inhibitor, remibrutinib, recently showed promising results in the phase 2 clinical trial LOUISse in primary SS (50).

In our study, *BTK* expression had a broad range in patients with primary SS, resulting in some overlap between groups. This observation could indicate that *BTK* overexpression does not involve all patients with primary SS and that there might be a distinct group of patients for whom therapeutic inhibition of BTK might be more relevant. To date, no data are available on the role of BTK in primary SS-NHL. NHL emergence in primary SS is thought to be driven by 2 main features: chronic lymphocytic activation in the target organs of the disease, which also explains the predominance of the MALT-MZL subtype arising from salivary glands (8), and antigen-induced B cell stimulation (6,51). Commonly found mutations or translocations involving MALT1, which activate NF- $\kappa$ B in sporadic MALT-MZL, are rarely seen in

MALT-MZL with RF-BCR (24,25), suggesting alternative pathways in primary SS-associated MALT lymphoma that may involve autoreactive B cell selection, promotion, and growth through chronic BCR activation. Chronic antigen stimulation by autoantigens (such as Ro and La or others) occurring in secondary and ectopic lymphoid structures can lead to the production of immune complexes exported from germinal centers to the marginal zone.

Subsequently, BTK is prone to be chronically induced after BCR engagement, following the recognition of these immune complexes, in B cells expressing a cell surface immunoglobulin (BCR) with RF activity (23,51). BTK expression and activation might result in NF- $\kappa$ B hyperactivation and aberrant B cell survival and growth. BTK might represent a key player in the transition from polyclonal B cell activation to monoclonal malignant proliferation, which should be further investigated. These data, along with previous results, increase the rationale to target BTK in primary SS and primary SS-related B cell lymphoproliferative disorders.

Our study had several limitations. First, lymphoma is a rare complication in primary SS patients. In addition, to rule out the effect of rituximab and cyclophosphamide on molecular signatures, we excluded patients who recently received these drugs. Therefore, the number of patients with lymphoma available for analysis was limited, reducing the statistical power. The lack of a definitive expression signature based on the primary analysis does not exclude that such a signature could be identified in a larger study sample. The limited statistical power for the primary analysis and for rigorously comparing the i-primary SS-NHL and h-primary SS-NHL groups to one another illustrates that larger studies are needed to fully elucidate the pathogenesis of primary SS-related lymphomas. Acknowledgment of the multiple comparisons/dimensionalities of these analyses is warranted, and confirmation of these results with larger cohorts is crucial.

This was a peripheral whole blood transcriptomic study, which is of interest because of the accessibility of blood samples in daily practice. However, the analysis of whole blood explains the relatively small differences in fold change in expression of DEGs, notably those for *BTK*, which resulted in wide ORs and CIs. These wide CIs reveal instability in the models and substantial imprecision in the resulting effect estimates. In addition, a single-cell approach or transcriptomic studies of sorted cell subpopulations might identify sets of genes different from those shown in the present study. Finally, analyzing target organs of autoimmunity in primary SS, such as salivary glands and lymphoma tissues, would be highly valuable.

In conclusion, this gene expression profiling study of peripheral whole blood highlights the role of B cell activation-related genes and sheds light on other pathways potentially involved in primary SS-related lymphomas. In this study, BTK, a pivotal transducer of BCR, and APRIL, a cytokine involved in B cell activation and growth, were shown to be overexpressed in peripheral

blood samples from patients with primary SS before the occurrence of lymphoma. The association between BTK, APRIL, and lymphoma in primary SS needs confirmation in other prospective cohorts.

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## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Duret had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Duret, Sarau, Devauchelle-Pensec, Seror, Sibilia, Nocturne, Mariette, Gottenberg.

**Acquisition of data.** Duret, Schleiss, Kawka, Meyer, Ye, Larroche, Perdriger, Nocturne, Mariette, Gottenberg.

**Analysis and interpretation of data.** Duret, Schleiss, Kawka, Meyer, Ye, Sarau, Devauchelle-Pensec, Seror, Sibilia, Vallat, Fornecker, Nocturne, Mariette, Gottenberg.

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## APPENDIX A: INVESTIGATORS OF THE ASSESS COHORT

Investigators of the ASSESS cohort are as follows: Drs. Emmanuelle Dernis, Valerie Devauchelle-Pensec, Philippe Dieude, Jean-Jacques Dubost, Anne-Laure Fauchais, Vincent Goeb, Eric Hachulla, Pierre Yves Hatron, Claire Larroche, Véronique Le Guern, Jacques Morel, Aleth Perdriger, Carinne Salliot, Stephanie Rist, Alain Saraux, Jean Sibia, Olivier Vittecoq, Gaétane Nocturne, Philippe Ravaud, and Raphaële Seror.