EXHIBIT D

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Hepatitis C and Risk of Lymphoma: Results of the European Multicenter Case-Control Study EPILYMPH

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Background & Aims: Increasing evidence points toward a role of hepatitis C virus (HCV) infection in the etiology of malignant lymphomas. However, previous epidemiologic studies were limited in size to establish an association between HCV infection and specific lymphoma subtypes. We performed a large, multicenter, case-control study to address this question. **Methods:** The study comprised 5 European countries and included newly diagnosed cases of any lymphoid malignancy recruited between 1998 and 2004. Controls were matched to cases by 5-year age group, sex, and study center. In-person interviews were conducted to collect data on demographic, medical, and family history as well as environmental exposures. Serum samples of 1807 cases and 1788 controls (excluding human immunodeficiency virus-positive and organ-transplantation subjects) were screened for HCV infection using an enzyme immunoassay. Positive as well as randomly selected negative samples were subjected to HCV RNA detection and HCV genotyping. **Results:** HCV infection was detected in 53 (2.9%) lymphoma cases and in 41 (2.3%) control subjects (odds ratio [OR], 1.42; 95% confidence interval [CI]: 0.93–2.15). Restricted to individuals who tested positive for HCV-RNA (indicating persistent infection and active viral replication), the OR was 1.82 (95% CI: 1.13–2.91). In subtype-specific analyses, HCV prevalence was associated with diffuse large B-cell lymphoma (OR, 2.19; 95% CI: 1.23–3.91) but not with chronic lymphocytic leukemia or follicular, Hodgkin's, or T-cell lymphoma. The sample size was not sufficient to derive any conclusions for rare lymphoma entities such as splenic marginal zone lymphoma. **Conclusions:** These results support a model that chronic HCV replication contributes to lymphomagenesis and establish a specific role of HCV infection in the pathogenesis of diffuse large B-cell lymphoma.

Hepatitis C virus (HCV) infection is estimated to affect more than 170 million individuals worldwide,[1] 70%–80% of whom become chronic carriers. Some differences in HCV prevalence have been identified, ranging from below 1% in Canada and Germany; to slightly higher rates (1%–2.5%) in the United States, Japan, and Italy; to more than 10% in Egypt (reviewed in Shepard et al[2]).

Individuals with chronic HCV infection are at increased risk of developing cirrhosis and hepatocellular carcinoma. Furthermore, HCV is a well-established risk factor of lymphoproliferative syndromes such as type II mixed cryoglobulinemia.[3] Since the early 1990s, increasing evidence suggests a role of HCV infection also in the etiology of malignant lymphoma (reviewed in Gisbert et al,[4] Matsuo et al,[5] Negri et al,[6] and Turner et al[7]). Two metaanalyses of epidemiologic studies on this topic reported a 5- to 10-fold increased risk of B-cell non-Hodgkin's lymphoma (B-NHL) associated with anti-HCV seropositivity.[4,5] In most studies,[6] the odds ratio (OR) of B-NHL for HCV infection ranged between 2 and 4. Only a few prospective studies have investigated the association between HCV infection and subsequent B-cell neoplasia.[8–10] A prospective study from Japan found an elevated risk of NHL among HCV-infected subjects.[9] In a cohort of HCV-infected patients in Sweden, the risk of NHL and multiple myeloma (MM) was significantly increased with standardized incidence ratios of 1.9 and 2.5, respectively, among individuals with more than 15 years of infection, supporting a role of HCV infection in lymphomagenesis.[10] However, the association was greatly reduced when human immunodeficiency virus (HIV)-positive cases were removed. Data from an American cohort do not support a substantial role of chronic HCV infection acquired during young adulthood in the etiology of B-cell lymphoma; however, the statistical power of this study was low.[8]

Lymphomas consist of a heterogeneous group of diseases with likely differences in etiology.[11] In most previous studies, small sample sizes or lack of information prevented an analysis of the relationship between HCV and single lymphoma entities. The aim of this study was to evaluate the association between HCV infection and development of the most frequent lymphoma subtypes within a large European multicenter case-control study, whose participants were recruited from areas of low as well as high prevalence for HCV.

Materials and Methods

The EPILYMPH multicenter case-control study was carried out in 7 countries (Germany, Italy, Spain, Ireland, France, Finland, and Czech Republic) from 1998 to 2004. Details of the study design have been provided elsewhere.[12] The present analysis is restricted to 1836 cases and 1793 controls from France,

Abbreviations used in this paper: B-NHL, B-cell non-Hodgkin's lymphoma; DLBCL, diffuse large B-cell lymphoma; MM, multiple myeloma.

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Germany, Ireland, Italy, and Spain, with available serum samples.

Cases were defined as all consecutive patients of lymphoid malignancy with an initial diagnosis during the study period. Cases were categorized according to the World Health Organization's (WHO) classification for neoplastic diseases of the lymphoid tissues and included all B-cell, T-cell and natural killer-cell neoplasms, including Hodgkin's lymphoma (HL).[13] Subjects with a diagnosis of uncertain malignant potential such as posttransplantation lymphoproliferative disorder or monoclonal gammopathies of undetermined significance were excluded. The participation rate of cases ranged between 82% and 93%. After exclusion of HIV-infected individuals (n = 20) and organ transplantation patients, (n = 14), the final study group included 1807 cases and 1788 controls.

The distribution of the 1807 cases by major histologic entities was as follows: 1405 B-cell lymphomas (including 27 precursor B-cell lymphomas, 221 MM, 342 chronic lymphocytic leukemias, 392 diffuse large B-cell lymphomas [DLBCL], 210 follicular lymphomas, 41 lymphoplasmacytoid lymphomas, and 172 other B-NHL), 101 T-cell lymphomas, 239 HL, 60 B-NHL not otherwise specified, and 2 NHL not otherwise specified.

In parallel with the recruitment of cases, controls were drawn randomly from population registers of the study regions in Germany and Italy. In the remaining countries, controls were recruited from the same hospital as cases. In all instances, controls were frequency matched to the cases by age (±5 years), sex, and study center. In the hospital-based studies, controls were excluded if the main reason for the hospitalization at the time of recruitment was cancer, organ transplantation, and/or systemic infection.

Informed consent was obtained from all subjects prior to enrollment, and the institutional review boards of participating centers approved the study. The overall participation rate of control subjects ranged between 60% and 96% for hospital controls and 44% and 66% for population controls. Standardized interviews following a common core protocol were conducted by trained interviewers to collect data on sociodemographic characteristics, lifetime medical history of common diseases, family history of cancer and genetic diseases, smoking, alcohol, lifetime x-ray exposure, regular use of medication, ultraviolet (UV) light exposure, and lifetime occupational history. All cases and controls were asked to provide a blood sample for subsequent serologic and genetic investigations. Blood samples were fractionated, aliquoted, and stored at −80°C. For subsequent detection of HCV, frozen sera were shipped from the Irish, French, Italian, German, and Spanish studies to a common laboratory.

HCV Infection

Samples were screened for anti-HCV antibodies with a third generation enzyme-linked immunosorbent assay (ELISA) on the AXSYM system according to the manufacturer's instructions (Abbott Laboratories, Wiesbaden, Germany) in the laboratory of the Department of Internal Medicine IV, University Hospital of Heidelberg, Germany. The assay detects antibodies to putative structural (core region) and nonstructural proteins of the HCV genome. Its sensitivity is estimated to be 98.9% (95% confidence interval [CI]: 94%–100%) in patients with chronic liver disease with specificity of 97.2% (95% CI: 92%–99%) in panels of sera.[14] In addition, HCV RNA was measured with the licensed Amplicor HCV version 2.0 (Roche Diagnostics, Basel, Switzerland) with a lower limit of detection of 50 IU/L. HCV RNA testing was carried out on all seropositive samples and a random sample of anti-HCV-negative sera (n = 954). HCV infection was defined as a positive test for either anti-HCV or HCV RNA. In a second analysis, risk associated with persistent HCV infection defined by the detection of HCV RNA was evaluated. Borderline serologic values were considered negative for HCV infection unless HCV RNA was detected. Genotyping was carried out using the Innogenetics Line Probe (Innogenetics, Zwijndrecht, Belgium).

Statistical Analyses

Relative risk was estimated by OR and associated 95% CI using the SAS procedure LOGISTIC for unconditional logistic regression. All models were adjusted by age (continuous), sex, and center of diagnosis. The logistic analysis for lymphoma entities was performed by comparing the respective cases against all controls adjusting for age, sex, and center of diagnosis and excluding the other lymphoma entities. Test of heterogeneity was conducted using the likelihood ratio test statistics for interaction between HCV infection and the variable of interest (country, age, sex) performed with the SAS procedure GENMOD. The analysis was conducted using the Statistical Software Analysis (SAS) version 9, SAS Institute, Inc., Cary, NC.

Results

Sociodemographic characteristics of our study population tested for HCV infection and the distribution of their reported lifetime history of blood transfusion and illicit drug use are summarized in Table 1. Cases and controls were similar in terms of sex, age, study center, education, and intravenous drug use. They differed in relation to transfusion history with more controls than cases having reported a blood transfusion up to 2 years prior to the date of interview and before 1990. When blood transfusions until the date of interview were considered, the difference was less pronounced.

In total, 94 individuals (2.6%) had a positive enzyme immunoassay for HCV antibodies, and 76 subjects (2.1%) tested positive for HCV RNA by Amplicor. Of 954 anti-HCV-negative subjects tested for viral RNA, 1 individual was HCV RNA positive. HCV prevalence among the controls ranged from 0.75% in Germany to 4.11% in Italy. The respective HCV prevalence for France, Ireland, and Spain were 1.74%, 1.47%, and 3.69% (see Table 2). When examining HCV prevalence in relation to predictors of HCV status among the controls, illicit drug use (OR, 14.1; 95% CI: 2.56–78.19), and transfusion history before 1990 (OR, 4.06; 95% CI: 1.99–8.27), but not transfusion history in 1990 and thereafter, were associated with a positive test (Table 2). HCV prevalence shows an inverse trend with educational level (P trend = .01). More HCV-positive individuals (78.1%) than HCV-negative individuals (50.9%) reported a low educational level. HCV status did not correlate with sex and age. More lymphoma cases (n = 51, 2.88%) than controls (n = 31, 1.76%) reported a history of hepatitis B infection (OR, 1.75; 95% CI: 1.11–2.77) (data not shown).

HCV infection defined as a positive test for anti-HCV or HCV RNA (n = 94) was associated with an increased risk for all lymphomas combined (OR, 1.42; 95% CI: 0.93–2.15) (Table 3).

Table 1. Characteristics of the Study Population

Category	Cases n = 1807 (%)	Controls n = 1788 (%)	*P* value
Sex			
Male	1011 (56.0)	957 (53.5)	.14
Female	796 (44.1)	831 (46.5)	
Age at interview, *y*[a]			
<30	142 (7.9)	135 (7.6)	.45
30–<40	167 (9.2)	188 (10.5)	
40–<50	216 (12.0)	223 (12.5)	
50–<60	335 (18.5)	324 (18.1)	
60–<70	492 (27.2)	483 (27.0)	
70–<80	401 (22.2)	381 (21.3)	
≥80	53 (2.9)	54 (3.0)	
Education[b]			
Low	914 (50.8)	920 (51.5)	.69
Medium	639 (35.5)	625 (35.0)	
High	248 (13.8)	242 (13.5)	
Countries			
France	275 (15.2)	172 (9.6)	.22
Ireland	148 (8.2)	136 (7.6)	
Italy	198 (11.0)	219 (12.3)	
Germany	681 (37.7)	664 (37.1)	
Spain	505 (28.0)	597 (33.4)	
Ever intravenous drug use[c]	5 (0.3)	9 (0.5)	.28
Ever transfusion	319 (17.9)	354 (19.9)	.12
Ever transfusion 2 years before interview[d]	192 (10.8)	245 (13.8)	.006
Ever transfusion before 1990[d]	129 (7.2)	177 (10.0)	.004

[a]N = 1 missing.
[b]N = 7 missing.
[c]N = 20 missing.
[d]N = 35 missing.

A statistically significant association between HCV infection and lymphoma was seen when only those subjects with detectable HCV RNA were considered. Presence of this marker of persistent and actively replicating HCV was associated with an OR of 1.82 (95% CI: 1.13–2.91; *P* value = .013).

In stratified analyses, the OR of lymphoma for HCV prevalence was similar among men and women (*P* value for test of heterogeneity = .832), but it was elevated in subjects aged 55 years or more (OR, 1.63; 95% CI: 1.02–2.61; *P* value = .040) but not in younger subjects (OR, 0.63; 95% CI: 0.22–1.76; *P* value = .377) (*P* value for test of heterogeneity = .178) (Table 3). Presence of HCV RNA was consistently associated with a 1.6- to 2.3-fold increased risk of lymphoma in all participating countries, although numbers in individual countries were small and risks not statistically significant. Adjustments by education, reported history of hepatitis B, drug use, transfusion history, and time since blood transfusion had only a marginal effect on the risk estimates (data not shown). In subtype-specific analyses, the elevated OR for lymphoma overall in relation to HCV RNA was largely explained by the association with B-cell lymphomas but not with the risk of T-cell and Hodgkin's lymphomas (Table 4).

DLBCL was the lymphoma subtype most clearly associated with indicators of HCV infection. The presence of anti-HCV and HCV RNA were both associated with a statistically significant 2.2-fold (95% CI: 1.23–3.91) and 3.3-fold (95% CI: 1.79–6.11) increased DLBCL risk. Based on only a few cases, HCV infection increased the risk of marginal zone lymphoma (OR, 2.42; 95% CI: 0.71–8.28), lymphoplasmacytoid lymphoma (OR, 2.97; 95% CI: 0.65–13.59), and B-cell lymphoma with other/unknown histology (OR, 6.88; 95% CI: 2.19–21.68) but not that of follicular lymphoma (OR, 0.74; 95% CI: 0.17–3.15), chronic lymphocytic leukemia (OR, 1.41; 95% CI: 0.62–3.17), or MM (OR, 1.57; 95% CI: 0.59–4.20). None of the subjects diagnosed with a precursor B-cell lymphoma and lymphoma not otherwise specified were HCV infected (data not shown).

The distribution of HCV genotypes among cases and controls is summarized in Table 5. HCV genotypes 1a, 1b, 2, 2a/c, 3a, 4c/d, and 5a were found. Genotype 1b was the most prevalent, resulting in a 2.6-fold increased risk of lymphoma (95% CI: 1.38–4.74). Among the rare genotypes, genotype 2 was associated with a 4.2-fold elevated risk (95% CI: 0.88–19.96).

Discussion

This study is the largest case-control study that has explored the relationship between indicators of HCV infection and lymphoma risk to date. Our results show a 2-fold increased risk for B-NHL and in particular a greater than 3-fold elevated risk for DLBCL in relation to HCV RNA. We found no evidence for a role of HCV infection in the etiology of T-cell lymphoma, follicular lymphoma, and Hodgkin's lymphoma. The sample size was not sufficient to explore the very rare lymphoma subgroups such as splenic marginal zone lymphoma. The strengths of this study lie in its size, its multicenter approach involving data from 5 European countries with large differences in HCV prevalence, and the confirmation of all anti-HCV-positive cases by HCV RNA detection.

Table 2. OR of Lymphoma for HCV Infection

	Anti-HCV+ or HCV RNA+								HCV RNA+		
	Cases			Controls							
	Total	HCV	Prevalence %	Total	HCV	Prevalence %	OR[a,b]	95% CI	Cases/ controls	OR[a,b]	95% CI
Total	1807	53	2.93	1788	41	2.29	1.42	0.93–2.15	47/29	1.82	1.13–2.91
Country											
France	275	3	1.09	172	3	1.74	0.61	0.12–3.06	3/1	1.91	0.20–18.6
Germany	681	7	1.03	664	5	0.75	1.38	0.44–4.39	7/3	2.32	0.60–9.01
Ireland	148	—	—	136	2	1.47	—	—	—	—	—
Italy	198	13	6.57	219	9	4.11	1.76	0.73–4.26	12/7	2.13	0.81–5.60
Spain	505	30	5.94	597	22	3.69	1.57	0.89–2.76	25/18	1.60	0.86–2.97
Sex											
Male	1011	27	2.67	957	21	2.19	1.31	0.73–2.35	24/13	1.99	1.00–3.95
Female	796	26	3.27	831	20	2.41	1.58	0.87–2.88	23/16	1.73	0.90–3.33
Age at interview, *y*											
>55	673	6	0.89	707	10	1.41	0.63	0.22–1.76	6/8	0.81	0.28-2.38
≥55	1134	47	4.14	1081	31	2.87	1.63	1.02–2.61	41/21	2.14	1.25-3.68

NOTE. Excluding HIV-positive and transplantation individuals.
[a]Adjusted for country and (when appropriate) age (continuous) and sex.
[b]HCV-negative subjects taken as reference category.

An approximately 2-fold elevated risk for lymphoma among HCV RNA-positive subjects was seen, albeit not statistically significant, in all countries except Ireland (where no HCV-positive cases and 2 HCV-positive controls were detected), regardless of the geographic differences in HCV prevalence among the controls. This result supports the specificity of the observed association. One potential weakness is the inclusion of hospital controls. Our study included population-based and

Table 3. OR and Corresponding 95% CI for HCV Positivity Among the Controls

Category	HCV positive n = 41 (%)	HCV negative n = 1747 (%)	OR[a] (95% CI)	*P* value
Sex				
Male	21 (51.2)	936 (53.6)		
Female	20 (48.8)	811 (46.4)		
Age at interview, *y*				
<45	8 (19.5)	421 (24.1)	1 (ref.)	
45–<55	2 (4.9)	276 (15.8)	0.36 (0.08–1.72)	.199
55–<65	9 (22.0)	376 (21.5)	1.26 (0.48–3.31)	.645
≥65	22 (53.7)	674 (38.6)	1.64 (0.72–3.74)	.238
Education[b]				
Low	32 (78.1)	888 (50.9)	2.49 (0.99–6.26)	.053
Medium	8 (19.5)	617 (35.3)	1 (ref.)	
High	1 (2.4)	241 (13.8)	0.34 (0.04–2.80)	.318
IV drug use[b]				
No	39 (95.1)	1737 (99.6)	1 (ref.)	
Yes	2 (4.9)	7 (0.4)	14.14 (2.56–78.19)	.002
Blood transfusion[b]				
No	26 (63.4)	1396 (80.5)	1 (ref.)	
Yes	15 (36.6)	339 (19.5)	2.05 (1.05–4.00)	.036
Year of transfusion[b]				
Before 1990	13 (31.7)	164 (9.5)	4.06 (1.99–8.27)[c]	<.001
In or after 1990	2 (4.9)	175 (10.1)	0.47 (0.11–2.04)[c]	.314
Reported history of hepatitis B[b]				
No	36 (90.0)	1694 (98.4)	1 (ref.)	
Yes	4 (10.0)	27 (1.6)	5.43 (1.74–17.0)	.004
Reported history of hepatitis other than B[b]				
No	24 (58.5)	1625 (93.7)	1 (ref.)	
Yes	17 (41.5)	109 (6.3)	14.23 (7.09–28.54)	<.001

[a]Adjusted for country and (when appropriate) age (continuous) and sex.
[b]Numbers do not add up to 1788 because of missing information on these variables.
[c]No transfusion taken as reference.

Table 4. OR of Lymphoma Subtypes for HCV Infection

Lymphoma type[a]	Anti-HCV+ or HCV RNA+				HCV RNA +			
	Cases/Controls	OR[b,c]	95% CI	*P* value	Cases/Controls	OR[b,c]	95% CI	*P* value
T-cell lymphoma (n = 101)	2/41	0.88	0.21–3.74	.864	2/29	1.29	0.30–5.55	.37
Hodgkin's lymphoma (n = 239)	3/41	0.97	0.27–3.48	.963	2/29	0.92	0.20–4.30	.915
B-cell lymphoma (n = 1465)	48/41	1.46	0.95–2.24	.086	43/29	1.91	1.18–3.09	.009
DLBCL (n = 392)	18/41	2.19	1.23–3.91	.008	18/29	3.30	1.79–6.11	.0001
FL (n = 210)	2/41	0.50	0.12–2.08	.338	2/29	0.74	0.17–3.15	.679
CLL (n = 342)	10/41	1.16	0.56–2.38	.689	8/29	1.41	0.62–3.17	.410
MM (n = 221)	7/41	1.40	0.61–3.24	.427	5/29	1.57	0.59–4.20	.367
LPL (n = 41)	2/41	1.94	0.43–8.66	.388	2/29	2.97	0.65–13.59	.162
Other B-cell lymphoma (n = 172)	4/41	1.03	0.36–2.93	.959	4/29	1.47	0.50–4.28	.483
Splenic marginal zone lymphoma (n = 35)	1/41	0.83	0.11–6.35	.861	1/29	1.13	0.15–8.72	.907
Other marginal zone lymphoma (n = 77)	3/41	1.76	0.52–5.91	.362	3/29	2.42	0.71–8.28	.160
B-NOS (n = 60)	5/41	4.65	1.66–12.97	.003	4/29	6.88	2.19–21.68	.001

NOTE. Excluding HIV-positive and transplantation individuals.
B-NOS, B-cell non-Hodgkin's lymphoma not otherwise specified; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; LPL, lymphoplasmacytoid lymphoma; MM, multiple myeloma.
[a]None of 27 cases of precursor B-cell lymphoma and none of 2 cases of not otherwise specified lymphoma were HCV infected.
[b]Adjusted for country, age (continuous), and sex.
[c]HCV-negative subjects taken as reference category.

hospital-based controls at almost equal proportions (49% vs 51%, respectively). An elevated risk of lymphoma overall and DLBCL in particular for individuals with measured HCV RNA has been found in the population-based (Germany, Italy; OR of all lymphomas, 2.12; 95% CI: 0.97–4.64) as well as hospital-based (France, Ireland, Spain; OR, 1.60; 95% CI: 0.90–2.98) study populations (*P* value for test of heterogeneity: $P = .746$). Overall, regardless of population-based and hospital-based sampling of cases and controls, HCV infection was consistently associated with B-cell lymphoma in our study. Our results are in line with results from several recent case-control studies[15–17] and further evidence from previous cohort and case-control studies as summarized in reviews on this topic.[5–7]

The comparison of our results on lymphoma subtypes with data from other studies is hampered by the fact that most previous studies were either too small to allow an entity-specific analysis or were not based on the WHO classification of lymphoid neoplasms. However, our results are in agreement with several studies mainly from Italy that also found an association of HCV with DLBCL,[15,18–21] with marginal zone lymphoma,[18,19,21] and with lymphoplasmacytoid lymphoma.[20–22] We and others[15] did not confirm the association of HCV infection with follicular lymphoma as was reported by Morton et al,[16] Engels et al,[17] and Luppi et al.[19] Other lymphoma subtypes, which do not originate from germinal center or postgerminal center B cells, such as mantle cell NHL, Burkitt's lymphoma, T-cell lymphoma, and HL, however, were not consistently linked to HCV infection. The lack of an association with these entities is in line with the notion that proliferation of specific B-cell clones because of chronic antigenic stimulation sustained by HCV is the likely mechanism that drives the HCV-mediated pathogenesis of B-cell lymphoma, in particular marginal zone lymphoma and DLBCL. In fact, the observed elevated risk in association with HCV infection is restricted to these entities in this study population.

Chronic infection with HCV is strongly associated with mixed cryoglobulinemia type II, which may evolve into an overt lymphoma in some patients.[23–25] Increasing evidence indicates that marginal zone lymphoma of mucosa-associated lymphoid tissue (MALT), nodal and splenic types, are associated with chronic antigenic stimulation by autoantigens and/or bacterial or viral pathogens. Of these, *Helicobacter pylori* is the best-characterized infectious agent and has repeatedly been associated with gastric MALT lymphoma.[26] In line with studies that demonstrated a regression of these tumors in the course of HCV therapy,[27] we and others[18,19,21] found some evidence for a

Table 5. HCV Genotype Distribution Among Cases and Controls

	Cases n (%)	Controls n (%)	OR[a,b]	95% CI	*P* value
HCV genotype					
1a	1 (1.9)	5 (12.2)	0.26	0.05–1.29	.104
1b	33 (62.3)	15 (36.6)	2.55	1.38–4.74	.003
2	8 (15.1)	2 (4.9)	4.19	0.88–19.96	.72
3a	1 (1.9)	0	—	—	
4c/d	0	2 (4.9)	—	—	
5a	1 (1.9)	0	—	—	
Missing	3	5			

[a]Adjusted for age (continuous), sex, and country.
[b]HCV-negative subjects taken as reference category.

role of chronic HCV infection in the etiology of marginal zone lymphoma; however, numbers were too small to provide convincing evidence. Pooling of data from recently conducted epidemiologic case-control studies with available data on *H pylori* status will clarify whether HCV is the infectious agent driving the pathogenesis of *H pylori*–negative marginal zone lymphomas.

The most important finding of this large study is the significant association of HCV infection with DLBCL. HCV does not integrate into host genomes and does not contain an obvious oncogene, and the exact pathogenetic mechanism through which the virus may lead to malignant transformation of B-cells is still unclear. HCV envelope protein E2 has been shown to bind to CD81, which is expressed on B-cells,[28] and to the B-cell receptor of HCV-associated lymphomas,[29] thereby activating the intracellular signalling of B-cells. Chronic stimulation of B cells, leading to polyclonal and later to monoclonal expansion of these immunoglobulin secreting cells, then leaves this population of cells prone to malignant-transforming mutational events.

Recently, it was shown that HCV infection activates the immunologic isoform of nitric oxide synthase and induces nitric oxide (NO) production in B-cells.[30] NO is a potent inducer of DNA breaks and was shown to enhance mutation frequencies of cellular genes. Acute and chronic HCV infection induces a mutator phenotype with a substantial increase in mutation frequency in Ig heavy chain, *BCL-6*, *TP53*, and β-catenin genes in vitro.[31] Interestingly, *BCL-6*, encoding a transcriptional repressor required for germinal center formation, is the hallmark gene affected by translocations and mutations specifically in DLBCL, the lymphoma subtype most strongly associated with HCV infection in this study.[11] Other evidence supports the role of HCV core proteins in activation of the nuclear factor-κB pathway,[32] in inducing reactive oxygen species,[33] and in impacting on the apoptotic pathway.[34]

HCV RNA was more strongly associated with lymphoma than anti-HCV antibodies. This may be related to an impaired antibody production among patients that results in an underestimation of the role of HCV in the pathogenesis of lymphomas. This also indicates that chronic rather than cleared HCV infection is required for lymphomagensis, consistent with the idea that chronic antigenic stimulation driven by chronic infection leads to lymphoma. Furthermore, the stronger association with HCV RNA may also suggest a more efficient clearing of the virus among the controls. It will be interesting to investigate to what extent host factors that control the natural course of HCV infection modulate lymphoma risk among HCV-infected individuals. Candidate genes, such as tumour necrosis factor-α TNF-α and interleukin-10 (*IL-10*), have already been shown to affect natural clearance and recovery from HCV.[35–37] Interestingly, some of these have also been identified as predisposing factors for lymphomas, particularly for DLBCL.[38–40]

HIV-1 infection is a well-recognized risk factor for NHL. Many individuals infected with HIV-1 are coinfected with HCV.[41] To exclude the possibility that HCV was only associated with lymphoma risk because of coinfection with HIV-1, individuals with a positive HIV-1 test were excluded from the analysis. In the context of this epidemiologic study, the individuals were not systematically screened for HIV-1. However, cases were tested for HIV-1 in the course of their diagnosis. It cannot be excluded that among the controls some may have been infected by HIV-1. If anything, this may have led to an underestimation of the true association between HCV and lymphoma. In the largest cohort study of HIV-1-positive individuals in Europe, HCV infection was not associated with systemic HIV-associated NHL.[42] HCV has not been shown to increase NHL risk among HIV-positive individuals.[43] Following the hypothesized mechanism of chronic antigen stimulation triggering lymphomagenesis, an effect of HCV infection in immunocompromised patients, such as HIV-positive subjects, was indeed not expected.

Many previous epidemiologic studies did not consider HCV genotypes in their evaluations. Therefore, data linking specific HCV genotypes with lymphomas have been scarce. Some evidence for a role of genotype 2 with lymphomas has been presented.[15,22] Other studies, however, did not support this finding.[44] We found an accumulation of genotypes 1b (statistically significant) and 2 among the cases, whereas genotype 1a was overrepresented among the controls. Whether the various HCV genotypes differ in their oncogenic potential would require further mechanistic research and an even larger study than ours.

The evidence from epidemiologic studies is strengthened by clinical data showing a regression of lymphoma after successful treatment of HCV infection.[27,45,46] In a recent systematic review including data from therapeutic studies in which α-interferon was administered to patients with lymphoproliferative disorders, a complete remission of this disorder was achieved in 75% of the cases.[45] Nonsplenic marginal zone lymphomas showed the same effect.[46] These data, including ours, collectively support a positive association between HCV infection and B-cell lymphoma and a role of viral replication in lymphomagenesis. However, a few questions remain, including the exact mechanism how HCV exerts its oncogenic potential, the specificity of the association with lymphoma subtypes, and the role of host and viral factors contributing to the pathogenesis of HCV-related lymphoma.

References

1. Global surveillance and control of hepatitis C. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. J Viral Hepat 1999;6:35–47.
2. Shepard CW, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. Lancet Infect Dis 2005;5:558–567.
3. Kayali Z, Buckwold VE, Zimmerman B, Schmidt WN. Hepatitis C, cryoglobulinemia, and cirrhosis: a meta-analysis. Hepatology 2002;36:978–985.
4. Gisbert JP, Garcia-Buey L, Pajares JM, Moreno-Otero R. Prevalence of hepatitis C virus infection in B-cell non-Hodgkin's lymphoma: systematic review and meta-analysis. Gastroenterology 2003;125:1723–1732.
5. Matsuo K, Kusano A, Sugumar A, Nakamura S, Tajima K, Mueller NE. Effect of hepatitis C virus infection on the risk of non-Hodgkin's lymphoma: a meta-analysis of epidemiological studies. Cancer Sci 2004;95:745–752.
6. Negri E, Little D, Boiocchi M, La Vecchia C, Franceschi S. B-cell non-Hodgkin's lymphoma and hepatitis C virus infection: a systematic review. Int J Cancer 2004;111:1–8.
7. Turner NC, Dusheiko G, Jones A. Hepatitis C and B-cell lymphoma. Ann Oncol 2003;14:1341–1345.
8. Rabkin CS, Tess BH, Christianson RE, Wright WE, Waters DJ, Alter HJ, Van Den Berg BJ. Prospective study of hepatitis C viral

infection as a risk factor for subsequent B-cell neoplasia. Blood 2002;99:4240–4242.
9. Ohsawa M, Shingu N, Miwa H, Yoshihara H, Kubo M, Tsukuma H, Teshima H, Hashimoto M, Aozasa K. Risk of non-Hodgkin's lymphoma in patients with hepatitis C virus infection. Int J Cancer 1999;80:237–239.
10. Duberg AS, Nordstrom M, Torner A, Reichard O, Strauss R, Janzon R, Back E, Ekdahl K. Non-Hodgkin's lymphoma and other nonhepatic malignancies in Swedish patients with hepatitis C virus infection. Hepatology 2005;41:652–659.
11. Harris NL, Stein H, Coupland SE, Hummel M, Favera RD, Pasqualucci L, Chan WC. New approaches to lymphoma diagnosis. Hematology (Am Soc Hematol Educ Program) 2001;194–220.
12. De Sanjose S, Benavente Y, Nieters A, Foretova L, Maynadie M, Cocco PL, Staines A, Vornanen M, Boffetta P, Becker N, Alvaro T, Brennan P. Association between personal use of hair dyes and lymphoid neoplasms in Europe. Am J Epidemiol 2006;164:47–55.
13. Jaffé ES, Harris NL, Stein H, Vardiman JWE. World Health Organization Classification of Tumours. Pathology and genetics of tumours of haematopoietic and lymphoid tissues. Lyon: IARC Press, 2001.
14. Colin C, Lanoir D, Touzet S, Meyaud-Kraemer L, Bailly F, Trepo C. Sensitivity and specificity of third-generation hepatitis C virus antibody detection assays: an analysis of the literature. J Viral Hepat 2001;8:87–95.
15. Talamini R, Montella M, Crovatto M, Dal Maso L, Crispo A, Negri E, Spina M, Pinto A, Carbone A, Franceschi S. Non-Hodgkin's lymphoma and hepatitis C virus: a case-control study from northern and southern Italy. Int J Cancer 2004;110:380–385.
16. Morton LM, Engels EA, Holford TR, Leaderer B, Zhang Y, Zahm SH, Boyle P, Zhang B, Flynn S, Tallini G, Owens PH, Zheng T. Hepatitis C virus and risk of non-Hodgkin lymphoma: a population-based case-control study among Connecticut women. Cancer Epidemiol Biomarkers Prev 2004;13:425–430.
17. Engels EA, Chatterjee N, Cerhan JR, Davis S, Cozen W, Severson RK, Whitby D, Colt JS, Hartge P. Hepatitis C virus infection and non-Hodgkin lymphoma: results of the NCI-SEER multi-center case-control study. Int J Cancer 2004;111:76–80.
18. Silvestri F, Pipan C, Barillari G, Zaja F, Fanin R, Infanti L, Russo D, Falasca E, Botta GA, Baccarani M. Prevalence of hepatitis C virus infection in patients with lymphoproliferative disorders. Blood 1996;87:4296–4301.
19. Luppi M, Longo G, Ferrari MG, Barozzi P, Marasca R, Morselli M, Valenti C, Mascia T, Vandelli L, Vallisa D, Cavanna L, Torelli G. Clinico-pathological characterization of hepatitis C virus-related B-cell non-Hodgkin's lymphomas without symptomatic cryoglobulinemia. Ann Oncol 1998;9:495–498.
20. Germanidis G, Haioun C, Pourquier J, Gaulard P, Pawlotsky JM, Dhumeaux D, Reyes F. Hepatitis C virus infection in patients with overt B-cell non-Hodgkin's lymphoma in a French center. Blood 1999;93:1778–1779.
21. Mele A, Pulsoni A, Bianco E, Musto P, Szklo A, Sanpaolo MG, Iannitto E, De Renzo A, Martino B, Liso V, Andrizzi C, Pusterla S, Dore F, Maresca M, Rapicetta M, Marcucci F, Mandelli F, Franceschi S. Hepatitis C virus and B-cell non-Hodgkin lymphomas: an Italian multicenter case-control study. Blood 2003;102:996–999.
22. Silvestri F, Barillari G, Fanin R, Pipan C, Falasca E, Salmaso F, Zaja F, Infanti L, Patriarca F, Botta GA, Baccarani M. Hepatitis C virus infection among cryoglobulinemic and non-cryoglobulinemic B-cell non-Hodgkin's lymphomas. Haematologica 1997;82:314–317.
23. Zuckerman E, Zuckerman T. Hepatitis C and B-cell lymphoma: the hemato-hepatologist linkage. Blood Rev 2002;16:119–125.
24. Misiani R, Bellavita P, Fenili D, Borelli G, Marchesi D, Massazza M, Vendramin G, Comotti B, Tanzi E, Scudeller G. Hepatitis C virus infection in patients with essential mixed cryoglobulinemia. Ann Intern Med 1992;117:573–577.
25. Pozzato G, Mazzaro C, Crovatto M, Modolo ML, Ceselli S, Mazzi G, Sulfaro S, Franzin F, Tulissi P, Moretti M. Low-grade malignant lymphoma, hepatitis C virus infection, and mixed cryoglobulinemia. Blood 1994;84:3047–3053.
26. Thieblemont C, de la FA, Coiffier B. Nongastric mucosa-associated lymphoid tissue lymphomas. Clin Lymphoma 2003;3:212–224.
27. Hermine O, Lefrere F, Bronowicki JP, Mariette X, Jondeau K, Eclache-Saudreau V, Delmas B, Valensi F, Cacoub P, Brechot C, Varet B, Troussard X. Regression of splenic lymphoma with villous lymphocytes after treatment of hepatitis C virus infection. N Engl J Med 2002;347:89–94.
28. Pileri P, Uematsu Y, Campagnoli S, Galli G, Falugi F, Petracca R, Weiner AJ, Houghton M, Rosa D, Grandi G, Abrignani S. Binding of hepatitis C virus to CD81. Science 1998;282:938–941.
29. Quinn ER, Chan CH, Hadlock KG, Foung SK, Flint M, Levy S. The B-cell receptor of a hepatitis C virus (HCV)-associated non-Hodgkin lymphoma binds the viral E2 envelope protein, implicating HCV in lymphomagenesis. Blood 2001;98:3745–3749.
30. Machida K, Cheng KT, Sung VM, Lee KJ, Levine AM, Lai MM. Hepatitis C virus infection activates the immunologic (type II) isoform of nitric oxide synthase and thereby enhances DNA damage and mutations of cellular genes. J Virol 2004;78:8835–8843.
31. Machida K, Cheng KT, Sung VM, Shimodaira S, Lindsay KL, Levine AM, Lai MY, Lai MM. Hepatitis C virus induces a mutator phenotype: enhanced mutations of immunoglobulin and protooncogenes. Proc Natl Acad Sci U S A 2004;101:4262–4267.
32. Kato N, Yoshida H, Kioko Ono-Nita S, Kato J, Goto T, Otsuka M, Lan K, Matsushima K, Shiratori Y, Omata M. Activation of intracellular signaling by hepatitis B and C viruses: C-viral core is the most potent signal inducer. Hepatology 2000;32:405–412.
33. Okuda M, Li K, Beard MR, Showalter LA, Scholle F, Lemon SM, Weinman SA. Mitochondrial injury, oxidative stress, and antioxidant gene expression are induced by hepatitis C virus core protein. Gastroenterology 2002;122:366–375.
34. Chung YL, Sheu ML, Yen SH. Hepatitis C virus NS5A as a potential viral Bcl-2 homologue interacts with Bax and inhibits apoptosis in hepatocellular carcinoma. Int J Cancer 2003;107:65–73.
35. Thio CL, Goedert JJ, Mosbruger T, Vlahov D, Strathdee SA, O'Brien SJ, Astemborski J, Thomas DL. An analysis of tumor necrosis factor α gene polymorphisms and haplotypes with natural clearance of hepatitis C virus infection. Genes Immun 2004;5:294–300.
36. Lio D, Caruso C, Di Stefano R, Colonna RG, Ferraro D, Scola L, Crivello A, Licata A, Valenza LM, Candore G, Craxi A, Almasio PL. IL-10 and TNF-α polymorphisms and the recovery from HCV infection. Hum Immunol 2003;64:674–680.
37. Mangia A, Santoro R, Piattelli M, Pazienza V, Grifa G, Iacobellis A, Andriulli A. IL-10 haplotypes as possible predictors of spontaneous clearance of HCV infection. Cytokine 2004;25:103–109.
38. Rothman N, Skibola CF, Wang SS, Morgan G, Lan Q, Smith MT, Spinelli JJ, Willett E, De Sanjose S, Cocco P, Berndt SI, Brennan P, Brooks-Wilson A, Wacholder S, Becker N, Hartge P, Zheng T, Roman E, Holly EA, Boffetta P, Armstrong B, Cozen W, Linet M, Bosch FX, Ennas MG, Holford TR, Gallagher RP, Rollinson S, Bracci PM, Cerhan JR, Whitby D, Moore PS, Leaderer B, Lai A, Spink C, Davis S, Bosch R, Scarpa A, Zhang Y, Severson RK, Yeager M, Chanock S, Nieters A. Genetic variation in TNF and IL10 and risk of non-Hodgkin lymphoma: a report from the InterLymph Consortium. Lancet Oncol 2006;7:27–38.
39. Warzocha K, Ribeiro P, Bienvenu J, Roy P, Charlot C, Rigal D, Coiffier B, Salles G. Genetic polymorphisms in the tumor necrosis

factor locus influence non-Hodgkin's lymphoma outcome. Blood 1998;91:3574–3581.
40. Cunningham LM, Chapman C, Dunstan R, Bell MC, Joske DJ. Polymorphisms in the interleukin 10 gene promoter are associated with susceptibility to aggressive non-Hodgkin's lymphoma. Leuk Lymphoma 2003;44:251–255.
41. Maier I, Wu GY. Hepatitis C and HIV co-infection: a review. World J Gastroenterol 2002;8:577–579.
42. Waters L, Stebbing J, Mandalia S, Young AM, Nelson M, Gazzard B, Bower M. Hepatitis C infection is not associated with systemic HIV-associated non-Hodgkin's lymphoma: a cohort study. Int J Cancer 2005;116:161–163.
43. Engels EA, Frisch M, Lubin JH, Gail MH, Biggar RJ, Goedert JJ. Prevalence of hepatitis C virus infection and risk for hepatocellular carcinoma and non-Hodgkin lymphoma in AIDS. J Acquir Immune Defic Syndr 2002;31:536–541.
44. De Rosa G, Gobbo ML, De Renzo A, Notaro R, Garofalo S, Grimaldi M, Apuzzo A, Chiurazzi F, Picardi M, Matarazzo M, Rotoli B. High prevalence of hepatitis C virus infection in patients with B-cell lymphoproliferative disorders in Italy. Am J Hematol 1997; 55:77–82.
45. Gisbert JP, Garcia-Buey L, Pajares JM, Moreno-Otero R. Systematic review: regression of lymphoproliferative disorders after treatment for hepatitis C infection. Aliment Pharmacol Ther 2005;21:653–662.
46. Svoboda J, Andreadis C, Downs LH, Miller WT Jr, Tsai DE, Schuster SJ. Regression of advanced non-splenic marginal zone lymphoma after treatment of hepatitis C virus infection. Leuk Lymphoma 2005;46:1365–1368.

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