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Role of Fms-like Tyrosine Kinase 3 Ligand as a Potential Biologic Marker of Lymphoma in Primary Sjögren's Syndrome

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Objective. **Patients with primary Sjögren's syndrome (SS) are at greater risk of developing lymphoma. This study was undertaken to evaluate whether the Fms-like tyrosine kinase 3 ligand (Flt-3L) might be associated with lymphoma in primary SS.**

Methods. **Serum levels of Flt-3L were measured in 369 patients with primary SS from the French Assessment of Systemic Signs and Evolution of Sjögren's Syndrome study cohort and in 10 patients with primary SS at the time of lymphoma diagnosis in an Italian cohort. Associations between increased levels of Flt-3L and a history of lymphoma, history of previously diagnosed criteria related to a high risk of lymphoma, and greater extent of disease activity were evaluated.**

Results. **Among patients with primary SS, higher levels of Flt-3L were significantly associated with a history of lymphoma ($P = 0.0001$). Previous markers for risk of lymphoma development, such as presence of purpura, low levels of C4, presence of lymphocytopenia, low levels of IgM, high levels of β_2-microglobulin, and a higher primary SS disease activity score, were all associated with higher levels of Flt-3L. The levels of Flt-3L were also increased in serum obtained from patients with primary SS at the time of lymphoma diagnosis. Furthermore, the Flt-3L levels were elevated in the serum of 6 patients up to 94 months (mean 46 months) prior to the diagnosis of lymphoma. Receiver operating characteristic curve analysis showed that an Flt-3L level of 175 pg/ml was the ideal cutoff value for demonstrating an association with lymphoma (specificity 97.5%, sensitivity 44%, negative predictive value 97%).**

Conclusion. **Flt-3L is associated with lymphoma in primary SS, and constitutes a good biologic marker. Higher levels of this cytokine are present several years before the diagnosis of lymphoma, and may be useful as a predictive marker of lymphoproliferative disorders in primary SS.**

Autoimmune epitheliitis, which is also known as Sjögren's syndrome (SS), occurs either alone, as primary SS, or in association with other autoimmune diseases, as secondary SS. SS predominantly affects women during the fourth and fifth decades of life (1,2). The spectrum of SS extends from an organ-specific autoimmune disorder to a systemic process that may involve the musculoskeletal system, lungs, kidneys, and blood vessels (3). Biologic abnormalities associated with B lymphocytes in primary SS are also a hallmark of the disease, and these

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abnormalities are characterized by the presence of rheumatoid factor (RF), hypergammaglobulinemia, anti-SSA and anti-SSB antibodies, and abnormal distribution of mature B lymphocytes in the peripheral blood, in addition to an increased risk of non-Hodgkin's lymphoma (NHL) in 5% of patients (4–6).

Several clinical and serologic markers have been recognized as predictors of the development of NHL in primary SS. Among these parameters, a higher risk of NHL has been observed in patients with primary SS whose clinical and serologic features include splenomegaly, persistent enlargement of the parotid glands (6–11), lymphadenopathy (6,9–11), palpable purpura (6,10,12–14), cryoglobulinemia (11,13–15), low levels of C4 (10–14,16,17), low levels of C3 (12,14,16,17), neutropenia (11), lymphocytopenia (6,11,12,17), anemia (6,11), monoclonal component (17), high levels of β_2-microglobulin (18), and hypogammaglobulinemia (17), as compared to patients without these risk factors.

The Fms-like tyrosine kinase 3 ligand (Flt-3L) is a type I transmembrane protein that can be released as a soluble homodimeric protein (19–21). Both the membrane-bound and the cell-free forms can activate Flt-3 and stimulate the growth of progenitor cells in the bone marrow and blood. Treatment of mice with Flt-3L results in significant stimulation of hematopoiesis, leading to bone marrow hyperplasia, splenomegaly, and enlargement of the lymph nodes and liver (19–21). In addition, Flt-3L levels are abnormally increased in the majority of leukemias (22), and Flt-3 mutations are present in 30% of patients with acute myeloid leukemia (23).

In a recent study, we demonstrated that serum levels of Flt-3L were elevated in 64 patients with primary SS as compared to healthy controls (24). These higher levels were correlated with abnormal B cell distribution in the peripheral blood (naive:memory B cell ratio $\geq$5) (5) and with the extent of disease activity as evaluated using the European League Against Rheumatism (EULAR) Sjögren's Syndrome Disease Activity Index (ESSDAI) (25). In addition, 2 patients who presented with lymphoma during the study had high serum levels of Flt-3L. Thus, in order to confirm that Flt-3L levels are a marker of lymphoproliferation in patients with primary SS, we evaluated the serum levels of Flt-3L in samples from 369 patients with primary SS enrolled in the prospective cohort of the Assessment of Systemic Signs and Evolution of Sjögren's Syndrome (ASSESS) study in France, and in samples from 10 patients with primary SS (obtained at the time of lymphoma development) in an Italian cohort.

PATIENTS AND METHODS

Patients and controls. Serum levels of Flt-3L were determined in the first 369 patients enrolled in the French ASSESS cohort of patients with primary SS, a cohort comprising a total of 395 patients with primary SS with or without extraglandular manifestations. Healthy control subjects consisted of 50 members of the staff and healthy residents of a home for the elderly. Healthy controls were sex- and age-matched to the patients with primary SS. All patients fulfilled the American–European Consensus Group amended classification criteria for SS (26). Connective tissue disease controls comprised 19 patients with systemic lupus erythematosus (SLE), 30 patients with rheumatoid arthritis (RA), and 10 patients with systemic sclerosis (SSc); all of these patients satisfied the diagnostic criteria for their respective diseases (27–29).

The ASSESS cohort is a nationwide cohort of patients with primary SS (recruited from a total of 15 centers in France) that was created in 2006 to evaluate predictive factors for the development of systemic complications and development of NHL in primary SS (30). All patients and controls gave their written informed consent to participate, and the study was approved in 2006 by the Ethics Committee of Hôpital Bichat and by the Commission Nationale Informatique et Libertés.

An aliquot of serum obtained at enrollment was immediately frozen, stored, and shipped to the Centre de Ressources Biologiques (Hôpital Bichat), which has received quality certification from the French Association for Quality Insurance (according to the norm NFS 96900, certification no. 2009/34457). In order to assess serum levels of Flt-3L in patients with primary SS at the time of lymphoma development, 10 patients with primary SS from an Italian cohort were enrolled. In addition, 4 of these patients, and 2 from the ASSESS cohort, were also analyzed for Flt-3L levels prior to the diagnosis of lymphoma.

Enzyme-linked immunosorbent assay (ELISA) for Flt-3L. Flt-3L levels were measured with a commercial kit that uses a 2-step sandwich ELISA (R&D Systems). The procedure was done according to the manufacturer's instructions, as has been described previously (24). An Flt-3L value of $\geq$120 pg/ml (i.e., 4 SD above the mean value in 50 healthy controls) was considered positive for increased Flt-3L levels (FL+). The test was done twice for each patient and control, and the mean levels were calculated. Three samples of known concentration were tested 20 times on our plate to assess intraassay precision, and 40 separate assays were performed to assess interassay precision. The intraassay coefficients of variation (CVs) ranged from 1.4% to 2.7%, and the interassay CVs ranged from 6.2% to 11.1%. The minimum detectable dose of Flt-3L was $<$7 pg/ml. No association between the RF titer and Flt-3L levels was found.

Clinical and serologic variables. The status of Flt-3L in the serum of patients with primary SS was evaluated for associations with the following factors: 1) previous occurrence of lymphoma; 2) history of previously diagnosed factors linked to a high risk of lymphoma, such as presence of purpura, low levels of C4, enlargement of the parotid glands, and presence of cryoglobulinemia; and 3) subjective patient-reported symptoms assessed with the EULAR Sjögren's Syndrome Patient-Reported Index (ESSPRI) (31), and extent of disease activity

evaluated with the ESSDAI (25). The measurement of Flt-3L levels by ELISA and the determination of all clinical and serologic characteristics in the patients were performed at the inclusion visit and collected from the clinical charts, using a predetermined protocol form. In addition, new clinical and/or serologic characteristics were recorded prospectively on the form. Special emphasis was placed on lymphoma-related risk factors and clinical characteristics of lymphoma. In the sera of 6 patients whose specimens were available prior to the development of NHL, the serum Flt-3L levels were measured in order to evaluate whether any previous elevation in Flt-3L levels could be predictive of lymphoma development.

Immunofluorescence staining of parotid gland biopsy tissue. Biopsy specimens of the parotid gland from 2 patients with primary SS (obtained at lymphoma diagnosis; Italian cohort) were analyzed as previously described (24). Briefly, a 2-color staining procedure was performed. In the first step, anti-human Flt-3 monoclonal antibodies (Santa Cruz Biotechnology) were used, in conjunction with rabbit anti-human CD20 antibodies (Interchim). After a 40-minute incubation and 3 washes in phosphate buffered saline (PBS), the second staining step consisted of incubating the first slide for a further 40 minutes with fluorescein isothiocyanate (FITC)–conjugated donkey anti-mouse antibodies along with tetramethylrhodamine isothiocyanate–conjugated donkey anti-rabbit antibodies (both from Jackson ImmunoResearch). After 3 washes in PBS, the sections were fixed with 4% cold paraformaldehyde, a coverslip was added (Vector), and the tissue was analyzed using a TCS-NT Leica confocal imaging system (Wetzlar). No background fluorescence was observed in cultures with control mouse IgG plus FITC-conjugated donkey anti-mouse antibodies.

Statistical analysis. In comparisons between FL+ and FL− patients and between patients with or without lymphoma in the ASSESS cohort, data were analyzed using SPSS version 15.0. The chi-square test (or Fisher's exact test, as appropriate) and Mann-Whitney test were used for univariate analyses of the data collected. Variables showing a significant association with lymphoma (defined as P values less than 0.1) in univariate analyses were entered into a multivariable regression model with forward selection. Logistic regression was used to identify independent markers of lymphoma in patients with primary SS. The sensitivity and specificity of Flt-3L levels for a diagnosis of lymphoma were assessed at different cutoff values, and results are presented as receiver operating characteristic (ROC) curves.

RESULTS

Increased Flt-3L levels in patients with primary SS. Among the 369 patients with primary SS from the French ASSESS cohort, 18 had previously received a diagnosis of lymphoma (mean ± SD 7.4 ± 3.4 years before inclusion in the cohort). In 15 of these patients, the lymphoma had been in complete remission, without treatment, for 7.0 ± 2.2 years before inclusion, while 3 of the patients were still receiving treatment for lymphoma at the time of inclusion.

Serum levels of Flt-3L were significantly higher in the 369 patients with primary SS compared to the 50 healthy control subjects in the ASSESS cohort (mean ± SD 107.1 ± 30.0 pg/ml versus 64.4 ± 14.5 pg/ml; $P < 0.01$). The serum levels of Flt-3L in the 18 patients with primary SS who had a history of lymphoma were found to be significantly higher than the levels in patients with primary SS without lymphoma ($P < 0.0001$), and also significantly higher than the levels in patients with other connective tissue diseases, such as SLE ($P < 0.02$), RA ($P < 0.0003$), or SSc ($P < 0.001$) (Figure 1A).

In addition, in the ASSESS cohort, 103 patients (28%) were considered to be FL+, having serum levels of Flt-3L that were ≥120 pg/ml. Whereas no association between the Flt-3L status and the ratio of men to women was observed, FL+ patients were older than FL− patients (mean ± SD 62.2 ± 11.3 years versus 56.3 ± 12.1 years) ($P < 0.0001$), and FL+ patients had a longer duration of disease (Table 1). In healthy controls (mean ± SD age 55.3 ± 10.4 years), the Flt-3L levels were not correlated with age ($P = 0.37$). Subjective symptoms of dryness, pain, or fatigue evaluated by the ESSPRI questionnaire were not statistically significantly associated with Flt-3L levels. In contrast, the ESSDAI scores of disease activity were significantly associated with levels of Flt-3L ($P = 0.03$). This correlation persisted even when we excluded the 3 patients with active lymphoma at the time of evaluation ($P = 0.05$). Finally, Flt-3L levels were not associated with the presence of RF, anti-SSA antibodies, or anti-SSB antibodies (Table 1).

Flt-3L levels and associations with clinical and serologic markers of NHL in primary SS. Several clinical and immunologic characteristics have been previously described as markers of lymphoproliferative disease in primary SS. Among these parameters, patients with primary SS who develop splenomegaly, persistent enlargement of the parotid glands, lymphadenopathy, palpable purpura, cryoglobulinemia, low levels of C4, neutropenia, or lymphocytopenia have a higher risk of NHL compared to patients with none of these risk factors. We therefore assessed correlations of the Flt-3L levels with every clinical and biologic parameter that has been previously described as a marker of lymphoproliferation in primary SS.

No association was observed between increased levels of Flt-3L and a history of splenomegaly, lymph-

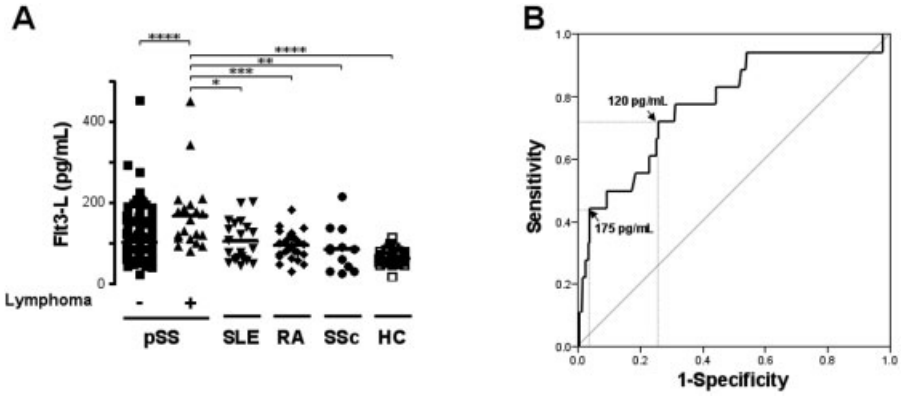

Figure 1. Elevated serum levels of Fms-like tyrosine kinase 3 ligand (Flt-3L) and lymphoma development in patients with primary Sjögren's syndrome (pSS) from the French Assessment of Systemic Signs and Evolution of Sjögren's Syndrome (ASSESS) cohort. **A,** Serum Flt-3L levels were compared between patients with primary SS without a history of lymphoma (n = 369), patients with primary SS with previous lymphoma (n = 18), patients with systemic lupus erythematosus (SLE) (n = 19), patients with rheumatoid arthritis (RA) (n = 30), patients with systemic sclerosis (SSc) (n = 10), and healthy control (HC) subjects (n = 50). Bars show the mean. The median serum Flt-3L concentrations were as follows: primary SS without lymphoma, 94.6 pg/ml; primary SS with lymphoma, 144.1 pg/ml; SLE, 86.9 pg/ml; RA, 95.0 pg/ml; SSc, 84.3 pg/ml; healthy controls, 61.6 pg/ml. * = $P < 0.02$; ** = $P < 0.001$; *** = $P < 0.0003$; **** = $P < 0.0001$, by Mann-Whitney test. **B,** Receiver operating characteristic curve analysis shows that an Flt-3L level of 175 pg/ml is the ideal cutoff value to detect an association with previous lymphoma in the ASSESS cohort, with a sensitivity of 44% and specificity of 97.5%. The cutoff value of ≥120 pg/ml was used to define elevated levels of Flt-3L (a status of FL+).

Table 1. Main demographic, clinical, and biologic characteristics of the 369 patients with primary Sjögren's syndrome from the French ASSESS cohort, according to Flt-3L status*

	FL+ (n = 103)	FL− (n = 266)	*P*
Demographic features			
Age, mean ± SD years	62.18 ± 11.3	56.25 ± 12.1	<0.0001
Sex, no. male/no. female	5/98	19/247	NS
Disease duration, mean ± SD years	7.53 ± 5.7	6.12 ± 5.7	0.007
Disease activity			
ESSPRI, mean ± SD	5.3 ± 2.3	5.3 ± 2.05	NS
ESSDAI, median (range)	4 (0–31)	2 (0–24)	0.03
Biologic parameters			
Anti-SSA antibodies	65/38	152/114	NS
Anti-SSB antibodies	31/72	88/178	NS
Rheumatoid factor titer, mean ± SD	20.18 ± 44	23.06 ± 75.92	NS
History of clinical features			
Enlargement of parotid glands	16/87	44/222	NS
Purpura	20/83	24/237	0.008
Splenomegaly	4/97	7/259	NS
Lymphadenopathy	19/84	33/233	NS
Previous lymphoma	13/90	5/261	0.0001
Biologic findings at inclusion			
Mixed monoclonal cryoglobulinemia	20/78	32/224	NS
Lymphocytopenia	16/84	8/248	0.004
IgM levels ≤0.5 gm/liter	20/82	27/238	0.024
β_2-microglobulin, median (range) mg/liter	2.3 (2.1–3.1)	2 (1.8–2.6)	0.001
C4, mean ± SD gm/liter	0.21 ± 0.088	0.24 ± 0.16	0.05

* A status of FL+ was defined as levels of Fms-like tyrosine kinase 3 ligand (Flt3-L) of ≥120 pg/ml. Except where indicated otherwise, values are the number of patients with/number of patients without the feature. ASSESS = Assessment of Systemic Signs and Evolution of Sjögren's Syndrome; NS = not significant; ESSPRI = European League Against Rheumatism Sjögren's Syndrome Patient-Reported Index; ESSDRI = European League Against Rheumatism Sjögren's Syndrome Disease Activity Index.

Table 2. History of clinical features and biologic findings at inclusion in patients with primary Sjögren's syndrome from the French ASSESS cohort, according to history of lymphoma*

	Lymphoma+ (n = 18)	Lymphoma− (n = 351)	*P*
History of clinical features			
Enlargement of parotid glands	5/13	55/296	NS
Purpura	6/12	38/308	0.007
Splenomegaly	4/14	8/341	0.0001
Lymphadenopathy	9/9	42/309	0.0001
Biologic findings at inclusion			
Flt-3L levels ≥120 pg/ml	13/5	90/261	0.0001
Mixed monoclonal cryoglobulinemia	5/13	55/281	NS
Lymphocytopenia	6/12	15/323	0.004
IgM levels ≤0.5 gm/liter	4/14	43/306	NS
C4, mean ± SD gm/liter	0.18 ± 0.1	0.24 ± 0.14	0.001
β_2-microglobulin, median (range) mg/liter	2.52 (2.1–3.1)	2.1 (1.8–2.6)	0.03

* Except where indicated otherwise, values are the number of patients with/number of patients without the feature. ASSESS = Assessment of Systemic Signs and Evolution of Sjögren's Syndrome; NS = not significant; Flt-3L = Fms-like tyrosine kinase 3 ligand.

adenopathy, enlargement of the parotid glands, or cryoglobulinemia (Table 1). In contrast, a history of purpura was significantly associated with elevated levels of Flt-3L. Similarly, biologic markers, such as low levels of C4, presence of lymphocytopenia, low levels of IgM, and high levels of β_2-microglobulin, were also associated with higher levels of Flt-3L.

Association of Flt-3L levels with previous lymphoproliferative disease in primary SS. The analysis of clinical parameters in the French ASSESS cohort showed that lymphoma was associated with antecedent purpura, splenomegaly, and lymphadenopathy. In addition, the analysis of biologic parameters showed that presence of lymphocytopenia, low levels of C4, and high levels of Flt-3L were all significantly associated with previous occurrence of lymphoma (Table 2). Among these biologic markers, the finding of elevated Flt-3L levels at inclusion was the most highly significantly associated with a history of lymphoma in univariate analyses.

Analysis of independent parameters associated with history of lymphoma in the French primary SS ASSESS cohort. When we compared risk factors associated with previous lymphoma by multivariate analysis, we observed that Flt-3L levels of ≥120 pg/ml, history of lymphadenopathy, and history of splenomegaly were all independent factors. Thus, we conducted a logistic regression analysis to determine which criterion was the most strongly associated with lymphoma. Based on the results of this analysis, only 2 items were selected as highly significant, history of splenomegaly (odds ratio [OR] 56.4, 95% confidence interval [95% CI] 14.1–223.6) and Flt-3L levels of ≥120 pg/ml (OR 17.3, 95% CI 5.8–50.9), confirming that an elevated Flt-3L level is the only biologic parameter associated with a history of lymphoma.

We then conducted a ROC analysis. Although Flt-3L levels of ≥120 pg/ml had a high sensitivity for detection of lymphoma (72%), we showed that an Flt-3L level of 175 pg/ml was the ideal cutoff value to detect an association with previous lymphoma. A serum Flt-3L level of 175 pg/ml had a sensitivity of 44%, specificity of 97.5%, positive predictive value of 38%, and negative predictive value of 97% (Figure 1B).

Flt-3L levels and associations with NHL in primary SS. Eighteen patients from the French primary SS cohort (4.9%) presented with a history of NHL, 12 of whom had previously been treated with rituximab. This clinical antecedent was statistically significantly associated with higher levels of Flt-3L ($P < 0.0001$). Among the NHL types, 8 patients presented with mucosa-associated lymphoid tissue (MALT)–type lymphoma, 4 had nodal marginal-zone B cell lymphoma, 3 had diffuse large B cell lymphoma, and 3 had other types of lymphoproliferative disease. All 8 patients with previous MALT-type lymphoma had levels of Flt-3L that met the cutoff for being FL+ (mean ± SD 216.5 ± 119.0 pg/ml). These levels in patients with previous MALT-type lymphoma were higher than those in patients with previous diffuse large B cell lymphoma (mean ± SD 167 ± 59.2 pg/ml) and patients with previous nodal marginal-zone B cell lymphoma (mean ± SD 134.0 ± 30.6 pg/ml). Flt-3L levels remained higher in patients with NHL even after treatment, which might indicate that there was persistent B cell activation.

Furthermore, high levels of Flt-3L (343.9 pg/ml, 211.5 pg/ml, and 157.0 pg/ml) were present in the 3 patients

Table 3. Clinical characteristics, treatments, and Flt-3L levels in the 18 patients with lymphoma from the French ASSESS cohort*

Patient/ sex	Lymphoma type	Lymphoma location	Clinical stage†	Main treatments	Relapse	Flt-3L, pg/ml
1/F	DLBCL	Skin	IE	Chemotherapy (interferon alfa)	No	99.5
2/M	MALT	Parotid gland	IV	Surgical	No	131.0
3/F	MALT	Parotid gland	IV	Chemotherapy (chlorambucil, rituximab)	Yes	343.9
4/F	NMZ	Lymphatic node	IV	Rituximab	Yes	157.0
5/F	MALT	Parotid gland	IV	Chlorambucil	Yes	211.5
6/F	NMZ	Lymphatic node + bone marrow	IV	Mini-CHOP	No	92.6
7/F	MALT	Submaxillary gland	IE	Mini-CHOP	No	178.0
8/M	NMZ	Lymphatic node + bone marrow	IV	Chlorambucil + fludarabine	No	176.9
9/F	NMZ	Spleen	I	Surgical	No	112.0
10/F	EBV-associated	Lymphatic node + bone marrow + spleen	IV	Chemotherapy (ACVBP + rituximab)	No	131.0
11/F	MALT	Gastric	IE	Chlorambucil, rituximab	No	120.4
12/F	DLBCL	Lymphatic node	III	CHOP, rituximab	No	208.0
13/F	MALT	Skin	IV	Surgical	No	121.0
14/F	MALT	Lung	IE	Chlorambucil	No	451.4
15/F	DLBCL	Lymphatic node + bone marrow	IV	Rituximab + autologous stem cell transplantation	No	195.9
16/F	MALT	Parotid gland + lymphatic node	IV	Chlorambucil + rituximab	No	185.5
17/F	Mycosis fungoides	Skin	IV	Local chlorethamine	No	130.0
18/F	MALT	Parotid gland	IE	Mini-CHOP	No	123.9

* Flt-3L = Fms-like tyrosine kinase 3 ligand; ASSESS = Assessment of Systemic Signs and Evolution of Sjögren's Syndrome; DLBCL = diffuse large B cell lymphoma; MALT = mucosa-associated lymphoid tissue; NMZ = nodal marginal zone; CHOP = cyclophosphamide, doxorubicin, vincristine, and prednisone; EBV = Epstein-Barr virus; ACVBP = doxorubicin, cyclophosphamide, vindesine, bleomycin, and prednisone.
† Clinical stages are defined according to the Ann Arbor staging system for lymphoma.

with relapsing disease who were still receiving treatment for lymphoma at the time of study inclusion. The clinical characteristics of all 18 patients with lymphoma from the ASSESS cohort are presented in Table 3.

High levels of Flt-3L were found in the serum of patients with primary SS at the time of diagnosis of lymphoma, but also found in the serum of patients prior to lymphoma development. To determine whether the level of Flt-3L might also be higher when patients develop active lymphoma, we analyzed Flt-3L expression

Table 4. Clinical characteristics, treatments, and Flt-3L levels in the 10 patients with current lymphoma in the Italian cohort*

Patient/ sex	Lymphoma type	Lymphoma location	Clinical stage†	Main treatments	Relapse/current treatment	Flt-3L, pg/ml
1/F	MALT	Parotid	IE	R-CHOP	Died (due to pancytopenia secondary to chemotherapy)	90.8
2/F	MALT	Parotid, nodal	IV	R-CHOP	No	144.0
3/F	MALT	Parotid	IE	R-CVP + radiotherapy (30.6 Gy)	No	99.9
4/F	MALT	Parotid, nodal	IV	Rituximab (375 mg/m^2 weekly for 8 weeks)	No	123.1
5/F	MALT	Parotid, nodal	IV	No treatment	No	68.5
6/F	MALT, then evolution into DLBCL	Parotid, lung, liver	IV	Lobectomy, R-CHOP, stem cell transplantation	No	176.5
7/M	MALT	Parotid, nodal	IV	R-CHOP	Died (due to pancytopenia secondary to chemotherapy)	123.2
8/F	MALT	Parotid, nodal	IV	R-CVP	No	180.4
9/M	MALT	Lacrimal glands	IE	No treatment	No	75.6
10/F	MALT	Parotid, nodal	IV	No treatment	No	141.6

* Flt-3L = Fms-like tyrosine kinase 3 ligand; MALT = mucosa-associated lymphoid tissue; R-CHOP = rituximab with cyclophosphamide, doxorubicin, vincristine, and prednisone; R-CVP = rituximab with cyclophosphamide, vincristine, and prednisone; DLBCL = diffuse large B cell lymphoma.
† Clinical stages are defined according to the Ann Arbor staging system for lymphoma.

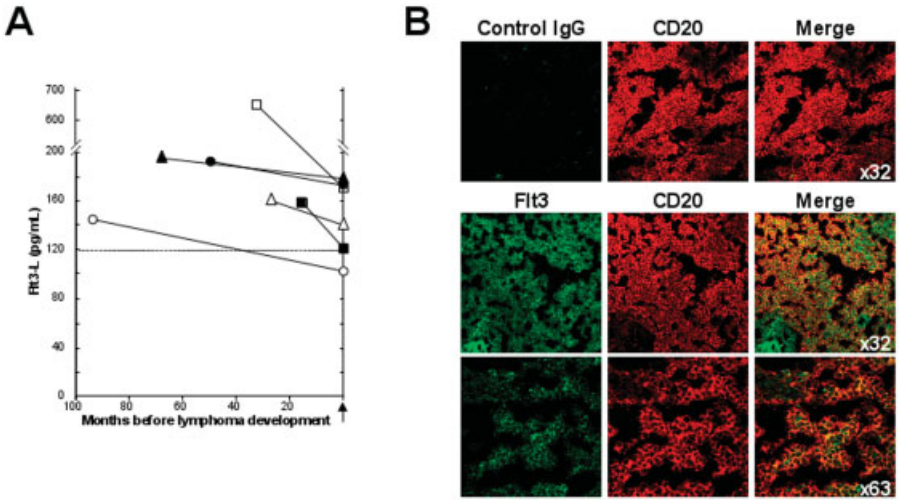

Figure 2. Elevated serum levels of Fms-like tyrosine kinase 3 ligand (Flt-3L) in patients with primary Sjögren's syndrome (SS) and a previous diagnosis of non-Hodgkin's lymphoma, and staining of lymphoma cells for Flt-3 expression. **A,** Six patients with primary SS showed increased levels of Flt-3L in the serum before the time of lymphoma diagnosis (indicated by **arrow**). Increased Flt-3L levels were defined as levels of ≥120 pg/ml, as detected by enzyme-linked immunosorbent assay. **B,** Biopsy sections of the parotid glands of patients with primary SS, obtained at the time of lymphoma diagnosis, were stained with rabbit anti-human CD20 antibodies, with results revealed using tetramethylrhodamine isothiocyanate–conjugated donkey anti-rabbit antibodies, along with either IgG isotype control (top) or anti–Flt-3 monoclonal antibodies (bottom), with results revealed using fluorescein isothiocyanate–conjugated donkey anti-mouse antibodies. The overlay of green-stained Flt-3 with red-stained B cells is visualized as yellow staining in the merged images.

in the serum of 10 Italian patients with primary SS collected at the time of lymphoma diagnosis. In this cohort, all cases of NHL were of the MALT type, and in 1 patient, NHL progressed to diffuse large B cell lymphoma (Table 4). Six patients were FL+; the mean ± SD level of Flt-3L in the whole cohort was 122.4 ± 37.0 pg/ml. Although none of the patients had splenomegaly, a history of parotid gland enlargement, history of lymphocytopenia, low levels of C4, and high levels of Flt-3L were all associated with current lymphoma. These results suggest that Flt-3L levels are also higher at the time of diagnosis of lymphoma.

In the 6 patients with primary SS (4 from Italy and 2 from the French cohort) whose serum samples were available prior to the development of lymphoma, the serum levels of Flt-3L were found to be elevated up to 94 months before the diagnosis of NHL (mean level 249 pg/ml at a mean 46 months prior to diagnosis) (Figure 2A). This interesting result highlights the possible utility of Flt-3L levels as a predictive factor for lymphoma development.

Expression of Flt-3 by lymphoma cells. To establish a causal relationship between Flt-3L levels and lymphoma, we investigated the presence of Flt-3 on lymphoma cells in 2 patients from the Italian cohort. Flt-3 expression was identified in infiltrating lymphoma cells from the parotid gland biopsy tissue of these patients at the time of diagnosis of MALT-type lymphoma (Figure 2B). These findings, as well as our previous observations that Flt-3L is also produced by epithelial cells (24), suggest that Flt-3L could be implicated in the survival of lymphoma cells that express the Flt-3 receptor.

DISCUSSION

Previous reported findings indicate that B cells have a major role in the pathogenesis of primary SS (16,18). In addition to biologic abnormalities in the B cell expression profile in this disease (5), 5% of patients may develop NHL. The clinical association between primary SS and NHL has been shown in several studies (6,7). Thus, it is important to determine which patients are at risk of progression to lymphoma. In this study, we established that Flt-3L levels are elevated and are correlated with a history of lymphoma in patients with primary SS.

Flt-3L plays a role in normal B cell development. Its level is increased in hematologic malignancies, and we previously demonstrated that serum levels of Flt-3L were correlated with the abnormal distribution of B cells in the peripheral blood and with the extent of disease

activity in patients with primary SS (24). Herein, in this larger study, we confirmed that the levels of Flt-3L were higher in patients with primary SS (n = 369) compared to healthy controls, and this was associated with the ESSDAI score of disease activity. In addition, a history of lymphoma was significantly associated with higher levels of Flt-3L ($P < 0.0001$). The cutoff value for the level of Flt-3L that was found to have the most specific association with NHL was 175 pg/ml (sensitivity 44.4%, specificity 97.5%). Higher expression of Flt-3L is also associated with other lymphoma risk factors that have been classically described, such as palpable purpura, low levels of C4, lymphocytopenia, low levels of IgM, and high levels of β_2-microglobulin. However, no association with persistent splenomegaly, cryoglobulinemia, low levels of C3, and neutropenia has been found. Among all of the biologic markers already described, the Flt-3L level was the biologic marker most significantly associated with lymphoma.

Flt-3L levels were also found to be elevated in small groups of patients with autoimmune diseases (e.g., SLE or RA) as compared to the levels in healthy controls (data not shown). The clinical associations of these levels have to be further studied. In contrast, patients with spondyloarthritides did not show higher levels of Flt3-L as compared to controls (data not shown).

In addition, the 3 patients previously treated with immunosuppressive drugs (cyclophosphamide, intravenous immunoglobulin, or rituximab) had higher levels of Flt-3L ($P = 0.002$, $P = 0.026$, and $P = 0.003$, respectively). This finding may be explained by the fact that these patients had more severe disease, including systemic complications and lymphoma, that required immunosuppressive treatment. The levels of Flt-3L seem to remain stable during disease evolution, since we observed that the serum levels of Flt-3L before and after B cell depletion by rituximab showed no difference in 8 patients (mean ± SD 106.2 ± 39.7 pg/ml and 104.2 ± 32.5 pg/ml, respectively). These results need to be confirmed in other studies, which would require a higher number of patients. Nevertheless, if Flt-3L levels are stable and not influenced by immunosuppressive drugs or biologic therapy, the determination of Flt-3L levels at the diagnosis of primary SS could be useful for distinguishing patients at high risk of developing severe disease, including lymphoma.

More interestingly, levels of Flt-3L remained during treatment in patients with lymphoma. In patients with hematologic malignancies, higher Flt-3L levels are associated with advanced disease before treatment and residual disease after treatment. Retzlaff and colleagues showed that before treatment, the serum levels of Flt-3L correlated with prognostic variables and a high-grade lymphoma subtype in patients with primary gastrointestinal NHL (32). In acute lymphoblastic leukemia, treatment with Flt-3L renders cell lines and primary leukemia cells resistant to chemotherapy agents (33), thus showing that high levels of Flt-3L may contribute, at least in part, to persistent minimal residual disease in the bone marrow. Thus, persistently high levels of Flt-3L after treatment of lymphoma may indicate the presence of minimal residual disease, B cell activation, and, probably, a high risk of relapse. In our study, 3 patients with relapsing disease after chemotherapy presented with high levels of Flt-3L. This observation needs to be confirmed in the prospective followup of our cohort. However, higher levels of Flt-3L were associated with previous MALT-type lymphoma, instead of the more aggressive subtype, diffuse large B cell lymphoma. This may explain the higher numbers of patients with MALT-type lymphoma compared to patients with diffuse large B cell lymphoma in both of our cohorts. Additional studies will be required to confirm that Flt-3L is a marker for the development of MALT-type lymphoma in patients with primary SS.

An alternative explanation for our results would be that Flt-3L is a marker of disease activity, and that patients with primary SS and lymphoma have a greater extent of disease activity, which remains at a higher level even after the lymphoma goes into remission. In support of this hypothesis, Flt-3L levels were correlated with the ESSDAI, a clinical activity score that has been recently developed and validated. Moreover, this correlation persisted even when we excluded the 3 patients with active lymphoma at the time of evaluation, since these latter 3 patients obviously had an ESSDAI score that was greatly increased (addition of 12 points) due to the presence of active lymphoma (25).

Flt-3L levels were also increased in the serum of patients with primary SS at the time of diagnosis of lymphoma. In these patients with primary SS (from an Italian cohort), the levels of BAFF (assessed by ELISA) were associated with lymphoma and the ESSDAI score (34). Interestingly, in the 10 patients analyzed at the time of lymphoma diagnosis, BAFF levels and Flt-3L levels were correlated (Pearson's correlation = 0.75, $P = 0.032$) (results not shown). Therefore, in the future, an observed association of the levels of BAFF, when quantified using a kit that is unable to recognize all forms of BAFF present in the serum (35), with the levels of

Flt-3L could support the diagnosis of lymphoma in patients with primary SS.

One of the most interesting findings of the present study, from a clinical perspective, was the presence of high serum Flt-3L levels prior to the development of lymphoma—up to 94 months preceding the diagnosis. This implies that followup assessments (both clinical and imaging) of patients with persistently high levels of Flt-3L are necessary in order to predict the risk of lymphoma development as early as possible. This result must be verified in the ongoing analysis of prospective data from the French ASSESS cohort.

Finally, high levels of Flt-3L may also imply the existence of a nonmalignant lymphoproliferative disorder many years before the diagnosis of NHL. This nonmalignant B cell proliferation indeed starts a long time before the diagnosis of NHL in patients with primary SS (36). Consequently, in addition to the possible role of Flt-3L as a predictor of NHL in patients with primary SS, high levels of Flt-3L could also indicate a predisposition for indolent, nonmalignant B cell proliferation to evolve into NHL.

In conclusion, although these results need to be verified in other cohorts, routine evaluation of Flt-3L levels in patients with primary SS seems to be useful in the assessment of the clinical severity and activity of the disease. The role of Flt-3L in B cell proliferation may explain the clinical evolution to B cell lymphoma in some patients, and targeting Flt-3L might therefore open new therapeutic options. Further followup of the ASSESS cohort will be required to assess whether the baseline level of Flt-3L might also be predictive of the occurrence of incident lymphoma.

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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. Pers 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.

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