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Pre-diagnosis plasma immune markers and risk of non-Hodgkin lymphoma in two prospective cohort studies

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ABSTRACT

Inflammation and B-cell hyperactivation have been associated with non-Hodgkin lymphoma development. This prospective analysis aimed to further elucidate pre-diagnosis plasma immune marker profiles associated with non-Hodgkin lymphoma risk. We identified 598 incident lymphoma cases and 601 matched controls in Nurses' Health Study and Health Professionals Follow-up Study participants with archived pre-diagnosis plasma samples and measured 13 immune marker levels with multiplexed immunoassays. Using multivariable logistic regression we calculated Odds Ratios (OR) and 95% Confidence Intervals (CI) per standard deviation unit increase in biomarker concentration for risk of non-Hodgkin lymphoma and major histological subtype, stratifying additional models by years (<5, 5 to <10, ≥10) after blood draw. Soluble interleukin-2 receptor-α, CXC chemokine ligand 13, soluble CD30, and soluble tumor necrosis factor receptor-2 were individually positively associated, and B-cell activating factor of the tumor necrosis factor family inversely associated, with all non-Hodgkin lymphoma and one or more subtypes. The biomarker combinations associated independently with lymphoma varied somewhat by subtype and years after blood draw. Of note, the unexpected inverse association between B-cell activating factor and chronic lymphocytic leukemia/small lymphocytic lymphoma risk (OR: 95%CI: 0.51, 0.43-0.62) persisted more than ten years after blood draw (OR: 0.70; 95%CI: 0.52-0.93). In conclusion, immune activation precedes non-Hodgkin lymphoma diagnosis by several years. Decreased B-cell activating factor levels may denote nascent chronic lymphocytic leukemia many years pre-diagnosis.

Introduction

Severe immune compromise is a strong risk factor for non-Hodgkin lymphoma (NHL), and B-cell activation and inflammation have been associated with an increased risk of AIDS-related NHL. Elevated pre-diagnosis plasma levels of markers of B-cell stimulation including CXC chemokine ligand 13 (CXCL13; a B-cell attracting chemokine),[1] interleukin (IL)-6 (a B-cell stimulatory cytokine), and soluble (s) CD30 (sCD30; a soluble receptor indicative of B- and T-cell activation) pre-

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dicted risk of an AIDS-NHL diagnosis in HIV-positive persons,[2-4] in some instances as early as five years pre-diagnosis.[5] Several of these markers have also demonstrated an association with NHL risk in immunocompetent people in prospective studies.[6-13] Of interest, plasma sCD30 levels were positively associated with NHL risk at 6-10 years[9] and even 15-23 years pre-diagnosis.[11] Another small nested case-control study reported a significant 2.5-fold increase in NHL risk in women with elevated soluble IL-2 receptor-α levels (sIL-2Rα; a marker of T-cell activation and IL-2 upregulation), and marginally significant increases in NHL risk in women with higher pre-diagnosis tumor necrosis factor (TNF)-α and soluble TNF-receptor-2 (sTNF-R2) levels.[14] These findings collectively suggest that chronic B-cell stimulation has a role in lymphomagenesis in immunocompetent persons.

Our study aimed to further characterize pre-diagnosis plasma immune marker profiles associated with risk of HIV-unrelated NHL and its major histological subtypes in two large US cohorts. This study represents one of the largest populations with prospectively collected pre-diagnosis blood samples to investigate the association between numerous immune markers and NHL risk, including those with specific NHL subtypes that are often precluded due to small sample size, and to assess the independence of biomarker-NHL associations for multiple immune markers.[11,12] The long-term follow up of the study population also allowed for examination of the influence of time since blood draw on observed immune marker-NHL associations, including an assessment of potential early markers of lymphomagenesis present ten years or more prior to diagnosis. The choice of immune markers was guided in part by the immune deregulation we sought to characterize and by reported findings in AIDS- or HIV-unrelated NHL. We hypothesized that pre-diagnosis levels of immune markers indicative of B-cell activation or inflammation would be positively associated with risk of developing NHL and major NHL subtypes, and that the use of multi-marker models will enhance characterization of the immune milieu associated with NHL risk and suggest subtle differences by histological subtype.

Methods

Study population

The study population comprised Nurses' Health Study (NHS, all female) and Health Professionals Follow-up Study (HPFS, all male) participants with archived plasma (*Online Supplementary Methods*).[15,16] Cancer diagnoses were identified *via* routine questionnaires or follow up after death[17,18] and confirmed by medical record review or tumor registry linkage.

Participants provided written informed consent at blood collection. The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Boards of the Brigham and Women's Hospital and Harvard T.H. Chan School of Public Health.

Case and control selection

We included all participants with confirmed incident NHL diagnosed three months or more after blood draw through 31st December 2010 with no other cancer history. Study pathologists (JCA, SJR) classified NHL histological subtype[19] according to World Health Organization[20,21] and International Lymphoma Epidemiology (InterLymph) Consortium guidelines.[22,23] We analyzed common B-cell (B-)NHL subtypes individually [diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL)], combined less common B-NHLs ("other B-NHL") and defined additional categories by cell type (T-NHL, B-NHL). We matched one control per case by sex (cohort), age, race, and blood draw details (*Online Supplementary Methods*).

Biomarker assessment

Assays were performed at the University of California, Los Angeles (LM, OMM), using multiplexed kits (Fluorokine® MAP, R & D Systems, Minneapolis, MN, USA), a Bio-Plex 200 Luminex instrument and Bio-Plex analysis software (Bio-Rad, Hercules, CA, USA). Blinded laboratory personnel measured sCD30, sIL-2Rα, B-cell activating factor of the TNF family (BAFF, a B-cell stimulatory cytokine), CXCL13, sIL-6Rα, sGP130, sCD14, sTNF-R2, C-reactive protein (CRP), IL-6, IL-8, IL-10, and TNF-α concentration according to the manufacturer's directions (*Online Supplementary Methods*). We set TNF-α, IL-8 and CXCL13 values to missing for samples with >24-hour processing delays (NHS: n=35; HPFS: n=23). Analyte concentrations were natural log-transformed for all analyses. We observed similar measured biomarker concentrations for the NHS and HPFS (*Online Supplementary Table S1*) and pooled the data.

Statistical analysis

We conducted batch calibration to diminish the potential influence of laboratory batch-related variability on biomarker-NHL associations.[24] Outlying biomarker values were identified using the Rosner extreme Studentized deviate method[25] and omitted from analyses of the marker.

The primary analysis assessed batch effect-corrected, log-transformed biomarker values continuously per Standard Deviation (SD) increase in concentration, with SD units calculated for log-transformed values in the pooled controls. We calculated Odds Ratios (OR) and 95% Confidence Intervals (CI) for the association of each biomarker with NHL risk (overall and for DLBCL, FL, CLL/SLL, other B-NHL, all B-NHL and all T-NHL) using unconditional logistic regression. Models adjusted for all matching factors unless small cell counts precluded adjustment for race. We evaluated but did not observe confounding by body mass index (BMI) and autoimmune disease history.

We intended *a priori* to identify multi-marker profiles associated with NHL risk *via* mutual adjustment of models for biomarkers that were individually associated. We also examined models stratified by follow-up interval (0 to <5, 5 to <10, ≥10 years) and assessed heterogeneity by time period using the contrast test.[26] The *Online Supplementary Methods* describe additional analyses designed *post hoc*.

Results

In total, 601 cases of NHL (345 NHS and 256 HPFS) were identified and individually matched to controls. Three cases were later excluded due to unconfirmed lymphoma status. The final analysis thus included 598 cases, including 114 DLBCL, 92 FL, 165 CLL/SLL, 132 other B-NHL (4 Burkitt lymphoma, 19 lymphoplasmacytic lymphoma, 20 mantle cell lymphoma, 44 marginal zone lymphoma, 20 other B-NHL, and 25 unclassified B-NHL) and 30 T-NHL, and 601 controls. The study population was 96% Caucasian and 58% female. Cases and controls had similar covariable distributions, due in part to the matched design (Table 1).

We omitted 109 individual biomarker measurements (<1% of all measurements) with implausible outlying values (NHS: 72; HPFS: 37), the majority (90%) of which were implausibly high for the particular marker. Omitted values ranged from one measure of IL-10 to 17 measures of IL-8. Spearman correlation coefficients ranged from -0.03 (IL-10 and CXCL13) to 0.58 (sIL-2Rα and sCD30) (*Online Supplementary Table S2*).

Individual immune marker models

Multivariable analyses of individual log-transformed immune markers revealed significant associations for all NHL per SD increment of log-transformed sTNF-R2, sIL-2Rα, CXCL13, sCD30 (all positive) and BAFF (inverse; Table 2). In subtype-specific analyses, sTNF-R2 levels were also positively associated with risk of all B-NHL, FL and CLL/SLL, while CXCL13 was positively associated with risk of all B-NHL, DLBCL and FL (Table 2). Levels of sIL-2Rα and sCD30 were positively associated with every NHL subtype, including T-NHL. Of interest, the association of BAFF with a 17% decreased risk of all NHL appeared to be driven by CLL/SLL, for which risk decreased by 49% per SD increase in log-transformed BAFF levels (OR: 0.51; 95%CI: 0.43, 0.62; $P<0.001$); BAFF was not associated with other NHL subtypes in single-marker models. We did not observe significant or consistent associations for the remaining immune markers with risk of any NHL end point. Results from cohort-specific models did not suggest marked differences by sex for these associations (*Online Supplementary Table S3*).

Multi-marker profiles

In the model that mutually adjusted for the five log-transformed immune markers that had significant individ-

Table 1. Characteristics of non-Hodgkin lymphoma cases and matched controls from two prospective cohort studies.

Variable	Cases	Controls	P*
Cohort			
NHS	344 (58%)	345 (57%)	0.97
HPFS	254 (42%)	256 (43%)	
Age, years, mean ± SD	60.8 ± 8.1	60.8 ± 8.1	0.87
Race/ethnicity			
Caucasian	573 (96%)	578 (96%)	0.75
Other	25 (4%)	23 (4%)	
BMI at blood draw, kg/m^2			
< 22.5	138 (23%)	118 (20%)	0.58
22.5-24.9	139 (23%)	166 (28%)	
25-29.9	217 (36%)	219 (36%)	
≥ 30	74 (12%)	74 (12%)	
Missing	30 (5%)	24 (4%)	
BMI in young adulthood, kg/m^2			
< 18.5	44 (7%)	54 (9%)	0.31
18.5-22.4	298 (50%)	299 (50%)	
22.5-24.9	126 (21%)	112 (19%)	
≥ 25	106 (18%)	101 (17%)	
Missing	24 (4%)	35 (6%)	
Autoimmune disease†			
Yes	97 (16%)	104 (17%)	0.62
No	501 (84%)	497 (83%)	
Years from blood draw to index date,			
Mean ± SD	9.6 ± 5.6	9.6 ± 5.6	0.99
Cell type/histological subtype of NHL‡			
B-NHL	503 (84%)		
DLBCL	114 (19%)		
Follicular lymphoma	92 (15%)		
CLL/SLL	165 (28%)		
Other B-cell subtypes§	132 (22%)		
T-NHL	30 (5%)		

SD: Standard Deviation; NHS: Nurses' Health Study; HPFS: Health Professionals Follow-up Study; BMI: Body Mass Index; NHL: non-Hodgkin lymphoma; B-NHL: B-cell NHL; DLBCL: diffuse large B-cell lymphoma; CLL/SLL: chronic lymphocytic leukemia/small lymphocytic lymphoma; T-NHL: T-cell NHL. *P-values from χ^2 test or ANOVA. Tests for BMI at blood draw and BMI in young adulthood did not include individuals' missing data for those variables. †Defined as any self-reported diagnosis of rheumatoid arthritis, ulcerative colitis, multiple sclerosis, psoriasis, or Sjögren syndrome. ‡Information on cell type was not available for 11% of NHL cases. §The other B-NHL subtypes include Burkitt lymphoma (n=4), lymphoplasmacytic lymphoma (n=19), mantle cell lymphoma (n=20), marginal zone lymphoma (n=44), other B-NHL (n=20), and unclassified B-NHL (n=25).

Table 2. Associations between pre-diagnosis concentrations of 13 individual immune markers and risk of non-Hodgkin lymphoma (NHL), overall and by major histological subtype.

	All NHL*		B-NHL Subtypes								All T-NHL		
			DLBCL		FL		CLL/SLL		Other B-NHL†				
Marker	N cases/ controls	OR (95% CI)‡	N cases	OR (95% CI)‡	N cases	OR (95% CI)‡	N cases	OR (95% CI)‡	N cases	OR (95% CI)‡	N cases	OR (95% CI)‡	P§
IL-6	597/600	0.97(0.87,1.08)	114	1.12(0.91,1.37)	92	0.90(0.73,1.12)	165	0.99(0.84,1.17)	131	0.89(0.74,1.08)	30	1.13(0.78,1.63)	0.46
IL-8	558/566	1.00(0.88,1.13)	106	0.96(0.77,1.22)	84	1.07(0.84,1.36)	156	0.98(0.81,1.20)	120	1.11(0.90,1.37)	29	0.82(0.54,1.26)	0.70
IL-10	596/597	1.00(0.89,1.11)	113	1.14(0.93,1.40)	91	0.98(0.78,1.22)	165	0.85(0.72,1.01)	132	1.04(0.86,1.25)	30	1.21(0.83,1.76)	0.18
TNF-α	566/571	1.02(0.91,1.14)	108	0.98(0.80,1.21)	87	1.16(0.92,1.47)	158	1.06(0.89,1.26)	120	0.82(0.68,1.00)	29	1.14(0.78,1.67)	0.17
CRP	596/599	1.06(0.94,1.19)	114	1.12(0.92,1.38)	92	1.12(0.89,1.40)	165	0.93(0.77,1.12)	130	1.15(0.94,1.40)	30	0.97(0.66,1.42)	0.50
sCD14	592/596	1.01(0.90,1.15)	114	0.90(0.72,1.13)	90	0.97(0.76,1.25)	164	0.91(0.75,1.11)	130	1.14(0.93,1.40)	30	0.94(0.62,1.43)	0.55
sGP130	592/596	1.03(0.89,1.18)	114	0.87(0.66,1.15)	91	1.15(0.90,1.47)	163	1.00(0.80,1.25)	130	0.99(0.79,1.26)	30	0.81(0.48,1.36)	0.57
sTNF-R2	592/601	1.25(1.12,1.40)	114	1.02(0.83,1.26)	90	1.37(1.10,1.70)	164	1.28(1.08,1.51)	129	1.35(1.13,1.62)	30	1.03(0.70,1.50)	0.20
sIL-6Rα	592/599	1.10(0.98,1.23)	114	0.89(0.72,1.10)	91	1.16(0.93,1.44)	165	1.15(0.97,1.36)	128	1.13(0.93,1.37)	30	1.01(0.69,1.46)	0.32
BAFF	592/601	0.83(0.75,0.92)	114	0.99(0.81,1.21)	92	0.93(0.73,1.17)	163	0.51(0.43,0.62)	128	0.87(0.73,1.05)	30	1.26(0.87,1.82)	<0.0001
sIL-2Rα	585/600	1.37(1.23,1.53)	114	1.26(1.04,1.54)	91	1.55(1.25,1.94)	162	1.49(1.27,1.76)	126	1.40(1.16,1.68)	30	1.97(1.37,2.85)	0.26
CXCL13	554/571	1.31(1.17,1.46)	107	1.25(1.03,1.52)	86	1.58(1.28,1.95)	156	1.10(0.93,1.31)	116	1.48(1.24,1.76)	28	1.23(0.86,1.76)	0.06
sCD30	590/600	1.37(1.23,1.52)	114	1.29(1.06,1.56)	90	1.76(1.44,2.15)	163	1.33(1.13,1.57)	131	1.29(1.09,1.54)	29	1.46(1.05,2.04)	0.15

N: number; NHL: non-Hodgkin lymphoma; B-NHL: B-cell non-Hodgkin lymphoma; DLBCL: diffuse large B-cell lymphoma; FL: follicular lymphoma; CLL/SLL: chronic lymphocytic leukemia/small lymphocytic lymphoma; T-NHL: T-cell NHL; OR: Odds Ratio; CI: Confidence Interval; IL: interleukin; TNF: tumor necrosis factor; CRP: C-reactive protein; sCD14: soluble CD14; sGP130: soluble GP130; sTNF-R2: soluble tumor necrosis factor receptor-2; sIL-6Rα: soluble interleukin-6 receptor-α; BAFF: B-cell activating factor of the TNF family; sIL-2Rα: soluble interleukin-2 receptor-α; CXCL13: CXC chemokine ligand 13; sCD30: soluble CD30. *The all B-NHL (n=503 cases) results were similar to the all NHL results. †Other B-NHL subtypes include Burkitt lymphoma (n=4), lymphoplasmacytic lymphoma (n=19), mantle cell lymphoma (n=20), marginal zone lymphoma (n=44), other B-NHL (n=20), and unclassified B-NHL (n=25). ‡Odds Ratios and 95% Confidence Intervals were calculated per 1 standard deviation increase in log biomarker concentration, based on batch-corrected values with outliers removed, for NHS and HPFS cohorts combined. All models except those for T-NHL were adjusted for age at blood draw (continuous), cohort, time of blood draw (continuous), race (Caucasian/other); the models for T-NHL were adjusted for age and cohort only. §*P*-values for heterogeneity by subtype from contrast tests comparing immune marker-specific estimates between DLBCL, FL, CLL/SLL, other B-NHL, and T-NHL.

ual associations with NHL end points (sTNF-R2, sIL-2Rα, CXCL13, sCD30, BAFF), sIL-2Rα, CXCL13 and sCD30 remained significantly associated with a 17-24% increased risk, and BAFF with a 26% decreased risk of all NHL per SD increase in log concentration, while sTNF-R2 was no longer significantly associated (Table 3). Results for all B-NHL risk were similar to those for all NHL, whereas mutual adjustment attenuated all the immune marker associations with DLBCL. In the multi-marker model of FL risk, sCD30 and BAFF remained independently associated, with a borderline association noted for CXCL13 (Table 4). In the multi-marker model of CLL/SLL risk, sIL-2Rα was significantly associated with a 50% increase (95%CI: 1.18-1.90), and BAFF with a significant 53% reduction (95%CI: 0.38, 0.58), per SD increase in log concentration. Lastly, only sIL-2Rα was independently associated with T-NHL risk (OR per SD increase in log concentration: 1.96; 95%CI: 1.22, 3.13) in mutually adjusted models.

The 5-marker models using the polytomous logistic regression (PLR) approach yielded essentially the same effect estimates as described above for biomarker associations with the NHL end points for the full follow-up period (*Online Supplementary Tables S4* and *S5*). sTNF-R2 had significantly different associations with B-NHL and T-NHL (*P*-value for heterogeneity by subtype=0.04) (*Online Supplementary Table S4*); the associations of CXCL13 and BAFF with individual B-NHL subtypes also showed evidence of significant heterogeneity (*P*-values for heterogeneity by subtype =0.007 and <0.0001, respectively) (*Online Supplementary Table S5*).

In the covariable-adjusted multi-marker models containing restricted cubic splines, there was evidence of non-linearity for two biomarkers, CXCL13 and BAFF, in their associations with risk of aggregated end points (all NHL, B-NHL and other B-NHL; *P*-value tests for significance of the curve <0.05), but not for biomarker associations with individual B-NHL subtypes or T-NHL.

In alternative models using semi-automatic stepwise selection, the final models for all NHL and all B-NHL included sIL-2Rα, CXCL13 and sCD30, which were positively associated with risk, as well as BAFF, which was inversely associated (*Online Supplementary Table S6*). In comparison, for DLBCL and FL, the stepwise procedure selected only sCD30 (*P*=0.004 and <0.0001, respectively), and for T-NHL the procedure selected only sIL-2Rα (*P*=0.002) as independently (positively) associated with risk. Of interest, the stepwise procedure identified four immune markers independently associated with risk of CLL/SLL, including BAFF and IL-10 with significant inverse associations and sIL-2Rα with a significant positive association. The stepwise procedure identified three immune markers associated with the combined category of other B-NHL subtypes, including significant positive associations for CXCL13 and sIL-2Rα, and a significant inverse association for BAFF.

In the model that included all 13 immune markers, sIL-2Rα , CXCL13, and sCD30 again had strong positive associations with risk of all NHL and all B-NHL (Table 5). In the CLL/SLL-specific model, we observed a significant inverse association with risk for BAFF and also for sCD14 and IL-10, and a positive association with sIL2-Rα. BAFF was also significantly inversely associated with FL risk, while sCD30 was significantly positively associated with FL risk. Only sIL-2Rα was significantly associated with an increased risk of T-NHL. We observed suggestive positive

Table 3. Independent associations of multiple pre-diagnosis plasma immune markers with risk of non-Hodgkin lymphoma (NHL), overall and by B- or T-cell type of origin, for the complete follow-up period and stratified by years of follow up.

Marker	Complete follow-up period		Years from blood draw to diagnosis/index date						$P^{\ddagger}$
			0 to less than 5		5 to less than 10		10 or more		
	N cases/ controls	OR (95% CI) per 1-SD *,†	N cases/ controls	OR (95% CI) per 1-SD *,†	N cases/ controls	OR (95% CI) per 1-SD *,†	N cases/ controls	OR (95% CI) per 1-SD *,†	
All NHL									
sTNF-R2	542/571	1.05 (0.91, 1.21)	133/140	0.83 (0.60, 1.14)	149/162	1.02 (0.77, 1.35)	260/267	1.18 (0.95, 1.46)	0.20
sIL-2Rα	542/571	1.20 (1.03, 1.39)	133/140	1.52 (1.09, 2.11)	149/162	1.16 (0.88, 1.53)	260/267	1.11 (0.88, 1.39)	0.28
CXCL13	542/571	1.17 (1.03, 1.32)	133/140	1.00 (0.78, 1.29)	149/162	1.30 (1.03, 1.62)	260/267	1.21 (1.01, 1.46)	0.32
sCD30	542/571	1.24 (1.06, 1.45)	133/140	1.52 (1.09, 2.13)	149/162	1.43 (1.07, 1.90)	260/267	0.98 (0.78, 1.23)	0.02
BAFF	542/571	0.74 (0.66, 0.83)	133/140	0.73 (0.59, 0.91)	149/162	0.61 (0.48, 0.78)	260/267	0.83 (0.69, 1.00)	0.15
All B-NHL									
sTNF-R2	454/570	1.07 (0.92, 1.25)	110/140	0.88 (0.63, 1.23)	118/161	1.15 (0.86, 1.54)	226/267	1.13 (0.90, 1.42)	0.40
sIL-2Rα	454/570	1.20 (1.03, 1.41)	110/140	1.51 (1.06, 2.14)	118/161	1.12 (0.84, 1.49)	226/267	1.15 (0.91, 1.46)	0.38
CXCL13	454/570	1.13 (1.00, 1.29)	110/140	0.96 (0.74, 1.25)	118/161	1.17 (0.92, 1.49)	226/267	1.24 (1.02, 1.50)	0.31
sCD30	454/570	1.24 (1.05, 1.46)	110/140	1.59 (1.10, 2.28)	118/161	1.58 (1.14, 2.20)	226/267	0.96 (0.75, 1.22)	0.02
BAFF	454/570	0.73 (0.64, 0.83)	110/140	0.67 (0.53, 0.84)	118/161	0.64 (0.50, 0.81)	226/267	0.84 (0.69, 1.02)	0.14
All T-NHL									
sTNF-R2	28/569	0.62 (0.37, 1.03)	11/140	0.44 (0.17, 1.19)	10/160	0.65 (0.27, 1.58)	7/267	0.73 (0.24, 2.21)	0.78
sIL-2Rα	28/569	1.96 (1.22, 3.13)	11/140	2.10 (0.95, 4.68)	10/160	2.20 (0.93, 5.20)	7/267	1.04 (0.36, 3.00)	0.50
CXCL13	28/569	1.11 (0.75, 1.65)	11/140	1.03 (0.48, 2.22)	10/160	1.37 (0.78, 2.42)	7/267	0.57 (0.24, 1.37)	0.26
sCD30	28/569	1.33 (0.84, 2.10)	11/140	1.68 (0.69, 4.08)	10/160	1.34 (0.56, 3.21)	7/267	1.77 (0.70, 4.43)	0.90
BAFF	28/569	0.88 (0.58, 1.32)	11/140	0.93 (0.52, 1.67)	10/160	0.55 (0.24, 1.28)	7/267	1.68 (0.64, 4.44)	0.23

N: number; NHL: non-Hodgkin lymphoma; B-NHL: B-cell NHL; T-NHL: T-cell NHL; OR: Odds Ratio; CI: Confidence Interval; SD: Standard Deviation; sTNF-R2: soluble tumor necrosis factor receptor-2; sIL-2Rα: soluble interleukin-2 receptor-α; CXCL13: CXC chemokine ligand 13; sCD30: soluble CD30; BAFF: B-cell activating factor of the TNF family. *Models were adjusted for age at blood draw (continuous), cohort (sex), time of blood draw (continuous) and race/ethnicity (Caucasian, non-Caucasian) and were mutually adjusted for all markers listed, except that models for all T-NHL were not adjusted for race. †Odds Ratios and 95% Confidence Intervals were calculated per 1-standard deviation increase in batch effect-corrected, log-transformed values (with cohort-specific outliers excluded) from the Nurses' Health Study and Health Professionals Follow-up Study combined. ‡*P*-values from tests for heterogeneity comparing immune marker-specific estimates across time strata.

associations of DLBCL risk with IL-6, CXCL13 and sCD30 in this 13-marker model.

Time-stratified analyses

The analyses stratified by time between blood draw and diagnosis/index date suggested that the individual biomarker associations with all NHL (*Online Supplementary Table S7*) and with NHL subtypes (*Online Supplementary Table S8*) varied somewhat by length of time after blood draw but did not strongly implicate any additional immune marker-NHL associations. The time-stratified 5-marker models (Table 3) also suggested variability by follow-up interval in the independent associations of those immune markers with future NHL risk. For example, the association of sIL-2Rα with risk of all NHL appeared to be restricted to a shorter-term interval, specifically within five years of blood draw (OR: 1.52, 95%CI: 1.09, 2.11) (Table 3), whereas significant associations of CXCL13 with risk of all NHL were evident only five or more years after blood collection (5-<10 years; OR: 1.23, 95%CI: 1.00, 1.52; and ≥10 years; OR: 1.21, 95%CI: 1.01, 1.45). sCD30 was most strongly associated with all NHL risk within ten years of blood draw, while BAFF was consistently inversely associated with all NHL across time periods. Of note, in subtype-specific time-stratified analyses, sCD30 levels were strongly positively associated with risk of FL within five years of blood draw (OR: 4.85, 95%CI: 2.02, 11.61), and the association decreased in magnitude with increasing follow-up time. In CLL/SLL-specific models, elevated sIL-2Rα was associated with a nearly 4-fold increased risk within five years of blood draw (OR: 3.71, 95%CI: 1.77, 7.76) but had no clear association with longer-term CLL/SLL risk. In contrast, BAFF had significant inverse associations with risk of CLL/SLL in all pre-diagnosis time periods, albeit with particularly strong associations with risk of CLL/SLL within five or ten years of blood draw (Table 4). When modeled using PLR, the effect estimates were virtually the same for time period-specific biomarker associations, both for the aggregated and the individual NHL end points (*Online Supplementary Tables S4* and *S5*). The most prominent differences between the two approaches for assessing heterogeneity by time period (PLR with interaction terms *vs.* time-stratified unconditional logistic regression) pertained to the statistical significance of apparent heterogeneity by follow-up period for the associations of sTNF-R2 with all B-NHL and FL. For example, for the association of sTNF-R2 with all B-NHL, the *P*-value for heterogeneity by follow-up time was 0.04 for the cross-product term in PLR (*Online Supplementary Table S4*) and 0.40 for the main model contrast test (Table 3). For the association of sTNF-R2 with FL, the *P*-value for heterogeneity by time period was 0.0007 for the cross-product term in PLR (*Online Supplementary Table S5*) and 0.11 for the main model contrast test (Table 4). Time-strat-

Table 4. Independent associations of multiple pre-diagnosis plasma immune markers with risk of non-Hodgkin lymphoma (NHL) by major histological subtype of B-cell NHL, for the complete follow-up period and stratified by years of follow up.

Marker	Complete follow-up period		Years from blood draw to diagnosis/index date: 0 to less than 5		5 to less than 10		10 or more		$P^{\ddagger}$
	N cases/ controls	OR (95% CI) per 1-SD*,†	N cases/ controls	OR (95% CI) per 1-SD*,†	N cases/ controls	OR (95% CI) per 1-SD*,†	N cases/ controls	OR (95% CI) per 1-SD*,†	
DLBCL									
sTNF-R2	107/570	0.81 (0.62, 1.07)	25/140	0.61 (0.34, 1.10)	25/161	1.05 (0.60, 1.85)	57/267	0.83 (0.56, 1.24)	0.42
sIL-2Rα	107/570	1.18 (0.91, 1.53)	25/140	1.83 (1.00, 3.37)	25/161	1.19 (0.67, 2.11)	57/267	1.09 (0.75, 1.58)	0.35
CXCL13	107/570	1.17 (0.95, 1.45)	25/140	0.71 (0.43, 1.19)	25/161	1.42 (0.95, 2.12)	57/267	1.30 (0.95, 1.79)	0.09
sCD30	107/570	1.23 (0.95, 1.59)	25/140	0.90 (0.48, 1.67)	25/161	1.76 (1.07, 2.89)	57/267	1.09 (0.75, 1.59)	0.19
BAFF	107/570	0.95 (0.76, 1.18)	25/140	0.96 (0.59, 1.55)	25/161	0.69 (0.44, 1.08)	57/267	1.08 (0.78, 1.50)	0.27
FL									
sTNF-R2	83/569	1.03 (0.77, 1.38)	18/140	0.45 (0.16, 1.25)	22/160	0.95 (0.53, 1.69)	43/267	1.35 (0.93, 1.96)	0.11
sIL-2Rα	83/569	1.06 (0.78, 1.46)	18/140	0.93 (0.35, 2.44)	22/160	1.09 (0.63, 1.89)	43/267	1.09 (0.70, 1.68)	0.95
CXCL13	83/569	1.24 (0.98, 1.58)	18/140	1.10 (0.58, 2.06)	22/160	1.12 (0.72, 1.75)	43/267	1.48 (1.03, 2.13)	0.56
sCD30	83/569	1.69 (1.26, 2.26)	18/140	4.85 (2.02, 11.61)	22/160	1.88 (1.04, 3.40)	43/267	1.06 (0.68, 1.64)	0.007
BAFF	83/569	0.76 (0.59, 0.98)	18/140	0.70 (0.38, 1.30)	22/160	0.72 (0.41, 1.24)	43/267	0.78 (0.55, 1.12)	0.94
CLL/SLL									
sTNF-R2	153/569	1.21 (0.96, 1.52)	36/140	0.98 (0.49, 1.93)	44/160	1.27 (0.81, 1.98)	73/267	1.16 (0.85, 1.58)	0.82
sIL-2Rα	153/569	1.50 (1.18, 1.90)	36/140	3.71 (1.77, 7.76)	44/160	1.39 (0.90, 2.15)	73/267	1.26 (0.87, 1.83)	0.04
CXCL13	153/569	0.90 (0.74, 1.10)	36/140	0.78 (0.48, 1.27)	44/160	0.80 (0.54, 1.20)	73/267	1.04 (0.77, 1.41)	0.48
sCD30	153/569	1.15 (0.89, 1.48)	36/140	1.43 (0.71, 2.87)	44/160	1.54 (0.96, 2.46)	73/267	0.90 (0.62, 1.30)	0.17
BAFF	153/569	0.47 (0.38, 0.58)	36/140	0.32 (0.19, 0.53)	44/160	0.39 (0.25, 0.61)	73/267	0.63 (0.46, 0.86)	0.05
Other B-NHL§									
sTNF-R2	111/569	1.17 (0.92, 1.49)	31/140	1.28 (0.78, 2.12)	27/160	1.17 (0.73, 1.90)	53/267	1.11 (0.77, 1.61)	0.90
sIL-2Rα	111/569	1.15 (0.89, 1.48)	31/140	1.07 (0.62, 1.83)	27/160	1.05 (0.63, 1.74)	53/267	1.29 (0.87, 1.91)	0.77
CXCL13	111/569	1.45 (1.19, 1.77)	31/140	1.50 (0.98, 2.30)	27/160	1.52 (1.07, 2.17)	53/267	1.36 (0.98, 1.88)	0.88
sCD30	111/569	1.08 (0.82, 1.41)	31/140	1.28 (0.73, 2.26)	27/160	1.37 (0.81, 2.32)	53/267	0.79 (0.52, 1.21)	0.20
BAFF	111/569	0.78 (0.64, 0.95)	31/140	0.74 (0.53, 1.04)	27/160	0.72 (0.48, 1.07)	53/267	0.89 (0.64, 1.24)	0.66

N: number; DLBCL: diffuse large B-cell lymphoma; FL: follicular lymphoma; CLL/SLL: chronic lymphocytic leukemia/small lymphocytic lymphoma; B-NHL: B-cell NHL; OR: Odds Ratio; CI: Confidence Interval; SD: Standard Deviation; sTNF-R2: soluble tumor necrosis factor receptor-2; sIL-2Rα: soluble interleukin-2 receptor-α; CXCL13: CXC chemokine ligand 13; sCD30: soluble CD30; BAFF: B-cell activating factor of the TNF family. *All models were adjusted for age at blood draw (continuous), cohort (sex), time of blood draw (continuous) and race/ethnicity (Caucasian, non-Caucasian), and were mutually adjusted for all markers listed, except that models for other B-NHL were not adjusted for race. †Odds Ratios and 95% Confidence Intervals were calculated per 1-standard deviation increase in batch effect-corrected, log-transformed values (with cohort-specific outliers excluded) from the Nurses' Health Study and Health Professionals Follow-up Study combined. ‡*P*-values from tests for heterogeneity comparing immune marker-specific estimates across time strata. §Other B-NHL subtypes included Burkitt lymphoma (n=4), lymphoplasmacytic lymphoma (n=19), mantle cell lymphoma (n=20), marginal zone lymphoma (n=39), other B-NHL (n=20), and unclassified B-NHL (n=25).

ified results for the multi-marker models identified with stepwise selection largely agreed with the main results described above (*Online Supplementary Table S6*).

Discussion

In this pooled analysis within the NHS and HPFS cohorts, we observed significant associations between NHL risk and pre-diagnosis levels of specific plasma immune markers, including a novel, inverse association between levels of BAFF and risk of CLL/SLL. Positive associations between levels of sIL-2Rα, CXCL13, and sCD30 and risk of all NHL and all B-NHL, as well as the inverse association of BAFF with risk of all NHL and CLL/SLL, were consistent and independent across several analytical approaches to constructing a multi-marker profile associated with risk. In contrast, the individual positive associations noted for sTNF-R2 with risk of all NHL and some B-NHL end points were attenuated upon adjustment for other immune markers, suggesting a lack of independence in the association between sTNF-R2 levels and NHL risk. Manual selection and automated stepwise selection of multi-marker profiles yielded fairly consistent results for all NHL, but also some differences for individual histological subtypes, particularly for CLL/SLL. We also observed some variation in the associations between NHL risk and immune markers by time between blood draw and diagnosis.

Our findings are in agreement with previous studies reporting associations between elevated CXCL13 and/or sCD30 levels and increased NHL risk in HIV-positive and immunocompetent populations, including several reports analyzing blood samples taken many years prior to NHL diagnosis.[2,3,8-13] In the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study, Purdue *et al.*[11] prospectively investigated multi-marker models similar to those in our analysis and observed independent positive associations

Table 5. Associations of pre-diagnosis plasma immune markers with risk of non-Hodgkin lymphoma (NHL), with mutual adjustment for all thirteen immune markers, for all NHL and by major histological subtype.

	All NHL		B-NHL Subtypes								All T-NHL	
			DLBCL		FL		CLL/SLL		Other B-NHL‡			
Marker	N Cases/ Controls	OR (95% CI) per 1-SD *,†	N cases	OR (95% CI) per 1-SD*,†	N cases	OR (95% CI) per 1-SD*,†	N cases	OR (95% CI) per 1-SD*,†	N cases	OR (95% CI) per 1-SD*,†	N cases	OR (95% CI) per 1-SD*,†
IL-6	523/550	1.06 (0.93, 1.21)	104	1.22 (0.96, 1.55)	78	0.86 (0.66, 1.12)	150	1.10 (0.89, 1.37)	106	1.03 (0.81, 1.31)	28	1.10 (0.71, 1.73)
IL-8	523/550	0.96 (0.83, 1.10)	104	0.92 (0.72, 1.18)	78	0.94 (0.71, 1.24)	150	1.00 (0.79, 1.26)	106	1.11 (0.88,1.38)	28	0.65 (0.40, 1.07)
IL-10	523/550	0.95 (0.84, 1.07)	104	1.14 (0.91, 1.43)	78	0.93 (0.72, 1.22)	150	0.80 (0.65, 0.99)	106	0.99 (0.79, 1.23)	28	1.19 (0.77, 1.82)
TNF-α	523/550	1.00 (0.87, 1.15)	104	0.84 (0.66, 1.08)	78	1.09 (0.81, 1.46)	150	1.09 (0.87, 1.37)	106	0.86 (0.67, 1.10)	28	1.18 (0.74, 1.89)
CRP	523/550	1.00 (0.87, 1.15)	104	1.07 (0.84, 1.37)	78	1.29 (0.96, 1.72)	150	0.82 (0.65, 1.04)	106	1.00 (0.77, 1.29)	28	0.85 (0.54, 1.34)
sCD14	523/550	0.84 (0.71, 1.00)	104	0.82 (0.61, 1.11)	78	0.84 (0.61, 1.18)	150	0.68 (0.51, 0.89)	106	0.92 (0.68, 1.24)	28	0.90 (0.51, 1.56)
sGP130	523/550	1.06 (0.85, 1.32)	104	1.05 (0.71, 1.55)	78	1.20 (0.78, 1.86)	150	1.11 (0.78, 1.58)	106	0.84 (0.57, 1.22)	28	0.76 (0.36, 1.60)
sTNF-R2	523/550	1.06 (0.86, 1.31)	104	0.96 (0.67, 1.37)	78	0.88 (0.57, 1.36)	150	1.33 (0.94, 1.87)	106	1.16 (0.81, 1.65)	28	0.75 (0.38, 1.46)
sIL-6Rα	523/550	1.03 (0.89, 1.20)	104	0.86 (0.66, 1.12)	78	1.02 (0.74, 1.40)	150	1.14 (0.90, 1.45)	106	1.19 (0.91, 1.56)	28	1.11 (0.67, 1.84)
BAFF	523/550	0.73 (0.64, 0.82)	104	0.98 (0.78, 1.23)	78	0.74 (0.56, 0.97)	150	0.46 (0.37, 0.57)	106	0.76 (0.62, 0.94)	28	0.88 (0.58, 1.34)
sIL-2Rα	523/550	1.19 (1.02, 1.40)	104	1.09 (0.82, 1.44)	78	1.09 (0.77, 1.54)	150	1.59 (1.23, 2.06)	106	1.10 (0.83, 1.46)	28	1.95 (1.22, 3.10)
CXCL13	523/550	1.18 (1.04, 1.34)	104	1.20 (0.96, 1.49)	78	1.22 (0.94, 1.59)	150	0.92 (0.74, 1.13)	106	1.45 (1.18, 1.78)	28	1.20 (0.80, 1.82)
sCD30	523/550	1.26 (1.06, 1.48)	104	1.27 (0.96, 1.66)	78	1.80 (1.30, 2.50)	150	1.10 (0.84, 1.45)	106	1.13 (0.85, 1.52)	28	1.25 (0.76, 2.05)

N: number; B-NHL: B-cell NHL; SD: Standard Deviation; OR: Odds Ratio; CI: Confidence Interval; DLBCL: diffuse large B-cell lymphoma; FL: follicular lymphoma; CLL/SLL: chronic lymphocytic leukemia/small lymphocytic lymphoma; T-NHL: T-cell NHL; IL: interleukin; TNF: tumor necrosis factor; CRP: C-reactive protein; sCD14: soluble CD14; sGP130: soluble GP130; sTNF-R2: soluble tumor necrosis factor receptor-2; sIL-6Rα: soluble interleukin-6 receptor-α; BAFF: B-cell activating factor of the TNF family; sIL-2Rα: soluble interleukin-2 receptor-α; CXCL13: CXC chemokine ligand 13; sCD30: soluble CD30. *From multivariable logistic regression models that include all 13 immune markers in each model, adjusted for age at blood draw, time of day of blood draw, race and cohort. T-NHL models were not adjusted for race due to sparse cell counts. †Odds Ratios and 95% Confidence Intervals were calculated per 1 standard deviation increase in biomarker concentration, based on batch effect-corrected log-transformed values with outliers removed, for Nurses' Health Study and Health Professionals Follow-up Study cohorts combined. ‡Other B-NHL subtypes included Burkitt lymphoma (n=4), lymphoplasmacytic lymphoma (n=19), mantle cell lymphoma (n=20), marginal zone lymphoma (n=44), other B-NHL (n=20), and unclassified B-NHL (n=25).

for sCD30 with risk of NHL and DLBCL when adjusted for other biomarkers. Those observations were detectable more than 15 years prior to diagnosis. Also similar to our findings, positive associations observed for sTNF-R2 with NHL did not persist upon adjustment for other immune markers.[11] In a prospective analysis in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial, individual associations of CXCL13 and sTNF-R2 with NHL both remained significant with mutual adjustment, with correction for multiple comparisons and with restriction to samples collected 8-13 years prior to diagnosis.[10]

We observed an unexpected yet consistently strong inverse association between BAFF levels and CLL/SLL risk. BAFF is a member of the TNF family involved with B-cell survival and maturation.[27] Pre-diagnosis serum BAFF concentrations were positively associated with AIDS-NHL, and BAFF overproduction has been associated with systemic autoimmune diseases, including systemic lupus erythematosus and Sjögren syndrome,[28,29] which are associated with an increased risk of NHL in HIV-negative persons.[30,31] However, systemic autoimmune disorders in HIV-negative individuals appear to be preferentially associated with NHL subtypes with a different natural history than CLL/SLL.[30,32] Nonetheless, CLL cells are known to express multiple BAFF receptors (including TNFRSF13B, TNFRS13C and TNFRSF17),[33] and the inverse association that we observed is biologically plausible if considered indicative of rapid uptake of circulating BAFF by nascent CLL/SLL clones,[34] reflecting subclinical progression of an indolent tumor whose natural course may extend multiple decades. Consistent with this interpretation, several clinical studies have observed lower levels of BAFF in sera from CLL/SLL patients than in healthy controls.[35-37] The mechanism for the latter findings is unknown; our observation suggests that those underlying physiological processes may commence early in CLL pathogenesis, even ten or more years pre-diagnosis. Concurrent measurement of soluble BAFF receptors and study of cell surface expression of those molecules and classification of cases into prognostic subgroups were not feasible for the present study. Confirmation of the present findings is warranted in larger populations with specimens suitable for determining cell surface marker or gene/protein expression. Additionally, prospective studies in patients with monoclonal B-lymphocytosis would be informative to evaluate whether circulating BAFF levels can enhance risk stratification for progression to malignancy.[38]

We also observed significant associations of elevated sIL-2Rα levels with increased risks of all NHL, B-NHL, DLBCL, CLL/SLL and T-NHL, primarily within five years of blood draw. One other study reported a positive association between sIL-2Rα levels and NHL risk in an HIV-negative population with prospective blood collection that persisted after incorporating lag-times greater than two years.[14] Of note, comparatively high sIL-2Rα levels at diagnosis were also associated with poor prognosis in patients with NHL.[39-41] Biologically, sIL-2Rα and sCD30 are highly correlated (r=0.58 in this study), and both can indicate B- and T-cell activation;[42] in the present analysis, both markers remained independently associated with a significant increased risk of all NHL and all B-NHL after mutual adjustment. In contrast, only sIL-2Rα was significantly associated with an increased risk of T-NHL in the multimarker models, although small sample size (n=30 cases) limited statistical power to detect significant independent associations for more strongly correlated biomarkers. Of

interest, we observed the strongest positive associations of sIL-2Rα with T-NHL risk within ten years of blood draw, a novel observation that requires confirmation in other populations.

We observed significant positive associations between CXCL13 and risk of all NHL, B-NHL, and FL, as well as borderline associations with DLBCL and other B-NHL, more than ten years after blood draw, suggesting an early role for an immune environment characterized by B-cell stimulation and aberrant B-cell trafficking. Consistent with this interpretation, a recent, large-scale genome-wide association study of FL identified CXCR5, which is the receptor for CXCL13, as a potential FL susceptibility locus.[43] Further, genetic variation in *CXCR5* and *CXCL13* was associated with serum CXCL13 levels in a study of AIDS-NHL, and elevated serum CXCL13 levels were observed in AIDS-NHL cases more than three years prior to diagnosis.[2] In contrast, elevated sCD30 levels were more strongly associated with increased risk of all NHL, B-NHL and FL within ten years of blood draw, with a particularly strong association with FL within five years of blood draw. These findings suggest sCD30 may be capturing a more proximal pre-diagnosis increase in immune activation.

When assessed with multivariable PLR models rather than the main unconditional logistic regression analysis, the associations between immune markers and NHL end points did not change substantially, whether for aggregated end points or the individual B-NHL end points. The minor discrepancies suggested somewhat improved precision in the PLR models, which yielded slightly narrower confidence intervals and slightly stronger *P*-values for heterogeneity by follow-up period for a few of the comparisons. None of the discrepant findings would suggest a different interpretation of the time- or subtype-specific model findings, however, and thus we retained the unconditional logistic regression models as our primary analysis for methodological consistency across the full series of analyses we conducted.

In the analyses with restricted cubic splines, we observed evidence of significant non-linearity for associations of CXCL13 and BAFF with aggregated NHL end points. Of note, those end points comprise small numbers of diverse histological subtypes of NHL which may have different etiologies. Thus, we believe that the observed non-linear associations more likely reflect sampling variability and/or an artifact of potentially heterogeneous subtype-specific associations for the subtypes in the end point groups than a true biological effect.

Together, our findings add new insight to previous publications on both AIDS-NHL and HIV-unrelated NHL risk, collectively suggesting that higher levels of immune activation, and in particular heightened B-cell stimulation, may affect B-cell lymphomagenesis. Interestingly, several markers of immune activation appear to be elevated many years prior to NHL diagnosis and thus could help identify populations at higher risk for developing NHL. It is important to note that some reported associations between immune markers and all NHL risk were not replicated in analyses of individual histological subtypes; this may be due in part to subtype-specific sample sizes that limited statistical power. Significant associations between immune markers and risk of all NHL may reflect commonalities in subtype etiologies; however, these findings may also conceal a more specific association with one or more of the less common subtypes, as illustrated by the present findings for BAFF and CLL/SLL.

This analysis of immune markers measured from prospectively collected blood specimens from two large US cohorts with lengthy follow up identified several statistically significant associations with the risk of developing NHL, including associations that remained statistically significant for blood samples collected five or more years prior to diagnosis.

Although our main results were fairly consistent across analytical approaches, slight variations in markers chosen by *a priori* and secondary analyses emphasize the importance of utilizing diverse panels of immune markers in future studies seeking to characterize conditions conducive to NHL development. Furthermore, our findings suggest that even though an activated immune milieu may contribute to the development of multiple types of NHL, there is evidence of subtle differences in the pathogenesis of individual NHL subtypes, some of which had not been previously reported. Larger pooled studies will be important to more accurately identify homogeneous and heterogeneous biomarkers of risk or early disease by NHL subtype and to elucidate which are more indicative of earlier or later pathogenic changes to the immune environment.

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