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EXPLORING THE EFFECT OF CHRONIC INFLAMMATION ON RESPONSE TO IMMUNE CHECKPOINT INHIBITORS IN CANCER

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EXPLORING THE EFFECT OF CHRONIC INFLAMMATION ON RESPONSE TO IMMUNE CHECKPOINT INHIBITORS IN CANCER

DISSERTATION

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Pharmacy at the University of Kentucky

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ABSTRACT OF DISSERTATION

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Precision medicine has allowed for the development of monoclonal antibodies that unmask the anti-tumor immune response. These agents have provided some patients durable clinical benefit. However, PD-1 and PD-L1 inhibitor therapies are effective in a small group (10-20%) of non-small cell lung cancer (NSCLC) patients when used as single-agent therapy. The approved companion diagnostic is expression of the immune cell surface molecule, programmed death ligand 1 (PD-L1), on tumors measured by immunohistochemistry (IHC). Studies in tumor biology and immune surveillance dictate that PD-1 inhibitor efficacy should depend on the level of PD-L1 expression; however, the literature has not followed with convincing evidence. The limitations of this test include timing of tissue acquisition, tumor heterogeneity, and timing of therapy relative to the expression of PD-L1. In addition, the requirement of analyzing tumor tissue biopsy samples from a patient is cumbersome. Thus, a peripheral blood biomarker that predicts efficacy of PD-1/PD-L1 inhibition would be optimal for precise and cost-effective treatment. A history of chronic inflammatory diseases may be advantageous for a cancer patient who is treated with PD-1/PD-L1 inhibitors and may allow them to then mobilize a swift immune response to tumor cells. Specific biological components of this persistent inflammation may predict PD-1 inhibitor response. We have taken a novel approach to leverage national healthcare claims data that couples patient history with response to therapy. We have identified potential peripheral blood biomarkers of response to PD-1/PD-L1 inhibitors using a combination of healthcare outcomes and molecular markers that correlate with therapeutic efficacy.

KEYWORDS: Immune checkpoint inhibitors, biomarkers of response, health outcomes research, cancer, PD-1/PD-L1
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CHAPTER 1

A. CANCER & THE IMMUNE RESPONSE OVERVIEW

Cancer Overview

Cancer is understood to be a disease of unchecked cell growth due to genetic mutation; a continuous series of genetic events that guide cells to uncontrolled proliferation and invasion into surrounding tissue. Tumor cells gain the ability to survive independently and metastasize to other organs within the body (1). Multiple organ involvement and ultimate failure is the final state of late-stage disease, resulting in death. Standard of care for cancer includes the resection of the malignant tissue, radiotherapy, and systemic chemotherapy. Resection of the malignancy, the only treatment which can be considered a cure among solid tumors, is typically only possible when the disease is localized and detected early (2). Unfortunately, patients with aggressive cancer types, including lung cancer, are diagnosed during late stage disease resulting in high mortality rates (3). Hanahan and Weinberg published the first iteration of *The Hallmarks of Cancer* in the year 2000 wherein they described six major factors influencing cancer biology; self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (1). Specifically, normal cells require mitogens to signal them to undergo growth and division; however, cancer cells are often able to grow despite the lack of external signals. Tumor suppressor genes are expressed to halt division in normal cells; however, cancer cells harbor mutated tumor suppressor genes resulting in aberrant progression through the cell cycle. Apoptosis is the programmed self-destruct function of nucleated cells to prevent the formation of abnormalities such as aberrant growth. Cancer cells can evade this programming. Normal cells have a finite number of divisions before a cell becomes unable to divide, known as senescence. Cancer cells overcome...
this limit by disabling the pRB, p53 tumor suppressor proteins and maintaining telomeres. Angiogenesis is the generation of new blood vessels from pre-existing vessels and is essential for the transportation of oxygen. New blood vessels are typically generated during wound repair and the development of embryos; however, cancer cells can trigger the production of new vasculature. Lastly, cancer cells can undergo transformation from an epithelial form to mesenchymal form to break away from the primary tumor site to metastasize to distant organs. In 2011, Hanahan and Weinberg supported the inclusion of four additional hallmarks; reprogramming of energy metabolism, genome instability, inflammation and evading the immune response (4). Specifically, cancer cells prevent mitochondria from normal aerobic respiration leading to the decreased production of ATP. The resulting low ATP: ADP ratio deactivates mitochondria and prevents triggering of apoptosis. Cancer cells can harbor chromosomal abnormalities such as tetraploidy or trisomy making DNA more susceptible to mutations. Local chronic inflammation can, over time, cause DNA damage leading to the development of cancer cells. The discovery that cancer cells use mechanisms to evade the immune response initiated investigations into medications that would retarget the immune response to cancer.

*Cancer immunosurveillance* describes a mechanism by which tumor cells are recognized and subsequently destroyed by the immune system (5, 6). Exploitation of immune mechanisms and evasion of immune surveillance are activities that survived selection in cells that underwent random mutagenesis to ensure the rise of a tumor. Tumor-associated antigens (TAAs) as well as neoantigens released by the cancer cells enter the blood circulation and initiate the triggering of the immune response (Figure 1.1). The tumor microenvironment contains multiple immune cell-types, including macrophages, dendritic cells, natural killer (NK) cells, mast cells, B cells, and T cells.
including T helper 1 (Th1) CD4+, T helper 2 (Th2) CD4+, regulatory T cells (TReg) and cytotoxic CD8+ T cells (7). The presence of the immune cells as well as cytokines and chemokines in the tumor microenvironment aid the growth and viability of the cancer cell. The immune response is differentiated into two types: the innate immune response and the adaptive immune response. The immune cells that are part of the innate response include dendritic cells, macrophages, NK cells and mast cells that are the first to respond to foreign agents. These cells, however, are not specially targeted for the cancer cells. The adaptive response typically follows the innate response. Its potency and effectiveness is greater than the innate response due to the priming and activation of these cells for the specific target. Certain adaptive immune cells can exert anti-tumor effects by recognizing TAAs or neoantigens presented on major histocompatibility complex (MHC) molecules (Figure 1.1). These antigens are presented to T cells via MHC class I or MHC class II on antigen presenting cells (APC) within lymph nodes. The presentation of the antigen requires additional co-stimulatory signals to induce T cell activation and expansion. Binding of B7 (CD80/CD86) on the APC to CD28 on the T cell leads to proliferation and differentiation via production of cytokines including interleukin (IL)-2. The effect is to drive clonal expansion of the activated T cell and to recruit other immune effector cells. Th1 and Th2 T cells secrete cytokines and chemokines that help to regulate this process with Th1 T cells activating CD8+ T cells and Th2 cells activating B cells. The primed T cells then exit the lymph node and are trafficked to the tumor site infiltrating the tumor. The cancer cells can then be destroyed by direct cell-mediated cytotoxicity. This adaptive immune response limits the establishment of cancer. However, in some cases tumor cells can escape the selective pressure from the immune system that allow tumor progression in the face of an ongoing immune response (8, 9).
Figure 1.1: Normal adaptive immune response to tumor associated antigens (TAAs).

Selection of tumor cells with the ability to increase the expression of certain markers that alter natural immune defenses of the host is favored, protecting themselves from destruction. Specifically, many tumors acquire the ability to modify cell surface epitopes and upregulate immune checkpoint molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed death-ligand 1 (PD-L1), lymphocyte activation gene 3 protein (LAG-3), and T cell immunoglobulin domain and mucin domain-containing protein 3 (TIM-3), thereby resisting an immune response (9-11). Programmed death cell protein 1 (PD-1), is normally expressed on CD4+ or CD8+ T cells. Antigen presenting cells (APC) normally express PD-L1 that, when bound by PD-1 on T cells, signal an exhausted active immune response. Evaluation of T cells in the tumor microenvironment show that these cells are often in the exhausted state leading to cancer immune evasion (Figure 1.2) (12). A growing approach to cancer therapy is the development of agents that block these immunosuppressive mechanisms by interfering with these checkpoint sites.

**Immune Response Deregulation**

Naïve T cells undergo maturity in response to acute events such as bacterial/viral infections or vaccinations over a 1-2 week period resulting in differentiation into T cells with effector functions (13, 14). Following clonal expansion and the clearance of the foreign antigen, a subset of T cells remain and become memory T cells. This pool of memory T cells retains the ability to rapidly reactivate effector functions against any future recurrence of the stimulating antigen. However, these functional, persistent memory T cells mature in the absence of continual antigen stimulation and after inflammation from the effector phase has subsided.

In stark contrast, chronic conditions, such as cancer, involve persistent antigen exposure and chronic inflammation which then alters the development of memory T cells (15-17).
The alteration of these memory T cells, known as T cell exhaustion, includes key characteristics such as sustained upregulation and co-expression of multiple inhibitory receptors. Another characteristic of T cell exhaustion is the loss of secreted cytokines including interleukin-2 (IL-2), interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNFα) (18, 19). T cells in this hypo-responsive state have been described in multiple conditions, including chronic viral infections such as lymphocytic choriomeningitis virus (LCMV), HIV and hepatitis C virus, in addition to cancer (20). Overexpression of inhibitory receptors PD-1 and CTLA-4, can be reversed to allow the immune response against these conditions (20, 21). The molecular mechanisms by which inhibitory receptors regulate T cell exhaustion remain unknown; however, there are several mechanisms discussed in the literature. First, inhibitory receptors may block target receptors or ligands preventing the formation of lipid rafts, as has been shown with CTLA-4 (22). Second, modulation of intracellular mediators downstream of receptor signaling can lessen the influence of activating signals from receptors such as TCR and co-stimulatory receptors (23). Lastly, induction of inhibitory genes such as basic leucine transcription factor, ATF-like (BATF) may lead to T cell exhaustion (24).

There are several cell surface interactions that mediate T cell exhaustion (Figure 1.2). Upon T cell activation due to an acute event, PD-1 expression is upregulated; however, during chronic infections, co-expression of other cell surface inhibitory molecules such as LAG-3, CD160, TIM-3 and CTLA-4 has been observed [(25, 26). LAG-3 is a cell surface protein that is structurally homologous to CD4 and binds to MHCII, inhibiting CD4-dependent downstream signaling (27). Immune checkpoints, consisting of stimulatory or inhibitory signals, regulate T cell activation and effector functions to sustain self-tolerance and minimize normal tissue damage (28). Blockade of more than one immune
checkpoint, such as PD-1 and LAG-3, results in improved reversal of T cell exhaustion (25, 29, 30).

In addition to the immune checkpoint receptors and proteins expressed on immune cell components, certain soluble factors found in the tumor microenvironment and periphery that also play a role in the induction and suppression of the exhausted T cells. Overexpression of inflammatory or immunosuppressive cytokines that induce T cell exhaustion are important soluble factors. Chronic interferon alpha and interferon beta (IFN-α/β) signaling has been shown to induce T cell exhaustion during chronic infection (31). IL-10 blockade has been shown to restore T cell function during chronic viral infections (32). Certain cytokines, such as IL-6 and IL-27, induce the exhausted T cell phenotype (33, 34). In addition, blockade of PD-1 and IL-10 simultaneously in mice synergistically reverses CD8+ T cell exhaustion (35). Lastly, in vivo inhibition of transforming growth factor-β (TGF-β) signaling in CD8+ T cells improved the function of exhausted T cells (36). Despite the present evidence that certain cytokines have direct or indirect influence on T cell exhaustion, the precise mechanism has not been defined.
Figure 1.2: Mechanisms of tumor immune evasion. Used with permission from Macmillan Publishers Ltd: Nature Reviews Cancer, 12, 252-264, copyright 2012.
B. CANCER IMMUNOTHERAPY

Immunotherapy describes several modes of treatment that aim to reorient the immune system to fight cancer. Early on, IL-2, an inducer of T cell expansion, was the first introduced in the treatment of cancer (37). IL-2 treatment is capable of mediating tumor regression in tumors; however, the toxicity profile manifests in multiple organ systems presenting as severe flu-like symptoms. Most common of these is capillary leak syndrome, a hypovolemic state and fluid accumulation the extravascular space leading to oliguria and ischemia. The toxicity profile of this treatment limited its clinical use. Another form of immunotherapy called adoptive cell transfer (ACT) was recently developed to utilize the patient’s own T cells with anti-tumor activity and expand those cells in vitro then reinfusing into the patient (38). Once reinfused, the T cells require binding to MHC molecules to induce their functions; however, tumor intrinsic functions may downregulate MHC on the surface of the tumor cell. Next, Chimeric antigen receptors (CAR) T cells were developed as engineered, patient-derived T cells with the ability to recognize and kill tumor cells without the MHC binding requirement (39). CAR T cells have additional co-stimulatory molecules that enhance its ability to proliferate. For example, a patient with advanced follicular lymphoma treated with CAR T-cell therapy targeting CD19 presented with dramatic regression after infusion (40). Finally, immune checkpoint inhibition (ICI) is a new mode of immunotherapy that has truly revolutionized cancer treatment and exhibits impressive responses in several tumor. The focus of my research explored markers of the immune response that may be associated with improved response to ICIs.

Inhibition of immune checkpoints has multiple FDA-approved indications for the treatment of various malignancies, including melanoma, non-small cell lung cancer
(NSCLC), bladder cancer and many more, and has allowed clinicians another tool in their armament of cancer treatments (41-44). This form of treatment shifts the perspective from giving a patient medication to destroy tumor cells to giving medication to aid their immune system in mounting a response against the tumor. The medications include agents that target immune checkpoints such as ipilimumab (Yervoy®, Bristol-Myers Squibb), an antibody to CTLA-4, nivolumab (Opdivo®, Bristol-Myers Squibb), and pembrolizumab (Keytruda®, Merck) which are antibodies to programmed cell death protein 1 (PD-1), the receptor for PD-L1, as well as atezolizumab (Tecentriq®, Genentech), avelumab (Bavencia®, Merck/Pfizer), and durvalumab (Imfinzi®, AstraZeneca), antibodies to PD-L1. The checkpoints these medications target act as mediators for the balance and escape phases of cancer immune editing, as discussed above. By targeting these inhibitory receptors, cancer cells lose the ability to suppress the antitumor response.

The use of ICIs in oncology has been met with excitement and hope as another pillar of treatment that is promising and results in long and durable responses. In the treatment of NSCLC patients, Nivolumab improved median overall survival as compared to Docetaxel treatment (9.2 mos vs. 6.0 mos; \( P < 0.001 \)) (42). Dual blockade of PD-1 and CTLA-4 in patients with melanoma has demonstrated improved tumor control with median duration of response at 22.3 months (45, 46). In addition to the improvement of overall survival, the treatment regimen is more tolerable as compared with docetaxel with less grade 3/4 adverse events. Importantly, in a long-term follow-up study of 129 nivolumab-treated patients with NSCLC, the 3-year OS rate was only 18% (47). The benefit of ICIs is the potential for long, durable responses, however, it's been shown that approximately 20% of the treated-population actually realizes benefit (48, 49). To improve that response, durable biomarkers of response are essential for clinical
management. Herein I review the body of scientific work assessing both tumor-specific and peripheral blood markers that are promising predictive biomarkers (Table 1).

C. TUMOR BIOMARKERS OF RESPONSE

Due to a low percentage of durable responders to ICI treatment, research efforts into developing predictive biomarkers of response began (Table 1.1). Given the mechanism of action of PD-1 and PD-L1 inhibitors, initial studies into biomarkers tried to determine whether the efficacy of these monoclonal antibodies correlated with PD-L1 expression in the tumor.

PD-L1 Expression
PD-L1 is a ligand of the PD-1 receptor, expressed on both tumor cells and APCs, and plays an important role in the inhibition of the normal T cell-mediated immune response. When PD-L1, expressed on the tumor cell, binds to PD-1 receptor on T cells, this leads to exhaustion of effector T cells and poorer prognosis of the patient (50). For the agents targeting the PD-1/PD-L1 axis, tumor expression of PD-L1 has been used as a method of selecting appropriate patients to be treated; however, it has not proven to be universal (51, 52). In cancers that express PD-L1, the interaction with the PD-1 receptor expressed on T cells, B cells, and natural killer (NK) cells of an activated immune system characterize an immune-evasive response by the tumor. Monoclonal antibodies that then bind to PD-1 or PD-L1 prevent cancer cells’ immune evasion (53). For this reason, expression of PD-L1 is currently utilized in the clinic as the best available biomarker to predict response to ICI targeting this checkpoint (51, 53, 54).

Interpretation of PD-L1 expression levels has caused confusion as there are several methodologies that utilize different cut-offs differentiating between positive and negative
expression. The currently utilized companion diagnostic tools were developed for use with pembrolizumab and nivolumab, both PD-1 inhibitors. Each measurement is completed using immunohistochemistry (IHC). However, there were two antibodies developed specifically for each of the medications; antibody 22C3, anti-PD-L1, for pembrolizumab and antibody 28-8, anti-PD-L1, for nivolumab. The Tumor Proportion Score (TPS) is the percentage of PD-L1 positive tumor cells showing partial or complete membrane staining relative to all viable tumor cells present in the sample. A positive TPS using pembrolizumab’s companion diagnostic (antibody 22C3) is considered to be greater than 50% whereas for nivolumab (antibody 28-8), expression cut-offs are set at >1%, >5% or >10%. There are ongoing efforts to standardize and optimize PD-L1 expression testing that include collaboration by the current ICI manufacturers to develop a universal PD-L1 testing platform (55).

From a simply biological perspective, measuring the expression of the membrane-bound PD-L1 ligand should indicate tumors that will respond to therapy. However, limitations of this diagnostic test, such as timing of tissue acquisition, tumor heterogeneity, and timing of therapy relative to the inducible expression of PD-L1, raise the question as to whether expression of this molecule truly represents an anti-PD-1 or anti-PD-L1 responsive tumor (52). The Keynote-001 trial sought to correlate response to pembrolizumab with expression of PD-L1. In the melanoma arm, PD-L1 expression correlated with increased response rate in 49% compared with 13% of PD-L1 negative patients. The NSCLC arm of this study showed that, when PD-L1 expression on tumor cells was greater than 50%, response to pembrolizumab was predicted (67% PFS rate at 6 months). Of these patients, nearly 25% demonstrated PD-L1 protein on greater than 50% of tumor cells. For all-comers, response rate to pembrolizumab was about 19%. Importantly, median progression free survival (PFS) was 14.1 months in PD-L1 strongly positive patients
compared with 9.3 months in the weakly positive population (56). Response rates appear to be higher in tumors with elevated PD-L1 expression; however, some tumors that do not express PD-L1 still exhibit a response to therapy (57). Given these observations, measuring PD-L1 expression in a tumor cannot serve as an exclusionary predictive biomarker of response. Development of robust and validated biomarkers of response for ICI therapy has become a priority in this burgeoning field.

Tumor-Infiltrating Lymphocytes and T-cell Receptors

The abundance of tumor-infiltrating lymphocytes (TILs), specifically CD8+ T-cells expressing PD-1 or CTLA-4, have previously been identified as indicators of successful checkpoint inhibition (58, 59). There is a demonstrated difference, however, between increased populations of CD8+ T cells found at the invasive tumor margin versus those in the tumor itself with the former being the most closely associated with increased PD-L1 expression. In addition, double positive PD-1+/CTLA-4+ CD8+ T cells within the melanoma were strongly associated with improved progression-free survival (PFS) (15.9 mos vs. 9.9 mos, $P=0.04$) after anti-PD-1 therapy (59). Out of 15 patients in a validation cohort assessing the predictive ability of CD8+ T cells abundance, quantified CD8+ T cell density accurately predicted 4 out of 5 patients in the true progression group and 9 out of 9 patients in the true response group. This data supports the notion that the abundance of TILs correlates with improved anti-PD-1 therapy response. Limitations of this assay include the need for assessing tumor tissue sample immediately prior to treatment. Most NSCLC patients undergo biopsies prior to resection and follow up with chemotherapy and/or radiotherapy. However, another biopsy would be necessary prior to addressing if the patient would be a candidate for ICI blockade. It is likely that the genomic and immune properties of the tumor would have changed since that initial biopsy so analyzing the original sample for TILs may not be accurate for subsequent
therapy. Further, it is cumbersome, and sometimes unreasonable, for a patient to undergo additional biopsies.

T-cell receptor (TCR) diversity, or the range of difference TCRs expressed, plays a vital role in host defense (60). Diversity refers to the degree of dispersion between clonotypes or phenotype of a clone of cells. TCR diversity is generated by mutations resulting from recombination, random insertion, deletion and/or mismatch in the genes that encode T-cell receptors, and there is potential to create between $10^6$ to $10^{20}$ TCR clonotypes. TCR diversity has been examined before and after checkpoint inhibition to assess its impact on T-cell clonal populations and its effect on response (61-63). In both peripheral blood as well as tumor samples, a substantial increase in “diversity” of unique TCR V-beta CDR3 sequences after anti-CTLA-4 treatment was observed and presumably led to pro-inflammatory or autoimmune hyper-responsiveness. Kvistborg et al monitored immune reactivity against a panel of 145 melanoma-associated epitopes in patients receiving anti-CTLA-4 treatment. Comparison of T cell reactivities prior to and after treatment for 40 melanoma patients demonstrated that anti-CTLA-4 treatment induces a significant increase in the number of detectable melanoma-specific CD8 T cell responses ($P =0.0009$) (63). There was an increase in the total number of unique sequences but no one specific clone became predominant. In a study of patients with metastatic melanoma treated with PD-1 blockade, tumors that illustrated expansion of pre-existing TCRs, a more “focused” repertoire, were most likely to respond to therapy (58). The “focused” repertoire, defined as a more restricted TCR beta chain population, correlated with clinical response after pembrolizumab therapy ($P=0.004$). Together, these data suggest a “diverse” profile of TCRs present in T cells infiltrating tumors is associated with improved response with anti-CTLA-4 treatment. A “focused” TCR profile is associated with improved response with anti-PD-1/PD-L1 blockade. Immuno-sequencing of TIL and
TCR diversity may be effectively used as predictors of response or for monitoring drug
efficacy and toxicity.

**Mutational Burden**

Mutations give rise to the development of cancer; however, the degree of burden differs
between different cancer types. Among all cancers, melanoma and lung cancers exhibit
the highest mutational burden as determined by cataloging somatic mutations (64).
Patients who harbor tumors exhibiting increased somatic mutations, such as
nonsynonymous variants (a nucleotide substitution that alters the amino acid sequence),
correlated with a benefit from CTLA-4 blockade (65). In a study of 64 malignant
melanoma patients treated with anti-CTLA-4, whole-exome sequencing was conducted
on tumor and matched blood samples. Somatic mutations and candidate neoantigens, a
new antigen developed within the tumor the immune system has not previously been
exposed to, were characterized. A discovery set of 11 patients who exhibited long-term
clinical benefit and 14 patients who presented with minimal benefit were characterized. A
neoantigen signature, determined by genome-wide somatic neoepitope analysis,
predicted strong response to CTLA-4 blockade. This signature was validated in a set of
39 patients. Tobacco smoke, which contains carcinogens such as arsenic and benzene
that induce mutations, was associated with benefit of PD-1 blockade in lung cancer
patients (66). A smoking signature was determined utilizing whole-exome sequencing of
NSCLC samples in two independent cohorts, n=16 and n=18. The smoking signature,
identified as transversion high (i.e. the substitution of a purine for a pyrimidine DNA base
or vice versa) and is associated with elevated neoantigen burden, lead to improved
objective response, durable clinical benefit, and progression-free survival (66). The
observation that nonsynonymous mutation burden is associated with anti-PD-1 efficacy
is consistent with the hypothesis that APC recognition of neoantigens, formed because
of somatic mutations, is important for the activity of anti–PD-1 therapy.
DNA repair mechanism mutations, especially in the mismatch repair (MMR) pathway, also contributed to the mutational burden leading to improved response to certain therapies. Patients exhibiting MMR-deficient tumors, which occurs in a small population of cancers, benefited from anti-PD-1 treatment (67). In 41 patients with metastatic carcinoma with or without MMR-deficient disease, anti-PD-1 treatment was administered. The 20-week immune-related progression free survival rate showed an improved response in patients with the MMR-deficient cancer (78% vs. 11%). Whole-exome sequencing revealed 1,782 somatic mutations per tumor in the MMR-deficient tumors compared to 73 in the MMR-proficient tumors ($P=0.007$). The resulting tumors express high levels of PD-L1 and possess a cytokine-rich microenvironment with immune infiltrates expressing PD-1, CTLA-4 or LAG-3, signaling a primed response. Since then, this study has been expanded to evaluate the efficacy of PD-1 blockade in patients with advanced MMR-deficient cancers across 12 tumor types and is currently still ongoing (68). Eighty-six patients with at least one prior therapy and evidence of progressive disease underwent treatment with a PD-1 inhibitor. Mismatch-repair deficiency was identified in all patients using either polymerase chain reaction or immunohistochemistry. The study is ongoing and has yet to reach the primary endpoint of overall survival. In May of 2017, Keytruda (pembrolizumab) received an indication for the treatment of adult and pediatric patients with unresectable or metastatic solid tumors that have a microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) hypermutated malignancy (69). These works have collectively led to the fast-tracked approval for the first cancer treatment for any solid tumor with a specific genetic feature.  

**Inflammatory Gene Signatures**

Other factors inherent to the nature of the tumor microenvironment may determine resistance or susceptibility to immunotherapy. Gamma interferon-inducible genes have
been highlighted in the literature in defining a “hot” tumor – or a tumor with an interferon responsive signature (70, 71). Class II MHC-positive melanomas that have this interferon responsive signature respond to PD-1 blockade. Other studies show the loss of this signature reduces efficacy of treatment (72). First, whole exome sequencing of tumors from 16 patients with melanoma who did not respond to ipilimumab therapy and had reduced overall survival, identified multiple-copy-number alterations resulting in the loss of interferon gamma pathway genes, including \textit{IFNGR1} (73). Next, patients from the KEYNOTE-012 (NCT01848834) study that assessed the use of pembrolizumab for the second-line treatment of head and neck squamous cell carcinoma (HNSCC) were tested using 4 multi-gene expression signatures (74). These 4 gene sets included “IFN-γ” (6-gene), “TCR signaling” (13-gene), “expanded-immune” (18-gene) and “de novo” (33-gene). The “IFN-γ” set included CXCL9, CXCL10, IDO1, IFN-γ, HLA-DRA, and STAT1. All 4 sets showed strong association with PFS \((P<0.0005)\) with “IFN-γ” the top performer with a positive predictive value (PPV) for response of 40.0%, and negative predictive value (NPV) of 95.0%. In a third study, patients treated with atezolizumab, a PD-L1 inhibitor, demonstrated an increased response when an INF-γ gene signature was highly expressed (75). In this open-label phase 2 trial, 286 patients with NSCLC previously treated with platinum chemotherapy were to be randomized to receive atezolizumab or docetaxel therapy. Overall survival for the atezolizumab group was 12.6 months (95% CI 9.7-16.4) versus 9.7 months (95% CI 8.6-12.0) for the docetaxel group. More impressively, in an additional exploratory analysis, patients treated with atezolizumab with high T-effector-interferon-γ-associated gene expression \(T_{\text{eff High}}\) improved overall survival \((HR 0.43; 95\% \text{ CI 0.24–0.77})\), the best improvement in response. End of the study was reached before overall survival could be assessed. \(T_{\text{eff high}}\) high was associated with a median follow-up of 15 months where \(T_{\text{eff low}}\) low had a median follow-up of 10 months (75). Another study assessed the regulation of a library of 209 different cytokines
from the human cytokine library, IFN-γ stood out due to its autocrine mechanism to elevate STAT1 and induce internalization of gp130, a component of many heterodimeric cytokine receptors (76).

These studies have identified the impact of inflammatory genes and pro-inflammatory cytokines on immune response. IFN-γ may be critical in this process due to its hypothesized role as a master checkpoint regulator for many other cytokines. STAT family proteins generally reside in the cytoplasm as inactive homodimers (77). Receptor-associated JAKs become activated upon ligand binding leading to phosphorylation of specific receptor tyrosine residues. These residues direct SH2-dependent recruitment of specific STATs which as then activated and released and reorient into antiparallel dimers where the SH2 domain on one STAT binds to the phosphotyrosine on the other STAT. Activated STAT dimers translocate into the nucleus. In response to IFN-γ, STAT3 protein phosphorylation is reduced in favor of increasing both expression and phosphorylation of STAT1. Once phosphorylated, STAT1 proteins form homo- or heterodimers and translocate to the nucleus where they act as transcription activators. This simple elevation of STAT1 and down-regulation of STAT3 by IFN-γ interferes with multiple cytokines using STATs as key signal transducers.

**Gut Microbiome**

Several studies have shown that the intestinal microbiome may modulate the anticancer effect of certain chemotherapies, such as cyclophosphamide (78-80). Cyclophosphamide alters the composition of the gut microbiota, specifically translocating select Gram positive bacteria into secondary lymphoid organs leading to generation of T helper 17 (Th17) cells and memory Th1 immune responses. The specific Gram-positive bacteria identified were *Lactobacillus johnsonii*, *Lactobacillus murinus* and *Enterococcus hirae*. When assessing the therapeutic efficacy of anti-PD-1 treatment alone or in
combination with anti-CTLA-4 treatment in mice with sarcoma or melanoma, mice were raised in specific pathogen-free conditions and then either treated for 14 days with broad-spectrum antibiotics (ampicillin, colistin, streptomycin) or left untreated (81). Mice that were exposed to antibiotics exhibited a worse prognosis when treated with an ICI. The researchers also examined the impact of antibiotics on 249 human patients with NSCLC, renal cell carcinoma (RCC) or urothelial carcinoma treated with ICI. Patients exposed to antibiotics two months prior to ICI treatment or one month after exhibited decreased PFS (3.5 mos vs. 4.1 mos, \( P=0.017 \)) and decreased overall survival (11.5 mos vs. 20.6 mos, \( P<0.001 \)) as compared to patients with no exposure to antibiotics. Analysis of the composition of the gut microbiota implicated \textit{A. muciniphila} with favorable clinical outcome (\( P=0.004 \)). In a separate prospective study, microbiome samples were collected from patients with metastatic melanoma patients prior to treatment with anti-PD-1 therapy (82). Thirty-five patients were classified as non-responders and fifty-four classified as responders based on Response Evaluation Criteria in Solid Tumors (RECIST) criteria at 6 months after treatment initiation. Diversity of the gut microbiome was significantly higher in the responder group compared to the non-responders (\( P<0.001 \)). \textit{R. faecalibacterium} was abundant in the responder group while \textit{Bacteroides thetaiotaomicron} was found present in non-responders. \textit{R. faecalibacterium} in the gut was associated with higher levels of effector CD4+ and CD8+ T cells in the systemic circulation with a preserved cytokine response to anti-PD-1 therapy, whereas patients with a higher abundance of \textit{Bacteroides} in the gut microbiome had higher levels of regulatory T cells (Treg) and myeloid derived suppressor cells (MDSC) in the systemic circulation, with a blunted cytokine response. The proposed mechanism by which the gut microbiome enhances systemic and anti-tumor immune responses suggests that increased antigen presentation improves effector T cell function in the periphery and the tumor microenvironment. Specific bacteria, such as \textit{E. hirae} and \textit{A. microsciphilia}, are
D. BIOMARKERS OF RESPONSE IN PERIPHERAL BLOOD

In the clinical setting, the ease of access to a patient sample with which to measure a potential biomarker is weighed heavily in addition to its precision of use. Currently, only a tumor-derived biomarker of response has been approved by the FDA for anticipating response to ICI. PD-L1 expression is typically assessed from a biopsy of the solid tumor and, as discussed earlier, the timing of tissue acquisition, tumor heterogeneity, and timing of therapy affect the PD-L1 expression value. PD-L1 expression is a dynamic parameter that cannot be adequately represented with a single snapshot (84). Liquid biopsies, in addition to being repeatable and easily accessible, allow for characterization of the dynamic changes of the tumor. Circulating tumor cells (CTCs) sampled from the primary tumor site likely share the immune escape mechanisms (85). In addition to PD-L1 expression, peripheral blood may allow for identification of other immune factors influencing the response to ICIs. Identification of a robust biomarker that is more readily available from peripheral blood would, not only avoid the need for additional biopsies, but could then be streamlined in the clinic to allow for more efficient treatment.

Peripheral Immune Populations

Tumor immune cell subtypes have been assessed within the tumor microenvironment, and specific cells are associated with improved response to ICIs (58, 59). These are typically measured from a tumor biopsy of the primary or metastatic tumor site. However,
these sites are often not very accessible and so immune cell subpopulations in the periphery can be explored using multi-parametric flow cytometry (86, 87). In a study of 209 advanced melanoma patients treated with ipilimumab, baseline frequencies of myeloid-derived suppressor cells (MDSC), regulatory T cells (Treg), serum lactate dehydrogenase (LDH), eosinophil count, and other clinical characteristics were measured then analyzed by Cox regression analysis to identify factors associated with improved overall survival (88). The study, conducted in two phases, aimed to first identify biomarker candidates that best fit a prognostic model and in the second phase validate the biomarker combination. Of 28 potential variables measured, a six biomarkers signature was confirmed to predict improved survival (low LDH count, elevated eosinophils, low absolute monocytes, high absolute lymphocytes, low Lin−CD14+HLA-DR^low MDSC frequencies, and elevated CD4+CD25+FoxP3+ Treg frequencies). The immunological rejection of cancer is dependent on the balance of interactions between T cells and regulatory cells (89). Eosinophils, lymphocytes, monocytes, Tregs and MDSCs are components involved in this regulatory network. Eosinophils are multifunctional white blood cells with cytosolic, large granules containing a variety of cytokines and chemokines. These cells play a part in tumor surveillance and tumor rejection. MDSCs and Tregs suppress the functions of T cells potentially counteracting the benefit of ICIs.

Another study investigating peripheral markers of response suggests that CD4+ T cells expressing PD-1 in peripheral blood are associated with poor clinical outcomes for NSCLC patients treated with a PD-1 inhibitor (90). Peripheral blood mononuclear cells (PBMC) retrieved from 42 NSCLC patients at diagnosis and from an additional 25 healthy donors were assessed for frequency of PD-1 expression on both CD4+ and CD8+ T cells. PD-1+ CD4+ T cells were elevated among NSCLC patients as compared
to healthy donors (13.3% vs. 8.8%, \(p=0.0045\)). However, there was no difference in PD-1 expression on CD8+ T cells among both populations. CD4+ T cell subgroups defined as high-PD-1 (PD-1 \(\geq 12.27\%\)) and low PD-1 (PD-1 <12.27%) were based on the mean expression levels of the 42 NSCLC patients. Lower rates of both OS and PFS were associated with the high-PD-1 groups as compared to the low-PD-1 group (median OS: 397 days vs. 721 days, \(p=0.028\); median PFS: 88 days vs. 391 days, \(p=0.044\)). In addition, the elevated PD-1 expression on either T cell subset isolated from peripheral blood was not associated with PD-L1 expression on tumor tissue. These results suggest a biomarker of response to checkpoint inhibition could be established based on cell surface markers of immune cell subsets in peripheral blood.

**Peripheral Immunoscore**

As discussed earlier, it has been reported that patients with high baseline TILs are associated with improved response to ICI therapy. Immunoscores are a signature of immune cell populations that indicate disease responsive to ICI treatment. Single parameters, like tumoral PD-L1 expression, are limited in their prognostic value, but the combination and interaction of several parameters may establish a robust predictor of response. Tumor immunoscore analyses have been primarily conducted on biopsies of primary or metastatic tumor lesions, but there may be promise in completing the same analyses of immune cell subsets in the periphery (91, 92). Further, others have hypothesized that immune cells in peripheral blood may be different among patients with metastatic disease leading to a different response to immunotherapy. Single marker studies have assessed the prevalence of circulating CCR7+ CD8+ T lymphocytes in head and neck cancers, or MDSC in advanced melanoma in relation to PFS (93, 94). However, a multivariate panel that measures immune cell subsets is more likely to be an efficient prognostic assay than any single immune effector cell (95). In a study by Farsaci et al, peripheral immunoscores were established from analysis of PBMC prior to
Tumor-derived Exosomes

Tumor cells produce more extracellular vesicles than normal cells, which may explain why plasma exosomes are substantially higher in patients with cancer than in normal donors (98). Tumor-derived exosomes, or “TEX”, carry molecular cargo from the tumor microenvironment and can also deliver suppressive or stimulatory signals to immune cells. TEX are typically isolated from supernatants of cultured human or murine tumor cell lines using ultracentrifugation or sucrose density gradient centrifugation; however, this method is time-consuming and is not reproducible in a scaled-up environment for exosome recovery from numerous human specimens. Taylor, et al. developed a size exclusion chromatography (SEC) approach to readily recover intact TEX from small
volumes of plasma (99). TEX do mirror some of the key molecules characteristic of the parent tumor cell and thus may serve as a surrogate of the tumor microenvironment (100). As such, they carry tumor-associated antigens, major histocompatibility complex (MHC) class I and II molecules, and a variety of cytokines that enable TEX to stimulate or suppress immune cells. However, in another study, antibody-based therapies such as ICIs can be less effective because TEX carry the antigen targets of ICI and can act as a sponge to “soak” up the antibodies. Therefore, diminishing concentrations of drug are measured at the tumor site (101). TEX may be used as a predictive model for treatment due to it serving as a surrogate for the tumor microenvironment; however, further validation is required.

**Angiopoietin-2**

Angiogenesis, the formation of new vasculature, is a hallmark of cancer. Angiogenic factors, such as angiopoietin-2 (ANGPT2), play significant roles in the inhibition of immune activities by inhibiting dendritic cell maturation, tumor infiltration by lymphocytes, promoting Tregs and MDSC expansion (102-104). Increased expression of Tregs and MDSCs directly suppresses the immune response. ANGPT2 is upregulated in tumor vasculature and confers resistance to anti-angiogenesis therapy targeting vascular endothelial growth factor (VEGF) (105). Although ANGPT2 had been previously identified as a prognostic marker of outcome. For certain cancers being treated with anti-VEGF therapy, it may have the potential to be used as a predictive biomarker for immune checkpoint inhibition. In metastatic melanoma patients, both high pretreatment concentrations and increases in serum ANGPT2 during treatment were associated with reduced overall survival in CTLA-4 and PD-1 blockade–treated patients (106). Further data indicates that the features of tumor blood vessels can limit the anti-tumoral functions of T cells, specifically cytotoxic CD8+ T cells. Dual blockade of VEGF and ANGPT2 as a tumor-conditioning strategy has been shown to increase the efficacy of
anti-PD-1/PD-L1 therapy in cancer (107). In both transgenic and transplant mouse tumor models, dual blockade delayed tumor progression and was found to expand tumor-associated-macrophages (TAMs) and dendritic cells. In addition, the proportion of CD8+ TILs were increased. When PD-1 blockage was introduced into the mouse model, the anti-tumoral activity of the dual blockade was further enhanced. The harmonizing actions of the antiangiogenic therapy with immune checkpoint inhibition may allow for synergistic efficacy in treatment. ANGPT2 addresses a lot of the criteria of a robust peripheral biomarker of response. It is easily sampled, validated in multiple tumor types in different combinations of ICIs, and is continually monitored during treatment. A comparison study of ANGPT2 levels and PD-L1 expression is needed to determine which one is a more robust biomarker.

E. RESEARCH PROPOSAL OVERVIEW

The identification of potent immune response inhibitory pathways, regulated by interactions managed by PD-1 and CTLA-4, have paved the way for revolutionizing immunotherapy for cancer treatment. Despite the development of immunotherapies utilized in the clinical setting today, we still understand little of the molecular mechanisms that initiate or subdue T cell exhaustion. With the increasing use of these agents in the clinic as well as many other ICIs currently in the pipeline, the demand for a validated and robust biomarker of response is high. Due to the potential for long durable responses, a validated and robust biomarker of response is needed to increase the rate of long-term responders from merely 20%. Further analyses of peripheral immune cells, cytokine production and the mechanisms influencing T cell exhaustion will be critical to defining a peripheral blood biomarker that is accessible, reproducible and validated.
I sought to identify a biomarker of response to ICIs that improves patient care using a combination of healthcare outcomes and molecular markers that correlate to therapeutic efficacy. Two testable research hypotheses were developed: First, I proposed that chronic inflammatory pretreatment comorbidities would impact baseline immune system function and regulation of response to cancer. This includes hyperlipidemia, hypertension, diabetes mellitus and obesity; collectively known as metabolic syndrome (MetS) as well as chronic obstructive pulmonary disorder (COPD). The comorbid history of MetS and/or COPD among NSCLC patients correlates with response to immune checkpoint inhibitor treatment. This hypothesis was assessed in both a retrospective single-center study as well as a national health outcomes observational study. Secondly, MetS and COPD comorbidities will provide additional understanding of the underlying biology of immune cell subsets and cytokine profiles in the periphery that influence response to ICIs.

The work herein documents the use of translational research and pharmacoepidemiology utilized to improve the clinical use of ICIs in cancer through improving patient selection via a more robust biomarker of response.
Table 1.1: Overview of candidate biomarkers of response for immune checkpoint inhibitors.

<table>
<thead>
<tr>
<th>TYPE</th>
<th>BIOMARKER</th>
<th>DISEASE SETTING</th>
<th>TREATMENT</th>
<th>CUT-OFF/MEASURE</th>
<th>CLINICAL SIGNIFICANCE</th>
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</thead>
<tbody>
<tr>
<td>Tumor-derived</td>
<td>Tumor-Infiltrating Lymphocytes (TILs)</td>
<td>Melanoma/NSCLC</td>
<td>Anti-PD-1</td>
<td>20%</td>
<td>PD-1+/CTLA-4+ CD8+ T Cells &gt;20% were associated with improved PFS (15.9 Mo vs. 9.9 Mos, ( P=0.04 )).(^{20})</td>
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<tr>
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<td>T-Cell Receptor (TCR) Diversity</td>
<td>Melanoma</td>
<td>Anti-CTLA-4</td>
<td>&quot;Diverse&quot;</td>
<td>Pre- and posttreatment T cell reactivity after anti-CTLA-4 treatment show significant increases in number of detectable melanoma-specific CD8+ T cell responses (( P=0.0009 )).(^{21,22})</td>
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<tr>
<td></td>
<td></td>
<td>Melanoma</td>
<td>Anti-PD-1</td>
<td>&quot;Focused&quot;</td>
<td>A more restricted TCR beta chain usage (less diverse and more clonal) correlated with clinical response after pembrolizumab therapy (( P=0.004 )).(^{19})</td>
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<td>Mutational Burden</td>
<td>Melanoma</td>
<td>Anti-CTLA-4</td>
<td>( \geq 100 ) mutations</td>
<td>Patients with long-term clinical benefit (PFS &gt; 6 months), had increased mutational burden (( P=0.04 )).(^ {25})</td>
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<tr>
<td></td>
<td></td>
<td>NSCLC</td>
<td>Anti-PD-1</td>
<td></td>
<td>Greater PFS in those with higher nonsynonymous mutation burden compared to those with lower nonsynonymous mutation burden (HR 0.19, 95% CI 0.08–0.47, ( P = 0.0004 )).(^{26})</td>
</tr>
<tr>
<td>Mismatch Repair (MMR)</td>
<td>Multiple</td>
<td>Anti-PD-1</td>
<td>MMR-deficient</td>
<td>Response rate and PFS for MMR-deficient colorectal cancers were 40% and 78% compared to 0% and 11% for MMR-proficient colorectal cancers, respectively. Current study expanded for 12 more tumor types and is ongoing.</td>
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<tr>
<td>IFN-γ Gene Signature</td>
<td>Melanoma</td>
<td>Anti-CTLA-4</td>
<td>High expression of IFN-γ gene signature</td>
<td>Strong associated with improved PFS ($P=0.0005$). PPV of 40.0%, NPV 95.0%.</td>
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<tr>
<td>NSCLC Anti-PD-L1</td>
<td>NSCLC</td>
<td>Anti-PD-L1</td>
<td>High expression of IFN-γ gene signature</td>
<td>NSCLC patients treated with atezolizumab with high T-effector-IFN-γ-associated gene expression ($T_{eff}$ IFN-γ High) improved overall survival (HR 0.43; 95% CI 0.24–0.77), compared to $T_{eff}$ IFN-γ Low (HR 1.10, 95% CI 0.68–1.76).</td>
<td></td>
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<tr>
<td>Gut Microbiome</td>
<td>mRCC/NS CLC</td>
<td>Anti-PD-1</td>
<td><em>E. hirae</em> &amp; <em>A. microscaphilid</em></td>
<td>Presence of certain gut flora has been associated with improved efficacy of nivolumab. In addition, antibiotic therapy 2 months or or month after nivolumab-treatment, resulted in reduced PFS (2.3 Mos vs. 9.1 Mos, $P&lt;0.001$).</td>
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<tr>
<td>Peripheral Blood</td>
<td>Immune Cell Populations</td>
<td>NSCLC</td>
<td>Anti-PD-1</td>
<td>PD-1 &gt;12.27% on CD4+ T Cells</td>
<td>Lower rates of both OS and PFS were associated with the high-PD-1 groups as compared to the low-PD-1 group (median OS: 397 days vs. 721 days, $P=0.028$; median PFS: 88 days vs. 391 days, $P=0.044$).</td>
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<tr>
<td>Peripheral Immunoscore</td>
<td>Breast</td>
<td>Immunotherapy Vaccines</td>
<td>Score based on % CD4, % CD8, % Treg, %MDSC, %NK, Ratio CD4:Treg, Ratio CD8:Treg, Ratio CD4:MDSC, Ratio CD8:MDSC</td>
<td>Peripheral immunoscore of refined subsets of immune cells revealed statistically significant differences in PFS ($P &lt; 0.001$) for breast cancer patients receiving docetaxel plus immunotherapy vaccine and in prostate cancer patients receiving radionuclide plus immunotherapy vaccine ($P = 0.004$).43</td>
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<tr>
<td>Tumor-derived Exosomes (TEX)</td>
<td>Multiple</td>
<td>N/A</td>
<td>TEX can act as a snapshot of the tumor microenvironment but easily assessed in peripheral blood.55</td>
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<tr>
<td>Angiopoietin-2 (ANGPT2)</td>
<td>Melanoma</td>
<td>Anti-CTLA-4/Anti-PD-1</td>
<td>ANGPT2 $\leq$ 3,175 pg/mL</td>
<td>Patients with low pretreatment ANGPT2 experienced improved survival as compared to those with low ANGPT2 concentrations (7.9 Mos vs. 34.6 Mos, $P&lt;0.0001$).61</td>
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</table>
CHAPTER 2

A. OVERVIEW

The utilization of pharmacoepidemiological techniques was a familiar task to myself and to our laboratory. To assess the ability of measuring survival and response from exposure to a certain treatment, we evaluated the role of statins in the cancer population. Our laboratory has prior experience in the analysis national healthcare claims datasets, such as Truven Marketscan (108). The role of HMG-CoA reductase inhibitors (statins) in chemoprevention and cancer treatment has been deliberated in the literature (109-113) as well as the mechanism by which they may exert their effect and improve overall survival (OS) in cancer patients (114-117). Recent cohort studies have shown that current statin use is associated with significantly lower risk of cancer death (111) while other studies have shown that statin use following diagnosis can reduce cancer-specific mortality in breast (118), colon (119, 120), and lung cancer (121) patients. However, some skepticism remains as mitigation of all confounding factors is not possible with cohort or pharmacoepidemiological studies (122, 123). Prospective evaluation of statins as monotherapy in cancer has been attempted, but a recent review of clinical trials of statin monotherapy in cancer revealed little effect (124). In contrast, prospective trials using a combination of statin with chemotherapy have shown improved survival in cancer patients (125-129). Given the relative safety profile of statins, rational combination therapies may provide cancer patients clinical benefit at the expense of minimal toxicity risk.

Mechanistically, statins inhibit 3-hydroxy-3-methylglutarylcoenzyme A (HMGCoA) reductase, the rate-limiting enzyme of the mevalonate pathway, which ultimately
produces cholesterol along with isoprenoids; intermediate pathway metabolites such as farnesyl pyrophosphate (FPP) and geranyl geranyl pyrophosphate (GGPP) (130, 131). The physiological importance of isoprenoids in normal and cancer cells is critical for facilitating membrane anchoring of numerous signaling molecules including G-proteins such as Ras and Rho (132). Furthermore, constitutive activation of signaling pathways in cancer is dependent on cholesterol availability for formation of lipid rafts (133, 134). Recognition of the biological importance of FPP and GGPP led to drug development efforts of isoprenylation enzyme inhibitors (135, 136), which ultimately stalled in early phase studies due to toxicity and lack of efficacy. Pharmacologically, bisphosphonates also act as isoprenylation inhibitors (137, 138) and are widely used to inhibit bone resorption and treat osteoporosis. Bisphosphonates have also been used extensively to treat cancer patients with bone metastases (139-142). Their effect on reducing bone metastasis is significant and is consistent with the observations that they cause apoptosis in tumor cells via inhibition of the mevalonate pathway as well as tumor cell invasion in vitro (143, 144). Reduced cholesterol levels trigger a negative feedback loop, which ultimately leads to the upregulation of mevalonate pathway genes via the transcriptional activity of SREBP transcription factors (145, 146). Based on this understanding, preclinical studies have demonstrated synergy when combining statins with bisphosphonates (114, 143, 147-151) and most recently dipyridamole, which has been shown to inhibit SREBP2 (152, 153). Thus, the use of combination therapies that can amplify the effect of statins or abrogate the development of statin related resistance may lead to synergistic clinical combinations. Here we used a large dataset of health claims to test the hypothesis that statins alone, or in combination with potentially synergistic therapies prolong survival in cancer patients. The combination of
epidemiological evidence and preclinical data may provide strong rational for future prospective clinical studies.

B. METHODS

We used the Truven Health MarketScan Commercial Claims and Medicare Supplemental Databases. The Marketscan database includes approximately 40 million individuals from over 160 large employers and health plans across the US and includes healthcare claims with diagnosis and procedure codes for medical encounters and all prescription medication fills. Data are de-identified in compliance with the Health Insurance Portability and Accountability Act regulations (HIPAA) and the University of Kentucky Institutional Review Board approved the use of the database for this study.

Patient Selection

Adults aged 18 years and older diagnosed with cancer between January 1, 2010 and November 31, 2013 were identified using ICD-9 codes in the primary or secondary positions. Patients with prostate and breast cancer were excluded due to the use of hormonal therapy affecting risk for thromboembolism. Patients were diagnosed with one of the following types of cancer: Stomach (ICD-9 codes: 151.xx), Pancreatic (157.xx), Brain (191.xx), Lung (162.2 – 162.9), Renal (189.0, 189.1), Lymphoma (200.xx – 202.xx), Leukemia (204.xx – 208.xx), Myeloma (203.0x), Colorectal (153.xx, 154.xx), or Gynecological (179.xx, 180.xx, 182.xx, 183.xx). At least 2 inpatient or outpatient diagnoses within 14 days were required, and the date of the first qualifying diagnosis of cancer was defined as the index date. Patients were required to have at least 12 months of pre-index and a minimum of one-month post-index continuous enrollment in the database.
Exposure groups were defined as: statin users with no history of non-statin cholesterol-lowering medication use; non-statin cholesterol-lowering medication users with no history of statin use (“non-statin users,” active control group); and those with no history of statin or non-statin medication use (“non-users,” control group). Medication use was based on having at least 90 cumulative days supplied in the 6 months prior to diagnosis. Specific statins include lovastatin, pravastatin, simvastatin, rosuvastatin, atorvastatin, fluvastatin and pitavastatin. Non-statins include fibric acid derivatives, bile acid sequestrants, and nicotinic acid.

**Measures**

Patient demographic characteristics included age, gender, geographic region and urban residence. Clinical characteristics measured during the 12-month pre-index period included the Charlson Comorbidity Index (CCI) and Elixhauser comorbidities (Elix) (154, 155). These include 17 and 31 categories of comorbid conditions, respectively, and are widely used for risk adjustment with health outcomes data. Additional medications accounted for in the pre-index period included anticoagulants, antihypertensives, antiplatelet, antiarrhythmics, and digoxin. Presence of metastatic disease was assessed on the index date.

**Statistical Methods**

Pairwise analyses were done between: statins vs. non-statins, statins vs. non-users, and non-statins vs. non-users. Propensity score matching was conducted using baseline comorbidities, medications, and demographic information to achieve balance between treatment groups. Propensity scoring mimics the randomization process of a clinical trial so that each matched pair has the same baseline probability to receive either treatment (156-158). Matched pairs should be similar in all baseline characteristics. Patients with the same cancer type were matched using a greedy, nearest neighbor algorithm with a
caliper set at 0.2 times the standard deviation of the propensity scores in the sample, allowing for up to five matches for each treated person (159). Standardized differences were calculated and shown in Appendix A. A standardized difference of <0.10 is generally considered to be non-significant (160). To address any residual confounding after propensity score matching, covariates were also incorporated in the final regression models (157). The final model included the following adjustment covariates: age, CCI score, region, anticoagulants, antihypertensives, antiplatelets, antiarrhythmics, digoxin, elixhauser index comorbidities and pre-index history of CHD, DVT, PE, atrial thrombosis and MI. Two sample t-test and chi-square tests were conducted to assess significant differences between treatment groups before and after matching.

The study cohort was followed until subjects died, were lost to follow-up due to loss of enrollment in the dataset, or the end of the study data. Cox proportional hazard regression models, accounting for correlation within matched pairs, were used to assess risk of death within one year of diagnosis among all cancers and then stratified among each cancer. Follow-up was terminated for those surviving beyond one year and were censored. Hazard ratios (HR) and 95% confidence intervals (CI) are reported. A p-value of <0.05 is considered statistically significant. All matching and statistical analyses were conducted in SAS.

**Subgroup Analyses**

A sensitivity analysis assessing the influence of age as an effect modifier was completed. Comparisons were made within age groups of less than 65 years old, 65-75 and greater than 75 as these groups may have different treatment patterns, responses and baseline survival prior to cancer diagnosis. The effect of dose intensity was evaluated by limiting the statin cohort to moderate or high dosage statins and comparing
again with non-statins and non-users (161). Patients remaining on statin therapy after
cancer diagnosis were assessed by observing the proportion of days where medication
was on-hand in the post-index period until end of follow-up. Statins were compared
individually and by type to ascertain differences among outcomes. Natural statins
include lovastatin, pravastatin and simvastatin and synthetic statins include rosvastatin,
atorvastatin, fluvastatin and pitavastatin (162). Patients using bisphosphonates or
dipyridamole, alone or in combination with statins, were compared to non-users to
assess effectiveness or synergy in terms of survival. Bisphosphonates assessed in the
analysis were alendronate sodium, etidronate disodium, ibandronate sodium,
pamidronate disodium, risedronate sodium and zoledronic acid. Bisphosphonates and
dipyridamole were subject to the same requirement of having at least a 90 cumulative
days supplied in the 6 months prior to cancer diagnosis. For all subgroup analyses, all
cohorts were rematched via propensity score methods described above.

C. RESULTS

Table 2.1 displays the baseline demographic and clinical characteristics of the study
population by treatment group. Due to the enrollment criteria, there were no missing
values on covariates used for propensity-score matching or survival analyses. Eligibility
criteria were met by 312,907 cancer patients. Three treatment groups were established
as outlined in the CONSORT diagram (Figure 2.1): statin-users (n=65,440), non-statin-
users who received non-statin cholesterol lowering medications (n=9,289) and non-users
(n=226,007). There were 8,198 patients who passed away within one year of diagnosis,
1,702 from the statin-users cohort, 216 among the non-statin users and 6,280 from the
no treatment cohort. The cohort contributed an average follow-up time of 359 days (SD
39.3) with no differences between cancer types or treatment groups. The mean age of patients in the statins, non-statins and non-users cohorts was 74.2 (7.8), 71.8 (9.3) and 60.8 (14.1), respectively. In all treatment groups, lung, lymphomas, and colorectal cancers accounted for the top three diagnosed cancers whereas stomach, pancreatic, and brain cancers were the least diagnosed.
Table 2.1: Baseline demographic and clinical characteristics of all cohorts included in the analysis.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Treatment Group</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Statins</td>
<td>Non-Statins</td>
<td>Non-Users</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N=65,440</td>
<td>N=9,289</td>
<td>N=226,007</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Mean: 74.72, Std. Dev: 7.84</td>
<td>Mean: 71.79, Std. Dev: 9.33</td>
<td>Mean: 60.8, Std. Dev: 14.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCI</td>
<td>Mean: 5.1, Std. Dev: 3</td>
<td>Mean: 5.09, Std. Dev: 3</td>
<td>Mean: 4.34, Std. Dev: 2.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (Male)</td>
<td>N: 31,006, %: 47.4%</td>
<td>N: 4,046, %: 43.6%</td>
<td>N: 129,670, %: 57.4%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urban</td>
<td>N: 55,634, %: 85.0%</td>
<td>N: 7,758, %: 83.5%</td>
<td>N: 188,958, %: 83.6%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHF</td>
<td>N: 10,456, %: 16.0%</td>
<td>N: 1,255, %: 13.5%</td>
<td>N: 16,016, %: 7.1%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arrhythmias</td>
<td>N: 18,941, %: 28.9%</td>
<td>N: 2,364, %: 25.4%</td>
<td>N: 37,160, %: 16.4%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Valvular disease</td>
<td>N: 10,749, %: 16.4%</td>
<td>N: 1,364, %: 14.7%</td>
<td>N: 19,602, %: 8.7%</td>
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</tr>
<tr>
<td>Pulmonary Circulation</td>
<td>N: 3,008, %: 4.6%</td>
<td>N: 414, %: 4.5%</td>
<td>N: 7,496, %: 3.3%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral Vascular</td>
<td>N: 20,169, %: 30.8%</td>
<td>N: 2,570, %: 27.7%</td>
<td>N: 33,064, %: 14.6%</td>
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<td></td>
</tr>
<tr>
<td>Hypertension uncomplicated</td>
<td>N: 46,024, %: 70.3%</td>
<td>N: 6,476, %: 69.7%</td>
<td>N: 108,426, %: 48.0%</td>
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<td></td>
</tr>
<tr>
<td>Hypertension complicated</td>
<td>N: 7,300, %: 11.2%</td>
<td>N: 1,026, %: 11.0%</td>
<td>N: 12,416, %: 5.5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Paralysis</td>
<td>N: 712, %: 1.1%</td>
<td>N: 86, %: 0.9%</td>
<td>N: 2,316, %: 1.0%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other neurological</td>
<td>N: 5,282, %: 8.1%</td>
<td>N: 666, %: 7.2%</td>
<td>N: 16,383, %: 7.2%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic pulmonary</td>
<td>N: 19,996, %: 30.6%</td>
<td>N: 2,599, %: 28.0%</td>
<td>N: 50,536, %: 22.4%</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes (Uncomplicated)</td>
<td>N: 21,848, %: 33.4%</td>
<td>N: 3,625, %: 39.0%</td>
<td>N: 41,544, %: 18.4%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes (Complicated)</td>
<td>N: 7,598, %: 11.6%</td>
<td>N: 1,230, %: 13.2%</td>
<td>N: 10,859, %: 4.8%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>N: 9,122, %: 13.9%</td>
<td>N: 1,378, %: 14.8%</td>
<td>N: 31,244, %: 13.8%</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Renal failure</td>
<td>N: 8,605, %: 13.1%</td>
<td>N: 1,433, %: 15.4%</td>
<td>N: 14,718, %: 6.5%</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Liver disease</td>
<td>N: 5,558, %: 8.5%</td>
<td>N: 943, %: 10.2%</td>
<td>N: 25,865, %: 11.4%</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Peptic ulcer disease</td>
<td>N: 1,278, %: 2.0%</td>
<td>N: 184, %: 2.0%</td>
<td>N: 3,776, %: 1.7%</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>HIV/AIDS</td>
<td>N: 49, %: 0.1%</td>
<td>N: 25, %: 0.3%</td>
<td>N: 1,035, %: 0.5%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastatic Cancer</td>
<td>N: 9,925, %: 15.2%</td>
<td>N: 1,362, %: 14.7%</td>
<td>N: 40,686, %: 18.0%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition</td>
<td>Value</td>
<td>Rate</td>
<td>Value</td>
<td>Rate</td>
<td>Value</td>
<td>Rate</td>
</tr>
<tr>
<td>---------------------------------</td>
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<td>-------</td>
</tr>
<tr>
<td>Rheumatoid Arthritis</td>
<td>3,231</td>
<td>4.9%</td>
<td>497</td>
<td>5.4%</td>
<td>11,365</td>
<td>5.0%</td>
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<tr>
<td>Coagulopathy</td>
<td>3,775</td>
<td>5.8%</td>
<td>527</td>
<td>5.7%</td>
<td>13,017</td>
<td>5.8%</td>
</tr>
<tr>
<td>Obesity</td>
<td>3,486</td>
<td>5.3%</td>
<td>676</td>
<td>7.3%</td>
<td>16,740</td>
<td>7.4%</td>
</tr>
<tr>
<td>Weight Loss</td>
<td>4,981</td>
<td>7.6%</td>
<td>656</td>
<td>7.1%</td>
<td>16,717</td>
<td>7.4%</td>
</tr>
<tr>
<td>Fluids and Electrolytes</td>
<td>9,179</td>
<td>14.0%</td>
<td>1,228</td>
<td>13.2%</td>
<td>30,267</td>
<td>13.4%</td>
</tr>
<tr>
<td>Blood Loss</td>
<td>4,005</td>
<td>6.1%</td>
<td>493</td>
<td>5.3%</td>
<td>10,277</td>
<td>4.5%</td>
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<tr>
<td>Deficiency Anemia</td>
<td>5,272</td>
<td>8.1%</td>
<td>767</td>
<td>8.3%</td>
<td>15,505</td>
<td>6.9%</td>
</tr>
<tr>
<td>Alcohol Abuse</td>
<td>522</td>
<td>0.8%</td>
<td>71</td>
<td>0.8%</td>
<td>3,212</td>
<td>1.4%</td>
</tr>
<tr>
<td>Drug Abuse</td>
<td>349</td>
<td>0.5%</td>
<td>51</td>
<td>0.5%</td>
<td>2,211</td>
<td>1.0%</td>
</tr>
<tr>
<td>Psychoses</td>
<td>1,252</td>
<td>1.9%</td>
<td>163</td>
<td>1.8%</td>
<td>3,705</td>
<td>1.6%</td>
</tr>
<tr>
<td>Depression</td>
<td>5,120</td>
<td>7.8%</td>
<td>751</td>
<td>8.1%</td>
<td>26,685</td>
<td>11.8%</td>
</tr>
<tr>
<td>CHD</td>
<td>24,187</td>
<td>37.0%</td>
<td>3,252</td>
<td>35.0%</td>
<td>26,015</td>
<td>11.5%</td>
</tr>
<tr>
<td>DVT</td>
<td>3,204</td>
<td>4.9%</td>
<td>442</td>
<td>4.8%</td>
<td>10,856</td>
<td>4.8%</td>
</tr>
<tr>
<td>PE</td>
<td>1,346</td>
<td>2.1%</td>
<td>208</td>
<td>2.2%</td>
<td>4,481</td>
<td>2.0%</td>
</tr>
<tr>
<td>AT</td>
<td>655</td>
<td>1.0%</td>
<td>92</td>
<td>1.0%</td>
<td>1,297</td>
<td>0.6%</td>
</tr>
<tr>
<td>MI</td>
<td>4,696</td>
<td>7.2%</td>
<td>569</td>
<td>6.1%</td>
<td>5,335</td>
<td>2.4%</td>
</tr>
<tr>
<td>Stomach CA</td>
<td>1,861</td>
<td>2.8%</td>
<td>245</td>
<td>2.6%</td>
<td>6,024</td>
<td>2.7%</td>
</tr>
<tr>
<td>Pancreas CA</td>
<td>3,011</td>
<td>4.6%</td>
<td>466</td>
<td>5.0%</td>
<td>9,770</td>
<td>4.3%</td>
</tr>
<tr>
<td>Brain CA</td>
<td>1,720</td>
<td>2.6%</td>
<td>272</td>
<td>2.9%</td>
<td>11,212</td>
<td>5.0%</td>
</tr>
<tr>
<td>Lung CA</td>
<td>15,812</td>
<td>24.2%</td>
<td>2,047</td>
<td>22.0%</td>
<td>36,891</td>
<td>16.3%</td>
</tr>
<tr>
<td>Kidney CA</td>
<td>6,308</td>
<td>9.6%</td>
<td>981</td>
<td>10.6%</td>
<td>19,799</td>
<td>8.8%</td>
</tr>
<tr>
<td>Lymphomas</td>
<td>9,926</td>
<td>15.2%</td>
<td>1,507</td>
<td>16.2%</td>
<td>40,114</td>
<td>17.7%</td>
</tr>
<tr>
<td>Leukemia</td>
<td>6,058</td>
<td>9.3%</td>
<td>948</td>
<td>10.2%</td>
<td>20,518</td>
<td>9.1%</td>
</tr>
<tr>
<td>Myeloma</td>
<td>3,031</td>
<td>4.6%</td>
<td>476</td>
<td>5.1%</td>
<td>10,367</td>
<td>4.6%</td>
</tr>
<tr>
<td>Colorectal CA</td>
<td>13,126</td>
<td>20.1%</td>
<td>1,817</td>
<td>19.6%</td>
<td>45,390</td>
<td>20.1%</td>
</tr>
<tr>
<td>Gynecologic CA</td>
<td>5,755</td>
<td>8.8%</td>
<td>752</td>
<td>8.1%</td>
<td>34,106</td>
<td>15.1%</td>
</tr>
</tbody>
</table>

CCI Charlson Comorbidity Index; CHF Congestive Heart Failure; CHD Coronary Heart Disease; DVT Deep vein Thrombosis; CA Cancer
Figure 2.1: CONSORT flow diagram. Flow diagram of the inclusion of patients from the national healthcare claims dataset.
The number of matched pairs post-propensity score matching were 39,989, 101,401 and 27,319 for the statins- vs. non-statins-users comparison, statins vs. no treatment comparison and the non-statins vs. no treatment comparison, respectively. While baseline differences existed among treatment groups, matching provided samples that had minimal differences as all standardized scores were below 0.1.

Figure 2.2 displays the HR and associated 95% CI for the effect of treatment group on survival within the propensity-matched sample. Overall, there were no differences in survival between statin-users and non-statin-users. Among all cancers, statin-use prior to diagnosis improved overall survival compared to no treatment (HR 0.85, 95% CI 0.80-0.91). When stratified by cancer type, this observation held true for lung cancer (HR 0.88, 95% CI 0.78-0.98), renal cancer (HR 0.63, 95% CI 0.44-0.90) and leukemia (HR 0.73, 0.58-0.92). Non-statin-use provided a similar reduction in overall survival compared to no treatment (HR 0.73, 95% CI 0.62-0.85); but when stratified, this held true only for pancreatic cancer (HR 0.53, 95% CI 0.29-0.98) and leukemia (HR 0.53, 0.30-0.94).

Dose intensity analysis determined that the effect observed is not dosage-dependent (Figure 2.3). By removing low-dosage statins and comparing to non-statins and non-users, overall survival was of the same magnitude and direction observed in the overall analysis. The sensitivity analysis assessing age (Table 2.2) also showed no difference in effect within age groups when comparing statins to non-statins or non-statins to non-users. However, patients under the age of 65 in the statins vs. non-users group had improved survival while those over the age of 65 did not.
Figure 2.2: One-year survival analysis. Hazard ratios (HR) and associated 95% confidence intervals (CI) for the effect of treatment group on survival within the propensity-matched sample. Matching was conducted for each treatment group analysis. (*) signifies statistically significant result.
Figure 2.3: The effect of dose intensity on one-year survival. Statin exposure groups limited to medium and high dosages. The distribution of low, moderate and high dosage statins prior to matching is 7,918, 46,152 and 11,307, respectively. Hazard ratios (HR) and associated 95% confidence intervals (CI) for the effect of treatment group are presented. (*) signifies statistically significant result.

<table>
<thead>
<tr>
<th>Group</th>
<th>HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Selected Cancers</td>
<td>1.09</td>
<td>0.93</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.17</td>
<td>0.68</td>
</tr>
<tr>
<td>Pancreas</td>
<td>1.49</td>
<td>0.74</td>
</tr>
<tr>
<td>Brain</td>
<td>0.88</td>
<td>0.25</td>
</tr>
<tr>
<td>Lung</td>
<td>0.90</td>
<td>0.70</td>
</tr>
<tr>
<td>Renal</td>
<td>0.56</td>
<td>0.28</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>1.07</td>
<td>0.62</td>
</tr>
<tr>
<td>Leukemias</td>
<td>1.32</td>
<td>0.73</td>
</tr>
<tr>
<td>Myeloma</td>
<td>1.02</td>
<td>0.48</td>
</tr>
<tr>
<td>Colorectal</td>
<td>1.76</td>
<td>1.06</td>
</tr>
<tr>
<td>Gynecological</td>
<td>1.40</td>
<td>0.49</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group</th>
<th>HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Selected Cancers</td>
<td>0.86</td>
<td>0.80</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.92</td>
<td>0.71</td>
</tr>
<tr>
<td>Pancreas</td>
<td>0.93</td>
<td>0.72</td>
</tr>
<tr>
<td>Brain</td>
<td>0.84</td>
<td>0.53</td>
</tr>
<tr>
<td>Lung</td>
<td>0.88</td>
<td>0.78</td>
</tr>
<tr>
<td>Renal</td>
<td>0.63</td>
<td>0.42</td>
</tr>
<tr>
<td>Lymphoma</td>
<td>0.85</td>
<td>0.68</td>
</tr>
<tr>
<td>Leukemias</td>
<td>0.70</td>
<td>0.54</td>
</tr>
<tr>
<td>Myeloma</td>
<td>0.92</td>
<td>0.64</td>
</tr>
<tr>
<td>Colorectal</td>
<td>0.92</td>
<td>0.76</td>
</tr>
<tr>
<td>Gynecological</td>
<td>0.78</td>
<td>0.55</td>
</tr>
</tbody>
</table>
Table 2.2: Age sensitivity analysis for all exposure group analyses. Hazard ratios (HR) and associated 95% confidence intervals (CI) for the effect of treatment group are presented.

<table>
<thead>
<tr>
<th>Age</th>
<th>Statins Vs. Non-Statins</th>
<th>Statins Vs. Non-Users</th>
<th>Non-Statins Vs. Non-Users</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR</td>
<td>95% CI</td>
<td>HR</td>
</tr>
<tr>
<td>&lt; 65 Yrs</td>
<td>0.67</td>
<td>0.40</td>
<td>1.10</td>
</tr>
<tr>
<td>65-75 Yrs</td>
<td>1.17</td>
<td>0.92</td>
<td>1.48</td>
</tr>
<tr>
<td>&gt;75 Yrs</td>
<td>1.22</td>
<td>0.96</td>
<td>1.56</td>
</tr>
</tbody>
</table>

DOI:10.1200/CCI.17.99919
To examine differences among individual statins (Figure 2.4), we stratified molecules by natural and synthetic origin. Compared to simvastatin as reference, the more hydrophilic rosvustatin (HR 1.21, 95% CI 1.03-1.43) and fluvastatin (HR 2.18, 95% CI 1.36-3.50) molecules were associated with a higher rate of death with no differences between other products, i.e. simvastatin is associated with a protective effect. When grouped by type, natural statins had a marginally protective effect on survival, but this was not statistically significant (HR=0.91, 95% CI 0.83-1.01)

Bisphosphonate users (n=4,528) were compared to non-users and stratified by statin use. Among all cancers, bisphosphonates had a non-significant reduction in death when compared to non-users (HR 0.82, 95% CI 0.65-1.03) (Figure 2.5). Stratification by cancer type could not be completed due to limited population size. A treatment group consisting of patients using both statins and bisphosphonates (n=4,090) exhibited a much larger improvement in survival compared to a subset of non-users that did not receive either medication (HR 0.60, 95% CI 0.45-0.81).

The majority of subjects remained on statin therapy with an average of 78.3% of days in the post-index period covered by statin therapy and only 19 out of 65,440 patients stopped statin therapy after cancer diagnosis. More than half of this treatment group received statins for the entire follow-up period. Generally, we observed that the statin cohort continued on statin therapy for the majority of their post-diagnosis follow-up time.
Figure 2.4: The effect of statin stratification by type and class on one-year survival. In comparison by type, simvastatin was used as reference. In comparison by class, synthetic statins were used as reference. The list of natural statins included lovastatin, pravastatin and simvastatin. Synthetic statins included rosvastatin, atorvastatin, fluvastatin and pitavastatin. Hazard ratios (HR) and associated 95% confidence intervals (CI) for the effect of treatment group are presented.

<table>
<thead>
<tr>
<th>Statin</th>
<th>N</th>
<th>HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simvastatin</td>
<td>28,714</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Lovastatin</td>
<td>3,935</td>
<td>0.97</td>
<td>0.77</td>
</tr>
<tr>
<td>Pravastatin</td>
<td>7,307</td>
<td>1.01</td>
<td>0.85</td>
</tr>
<tr>
<td>Rosuvastatin</td>
<td>6,492</td>
<td>1.21</td>
<td>1.03</td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>18,443</td>
<td>1.04</td>
<td>0.92</td>
</tr>
<tr>
<td>Fluvastatin</td>
<td>372</td>
<td>2.18</td>
<td>1.36</td>
</tr>
<tr>
<td>Pitavastatin</td>
<td>112</td>
<td>0.41</td>
<td>0.06</td>
</tr>
<tr>
<td>Synthetics</td>
<td>25,419</td>
<td>Ref</td>
<td>Ref</td>
</tr>
<tr>
<td>Natural</td>
<td>39,956</td>
<td>0.91</td>
<td>0.83</td>
</tr>
</tbody>
</table>
Figure 2.5: The effect of synergistic statin combinations on one-year survival.

Bisphosphonates assessed in the analysis were alendronate sodium, etidronate disodium, ibandronate sodium, pamidronate disodium, risedronate sodium and zoledronic acid. Bisphosphonates and dipyridamole users were required to have at least a 90 cumulative days supplied in the 6 months prior to cancer diagnosis. Hazard ratios (HR) and associated 95% confidence intervals (CI) for the effect of treatment group are presented. (*) signifies statistically significant result.
D. DISCUSSION

This observational cohort study used epidemiological data and identified a significant effect on the overall survival of cancer patients who receive statins at the time of diagnosis. This advantage was specific to cancers of the lung, kidney, and leukemias. In addition, the concurrent use of a bisphosphonate with a statin was associated with an improvement in overall survival, but stratification by cancer type was not possible due to the small sample size. This observation is consistent with the mechanisms of action of the two agents suggesting the theoretical potential for synergy in a prospective study. The effect of dipyridamole in combination with statin was not significant in the small cohort of patients receiving this combination (n=651). The effect of statins was comparable to other cholesterol-lowering medications, which is consistent with a recent cohort study in postmenopausal women showing that regular use of statins or other-lipid-lowering medications was associated with decreased cancer death (111). Multiple systems or pathways may explain the mechanism by which statins induce their effect on cancer survival including inhibition of Ras, improvement of immune surveillance, and the reduction of venous thromboembolisms (VTE).

Statins impact normal cell survival mechanisms including cell proliferation, pro-apoptotic effects, induction of autophagy, and anti-invasive and anti-migration effects that have been systematically studied in in vitro and in vivo models systems. The overarching hypothesis is the potential effect of statins on the Ras signaling pathway (163-169). Lung cancer, pancreatic cancer and hematopoietic/lymphoid cancers are associated with high rates of K-Ras mutation (38%, 63% and 21%, respectively) (170). High frequency of mutation may explain why patients with these cancers benefited in our analysis.
However, our results did not show an effect in colorectal cancer, which also has a high frequency in K-Ras mutation (170). Stain-use following diagnosis was previously shown to reduce colorectal cancer–specific mortality (119) and the prospective use of simvastatin with cetuximab/irinotecan in K-Ras mutant patients had favorable disease control rate (129). Considering in vitro evidence, statin-mediated modulation of protein prenylation in cancer cells requires suprapharmacologic concentrations (e.g., 1-25µM) for prolonged periods (171-174). Previous studies from our group demonstrated high (7.5mg/kg b.i.d.) simvastatin doses, ~25-fold of the typical daily dose (i.e., 40mg), achieved maximum plasma concentrations that were in the range of 0.08-2.2µM (175). Consistent with this, our results show that the dose-intensity of typical dosages cholesterol lowering treatment does not impact overall survival, suggesting other mechanisms may be involved.

Prior studies have suggested the immuno-stimulatory effects of statins as the mechanism for anti-cancer activity. By inhibiting the mevalonate pathway, statins can induce innate lymphocyte activation and increase immune surveillance. Depletion of prenyl pyrophosphates in human dendritic cells generates danger signals that can translate into caspase-1 activation. Caspase-1 cleaves interleukin-1-beta (IL-1β) and IL-18 into their activated forms allowing the release of cytokines that include interferon-γ and IL-2 (176). Statin-induced activation of IL-2 primed γδ T cells and natural killer cells exhibit potent antitumor cytotoxicity and ectopic GGPP reintroduced into the cell culture abolishes this effect (177, 178). Immunomodulation may be an important contributor as immune cells are more likely to be exposed to higher statin concentrations than cancer cells.
Finally, given the lack of difference between statin and non-statin drugs, the survival effect may in part be due to reductions in thrombotic events. Observational studies show that statins lower the risk of VTE in the cancer population, thereby increasing OS (179). In a prospective observational cohort of 1,434 cancer patients, VTEs occurred in 2.94% of patients at 12 months and 3.54% at 24 months for statin users. In comparison, those who were not treated with statins had elevated rates of VTE of 7.13% at 12 months and 8.13% at 24 months ($P=0.04$). Among newly-diagnosed cancer patients who were prospectively followed for a two-year period, statins users had a lower risk of VTE than non-users (HR 0.43, CI 0.19 to 0.98). In contrast, a meta-analysis of 27 trials assessing the effect of statin use and the lowering of LDL cholesterol on cancer incidence and mortality found a lack of effect after a median of five years of therapy, but a small effect within the first year after diagnosis (180). In the ACALM study, hyperlipidemia was associated with a significantly reduced mortality rate in lung, breast, prostate and bowel cancers (181). With respect to age, our analyses shows that those over 65 had no significant benefit from statin treatment, which may be the result of an overall increased VTE risk in that population. More work is needed to understand how cholesterol lowering medications impact the risk of thrombotic events in cancer and whether this effect explains part of the overall protective effect observed in this study.

This study is subject to the limitations of all claims-based studies (182, 183). Claims data lack detailed information on laboratory values or tumor staging, which may have influenced the outcomes of this study. This study was limited to a one-year follow-up due to the availability of data and the heterogeneity and time-varying confounded with longer follow-up. Lastly, while propensity score matching is known to reduce selection bias in non-randomized studies, it is possible that residual bias is present or that unmeasured
confounders may have impacted these findings (158). This study is strengthened by a large sample size, inclusion of minimum medication exposure criteria (e.g. 90 days supplied), and by inclusion of an active control group, which are often lacking in similar studies.

E. CONCLUSION

Epidemiological health outcomes data can be used to test hypotheses based on the effect of drugs on specific biological pathways and processes. Our work shows that the use of statins alone and in combination with bisphosphonates could provide a survival benefit in certain cancers. We have shown the applicability of health outcomes research in the assessment of response dependent on differing exposure groups.
CHAPTER 3

A. OVERVIEW

Immune checkpoint inhibitors (ICI) are designed to restore a patient’s antitumor immune response, which has been attenuated during the process of tumor development. Antigen presenting cells (APC) normally express programmed death ligand 1 (PD-L1) that, when bound by PD-1 on T cells, signal an exhausted active immune response. In cancers that express PD-L1 on tumor cells, PD-1 receptor expressed on T cells, B cells, and NK cells of an activated immune system, and the interaction of PD-1 and PD-L1 characterize an adaptive and immune-evasive response by the tumor. This immune-evasive interaction can be reversed by addition of ICIs that inhibit either molecule (53). Recently, agents that modulate the tumor immune response have provided durable clinical benefit to patients with late-stage or recurrent disease. Nivolumab (Opdivo®) has received FDA approval for the treatment of squamous and non-squamous metastatic non–small cell lung cancer (NSCLC) and metastatic renal cell carcinoma with progression on or after chemotherapy (184). Nivolumab is a human IgG4 antibody that blocks programmed death 1 (PD-1) receptor and potentiates activation of T cells (185). Nivolumab therapy demonstrated improved tumor-related outcomes in multiple types of cancer (186). Pembrolizumab (Keytruda®), another PD-1 inhibitor, received FDA approval to treat advanced (metastatic) NSCLC whose disease has progressed after other treatments and with tumors that have 50% programmed death ligand 1 (PD-L1) expression (187). PD-1 inhibition facilitates activation of potentially autoreactive T cells, leading to inflammatory adverse events across a range of tissues. Patients with a history of autoimmune diseases were excluded from clinical trials of PD-1 inhibitors (188). Exclusions included multiple sclerosis, autoimmune neuropathy, Guillain-Barre
syndrome, myasthenia gravis, systemic lupus erythematosus, connective tissue diseases, scleroderma, inflammatory bowel disease, Crohn’s disease, ulcerative colitis, and hepatitis. Patients with rheumatoid arthritis, Sjogren’s syndrome, and psoriasis were included if disease was well-controlled. Although these therapies hold great promise, ICIs can trigger a variety of immune-related adverse events (irAEs). These include dermatologic, gastrointestinal, hepatic, endocrine, and other inflammatory conditions and they are believed to result from general immune response enhancement (189). Khan et al have shown a relatively high rate of autoimmune diseases, approximately 14%, among lung cancer patients, and these patients were more likely to be older females (190). The objective of this study was to confirm findings of Khan and colleagues in a more diverse cohort and identify whether cancer patients with autoimmune disease exhibit different baseline characteristics and comorbidities.

B. METHODS

We identified lung and renal cancer patients using Truven Health MarketScan Commercial Claims and Medicare Supplemental Database. The Marketscan database includes approximately 40 million individuals from over 160 large employers and health plans across the US and includes healthcare claims with diagnosis and procedure codes for medical encounters and all prescription medication fills. These data are de-identified in compliance with the Health Insurance Portability and Accountability Act regulations (HIPAA) and the University of Kentucky Institutional Review Board approved the use of the database for this study.
Adults 18 years and older diagnosed with cancer between January 1, 2010 and November 31, 2013 were identified. At least 2 inpatient or outpatient diagnoses separated by at least 14 days were required and the date of the first qualifying diagnosis of cancer was defined as the index date. We directed our analyses to only lung and renal cancer due to the initial approvals of the immune checkpoint inhibitor, nivolumab. Nivolumab received approval for the treatment of metastatic non-small cell lung cancer on March 4, 2015 and for metastatic renal cell carcinoma on November 23, 2015.

Patients were required to have at least 12 months of pre-index and a minimum of one-month post-index continuous enrollment in the database. We assessed patients for diagnosis of autoimmune diseases prior to or after diagnosis of cancer using ICD-9 codes for 41 autoimmune diseases. It is necessary to assess autoimmune disease before and after diagnosis because newly diagnosed autoimmune conditions would still have bearing on therapeutic decision-making practices. Prevalence was determined by the presence of 2 or more claims to autoimmune diseases separated by at least 30 days. Baseline characteristics and Elixhauser and Charlson comorbidity indexes of patients with and without autoimmune diseases were compared. These indexes include 17 and 31 categories of comorbid conditions, respectively, and have been widely used for risk adjustment with health outcomes data (154, 155). Two sample t-test and chi-square tests were conducted to assess significant differences between groups. Bonferroni correction was applied due to multiple comparisons.

C. RESULTS

We identified 53,783 lung cancer patients and 27,349 renal cancer patients of whom 13,156 (24.5%) and 8,217 (30.1%) also had an autoimmune disease, respectively.
Hypothyroidism (55.8%, 56.7%), rheumatoid arthritis (20.2%, 18.1%) and type 1 diabetes mellitus (11.5%, 14.5%) were the most common for both lung and renal cancer patients respectively (Table 3.1). Baseline characteristics and comorbidities are listed in Table 3.2. Cancer patients with autoimmune disease were more likely to be female, older and had higher prevalence of comorbidities than cancer patients without autoimmune disease (Table 3.2).
Table 3.1: Autoimmune (AI) disorders in lung and renal cancer patients between the years 2009-2013.

<table>
<thead>
<tr>
<th>Autoimmune Disorder</th>
<th>Among Lung CA Patients with AI Disease. N (%)</th>
<th>Among Renal CA Patients with AI Disease. N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rheumatoid Arthritis</td>
<td>2,653 (20.2)</td>
<td>1,490 (18.1)</td>
</tr>
<tr>
<td>Psoriasis</td>
<td>527 (4.0)</td>
<td>402 (4.9)</td>
</tr>
<tr>
<td>Systemic Lupus Erythematosus</td>
<td>225 (1.7)</td>
<td>120 (1.5)</td>
</tr>
<tr>
<td>Systemic Sclerosis</td>
<td>78 (0.6)</td>
<td>20 (0.2)</td>
</tr>
<tr>
<td>Sicca Syndrome</td>
<td>115 (0.9)</td>
<td>70 (0.9)</td>
</tr>
<tr>
<td>Autoimmune NOS</td>
<td>37 (0.3)</td>
<td>20 (0.2)</td>
</tr>
<tr>
<td>Autoimmune Hepatitis</td>
<td>59 (0.5)</td>
<td>72 (0.9)</td>
</tr>
<tr>
<td>Primary Biliary Cirrhosis</td>
<td>38 (0.3)</td>
<td>33 (0.4)</td>
</tr>
<tr>
<td>Celiac Disease</td>
<td>71 (0.5)</td>
<td>51 (0.6)</td>
</tr>
<tr>
<td>Ankylosing Spondylitis</td>
<td>506 (3.9)</td>
<td>402 (4.9)</td>
</tr>
<tr>
<td>Polymyalgia Rheumatica</td>
<td>227 (1.7)</td>
<td>141 (1.7)</td>
</tr>
<tr>
<td>Addison's Disease</td>
<td>357 (2.7)</td>
<td>196 (2.4)</td>
</tr>
<tr>
<td>Ulcerative Colitis</td>
<td>352 (2.7)</td>
<td>238 (2.9)</td>
</tr>
<tr>
<td>Crohn's Disease</td>
<td>258 (2.0)</td>
<td>208 (2.5)</td>
</tr>
<tr>
<td>Meniere's Disease</td>
<td>89 (0.7)</td>
<td>67 (0.8)</td>
</tr>
<tr>
<td>Hashimoto's Disease</td>
<td>89 (0.7)</td>
<td>67 (0.8)</td>
</tr>
<tr>
<td>Polyartheritis Nodosa</td>
<td>174 (1.3)</td>
<td>89 (1.1)</td>
</tr>
<tr>
<td>Giant Cell Arthritis</td>
<td>93 (0.7)</td>
<td>45 (0.6)</td>
</tr>
<tr>
<td>Pernicious Anemis</td>
<td>710 (5.4)</td>
<td>301 (3.7)</td>
</tr>
<tr>
<td>Autoimmune Hemolytic Anemia</td>
<td>39 (0.3)</td>
<td>22 (0.3)</td>
</tr>
<tr>
<td>Idiopathic Thrombocytopenic Purpura</td>
<td>152 (1.2)</td>
<td>82 (1.0)</td>
</tr>
<tr>
<td>Thyrotoxicosis</td>
<td>157 (1.2)</td>
<td>100 (1.2)</td>
</tr>
<tr>
<td>Multiple Sclerosis</td>
<td>200 (1.5)</td>
<td>103 (1.3)</td>
</tr>
<tr>
<td>Iridocyclitis</td>
<td>280 (2.1)</td>
<td>209 (2.5)</td>
</tr>
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<td>Pemphigus</td>
<td>32 (0.2)</td>
<td>33 (0.4)</td>
</tr>
<tr>
<td>Eczema</td>
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<td>288 (3.5)</td>
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<tr>
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<td>26 (0.3)</td>
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<tr>
<td>Vitiligo</td>
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<td>Wegener's Granulomatosis</td>
<td>28 (0.2)</td>
<td>21 (0.3)</td>
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<tr>
<td>Dermatopolymyositis</td>
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<td>10 (0.1)</td>
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<tr>
<td>Myasthenia Gravis</td>
<td>89 (0.7)</td>
<td>58 (0.7)</td>
</tr>
<tr>
<td>Scleroderma</td>
<td>62 (0.5)</td>
<td>47 (0.6)</td>
</tr>
<tr>
<td>Antiphospholipid</td>
<td>3 (0.0)</td>
<td>5 (0.1)</td>
</tr>
<tr>
<td>Guillian-Barre Syndrome</td>
<td>28 (0.2)</td>
<td>23 (0.3)</td>
</tr>
<tr>
<td>Type 1 Diabetes Mellitus</td>
<td>1,507 (11.5)</td>
<td>1,189 (14.5)</td>
</tr>
<tr>
<td>Condition</td>
<td>First Dataset Value</td>
<td>Second Dataset Value</td>
</tr>
<tr>
<td>----------------------------</td>
<td>---------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>Hypothyroidism</td>
<td>7,334 (55.8)</td>
<td>4,661 (56.7)</td>
</tr>
<tr>
<td>Hyperthyroidism</td>
<td>157 (1.2)</td>
<td>100 (1.2)</td>
</tr>
<tr>
<td>Sweet's Syndrome</td>
<td>215 (1.6)</td>
<td>183 (2.2)</td>
</tr>
<tr>
<td>Sjogren's Syndrome</td>
<td>115 (0.9)</td>
<td>70 (0.9)</td>
</tr>
<tr>
<td>Pyoderma Gangrenosum</td>
<td>7 (0.1)</td>
<td>1 (0.0)</td>
</tr>
<tr>
<td>Sarcoidosis</td>
<td>249 (1.9)</td>
<td>129 (1.6)</td>
</tr>
</tbody>
</table>
Table 3.2: Baseline characteristics and comorbidities between lung and renal cancer patients with or without autoimmune disease.

<table>
<thead>
<tr>
<th></th>
<th>Cancer W/ Autoimmune</th>
<th>Cancer W/O Autoimmune</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age Categories</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 65 Yrs</td>
<td>8,393 39.3</td>
<td>25,672 43.0</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>65 to 74 Yrs</td>
<td>6,439 30.1</td>
<td>16,762 28.1</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>75 to 80 Yrs</td>
<td>3,545 16.6</td>
<td>9,276 15.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>&gt;80 Yrs</td>
<td>2,996 14.0</td>
<td>8,049 13.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Gender (F)</strong></td>
<td>12,133 56.8</td>
<td>25,140 42.1</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>CHF</strong></td>
<td>2,938 13.8</td>
<td>6,860 11.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Arrhythmias</strong></td>
<td>5,530 25.4</td>
<td>13,659 22.9</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Valvular disease</strong></td>
<td>2,986 14.0</td>
<td>6,920 11.6</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Pulmonary Circulation</strong></td>
<td>1,150 5.4</td>
<td>2,953 4.9</td>
<td>0.0119</td>
</tr>
<tr>
<td><strong>Peripheral Vascular</strong></td>
<td>5,812 27.2</td>
<td>14,187 23.7</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Hypertension uncomplicated</strong></td>
<td>13,814 64.6</td>
<td>35,614 59.6</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Hypertension complicated</strong></td>
<td>2,375 11.1</td>
<td>5,067 8.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Paralysis</strong></td>
<td>211 1.0</td>
<td>584 1.0</td>
<td>0.899</td>
</tr>
<tr>
<td><strong>Other neurological</strong></td>
<td>1,919 9.0</td>
<td>4,231 7.1</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Chronic pulmonary</strong></td>
<td>9,063 42.4</td>
<td>25,053 41.9</td>
<td>0.2219</td>
</tr>
<tr>
<td><strong>Renal failure</strong></td>
<td>3,085 14.4</td>
<td>6,130 10.3</td>
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<tr>
<td><strong>Liver disease</strong></td>
<td>2,528 11.8</td>
<td>6,767 11.3</td>
<td>0.047</td>
</tr>
<tr>
<td><strong>Peptic ulcer disease</strong></td>
<td>308 1.4</td>
<td>772 1.3</td>
<td>0.1032</td>
</tr>
<tr>
<td><strong>HIV/AIDS</strong></td>
<td>33 0.2</td>
<td>116 0.2</td>
<td>0.2445</td>
</tr>
<tr>
<td><strong>Metastatic Cancer</strong></td>
<td>4,340 20.3</td>
<td>15,952 26.7</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Coagulopathy</strong></td>
<td>1,145 5.4</td>
<td>2,332 3.9</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Obesity</strong></td>
<td>1,598 7.5</td>
<td>3,402 5.7</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Weight Loss</strong></td>
<td>1,599 7.5</td>
<td>4,778 8.0</td>
<td>0.0165</td>
</tr>
<tr>
<td><strong>Fluids and Electrolytes</strong></td>
<td>3,549 16.6</td>
<td>8,679 14.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Blood Loss Anemia</strong></td>
<td>990 4.6</td>
<td>2,075 3.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Deficiency Anemia</strong></td>
<td>1,358 6.4</td>
<td>2,835 4.7</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Alcohol Abuse</strong></td>
<td>278 1.3</td>
<td>1,087 1.8</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>Drug Abuse</strong></td>
<td>246 1.2</td>
<td>624 1.0</td>
<td>0.1933</td>
</tr>
<tr>
<td><strong>Psychoses</strong></td>
<td>397 1.9</td>
<td>1,148 1.9</td>
<td>0.5595</td>
</tr>
<tr>
<td><strong>Depression</strong></td>
<td>2,713 12.7</td>
<td>5,888 9.9</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td><strong>CHD</strong></td>
<td>5,791 27.1</td>
<td>14,702 24.6</td>
<td>&lt;.0001</td>
</tr>
</tbody>
</table>
More than a quarter of patients diagnosed with lung and renal cancer were found to have a comorbid autoimmune condition. When considering that immune checkpoint inhibition is only approved in late stages of cancer, it is not clear whether the benefits of pursuing treatment in patients with autoimmune disease outweigh the risk of inducing worse irAEs. Several case reports have been published showing that while discontinuation of the immune checkpoint inhibitor results in resolution of the irAE, long courses of medications specific to the autoimmune reaction may be needed to mitigate the effects of ICI therapy (191-193). In a large systematic review of 251 cases involving anti-CTLA-4 and anti-PD-1 agents, approximately 52% of treated patients discontinued ICI therapy due to the irAEs. (191) Less than 10% required no treatment for the irAE, while the remainder was treated with corticosteroids, infliximab (an anti-tumor necrosis factor (TNF) agent), or disease-modifying anti-rheumatic drugs (DMARDs). Death due to the irAEs occurred in 4.7% of patients. Cutaneous autoimmune reactions are commonly associated with ICI therapy, but a case report on two patients with metastatic melanoma illustrated that irAEs may not appear until long after initiation of therapy. (193) An autopsy study presented an elderly melanoma patient exhibiting a systemic inflammatory response that affected multiple organ sites ultimately resulting in the death of the patient (194).

This study is subject to the limitations of all claims-based studies (182, 183). Notably, claims data lack detailed information on laboratory values or information on tumor staging, which may have influenced the outcomes of this study. This study was limited to a one-year follow-up due to the availability of data and the heterogeneity and time-varying confounded with longer follow-up. This study is strengthened by a large sample size and the inclusion of both commercial and medicare claims.
D. CONCLUSION

The exclusion of patients with autoimmune conditions from the approval studies of nivolumab and pembrolizumab resulted in a lack of clinical guidance for a large population of patients that oncologists must decide whether to treat or not. In late stage treatment of these cancers, the potential durable response associated with immune checkpoint inhibitors will need to be weighed against the worsening of the patient’s autoimmune condition, a decision for which clinical trials have not provided a concrete answer. Future evaluation of real-world treatment patterns will be needed to assess ICI usage and response in patients with autoimmune conditions.
CHAPTER 4

A. OVERVIEW

Immune checkpoint inhibitors (ICI) are designed to restore a patient’s antitumor immune response, which has been attenuated during the process of tumor development. Antigen presenting cells (APC) normally express programmed death ligand 1 (PD-L1) that, when bound by programmed death 1 receptor (PD-1) on T cells, signal an exhausted active immune response. In cancers that express PD-L1 on tumor cells, PD-1 receptor expressed on T cells, B cells, and NK cells of an activated immune system, and the interaction of PD-1 and PD-L1 characterize an adaptive and immune-evasive response by the tumor. Cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) is a homolog to CD28 and competitively binds to B7 on antigen-presenting cells providing a co-inhibitory signal, preventing the priming and activation of CD8+ T cells (195). These immune-evasive interactions can be reversed by addition of ICIs (53). These agents that modulate the tumor immune response have recently provided durable clinical benefit to patients with late-stage or recurrent disease.

To date, nivolumab (Opdivo®) has received FDA approvals for the treatment of squamous and non-squamous metastatic non–small cell lung cancer (NSCLC), metastatic renal cell carcinoma, advanced melanoma, Hodgkin lymphoma, previously treated locally advanced or metastatic urothelial carcinoma, hepatocellular carcinoma patients previously treated with sorafenib and microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) metastatic colorectal cancer (mCRC) that has progressed following treatment with a fluoropyrimidine, oxaliplatin and irinotecan (184, 186). Nivolumab is a human IgG4 antibody that blocks PD-1 and potentiates activation
of T cells (185). Pembrolizumab (Keytruda®), also a PD-1 inhibitor, received FDA approval for the treatment of advanced (metastatic) NSCLC that has progressed after other treatments and for tumors with at least 50% expression of programmed death ligand 1 (PD-L1) by tumor cells. Specifically, advanced melanoma, metastatic head and neck squamous cell carcinoma, classical Hodgkin lymphoma, advance or metastatic urothelial carcinoma, gastroesophageal junction cancer whose tumors express PD-L1, and metastatic solid tumors that are MSI-H or dMMR are eligible for this therapy (187).

The only approved biomarker of response for any ICI is measurement of PD-L1 expression from the tumor. The Keynote-001 trial sought to correlate response to MK-3475, a PD-1 inhibitor, with expression of PD-L1. In the melanoma arm, PD-L1 expression on tumor cells increased the response rate in 49% compared with 13% of PD-L1 negative patients. The NSCLC arm of this study showed that when greater than 50% of tumor cells expressed PD-L1, response to MK-3475 was predicted and this was observed in nearly 25% patients. For all patients, response rate to MK-3475 was about 19%, irrespective of PD-L1 expression in the tumor. Median progression free survival (PFS) was 14.1 months in PD-L1 strongly positive patients compared with 9.3 months in the weakly positive population (56). These findings preclude PD-L1 expression from being an exclusionary predictive biomarker. The limitations of this assay include timing of tissue acquisition, tumor heterogeneity, and timing of therapy relative to the expression of PD-L1 suggesting that finding a more robust biomarker of response is needed.

Applying precision medicine principles to immuno-oncology requires the discovery of biomarkers that can identify the patients most likely to benefit from this class of therapies.
treatment. Robust biomarkers need to encompass an efficient, repeatable and easily accessible source of biological data from the patient. Determination of PD-L1 expression requires direct sampling of tumor tissue from a patient which necessitates an invasive procedure. If samples banked early during treatment and then used later to determine expression levels, the assayed expression levels may not represent the tumor or metastases in the current state. Predictive biomarkers that can be measured from peripheral blood sampling allow the inclusion of this tool into clinical practice with minimal workup, allow for assay immediately prior to initiation of ICI treatment, and can be repeated throughout therapy for monitoring purposes. I sought to determine whether peripheral factors associated with the patient’s immune response or malignancy that have the precision and robustness to be more predictive than PD-L1 expression for anticipating response to PD-1 inhibitor therapy.

Several comorbid conditions have been associated with the development and progression of cancer but the long-term effect on the immune system may also impact response to ICI treatment (196). It is estimated that potentially 25% of all malignancies develop after exposure to chronic inflammation and to viral and bacterial infections that initiate an immune response (197). Chronic inflammation encompasses multiple conditions. Metabolic syndrome, one family of chronic inflammatory diseases, has consistently been correlated with the development of several tumor types. Worldwide there are over 312 million people with a BMI>30 kg/m² and within the last four decades, the prevalence of obesity has amassed to approximately 50% of adults within the US (198). Obese patients develop more localized tumors, have earlier relapse, and a diminished overall survival (199). Hyperlipidemia includes low high-density-lipoprotein (HDL) cholesterol and elevated low-density lipoprotein (LDL) cholesterol. Low HDL and
high serum levels of total cholesterol have been associated with higher incidence of lung, prostate and post-menopausal breast cancer (200, 201). This study followed over 1 million patients for 16 years who had no reported history of cancer and identified that, independent of high body mass, type 2 diabetes mellitus (T2DM) is a predictor of mortality in pancreatic, breast, liver and bladder cancer (202). The potential causal link between metabolic syndrome, inflammation and cancer is adipose tissue hypoxemia (203). This phenotype is characterized by inflammatory cytokines in plasma and the adipose tissue itself as well as macrophage infiltration and activation.

Immune cells secrete specific cytokines and chemokines that act as survival and proliferation factors for the promotion of malignant tumor cells. The presence of inflammation and the secretion of inflammatory mediators can lead to the induction of transcription factors such as NF-κβ. In the initial phase of tumor development, inflammatory mediators such as cytokines, reactive oxygen species (ROS), and reactive nitrogen species (RNS) derived from tumor-infiltrating immune cells induce epigenetic alterations in pre-malignant lesions and silence tumor suppressor genes (204).

Accumulation of microbial pathogens and tissue necrosis activate transcription factors that are necessary for the expression of pro-angiogenic factors (IL-8, VEGF), growth factors (IL-6, GM-CSF), anti-apoptotic factors (Bcl-XL, c-FLIP), invasion-promoting factors (MMP-2, MMP-7, MMP-9, uPA), inflammatory enzymes (PGHS-2, LOX), prostaglandins, iNOS, chemokines (CCL2, CCL20, IL-8), and pro-inflammatory cytokines (IL-1, IL-6, IL-23, TNF, TGF-β, EGF) that support the malignant phenotype (205). Of the cytokines released, transforming growth factor beta (TGF-β) and tumor necrosis factor alpha (TNF-α) help activated NF-κβ in a process called epithelial-mesenchymal
transition (EMT). EMT is a process necessary for tumor invasiveness and metastasis to other sites (206).

While we understand that a pro-inflammatory cytokine-rich environment promotes the formation of some tumor types, we are interested in understanding whether these cytokines induce adaptive immune resistance in lung cancers. If true, patients with an inflammatory cytokine-rich periphery may respond well to certain immune checkpoint inhibitors. Importantly, PD-L1 expression is induced by interferon (IFN) activity, and indirectly by IL12, in both endothelial and breast cancer cells (207, 208). Further, Taube and colleagues investigated the signaling events that induce PD-L1 expression and demonstrated that many cytokines were overexpressed expression in melanoma cell lines, including RANTES, CXCL1, IL10, IL18 and IL21. In vitro stimulation of melanoma cells by IFN-γ induced PD-L1, but recombinant forms of the other cytokines failed to increase cell surface expression of PD-L1 with or without IFN-γ (54). In a separate study, incubation of activated T-cells with IL10 (+/- IFN-γ) induced expression of PD-L1 on monocytes thereby reducing T-cell activation (51). Increased expression of these molecules correlates with increased T cell infiltration (209, 210). Because PD-L1 expression is modulated by the interferons (α, β, and γ) in a dose- and time-dependent manner as measured by both mRNA and cell surface expression, regulation of this molecule is important for maintaining control of the immune response and is dependent on peripheral cytokines (207).

Inflammatory diseases are, in part, caused by poor lifestyle choices and can contribute to cancer development. Paradoxically, the presence of inflammatory diseases may serve as biomarkers of durable response to immune checkpoint inhibitors. Given these
studies, there is a fundamental gap in understanding of the timing of expression of PD-L1 in NSCLC tumors and PBMC and how those levels relate to the state of the inflammatory response and response to ICI. To measure the relationship between chronic inflammation and the response to ICIs, we investigated the following two aims:

- Retrospective analysis of local Markey Cancer Center (MCC) data for a history of chronic inflammation and correlate with response to nivolumab therapy in NSCLC patients
- Assess national healthcare claims data to determine if ICI-treated patients with a history of chronic inflammation exhibited improved overall survival

Health outcomes research is a methodology used to identify and measure the link between treatments or interventions and the desired outcome of interest. With the limited, real-time clinical applicability of basic science research and the constraints of expensive, time-consuming and limited populations of clinical trials, health outcomes data research bridges the gap to identify the most effective intervention. Health outcomes research can be used to identify disparities among different populations and further patient-centered outcomes. Advances in bioinformatics using a “Big Data” approach provide an opportunity for novel insights regarding the discovery of biomarkers of response (211). Researcher are now able to use real-world data to conduct high quality investigations that demonstrate the value of this novel class of treatment and the variables that influence its success. The combination of epidemiological evidence and preclinical data will provide strong rationale for future prospective clinical studies.
B. METHODS

Retrospective Study Subjects and Methods

Metastatic NSCLC-diagnosed patients treated at the MCC with nivolumab were retrospectively identified between March 2015 and February 2016 using an institutional IRB-approved study. The study was deemed as minimal risk and was exempt from obtaining informed consent from study patients. The range of dates chosen covered the span of time from the FDA approval of nivolumab treatment for patients diagnosed with metastatic NSCLC with progression on or after platinum-based chemotherapy in NSCLC to the date this study was conducted. The data were gathered by reviewing electronic medical records. Data collected included gender, age, comorbid condition history, previous chemotherapy treatment, nivolumab treatment start date and number of cycles of therapy completed. Cycles of nivolumab therapy received were utilized as a surrogate for response. Patients treated with 6 cycles or more of therapy were considered to have a robust, objective response. Chronic inflammatory conditions collected included the diagnosis of hyperlipidemia, hypertension, diabetes mellitus, obesity or chronic obstructive pulmonary disorder (COPD). No statistical analyses or power calculations was undertaken.

Tumor Acquisition & PD-L1 Expression

Using the identified patients treated with nivolumab, the MCC Biospecimen and Tissue Procurement Shared Resource Facility was used as an honest-broker to query for formalin-fixed, paraffin-embedded (FFPE) tumor tissue samples associated with each patient. Of the 45 patients for which health history was collected, PD-L1 expression was assessed for each tumor sample retrieved. The PD-L1 IHC 28-8 pharmDx assay is approved by the US Food and Drug Administration (FDA) as a complementary
diagnostic for non-squamous NSCLC and melanoma in the USA (212). The rabbit monoclonal antihuman PD-L1 antibody 28-8 was produced by Abcam (San Francisco, CA, USA). The 28-8 antibody was tested using Dako PD-L1 IHC 28-8 pharmDx (Dako North America; SK005) and the Dako-recommended protocol, as previously described (213). Tumor tissue sections were de-paraffinized and antigen-retrieval was initiated at 97 °C for 20 min. Detection of PD-L1 protein was conducted using 2 µg/mL of the antibody on the Autostainer Link 48 according to Dako instructions. Upon completion, the stained sample slides were sent to Kimberly Abshear M.D. of UK Pathology to measure the expression level of PD-L1 and to determine the histology as either squamous or non-squamous carcinoma.

Outcomes Study Subjects
For the health outcomes research aim of this study, the Truven Health MarketScan Commercial Claims and Medicare Supplemental Databases was used to complete the outcomes aim of this study. The Marketscan database includes approximately 40 million individuals from over 160 large employers and health plans across the United States and includes healthcare claims with diagnosis and procedure codes for medical encounters and all prescription medication fills. Data are de-identified in compliance with the Health Insurance Portability and Accountability Act regulations (HIPAA), and the University of Kentucky Institutional Review Board previously provided blanket approval the use of the database for studies conducted by UK researchers. Adults aged 18 years and older treated with an ICI between January 1, 2013 and December 31, 2015 were identified using J codes associated with the medications. The healthcare common procedure coding system (HCPCS) is a standardized coding system, including current procedural terminology (CPT) codes and J codes, which is used to identify products, supplies or services. Patients were required to have at least 24 months of pre-index and a minimum
of one-month post-index continuous enrollment in the database (Figure 4.1). Patients were diagnosed with one of the following types of cancer as categorized using the international classification of diseases, ninth revision (ICD-9) codes: Lung cancer (162.2 – 162.9) or Melanoma (172.0-172.9) (214). The ICIs included were nivolumab (C9453, J9299), pembrolizumab (C9027, J9271), and ipilimumab (J9228). At least two inpatient or outpatient diagnoses within 14 days were required, and the date of the first qualifying diagnosis of cancer was defined as the index date. Exposure groups were defined as: ICI-treated patients with a history of chronic inflammatory conditions or ICI-treated patients without a history of chronic inflammatory conditions. Chronic inflammatory conditions included the diagnosis of hyperlipidemia, hypertension, obesity, diabetes mellitus or chronic obstructive pulmonary disorder (COPD).
Figure 4.1: Health outcomes data analysis timeline and process.

**Truven claims data stratification and analysis approach.** An illustration representing the manner in which the desired patient population was identified under Truven and how the relationship between chronic inflammatory conditions and ICIs are assessed.
Outcomes Data Measures & Statistical Methods

Patient demographic characteristics included age, gender, geographic region and urban residence. Clinical characteristics measured during the 24-month pre-index period included previous chemotherapy treatments, previous radiation exposure, and the Charlson Comorbidity Index (CCI) (154). CCI includes 17 categories of comorbid conditions, respectively, and are widely used for risk adjustment with health outcomes data. Presence of metastatic disease was assessed on the index date. The outcome of interest is one-year overall survival. Pairwise analyses were carried out between the two exposure groups: ICI-treated patients with a history of a chronic inflammatory disorders versus ICI-treated patients without a history of chronic inflammatory disorders.

Propensity score matching was conducted using baseline comorbidities, medications, and demographic information to achieve balance between treatment groups. Propensity scoring mimics the randomization process of a clinical trial so that each matched pair has the same baseline probability to receive either treatment (156-158). Matched pairs should be similar in all baseline characteristics. Patients with the same cancer type were matched using a greedy, nearest neighbor algorithm with a caliper set at 0.2 times the standard deviation of the propensity scores in the sample, allowing for up to five matches for each treated person (159). Standardized differences were calculated. A standardized difference of <0.10 is generally considered to be non-significant (160). To address any residual confounding after propensity score matching, covariates were also incorporated in the final regression models (157). The final model included the following adjustment covariates: age, CCI score, gender, region, urban, myocardial infarction, congestive heart failure, peripheral vascular disease, cerebrovascular disease, chemotherapy and radiation.
Cox proportional hazard regression models, accounting for correlation within matched pairs, were used to assess risk of death within one year of diagnosis among all cancers and then stratified among each cancer. Follow-up was terminated for those surviving beyond one year and were censored. Hazard ratios (HR) and 95% confidence intervals (CI) are reported. A p-value of <0.05 is considered statistically significant. All matching and statistical analyses were conducted in SAS.

C. RESULTS

Identification of chronic inflammatory diseases as precursor to response in MCC patients

We identified 45 patients diagnosed with metastatic NSCLC treated with nivolumab between the dates of March 2015 and February 2016. The average number of cycles of nivolumab therapy administered for all 45 patients is 5.49 with a standard deviation of 5.03 (Figure 4.2). Nine patients achieved long-term objective response (Mean=8.5 cycles, SD=1.2), representing 20% of the patient population. Consistent with prior studies, approximately 20% of treated patients achieved an objective response/clinical benefit (≥6 cycles of nivolumab) in the absence of molecular selection for therapy (47, 215). Of the 45 patients, twenty-nine had at least one, pre-existing, comorbid, chronic inflammatory condition. Eleven patients had two or more chronic inflammatory conditions. Patients with no history of chronic inflammatory conditions averaged 3.88 cycles of nivolumab treatment (SD = 0.28), those with at least one chronic inflammatory condition averaged 6.38 cycles of therapy (SD = 0.89), and lastly those patients with a history of two or more chronic inflammatory conditions averaged 8.45 cycles of nivolumab treatment (SD = 1.02) (Figure 4.3).
Figure 4.2: MCC nivolumab-treated patients treated March 2015 to February 2016.
Figure 4.3: The effect of chronic inflammatory conditions on cycles of nivolumab.
**PD-L1 expression of tumor tissue analysis**

Seven patients were found to have banked FFPE lung tumor tissue samples in the MCC biospecimen core. No information was present as to when sample was isolated during the patient's treatment history. Three patients demonstrated no PD-L1 expression, two had expression on macrophages only, one showed less than 10% expression, and one had greater than 20% PD-L1 expression (Table 4.1). Three of those seven patients exhibited objective response to nivolumab treatment (i.e. >6 cycles of therapy); however, response was not correlated with PD-L1 expression. The patients that exhibited a positive tumor PD-L1 expression had a histology of squamous cell carcinoma. The patient whose sample had a PD-L1 expression greater than 20% had a history of chronic inflammatory conditions while the patient with tumor PD-L1 expression less than 10% did not have a history of chronic inflammatory conditions.
Table 4.1: PD-L1 expression and history of inflammation for nivolumab-treated NSCLC patients.

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Gender</th>
<th>Histology</th>
<th>Response on Nivolumab</th>
<th>Inflammatory Disease</th>
<th>PD-L1 expression by IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td>BH556</td>
<td>F</td>
<td>adenocarcinoma</td>
<td>NR-expired</td>
<td>yes</td>
<td>macrophages only</td>
</tr>
<tr>
<td>BH2022</td>
<td>F</td>
<td>adenocarcinoma</td>
<td>R</td>
<td>yes</td>
<td>macrophages only</td>
</tr>
<tr>
<td>BH2023</td>
<td>M</td>
<td>squamous cell carcinoma</td>
<td>NR-expired</td>
<td>no</td>
<td>&lt;10%</td>
</tr>
<tr>
<td>BH2024</td>
<td>F</td>
<td>adenocarcinoma</td>
<td>Lost to follow-up</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>BH2025</td>
<td>F</td>
<td>adenocarcinoma</td>
<td>Lost to follow-up</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>BH2026</td>
<td>M</td>
<td>squamous cell carcinoma</td>
<td>NR-expired</td>
<td>yes</td>
<td>~20%</td>
</tr>
<tr>
<td>BH2027</td>
<td>F</td>
<td>N/A</td>
<td>NR-expired</td>
<td>yes</td>
<td>no</td>
</tr>
</tbody>
</table>
**Outcome Data Results**

The health outcomes research aim provided a much larger pool of patients to assess. Table 4.2 displays the baseline demographic and clinical characteristics of the study population by exposure group. Eligibility criteria were met by 3,252 ICI-treated patients from whom a complete dataset was available. Among the ICI-treated cancer patients identified, 2,339 had a history of chronic inflammation and 913 patients did not. There were 432 patients who passed away within one-year of diagnosis. The mean age of patients in the chronic inflammation cohort was 62.3 (SD = 12.31) and in the no chronic inflammation cohort was 52.6 (SD=12.72). In both exposure groups, melanoma and lung cancer were the only two diagnosed cancers, and the majority of patients were diagnosed with melanoma (lung cancer = 815 and melanoma = 2,612 patients). Over 95% of the entire patient population was previously treated with cytotoxic chemotherapy. Of the chronic inflammation cohort, 42% were treated previously with radiotherapy whereas 36% of the no chronic inflammation cohort was treated with radiotherapy. In the chronic inflammation cohort, 1,987 were obese, 1,761 were diagnosed with diabetes, 609 with hypertension, 854 with hyperlipidemia and 1,582 with COPD.

The number of matched pairs post-propensity score matching were 1,948 for the chronic inflammations versus no chronic inflammation comparison. Upon stratification by cancer type, the number of matched pairs for lung cancer and melanoma were 367 and 1,531, respectively. While baseline differences existed among exposure groups, matching provided samples that had minimal differences (Table 4.3). Four clinical variables (myocardial infarction, congestive heart failure, peripheral vascular disease and cerebrovascular disease) did not meet standardized differences scores below 0.10, that which is accepted to be balanced between cohorts. To account for these differences,
these four variables were included as covariates in the Cox proportional hazard regression model. Table 4.4 displays the HR and associated 95% CI for the effect of the exposure group on on-year survival within the propensity-matched sample. Pre-existing chronic inflammation improved overall survival compared to no history of chronic inflammation (HR 1.23, 95% CI 1.01-1.50). When stratified by cancer, this observation held true specifically in patients with melanoma (HR 1.26, 95% CI 1.01-1.58). No differences were observed in patients diagnosed with lung cancer (HR 1.35, 95% CI 0.82 – 2.23).
Table 4.2: Baseline demographics for all immune checkpoint inhibitor treated patients.

<table>
<thead>
<tr>
<th></th>
<th>Chronic Inflammation</th>
<th>No History</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Std. Dev</td>
</tr>
<tr>
<td>Age</td>
<td>62.3</td>
<td>12.31</td>
</tr>
<tr>
<td>CCI</td>
<td>9.65</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>N (2,339)</td>
<td>%</td>
</tr>
<tr>
<td>Gender (M)</td>
<td>1518</td>
<td>64.9%</td>
</tr>
<tr>
<td>Urban</td>
<td>398</td>
<td>17.0%</td>
</tr>
<tr>
<td>CCI Category</td>
<td>2,339</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>37</td>
<td>1.6%</td>
</tr>
<tr>
<td>2</td>
<td>67</td>
<td>2.9%</td>
</tr>
<tr>
<td>3</td>
<td>48</td>
<td>2.1%</td>
</tr>
<tr>
<td>4</td>
<td>2187</td>
<td>93.5%</td>
</tr>
<tr>
<td>Obesity</td>
<td>1987</td>
<td>85.0%</td>
</tr>
<tr>
<td>Diabetes</td>
<td>1761</td>
<td>75.3%</td>
</tr>
<tr>
<td>Hypertension</td>
<td>609</td>
<td>26.0%</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>854</td>
<td>36.5%</td>
</tr>
<tr>
<td>COPD</td>
<td>1582</td>
<td>67.6%</td>
</tr>
<tr>
<td>Myocardial Infarction</td>
<td>140</td>
<td>6.0%</td>
</tr>
<tr>
<td>Congestive Heart Failure</td>
<td>229</td>
<td>9.8%</td>
</tr>
<tr>
<td>Peripheral Vascular Disease</td>
<td>657</td>
<td>28.1%</td>
</tr>
<tr>
<td>Cerebrovascular Disease</td>
<td>705</td>
<td>30.1%</td>
</tr>
<tr>
<td>Dementia</td>
<td>145</td>
<td>6.2%</td>
</tr>
<tr>
<td>Rheumatism</td>
<td>53</td>
<td>2.3%</td>
</tr>
<tr>
<td>Peptic Ulcer Disease</td>
<td>56</td>
<td>2.4%</td>
</tr>
<tr>
<td>Mild Liver Disease</td>
<td>713</td>
<td>30.5%</td>
</tr>
<tr>
<td>Paralysis</td>
<td>69</td>
<td>2.9%</td>
</tr>
<tr>
<td>Renal Disease</td>
<td>189</td>
<td>8.1%</td>
</tr>
<tr>
<td>Cancer</td>
<td>2325</td>
<td>99.4%</td>
</tr>
<tr>
<td>Severe Liver Disease</td>
<td>19</td>
<td>0.8%</td>
</tr>
<tr>
<td>Metastatic Cancer</td>
<td>2,184</td>
<td>93.4%</td>
</tr>
<tr>
<td>HIV/AIDS</td>
<td>7</td>
<td>0.3%</td>
</tr>
<tr>
<td>Lung Cancer</td>
<td>679</td>
<td>29.0%</td>
</tr>
<tr>
<td>Melanoma</td>
<td>1,839</td>
<td>78.6%</td>
</tr>
<tr>
<td>Previous Chemotherapy</td>
<td>2,272</td>
<td>97.1%</td>
</tr>
<tr>
<td>Previous Radiation</td>
<td>971</td>
<td>41.5%</td>
</tr>
</tbody>
</table>
Table 4.3: Standardized differences post-propensity score matching.

<table>
<thead>
<tr>
<th>Obs</th>
<th>Variable</th>
<th>Std. Diff</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Age</td>
<td>0.117</td>
</tr>
<tr>
<td>2</td>
<td>Charlson Comorbidity Index (CCI)</td>
<td>0.517</td>
</tr>
<tr>
<td>3</td>
<td>Gender</td>
<td>0.035</td>
</tr>
<tr>
<td>4</td>
<td>Region</td>
<td>0.050</td>
</tr>
<tr>
<td>5</td>
<td>Urban</td>
<td>0.046</td>
</tr>
<tr>
<td>6</td>
<td>CCI Category</td>
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<td>7</td>
<td>Myocardial Infarction</td>
<td>0.231</td>
</tr>
<tr>
<td>8</td>
<td>Congestive Heart Failure</td>
<td>0.298</td>
</tr>
<tr>
<td>9</td>
<td>Peripheral Vascular Disease</td>
<td>0.299</td>
</tr>
<tr>
<td>10</td>
<td>Cerebrovascular Disease</td>
<td>0.228</td>
</tr>
<tr>
<td>11</td>
<td>Dementia</td>
<td>-0.011</td>
</tr>
<tr>
<td>12</td>
<td>Rheumatism</td>
<td>-0.006</td>
</tr>
<tr>
<td>13</td>
<td>Peptic Ulcer Disease</td>
<td>0.003</td>
</tr>
<tr>
<td>14</td>
<td>Mild Liver Disease</td>
<td>0.024</td>
</tr>
<tr>
<td>15</td>
<td>Paralysis</td>
<td>0.021</td>
</tr>
<tr>
<td>16</td>
<td>Renal Disease</td>
<td>0.021</td>
</tr>
<tr>
<td>17</td>
<td>Cancer</td>
<td>0.021</td>
</tr>
<tr>
<td>18</td>
<td>Severe Liver Disease</td>
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</tr>
<tr>
<td>19</td>
<td>Metastatic Cancer</td>
<td>0.021</td>
</tr>
<tr>
<td>20</td>
<td>HIV/AIDS</td>
<td>0.021</td>
</tr>
<tr>
<td>21</td>
<td>Chemotherapy</td>
<td>0.0722</td>
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</tbody>
</table>
Table 4.4: One-year survival analysis for ICI-treated patients. Hazard ratios (HR) and associated 95% confidence intervals (CI) for the impact of chronic inflammation on survival within the propensity-matched sample. Results include entire treated group (All Selected Cancers) and stratified by cancer type.

<table>
<thead>
<tr>
<th>Stratification</th>
<th>Patients with History of Chronic Inflammation</th>
<th>Patients without history of Chronic Inflammation</th>
<th>HR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Selected Cancers</td>
<td>1,218</td>
<td>730</td>
<td>1.23</td>
<td>1.01</td>
</tr>
<tr>
<td>Lung</td>
<td>240</td>
<td>127</td>
<td>1.35</td>
<td>0.82</td>
</tr>
<tr>
<td>Melanoma</td>
<td>949</td>
<td>582</td>
<td>1.26</td>
<td>1.01</td>
</tr>
</tbody>
</table>
D. DISCUSSION

ICIs have been enthusiastically accepted by clinicians as a promising option for patients well into their malignancy treatment pathway. PD-1 inhibitors such as nivolumab and pembrolizumab are gaining additional FDA approvals for several malignancies, including becoming first line treatment options in metastatic disease (216). The high financial costs of these agents justify careful consideration when selecting a patient to be treated. The one guiding principle presently used clinically is PD-L1 expression on tumors. This diagnostic has inherent flaws and has not been able to objectively determine patients exhibiting long-term durable responses. Therefore, we undertook these studies with the hypothesis that comorbid conditions, specifically those that affect the inflammatory conditions in the periphery and in the tumor microenvironment, would correlate with response to nivolumab in NSCLC patients. Furthermore, closer examination of these comorbid conditions, may allow us to identify a molecular signature going forward that serves as a robust predictor of response. Our hypothesis is anchored in the concept that while chronic inflammation may create genetic alterations that induce the growth of tumor cells, the presence of those immune factors in peripheral blood, and in the tumor microenvironment, may prime an immune response once the inhibition of the immune checkpoint is released.

The first local, retrospective study presented herein identified an association of chronic inflammatory conditions with the response of cancer patients treated with nivolumab. Our patient population consisted of a small group of adult patients with metastatic NSCLC disease who progressed after platinum chemotherapy. As expected, twenty percent of our population exhibited long-term durable responses matching the results of
other long-term nivolumab treatment studies. Patients with a history of chronic inflammatory conditions, including metabolic syndrome disorders or COPD, stayed on nivolumab therapy for a longer period averaging a higher number of cycles received. In addition, those patients with a history of more than two of these conditions received an average number of nivolumab cycles greater than those patients with only one condition. Of the seven tumor samples analyzed for PD-L1 expression, two exhibited positive expression and neither correlated with improved response. PD-L1 expression alone did not correlate with response however the patient with PD-L1 expression greater than 20% also had a comorbid history of chronic inflammation.

Components of the circulating immune response might better indicate response to ICI agents than PD-L1 expression alone. Systemic inflammation has been shown to increase oxidative stress, activate circulating neutrophils and lymphocytes, and alter levels of inflammatory mediators (i.e. TNF-α, IL-6, IL-8 & C-reactive protein) (217, 218). The tumor microenvironment may have altered levels of cytokines, growth factors, and chemokines that affect tumor cell proliferation, survival and immune evasion due to chronic inflammation (4). Chronic obstructive pulmonary disease (COPD), a chronic inflammatory condition, is associated with elevated IFN-γ + and TNF-α+ CD8+ T-cells when compared with healthy controls (219). TNF-α is recognized by two receptors, TNF-α R-1 and TNF-α R-2, the latter of which is expressed mainly on immune cells (220). It has been shown that high concentrations of this cytokine can induce an anti-tumor response in sarcoma mouse models (221). In addition, NSCLC patients with elevated TNF-α in tumor islets have had favorable survival rates (222). The interplay between chronic inflammation and immune response has been theorized as an important part of immune checkpoint inhibition (223, 224). PD-L1 expression can be induced by interferon
(IFN) activity, and indirectly by IL-12, in both endothelial normal cells and breast cancer cells (207, 208). Importantly, activated oncogenes can transduce intracellular signaling events leading to aberrant PD-L1 expression in cancer cells (225). Taube and colleagues investigated the signaling events that induce PD-L1 expression and demonstrated that many cytokines were overexpressed in melanoma cell lines, including RANTES, CXCL1, IL-10, IL-18 and IL-21. In vitro stimulation of melanoma cells by IFN-γ induced PD-L1, but recombinant forms of the other cytokines failed to increase cell surface expression of PD-L1 with or without IFN-γ (54). However, incubation of activated T-cells with IL10 (+/- IFN-γ) induced expression of PD-L1 on monocytes thereby reducing T-cell activation (51). In an open-label, phase 2 randomized controlled trial, patients with NSCLC who progressed post-platinum chemotherapy were allocated to treatment with atezolizumab (PD-L1 inhibitor) or docetaxel (75). In an exploratory analysis, effector T cell INF-γ gene signatures, defined by CD8A, GZMA, GZMB, IFNγ, EOMES, CXCL9, CXCL10, and TBX21 were assessed. Patients with high expression of the IFN-γ signature had improved overall survival with atezolizumab treatment compared to patients with low expression of this cytokine gene signature (HR 0.43, 95% CI 0.24-0.77).

Our second aim was an observational cohort study and identified a significant effect on the overall survival of cancer patients who received immune checkpoint inhibitor treatment with previous comorbidity of a chronic inflammatory condition, replicating the institutional study. Upon stratification by cancer type, the improved survival advantage held true only in the melanoma population. Our Truven population of ICI-treated patients were identified from early 2011 to the end of December 2015. The earliest FDA approval of an ICI for the treatment of lung cancer was granted to nivolumab on March 4th, 2015.
for the treatment of metastatic squamous NSCLC with progression on or after platinum-based chemotherapy (184). This limited our follow-up and none of these patients completed one full year of follow-up to assess one-year mortality. Thus, additional years of data are necessary to fully vet the lung cancer population for the effect of chronic inflammation on response to ICI. This population suggests however that chronic inflammation does impact overall survival for ICI-treated patients. This health outcomes study improved and extended the results of the prior analysis with the inclusion of a large sample size and with the use of one-year overall survival as a measure of response.

Limitations of the local retrospective study include the small population size as well as the use of cycles of nivolumab therapy as a surrogate for survival. The population size assessed in this study is 45 patients, which is enough to assess trends and correlations, but a much larger sized population of real patient data will be required to validate the findings of this study. Clinically, response is measured by two outcomes, PFS and OS, each allowing direct interpretation of how ICIs affect the tumor size and growth. Cycles of therapy, although may reflect the length of the treatment period and tolerance of the regimen, does not give us insight on the effect of ICIs on the tumor. The national health outcome study is subject to the limitations of all claims-based studies (182, 183). Claims data lack detailed information on laboratory values or tumor staging, which may have influenced the outcomes of this study. This study was limited to a one-year follow-up due to the availability of data. Lastly, while propensity score matching is known to reduce selection bias in non-randomized studies, it is possible that residual bias is present or that unmeasured confounders may have impacted these findings (158). This study is strengthened by a large sample size and the assessment of all-cause mortality.
**Obesity and Inflammation**

Obesity is a worldwide epidemic which is characterized by inflammation of adipose tissue eventually leading to the development of type 2 diabetes, cardiovascular disease and cancer (226). Immunologically, adipocytes represent a large source of immune cells and inflammatory cytokines including T cells, B cells, macrophages and neutrophils (227). The number of leukocytes in the blood is increased as compared to patients with a normal body surface area (BSA) (228). Monocytes, specifically macrophages, migrate to adipose tissue and overwhelming have an M1 configuration relative to M2, leaning into a pro-inflammatory function (229). The increased release of leptin from adipocytes of visceral fats act upon Th1 and Th2 cells inducing the production of IL-2 and IL-4 activating the proliferation of T cells (230). In addition to the release of leptin, resistin and visfatin induce the production of IL-1β, IL-6, IL-8 and IL-12 (231). Adipose tissue from obese patients with colorectal cancer expressed elevated levels of PD-L1 and PD-L2 (232). The presence of adipose tissue from obese patients that is present throughout the development of the tumor may instill a pro-inflammatory tumor microenvironment. The adipose tissue surrounding the tumor site would potentially secrete the same cytokines and induce the immune cell populations present in the periphery as well.

**Diabetes and Inflammation**

Obesity is closely linked to the advent of type 2 diabetes mellitus (T2DM) which itself is associated with several macrovascular (coronary artery disease, stroke) and microvascular complications (diabetic retinopathy, nephropathy and neuropathy) (233). In an analysis of inflammatory cytokine concentrations from monocytes and neutrophils in patients with T2DM, it was observed that the concentrations of TNF-α, IL-6, IL-1β, IL-10, IL-12, IL-8, IFN-β and IFN-γ were all normal compared to non-diabetic volunteers (234). However, when assessing the gene expression, cytokine and TLR gene profiles...
were enhance in T2DM patients compared to non-diabetic volunteers. TNF-α, IL-6 and IFN- β mRNA levels in monocytes and neutrophils were elevated in patients with good glycemic control however patients with poor glycemic control presented with a reduced inflammatory expression and did not differ from the non-diabetic volunteers. Genome-wide expression profiling of patients with diabetes shows an up-regulation of CD274, otherwise known as PD-L1 (235). This is to prevent the pancreatic islets from autoimmune destruction but may be allowing for the up-regulation of PD-L1 on tumors increasing the likelihood of success with ICI therapy. The control of the diabetic state also directly influences the inflammatory environment. The diagnosis alone of diabetes prior to ICI therapy may not be a robust measure of predicting response without the assessment of glycemic control.

**COPD and Inflammation**

COPD is typically caused by exposure to inhaled toxins such as tobacco smoke or dust and has been deemed a risk factor for the development of lung cancer. Just like with T2DM, the severity of COPD dictates the inflammatory cytokines present in the lung (236). IL-6, IL-8 and IL-10 were independently associated with worse airflow obstruction \((P<0.05)\). In a full regression model with all clinical covariates including IL-2, IL-6, IL-8, IL-10, TNF-α and INF-γ, IL-6 accounted for the largest portion of the variance of forced expiratory airflow at 1 sec \((FEV_1\%)\). In a cohort of 10,300 COPD patients, blood was drawn and spun down during their initial visit and then again in their 5-year follow-up visit (237). Elevated IL-6 was associated with rapid decline of airflow at 5 years. This observations held despite stratification by COPD treatments used. In malignancies, IL-6 was seen to play a part in tumor microenvironment regulation and the induction of metastasis through down-regulation of E-cadherin (238, 239). IL-6 exposure in cervical carcinoma cell lines induced IL-6R and STAT3 expression resulting in the down-
regulation of E-cadherin. IL-6 complexes with IL-6R then associated with signal-
transducing membrane protein gp130. GP130 dimerization occurs and is followed by
rapid activation of the Janus kinase (JAK) family. Activated JAKs then phosphorylate
tyrosine residues on the receptor. Signal transducer and activator of transcription
proteins (STATs) with SH2 domains are recruited to the receptor where they are
tyrosine-phosphorylated by JAKs. The activated STATs then dimerize and translocate to
the cell nucleus where they induce transcription of target genes. STAT3 activation
suppresses Toll-like receptor 4 (TLR4) ligand and lipopolysaccharide (LPS)-mediated
dendritic cell (DC) maturation and activation (240). In addition, STAT3 activation by IL-6
suppresses MHC class I expression on DCs and attenuates CD4+ Th1 helper T cell
response through activation of lysosomal protease (241). Th1 cells produce IL-2 and
IFN-γ which are involved in the activation of cytotoxic T lymphocytes (242).
Augmentation of IFN-γ levels directly impact PD-L1 expression on tumor cells (243).

E. CONCLUSIONS

Our data show that a patient history of chronic inflammation correlates with ICI
response. In an institutional IRB-approved study, former and current NSCLC patients
treated with a PD-1 inhibitor were assessed for a correlation between their history of
chronic inflammation and ICI response, and those with a history of at least one chronic
inflammatory condition received more cycles of nivolumab therapy than those patients
who did not have these conditions. Using the national health outcomes data, our work
shows that patients with a history of chronic inflammatory comorbidities have improved
one-year survival rates as compared to ICI-treated patients without a history of chronic
inflammation. These results are in accordance with the findings of the single-center
retrospective study.
CHAPTER 5

A. OVERVIEW

My work that determined that cancer patients with a history of a chronic inflammatory condition improves the response to immune checkpoint inhibitors (ICIs) was an initial step in the development of a blood-based biomarker of response to ICI therapy. Currently in clinical practice, the gold standard for isolating samples to develop biomarkers of response in cancer is tumor tissue analysis. It is amenable to many measures of biological activity including nucleic acid sequencing and assessment of protein expression by immunohistochemistry (IHC) (244). Characterization of the tumor specimen, from a biopsy or from resected material, can measure molecular features specific to the patient; however, it represents a static image and does not characterize the dynamic changes to the tumor over time. Importantly, even though this snapshot is rich in information, it does not represent the inter- and intratumoral heterogeneity of a tumor (245). Further, logistical issues may prevent acquisition of tumor tissue. Eighty percent of metastatic non-small cell lung cancer (NSCLC) patients have limited tissue availability and up to 31% do not have accessible tissue at all (246) . The performance status of NSCLC patients may not allow for interventional biopsy procedures at the moment needed for biomarker evaluation (247). Considering the limitations of tumor tissue, liquid biopsies have become more appealing as they are much more accessible for sampling, amenable to serial sampling throughout treatment, and flexible for multiple testing platforms (flow cytometry, ELISA, mass spectrometry, etc.). Liquid biopsies may contain circulating tumor cells (CTCs), non-hematological cells with malignant features encompassed in the tumor microenvironment, and cells of the immune system (248). Large comparison studies that assess the equality of DNA analysis of CTCs and tumor
tissue biopsies demonstrate that the liquid biopsy approach gives a faithful measure of DNA features compared with tumor tissue (249, 250). In addition to CTCs and cells of the immune response, sampling the periphery can also measure tumor-derived exosomes, extracellular vesicles that contain cytokines present in the tumor microenvironment. Serial sampling of the periphery using liquid biopsies permits tracking of efficiency and toxicity of treatment allowing the oncologist to anticipate the most effective subsequent treatments (251).

I believe that liquid biopsy of NSCLC patients can provide a sample(s) for development of a biomarker of response to ICI that exceeds the performance of the companion diagnostic (IHC for PD-L1 expression) for PD-1 and PD-L1 inhibitors. Development of a robust biomarker of response can improve efficacy and control the cost of therapy in patients who have few therapeutic options, like recurrent lung cancer patients. We hypothesized that chronic inflammation creates an environment that promotes an anti-tumor immune response and the molecular marker of that response can predict an anti-tumor response in NSCLC patients to immune checkpoint inhibitors. The primary goal for this study was to measure pro-inflammatory cytokine levels and immune cell subtype populations in healthy subjects and patients with metabolic syndrome, chronic obstructive pulmonary disorder (COPD), and NSCLC to develop predictive biomarkers of response to PD-1 and PD-L1 inhibitors. We expect this work to also be impactful in further understanding the biological role of chronic inflammation in response to ICI.
B. METHODS

Patient Selection and Monitoring

We received institutional IRB approval for this clinical study to recruit patients diagnosed with metabolic syndrome disorders (MetS; hyperlipidemia, hypertension, diabetes mellitus or obesity), COPD or NSCLC. These cohorts were selected as COPD and MetS populations are expected to have a high degree of chronic inflammation, healthy subjects were expected to have no history of chronic inflammation and NSCLC to have a mixed history. Healthy subjects were included in this study who did not self-report any of the aforementioned conditions and history of smoking. All subjects/patients were aged 50 years or older. We will enroll 20 healthy subjects and 20 each of the MetS and COPD cohorts. We will enroll 90 NSCLC patients. All patients enrolled are 50 years or older and women will be postmenopausal. Enrolled patients were excluded if they exhibited flu or cold symptoms or the use of antibiotics in the two weeks prior to clinic visit, diagnosis of an autoimmune disorder or treatment with any immune modulating therapies or lastly, smoking history (only for healthy volunteer cohort). All patients gave written informed consent. The study protocol was approved by the institutional review board (IRB) of the University of Kentucky. NSCLC cohort patients were recruited at the time of treatment with a PD-1 or PD-L1 inhibitor. These included nivolumab, pembrolizumab and atezolizumab. Blood samples were taken from patients with histologically confirmed NSCLC. Blood samples were drawn right prior to ICI infusion. All NSCLC patients were monitored via monthly (Months 1-3) and 3-month (Months 6, 9, and 12) chart review. Data collected includes number of treatment cycles, CT scan results, objective response, patient survival, and disease state measures (recurrence, progression, etc.). Durable response defined as ongoing treatment with complete disappearance of all lesions or decrease in tumor burden.
by $\geq 50\%$ relative to baseline after 6 cycles of therapy per the immune-related response evaluation criteria in solid tumors (irRECIST) (252).

**Data Collection and Analysis**

Enrollment of 20 subjects/patients of the healthy, MetS and COPD cohorts will provide 90% power to detect difference in expression of several inflammatory cytokines (see power analysis below) using a two-sided, two-sample t-test with 1% significance level. Adjustment in significance level was employed due to multiple comparisons between healthy versus MetS and COPD cohorts. Frequency matching will be employed to ensure similar distribution with respect to gender among the subject cohorts. Assuming a moderate correlation coefficient equal to 0.30 between each cytokine level and PD-L1 expression levels, enrollment of 90 NSCLC patients will provide 82% power based on a two-sided t-test with 5% significance level.

**Power calculations**

Power was determined by reviewing the literature for evidence of whether physiological levels of cytokines were significantly different among COPD and metabolic syndrome patients, and healthy adults (e.g. IL-4, IL-5, and TNFα). From these reports, we determined that a small sample size per group provide power to detect differences in cytokine levels (253-255). Specifically, we will compare each cytokine level between NSCLC and each of the MetS, COPD and healthy cohorts. Statistical power for the NSCLC cohort was primarily based on the association of PDL1 expression with clinical outcome and this will be carried out as an exploratory analysis. Other data analysis plans include two group comparisons of each cytokine between healthy and each of the MetS, COPD and NSCLC groups using two sample t-test, and analysis of variance (ANOVA) for comparison across groups.
**Multiplexed ELISA**

Consented patients had two blood samples drawn in 6-mL K2+EDTA+ in the clinic and, those were transported to the lab. Each blood sample (10-12ml) was immediately centrifuged at 250 g for 10 minutes at room temperature. The plasma from each patient or normal donor was aliquoted in cryotubes and stored in -80°C. Cytokine profiles were assessed by multiplex human cytokine ELISA assay (Quansys Biosciences, Logan, UT, USA) to determine the relative levels of 15 pro-inflammatory cytokines. The 15 cytokines included were IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, IL-13, IL-15, IL-17, IL-23, IFN-γ, TNF-α and TNF-β. The multiplexed assay was used to reduce volume of sample needed and to standardize the assay using purified cytokines as controls. Cytokine concentrations were measured then calculated by Q-View software version 3.09. All samples were assayed in duplicate and the mean value was reported.

**Flow Cytometry**

Due to known degradation of cell surface markers such as PD-L1 when samples are cryopreserved, flow cytometry was conducted promptly after blood sample collection (256). Following plasma removal, the remaining blood sample was processed through a Ficoll Hypaque gradient and centrifuged at 400 g for 20 minutes at room temperature to separate plasma from the buffy coat which contains peripheral blood mononuclear cells (PBMC) (257). Cells were washed twice in buffer (PBS). After PBMC isolation, 1 x 10^6 cells were distributed to 10 FACs tubes. Cells were pelleted by centrifugation, resuspended in 400 μl of buffer (PBA). Cell suspensions were stained with Phycoerythrin-conjugated anti-human CD279 (PD-1), fluorescein isothiocyanate-conjugated anti-human CD3, phycoerythrin-cyanine 5-conjugated anti-human CD4, phycoerythrin-cyanine 7-conjugated CD8, phycoerythrin-conjugated anti-human CD274 (PD-L1), fluorescein isothiocyanate-conjugated anti-human CD14 and allophyocyanin-conjugated anti-human CD45 antibodies. Secondary antibodies for CD3, CD4, CD8,
CD14, CD45, PD-1 and PD-L1 bound to fluorophores were added to FACs tubes and incubated in cold room over 20 minutes. Immune cell subtype populations sorted using the Attune™ flow cytometer. Immune cell populations analyzed included CD3+ only, CD3+CD4+, CD3+CD8+, CD4+PD-1+, CD8+PD-1+, CD14+CD45+ and CD14+CD45+PD-L1+. Flow cytometry data analysis was performed using FlowJo version 7.6.5 (Tree Star).

Statistical analyses

Statistical analyses were conducted in collaboration with Katherine Thompson, PhD of the Department of Statistics at the University of Kentucky. Distribution of continuous variables, including cytokine concentrations and immune cell populations, were described by their mean and standard deviation. Four cohort comparisons (Healthy, MetS, COPD, NSCLC) were done using analysis of variance (ANOVA). A p-value < 0.05 was considered as statistically significant. All analyses were performed using SAS software (SAS, Cary, NC, USA) and R Package software (Version 3.40) (258). Cytokine concentrations, expressed in pg/ml, were first assessed by Fisher’s exact test for detection in plasma. Cytokines that were detectable were then compared by ANOVA followed by post-hoc t tests with reference to NSCLC cohort. Cytokine concentrations were natural log-transformed. Immune cell subtype populations derived from flow cytometry were compared using ANOVA followed by post-hoc t tests with reference to NSCLC cohort. Percentages of specific immune cell populations were transformed by taking the arcsine of the square root for ease of depiction. ANOVAs followed by post-hoc t tests were used for assessment with reference to NSCLC to determine if any cohort significantly differed in immune cell subtypes. Finally, to determine whether immune cell subpopulations and/or cytokine levels predict response to ICI in NSCLC patients, a logistic regression model was employed. It is likely that this model would not be appropriate with these data due to perfect separation of responders from non-responders, so feasible solutions algorithm (FSA) with the
criterion of Bhattacharrya distance (B-distance) will be utilized to assess pairs of observations that can provide “feasible solutions” for prediction of ICI response in the NSCLC cohort (259-261). Perfect separation typically occurs in small samples with unbalanced and/or highly predictive variables. Separation occurs if the predictor is associated with only one outcome value when the predictor is greater than some constant (262). This means the maximum likelihood estimate of the logistic slope coefficient does not exist. FSA is a technique that allows the assessment of predictive capabilities of different combinations of pairs of variables to determine the pair(s) with the best predictive ability of the outcome of interest. This technique serially tests pairs based on a selected criterion. FSA in turn can employ B-distance, a measure of the relative proximity of two samples taking into account shape, range and direction of the sample data. The center point of two samples may overlap, but when considering each individual data value, the B distance may be different and highlight variables of interest.
Figure 5.1: Clinical study protocol

1. Measure Circulating Cytokines
2. Measure Lymphocyte & Monocyte Populations
   a) Including PD-1+ CD4+ or PD-1+ CD8+ Cells and PD-L1+ Monocytes

Identify cytokine & immune cell profile that correlates with improved response to therapy
C. RESULTS

The study is ongoing and has recruited 36 patients to date, six in the COPD cohort and ten in each of the remaining cohorts. The study will continue to achieve target accrual; however, herein I discuss the interim analysis of the data collected. First, not all cytokines could be detected (Detect: Y/N) from plasma using multiplexed ELISA (Table 5.1). Cytokines that were detectable in greater than 50% of the subject/patient samples included IL-4, IL-6, IL-13, IL-23, IFN-γ and TNF-α. IFN-γ was evaluable in all patient samples. Certain cytokines were more readily detectable in certain cohorts. IL-4 concentrations were detectable in most healthy subjects but not in any other cohort. IL-6 concentrations were detectable in patients with NSCLC and healthy subjects. Six detectable cytokines were compared for differences among the cohorts (Figure 5.2). In NSCLC patients, IL-4 levels were significantly decreased compared to the healthy subject cohort ($P=0.0066$). In addition, the COPD cohort trended toward increased IL-4 concentrations compared to NSCLC patients ($P=0.1888$). IL-6 demonstrated lower concentrations in the MetS cohort compared to the NSCLC cohort ($P=0.0691$). IL-13 levels are decreased in NSCLC patients compared to healthy subjects ($P=0.0788$). Although IFN-γ was detectable in all patients, there were no significant differences among the four cohorts. TNF-α did not show any significant differences among the cohorts.
Table 5.1: Cytokine measurements organized by cohort.

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<th>Cytokine (pg/ml)</th>
<th>Detect</th>
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<th>MetS</th>
<th>NSCLC</th>
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</table>
Figure 5.2: Comparison of IL-4, IL-6, IL-13, IL-23, IFN-γ, and TNF-α levels by cohort.
The second goal was to investigate the proportions of relevant immune cell subtypes in each cohort and compare among cohorts. The immune cell subsets chosen for study include CD8+ T cells, CD4+ T cells, CD14+CD45+ monocytes, PD-1+ T cells and PD-L1+ monocytes. Lymphocyte populations were assessed using CD3, CD4 and CD8 markers. Monocyte populations were then assessed using the markers CD14 and CD45. We found significant differences among the several cohorts (Figure 5.3). Healthy subjects, patients diagnosed with MetS, and those with COPD all had significantly elevated levels of CD3+ lymphocytes compared to patients with NSCLC ($P=0.0094$, $P=0.0043$, $P=0.0026$, respectively). There were no detectable differences between CD3+CD4+ lymphocyte populations among all four cohorts; however, there was a significantly increased level of CD3+CD8+ lymphocytes in the NSCLC cohort compared to both healthy subjects and MetS patients ($P=0.0344$, $P=0.0099$, respectively). Interestingly, the CD3+CD8+ immune cell subtype did not differ between NSCLC and COPD patients. There were no distinguishable differences in CD4+PD-1+ or CD8+PD-1+ populations among the cohorts. Of note, monocyte populations were significantly elevated in patients with NSCLC compared to the MetS and COPD cohorts ($P=0.0293$, $P=0.0025$, respectively). However, the comparison of CD4+CD45+PD-L1+ immune cell subtypes showed no difference among cohorts.

Finally, we examined whether any of the measured parameters might predict durable response to ICI in the NSCLC cohort. Of the ten patients in the NSCLC cohort, seven met our criteria for durable response. Eleven variables were selected to be included in a logistic regression model; six cytokines (IL-4, IL-6, IL-13, IL-123, IFN-γ, TNF-α) and 5 immune cell populations (CD3+ Only, CD3+CD8+, CD8+PD-1+, CD14+CD45+, CD14+CD45+PD-L1+). Logistic regression models could not be fit due to perfect separation between the 7 responders and 3 non-responders. In this case, all durable
responders are associated with a cytokine concentration above or below a certain level. For maximum likelihood estimates to exist, there must be some overlaps in the two distributions. We then used FSA, with a criterion of B distance, to find possible solutions that might be predictive. Out of 25 times FSA was run, two pair of measures were determined to be predictive of response (Figure 5.4A). The combination pair of CD3+ only and CD8+PD-1+ immune cell populations had the largest B distance of 2.88. This pair was chosen 15 of the 25 trials. Elevated CD8+PD-1+ populations and low CD3+ only populations were predictive of non-responders (Figure 5.4B). Another pair was chosen for the remaining 10 trials and that is the combination pair of CD14+CD45+ monocyte population and IFN-γ cytokine concentration with a B distance of 2.34. Increased concentrations of IFN-γ and elevated monocyte populations were indicative of response (Figure 5.4C).
Figure 5.3: Comparison of immune cell populations by cohort.
Figure 5.4: Feasible solutions algorithm (FSA) assessment to predict durable response in NSCLC cohort.

<table>
<thead>
<tr>
<th>Variable 1</th>
<th>Variable 2</th>
<th>B Distance</th>
<th>Trials</th>
</tr>
</thead>
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<tr>
<td>CD3+</td>
<td>CD8+ PD-1+</td>
<td>2.878134</td>
<td>15</td>
</tr>
<tr>
<td>CD14+ CD45+</td>
<td>IFN-γ</td>
<td>2.340578</td>
<td>10</td>
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D. DISCUSSION

Recent literature has revealed that the presence of inflammatory cells within the tumor microenvironment is associated with an improved clinical outcome after treatment with ICIs (263). The concept of “immune contexture” was established, assessing the type, density, functional orientation and location of the immune cells within distinct tumor regions (264). To establish a viable clinical utility for this concept in ICI-treated patients, the immunoscore was formed that is based on the immune cell subtype populations. The immunoscore can be based on any combination of the lymphocyte or monocyte populations but most researchers noted the use of CD3+ and CD8+ tumor-infiltrating lymphocytes (TILs).

To date, there has been no standardized scoring system of immune cell profiles combined with cytokines concentrations from peripheral blood to predict response from ICI treatment in NSCLC patients. Our goal was to use our prior work that established that chronic inflammation associates with response to PD-1 and PD-L1 inhibition and extend that work to measure molecular markers of inflammation, specifically inflammatory cytokines and immune cell subsets in the periphery of patients with known chronic inflammation or NSCLC. The inclusion of healthy, MetS and COPD cohorts was to compare different states of known chronic inflammation to the mixed state expected within the NSCLC cohort with the expectation that NSCLC patients that are responders to ICI would have an immune profile similar to those with chronic inflammation. We sought to determine whether peripheral biomarkers could also predict response to ICI therapy in NSCLC and whether the inflammatory markers are different in NSCLC compared with other cohorts of non-cancer, chronically inflamed patients.
While this study is ongoing, preliminary analysis of the data has provided insights into the molecular interactions of immune response and cancer. The primary endpoints analyzed are the differential expression of cytokine concentrations and immune cell subsets in NSCLC patients compared to patients with MetS or COPD. In our exploratory analysis, we have shown that immune cell subtype populations consisting of elevated CD3+ lymphocytes as well as low CD8+PD-1+ T cells, which distinguishes an exhausted cytotoxic T cell population, were predictive of response in NSCLC. CD3 is a T cell co-receptor present on both T helper cells and cytotoxic T cells and is a general indicator of proliferation of lymphocytes (265). Elevated CD3+ expression suggests that the proliferative status of the immune response in NSCLC patients may indicate a readily actionable response to tumor cells following inhibition of the co-inhibitory signal. Low PD-1 expression on CD8+ cells has previously been shown to be a distinctive feature of nivolumab-treated patients showing clinical benefit with prolonged progression-free survival (HR 4.51; 95% CI 1.45-13.94) (266). With the development of PD-1 inhibitors that bind directly to the PD-1 receptor, it was hypothesized that the more PD-1 expressed on T cells, the more anti-PD-1 antibody binding will occur. However, it is likely that elevated PD-1 expression indicates an exhausted state that, even with the binding of anti-PD-1 antibodies, the cytotoxic T cells cannot surmount a strong immune response to the tumor cells. With the variability of PD-L1 expression, PD-1 negative effector T lymphocytes provides an immune-privileged microenvironment with a positive impact on survival.

We also identified a second combination of predictive markers, consisting of an immune cell subtype population and concentration of a certain cytokine was also predictive of response: increased CD14+CD45+ macrophage populations and IFN-γ concentrations. These results suggest activation of the innate immune response in NSCLC patients that
respond to ICI therapy. Specifically, innate immune response includes the activation of macrophages and the boosting of natural killer cell activity. Inflammatory monocytes selectively traffic to the sites of inflammation, produce inflammatory cytokines and contribute to local inflammation (267). CD14+ monocyte count increases correlate with presence of inflammatory conditions. Antitumor M1-polarized macrophages have the ability to direct cytostatic and cytotoxic effects on tumor cells, secret pro-inflammatory cytokines, and stimulate T cell immunity (268, 269). Investigations into the cooperation of lymphoid cells and macrophages led to the identification of IFN-γ as a regulator of macrophage tumoricidal activity (270). Two molecular signals are required for efficient induction of the M1 phenotype, TLR4 agonist LPS and IFN-γ (271). Importantly, IFN-γ has been identified as a cytokine that specifically upregulates PD-L1 expression on tumor cells, but it also plays a key role in the PD-L1 expression on macrophages.

Completion of this study is required to ascertain whether these pairs of peripheral immune properties significantly predict responders to ICI treatment. Once accrual to all cohorts is complete, a validation cohort study will be required to compare the precision of this assay compared to the current standard of care, PD-L1 expression in the tumor.

E. CONCLUSION

Preliminary analysis of peripheral cytokine profiles and immune cell subset populations has identified several immune response molecular markers that describes differences among inflamed patients and healthy subjects and may be effective in predicting response to ICI therapy in NSCLC patients. We have shown that IL-4, IL-6, IL-13, IL-23, IFN-γ and TNF-α are detectable in peripheral blood. The cytokine profile present in NSCLC patients closely resembles that of COPD patients. Immune cell subset populations, including those with PD-1 receptor or PD-L1 can be assessed from PBMC
however there were no significant differences among cohorts. These data have given us insight into the alteration of the innate and adaptive immune responses that may be important for response to immune checkpoint inhibition. Importantly, these markers were generated from a minimally-invasive sample from peripheral blood and could be readily used by clinicians to continually monitor the patient for response and toxicity. Complete analysis of the entire study population will be required to confirm these findings and extend these observations.
A. SUMMARY OF RESULTS

By coupling an observational clinical study design with a pharmacoepidemiological analyses, I have developed a testable hypothesis that chronic inflammation predicts response to ICI treatment in NSCLC patients. I then hypothesized that a liquid biopsy could be used to generate a biomarker of response to immune checkpoint inhibitors (ICI) in NSCLC patients by measuring molecular markers of inflammation. I concluded this work by demonstrating that this strategy could be utilized for the development of a peripheral blood biomarker assessment to predict response to ICI treatment.

The first hypothesis that chronic inflammatory comorbidities, explored in Chapter 4, was tested by utilizing health outcomes research and pharmacoepidemiology principles to address the direct impact of chronic inflammatory comorbid conditions on response to ICI treatment without respect to a particular cancer. I developed an experimental strategy to identify a novel biomarker of response to ICI therapy. I initially utilized these principles to test hypotheses using large national databases based on the impact of treatment or exposure on specific biological pathways and processes. Specifically, in two previous studies, I measured the impact of statin therapy on survival in cancer and then in the next study, calculated the incidence of autoimmune diseases in lung and renal cancer patients (108, 272). In the study outlined in Chapter 4, I found that, among the 3,252 ICI-treated patients between 2011 and 2015, 2,339, with a history of chronic inflammation, had an improved one-year overall survival compared to those without chronic inflammation. This observation was in agreement with the findings of our local retrospective study analysis. Those results suggest that comorbidities that elicit a
constant, low-grade inflammatory state have a positive impact on the patient response to ICI treatment. It is anticipated that through further analysis and validation, the immune cell and cytokine profile brought about by this low-grade inflammatory state will yield a predictive diagnostic, achieved by liquid biopsy, to identify responders to ICI treatment in lung cancer.

In Chapter 5, I analyzed inflammatory cytokine concentrations and immune cell populations in peripheral blood mononuclear cells (PBMCs) sampled from peripheral blood of non-small cell lung cancer (NSCLC) patients and identified differences among cohorts with and without inflammation and/or NSCLC and molecular profiles that may be predictive of response to ICIs. I characterized the inflammatory profile of NSCLC patients treated with programmed death receptor 1 (PD-1) or programmed death ligand 1 (PD-L1) inhibitors and compared to the profile present in patients with chronic inflammatory disorders, either metabolic syndrome (MetS) or chronic obstructive pulmonary disorder (COPD). I was able to identify six cytokines from peripheral blood in all cohorts including interleukin 4 (IL-4), IL-6, IL-13, IL-23, IFN-γ and tumor necrosis factor alpha (TNF-α) that were expressed above baseline. Of the six differentially-expressed cytokine, significant differences were observed between certain cohorts. The cytokine profile of NSCLC most closely resembled that of the COPD cohort. NSCLC patients exhibited a smaller population of CD3+ lymphocytes relative to the other cohorts. Cytotoxic T cell (CD8+) levels in the NSCLC cohort were elevated compared to healthy and MetS populations, but did not differ from the COPD population. In addition, patients with NSCLC had a higher concentration of monocytes compared to MetS or COPD patients. Of these observations, I identified two pair of peripheral blood markers that may be predictive of response to ICI treatment; high CD3+ lymphocyte and low PD-1 + cytotoxic T cell population levels or high monocyte levels coupled with high IFN-γ
concentrations. Finally, these results suggest that the peripheral blood can provide a means for minimally-invasive assessment of the state of the innate and adaptive immune response that may serve as a predictor of response to ICIs. This enables clinicians to sample for the predictor immune response from a peripheral blood test right prior to ICI treatment and determine whether this treatment path is suitable for this patient. Extending these observations to a more complete understanding of the underlying mechanisms that influence patient response to immunotherapies including ICIs will help shape future targeting strategies of the immune response.

A. EXPERIMENTAL CONSIDERATIONS

I utilized health outcomes data analysis to drive the design and analysis of the observational clinical study to test the central hypothesis that peripheral blood markers could be used to select patients primed for durable response for ICI therapy. Using health outcomes approaches, we were able to assess specific health outcomes from a large national population and make broad inferences about the underlying interaction of cancer and chronic inflammation. Incorporating pharmacoepidemiology allowed us to then focus on specific properties of chronic inflammation to analyze in the clinical study. Finally, the use of clinical study model allowed us to directly explore the impact of specific cytokine levels and of immune cell populations in a prospective manner in patients undergoing therapeutic interventions.

Although the interim results are promising, it is important to note that the clinical study has not met accrual goals and is thus not powered to address our central hypothesis that chronic inflammation improves the response to ICI treatment in NSCLC patients.
Completion of patient enrollment and peripheral blood assessment will be needed to identify the appropriate immune markers to further validate in future studies.

**Health Outcomes Data Models**

National healthcare claims data from Truven Marketscan were used in these analyses. This data encompasses both commercial and Medicare claims for over 65 million patients across the United States. Due to the limited history of ICI use in clinical practice, analysis of “big data” allowed us to study a large population of patients who otherwise would not be evaluable. However, these data are limited to what is billed per claim for each patient visit. Pathology of tumors and diagnostic laboratory values, which are not present in the dataset, would allow for further insights into the underlying molecular mechanisms. Importantly, health outcomes research is understood to identify correlations as opposed to causation. Thus, continued validation in human peripheral blood samples will be important for further development as a useful clinical biomarker of response.

**Prospective Clinical Study Model**

We used a single site for prospective evaluation of biological markers of immune response from peripheral blood. There are important considerations associated with conclusions drawn from this clinical study. Peripheral blood samples were drawn only once prior to treatment limiting the analysis to a single measure of cytokine concentrations and immune cell populations. Also, peripheral blood concentrations of cytokines are notably less than within the tumor microenvironment (255, 273, 274). Of note, blood was drawn from a central line in NSCLC patients however was drawn by venipuncture in the other cohorts. Blood drawn by venipuncture may introduce inflammatory mediators not present from a central line blood draw. In order to reduce the impact of these limitations, we performed all ELISA experiments using a multiplexed kit.
with the lowest level of detection (LLD) on the market. Although the kit allowed for levels of 15 different cytokines, only 6 could be ascertained above the LLD.

Another experimental consideration in the prospective clinical study is the lack of PD-L1 expression data on the tumor from prior biopsies of the included NSCLC patients. A comparison of the predictive ability of the peripheral immune profiles identified in this clinical study to the clinical assessment of PD-L1 would have aided in determining the value of the peripheral blood analysis. PD-L1 status is not routinely ordered for patients to be treated with a PD-1 or PD-L1 inhibitor in the clinic. To address this limitation, we assessed PD-L1 status expressed on PBMC in peripheral blood as a surrogate as it has been previously shown to be readily detectable from peripheral blood (275).

B. CONTRIBUTION TO THE FIELD

This work makes a substantial contribution to an improved understanding of immune checkpoint inhibition and immune response to cancer. The rising costs of immunotherapy treatment are a burden for patients to bear. The identification of effective ways of minimizing non-beneficial medication use and maximizing outcomes would aid in their decision to proceed with treatment. Our work and the work of others have identified multiple potential peripheral blood-sampled biomarkers that could underlie responsiveness to ICIs (93, 94, 99, 102-104, 276). (see also Chapter 1). In a study by Farsaci et al, peripheral immunoscores were established from analysis of PBMC prior to treatment with vaccine therapy to prostate specific antigen (PSA) to define whether there was a correlation of what with efficacy of immune-based treatment (87). Their approach similarly assessed PBMC for immune cell populations but did not assess cytokine concentrations from plasma. In addition, the Farsaci, et al. study did not assess a
peripheral immunoscore prior to the use of ICIs. Importantly, their study was able to identify a peripheral immunoscore capable of predicting improvements in progression-free survival (PFS). Martens et al explored combinations of 28 potential biomarkers sourced from peripheral blood prior to cytotoxic T lymphocyte antigen-4 (CTLA-4) inhibitor (88). A six-candidate combination biomarker was identified to be predictive of improve overall survival; low LDH count, elevated eosinophils, low absolute monocytes, high absolute lymphocytes, low Lin−CD14+HLA-DRlow myeloid-derived suppressor cells (MDSC), frequencies, and elevated CD4+CD25+FoxP3+ Treg frequencies. Similarly to Farscai et al, this study did not evaluate cytokine concentrations. Another difference is the assessment of baseline markers prior to anti-CTLA-4 treatment in melanoma patients whereas our focus was on anti-PD-1 or anti-PD-L1 treatment in NSCLC patients.

To our knowledge, our study is the first to identify potential biomarkers of response to ICI therapy utilizing health outcomes research data. Our own mining of this dataset was successful in identifying chronic inflammation as a precursor to durable response. I have shown that the analysis of national patient data can be used to inform biomarker characterization and drive clinical study design, limiting the expensive cost of large prospective adequately-powered clinical trials. This concept of using health outcomes data principles to drive translational research efforts remains to be rigorously tested, but provides a hypothesis to be further explored by those in the field.

**C. TRANSLATIONAL AND CLINICAL RELEVANCE**

The findings presented in this work are fully translatable for clinical application. Each of the formulated hypotheses and aims in this work were made with particular concern for
their impact on patient care. Furthermore, the approaches used bridged both basic science and clinical fields, thus being largely translational in nature.

Improving the methods by which appropriate patients are selected for treatment with ICIs can properly balance cost-efficiency of this class of medications in the clinic. The cost of nivolumab treatment is estimated to be greater than $100,000 per year due to the continuous administration schedule (277). The median PFS of 9 months or longer, depending on the agent used, may be worthwhile for durable responders but the cost may be too great for those patients that gain little to risk no benefit. The impact of failed treatment on health care costs, quality of life, and outcome are driving forces in the focus on predicting response to cancer therapies. I have made a significant effort in understanding the relationship of inflammation and response to ICI therapy in a clinical setting, but more validation is necessary prior to implementation into practice. The ongoing clinical study requires completion with accrual targets for all cohorts to firmly assess significance of results in an appropriately powered cohort study. If our findings of the two combination pairs of peripheral immune data remain predictive of response, a prospective validation study will be conducted to affirm their utility. Translating our findings to a clinically useful diagnostic is difficult, but addresses the broader clinical needs of the health care system. Successful implementation of the model could significantly impact health care costs and outcomes associated with ICI use.

One common practice utilized in anti-cancer therapy is the implementation of drug combinations to combat resistance and to synergistically improve outcomes compared to each agent separately. The probability of a tumor cell becoming resistant to a combination therapy of two agents with differing mechanisms of action is far less than
the probabilities of the development of resistance to each individual agent alone (278, 279). If patients, at baseline, do not have the immune profile deemed as predictive as a response, a secondary agent could be used to “prime” the patient’s immune profile to become responsive to ICI therapy. Thus, identifying effective drug combinations for treating NSCLC is paramount.

D. CONCLUSIONS

I conclude that chronic inflammation can be defined by a specific immune profile consisting of cytokines and immune cell populations and believe that chronic inflammation is predictive of response to ICI in NSCLC. This profile could aide in identifying and stratifying NSCLC patients who will benefit from ICI therapy. Enrichment of the treated population for responders will significantly impact the clinical utility of these agents. The research methods employed here allowed us to characterize potential biomarkers from peripheral blood without the cost of a large prospective clinical study. Furthermore, we conclude that coupling bioinformatics principles with basic science experimental approaches can bridge the gap of understanding of translating findings to clinical application.
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