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Assessment of Pre-Diagnosis Biomarkers of Immune Activation and Inflammation – Insights on the Etiology of Lymphoma

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Abstract

The DNA-modifying processes that are involved in B lymphocyte activation, somatic hypermutation (SHM) and *IgH* class switch recombination (CSR), have the potential to lead to genetic errors that lead to the genesis of B cell cancers, such as lymphoma. Given the potential contribution of these immune mechanisms to the development of cancer, assessment of the expression of cytokines and other immune stimulatory molecules that drive B cell activation, prior to lymphoma diagnosis, may provide insights into the etiology of these cancers. Here, we review studies that have examined pre-diagnosis protein biomarkers for non-Hodgkin lymphoma (NHL), both AIDS-related NHL, as well as NHL seen in immunocompetent populations. Overall, these studies provide support for the notion that B cell hyper-activation is elevated preceding the appearance of AIDS-NHL, particularly those forms of AIDS-NHL that are not driven by EBV infection, and which presumably arise from errors in *IgH* CSR and SHM. In more limited studies, it appears that dysregulation of cytokine production also precedes the diagnosis of NHL in HIV-negative persons. The availability of pre-diagnosis serum/plasma from cohort studies provides unique opportunities for proteomic approaches to identify novel pre-diagnosis etiologic biomarkers for NHL.

Introduction

The immune system plays a central role in protection from infection. This involves the orchestrated interactions of subpopulations of immune cells that detect microbes, and then respond to these invading microorganisms with appropriate effector responses. The ability of the immune system to identify and respond to cells seen as foreign led to the widespread belief that it also played a central role in guarding against the development of cancer, and in fact, the immune system can effect anti-tumor responses. However, immune responses also have the potential to promote cancer development and growth. Chronic inflammatory responses can contribute to the development of cancer ¹. Additionally, the physiological processes that are involved in the activation of immune cells place these cells at risk for cancer. For example, the activation of B lymphocytes requires that these cells undergo various DNA modifying activities (somatic hypermutation, *IgH* class switch recombination), errors in which can result in molecular lesions (oncogene mutation, chromosomal translocations) that lead to B cell cancers ². Given the potential contribution of immune mechanisms to the development of cancer, assessment of the expression of molecules involved in immune responses, including cytokines and other immune stimulatory

molecules, may provide insights into mechanisms involved in the development and/or growth of cancer. This is especially true for lymphomas, which are cancers that arise from mature antigen-activated lymphocytes.

In this review, we will provide an overview of work assessing the expression of immune system molecules preceding the diagnosis of lymphoma, focusing on serum/plasma protein biomarkers. By virtue of requiring access to pre-diagnosis specimens, all of these studies have been done using archival specimens collected in prospective cohort studies, or as part of serum repositories created for various purposes. Virtually all studies done so far examining pre-diagnosis materials have been hypothesis-testing efforts focusing on candidate molecules, assessed by conventional assays or by various newer multiplexed assays. Extension of these studies to non hypothesis-limited proteomic approaches, utilizing cohort and repository specimens, has the potential to identify new proteins associated with lymphoma etiology and/or biomarkers for the early detection of these cancers.

Pathogenesis of Non-Hodgkin Lymphoma (NHL)

Lymphomas are solid tumors of lymphocyte origin and are the most common hematopoietic cancers, representing the fifth most common cancer, in terms of incidence rate, and the sixth in terms of cancer deaths, in both men and women in the United States³. Lymphomas represent diverse and heterogeneous groups of cancers, consisting of Hodgkin lymphoma, T cell and natural killer cell lymphoma, and various types of B cell non-Hodgkin lymphoma^{4, 5}. Most types of B cell NHL correspond to B cells that have undergone various molecular changes that occur after initial encounter with antigen and exposure to helper T cells⁶. As these activation-promoting interactions occur in the germinal centers of lymph nodes and other lymphoid organs, these cells are referred to as post-germinal center B cells. The germinal center reaction involves rapid B cell proliferation and somatic DNA changes that result in both changes in the isotype of the Ig that is produced by these cells (from IgM to IgG or other isotypes), as well as enhanced antigen-binding affinity. These DNA-modifying processes consist of Ig class switch recombination (CSR), which involves the recombination of the Ig variable region segment in Ig heavy chain genes (IgH) from IgM to another isotype (i.e., IgG), with the looping out and excision of intervening DNA, and somatic hypermutation (SHM), the active mutation of the variable region of Ig genes, which, when combined with selection by antigen, results in the production of antibodies with enhanced antigen-binding affinity. Errors in both *IgH* CSR and SHM are believed to lead to the seminal molecular lesions for many forms of B cell lymphoma, as well as other mature B cell cancers. For example, the *MYC:IgH* chromosomal translocation is believed to be the seminal event in the genesis of NHL of the Burkitt's lymphoma subtype⁷. Oncogene translocations/mutations involving other oncogenes, such as *BCL2* or *BCL6*, which are involved in other forms of NHL (follicular lymphoma and diffuse large B cell lymphoma, respectively) are thought to result from errors in SHM. Activation-induced cytidine deaminase (AID) is a DNA-mutating enzyme that plays a central role in both *IgH* CSR and SHM⁸. The expression of *AID*, either aberrant, or that driven by B cell activation, is believed to result in undesired DNA modifying events that lead to the development of lymphoma⁹. In our own work, we noted that elevated levels of *AID* expression are seen for several years preceding the development of AIDS-associated NHL (AIDS-NHL)¹⁰

The risk of developing lymphoma is greatly increased in HIV infection⁷. Some AIDS-associated lymphomas, including virtually all primary CNS lymphomas, are derived from EBV-transformed B cells, which grow in an uncontrolled fashion in an environment devoid of T cell-mediated immunoregulation. In fact, loss of EBV-specific T cell immunity has been seen to precede the development of EBV+ AIDS-NHL¹¹. However, many AIDS-NHL, including Burkitt's lymphomas (BL) and diffuse large B cell lymphomas (DLBCL),

are often EBV-negative¹². AIDS-NHL of the Burkitt's subtype are characterized by a chromosomal translocation that involves the rearrangement of the *c-MYC* oncogene and the *IgH* switch region¹². Lymphomagenesis results from the accumulation of several genetic lesions, the *c-MYC:Ig* translocation is a seminal event, since it can result in a perpetually proliferative state. As noted above, the *MYC:IgH* rearrangement is believed to result from an error in *IgH* CSR. AIDS-NHL of the DLBCL subtype are associated with the mutation/translocation of the *BCL-6* oncogene and other oncogenes. The mutation of *BCL-6* and other NHL-associated oncogenes is believed to occur during *Ig* SHM. Therefore, at least two general types of pathogenetic mechanisms appear to contribute to the genesis of AIDS-NHL: 1) loss of immunoregulation of EBV-infected B cells, due to the loss of T cell function that is associated with HIV infection, and 2) chronic B cell hyper-activation, which is associated with DNA-modifying events (SHM and *IgH* CSR) that can contribute to lymphomagenic molecular lesions (oncogene mutations/translocations)⁷.

The study of biomarkers for AIDS-NHL has been fruitful for two reasons. First, the risk for developing NHL is greatly increased in HIV infection¹³⁻¹⁵. Second, several large prospective cohort studies, established to better define the natural history of HIV/AIDS, have methodologically collected and stored appropriate specimens (cells, serum/plasma, tumor tissue) as part of their study design. Together, this has made available appropriate stored, well curated biospecimens, collected prior to NHL diagnosis, from a significant number of subjects.

Cytokines and Immune Stimulatory Molecules

Immune responses involve the production of cytokines, which are soluble mediator molecules. Cytokines are secreted proteins that can have multiple biologic effects, are extremely potent, and interact with high-affinity cellular receptors specific for each cytokine. Cytokines binding to the appropriate receptor results in signal transduction, followed by changes in gene expression, and ultimately, in altered target cell behavior. Characteristic patterns of cytokine production have been recognized. For example, in innate immune responses, macrophages activated by exposure to microbial molecules, such as endotoxin, produce pro-inflammatory cytokines, including IL6, TNF α , and chemokines. These cytokines enhance inflammation, chemotaxis, and promote the development of subsequent adaptive immune responses. Inflammatory cytokines have been seen to promote the development of cancer¹.

Subsets of CD4 helper T cells also have been identified, and are defined primarily by the pattern of cytokines produced, as well as the expression of unique molecular transcription factors: T_{H1}, T_{H2}, T_{H17}, and T follicular helper cells (T_{FH})^{16, 17}. Other T cell subsets also display distinct patterns of cytokine production. Regulatory T cells (T_{REG}), a functionally-defined subset of T cells that can inhibit other immune cells, have been the focus of much attention. T_{REG} cells, which are CD4+, CD25+ and Foxp3+ T cells, produce IL10 and TGF β , cytokines that down-regulate many immune responses. It is believed that T_{REG} play an important role in preventing anti-self responses and in regulating immune responses, and T_{REG} dysfunction also may contribute to the growth of cancer.

Cytokines and immune stimulatory molecules as cancer biomarkers

The host immune/cytokine environment may promote the development of certain cancers, including lymphomas. For instance, an immune system environment that is characterized by higher levels of T_{FH} activity, characterized by elevated levels of B cell stimulatory cytokines such as IL6, TNF α or CXCL13, would be expected to contribute to the development of B cell lymphoma by promoting B cell activation and associated high-risk DNA-modifying activities, such as somatic hypermutation^{2, 7, 18}. For these reasons, cytokines can contribute

to the etiology of NHL and have the potential to serve as biomarkers for an immune system environment that promotes the emergence of these tumors. Additionally, cytokines and immune activation molecules may be produced by nascent tumor cells and/or by reactive tumor-associated immune cells, and thereby serve as early biomarkers for these cancers.

Measurement of cytokine levels in serum or plasma is usually done by ELISA, although there are various alternative assays, including multiplexed assays that allow the simultaneous determination of multiple molecules in a single sample. While the quantification of cytokines in serum/plasma, collected prior to the clinical recognition of cancer, may provide insights into the nature of a host immune system environment that contributes to cancer development and/or growth, such studies need to be designed with knowledge of the biology of the cytokines to be measured, and of the limitations inherent in the measurement of these molecules. For example, most cytokines are present at low levels in serum, near the limit of detection for conventional assays. Also, many cytokines are labile and/or are produced locally in immune system organs and not distributed systemically, so they cannot be detected in serum/plasma. This has led to the utilization of surrogate biomarkers for cytokine activity, such as CRP (a surrogate marker for IL6 and other inflammatory cytokines)¹⁹, soluble TNF receptor II (co-regulated with TNF α)^{20, 21}, or the measurement of antibody isotype ratios as a surrogate for T_{H1}/T_{H2} cytokines²².

In addition to the direct assessment of cytokine levels, it is possible to determine cytokine and cytokine receptor genotype, which requires DNA, but not serum or other biological specimens. Cytokine and cytokine receptor genes have genetic polymorphisms, including single nucleotide polymorphisms (SNP), which affect the level of expression, or less commonly, the function of these genes. Therefore, cytokine genotype provides information on the genetic propensity of an individual to be a high- or low-producer/responder for a given cytokine. Assessment of the genotype of immune system genes in population-based studies is increasingly attractive, given the fast pace of development of technologies to assess multiple genetic polymorphisms inexpensively and rapidly, and the added information that can be obtained when cytokine levels are compared to cytokine genotype (genotype:phenotype correlations).

Pre-diagnosis serum/plasma biomarkers for AIDS-NHL

While HIV infection is characterized by a decrease in the function/number of CD4 T cells, a marked increase in B cell activation also is seen in HIV infection, which is driven by the over-production of B cell-stimulatory cytokines, such as IL6 and IL10, as well as by stimulation of B cells by HIV and other microbial antigens²³⁻²⁵. Additionally, HIV itself has been seen to have direct B cell-stimulatory activity^{26, 27}. Various factors associated with B cell activation, including B cell-stimulatory cytokines, as well as soluble serum molecules that are associated with B cell activation, including serum immunoglobulins (Ig) and Ig components, such as free light chains²⁸, have been seen to be elevated preceding the appearance of AIDS-lymphoma (Table 1). Elevated circulating levels of IL6 were noted prior to AIDS-NHL diagnosis in two studies, an early report from an NIH clinical cohort²⁹ and in studies from the Multicenter AIDS Cohort Study³⁰. Elevated levels of IL10 have been seen pre-AIDS-NHL³¹. In addition to this, an IL10 genotype associated with higher levels of *IL10* expression was seen to be associated with an elevated risk for AIDS-NHL³¹. IL6 and IL10 are potent B cell-stimulatory cytokines. Elevated levels of soluble CD23 (sCD23), a B cell activation molecule that has cytokine-like B cell-stimulatory properties, were seen prior to AIDS-NHL diagnosis in studies from the MACS³²⁻³⁴. Interestingly, elevated levels of sCD23 were associated with EBV-negative forms of lymphoma, but not with EBV+ tumors³³. Recently, elevated serum levels of CXCL13, a B cell-stimulatory chemokine that is produced by T_{FH} cells, and that is responsible for inducing the homing of

B cells to germinal centers¹⁶, were noted pre-AIDS-NHL diagnosis, in studies carried out in two cohorts, the MACS and the Women's Interagency HIV Study (WIHS)^{35, 36}.

Elevated levels of other immune activation-associated molecules also have been seen preceding the diagnosis of AIDS-NHL. In an Australian case-control study of >200 AIDS-NHL cases, the duration of immunodeficiency, assessed by CD4 count, and elevated serum globulin levels (a surrogate marker for serum Ig) both were pre-diagnostic factors related to AIDS-NHL risk³⁷. These workers concluded that these markers of long-standing immune deficiency and B-cell stimulation were associated with an increased risk of developing NHL. In an earlier study in the MACS, Yawetz et al³² found elevated serum levels of IgE preceding AIDS-NHL, although this was not confirmed in more recent larger studies³⁸, while Breen et al³⁹ found that AIDS-NHL was preceded by lower levels of IgG, when compared to CD4-matched HIV+ control subjects.

More recently, Landgren and co-workers²⁸ found that elevated serum free light chain levels were associated with an increased risk for AIDS-NHL. These workers assessed IgG, IgM, and IgA levels, monoclonal Ig, and kappa (κ) and lambda (λ) free light chain (FLC) levels in pre-NHL diagnosis serum/plasma specimens from 66 individuals who developed NHL and 225 HIV+ lymphoma-free controls. κ and λ FLC levels in specimens collected 2-5 years pre-NHL both were associated with an increased risk for AIDS-NHL, while, in contrast, IgG, IgM, and IgA levels were similar in patients and controls. These workers concluded that elevated FLC may reflect polyclonal B-cell activation and immune dysfunction and may be useful for identifying HIV+ persons at increased risk for developing AIDS-NHL.

In studies carried out in the MACS, elevated serum levels of soluble CD27 (sCD27) and CD30 (sCD30), both members of the TNF receptor superfamily and both biomarkers for immune activation, were seen prior to AIDS-NHL diagnosis^{39, 40}. Additionally, serum levels of sCD27 were inversely correlated with the cell surface expression of sCD27 on B cells⁴⁰. Interestingly, CD27 is a marker for memory B cells⁴¹. Elevated serum levels of soluble CD44 (sCD44) also were seen preceding AIDS-NHL, in another study in the MACS⁴².

In recent studies, we have assessed serum levels of several immune system molecules using a combination of ELISA and Luminex-based multiplexed assays, longitudinally over a period extending out to five years prior to AIDS-NHL diagnosis, in two large prospective cohort studies, the MACS and the Women's Interagency HIV Study (WIHS). The results of these studies are still being analyzed. However, preliminary analyses indicate that elevated serum levels of several of these, and other, B cell stimulatory molecules are seen preceding AIDS-NHL diagnosis. For example, elevated serum levels of IL6, sCD23, sCD27, sCD30, CXCL13, and TNF α , as well as elevated levels of both κ and λ FLC, were seen up to five years preceding AIDS-NHL diagnosis (unpublished observations)^{35, 36, 38}. CXCL13 (BCA-1, BLC) is a B cell-stimulatory chemokine, which is typically produced by T_{FH} and by germinal center follicular dendritic cells, and which acts to attract B cells to follicular areas of secondary lymphoid organs^{16, 43, 44}. Elevated levels of serum IL10, IL5, and GM-CSF also were seen, but only at time points within one year of AIDS-NHL diagnosis (unpublished observations)³⁸. Importantly, elevated pre-diagnosis levels of most of these biomarkers were associated with a greater risk for the development of non-CNS forms of NHL, with a lower risk seen for CNS (EBV+) NHL. Additionally, *AID* expression in peripheral blood mononuclear cells was seen preceding the diagnosis of AIDS-NHL of non-CNS origin, but not in those who developed CNS (EBV+) lymphomas¹⁰. Rabkin and co-workers⁴⁵ recently reported elevated pre-AIDS-NHL diagnosis serum/plasma levels of several cytokines, including GM-CSF, IL15, IL1 α , IL4, IL6, IL8, IL12p40, IL12p70 and

IL-13, using a Millipore Luminex-based assay to assess these cytokines in specimens from 66 AIDS-NHL cases, with specimens collected 0.1 - 2.0 years (median 1.0) pre-NHL.

Overall, the results of these studies provide ample support for the notion that B cell hyper-activation is particularly elevated preceding the appearance of AIDS-NHL, particularly those forms of AIDS-NHL that are not driven by EBV infection, and which presumably arise from errors resulting from *IgH* CSR and SHM.

Pre-diagnosis serum/plasma biomarkers for NHL not associated with HIV infection

There has been increasing interest in conducting similar studies examining pre-diagnosis biomarkers in NHL in presumably immunocompetent, HIV-negative subjects (Table 2). A study by Purdue and co-workers assessed pre-diagnosis serum sCD30 levels⁴⁶, following up on our initial observation that prediagnostic serum sCD30 was associated with increased risk of developing AIDS-NHL³⁹. This nested case-control study was done using specimens from 234 cases and 234 individually matched controls in the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial, and found a strong relationship between prediagnostic sCD30 concentration and NHL risk that persisted among cases diagnosed 6-10 years after providing a blood sample. Those investigators concluded that these findings suggest that chronic B-cell stimulation may be an important mechanism involved in B-cell lymphomagenesis in healthy populations, as well as in those who are HIV+.

In an ongoing study, using specimens from the U.S. Department of Defense Serum Repository (DoDSR), we are assessing serum levels of various cytokines and immune stimulatory molecules in prediagnostic sera from HIV-seronegative persons who developed NHL. Cases were identified from the Armed Forces Institute of Pathology (AFIP) National Pathology Repository and the military Automated Centralized Tumor Registry (ACTUR). To determine if serum cytokine levels were elevated in those who went on to develop several subtypes of NHL, a nested case-control study was conducted among active-duty military personnel with archived serum in the DoDSR collected several years prior to diagnosis of NHL. Each case was matched to two controls on age, sex, race, and dates of blood collection. Cytokine levels were quantified in one to three pre-NHL diagnosis serum specimens and in matched control specimens. Analysis of the results obtained in this study is ongoing. Preliminary analyses indicate that serum CXCL13 levels were clearly and significantly elevated preceding the clinical recognition of several subtypes of NHL, including diffuse large B cell lymphoma (DLBCL) (n=265) and follicular lymphoma (n=134), the two most common forms of B cell NHL seen in presumably immunocompetent persons, with NHL cases showing mean serum CXCL13 levels that were approximately three or four-fold higher than those seen in controls⁴⁷. These results suggest that CXCL13-driven B cell activation may contribute to the etiology of these cancers, and that CXCL13 has the potential to serve as a biomarker for these lymphomas. As T_{H17} and T_{FH} cells produce CXCL13⁴⁸, this finding also suggests that these T cell subsets may contribute to the genesis of these forms of B cell NHL. Furthermore, T_{H17} cells are known to contribute to autoimmunity^{49, 50}, elevated levels of CXCL13 are seen in some autoimmune diseases⁵¹⁻⁵³, and a recent large pooled InterLymph study showed that autoimmunity was associated with a marked increase in risk for B cell NHL⁵⁴. Studies are currently underway to define serum levels of various other B cell-stimulatory cytokines preceding NHL diagnosis using Luminex-based multiplexed assays, as well as to better define the role of CXCL13 and its receptor CXCR5 in lymphomagenesis.

Two recent studies have assessed levels of cytokines in pre-NHL diagnosis serum/plasma using multiplexed assays. A recently published study by Saberi Hosnijeh, et al⁵⁵ assessed

pre-diagnosis cytokine levels and determined their association with risk for the development of NHL in the general population, to assess the potential role of these immune system stimulatory molecules in the etiology of NHL in immunocompetent populations. These investigators used a Luminex bead-based multiplexed assay panel of their own construction to quantify plasma levels of 15 cytokines and chemokines (IL1 α , IL1 β , IL2, IL4, IL5, IL6, IL10, IL12, IL13, IFN γ , TNF α , IL8, RANTES, eotaxin, IP10), and an adhesion molecule (ICAM) in stored citrate plasma from prediagnostic blood samples from the Italian European Prospective Investigation into Cancer and Nutrition cohort (86 NHL cases and 86 matched controls, a single determination in plasma collected on average 4.5 years pre-NHL diagnosis) in a nested case-control study. They observed a significant association of the development of B cell NHL with lower plasma levels of IL2 and TNF α and with elevated ICAM levels, which they took to suggest that risk of NHL might be related to a downregulation of T helper 1 cytokines.

Another recent study by Gu et al.⁵⁶ also utilized multiplexed assay technology to assess cytokine levels preceding NHL diagnosis, in a nested case-control study using specimens from a prospective cohort of non-HIV-infected, healthy women, the New York University Women's Health Study. These investigators quantified serum levels of fifteen cytokines in samples (a single pre-diagnosis serum specimen from 92 NHL cases, collected a median of 8.2 years prior to diagnosis, and sera from controls). An increased risk of developing NHL was seen to be associated with elevated serum soluble IL-2 receptor (sIL-2R), and a decreased risk for NHL with serum IL13. The other cytokines tested (IL1 β , IL1RA, IL2, IL4, IL6, IL10, IL12, IL12, CRP, sTNF-R1) were not significantly associated with NHL risk, or were marginally and non-significantly associated with NHL risk (TNF α , sTNF-R2).

Overall, while it is clear from these studies that dysregulation of cytokine production may precede NHL diagnosis in immunocompetent persons, there does not yet appear to be a clear pattern associated with the development of NHL. Larger longitudinal studies are needed to provide more complete and reliable information on the pattern of cytokine production seen pre-diagnosis in those who develop non-AIDS-associated NHL. Additionally, cross-comparison and validation of the multiplexed assays used in these different studies needs to be carried out by the different groups who are working in this area. Eventually, pooled multi-cohort studies should be done in the future to maximize the information that is available on pre-diagnostic, etiologic biomarkers for NHL.

Future directions – biomarkers for NHL

While the studies mentioned above are informative on the immunological changes that accompany the development of NHL in HIV positive and negative individuals, the precise role of each individual biomarker in the etiology of these cancers is still not completely unraveled. Nevertheless, it appears clear that all the cytokines mentioned above may represent useful markers for early NHL diagnosis. This is particularly relevant in the case of HIV positive individuals, who are known to be at highly increased risk for NHL, and for whom an early detection, and therefore an early initiation of therapy, would be particularly important. Measurement of serum FLCs and globulins is already a standard procedure for early detection or follow up of multiple myeloma. For this reason, and also considering their widespread availability and standardization in clinical laboratories, these markers could be easily and quickly implemented as part of a screening protocol for NHL in HIV positive individuals. In this way, testing for FLCs has the potential to become routine in the management of HIV positive patients, analogous to measurement of CD4⁺ cell count or HIV viral load. Among the other markers reviewed, we believe that, given the strong correlation of their serum levels with subsequent AIDS-NHL diagnosis, IL6, IL10, TNF α and CXCL13 are good potential candidates for use in patient management. However, at this

time there are no standard procedures employed for the measurement of these biomarkers in clinical laboratories, which is an impediment to the rapid clinical application of these cytokines as early diagnostic markers for NHL. Given that high levels of these molecules can be seen for several years before NHL diagnosis, their detection might precede tumor development, thus identifying a high risk subpopulation of HIV positive subjects, who would then merit ongoing observation. Interestingly, in unpublished work from an AIDS Malignancies Consortium study, high levels of CXCL13 were found to be strongly correlating with a poor response to therapy. Similarly, IL10 and IL6 have also been seen to correlate with response to chemotherapy. For this reason, these markers may be useful not only for the early detection of lymphoma, or identifying those persons who are at the highest risk for developing these cancers, but also to identify those individuals who will not completely respond optimally to standard therapy, and who may be good candidates for alternative therapeutic regimens.

Work by several groups on pre-diagnosis biomarkers for NHL is continuing, using specimens from several cohorts. This includes studies toward the development of novel nucleic acid based biomarkers, such as those that aim to detect clonal Ig DNA in serum/plasma as a marker for the presence of an emerging clone of nascent NHL⁵⁷. In our own group, we are currently beginning to assess salivary biomarkers for AIDS-NHL, in collaboration with Dr. David Wong at UCLA, whose group has done extensive studies assessing saliva for tumor biomarkers⁵⁸. Combined with emerging insights from the NHL proteome and secretome^{59, 60}, such approaches may result in the identification of novel pre-diagnostic protein and nucleic acid biomarkers for NHL.

Additionally, the availability of pre-diagnosis serum/plasma from several cohort studies, including the MACS, provides unique opportunities to utilize proteomic, non-hypothesis encumbered approaches to attempt to identify novel protein pre-diagnosis etiologic biomarkers for NHL. The development of such studies, while of tremendous potential value, will need to be done with great care taken in the selection of both NHL cases and controls to avoid bias and spurious results. This will necessitate grouping of well-curated, pathology-confirmed NHL cases by lymphoma subtype, as well as careful selection of controls to avoid the introduction of biases that might skew the outcome of such studies. Given the extensive recent interest in pre-diagnosis biomarkers for NHL, it is anticipated that the future will bring much new information on this topic, and that this will enhance both knowledge about the etiology of these cancers, and improve their early detection and treatment.

We look forward to such studies in the future, and ongoing work with multiplexed assays, to identify novel biomarkers for lymphoma, and to extend this work to the clinic. It is encouraging to have witnessed the exceptional scientific and technical advances that have occurred since we began our work on biomarkers, which was initiated using bioassays^{25, 61, 62}, prior to the availability of dependable immunometric assays, was extended using continually evolving ELISA assays, is now in the ear of proteomics and multiplexed assays for both protein and nucleic acid biomarkers.

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Table 1
Pre-diagnosis serum/plasma biomarkers and risk for AIDS-NHL

Molecule	Change seen prior to AIDS-NHL diagnosis	References
Cytokines:		
IL6	elevated	29, 30, 38, 45
IL10	elevated	31, 38
CXCL13	elevated	35, 36
GM-CSF	elevated	unpublished results, 45
TNF α	elevated	unpublished results, 45
IL15	elevated	45
IL1 α	elevated	45
IL4	elevated	45
IL8	elevated	45
IL12	elevated	45
IL13	elevated	45
Cytokine-like molecules:		
sCD23	elevated	30, 32-34, 38
Soluble cytokine receptors:		
sCD27	elevated	38, 40
sCD30	elevated	38, 39
Immunoglobulins:		
Serum globulins	elevated	37
IgE	elevated or unchanged	32, 38
IgG	decreased or unchanged	28, 39
free light chains (FLC)	elevated	28
Others:		
sCD44	elevated	42
<i>AID</i>	elevated	10

Table 2
Pre-diagnosis biomarkers and risk for NHL not associated with HIV infection

Molecule	Change seen prior to NHL diagnosis	References
Cytokines:		
IL2	decreased	55
TNF α	decreased	55
IL13	decreased	56
CXCL13	elevated	47
Soluble cytokine receptors:		
sCD30	elevated	46
sIL2R	elevated	56
Others:		
ICAM	elevated	55