Title (Limit = 50 words characters, 14):

The extent of B-cell activation and dysfunction preceding lymphoma development in HIV positive people

Short title (limit = 50 characters, 36): Markers of B-cell dysfunction prior to lymphoma

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Abstract

Objective

B-cell dysfunction and activation is thought to contribute to lymphoma development in HIV-positive people, however the mechanisms are not well understood. We investigated levels of several markers of B-cell dysfunction (free light chain[FLC]-κ, FLC-λ, immunoglobulin[Ig]G, IgA, IgM and IgD) prior to lymphoma diagnosis in HIV-positive people.

Design

A nested-matched-case-control study within the EuroSIDA cohort, including 73 HIV-positive people with lymphoma and 143 HIV-positive lymphoma-free controls.

Methods

Markers of B-cell dysfunction were measured in prospectively stored serial plasma samples before lymphoma (or selection date in controls). Marker levels ≤2 and >2 years prior to diagnosis were investigated.

Results

A 2-fold higher level of FLC-κ(OR: 1.84 95%CI: 1.19, 2.84), FLC-λ (2.15 95%CI: 1.34, 3.46), IgG(3.05 95%CI: 1.41, 6.59) and IgM(1.46 95%CI: 1.01, 2.11) were associated with increased risk of Lymphoma >2 years prior to diagnosis, but not ≤2 years prior. Despite significant associations >2 years prior to diagnosis, predictive accuracy of each marker was poor, with FLC-λ emerging as the strongest candidate with a c-statistic of 0.67 (95%CI: 0.58, 0.76).

Conclusions

FLC-κ, FLC-λ, and IgG levels were higher >2 years before lymphoma diagnosis, suggesting that B-cell dysfunction occurs many years prior to lymphoma development. However, the predictive

value of each marker was low and are unlikely candidates for risk assessment for targeted intervention.

Key words

HIV, Free light chains, Immunoglobulins, Lymphoma, B-cell dysfunction, biomarkers

Introduction

HIV-positive (HIV+) persons are known to have higher rates of infection-related malignancies due to increased immune deficiency [1-4]. Epstein-Barr Virus (EBV) has been associated with almost all cases of Hodgkins lymphoma (HL) and between 30-100% of non-Hodgkins lymphoma (NHL) occurring in the setting of HIV infection . Effective combination antiretroviral treatment (cART) has led to a significant decline in the incidence of all subtypes of NHL (except Burkitt) [5-12]. Despite this, NHL still accounts for approximately half of all AIDS related malignancies and incidence remains around 10-fold higher in HIV+ compared to HIV-negative (HIV-) people. Conversely, HL incidence has remained stable or even increased in HIV+ people since the introduction of cART , and is estimated to be 11-fold higher (with estimates ranging from 5 – 15) than in the HIV- .

Despite being common cancers in HIV+ populations, the mechanisms driving pathogenesis of lymphoma in the HIV+ setting is poorly understood. Untreated HIV infection causes disruption to the immune system , characterised by hypergammaglobulinaemia, immune deficiency, immune dysfunction, senescence, chronic immune activation (or T cell activation) and inflammation, several of which are thought to be drivers in the development B-cell malignancies [4, 13-17]. Increased B–cell activation and proliferation also leads to increased synthesis of antibodies [18], which consist of 2 light chain immunoglobulins bound to 2 heavy-chain immunoglobulins (IgG, IgA, IgM, IgD, or IgE). During immunoglobulin production, more light chains are produced than heavy chains, and excess unbound light chains (known as free light chains, FLCs) levels enter circulation, where both immunoglobulins and FLCs can be detected in serum [19]. There are two types FLCs: kappa (FLC-κ) and lambda (FLC-λ) [20], which are markers of non-specific polyclonal B-cell activation and hypergammaglobulinemia, both of which have been linked to HIV disease severity and lymphoma development [19, 21-24].

While HIV-associated immune deficiency, B-cell dysfunction, B-cell activation, as well as reactivation of latent EBV infection all play a role in lymphoma development, it is unclear whether HIV-related factors facilitate B-cell proliferation which promotes EBV expansion or if ongoing EBV replication directly causes immune activation

prior to lymphoma development, or a combination of both. This study aims to investigate the relationship between B-cell activation, as demonstrated by increased levels of immunoglobulins and FLCs, and the subsequent risk of lymphoma development in HIV+ people.

Methods

The EuroSIDA study

EuroSIDA is a prospective, observational, open cohort of more than 22,000 HIV-positive people aged over 16 years in 107 centres across 35 European countries, Israel and Argentina recruited since May 1994 (details at www.cphiv.dk). Informed consent was obtained from all patients. Basic demographic, clinical and laboratory data are collected every six months, including all CD4 counts and HIV-RNA viral load measured since last follow-up, starting and stopping dates of all antiretroviral drugs, dates of AIDS-defining diagnoses (using the 1993 CDC clinical definition, including AIDS-defining malignancies [ADM]). All new non-AIDS-defining diagnoses (including HL) [25] have been collected since 2001. All reported malignancies were source verified against case notes at the sites by members of the coordinating office to ensure data accuracy. Loss to follow-up in EuroSIDA is<5% per 100 PYFU and consistent over time [26]. EuroSIDA has an established biobank, where prospective plasma samples have been collected at approximate 6 monthly intervals. This sample repository currently holds more than 78,000 plasma samples on 8,300 patients.

Nested case control study

A 1:2 nested case control study was performed within the EuroSIDA cohort utilising stored plasma samples to investigate the kinetics and predictive value of several markers of immune activation: FLC-κ, FLC-λ, IgG, IgA, IgM and IgD. Both cases and controls were selected from HIV-positive people enrolled in EuroSIDA with prospective follow-up after the 1 January 2001. Eligible people with a primary diagnosis of lymphoma after 1 January 2001 were considered as cases. For each case, 2 matched controls (where available) were selected from eligible people with no

history of NHL or HL at the time of diagnosis for each case (referred to as the "selection date" of the matched controls). Both cases and controls were required to have at least one plasma sample available prior to the diagnosis date (or selection date in controls). Cases and controls were matched on region of Europe, gender, date of earliest plasma sample (±2years), date of latest plasma sample (±2years), age at earliest plasma sample (±5 years), and CD4 cell count at earliest plasma sample (±200 cells/mm^3). The windows used for matching were selected to allow suitable identification of controls while ensuring as few cases as possible were excluded from analyses. All available serial samples for cases and controls prior to the date of lymphoma diagnosis or selection date in controls were considered for inclusion. Where more than one plasma sample was available during the same calendar year, one plasma sample was randomly selected. Date of earliest plasma sample was considered as baseline. We initially selected 73 cases and 143 controls (3 cases had only 1 suitable control available) with 600 samples for inclusion. However 6 samples were not available and 594 samples were analysed.

Laboratory markers

Serial samples for cases and controls were analysed for FLC-κ, FLC-λ, IgG, IgA, IgM and IgD. All biomarkers were centrally measured by a technician blinded to case control status on frozen stored plasma at the Department of Clinical Biochemistry at Rigshospitalet. FLC (the κ and λ Freelite® turbidimeteric/nephelometric immunoassay, product code: LK016.S and KL018.S), IgG (NK004.S), IgA (NK010.S), IgM (NK012.S), and IgD (LK013.S) concentrations were measured on plasma in all patients using Immunoassay from Binding Site Group Ltd, Birmingham, UK on the SPAPLUS ®.

Statistical analysis

We considered the relationship between FLC-κ, FLC-λ, the ratio of FLC-κ to FLC-λ (FLC-κ/λ), the sum of FLC-κ and λ (FLC-κ+λ), IgG, IgA, IgM and IgD and lymphoma development.

Unadjusted conditional logistic regression models were used to investigate the association between the odds of developing lymphoma and a higher level of each marker using samples that were collected ≤2 and >2 years prior to lymphoma diagnosis or selection date in controls. In the case where ≥ 1 sample was available within the same time period, one was randomly selected. Many other studies have used the upper limit of the normal to identify high marker levels, however, it was decided not to use this approach as these limits have been validated in the HIV-negative population only. Instead, marker levels were investigated on the log2 scale, giving an odds ratio (OR) which corresponding to a 2-fold higher (or a doubling of) of each marker level. Polyclonal elevations in FLCs refer to proportionately elevated levels of FLC-κ and FLC-λ, defined as FLC-κ >19.4 mg/L or FLC-λ >26.3 mg/L and FLC-κ/λ between 0.26 mg/L – 1.65 mg/L. Monoclonal elevations in FLCs were refer to a disproportionately higher level of one FLC, defined as FLC-κ >19.4 mg/L or FLC-λ >26.3 mg/L and FLC-κ/λ not between 0.26 – 1.65.

The area under the receiver operator curve statistic (c-statistic) was calculated to determine the predictive value of each marker. Predictive ability was classified as follows (c-statistic 0.51 – 0.6: Poor; 0.61 – 0.7: Poor-moderate; 0.71 – 0.8: Moderate, 0.81 – 0.9; Good, 0.9 – 1: Excellent). This was performed for each marker using plasma samples collected ≤2 and >2 years prior to lymphoma diagnosis or selection date in controls.

The percentage change for each marker over calendar time (% change per year) in the period prior to diagnosis or selection date in controls was investigated using mixed models with random slopes and intercepts (accounting for multiple measurements within each person).

Patient factors associated with higher B-cell activation marker levels were assessed using mixed models. In order to minimise bias due to our nested-case control study design (leading to a non-representative patient population where 1 in 3 develop a lymphoma), this analysis of factors was restricted to controls only. Factors investigated included current age, gender, region of Europe, current use of cART (defined as ≥ 3 antiretroviral drugs), current and nadir CD4 cell count, and current and area under the curve (AUC) of HIV viral load (HIV-VL). AUC of HIV-VL is a measure of accumulated exposure to replicating HIV [27]. All statistical tests were two sided with a type I error rate of 5%. All statistical analyses were performed using SAS 9.4 (Statistical Analysis Software, Cary NC, USA).

Results

Baseline characteristics of cases and controls

Characteristics of cases (N=73, 52 non-Hodgkin lymphoma and 21 Hodgkin lymphoma) and controls (N=143) are shown in Table 1. There was a median of 2.0 years (IQR 0.4,3.1) between first and last plasma sample (cases: 2.1 years IQR: 0.6,4.4; controls: 1.8 years IQR: 0.0,4.3, P=0.72) and 1.3 years (IQR: 0.3,2.9) between last sample and date of lymphoma diagnosis in cases. Cases differed from controls according to HIV-related factors, including HIV-VL and treatment. Median HIV-VL level was higher in cases than controls and a lower proportion of cases were on cART at baseline (63.0 vs 78.3%). The duration of cART was also less in cases (0.9 years IQR: 0.0, 2.5) than controls (1.7 years IQR: 0.4 ,3.3). At earliest sample, median levels of FLC-κ, FLC-λ and FLC-κ+λ were elevated in cases relative to controls, however the median FLC-κ/λ ratio was similar. Levels of IgG and IgM were also elevated in cases, however levels of IgA and IgD were similar in cases and controls. In addition, a lower proportion of cases were HCV positive compared to controls (9.6 vs 27.3%). Cases and controls were well balanced on other non-matched demographic characteristics. There was no difference in levels of any B-cell markers at latest sample (all P>0.05).

Odds ratios (OR) of developing a lymphoma during prospective follow-up

The OR of developing lymphoma for a 2-fold higher marker level both ≤2 and >2 years before lymphoma diagnosis or selection date in controls are shown in Figure 1. A 2-fold higher level of FLC-κ (OR: 1.84 95%CI: 1.19,2.84), FLC-λ (OR:2.15 95%CI: 1.34,3.46), IgG (OR:3.05 95%CI: 1.41,6.59), and IgM (OR:1.46 95%CI: 1.01,2.11) were predictive of lymphoma development >2 years prior to diagnosis. However, associations were not evident ≤2 years prior to diagnosis. No association was found for 2-fold higher IgA or IgD (although, the p-value was close to 0.05 for the association >2 years prior for IgD) at either time point. FLC-κ+λ (OR: 2.08 95%CI: 1.30,3.35) was predictive >2 years but not ≤2 years prior to diagnosis prior to lymphoma diagnosis. The ratio of FLC-κ/λ was not predictive at either >2 years or ≤2 years prior to diagnosis. Proportionately high levels of both FLC-κ and FLC-λ was associated with lymphoma >2 years prior to diagnosis (OR: 4.74 95%CI: 1.71,27.56), but not ≤2 years prior (1.62 94%CI: 0.54,5.05). Having a disproportionately high level of one FLC was not associated at either time point.

Of the HIV related markers, HIV-VL AUC was associated with a higher risk in sample ≤ 2 years prior to diagnosis, however HIV-VL was predictive >2 years prior. In those who had HIV-VL measured, a 10-fold higher HIV-VL was predictive of lymphoma development >2 years prior to development (OR: 1.51 95%CI: 1.1,2.08), but not ≤2 years prior (OR: 1.31 95%CI: 0.99,1.75). Whereas for a 10–fold higher AUC of HIV-VL, there was no association >2 years (OR: 1.25 95%CI: 0.93,1.67), but a 1.68-fold higher odds of lymphoma ≤2 years of diagnosis (95%CI: 1.08,2.62).

Predictive value of B-cell marker markers

The marker with the best predictability >2 years prior to lymphoma diagnosis was FLC-λ (Table 2). This marker predicted lymphoma diagnosis with better accuracy than chance alone (P<0.01), however the c-statistic of 0.67 suggests only poor-moderate classification power. The following markers also had some predictive power (all P<0.05), however prediction was poor to moderate at best: FLC-κ+λ (c-statistic= 0.67, poor-moderate prediction), and IgG (c-statistic: 0.64, poor-moderate). Only FLC-λ (c-statistic: 0.61) predicted lymphoma ≤2 years of diagnosis, however accuracy was poor. No other markers were predictive (all P>0.05).

Trajectories of B-cell markers prior to lymphoma diagnosis

The trajectories of each marker in the cases and controls and unadjusted % change per year for each marker in the time leading up to diagnosis or selection date in controls are shown in Figure 2. In unadjusted analysis, the largest difference was observed for IgM, which was declining in cases by 6.42% (95%CI: 3.12, 9.61) per year, but levels were stable in controls (%Change per year: 0.40 95%CI:-2.09, 2.95%). The difference in the rates of change per year between cases and controls was statistically significant (P for interaction<0.01). Levels of IgG were also declining in cases, but stable in controls, which was a borderline significant difference in the rates of change per year (P for interaction=0.10). Although levels of FLC-κ were stable in cases but increasing in controls, the difference in the rates of change per year between cases and controls were non-significant (P for interaction = 0.20). The ratio of FLC-κ/λ was increasing in cases, but stable in controls, and conversely, FLC-κ+λ level was increasing in controls but stable in cases, however the differences in the rates of change per year between cases and controls were not-significant (P for

interaction = 0.44 and 0.16, respectively). Levels of FLC-λ, IgA, and IgD did not change over time in either cases or controls. In those who had a HIV-VL measured (N= 586 samples in 214 people), HIV-VL levels were high many years prior to diagnosis (Figure 2), and significantly declined in cases in the time leading up to diagnosis, whereas levels were stable in controls, however this difference was not significant (P=0.11). The level of current CD4 cell count was stable in both cases and controls. The results were similar after adjustment for matching variables (data not shown). Further adjustment for current CD4, age and HIV-treatment variables also produced consistent results (data not shown).

Patient factors associated with higher B-cell activation marker levels in controls

Demographic and HIV-related factors which were associated with B-cell marker levels in the control population are shown in table 3. For each factor, the adjusted fold change in marker level is presented. For example, those from southern Europe had on average a 1.46-fold higher marker level compared to those from west-central Europe. Higher levels FLC-κ, FLC-λ, and IgG were associated with HIV transmission modes other than MSM, lower current CD4 cell count, higher current HIV-VL, and not being on cART (borderline for FLC-λ). FLC-κ and FLC-λ levels also increased with older age. Higher IgA level was associated with lower CD4 cell count and higher AUC of HIV-VL. Higher IgM level was associated with higher HIV-VL, not being on cART, and HIV transmission mode other than MSM. Higher IgD level was associated with higher AUC of HIV-VL only.

Discussion

This study investigated the trajectories of FLC-κ, FLC-λ, IgG, IgA, IgM, and IgD over time prior to lymphoma diagnosis. We show that the strength of association diminishes consistently with time leading up to diagnosis. Levels of FLC-κ, FLC-λ, and IgG were associated with lymphoma development in HIV+ people >2 years prior to lymphoma development. However, The magnitude of the associations was moderate, and poorly predicted lymphoma development. The markers investigated in this study, therefore, are unlikely to be strong candidates for risk assessment for targeted interventions.

Proportionately higher levels of both FLC-κ and FLC-λ (indicating polyclonal expansion) were associated with lymphoma >2 years prior to diagnosis. Two main studies have also demonstrated that elevated levels of FLC-κ and FLC-λ are associated with a higher likelihood of lymphoma in HIV+ people [23, 24]. The study by Landgren *et al* (2010) [24] found that elevated FLC-κ and FLC-λ were associated with NHL 2–5 years prior to diagnosis, conversely only FLC-λ was associated 0–2 years prior [24]. However, a later study by the same group subsequently found FLC-κ and FLC-λ levels to be similarly predictive of all AIDS defining events and not specifically NHL [22]. Results were consistent in the more recent study by Bibas *et al* (2012) , who found FLC-κ and FLC-λ to be predictive of both NHL and HL independently of CD4 cell count and HIV-VL . Our finding that FLC-κ and λ are predictive of lymphoma in the long term are somewhat concurrent with those of Landgren *et al* and Bibas *et al* , however, we did not find an association with FLC-λ closer to diagnosis. Furthermore, our finding that polyclonal FLC elevations preceded lymphoma development is also concurrent with both studies [23, 24].

Our results demonstrated an association between IgG and IgM >2 years prior to date of lymphoma diagnosis (although the association with IgM was borderline), which attenuated closer to this date. This was driven by a faster decline in levels in cases while controls remained stable. Studies have found mixed associations between immunoglobulins and lymphoma in HIV. For example, an Australian study found high levels of serum globulin, mainly IgG, were predictive of NHL [28]. However, other studies found no association between serum globulin, immunoglobulins and NHL [24, 29, 30].

The attenuation of associations between markers of B-cell activation and lymphoma ≤2 years of diagnosis may simply be reflecting a 2 year lag period for an increase in B-cell activity to manifest as a clinically detectable lymphoma [23]. However, it is more likely that the observed trends are signifying the concurrent, but very different, immune consequences induced by HIV infection and lymphoma development and disentangling this relationship is not straight forward. HIV associatied immune dysfunction is associated with elevated serum levels of immunoglobulins, mainly IgG but also IgA and IgD [16]. Conversely, Studies in the general population have found lower levels of IgM, IgA and IgG prior to lymphoma diagnosis [31], and levels declined with more advanced disease

[32], speculated to be driven by the developing lymphoma. Additionally, transformed B-cells may have compromised immunoglobulin production and levels may not reflect the immune environment in which the lymphoma initiated [32]. Therefore, it is possible the decline in marker levels ≤2 years may be a consequence of early undiagnosed lymphoma. A similar phenomenon has been reported for undiagnosed HL and declining CD4 cell counts within 1–2 years prior to diagnosis [33, 34].

Current HIV-VL was found to be a strong predictor of lymphoma risk >2 years prior to diagnosis, however, AUC of HIV-VL was a predictor ≤2 years prior to diagnosis. Our results support the strong association between cumulative HIV-VL and AIDS-related lymphoma identified in previous studies [35]. Furthermore, elevated levels of FLC-λ, FLC-κ, and IgG were associated with higher current HIV-VL and lower current CD4 in controls, which is consistent with other studies [23, 36]. This may indicate that a history of uncontrolled HIV-VL is playing an integral role in lymphoma development , and elevated FLCs are reflecting HIV-specific B-cells dysfunction [37] occurring long before diagnosis. Furthermore, HIV-specific B-cells dysfunction may contribute to lymphoma development by facilitating the reactivation of latent EBV, resulting in the long term stimulation and proliferation of impaired B-cells[38].

The major strength of our study is the availability of serial plasma samples collected prior to and independently of lymphoma diagnosis, as well as the inclusion of a comparatively large number of lymphomas from contemporary HIV+ individuals. However, the limitations need to be considered. Firstly, it is possible that changes in FLCs and immunoglobulins reflect undiagnosed or late diagnosed cancer rather than preceding cancer development. In this study we grouped NHL and HL together as we did not have the numbers to investigate them separately. Furthermore, FLCs are excreted from the kidneys and therefore levels are possibly affected by renal function [39]. Measurements of eGFR were only available on one third of measurements (175/592, prospective collection of serum creatinine to calculate eGFR started in 2004), however in people with eGFR measurements available, there was no evidence of a difference in eGFR between cases and controls at baseline. Cases were less likely to be on treatment than controls and a lower proportion were HCV-positive. HCV infection is associated with several B-cell disorders, including cryoglobulinaemia and B-cell NHL, and elevated levels of FLC-κ and an abnormal FLC-ratio have been

associated with the severity of B-cell dysfunction in HCV-positive people [40, 41]. Therefore, the higher HCV prevalence in the control group may result in an underestimation of the effects. Further adjustment for potential confounders was not possible, although no other significant imbalances were evident at baseline. Baseline CD4 was included as a matching factor in order to investigate the independent associations between B-cell activation and lymphoma development, however, it should be kept in mind that this may result in an underestimate of the association between markers of B-cell activation and lymphoma development.

In conclusion, FLC-λ, FLC-λ and IgG were higher >2 years before lymphoma diagnosis, but the difference diminished nearer diagnosis. B-cell dysfunction, as demonstrated by polyclonal hyperglobulinemia, occurs many years prior to lymphoma development. The trajectories of FLC-κ, FLC-λ IgG, IgA, IgM, IgD over time prior to lymphoma diagnosis show that the strength of association diminishes consistently with time leading up to diagnosis. The magnitude of the associations was moderate at best, and poorly predicted lymphoma development. The markers investigated are unlikely to be strong candidates for risk assessment for targeted interventions.

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Disclosure of Conflicts of Interest

AM has recieved lecture fees, travel support, consultancy fees or honoraria from ViiV, BMS, BI, Pfizer, Merck and Wragge LLC. All other authors report no conflicts of interest.

Previous presentations

This work was presented as a thistle presentation (poster + 7 minute speed presentation) at HIV drug therapy, Glasgow UK, October 2016 (P189).

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