

Using Immune Marker Panels to Evaluate the Role of Inflammation in Cancer: Summary of an NCI-Sponsored Workshop

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Abstract

Chronic inflammation is recognized to play a role in the development of several cancers. Past investigations of inflammation and cancer have typically been small, used varied assay platforms, and included a narrow range of analytes. Multiplex technologies have now been developed to measure larger numbers of inflammatory markers using small volumes of specimens. This has created an opportunity for systematic, large-scale epidemiologic studies to evaluate the role of inflammation in cancer. However, lack of consensus on the approach to these studies, the technologies/assays to be used, and the most adequate analysis/interpretation of findings have thus far hindered progress. In June 2014, the National Cancer Institute (Bethesda, MD) convened a workshop involving epidemiologists, immunologists, statisticians, and laboratory biologists to share their experiences with new inflammation marker technologies and findings from association studies using such methods and technologies (<http://epi.grants.cancer.gov/workshops/>). Consensus and gaps in our understanding of the role of chronic inflammation in cancer were identified and recommendations were made to improve future efforts in this area. These recommendations are summarized herein, along with specific suggestions for how they may be implemented. By facilitating discussions among various groups and encouraging interdisciplinary collaborations, we anticipate that the pace of research in this field will be accelerated and duplication of efforts can be minimized. *Cancer Epidemiol Biomarkers Prev*; 24(9); 1427–33. ©2015 AACR.

Chronic inflammation is recognized to play a role in the development of several cancers. Past investigations of inflammation and cancer have typically been small, used varied assay platforms, and included a narrow range of analytes. Multiplex technologies have now been developed to measure larger numbers of inflammatory markers using small volumes of specimens. This has created an opportunity for systematic, large-scale epidemiologic studies to evaluate the role of inflammation in cancer. However, there has been limited coordination of work in this area and a lack of consensus on best approaches to evaluate the role of inflammation within and across cancer types, best analysis platforms to use, protein markers to measure, and ideal approaches for evaluation and interpretation of the results. As these new technologies are implemented within carefully designed epidemiologic studies, it is important to create a forum where groups involved in the field can share experiences and findings. With this in mind, the National Cancer Institute (NCI, Bethesda, MD) convened a workshop in June 2014, the Inflammation and Cancer Epidemiology (ICE) Workshop (<http://epi.grants.cancer.gov/events/immune-marker-panels/>). The goals of the meeting were to (i) share experiences with the use of various technologies to measure inflammatory protein markers in population-based samples; (ii) compare results from studies utilizing such technologies to evaluate the associations between inflammation markers and cancer; (iii) discuss possible biologic interpretations of findings from presented studies; and (iv) explore laboratory, epidemiologic, and statistical issues related to the conduct, analyses, and interpretation of inflammation and cancer studies. Participants were charged with identifying areas of consensus and gaps in understanding the role of inflammation in cancer so that recommendations could be made for ways to accelerate discovery in this area of research, while avoiding duplication of efforts. More specifically, participants were asked to identify consensus and gaps in the following three areas: (i) epidemiologic studies and statistical considerations, (ii) laboratory testing methods, and (iii) biologic interpretation of and functional follow-up to epidemiologic findings.

Introduction

Inflammatory factors have been associated with increased risk for several cancers; several reviews over the past 10 years describe such associations (1–4). However, past epidemiologic investigations of inflammation and cancer risk have typically been small, utilized varied analysis platforms, and included a narrow range of analytes. For instance, epidemiologic studies of non-Hodgkin lymphoma (5–10), colorectal (11–13), gastric (14, 15), endometrial (16), ovarian (17–21), and lung cancer (22–24) examined a select number of inflammatory markers in various cohorts. Markers evaluated, however, have varied between studies, making interpretation of results difficult. Consequently, the precise molecular markers and pathways involved in carcinogenesis remain unclear.

Multiplex technologies have now been developed to measure larger numbers of inflammatory protein markers using small volumes of serum, plasma, and other specimens (25–31). This

has created an opportunity for systematic, large-scale epidemiologic studies to evaluate the role of inflammation in cancer. However, there has been limited coordination of work in this area and a lack of consensus on best approaches to evaluate the role of inflammation within and across cancer types, best analysis platforms to use, protein markers to measure, and ideal approaches for evaluation and interpretation of the results. As these new technologies are implemented within carefully designed epidemiologic studies, it is important to create a forum where groups involved in the field can share experiences and findings.

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It should be noted that this report is not intended to provide a systematic review of the literature in the area of inflammation and cancer; for that readers are referred to recent reviews on this topic (1–4). Herein, we summarize the main issues discussed at the ICE Workshop, the areas of consensus/gaps identified, recommendations made, and suggestions for how these recommendations might be implemented.

Materials and Methods

Organization of workshop

The ICE Workshop was designed as a one and a half day meeting in which individuals actively involved in the evaluation of the role of inflammation in cancer using multiplex proteomic technologies were invited to present their results and share their experiences. In addition to epidemiologists involved in studies of inflammation and cancer, immunologists, other laboratory scientists, and statisticians were invited to participate and contribute to the discussion. The Workshop was organized into four sessions, as follows: (i) presentation of results from disease association studies that have evaluated multiplex inflammation markers and cancer; (ii) biologic interpretation of findings from these studies; (iii) evaluation of available assay platforms and their performance; and (iv) consideration of statistical design and analysis issues that impact studies of multiplex inflammation markers and cancer. Following these initial sessions, three concurrent breakout groups convened and focused on identifying areas of consensus and research gaps. A final session reviewed the issues identified during the breakout sessions to help formulate recommendations within each of these areas.

Results

Epidemiologic studies and statistical considerations

Summary of presentations and discussions. Multiplex inflammation marker panels are now being used to assess large numbers of inflammation markers in cancer association studies (32–34). Two sessions were devoted to epidemiologic and statistical considerations. In the session on epidemiologic studies, presenters reviewed results from recent studies as well as ongoing, unpublished studies of inflammation markers and a range of cancers, including lung (33), non-Hodgkin lymphoma (32, 35), ovary (21), endometrium, stomach (15), gallbladder, colorectal, breast, and childhood leukemia (36). Presentations also covered the association of circulating inflammation protein marker levels with exposures relevant for carcinogenesis, such as smoking (37), body mass index (38), and use of non-steroidal anti-inflammatory drugs (39). The discussed studies represented several large, population-based US and international cohorts—Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO); Alpha-tocopherol and Beta-Carotene Trial (ATBC); Women's Health Initiative (WHI); European Prospective Investigation into Cancer (EPIC); California birth cohort; and Shanghai Women's and Men's Cohort Studies.

The statistical considerations session included presentations of epidemiologic design and interpretation, evaluation of reliability of assays and methods for measurement error correction, and analysis methods for multidimensional data. Discussions on epidemiologic design issues included several topics—the impact of temporal intraclass correlation coefficients (ICC) of circulating inflammation markers on attenuation of risk estimates, the importance of measuring inflammation markers in an etiologi-

cally relevant window before the development of cancer, issues of reverse causation in retrospective and prospective studies, correlation of systemic versus local inflammation, residual confounding from known/strong risk factors, control sampling, genotype-phenotype associations, and studies of cancer outcomes. Discussions on assay reliability and measurement error included the relative merits of coefficients-of-variation (CV) and ICCs in evaluating the utility of assays for epidemiologic studies, the impact of measurement error and batch effects, and methods for correction of measurement error, such as regression calibration through the use of validation subsets and test-retest of marker measurements (40). Discussions of dimensionality-reduction approaches included several analytic methods, such as Lasso regression, principal components analyses, factor analyses, and linear discriminant analyses.

Consensus and gaps. There was a general consensus that continued, systematic evaluation of the association between circulating inflammation markers and cancer is warranted at this time given the availability of stored specimens from well-established studies and technologies with demonstrated analytical repeatability to measure large numbers of markers with limited specimen volumes. There was also consensus among the Workshop participants regarding design/analytic issues to be considered in the conduct of etiologic studies of inflammation markers as well as gaps in the current knowledge base. These are described below:

Epidemiologic design Studies need to be carefully designed to guard against standard threats to epidemiologic validity. These design considerations include well-known rules, such as the use of appropriately powered studies to reduce chance associations, assessments of and accounting for potential bias from measurement error in inflammation marker measurements, and adequate adjustments for potential confounders. In addition, because several carcinogenic exposures, such as smoking, are also associated with inflammation (37), studies should be designed to investigate exposure-mediated and exposure-independent components of inflammation–cancer associations.

"Omics" considerations In addition to standard features for the design of molecular epidemiologic studies, consideration is needed for pitfalls inherent to "omic" studies. These include design features, such as the measurement of cases and controls in the same assay batch (see Laboratory Methods section for additional details), and analytic features, such as corrections for multiple-testing through the use of false discovery rate (41), Bonferroni corrections, and false-positive report probability (42). Likewise, akin to genome-wide association studies, independent replication efforts need to be planned from the outset (43). In addition, in view of the pleiotropic, epistatic, and redundant nature of several inflammation markers (44, 45), analyses need to consider interrelationships across markers and investigate associations for groups of inflammation markers. These analyses can be accomplished through the use of several data analytic methods, including principal components analyses, factor analyses, and linear discriminant analyses. Furthermore, biologic pathway-based analytic methods, such as the Database for Annotation, Visualization and Integrated Discovery (DAVID; refs. 46, 47) for microarray data, need to be developed to gain mechanistic insight into inflammation–cancer associations.

Use of prospective study designs with multiple time-point measurements Reverse causation is an important issue for inflammation marker levels. The presence of cancer alters the circulating levels of a wide range of inflammation markers due to local tissue-level influences as well as generalized systemic effects of cancer. Therefore, prospective epidemiologic designs are preferred over retrospective studies. The understanding of the relevant etiologic window for the role of carcinogenesis is still evolving and could differ by cancer site. Thus, prospective studies need to include a broad range of time periods between blood collection and cancer diagnosis in order to understand the relevance of inflammation in the natural history of carcinogenesis. The temporal variability is not known for a large number of markers included in currently available multiplexed assays (29, 48). Because low temporal ICCs could bias risk associations toward the null (40), studies need to incorporate inflammation marker measurements at multiple time points to accurately estimate associations with cancer.

The use of validation substudies to evaluate assay reproducibility Methodologic studies conducted to date show that a large number of markers included in current multiplexed assays can be measured with demonstrated analytical repeatability, as evidenced by low CVs and high ICCs in blinded replicate samples (25). However, individual studies should include blinded quality control (QC) samples to investigate within- and across-batch reproducibility of inflammation marker measurements, and report results of such evaluations. In addition, estimates of ICCs from adequately sized substudies can be utilized to conduct formal corrections for measurement error through several analytic methods, such as regression calibration (40).

The use of marker association results for cancer risk stratification Use of inflammation marker levels or scores for risk stratification and cancer risk prediction might be considered a long-term goal of studies on inflammation and cancer. However, before use of such markers or scores can be contemplated for this purpose, several conditions need to be met. First, etiologic studies need to be completed to identify robust and consistent associations between inflammation markers and cancer. Second, evidence of good discriminatory ability (i.e., sensitivity, specificity, and positive/negative predictive value) should be shown for marker associations identified from etiologic studies. Finally, additional collaboration with laboratories will be needed to ensure that absolute marker levels are meaningful and highly reproducible across batches manufactured over time to allow for use of such markers in a clinical setting.

Laboratory testing methods

Summary of presentations and discussions. One workshop session was devoted to the state of the science on laboratory methods for multiplexed immune/inflammation marker measurements. Presentations included a review of currently available technologies, including multiplexed bead-based arrays, electrochemiluminescence, and multiplexed aptamer-based proteomic assays, as well as comparability of multiplexed assays versus high-sensitivity singleplexed ELISA. Presentations and discussions also covered technical sources of assay variability, such as the use of different antibodies by different manufacturers, lot-to-lot variability in assay reagents and standards, within- and-across batch test-retest variability, and variability arising from collection, processing, and storage of specimens, as well as biologic sources of variability in

marker measurements, such as diurnal/seasonal variation, the use of specimens collected with or without fasting, and demographic factors (e.g., age, gender, race). Discussions also centered around the appropriateness of serum versus plasma specimens for marker measurements, differences in assay sensitivity and lower limits of detection for the measurement of cytokines, chemokines, and soluble receptors.

Consensus and gaps. Technical sources of variability in marker measurements Several technical aspects influence inflammation marker measurements, including specimen collection, processing, and storage protocols as well as specific assay features (2). The time between blood draw and specimen processing, which potentially influences *ex vivo* production of cytokines and chemokines, processing protocols (e.g., centrifugation speed), duration of storage, and number of freeze-thaws are all known to influence inflammation marker measurements (2, 49). However, the influence of these factors has been evaluated for a select few markers and remains unknown for a large number of markers. Inflammation marker measurements also show wide variability across analysis platforms, perhaps due to the use of different antibodies and assay matrices, and even across production lots from the same manufacturer (26). Given this latter variability, individual studies should utilize reagents from a single production lot.

Biologic sources of variability in marker measurements Several biologic factors also influence inflammation marker measurements, including time and season of blood collection, fasting versus non-fasting blood samples, demographic (age, gender, race), anthropometric (BMI), and behavioral factors (smoking, alcohol use; ref. 2). Because several of these biologic sources of variability are also associated with cancer risk, studies need to carefully account for confounding and examine mediation in marker-cancer associations.

Appropriateness of specimen types (serum vs. plasma) Serum and plasma samples differ significantly in inflammation marker levels (25, 50, 51). The use of serum samples is generally preferable to avoid *ex vivo* production of cytokines/chemokines after specimen collection. However, platelet degranulation in serum samples can distort levels of certain analytes; thus, the use of platelet-free plasma samples is ideal for the measurement of platelet-derived analytes (e.g., IL8, TGF- β , EGF, VEGF, CXCL4). Furthermore, inflammation marker levels also differ significantly across different plasma anticoagulants (e.g., acid citrate, EDTA, heparin), with heparinized plasma being more similar to serum when compared with plasma obtained using other anticoagulants (25, 50, 51). Thus, studies need to utilize the same material type for cases and controls to ensure comparability, and specimen type needs to be considered when comparing results across studies.

Comparability of multiplexed inflammation marker assays The performance characteristics (lower limits of detection and test-retest reproducibility) as well as absolute marker levels for a given analyte differ significantly across analysis platforms (26). Few efforts have been made to benchmark multiplexed assays across different platforms. These issues reduce comparability of results in the literature.

Utility of multiplexed inflammation marker assays Currently available antibody-based multiplexed assays allow the measurement of a sizeable number of markers and therefore enable studies to better

characterize the role of inflammation in cancer etiology. Yet, the extent to which currently measurable assays/analytes adequately capture the general processes of inflammation and immune dysregulation is unknown, and perhaps low. Novel platforms that measure an increasing number of markers are likely to be developed and will need to be considered for future epidemiologic studies in this area.

Epidemiology/immunology interface

Summary of presentations and discussions. One session in the Workshop was devoted to discussions of potential biologic implications of markers identified from epidemiology association studies to date and to future needs to follow-up on epidemiologic studies to elucidate the biologic underpinnings of robust, reproducible associations. Immunologists invited to present at the Workshop were provided with a summary of findings from published and ongoing studies of inflammation markers and asked to discuss their implications. Presenters highlighted the fact that inflammatory responses are complex and involve a network of cells, receptors, and soluble factors that together define the pattern of response induced by external exposures and tissue disruption/damage (52, 53). They stressed that although epidemiologic studies to define consistent associations between individual and groups of inflammation protein markers and cancer are necessary, they are not sufficient to fully understand the biologic role of inflammation in cancer and that laboratory studies to define the biologic underpinnings of epidemiologic associations will be needed.

Cytokines, chemokines, soluble receptors, and acute phase markers were discussed by presenters, and their respective roles in the innate inflammatory response were summarized. Presentations focused on markers that have been reported to be associated with one or more cancers in published and ongoing studies. Patterns of inflammatory responses at the local/tissue level were also described and how these local responses might be reflected (or not) in systemic patterns were discussed (54).

Consensus and gaps. Marker levels for different classes of inflammation markers. Bioactive concentrations, circulating levels, and half-life all differ significantly for different classes of inflammation markers (29). It is known that circulating levels of soluble receptors and chemokines far exceed those of several cytokines, which often lead to undetectable or low levels for cytokines of general interest in cancer association studies. This is particularly true for several anti-inflammatory cytokines (e.g., IL4, IL10, IL13). This issue manifests as varying statistical power to find significant associations for the different analytes. In reviewing results from published and ongoing studies, it was noted that reproducible marker associations observed for individual cancers were often chemokines (e.g., CXCL9 and lung cancer; ref. 33), soluble receptors (e.g., CD23, CD30, and NHL; refs. 7, 10, 35), or acute phase markers (e.g., CRP and SAA and lung cancer; refs. 24, 33). These analytes are part of networks that include important upstream cytokines that occur naturally at low levels (e.g., CXCL9 is induced preferentially by IFN γ ; CRP and SAA are induced by IL1/6). Therefore, findings that point to these downstream effectors should be viewed as "marking" specific pathways/networks and not simply as reflecting individual associations in isolation.

Interpretation of systemic marker associations Systemic levels of inflammation markers may not always mirror levels at individual

tissue sites, particularly early in the natural history of a cancer. Interpretation of systemic associations should be made with caution. The associations might reflect residual confounding by exposures that induce, separately, inflammatory responses, and cancer. They may also reflect late disease effects (i.e., reverse causality; see Results, Epidemiology Studies and Statistical Considerations: Consensus and Gaps, Point #3) rather than etiologic factors. In some instances, the associations might reflect true biologic associations. In such cases, systemic levels of inflammation markers might reflect an individual's inherent predisposition to be a strong or weak responder to external stimuli, or more directly reflect tissue levels that, themselves, are causally linked to cancer development. Distinguishing between these various alternatives will be important in future studies.

Incomplete understanding of inflammatory response patterns Our understanding of the complex nature of inflammatory responses *in vivo* remains incomplete. As our ability to measure analytes involved in the inflammatory response increases (55), so does the need for studies to understand temporal patterns of these analytes in response to specific classes of exposure at different body sites and the interplay between individual analytes. This will require carefully designed, multidisciplinary molecular epidemiology studies with repeated measures and intensive biologic specimen collection/testing.

Discussion

Recommendations and their implementation

On the basis of the consensus/gaps identified in each of the discussion sections summarized above, a set of broad recommendations was developed. These recommendations are summarized in Table 1. After the ICE Workshop, the recommendations were reviewed and the following steps were defined as possible mechanisms for their implementation.

Epidemiologic studies and statistics. Guidelines for designing and describing high-throughput "omics" technologies in clinical trials (56), designing and reporting on biomarker studies (57, 58), and reporting on studies of diagnostic test accuracy (59) have been developed with the goal of improving reproducibility and interpretation of research results in those areas. Similarly, a set of specific guidelines for designing and reporting future epidemiology studies of inflammatory protein biomarkers and cancer should be developed. The guidelines would include requirements for adequate study power, use of assays with known/acceptable performance that have been benchmarked against standard assays, analytical plans that specifically address multiple comparisons and adjustment for relevant confounders, and an explicit plan for independent replication. These guidelines could be used by consortium groups (e.g., NCI EGRP Cohort Consortium; <http://epi.grants.cancer.gov/Consortia/cohort.html>) and funding entities (e.g., NIH, Bethesda, MD) to guide their review and funding decisions.

Laboratory testing methods. A coordinated effort, such as the Early Detection Research Network (EDRN) biomarker validation reference sets (<http://edrn.nci.nih.gov/resources/sample-reference-sets>), should be established to facilitate the development and implementation of reference standards to be used across laboratories that test biospecimens from epidemiologic studies aimed at

Table 1. Summary of main recommendations identified at the Inflammation and Cancer Epidemiology (ICE) Workshop sponsored by the National Cancer Institute, June 2014

Topic area	Recommendations
Epidemiologic studies and statistical considerations	<ol style="list-style-type: none"> 1. Studies using multiplex assays to measure inflammation markers should include discovery followed by replication studies so that reproducible findings can be identified. 2. Studies should be designed and powered to control for multiple comparisons and, ultimately, for mediation analyses to understand whether inflammation explains established exposure–cancer associations. 3. Studies should measure marker levels over time before the diagnosis of disease to help uncover the underlying mechanism(s) linking inflammation and cancer. 4. Use of pathway-based analyses/approaches to interpret individual marker associations should be encouraged. 5. Studies should be coordinated/integrated so that results can be directly compared. 6. Efforts to translate findings to clinical use (e.g., risk stratification) will need to await completion of carefully conducted and replicated etiologic studies and will require additional standardization of assays targeting informative markers.
Laboratory testing methods	<ol style="list-style-type: none"> 1. Efforts should continue to develop new assays and to understand/refine existing ones to more reliably/broadly assay inflammation markers and more fully understand markers that are being measured. 2. Efforts should be promoted to develop standards and establish a set of common practices to ensure that data derived from specific assays and laboratories are reliable/reproducible and to enable calibration of results across labs and testing platforms. 3. Efforts should be made to incorporate new platforms/technologies into epidemiological studies, as they become available; such efforts will require bridging of new studies to previously completed work.
Epidemiology–immunology interface	<ol style="list-style-type: none"> 1. Interdisciplinary research should be encouraged and facilitated. 2. Studies should consider measuring local (tissue) markers of inflammation. 3. New, biological specimen intensive studies should be considered to more comprehensively evaluate the underlying mechanism(s) for the strongest and most consistent associations observed from epidemiological studies.

evaluating the association between circulating protein markers of inflammation and cancer risk. Such an effort should also support continued methodologic work to better understand the impact of preanalytic variables (specimen collection, processing, and storage) on inflammatory analyte levels, calibration studies to formally bridge newer assays (e.g., assays designed to capture a larger numbers of inflammation markers/analytes) against existing assays from published work, and efforts to characterize novel, scalable laboratory assays to measure inflammation at the tissue level. This will maximize comparability of findings across studies in the future and allow for bridging between blood and tissue-based studies.

Epidemiology/immunology interface. Private and public funding entities should develop mechanisms to formally promote small but specimen-intensive studies involving repeated measurements over time to interrogate the inflammatory process at the cellular level in more detail. This would include the interplay between the various components of the inflammatory response. Such studies should be interdisciplinary; should involve expertise in immunology, systems biology, epidemiology, and statistics among others; and should be designed to inform results from larger epidemiologic association studies.

Conclusions

Research groups have begun to incorporate multiplex immune protein marker panel testing into epidemiologic studies designed to evaluate the association between inflammation and cancer. Interdisciplinary work involving immunologists, epidemiologists, and laboratory analysis scientists will be required if we are to extend results from epidemiologic association studies into a better understanding of the underlying mechanisms linking inflammation to cancer. Coordination of efforts will become important to maximize what we learn from these studies and to

avoid unnecessary duplication of efforts. This coordination should encourage sharing of results from both methodologic and analytical studies. The development of a set of agreed upon common practices and reference standards would be helpful. As efforts in this field evolve, there will undoubtedly be new technological advancements that will need to be considered and incorporated into new studies. We hope that the recommendations generated from the NCI-sponsored ICE Workshop and summarized herein will be helpful in these respects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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