Quantification of the PD-1/PD-L1 Axis in Various Cancer Types by Immuno-Multiple Reaction Monitoring

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ABSTRACT

Introduction

Although therapeutic antibodies targeting checkpoint inhibitors like PD-L1 (Programmed Death-Ligand 1) have revolutionized cancer treatment, PD-L1 immunohistochemistry (IHC) is the only approved companion diagnostic. However, relying on IHC as the sole biomarker has limitations including lack of antibody specificity, difficulty preserving samples, and problems in detecting post-translational modifications such as glycosylation. Measuring other constituents of the PD-L1 signaling pathway could improve prediction. We used anti-peptide antibodies, liquid chromatography, and multiple reaction monitoring to quantify PD-L1, PD-1, PD-L2, NT5E, LCK, and ZAP70. Despite challenges posed by low protein concentrations in the PD-1/PD-L1 axis, mass spectrometry-based assays showed promise in overcoming the limitations of IHC.

Methods

We used an immuno multiple reaction monitoring (iMRM) assay to explore the PD-L1 axis in clinical formalin-fixed paraffin-embedded samples from 175 cancer patients with cancers in a variety of different sites including lung, esophageal, urothelial, laryngeal, oropharynx, pancreatic, renal, breast, and oral. First, we treated protein extracts with peptide N-glycosidase F. Next, we used anti-peptide antibodies to enrich surrogate tryptic peptides from low-abundance proteins (8 peptides from PD-L1, PD-1, PD-L2, NT5E, LCK, and ZAP70) and quantified concentrations of each peptides within the enriched samples using liquid chromatography mass spectrometry (LC-MS). Finally, we evaluated correlations between: i. PD-1 and PD-L1 peptide concentrations in patients above the limit of detection, ii. correlations between peptide concentrations and clinical benefit as defined by RECIST criteria (i.e., values of 1, 2, or 3 with 6 months of survival defined as “benefit”, while values of 4 were defined as “no benefit” using t-

tests), and iii. correlations between peptide levels and survival time after immunotherapy with Kaplan-Meier methods and Cox regression.

Results

We examined samples from 175 patients with different cancer types. The proportions of samples with detectable amounts of each peptide varied substantially: 92% had detectable NIIQ (PD-L1), 80% had detectable LQDA (PD-L1), 60% had detectable LFDV (PD-L1), 54% had detectable ATLL (PD-L2), 76% had detectable GPLA (NT5E), 89% had detectable LAAF (PD-1), 68% had detectable ITFP (LCK), and 53% had detectable LIAT (ZAP70). Correlations between each pair of the PD-L1 peptides (LQDA, NIIQ, and LFDV) were high at R=0.70 between (LQDA and NIIQ), R=0.65 between (NIIQ and LFDV), and R=0.92 (between LFDV and LQDA), with p-values for these correlations of less than 0.001.

Of the 175 samples, 83 were linked to clinical data. Of those samples, 51 patients received immunotherapy as at least one of their lines of therapy and 26 of those 51 patients benefited from immunotherapy. P-values from t-tests comparing single peptide concentrations between patients who experienced clinical benefit and those who did not were all greater than 0.05. P-values from the Cox regression comparing patients above vs below cut-offs based on individual peptide concentrations were also not statistically significant.

Conclusion

We were able to determine the concentrations of multiple peptides that contributed to proteins in the PD-1/PD-L1 signaling axis by using anti-peptide antibodies. The strong correlation between the PD-L1-associated peptides and the strong correlations between those peptides and the peptide

associated with PD-1 both suggest the assay functioned well. That being said, the lack of significant differences between peptide levels in patients who benefited from immunotherapy and those who did not indicates that single peptide concentrations may not be sufficient to predict clinical outcomes in patients receiving immunotherapy. Research into algorithms for combining information on peptide concentrations may demonstrate the potential of optimized iMRM and LC-MS. Multiplexed mass spectrometry-based assays like optimized iMRM may prove an effective alternative to PD-L1 IHC.

RÉSUMÉ

Quantification de l'axe PD-1/PD-L1 dans divers types de cancer par surveillance immuno-multiple des réactions

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Introduction

Les anticorps thérapeutiques ciblant les inhibiteurs de point de contrôle comme PD-L1 (Programmed Death-Ligand 1) ont révolutionné le traitement du cancer. L'immunohistochimie (IHC) de PD-L1 est le seul diagnostic compagnon approuvé. Cependant, se fier uniquement à l'IHC comme biomarqueur présente des limites (par exemple, manque de spécificité des anticorps, difficulté à préserver les échantillons, et détection des modifications post-traductionnelles comme la glycosylation). Mesurer d'autres composants de la voie de signalisation de PD-L1 pourrait améliorer la prédiction. Nous avons utilisé des anticorps anti-peptides, la chromatographie liquide et la surveillance par réactions multiples pour quantifier PD-L1, PD-1, PD-L2, NT5E, LCK et ZAP70. Malgré les défis posés par les faibles concentrations de protéines dans l'axe PD-1/PD-L1, les tests basés sur la spectrométrie de masse montrent un potentiel pour surmonter les limitations de l'IHC.

Méthodes

Nous avons utilisé un test d'immuno-surveillance par réactions multiples (iMRM) pour explorer l'axe PD-L1 dans des échantillons cliniques fixés au formol et inclus en paraffine provenant de 175 patients atteints de cancers de différents sites, notamment le poumon, l'œsophage,

l'urothélium, le larynx, l'oropharynx, le pancréas, le rein, le sein et la cavité buccale. Tout d'abord, nous avons traité les extraits de protéines avec de la peptide N-glycosidase F. Ensuite, nous avons utilisé des anticorps anti-peptides pour enrichir des peptides tryptiques substituts pour les protéines de faible abondance (8 peptides de PD-L1, PD-1, PD-L2, NT5E, LCK et ZAP70) et quantifié les concentrations de chaque peptide dans les échantillons enrichis en utilisant la chromatographie liquide couplée à la spectrométrie de masse (LC-MS). Enfin, nous avons évalué les corrélations entre les concentrations de peptides PD-1 et PD-L1 chez les patients au-dessus de la limite de détection, les corrélations entre les concentrations de peptides et les bénéfices cliniques définis selon les critères RECIST (valeurs de 1, 2 ou 3 avec une survie de 6 mois définie comme « bénéfice », valeurs de 4 définies comme « absence de bénéfice » à l'aide de tests t), et les corrélations entre les niveaux de peptides et le temps de survie après l'immunothérapie à l'aide des méthodes de Kaplan-Meier et des régressions de Cox.

Résultats

Nous avons examiné des échantillons de 175 patients atteints de différents types de cancer. Les proportions d'échantillons avec des quantités détectables de chaque peptide variaient considérablement : 92 % avaient un NIIQ détectable (PD-L1), 80 % un LQDA détectable (PD-L1), 60 % un LFDV détectable (PD-L1), 54 % un ATLL détectable (PD-L2), 76 % un GPLA détectable (NT5E), 89 % un LAAF détectable (PD-1), 68 % un ITFP détectable (LCK) et 53 % un LIAT détectable (ZAP70). Les corrélations entre chaque paire de peptides PD-L1 (LQDA, NIIQ et LFDV) étaient R=0,70 entre (LQDA et NIIQ), R=0,65 entre (NIIQ et LFDV), et R=0,92 (entre LFDV et LQDA), avec des p-values pour ces corrélations inférieures à 0,001.

Parmi les 175 échantillons, 83 étaient liés à des données cliniques. Parmi ces échantillons, 51 patients ont reçu une immunothérapie dans au moins une ligne de traitement et 26 de ces 51

patients ont bénéficié de l'immunothérapie. Les p-values des tests t comparant les concentrations individuelles de peptides entre les patients ayant bénéficié d'une immunothérapie et ceux n'en ayant pas bénéficié étaient toutes supérieures à 0,05. Les p-values de la régression de Cox comparant les patients au-dessus ou en dessous des seuils basés sur les concentrations individuelles de peptides n'étaient également pas significatives.

Conclusion

Nous avons pu quantifier la concentration de plusieurs peptides contribuant aux protéines de l'axe de signalisation PD-1/PD-L1 en utilisant des anticorps anti-peptides. La forte corrélation entre les peptides associés à PD-L1 et les fortes corrélations entre ces peptides et le peptide associé à PD-1 suggèrent que le test fonctionne bien. Cela dit, l'absence de différences significatives entre les niveaux de peptides chez les patients ayant bénéficié de l'immunothérapie et ceux n'en ayant pas bénéficié indique que les concentrations de peptides individuels pourraient ne pas suffire à prédire les résultats cliniques chez les patients recevant une immunothérapie. Des recherches sur des algorithmes combinant les informations sur les concentrations de peptides pourraient démontrer le potentiel des tests iMRM et LC-MS optimisés. Les tests basés sur la spectrométrie de masse comme les iMRM optimisés pourraient s'avérer une alternative efficace à l'IHC de PD-L1.

CONTRIBUTIONS OF AUTHORS

Neda Boushehri, DDS

I wrote the entirety of all sections of this thesis. I analyzed approximately 100 of the 175 samples and performed data analysis within R to obtain the final results for the project. I created the final versions of all figures using customized R scripts and combined the figures myself where necessary.

Dr. Christoph Borchers, MD

Dr. Borchers supervised me throughout the project, helped me prepare for various conference abstracts, and gave me consistent feedback on my results and the figures I was creating. He also provided feedback on the body of the thesis as well as helped me understand the nuances of my results.

Vincent Lacasse

Vincent Lacasse helped me greatly throughout the duration of my thesis project. His work developing the assay was key to the success of my project. He helped me with sample preparation as well as coding within Skyline and R. He also provided feedback on numerous abstracts and visualizations, as well as comments on the methods section of this thesis.

Other committee members

My Master's committee members provided essential feedback on my proposed project before, during, and after my master's committee meetings and will be instrumental in the preparation of the final academic manuscript.

Clinical collaborators

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ABBREVIATIONS

ACS - American Cancer Society

ACN - Acetonitrile

AFA - Adaptive Focused Acoustics

ALK - Anaplastic Lymphoma Kinase

BCA - Bicinchoninic Acid

CHAPS - 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate

COPD - Chronic Obstructive Pulmonary Disease

CTLA-4 - Cytotoxic T Lymphocyte Antigen 4

CV - Coefficient of Variation

EGFR - Epidermal Growth Factor Receptor

FA - Formic Acid

FFPE - Formalin-Fixed Paraffin-Embedded

IHC - Immunohistochemistry

ICP - Immune Checkpoint Protein

ICIs - Immune Checkpoint Inhibitors

IPA - Isopropanol

IRB - Institutional Review Board

KRAS - Kirsten Rat Sarcoma Viral Oncogene Homolog

LAG-3 - Lymphocyte-Activation Gene 3

LCK - Lymphocyte-Specific Protein Tyrosine Kinase

LLOQ - Lower Limit of Quantification

LOD - Limit of Detection

MRM - Multiple Reaction Monitoring

NAT - Natural Peptides

NSCLC - Non-Small Cell Lung Cancer

PAC - Protein Aggregation Capture

PD-1 - Programmed Cell Death Protein 1

PD-L1 - Programmed Death-Ligand 1

PD-L2 - Programmed Death-Ligand 2

pCR - Pathological Complete Response

PI3K - Phosphoinositide 3-Kinase

PNGase F - Peptide-N-Glycosidase F

PSBC - Phosphate Buffer Saline with CHAPS

PTMs - Post-Translational Modifications

RAS - Rat Sarcoma

RECIST - Response Evaluation Criteria in Solid Tumors

ROS1 - c-ros Oncogene 1

RT - Room Temperature

SAEs - Serious Adverse Events

SCLC - Small Cell Lung Cancer

SHP2 - Src Homology Region 2-Containing Protein Tyrosine Phosphatase-2

SIS - Stable Isotope Standards

SPD - Samples Per Day

TCR - T-cell Receptor

TIGIT - T-cell Immunoreceptor with Ig and ITIM Domains

TIM-3 - T-cell Immunoglobulin and Mucin-Domain Containing-3

TOF - Time-of-Flight

T-VEC - Talimogene Laherparepvec

ULOL - Upper Limit of Linearity

ZAP70 - Zeta-Chain Associated Protein Kinase 70

CHAPTER 1: INTRODUCTION

1.1: Cancer

Cancer is defined by the National Cancer Institute as "a disease in which some of the body's cells grow uncontrollably and spread to other parts of the body."(1) If the body fails to manage this cell growth, the body's tissues can be damaged leading to a variety of systemic problems and, ultimately, to death.

1.1.1: Cancer nomenclature, classification, and staging

General cancer nomenclature

To distinguish between the many different types of abnormal cell proliferation, cancers are typically named and classified by their origin organ and severity.(2) Benign tumors include adenomas (epithelial), fibromas (mesenchymal), and teratomas (mixed type). Malignant tumors are called carcinomas (epithelial) and sarcomas (mesenchymal). Adenocarcinomas, for example, are malignant cancers that occur in epithelial cells. Adenocarcinomas can occur in different places within the body, including the breast, the colon, the prostate, and the lung, though these sites can also be affected by other tumor types.(3-6)

Further classification: the example of lung cancer

Specific types of cancer are often classified further. Within lung cancer, for example, lung cancer is divided into adenocarcinomas (often found in the outer parts of the lungs and more common in non-smokers), squamous cell carcinomas (found in the central airways and more common in smokers), large cell carcinomas (a rarer cancer type that can occur in a number of different places throughout the lungs), and small cell lung cancer (SCLC, a cancer type that is

somewhat rare but grows and spreads faster than other types of lung cancer and is also more common in smokers). Historically, lung cancer tumors were simply split into SCLC and non-SCLC. In 2015, however, the World Health Organization decided to create a new and more detailed classification system specifically separating adenocarcinoma, squamous cell carcinoma, and neuroendocrine tumors rather than referring to them all as “non-SCLC.”(7)

Cancer staging

In addition to a classification based on origin organ and severity, cancer is also generally referred to using a staging system. These stages are crucial when evaluating patient’s prognoses and treatment options. Again, within lung cancer, for example, a patient’s tumor can be any one of five numbered stages (0, 1, 2, 3, and 4) with further categorization in substages using letters (A, B and sometimes C)(8). Staging primarily depends on the tumor's size and its spread to other areas, such as multiple lung lobes or lymph nodes. Stage 0, known as *in situ*, indicates that the tumor is very small and remains localized within the airway lining. A tumor that is visible but no larger than 3 cm is classified as stage 1A, while a size between 3 cm and 4 cm is considered stage 1B. Stage 2 encompasses tumors that measure between 3 cm and 7 cm with little to no lymph node involvement. Stages 3A, 3B, and 3C involve tumors larger than 5 cm with more significant local lymph node invasion. Stage 4 is characterized by metastasis, where the cancer has spread beyond the lung.

1.1.2: Epidemiology of lung cancer

Samples from patients with a variety of different cancer types including lung, breast, pancreatic, oral, esophageal, and several other cancer types were included in this research. While

a full overview of the epidemiology of all these different cancer types is beyond the scope of this research, we have elected to provide an overview of the epidemiology and treatment options available for lung cancer (as it is the most common cancer type in our samples) to provide some context for potential benefits of a better risk evaluation tool for immunotherapy.

Global burden of lung cancer

Lung cancer is a significant public health concern globally, characterized by high morbidity and mortality rates. In 2020, there were approximately 2.2 million new cases of lung cancer worldwide, accounting for 18% of all cancer deaths.(9) In Canada, lung cancer remains the most diagnosed cancer and the leading cause of cancer death.(10) The economic impact of lung cancer on the Canadian healthcare system is substantial. An analysis of the 2021 economic burden of different types of cancer across Canada found that lung cancer had the largest direct costs (2.7 billion CAD) and indirect costs (243 billion CAD).(11) A great deal of research has been done to explore the etiology of, and treatments for, lung cancer.

Causes of lung cancer

There are a number of toxins and environmental exposures known to have causal relationships with the development of lung cancer. Smoking tobacco, for example, was suspected as a cause of lung cancer based as early as 1930s and 1940s based on the results of studies using nonexperimental data. Currently, smoking is estimated to increase the risk of lung cancer by approximately 20 times (with even second-hand smoke increasing the risk by about 25%).(12) Environmental exposures such as radon and asbestos have also been shown to increase the risk of lung cancer with multiplicative effects in smokers.(13) Respiratory diseases are another

potential cause of lung cancer.(14) Patients with chronic obstructive pulmonary disease (COPD), for example, appear to have an elevated risk of lung cancer even after accounting for their higher rates of smoking(15). Asthma also appears to be associated with lung cancer,(16) as do different types of lung infections. While some of these associations may be the result of greater surveillance and screening within populations with health conditions impacting the lungs, each has also been linked to immune dysfunction (a major carcinogenic factor).(17)

1.1.3: Lung cancer treatments

Surgery

As one might expect, one of the most effective ways to eliminate the threat posed by cancer is removing the cancerous tissue from the body via surgical intervention.(18, 19) With early-stage lung cancers (e.g., stage 0 or 1), the cancerous tissue can be removed via endobronchial therapy with no need to remove large portions of the lung. Stage 2 and 3A tumors, on the other hand, typically require entire lobes to be removed. In later cancer stages (e.g., 3B, 3C, or 4) where the cancer is no longer localized to a small area and which tends to be more common in lung cancer patients, alternative treatments are necessary.

Radiation and chemotherapy

In cases where surgical interventions are not feasible, radiation and chemotherapy have historically been the most widely used treatments among lung cancer patients.(19, 20) While treatment durations and modalities vary depending on specific cancer stages, radiation therapy(21) with or without platinum-based adjuvant chemotherapy is currently accepted as best practice in treatment of lung cancers that cannot be treated with surgery along, and the most

common chemotherapy regimens have historically been platinum-based.(22, 23) These treatments make the body inhospitable for tumor cells but also cause substantial damage to the rest of the body. Due to the toxic effects of these treatments, alternative options are constantly being investigated.

Targeted therapy and angiogenesis inhibitors

"Molecular profiling" is a process involving in-depth analysis of tumor tissue.(24) After creating these profiles, several different therapies specifically **target** (hence "targeted therapies) different aspects of the tumor. These include anti-EGFR antibodies(25) as well as small molecule tyrosine kinase inhibitors(26) that target different proteins within the tumor such as ALK, KRAS, or ROS1. Another option is angiogenesis inhibitors,(27) which can treat lung cancer by blocking the signaling pathways that generate new blood vessels which facilitate the growth of the tumor. These treatments still have considerable side effects, however.

Immunotherapy

Some of the most recent therapeutic additions to this suite of therapeutic options have been immunotherapies,(28) a general name for a wide class of drugs and treatments that help the immune system conduct its own battle against the tumor cells and may have fewer toxic side effects than radiation, chemotherapy, targeted therapies, or angiogenesis inhibitors. Immunotherapy includes a wide array of different types of molecules and includes everything from monoclonal antibodies (e.g., cetuximab)(29) to non-specific immunotherapies (e.g., CpG oligonucleotides)(30), to cancer vaccines to oncolytic viruses(31) (e.g., talimogene laherparepvec (T-VEC)), and to T-cell therapy and immune checkpoint inhibitors (e.g., pembrolizumab and

nivolumab).(32) This last category of drugs, which represent the focus of this work, are antibodies which bind to specific proteins to prevent tumor cells from inhibiting the immune system's typical paths to combating tumor growth.

1.2: The immune system and the regulation of cancerous cells

The immune system plays an essential role in the fight against cancer. Under ordinary conditions, the immune system identifies and eliminates atypical cells which may develop into tumors.(33) As part of this process, T-cells must identify which cells to eliminate using molecules referred to as immune checkpoint proteins (ICPs).(34)

1.2.1: Immune checkpoint proteins (ICP)

Simply put, when a T-cell's receptors see an abnormal antigen on the major histocompatibility complex of another cell, the T-cell begins acting to eliminate the cell presenting the abnormal antigen. If a T-cell detects an ICP (for example, PD-1 or CD28) and a corresponding ligand (for example, CD-80 or PD-L1) along with the abnormal antigen, however, the normal immune response is suppressed. This helps facilitate the body's delicate balance between activating and inhibiting immune responses to avoid damage to healthy tissues while fighting cancer.

One of the first identified ICPs was Cytotoxic T lymphocyte antigen 4 (CTLA-4), which was shown to function as an inhibitory molecule limiting T-cell responses in the early 1990s.(35) Today, several different immune checkpoint proteins have been identified, including PD-1 (Programmed cell death protein 1),(36) PD-L1 (Programmed death-ligand 1), LAG-3 (Lymphocyte-activation gene 3), TIM-3 (T-cell immunoglobulin and mucin-domain containing-

3), and TIGIT (T-cell immunoreceptor with Ig and ITIM domains). Notably, while all ICPs act to prevent cytotoxic actions against the target cell, each interferes with the process in a unique way.

1.2.2: Immune checkpoint inhibitors (ICIs)

Mechanisms and therapeutic roles of ICIs

Immune checkpoint inhibitors (ICIs) are immunotherapy treatment options that interfere with the activity of ICPs.(32) ICIs are typically monoclonal *hybrid* or *humanized* antibodies, meaning they are engineered from animal antibodies. These antibodies (such as pembrolizumab, nivolumab, atezolizumab, cemiplimab, and durvalumab) are designed to target the immune checkpoint proteins on the T-cell such as PD-1, PD-L1, and CTLA-4.(37) After binding to their respective ICPs, ICIs block interactions with the "natural" ligands expressed by the antigen-presenting cell, reducing inhibitory signals and aiding the immune response's ability to destroy abnormal cells.(32)

These types of interactions often involve a number of different proteins (see **Figure 1**). When the PD-1 on T-cells binds to PD-L1 on tumor cells, for example, it recruits the protein SHP2(38). The recruitment of SHP2 in turn leads to dephosphorylation of downstream signaling proteins of the TCR such as LCK, ZAP70, RAS and PI3K to dampen T-cell activation and function(39). This

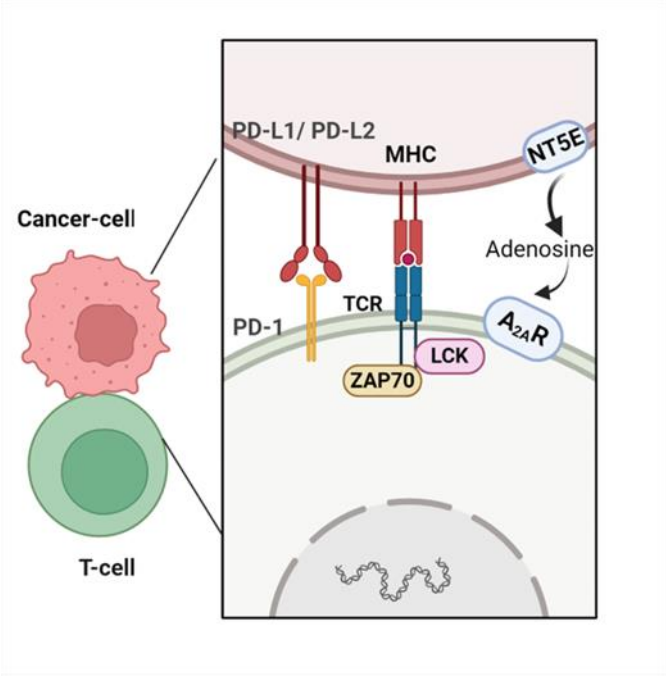

Figure 1: Graphical representation of how the interactions between T-cells and cancerous cells are mediated by components of the PD-1/PD-L1 axis.

negative regulation of T-cell activation mechanism has been shown to be a key factor in immune evasion by tumors. Simultaneously, NT5E (sometimes referred to as CD73) can generate adenosine to further suppress the immune response and contribute to the growth of the tumor, and PD-L2 (an alternative ligand for PD-1) can result in immune suppression even in the absence of high concentrations of PD-L1.(40, 41)

Interfering with any component of this pathway has the potential to boost immune response to tumors. Topalian et al.(42) showed in 2012 that preventing PD-1 from binding to the PD-L1 on tumor cells was one way to enhance T-cell responses against tumors and had the potential to improve patient outcomes across a wide variety of cancer types. ICIs have been shown to be particularly beneficial in the treatment of melanoma, lung cancer, renal cell carcinoma, and head and neck cancers(43).

Response rates to ICIs

ICIs have been shown to be associated with improved survival overall and durable response in randomized trials. A meta-analysis done by Gettinger et al concluded that nivolumab treatment could result in increases in long-term overall survival (13.4% of 5-year overall survival rate with nivolumab treatment compared to 2.6% for those receiving docetaxel, a standard chemotherapy agent) and durable responses for patients with pretreated advanced non-small-cell lung cancer (NSCLC)(44). However, ICIs, like any medical intervention, do not work in all patients. For example, a study by Cai et al examining ICI efficacy in patients with lung cancer treated prior to surgical resection estimated a pooled complete pathological complete response (pCR) rate of 39%. Within that same meta-analysis, 97 patients (29%) experienced serious adverse events (SAEs)(45). These side effects can lead to significant morbidity and mortality,

meaning that the best solution is to provide ICIs only to patients who are most likely to benefit. At present, the only way to identify potential responders is through predictive biomarkers and PD-L1 immunohistochemistry (IHC).(46, 47)

1.2.3: Biomarkers and predicting response to ICIs

Biomarkers are an essential part of drug and medical device development, especially for targeted therapies. Unfortunately, the term "biomarker" is often used without being clearly defined. In 2017, the US National Institutes of Health and the US Food and Drug Administration sought to remove that ambiguity by formally defining a biomarker as "a characteristic that is measured as an indicator of normal biological processes, pathogenic processes or responses to an exposure or intervention."(48) In the context of response to ICIs, biomarkers should be something that can be measured prior to treatment which will successfully separate responders from non-responders, with PD-L1 expression within the tumor cells in different cancer types such as Non-Small Cell Lung Cancer (NSCLC), Melanoma, and Breast Cancer being the most examined biomarker to date.(49)

1.3: Immunohistochemistry

Generally speaking, immunohistochemistry (IHC) is a technique used by pathologists to check for signs of disease following a biopsy.(46, 47) The pathologist uses antibodies to detect antigens in a tissue sample, with the antigens acting as markers indicating the presence of a particular disease. When the antibody recognizes the antigen, it binds to it. If binding occurs, the tissue sample is stained, allowing the antigen to be visualized under a microscope. In the context of PD-L1 expression, validated IHC assays like 28-8 (Dako), 22C3 (Dako), SP142 (Ventana),

and SP263 (Ventana) are commonly used in measuring PD-L1 expression in tumor cells and immune cells to diagnose and prognosis of cancer disease, and predict treatment response to ICIs.(50-52)

1.3.1: Known issues with immunohistochemistry as a predictive biomarker

Lack of standardization

Unfortunately, the above-mentioned IHC assays exhibit variable sensitivity and specificity resulting from their use of differing antibodies and scoring systems. This variation in turn leads to substantial variability between assays whether a particular patient will be classified as an ICI responder or non-responder(53). For example, Hirsch et al.'s Blueprint study demonstrated significant discrepancies in PD-L1 expression assessment among IHC assays, with up to 37% disagreement regarding patient eligibility for immunotherapy(54). As a result of this inconsistency, standardizing PD-L1 expression as a predictive biomarker for ICI response has been extremely difficult.

Other issues with IHC

Standardization is not the only problem with relying entirely on PD-L1 IHC as a method for predicting response to ICIs. First, tumors can display heterogeneous expression of PD-L1 throughout the tumor so that even *within* a single tumor sample some portions may express high levels of PD-L1 whereas others show little to no PD-L1 expression(55). In 2020, Tuminello et al demonstrated how the potential for heterogeneous PD-L1 expression across tumors can lead to misclassification of non-responders as responders and vice versa(56).

Second, it is true that the probability of response to immunotherapy increases with higher PD-L1 expression, but this association is not universal and PD-L1 expression levels do not always correlate perfectly with clinical outcomes. Fundytus et al (2021) and Davis et al (2019) both identified cases in which patients with low PD-L1 expression responded to immunotherapy, while others that appeared to have high PD-L1 expression did not(57, 58). As a result, relying solely on PD-L1 IHC as a predictive tool for identifying patients eligible for ICI can result in inappropriate treatment decisions and reduced overall effectiveness of immunotherapy. If an improved predictive tool or additional biomarker were able to be identified, it could result in more effective, safer, and less costly use of immunotherapy.

1.3.2: Post-translational modifications of PD-L1 and PD-1

Proteins like PD-L1 and PD-1 undergo a specific form of post-translational modification known as *glycosylation.*(59) Post-translational modifications, or PTMs, occur when proteins are altered by enzymes or other process after protein translation. A single PTM can alter a protein's activity, its structure, and its capacity for interaction with other molecules either within or outside the cell.(60, 61) More than 400 PTMs have been identified for a wide range of proteins. They include sumoylation, ubiquitination, nitrosylation, acetylation, and-most importantly for the purposes of this project-glycosylation.(62)

Glycosylation is an enzymatic process involving the formation of molecules known as glycoconjugates via the addition of sugar or carbohydrate chains (known as glycans) to target molecules (usually proteins or lipids). The two primary classes of glycoconjugates include N-linked and O-linked glycoproteins, where glycans are covalently attached to a polypeptide backbone via a nitrogen atom (N) of asparagine (Asn) or an oxygen atom (O) of serine (Ser) or

threonine (Thr). Glycans that have undergone glycosylation with nitrogen are referred to as N-glycans, while those that undergo glycosylation with oxygen are referred to as O-glycans.(60)

When PD-L1 and PD-1 undergo glycosylation, it changes both the function and stability of the two proteins. Specifically, N-linked glycosylation of PD-L1 contributes to approximately half of its observed molecular weight of around 33 kilodaltons (kDa)(63). Lee et al. hypothesized that heavy glycosylation of PD-L1 might hinder recognition of polypeptide antigenic regions by PD-L1 diagnostic antibodies. Because these heavily glycosylated regions would be less accessible to antibody binding, inaccurate PD-L1 bioassay readouts in some patient samples might result in inaccurate results and conflicting or incorrect therapeutic predictions. Heng-Huan Lee and the colleagues demonstrated that enzymatic digestion to remove PD-L1 N-linked glycosylation from tissue samples increased antibody-based PD-L1 detection and had the capability to avoid false-negative IHC readouts(64). This argues for deglycosylation of PD-L1 before quantification as a more accurate method for the quantification of PD-L1 expression than conventional IHC, and measuring after deglycosylation would therefore more accurately identify patients who would receive the most benefit from ICIs.

1.4: Liquid chromatography and mass spectrometry

One alternative to IHC that could address the disadvantages discussed above while accounting for the impacts of the glycosylation of PD-L1 and PD-1 proteins on clinical response is the use of mass spectrometry (MS). MS is an analytical technique that can be used for analyses for qualitatively identifying and quantifying the amount of a diverse array of substances in a given panel.(65)

1.4.1: An overview of mass spectrometry

While initially developed in a chemistry context, MS has gone on to be used in clinical settings specifically focusing on a wide variety of biologically relevant molecules ranging from proteins to peptides and to other metabolites(66, 67). Data obtained from MS is primarily expressed in terms of the mass-to-charge ratio (m/z), where "m" represents the molecular weight of the ion in Daltons and "z" indicates the number of charges on the molecule examined in the analysis(66). Small molecules under 1000 Daltons, when analyzed by electron impact, typically possess only a single charge, meaning that the mass-to-charge ratio is equal to their molecular weight. Larger molecules, like proteins, analyzed by “soft ionization” methods such as electrospray ionization or matrix-assisted laser desorption/ionization (MALDI) often carry multiple charges and will typically have a mass-to-charge-ratios less than their molecular weights. Because MS can detect a wide range of molecular weights and molecular types, it is an invaluable tool in the analysis of complex biological samples, allowing for comprehensive biomarker discovery and profiling in the context of immuno-oncology.(68)

1.4.2: Mass spectrometry as a superior tool for detailed protein analysis

In the 1990s, advances in MS techniques allowed the targeting of biomolecules, and enhanced its sensitivity and reliability, making it a better tool for detailed protein analysis than older more traditional gel-based methods like Western Blotting and SDS-PAGE. These advances made MS essential for the study of the proteome (a term introduced by Marc Wilkins in 1995 referring to complete sets of proteins produced by cells, tissues, and organisms)(69). The sensitivity and the consistency of the results from biological MS analyses has allowed MS to play a key role in the characterization of recombinant proteins, the identification proteins with

high accuracy, and in the detection and analysis of post-translational modifications (PTMs). MS-based proteomics has thus emerged as a major asset for immuno-oncology as it offers important insights into patient responses to therapies and the biological reasons for these responses. Because of its high sensitivity and its ability to detect subtle changes in molecular mass it is particularly valuable for immuno-oncology, as shown by Scigelova and Makarov.(70)

1.4.3: An overview of liquid chromatography

In order to apply mass spectrometry to a tissue sample, the different proteins, peptides, and small molecules must be somehow separated from one another. One of the most common methods to achieve this separation is liquid chromatography (LC)(71). This chromatographic approach relies on separating analytes between two phases, a liquid mobile phase and a stationary phase(71, 72). The method was first used early in the 20th century by Russian botanist Mikhail Tsvet. While researching plant pigments, he used a column filled with solid adsorbents to separate the different pigments from one another(72), and column chromatography is still the most common form of chromatography, altho the columns and particle sizes used for these separations are now much smaller in diameter. Two distinct phases are used for column chromatography: the first phase (the stationary phase) is composed of solid particles or beads tightly packed into a column. The second phase (the mobile phase) is a liquid solvent carrying the mixture of analytes that, together, are pumped through the column comprising the stationary phase. As the mobile phase goes through the column, the different analytes each interact with the solid particles within the column, separating them from one another based on their molecular size, and (in reversed-phase chromatograph) their polarity and their affinity for water.(73-75)

1.5: Key considerations for protein and peptide analysis with LC-MS

There are several essential considerations when using LC-MS for protein and peptide detection and quantification. These include deciding whether to target specific proteins, selecting the appropriate chromatography column, ionization method, and understanding the specific characteristics of different mass analyzers.

1.5.1: Targeted vs. untargeted mass spectrometry

There are two different approaches to analyzing proteins with mass spectrometry: targeted and untargeted proteomics.

Targeted proteomics:

In targeted proteomics, the focus is on analyzing specific proteins or peptides of interest. Typically, researchers utilize labeled standards and sometimes tailored antibodies to determine the concentrations of the specific proteins of interest. Because this approach uses well-understood standards and specific antibodies, it generally has relatively high sensitivity and specificity for detecting the presence or absence of the proteins of interest, and their amounts. The use of labeled internal standards also allows *absolute quantification* (e.g., obtaining concentrations of a given protein or peptide in terms of femtomoles per microgram)(76-78). One such targeted method is multiple reaction monitoring (MRM), and the analogous parallel reaction monitoring (PRM) method, performed on triple-quadrupole or Orbitrap instruments, respectively.(79, 80). MRM was originally developed to quantify small molecules and uses triple-quadrupole instruments to obtain high sensitivity and specificity while monitoring

precursor and product ions, and can be especially useful when analyzing biomarkers, particularly in complex biological systems.(79)

Untargeted proteomics:

Untargeted proteomics aims to analyze and evaluate every protein within a sample with no prior knowledge about the proteins with that sample. Because untargeted proteomics involve examining the entire range of proteins, it can be extremely difficult to identify rare proteins that are only present in small concentrations. That being said, the fact that it does not require labeled standards or targeted antibodies makes untargeted approaches ideal for finding other proteins in a sample, understanding the central biological paths in a given biological response or therapy response, and identifying when various conditions resulting in changes in protein expression.(81, 82)

Combining targeted and untargeted proteomics

A holistic approach that integrates both targeted and untargeted proteomic techniques is a key part of discovering, and subsequently validating, the clinical utility of different biomarkers. Conventionally, research into biomarkers using MS starts with untargeted proteomics to identify potentially useful biomarkers, followed by targeted assays to validate the performance of those biomarkers. This allows the highest potential of identify new and interesting biomarkers (thanks to the wide range of the untargeted approach). (83, 84)

1.5.2: Mass spectrometry ionization methods

In order to measure the mass of a specific molecule and identify how often it is detected within a given sample using MS, the molecules must be ionized (i.e., given an electrical charge). After charging, molecules are guided and separated based on their mass-to-charge ratio before they ultimately reach a detector. These detectors then generate electrical signals in proportion to the quantity of the molecules within the sample.(85) While a number of ionization techniques are available in mass spectrometry, "soft ionization techniques" (which involve the addition of protons to the target molecules) are typically used in immuno-oncology to prevent degradation and denaturing of proteins and peptides. The two most prominent "soft ionization techniques" are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI).(86, 87)

Electrospray ionization:

In ESI, protons (i.e., H^+ ions) are added to the molecules in an acidic solution, giving those molecules a positive charge(88). A basic solution could also be used, in which case, protons are removed to generate negatively charged molecules. Because the number of protons added can vary depending on a molecule's structure, multiple protons are typically attached to a given molecule, creating multiply charged ions. In electrospray ionization, molecules are sprayed from the tip of a capillary needle at high voltage, which results in a fine spray of droplets in a heated chamber at which point the solvent evaporates in a process called **desolvation**. After desolvation, all that remains are the ionized molecules in a gas phase. Placing an electric field of opposite charge (negative for positively charged ions, positive for negatively charged ions) at the entrance to the mass spectrometer then draws desolvated ionized molecules into the vacuum

system of the instrument, at which point they are analyzed based on their mass-to-charge ratio.(89, 90)

1.5.3: Different types of mass analyzers

A number of different types of mass analyzers are used in mass spectrometry, each with its own unique advantages and disadvantages including orbitrap, time of flight, and quadrupole analyzers.(91) Our analysis used quadrupole analyzers.

Quadrupoles:

Quadrupoles are valued for their robustness, low maintenance, and reliability, making them ideal for quantification in many routine applications especially in targeted proteomics, where quantification of specific peptides or proteins is required.(92, 93). Quadrupoles consist of four electrodes, with two fixed and two oscillating.(94) The fixed pair keeps ions in a defined plane, while the oscillating pair causes ions to move in a wave-like pattern. The amplitude of this wave depends on the ion's mass and charge, which allows the analyzer to filter ions based on their m/z ratio. Molecules that are too heavy or too light will collide with the rods or be ejected from the analyzer. Quadrupoles excel where reliability and cost-effectiveness are important. This makes quadrupoles particularly popular in clinical settings and high-throughput analysis, where frequent maintenance and recalibration are less desirable.(95)

1.5.4: Chromatography column:

Choosing the right column for LC-MS in protein and peptide detection is important for achieving optimal separation, sensitivity, and reproducibility. Here we chose C18 (an 18-carbon

alkyl chain) column which is a type of stationary phase consisting of silica particles with non-polar hydrocarbon chains (octadecyl chains, containing 18 carbon atoms) chemically bonded to their surface, creating a hydrophobic surface used in reverse-phase chromatography and making it a great choice for peptide separation due to its strong hydrophobic interactions.(96)

1.6: Tandem mass spectrometry, protein sequencing, and fragmentation analysis

Tandem mass spectrometry (MS/MS) is a key technique for protein or peptide sequencing and the analysis of post-translation modifications.(86) By separating, fragmenting, and analyzing ions, tandem mass spectrometry enhances the ability to accurately identify and characterize proteins or peptides, even those with very similar masses. Most research-grade mass spectrometers utilize MS/MS, which typically comprises three main components.(97)

First, a mass analyzer separates ions based on their mass-to-charge ratio. Measuring the mass of a molecule provides valuable information for identification purposes. However, many molecules have very similar masses, making it challenging to distinguish between them. To address this challenge, selected ions are subsequently directed into the collision cell—a chamber where a neutral gas is introduced to induce high-energy collisions(98). These collisions fragment the molecules, causing fragmentation of the peptide amide backbone at the CO–NH bonds and generating a series of b- and y-type fragment ions (the nomenclature being based on whether the charge is on the N- or C-terminus of the peptide(99). This fragmentation enables more precise

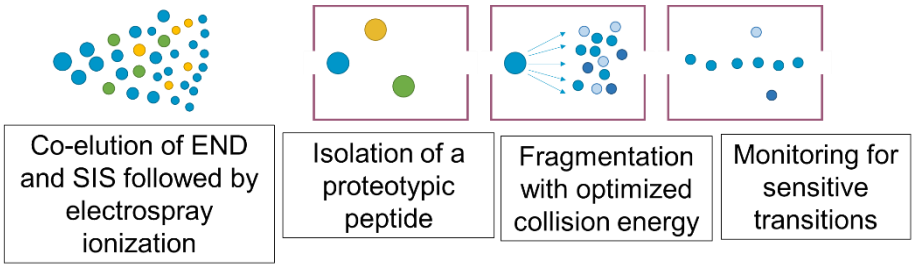

Figure 2: Electrospray ionization and key steps in the mass spectrometry process.

identification since peptides tend to fragment between amino acids, so a partial amino acid sequence is obtained. The fragmented ions are then further analyzed by a second mass analyzer to provide detailed molecular information based on the fragments. After this stage the data generated from the MS/MS analysis can be interpreted to identify and quantify peptides, proteins, and PTMs.(100, 101)

1.6: Summary and objectives

The global burden of cancer is high, and new treatments and therapies are constantly being developed to help reduce morbidity and mortality. One such therapy, immunotherapy, helps boosts the body's natural response to fighting off malignancies. However, immunotherapy is not always the best choice for every patient because some patients may experience immune-related adverse effects or may not receive any benefit from the therapy. As a result, biomarkers like PD-1 and PD-L1 (both highly expressed across different cancer types) are needed to predict which patients will clinically benefit. While PD-L1 expression as measured by immunohistochemistry (IHC) is the only approved companion diagnostic for immunotherapy, relying on IHC alone has limitations. These include a lack of antibody specificity, the qualitative nature of the assay, issues preserving samples, and problems properly detecting post-translational modifications like glycosylation. This approach also fails to leverage the knowledge that the PD-1/PD-L1 pathway includes more than just those two proteins, and in fact includes PD-L2, NT5E, LCK, and ZAP70, all of which could be investigated and evaluated with a multiplexed assay. A better method could also address the current limitations of IHC by using immuno-multiple reaction monitoring to measure other constituents of the PD-1/PD-L1 signaling pathway.

Objectives:

Our goal was to evaluate the ability of immuno-multiple reaction monitoring to quantify components of the PD-1/PD-L1 signaling pathway in samples from tumors of patients with various cancers, by checking correlations between the concentrations of different peptides. We also aimed to evaluate the extent to which values of those components were associated with clinical benefit and overall survival after patients were treated with immunotherapy (specifically, immune checkpoint inhibitors) by obtaining clinical information on the patients from which the samples were obtained.

CHAPTER 2: METHODS

2.1: Data source and sample collection

Patients with different types of cancer receiving care within the Allegheny Health Network in Western Pennsylvania were recruited for the study. After patients provided informed consent, their tumor cells were released to be analyzed at the Lady Davis Institute in Montreal, Quebec, Canada. The project received ethical approval from the institutional review board of the approved by the Jewish General Hospital Research Ethics Board (IRB number: MP-37-2021-7721). If patients received at least one dose of pembrolizumab, nivolumab, durvalumab, atezolizumab, or the equivalent brand-name immunotherapies, they were categorized as "immunotherapy-treated." Samples from the tumors were preserved within formalin-fixed paraffin embedded blocks in a tissue sample archive. Hematoxylin and eosin staining was performed after sectioning on each block to identify tumor-rich sections and 1-millimeter cores were punched through these section, which were in turn sent to the Lady Davis Institute and housed at 4 degrees Celsius.

2.2: Mass spectrometry assay parameters

We used a previously developed targeted multiplexed immuno-MRM method developed by Lacasse et al. This method was developed and optimized for the quantitation of PD-L1, PD-L2, NT5E, PD-1, LCK, and ZAP70 proteins and focused on 9 tryptic peptides (8 non-modified peptides and 1 glycosylated peptide, **Table 1**). Peptides were chosen for their uniqueness, detectability, and digestion efficiency based on bioinformetric information obtained from Peptide Picker(102). The resulting set of peptides (four PD-L1 related peptides and five additional peptides associated with each of PD-L2, NT5E, PD-1, LCK, and ZAP70) was chosen to

minimize LC-MS run time without sacrificing the sensitivity, precision, or recovery of the fully multiplexed assay.

Table 1: Peptide sequences and information on the quantities and concentrations of heavy and medium stable isotope standards (SIS1 and SIS2), as well as the stock natural peptides, used in the assay.

Protein	Peptide modified sequence	PTM	SIS1 amount (fmol)	SIS2 amount (fmol)	Stock SIS (pmol/µl)	Stock SIS2 (pmol/µl)	Stock natural peptides (pmol/µl)
PD-L1	NIIQFVHGEEDLK	Non-modified	0.62	16	1	1.1	1.2
PD-L1	LQDAGVYR	Non-modified	0.62	40	1	2.8	1.2
PD-L1	LFNVTSTLR	Glycosylated	0.62	16	1	1.1	1.2
PD-L1	LFN[+1]VTSTLR	De-glycosylated	1.9	16	3	1.1	2.8
PD-L2	ATLLEEQLPLGK	Non-modified	1.9	40	3	2.8	4
PD-1	LAAFPEDR	Non-modified	0.62	40	1	2.8	1.2
LCK	ITFPGLHELVR	Non-modified	5.6	16	9	1.1	2.4
ZAP70	LIATTAHER	Non-modified	1.9	40	3	2.8	4
NT5E	GPLASQISGLYLPYK	Non-modified	8	50	13.5	3.4	6.8

In the final assay, the internal standards were mixed at concentrations such that they would be approximately three times the respective lower limit of quantification for stable isotope labeled standard 1 (SIS1) and approximately two to three times the upper limit of linearity for stable isotope labeled standard 2 (SIS2). SIS1 mix and SIS2 mix were specifically chosen in such a way that the peptides with the lowest concentration would be at at least at 1 picomole per microliter in the stock solution.

The analytical setup was an Agilent 1290-6495A LC-MS system with flow-rate of 400 400 µL/min and a 2.1mm inner diameter Zorbax C18 column. This setup is representative of

robust triple quadrupole LC-MS setups routinely being used in clinical laboratories. **Table 2** lists values for the limit of detection (LOD), which was set at the average blank value plus three standard deviations, the lower limit of quantification (LLOQ) which was set at the lowest point of the receiver operating characteristic (ROC) curve that yielded an accuracy between 80% and 120%,

and the upper limit of linearity (ULOL) which was set at the corresponding highest part of the curve, as recommended by CPTAC.(103). Both the intra-day and inter-day reproducibility of the assay were below 10% and 13%, respectively, for all peptides.

Table 2: Peptide sequences and LC-MRM assay showing the assay's quality and reliability as represented by the limit of detection (LOD), lower limit of quantification (LLOQ), upper limit of linearity (ULOL), and coefficient of variation (CV).

Protein	Peptide modified sequence	PTM	LOD (fmol)	LLOQ (fmol)	ULOL (fmol)	Average intra-day CV (%)	Average inter-day CV (%)
PD-L1	NIIQFVHGEEDLK	Non-modified	0.1681	0.4115	20	10±10	13±8
PD-L1	LQDAGVYR	Non-modified	0.0047	0.0457	50	4±2	4±2
PD-L1	LFNVTSTLR	Glycosylated	0.0092	0.0457	20	4±2	6±3
PD-L1	LFN[+1]VTSTLR	De-glycosylated	0.0086	0.0457	20	4±2	6±2
PD-L2	ATLLEEQLPLGK	Non-modified	0.1210	0.4115	50	4±2	5±2
PD-1	LAAFPEDR	Non-modified	0.0315	0.0457	50	6±4	7±3
LCK	ITFPGLHELVR	Non-modified	0.3217	3.7037	20	3±1	4±2
ZAP70	LIATTAHER	Non-modified	0.0822	0.4115	20	5±2	6±2
NT5E	GPLASQISGLYLPYK	Non-modified	3.6008	11.1111	50	4±2	5±3

2.3: Sample preparation

2.3.1: Anti-peptide immunoprecipitation

For each peptide in the assay, 8 antibodies [Ab] targeting 9 individual peptides (8 non-modified peptides and one glycosylated peptide were obtained from Signatope GmbH (Reutlingen, Germany).

2.3.2: Deparaffinization

Excess paraffin was eliminated from each FFPE scroll. Then, 1 ml of MS-grade water at 80°C was added to each scroll for incubation for 1 minute at the same temperature while shaking at 2000 rotations per minute. After shaking, samples were centrifuged at 20,000 x g for 2 minutes at room temperature and the supernatant containing the excess paraffin was discarded. This 80°C wash step was repeated, at which point cores were transferred to new 1.5 ml LoBind Eppendorf tubes.

2.3.3: Protein extraction

To perform protein extraction, we added 250 µl of extraction buffer containing 50 mM Tris (pH 8.5), 2% sodium deoxycholate and 10 mM tris(2-carboxyethyl) phosphine to each sample. This scroll was then finely crushed in a 1.5 ml LoBind Eppendorf tube using a disposable pestle until no pieces were visible. The resulting homogenate was transferred to a 130 µl adaptive focused acoustic (AFA) microtube (specifically, a Covaris 520185) and subjected to AFA sonication for 5 minutes in a Covaris M220 focused-ultrasonicator with the chiller set to 20°C, the water level at 15 peak, incident power at 50W, the duty factor at 20%, the cycle burst at 300, and the instrument temperature held constant at 20°C. After this, samples were incubated

at 99°C for 20 minutes while shaking at 700 rotations per minute in a thermomixer. This was followed by a second AFA sonication for 6 minutes using the same parameters. The sonicated samples were then incubated at 80°C for 2 hours while shaking at 800 rpm. After the second incubation, samples were cooled on ice for 1 minute and then centrifuged for 15 minutes at 21,000 x g at 4°C. The supernatant was then carefully transferred to a new 1.5 ml LoBind Eppendorf tube. Finally, the protein concentration was measured using the bicinchoninic acid assay with a Pierce reducing-agent-compatible kit (category 3250).

2.3.4: Reduction and alkylation

A total of 80 µg of protein was placed into a 1.5 ml LoBind Eppendorf tube. These samples were heated to 60°C for 15 minutes with shaking at 800 rotations per minute. After this, 30 mM iodoacetamide was added to each sample and the sample was incubated for 30 minutes at room temperature without light. A quench reaction was then performed by adding tris(2-carboxyethyl) phosphine to achieve a final concentration of 20 mM.

2.3.5: Deglycosylation

Samples were heated to 99°C for 10 minutes with shaking at 800 rotations per minute in the thermomixer. After shaking, we cooled them with ice for 1 minute and then centrifuged them for 10 seconds at 500 rotations per minute. A final concentration of 50 mM glyco buffer (NEB GlycoBuffer 2 [10X]) and 10% IGEPAL CA-630 (NP-40 substitute, Thermo Scientific p/n J19628-AP) was added to each tube, along with 2 µl of PNGase F (P0704) unless working on 50µg proteins or less, in which case1µl was PNGase F was added. Samples were then mixed gently by hand and incubated at 37°C for 1 hour without shaking.

2.3.6: Protein digestion

After deglycosylation, samples were digested after protein aggregation capture.(104) Briefly, amine functionalized magnetic beads (ReSyn Bioscience, MR-AMN005) were equilibrated with 70% acetonitrile (ACN) in water three times. The deglycosylated samples were added to the beads at a 1:20 protein-to-bead ratio and 100% ACN was added to give a final concentration of 70%. Samples were vortexed for 10 seconds and incubated at room temperature for 10 minutes to allow protein aggregation to occur. Samples were then washed with 95% ACN and 70% ACN once each, followed by addition of trypsin (Promega, V5117) at a 20:1 protein-to-trypsin ratio. Digestion proceeded overnight at 37°C without agitation in a final volume of 200 µl of digestion buffer composed of 50mM ammonium bicarbonate, 200mM guanidinium chloride, and 200µM calcium dichloride.

2.3.7: Acidification and SIS addition

To stop the digestion process, the samples were acidified with a 2% solution of trifluoroacetic acid. Next, stable-isotope labeled standards (both medium and heavy) were added at designated concentrations (as described in **Table 1**). The samples underwent desalting using in-house made R3-C18 stage tips and were subsequently dried after the elution step.

2.3.8: Antibody-bead conjugation and peptide immunoenrichment

Based on the previous work done by Vincent Lacasse, 9 antibodies that demonstrated the highest levels of recovery and reproducibility were acquired from Signatope GmbH (Reutlingen, Germany), including 8 antibodies targeting 8 non-modified peptides (LAAF-PD-1, GPLA-ZAP70, ITFP-LCK, LFDV-PD-L1, LQDA-PD-L1, NIIQ-PD-L1, ATLL- PD-L2) and one

antibody targeting a non-modified peptide (LFNV-PD-L1), covering 9 peptides in total. After choosing these antibodies, we conjugated them individually to the Magnetic Protein G Dynabeads (Invitrogen, 10004D) and incubated them for 1 hours at RT with end over end rotation (15-20 rpm). Beads were then washed 3 times with 1x phosphate buffer saline (Sigma Life Science, D1408) containing 0.02% 3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate (CHAPS); subsequently referred to as PSBC. Then, each antibody was added at a 0.4:1 weight-to-volume ratio and the final volume was adjusted to 240µl. Antibody-bead conjugation was tested at room temperature with end-over-end rotation at 15 rpm, after which the supernatant was removed and the beads washed once with PBSC. Then, we prepared 8-plex stock AB by pooling 30 µl of each Ab-bead conjugate into a LoBind tube. Finally, we resuspended peptides for each sample in 200µl of PBSC and added 20 µl of 8-plex mix to each sample and shake the tubes by hand. Peptide capture was performed overnight at 4°C with end-over-end rotation at 15 rpm.

2.3.9: Immunoaffinity wash and elution

The following day, beads were retrieved using the magnetic rack and the supernatant was transferred to a protein low-bind microtube for storage at -80°C. The beads underwent a wash with 200 µl of PBSC, were recovered, and then resuspended in 200 µl of 0.1x PBSC. To minimize CHAPS contamination, the suspension was transferred to a new tube before being washed again with 200 µl of MS-grade water. Peptides were eluted from the beads using 50 µl of immunoenrichment elution buffer (5% acetic acid and 3% ACN in water) and incubated in a thermomixer at room temperature (1300 rpm, 5 minutes). The beads were removed, and the

supernatant containing the target peptides was transferred to a new tube, dried by using a speedvac, and resuspended in 20 µl of 0.1% FA in water (LC phase A).

2.3.10: Preparation, conditioning, and handling of Evotips during sample analysis

Using a multichannel pipette, 100 µl of 1-propanol was added to each well of a 96-well microtiter plate (MTP). To wash Evotips, we transferred 20 µl of Solvent B (0.1% formic acid in acetonitrile) to all Evotips, which were then centrifuged at 700 x g for 60 seconds. We emptied the 96-well box for Solvent B after centrifugation. We then placed the Evotips in an adapter rack on top of the MTP containing 1-propanol, soaked for a minimum of 10 seconds, and visually inspected to ensure they turned pale white. During equilibration, 20 µl of Solvent A (0.1% formic acid in water) was added to each Evotip, followed by centrifugation at 700 x g for 60 seconds. For sample loading, the Evotips were returned to their original tray, and 20 µl of the sample was transferred to each tip. The loaded tips were centrifuged at 700 x g for 60 seconds. During the final wash step, 20 µl of Solvent A was added to each Evotip, followed by centrifugation at 700 x g for 60 seconds. To keep the tips wet, 100 µl of Solvent A was added, and the tips were centrifuged at 700 x g for 10 seconds. For storage, sufficient Solvent A was added to the tray to ensure that the bottoms of the Evotips were submerged, and the tray was stored at 4 °C, covered with a lid, until analysis.

2.4: Analytical Setup for LC-MS

We used natural (NAT) and stable isotope-labeled (SIS) versions of the analytes dissolved in solvent A in water to optimize the best settings for both the chromatographic separation and the mass spectrometry detection. Chromatography was performed using the EvoSep system. We

used the EV1064 Endurance column, which measures 8 cm in length with an inner diameter of 100 µm, maintained at an ambient temperature of 23 °C. The Evosep One system was configured for the 100 samples per day (100 SPD) method. The 100 SPD method featured a 13-minute gradient and a cycle time of 14 minutes. At the beginning of the day, we loaded the work method and installed the capillary while the column was disconnected. The binary pump was activated and run at 2% solvent B (0.1% formic acid in acetonitrile) for 30 minutes to remove any contamination, particularly if the system had previously been used with solvents containing isopropanol (IPA). The analytical column was equilibrated at a flow rate of 2 µl/min, while the gradient flow during operation was maintained at 1.5 µl/min.

For column clean-up, the concentration of solvent B was increased to 20% over 7.2 minutes, followed by a ramp-up to 80% solvent B for 0.8 minutes to wash the column. The column was then equilibrated at 2% solvent B for 5 minutes before the next injection. Operating pressures during sample runs ranged from 300 to 500 bar. The LC system was coupled online to a mass spectrometer, operated in dynamic MRM mode. The gas temperature was set to 150 °C, with a gas flow rate of 15 L/min. The nebulizer pressure was 30 psi, sheath gas temperature was 250 °C, and sheath gas flow rate was 11 L/min. The capillary voltage was set at 3,500 V, while the nozzle voltage was maintained at 300 V. The NAT, SIS, and SIS2 peptides exhibited identical retention times as expected.

2.5: MRM data analysis

First, we imported the raw mass spectrometry files from the analyzed samples into Skyline (an open source software for proteomics)(105) for data processing and quality control. Peaks were checked for misassignment by Skyline's automatic peak assignment tool and, since

three different transitions were monitored for each of the peptides (specific values listed in Appendix A), any mismatches in the order of transition peaks were flagged and removed. Finally, Skyline calculated the areas under the curve (AUCs) for each transition to be exported to R for processing, linkage to the clinical data, and visualization.

To simplify processing, we only examined the quantitative transition when exploring quantification. As an additional check, any NAT, SIS and SIS2 AUCs that were below the average AUC of the lowest point in the response curve (typically corresponding to one to two points below the limit of detection) were replaced with 0s. Quantification was based on NAT divided by the sum of SIS1 and SIS2 standards to obtain additional precision compared to relying on only one internal standard or using two different standards for internal calibration (as reported by Ibrahim et al (106). Because the total protein in each sample varied, the quantification results were normalized to the amount of total protein used to obtain final peptide concentrations as attomoles per microgram of total protein.

2.6: Statistical analysis

We were interested in three core questions: are peptide concentrations correlated with one another in the way that we would expect, are concentrations of any individual peptides associated with a higher probability of experiencing clinical benefit, and are higher-than-median vs lower-than-median concentrations of any individual peptides associated with longer survival times after starting immunotherapy.

2.6.1: Correlations between peptide concentrations

If the LC-MS system is working properly and we are obtaining accurate quantification of the concentrations of the different peptides, we should observe high correlations between the concentrations of pairs of peptides that are present within a given protein. To evaluate whether this is the case, we calculated log-transformed correlation coefficients(107) and associated p-values for each possible pair of PD-L1 constituent peptides (i.e., NIIQ, LQDA, and LFDV) using R version 9.4. Positive correlations imply that higher concentrations of one peptide are associated with higher concentrations of another, correlations close to 0 suggest so association between the peptides, and negative correlations suggest that higher levels of one peptide are associated with lower levels of another. We calculated these coefficients in the full cohort of patients as well as the patients with linked clinical data. In order to contribute to the estimation of this correlation coefficient, values of both peptides had to be above the limit of detection. We expected to observe very strong and very statistically significant correlations between these pairs of peptides.

We were also interested in evaluating whether there were correlations between the concentrations of these three PD-L1 associated peptides and the PD-1 associated peptide (LAAF), given that PD-L1 is a ligand for the PD-1 receptor. Just as when assessing correlations between the three peptides that were digestion products of PD-L1, we calculated log-transformed Pearson correlations between the concentrations of the three PD-L1-associated peptides (NIIQ, LFDV, LQDA) and LAAF, repeating the analysis once within the full cohort and once within the linked samples with clinical data. We expected to observe a positive linear correlation between these peptides and LAAF.

2.6.2: Correlations between peptide concentration and PD-L1 expression

In addition to checking whether peptides were correlated with one another, we were also interested in evaluating to what extent the concentration of each peptide was correlated with the IHC values within the clinical data among patients who received immunotherapy using a linear version of the PD-L1 expression in the clinical data and log-transformed peptide concentrations. If these correlations were weak, it would suggest that the peptide concentrations may contain additional information useful for predicting risk above and beyond IHC results.

2.6.3: Peptide concentrations and clinical benefit

After establishing the face validity of the assay and evaluating whether peptide concentrations are correlated with IHC, our goal was to determine the extent to which individual peptide concentrations were associated with clinical benefit among the patients that ultimately received immunotherapy. Clinical benefit was based on the clinical data using a modified version of the RECIST criteria(108). Patients with values of 1 or 2 for their immunotherapy regimen (indicating a partial or complete response) were classified as receiving clinical benefit, as were patients who had values of 3 but survived more than six months following immunotherapy initiation. Patients with values of 4 were classified as not benefiting. Patients who did not have response values for their immunotherapy in the linked clinical data, but survived more than six months following immunotherapy initiation, were also classified as receiving clinical benefit. We used this measure of clinical benefit to conduct t-tests comparing the concentration of each peptide (above the limit of detection) between patients who experienced clinical benefit and patients who did not benefit clinically after immunotherapy. We expected to observe potential associations between peptide concentrations and clinical benefit.

2.6.4: Peptide concentrations and overall survival

In addition to evaluating associations with clinical benefit as established by the RECIST criteria, we were also interested in assessing the extent to which concentrations of each peptide were associated with overall survival. We identified the two peptides that were most commonly above the limit of detection in patients who received immunotherapy and split the patients who received immunotherapy based on whether they were above or below the median concentration of that peptide. We then performed a survival analysis (censoring when patients were lost to follow-up) evaluating all-cause mortality after the date of immunotherapy initiation and used the log-rank(109) test to assess if patients above the median value of the peptide were statistically significant in terms of all-cause survival to patients below the median value of the peptide. For the sake of comparing the performance of single-peptide prediction with IHC, we repeated this analysis classifying patients as above or below 1% PD-L1 expression, as detected by whatever assay was used within the clinical data.

CHAPTER 3: RESULTS

3.1: Patient and cancer characteristics

In total, we received and analyzed samples from the tumors of 175 different patients diagnosed with cancer between April 2008 and April 2022. Of those 175 samples, 83 (47%) were linked to clinical records, including results for 44 female patients and 39 male patients. Most linked patients (58) had lung cancer, with the remaining 25 patients having cancer in a wide variety of sites including the breast, esophagus, larynx, pancreas, and kidneys. With respect to clinical staging, the most common (54) samples were from stage IV patients, followed by stage III (14 patients), stage II (7 patients) and stage I (6 patients), with the remaining 2 patients missing information on cancer stage. The median age at diagnosis of the linked patients was 68 years (interquartile range: 62, 76) with 52 out of the 83 patients being over the age of 65 when diagnosed. The median follow-up before patients died or were lost to follow-up (i.e., no longer received care within the Allegheny Health Network) within the clinical data was highly variable, with a range of 1 to 197 months, a median of 21 months, and an interquartile range of 6 to 40 months.

Of the 83 linked patients, 51 (61%) received immunotherapy at some point during their cancer treatment and 27 patients received immunotherapy as part of their first-line therapy. The most common type of immunotherapy was pembrolizumab (40 patients), with smaller numbers receiving nivolumab, avelumab, durvalumab, or atezolizumab. While death dates were known for 31 of these patients, the remaining 20 either survived until the time of linkage or were lost to follow-up. Overall, the follow-up times after diagnosis in patients who received immunotherapy were very similar to the full population with clinical data, with a median follow-up of 21 months, a range of 0-197 months and an interquartile range of 11 to 40 months.

3.2: Peptide quantification

We estimated the concentrations in attomoles per microgram for the nine peptides in three key groups of samples: all samples, samples with clinical data, and samples that received immunotherapy.

3.2.1: All samples

We analyzed a total of 175 samples. Because the LOD, LLOQ, and ULOL varied for each different peptide, the number of samples within the detectable range varied greatly. Of the PD-L1 peptides we examined, 161 (92%) of samples had detectable NIIQ, 140 (80%) of samples had detectable LQDA, and 106 (60%) of samples had detectable LFDV. With respect to peptides associated with other proteins, the PD-1 associated peptide was the next most commonly detectable (156 samples with detectable LAAF, 89%), followed by the NT53 associated peptide (134 samples with detectable GPLA, 77%), the LCK associated peptide (119 samples with

Table 3: Number of samples above the limit of detection (LOD) for each of the 9 different peptides among all 175 analyzed samples, along with ranges, medians, means, and standard deviations of their concentrations in attomoles per microgram (amol/µg).

	Number above LOD (%)	Range (in amol/µg)	Median (in amol/µg)	Mean (in amol/µg)	Standard deviation (in amol/µg)
NIIQ	161 (92%)	8.5 – 815.9	25.2	52.0	98.5
LQDA	140 (80%)	0.1 – 774.2	9.5	32.1	91.8
LFNV	17 (10%)	0.6 – 5.8	1.9	2.6	1.8
LFDV	106 (60%)	1.0 – 714.3	16.7	49.6	120.5
LAAF	156 (89%)	1.3 – 235.2	10.7	21.9	33.0
ITFP	119 (77%)	8.5 – 1310.9	64.3	137.6	185.6
ATLL	95 (54%)	2.9 – 375.7	14.6	27.1	47.2
LIAT	93 (53%)	2.8 – 283.7	15.2	35.9	48.3
GPLA	134 (77%)	50.2 – 1801.8	156.1	270.7	310.6

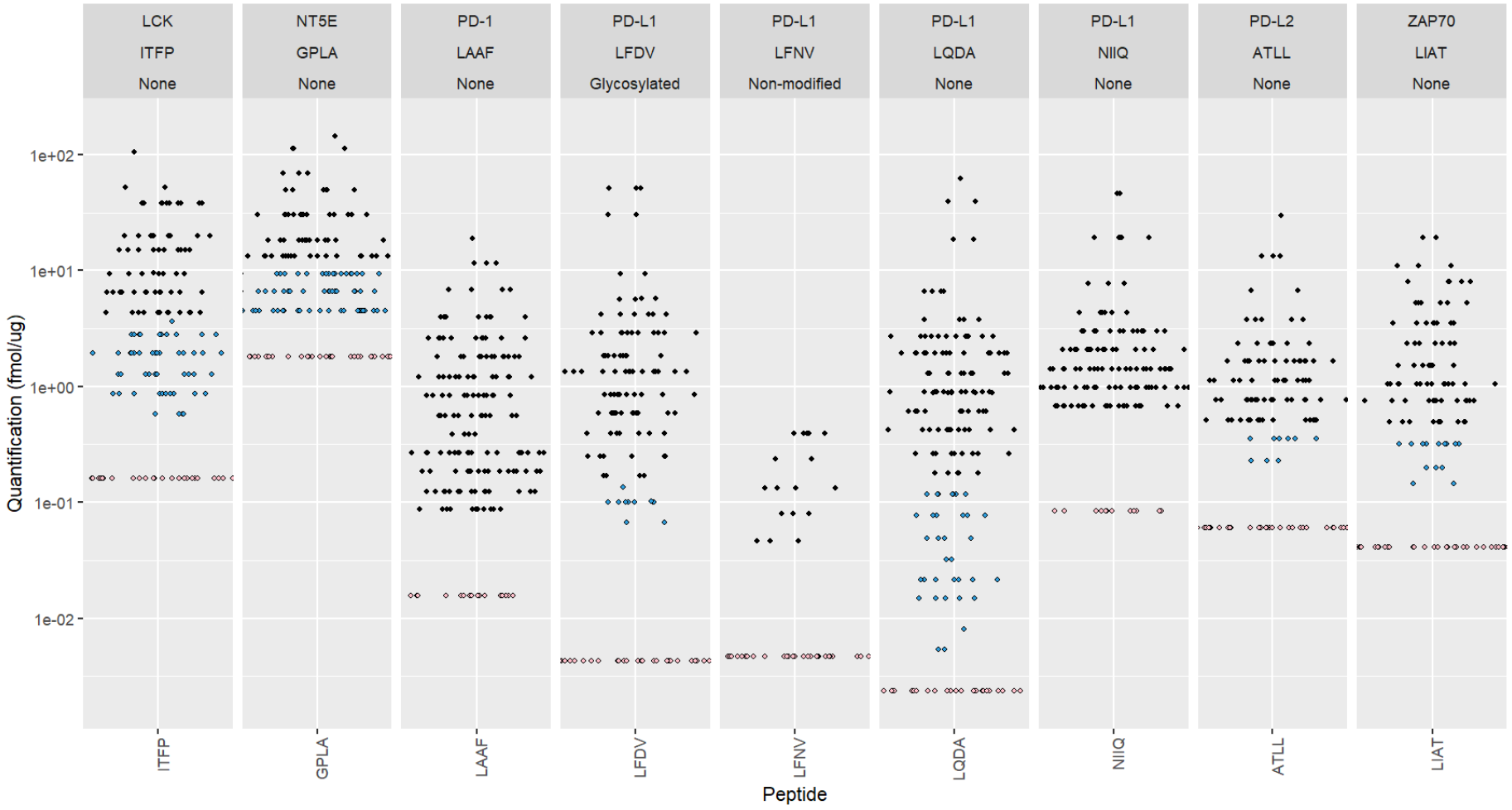

Figure 3: PD-1/PD-L1 axis protein quantification across 175 cancer samples. Black dots are above the lower limit of quantification (LLOQ), blue dots are between the LLOQ and the limit of detection (LOD), and pink dots are below the LOD.

detectable ITFP, 68%), the PD-L2 associated peptide (95 samples with detectable ATLL, 54%) and finally the ZAP70 associated peptide (93 samples with detectable LIAT, 53%). The non-glycosylated form of LFDV, LFNV, was only detectable in 17 samples (10%).

Table 3 presents ranges, medians, means, and standard deviations of the concentration of each peptide in attomoles per microgram across these 175 samples. **Figure 3** visualizes those concentrations (except for LFNV, the glycosylated form of PD-L1). The peptide with the widest range was GPLA (concentrations ranging from 50.2 attomoles per microgram to 1801.8 attomoles per microgram with a median value of 156.1 attomoles per microgram), while the peptide with the smallest range was LAAF (concentrations ranging from 1.3 attomoles per microgram to 235.2 attomoles per microgram with a median value of 10.7 attomoles per microgram). These greater ranges were associated with greater standard deviations as well, with GPLA having the largest standard deviation of 310.6 attomoles per microgram and LAAF having the smallest standard deviation of 33.0. Mean values were higher than median values for every single peptide suggesting a very skewed distribution, with some peptides (NIIQ, LQDA, LFDV, and LIAT) having mean values more than twice their median values.

3.2.2: Samples from patients with clinical data

The proportion of the samples with peptide concentrations above the limit of detection in the 83 patients that were linked to clinical records was lower than the proportion in the full sample. For PD-L1 protein-associated peptides, only 70% of linked patients had detectable NIIQ concentrations, 44% had detectable LQDA concentrations, and 35% had detectable LFDV concentrations. With respect to other protein-associated peptides, 60% had detectable LAAF (PD-1) concentrations, 47% had detectable GPLA (NT5E) concentrations, 40% had detectable

ITFP (LCK) concentrations, 25% had detectable ATLL (PD-L2) concentrations, and 24% had detectable LIAT (ZAP70) concentrations. Only 7% had detectable LFNV concentrations, the glycosylated peptide associated with PD-L1. Notably, the relative ranking of the peptides by proportion detectable was identical in both the full population and those with linked clinical data. Also worth noting is that, as can be seen when comparing **Table 4** (the ranges, medians, means, and standard deviations for each peptides' concentration in the linked clinical data) to **Table 3**, the medians, means, and standard deviations of the samples above the limit of detection when up for every single peptide and the upper limit of the ranges stayed constant. The distributions were still heavily skewed with markedly higher means than medians across the board.

Table 4: Number of samples above the limit of detection (LOD) for each of the 9 different peptides among the 83 samples linked to clinical data, along with ranges, medians, means, and standard deviations of their concentrations in attomoles per microgram (amol/µg).

Short peptide sequence (associated protein)	Number above LOD	Range (in amol/µg)	Median (in amol/µg)	Mean (in amol/µg)	Standard deviation (in amol/µg)
NIIQ (PD-L1)	58 (70%)	8.5 – 815.9	42.2	74.5	125.0
LQDA (PD-L1)	37 (45%)	0.4 – 774.2	13.8	66.2	165.0
LFNV (PD-L1)	6 (7%)	0.8 – 5.8	4.7	4.1	2.0
LFDV (PD-L1)	29 (35%)	1.8 – 714.3	35.1	111.1	204.7
LAAF (PD-1)	50 (60%)	2.4 – 235.2	14.0	28.7	43.5
ITFP (LCK)	33 (40%)	13.6 – 1310.9	112.4	201.3	269.0
ATLL (PD-L2)	21 (25%)	5.4 – 375.7	22.2	46.5	81.8
LIAT (ZAP70)	20 (24%)	4.1 – 283.7	22.6	50.1	71.6
GPLA (NT5E)	39 (47%)	51.1 – 1801.8	209.8	373.3	412.2

3.2.3: Samples from patients that received immunotherapy

As mentioned previously, only 51 out of the 83 samples with linked clinical data received immunotherapy. The proportions of those 51 patients with detectable amounts of each peptide

were closer to the proportions in the linked clinical cohort than μthe full samples; with respect to PD-L1 associated peptides, 71% of patients had detectable NIIQ, 37% had detectable LQDA, and 33% had detectable LFDV. With respect to other protein-associated peptides, 31% had detectable LAAF (PD-1), 43% had detectable GPLA (NT5E), 41% had detectable ITFP (LCK), and 24% had detectable ATLL (PD-L2) or LIAT (ZAP70). Only 4% had detectable LFNV, the glycosylated peptide associated with PD-L1.

Table 5 (which describes the distributions of peptide concentrations in patients who received immunotherapy) is generally more similar to **Table 4** (which includes all 83 patients with clinical data) than **Table 3** (which includes all 175 samples). The main difference is that standard deviations and means in **Table 5** are generally lower than the corresponding means and standard deviations in **Table 4**, while still being larger than those in **Table 3**.

Table 5: Number of samples above the limit of detection (LOD) for each of the 9 different peptides among the 51 patients who received immunotherapy along with ranges, medians, means, and standard deviations of concentrations in attomoles per microgram (amol/μg).

Short peptide sequence (associated protein)	Number above LOD	Range (in amol/μg)	Median (in amol/μg)	Mean (in amol/ug)	Standard deviation (in amol/μg)
NIIQ (PD-L1)	36 (71%)	8.5 – 815.9	39.9	75.0	136.8
LQDA (PD-L1)	19 (37%)	0.4 – 443.1	11.4	45.4	101.3
LFNV (PD-L1)	2 (4%)	0.8 – 5.2	3.0	3.0	3.1
LFDV (PD-L1)	17 (33%)	1.8 – 585.2	38.3	88.3	155.0
LAAF (PD-1)	31 (61%)	2.4 – 163.9	16.3	29.7	38.1
ITFP (LCK)	21 (41%)	13.6 – 688.3	102.5	156.4	170.3
ATLL (PD-L2)	12 (24%)	5.4 – 143.9	23.2	36.8	40.8
LIAT (ZAP70)	12 (24%)	4.1 – 200.4	22.6	40.5	53.6
GPLA (NT5E)	22 (43%)	51.1 – 1613.9	184.5	338.9	418.2

3.3: Peptide correlation results

We focused on three distinct types of correlations: one-way log-scale correlations between PD-L1 associated peptides NIIQ, LQDA, and LFDV (to establish face validity of the assay), one-way log-scale linear correlations between PD-L1 associated peptides (NIIQ and LQDA, specifically) and the PD-1 associated peptide LAAF (to evaluate whether the two proteins appeared to be correlated in their expression), and correlations between the individual peptides and results from the IHC within the clinical data. The inter-peptide correlations were examined within all 175 samples as well as within the 83 patients with linked clinical data.

3.3.1: Correlations between PD-L1 peptides

Among the 175 samples, there were 102 samples with detectable levels of both NIIQ and LQDA, 88 samples with detectable levels of both LQDA and LFDV, and 93 samples with detectable levels of both LFDV and NIIQ. Panels A, B, and C in **Figure 4** show that the PD-L1 peptides exhibited high log-scale correlation, with R values of 0.7 for the NIIQ-LQDA correlation, 0.65 for the NIIQ-LFDV correlation, and 0.92 for the LQDA-LFDV correlation. Among the 51 samples linked with the clinical data of patients who received immunotherapy, 24 samples had detectable levels of both NIIQ and LQDA, 23 had detectable levels of LQDA and LFDV, and 28 had detectable levels of LFDV and NIIQ. Panels D, E, and F in **Figure 4** show that the log-scale correlations were universally higher in the patients with clinical data, with R values of 0.82 for NIIQ vs LQDA, 0.82 for NIIQ vs LFDV, and 0.96 for LQDA vs LFDV. All correlations were statistically significant with p values of much lower than 0.05.

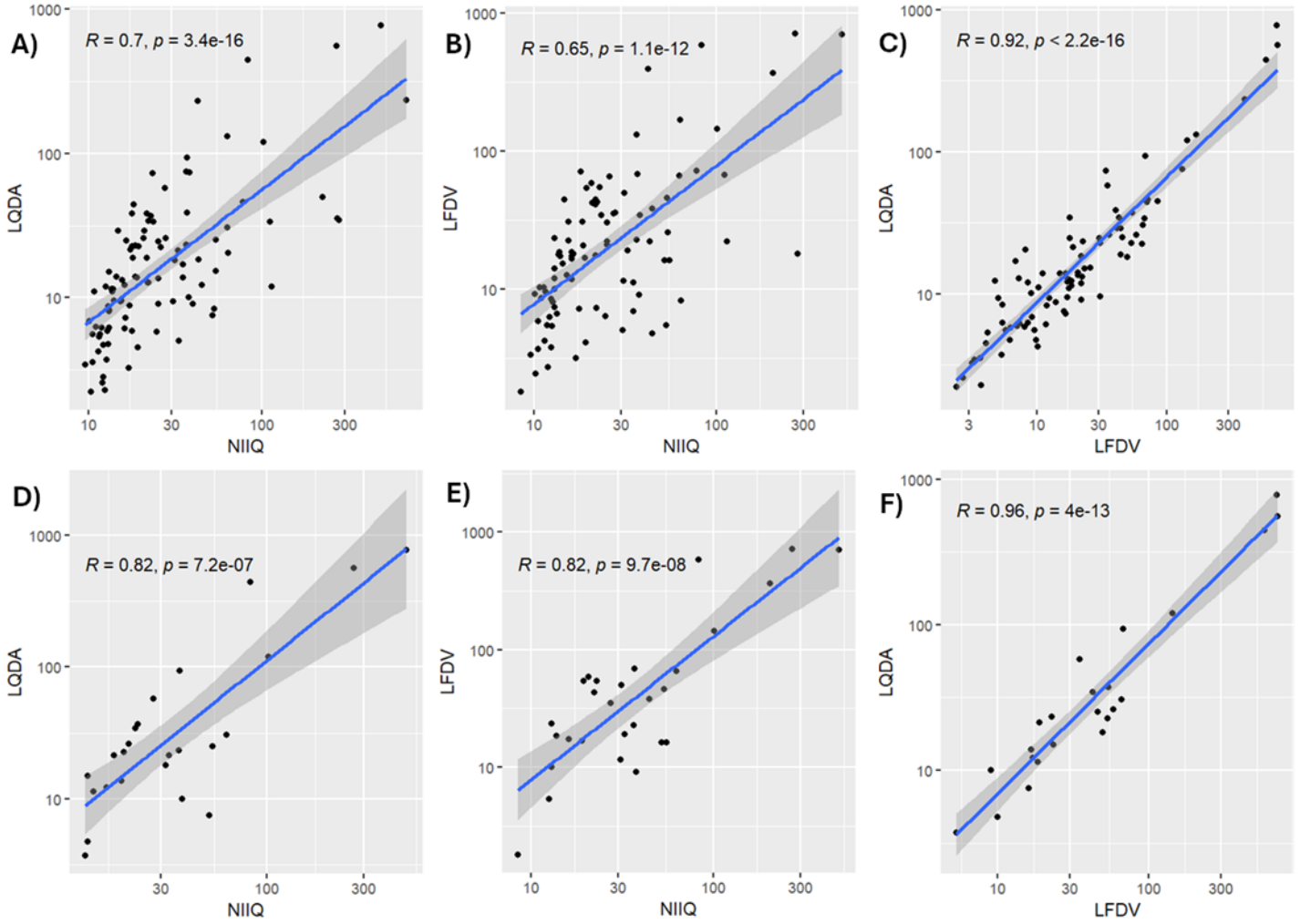

Figure 4: Correlations between concentrations (in attomoles per microgram) of PD-L1 associated peptides across all 175 samples (panels A-C) and within the 83 samples with clinical data (panels D-F). Panels A and D show NIIQ vs LQDA correlations, panels E and B show NIIQ vs LFDV, and panels C and F show LFDV vs LQDA.

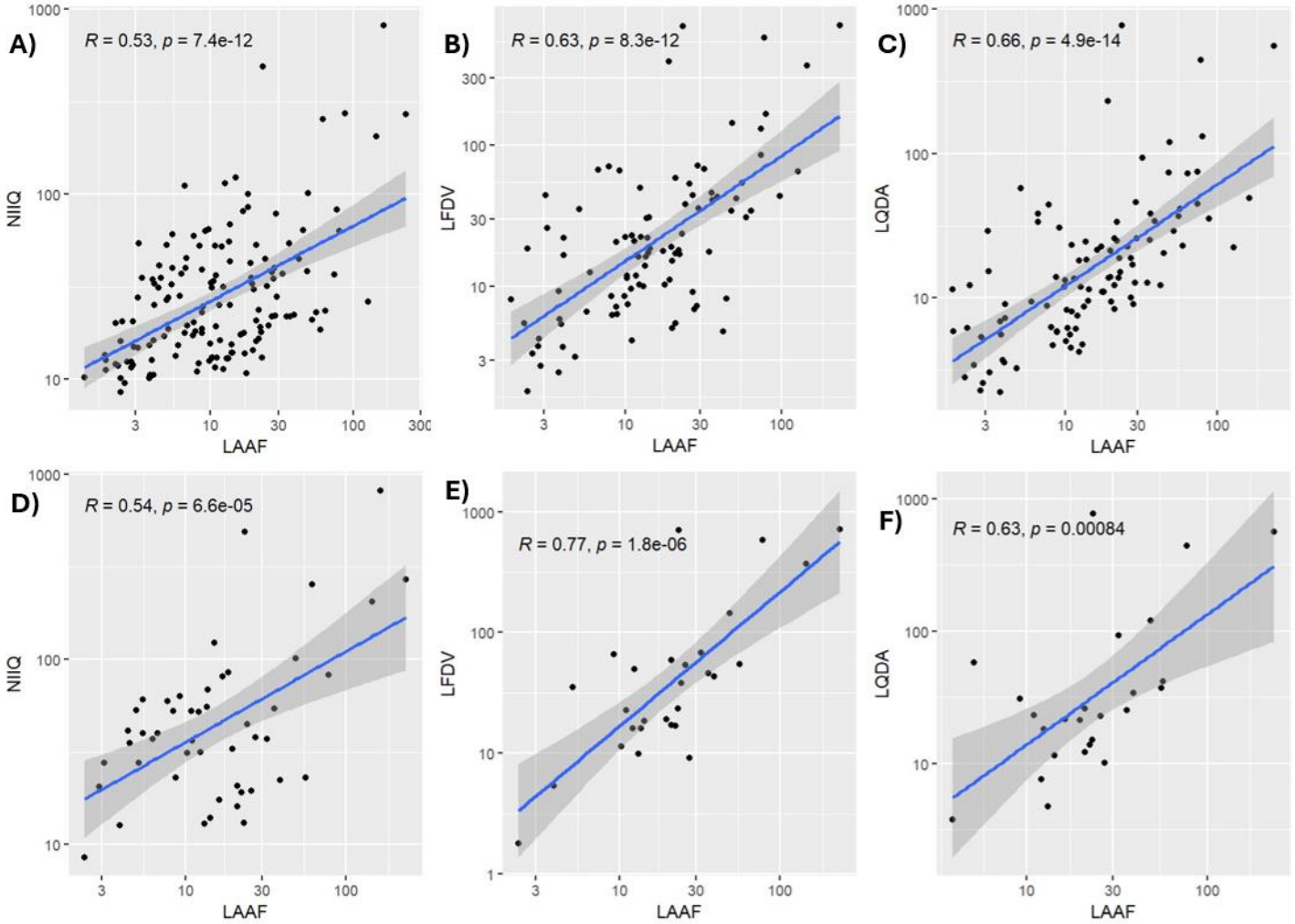

Figure 5: Correlations between concentrations (in attomoles per microgram) of PD-L1 associated peptides and the PD-1 associated peptide LAAF across all 175 samples (panels A-C) and within the 83 samples with clinical data (panels D-F). Panels A and D show LAAF vs NIIQ correlations, panels B and E show LAAF vs LFDV, and panels C and F show LAAF vs LQDA.

3.3.2: Correlation between PD-L1 peptides and the PD-1 peptide

Among the 175 samples, there were 147 samples with detectable NIIQ and LAAF, 94 samples with detectable LFDV and LAAF, and 102 samples with detectable levels LQDA and LAAF. As with the correlations between PD-L1 associated peptides, the correlations between LAAF and the PD-L1 associated peptides were all positive as shown in Panels A, B, and C of **Figure 5** (LAAF vs NIIQ R = 0.53; LAAF vs LFDV R = 0.63; and LAAF vs LQDA R = 0.66). In the samples with linked clinical data, there were 48 samples with detectable NIIQ and LAAF, 28 samples with detectable LFDV and LAAF, and 25 samples with detectable LQDA and LAAF. The LAAF vs LFDV correlation was stronger in the samples with linked clinical data, but other correlations were relatively unchanged as can be seen in panels D, E, and F of Figure X (LAAF vs NIIQ R=0.54; LAAF vs LFDV R value = 0.77; LAAF vs LQDA R value = 0.63). As when examining the correlations between PD-L1 associated peptides, all correlations were very statistically significant with p-values of much less than 0.01.

3.3.3: Correlation between peptides and IHC

Finally, we evaluated potential correlations between the various peptides concentrations and PD-L1 expression based on IHC results for the 83 patients with linked clinical data (see **Figure 6**). Of the eight peptides studied, three had statistically significant correlations with IHC-based PD-L1 expression: LQDA (R = 0.55, p-value < 0.001), LFDV (R = 0.46, p-value = 0.013), and ITFP (R = 0.36, p-value 0.046). Interestingly, NIIQ -- despite being flagged as a PD-L1 associated peptide -- did not appear to be associated with PD-L1 expression (R = 0.13, p-value = 0.37) in our study.

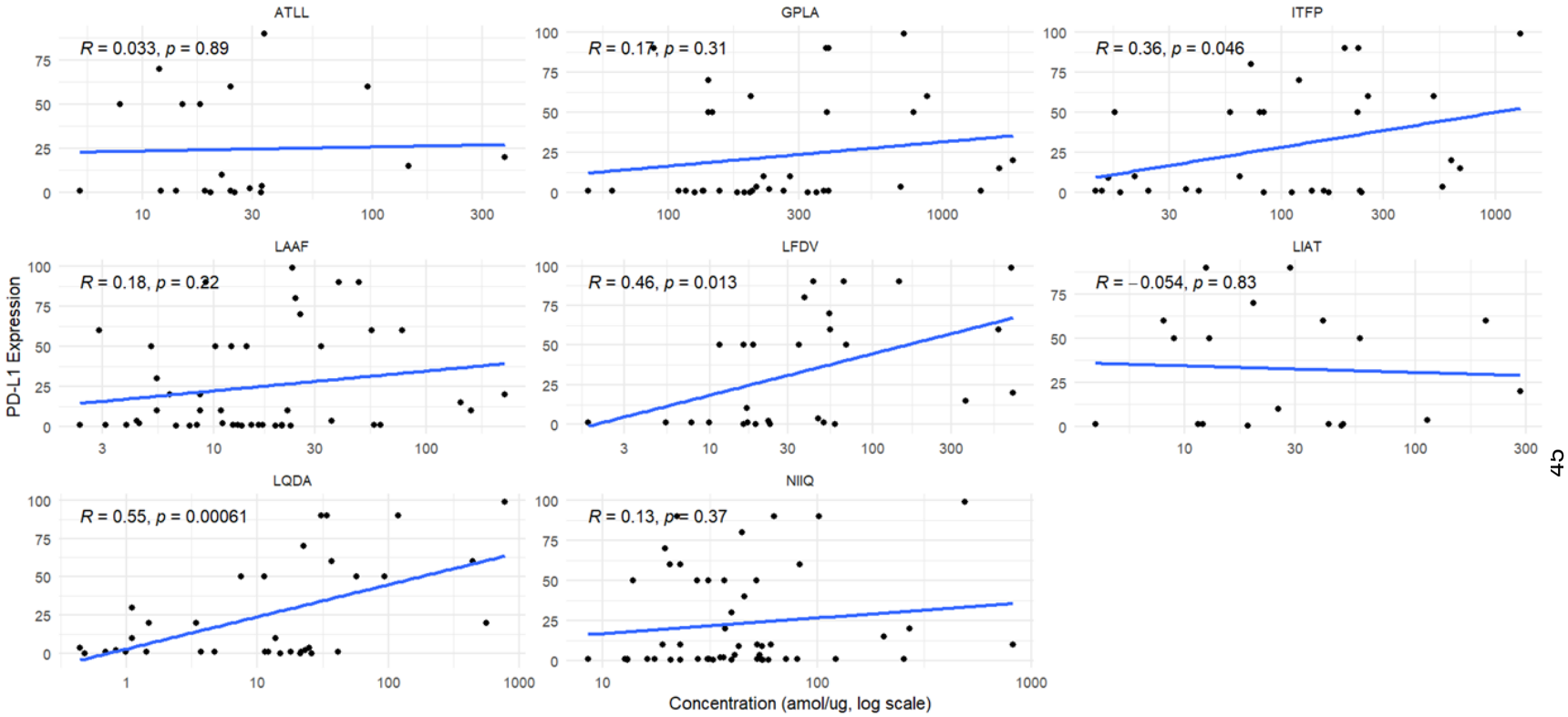

Figure 6: Correlations between concentrations of PD-L1 associated peptides (in attomoles per microgram) and PD-L1 expression calculated from IHC in the 83 samples linked to clinical data.

3.4: Association with benefit and survival

Finally, we evaluated the extent to which individual peptide concentrations were correlated with clinical benefit (based on the RECIST scale) and survival (in terms of all-cause mortality) after receiving immunotherapy.

3.4.1: Associations with clinical benefit

Of the 51 patients who received immunotherapy, 26 patients benefited from immunotherapy, 24 patients did not benefit, and 1 patient could not be classified due to the lack of data for the six-month of follow-up after immunotherapy and having no direct RECIST information in their record. The total number of patients with detectable peptide levels that benefited or did not benefit also varied across peptides, with the precise numbers of patients with values above the LOD who did and did not benefit listed in **Table 6.** The peptides that were most commonly above the LOD were, once again, NIIQ (37 patients) and LAAF (30 patients).

Table 6: Number of patients with detectable amounts of each of 8 different peptides that received immunotherapy that did and did not experience clinical benefit.

Short peptide sequence	Number above the limit of detection with benefit	Number above the limit of detection without benefit
NIIQ (PD-L1)	20	15
LQDA (PD-L1)	11	7
LFDV (PD-L1)	9	7
LAAF (PD-1)	18	12
ITFP (LCK)	11	10
ATLL (PD-L2)	4	7
LIAT (ZAP70)	6	6
GPLA (NT5E)	12	9

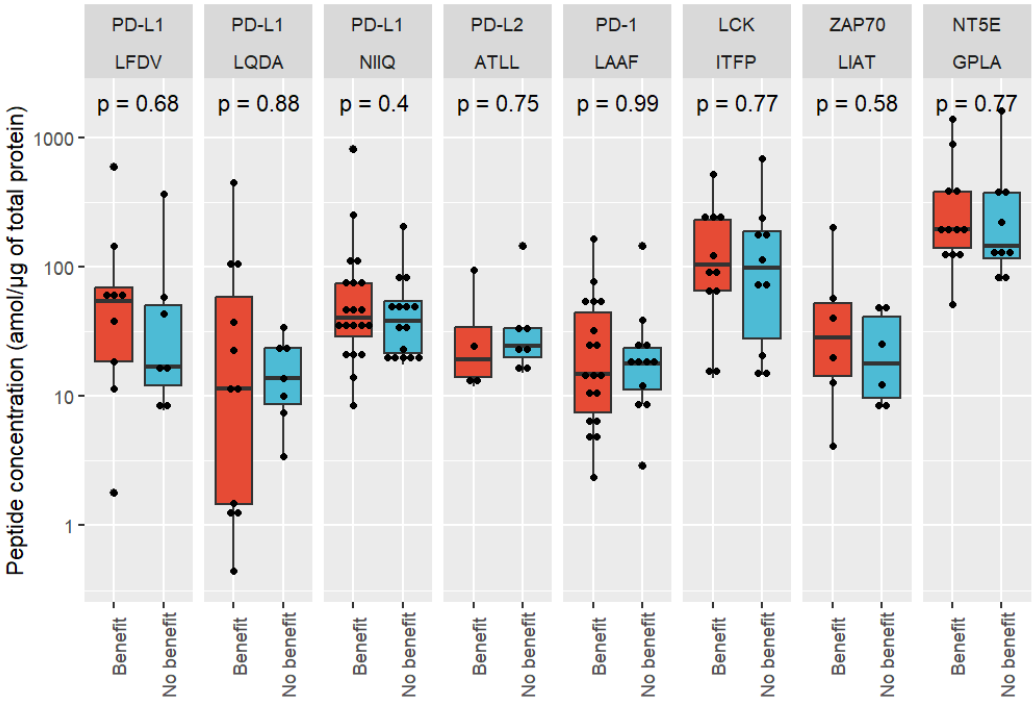

Figure 7: Box plots showing the distribution of the concentrations of each of the 8 different peptides among patients who benefited from immunotherapy (red boxes) or did not benefit (blue boxes), along with p values for the associated t-tests.

Figure 7 presents box plots showing how peptide concentrations were distributed among patients who benefited (in red on the left) and among patients who did not benefit (in blue on the right) among patients with peptide values above the LOD, with p values for the associated t tests. None of the t-tests showed statistically significant differences between the concentrations of the individual peptides in patients who did and did not appear to benefit from immunotherapy, although the number of patients with values above the limit of , information on clinical benefit was available, was very small for some peptides.

3.4.2: Associations with survival

We performed three total survival analyses, all of which are included in **Figure 8**. The first (panel A) compared patients who received immunotherapy with NIIQ concentrations above the median NIIQ concentration in the population (42 attomoles per microgram) with those with NIIQ concentrations below the median NIIQ concentration. There was no statistically significant difference between the two groups (p-value from the log rank test = 0.7). Similar results were observed when comparing patients who received immunotherapy with LAAF concentrations above vs below the median LAAF concentration of 16 attomoles per microgram (panel B), with a p-value from the log rank test of 0.5 that was not statistically significant. Interestingly, the p value from the log rank test comparing the survival of patients with PD-L1 IHC results above 1% compared with that of patients whose PD-L1 IHC results were below 1% was also not statistically significant (panel C).

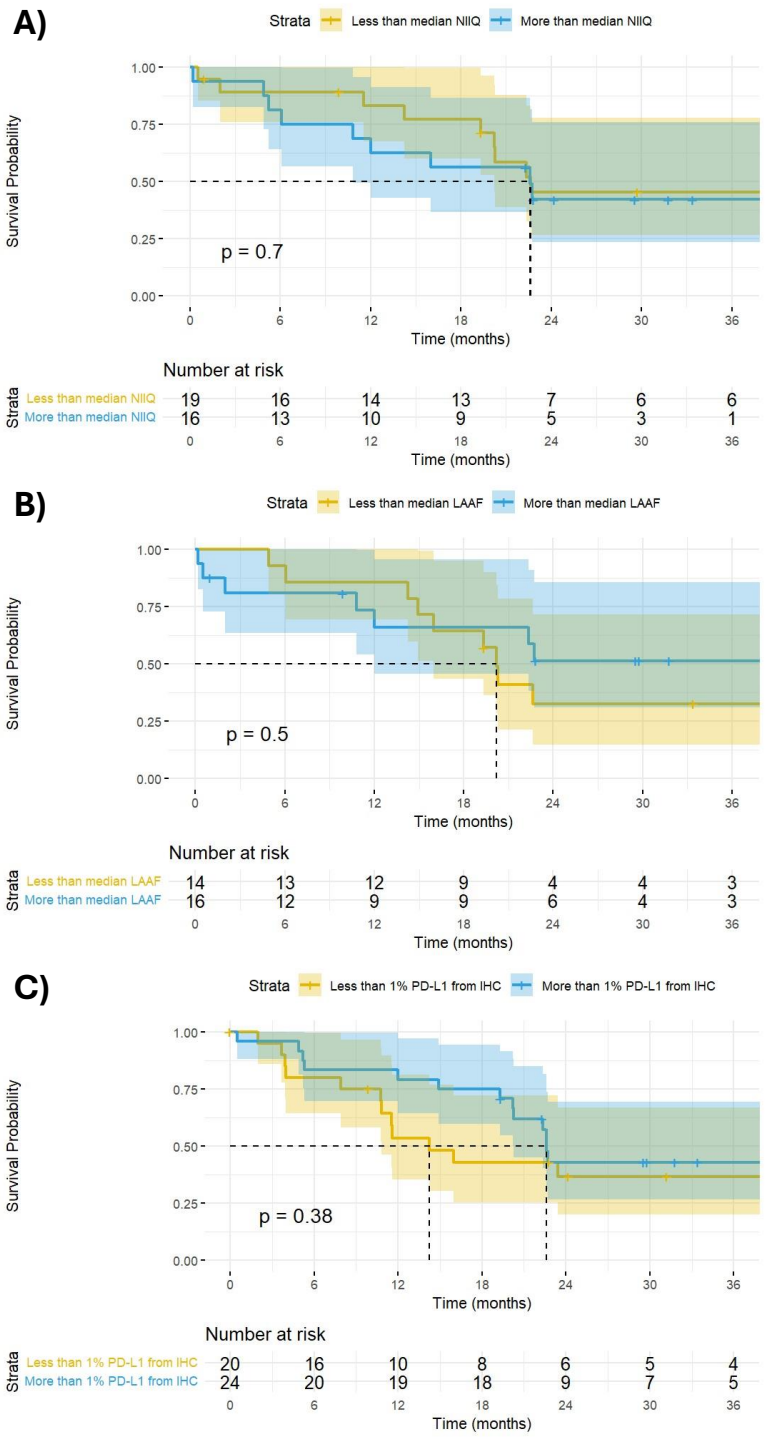

Figure 8: Survival curves for all-cause mortality in patients who received immunotherapy above vs below the median NIIQ concentration (panel A), above vs below the median LAAF concentration (panel B), and above vs below a 1% PD-L1 result from IHC (panel C).

CHAPTER 4: DISCUSSION

4.1: Our findings

In this project, we laid the foundation for future research using immuno-multiple reaction monitoring, and LC/MS to evaluate clinical responses to immunotherapy in patients with different types of cancer. We analyzed a total of 175 samples taken from the tumors of patients with cancer who were treated within Pennsylvania's Allegheny Health Network. A total of 81 samples had linked clinical data, and 51 of the samples that had linked clinical data were from patients who had received immunotherapy.

We were able to apply our immuno-MRM method to quantify 9 different peptides (specifically NIIQ, LFDV, LQDA, LFNV, ATLL, LAAF, ITPF, LIAT, and GPLA) which were associated with different proteins within the PD-1/PD-L1 axis (specifically including PD-L1, PD-1, PD-L2, NT5E, ZAP70, and LCK). While some patients had values below the limit of detection for each peptide, NIIQ, LAAF, and LQDA were the most consistently quantified peptides whether examining *all* of the samples (92%, 89%, and 80% quantified, respectively), the samples with linked clinical data (70%, 60%, and 45% quantified, respectively), or the samples from patients known to have received immunotherapy (71%, 61%, and 37% quantified, respectively). Also, the deglycosylated peptide of PD-L1, LFDV, was much more frequently quantifiable than its non-modified equivalent, LFNV. Among all 175 samples, for example, 60% of samples had quantifiable LFDV while only 10% had quantifiable LFNV, and this trend also held across the samples linked with clinical data and the samples from patients who had received immunotherapy. This shows that when PD-L1 is heavily glycosylated it is less accessible to antibody binding than when it was deglycosylated.(60)

We also established the face validity of the assay by confirming that -- among patients with detectable levels of the PD-L1 associated peptides NIIQ, LFDV, and LQDA -- the concentrations of the peptides were strongly positively correlated with one another in the full set of samples (NIIQ vs LQDA R value: 0.70; NIIQ vs LFDV R value: 0.65; LFDV vs LQDA R value: 0.92) as well as in the samples that were linked to clinical data (NIIQ vs LQDA R value: 0.82; NIIQ vs LFDV R value: 0.82; LFDV vs LQDA R value: 0.96). We also found that the concentrations of these different peptides were all strongly associated with the concentrations of the PD-1 associated peptide LAAF, both when examining all 175 samples (LAAF vs NIIQ R value: 0.53; LAAF vs LFDV R value: 0.63; LAAF vs LQDA R value: 0.66) and when examining the samples linked to clinical data (LAAF vs NIIQ R value: 0.54; LAAF vs LFDV R value: 0.77; LAAF vs LQDA R value: 0.63) suggesting a pairing between the expression of the ligand and its corresponding receptor.

That said, our analyses exploring the extent to which the concentrations of each of the different peptides were associated with clinical benefit in patients receiving immunotherapy did not identify any single peptide as an excellent predictors of clinical benefit or overall survival based on the p-values from the t-tests (when examining clinical benefit) or log-rank tests (when examining overall survival) which we performed. Based on these results, individual peptide concentrations do not seem to serve as a "silver bullet" to predict whether patients respond to immunotherapy. The associations between the different peptides and the expression of PD-L1 as assessed by IHC in the linked clinical data were also fairly weak, with the exception of LQDA (R value: 0.55 and p-value < 0.01), LFDV (R value: 0.46 and p-value $= 0.013$) and ITFP (R value: 0.36 and p-value 0.046). Despite its strong association with both LQDA and LFDV, NIIQ was not statistically significantly associated with IHC-determined PD-L1 expression (R value: 0.13,

p-value = 0.37). Even more intriguingly, the IHC-determined PD-L1 in the clinical data did not seem to be strongly predictive of overall survival, meaning that none of these biomarkers appear to perform exceptionally well on their own and reinforcing the potential need for evaluating alternative predictive and prognostic approaches that incorporate information from across the entirety of the PD-1/PD-L1 immune axis.

4.2: Relationships to previous research

Due to the novelty of immuno-multiple reaction monitoring as a method for predicting patient response to immunotherapy and the fact that we were relying on a newly developed assay, it is difficult to establish the extent to which our findings agree or conflict with the published literature. The positive correlations we observed between concentrations of the three PD-L1 associated peptides and the PD-L1 associated peptide are in line with previous literature where upregulation of PD-L1 has sometimes been observed alongside increased PD-1 expression(110-112). Notably, however, this relationship is complex and often varies depending on the type of tumor, the tumor microenvironment, and other patient-specific characteristics.(113)

The lack of an association between PD-L1 expression in the clinical data and survival in patients receiving immunotherapy may seem counterintuitive at first given PD-L1's use as a predictive biomarker for immunotherapy response. A past study by Shah et al(114), however, did show that very high (>90%) PD-L1 expression was associated with longer survival compared to lower, but still elevated, levels of PD-L1 expression (50-90%). Previous work by Fenton et al.(115) using medical-record data, however, was not able to identify an association between PD-L1 expression and overall survival. Potential reasons for this lack of association include

heterogeneity in PD-L1 expression across different cancer types, across time (meaning that its expression can vary across cancer stages), and across different regions of a given tumor. Our relatively small sample size of patients receiving immunotherapy and the fact that PD-L1 IHC levels in the clinical data were determined using a variety of different assays may also explain our inability to obtain a statistically significant association between the expression of PD-L1 and overall survival following immunotherapy.

4.3: Strengths and limitations

This is among the first projects to use immuno-multiple reaction monitoring to quantify the concentrations of different peptides in the context of the PD-L1 axis. We analyzed samples from 175 different patients suffering from a wide range of cancer types and demonstrated that the concentrations of peptides from the assay were correlated as expected. We were able to leverage the multiplexing capability of immuno-multiple reaction monitoring to evaluate the concentrations of multiple peptides at once, rather than attempting to do so via immunohistochemistry. We were also able to link the results from the quantitative analysis to clinical data for many of the patients, to check whether these correlations still held in patients who received immunotherapy, and to evaluate the extent to which these peptide correlations could predict clinical benefit and overall survival. This linkage to clinical data drew on the expertise of local oncologists familiar with the relevant electronic medical record system in the Allegheny Health Network.

Unfortunately, the project was not without its limitations. We were not able to obtain clinical data for the patients associated with all of the 175 samples, and only 51 of the patients for whom we were able to obtain clinical data (most of whom were diagnosed with lung cancer),

ultimately received immunotherapy. Because data on whether the immunotherapy regimen, specifically, led to clinical benefit was not available for every patient, we had to apply a modified version of the RECIST criteria based on the survival of those patients where there was no information on immunotherapy response. As a result, some of the patients that we classified as benefiting from immunotherapy may actually have failed to respond. We were also limited by the fact that the structure of the assay and the quantitative analyses we were performing made it difficult-to-impossible to incorporate results that were below the limit of detection when determining correlations between peptides as well as associations of peptide concentrations with clinical benefit and overall survival.

4.4: Future avenues for research

While we did not observe a strong predictive value for these individual peptides with respect to predicting a positive response to immunotherapy, thus far, we have only examined the of *individual* peptide concentrations with clinical benefit. One of the most useful aspects of immuno-multiple reaction monitoring is that it has the ability to quantify multiple proteins simultaneously, within a single sample, meaning that developing a more appropriate model which takes advantage of concentration data from multiple proteins *simultaneously* to create a prognostic score may perform better than examining individual proteins one by one.(116) Developing a more appropriate statistical analysis, or applying more complex analytical methods to include those samples below the lower limit of detection could simultaneously boost statistical power (by including more samples in the analysis) while also minimizing the potential selection bias resulting from including only cases above lower limit of detection. Finally, obtaining clinical information for the remaining 98 patients would also help boost sample size and allow

the examination of heterogeneity across different types and stages of cancer, instead of treating all types and stages of cancer as interchangeable with one another.

CHAPTER 5: CONCLUSION

Cancer is a common disease with exceptionally high morbidity and mortality. Immunotherapy is an important weapon in the arsenal of modern oncologists -- in addition to or in combination with surgery, radiation, and chemotherapy. Not all patients, however, will benefit from immunotherapy. While PD-L1 immunohistochemistry has been approved for use as a biomarker for predicting clinical response to immunotherapy, it has a number of limitations ranging from a lack of standardization across assays, to inherent variability within and across tumor cells, to difficulty incorporating information from across the entirety of the PD-1/PD-L1 axis. We were able to show that immuno-multiple reaction monitoring mass spectrometry, a promising alternative to immunohistochemistry that addresses many of these limitations, can reliably quantify the concentrations of specific peptides related to PD-L1 axis proteins in tumor samples from patients with cancer, specifically peptides related to PD-L1 and PD-1.

That being said, we did not observe statistically significant associations between the concentrations of the *individual* peptides studied and the clinical benefit of immunotherapy. This means that the concentrations of individual peptides obtained from immune-multiple reaction monitoring may not be sufficient to act as a clinically useful predictive or prognostic biomarker for response to immunotherapy. In order to improve the utility of immune-multiple reaction monitoring as a method to predict patient response to immunotherapy while taking advantage of its ability to quantify concentrations of multiple molecules in a single sample, future research should focus on developing more sophisticated statistical algorithms and classification systems that incorporate information from multiple peptides simultaneously.

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CHAPTER 7: APPENDICES

Appendix A: Transition values for each peptide

Compound Group	Peptide Modified Sequence isotope label	Internal Standard	Precursor Ion	Product Ion	Dwell	Fragmentor	Collision Energy
PD-L1	NIIQFVHGEEDLK.light	FALSE	514.6035	827.3894	20	130	13.7
PD-L1	NIIQFVHGEEDLK.light	FALSE	514.6035	657.8381	20	130	13.7
PD-L1	NIIQFVHGEEDLK.light	FALSE	514.6035	601.296	20	130	13.7
PD-L1	NIIQFVHGEEDLK.heavy	TRUE	517.2749	835.4036	20	130	13.7
PD-L1	NIIQFVHGEEDLK.heavy	TRUE	517.2749	661.8452	20	130	13.7
PD-L1	NIIQFVHGEEDLK.heavy	TRUE	517.2749	605.3031	20	130	13.7
PD-L1	NIIQFVHGEEDLK.medium	TRUE	519.2816	834.4065	20	130	13.7
PD-L1	NIIQFVHGEEDLK.medium	TRUE	519.2816	664.8552	20	130	13.7
PD-L1	NIIQFVHGEEDLK.medium	TRUE	519.2816	604.8046	20	130	13.7
PD-L1	LQDAGVYR.light	FALSE	461.2431	680.3362	20	130	15.3
PD-L1	LQDAGVYR.light	FALSE	461.2431	565.3093	20	130	15.3
PD-L1	LQDAGVYR.light	FALSE	461.2431	338.1823	20	130	15.3
PD-L1	LQDAGVYR.heavy	TRUE	466.2472	690.3445	20	130	15.3
PD-L1	LQDAGVYR.heavy	TRUE	466.2472	575.3175	20	130	15.3
PD-L1	LQDAGVYR.heavy	TRUE	466.2472	348.1905	20	130	15.3
PD-L1	LQDAGVYR.medium	TRUE	464.7516	680.3362	20	130	15.3
PD-L1	LQDAGVYR.medium	TRUE	464.7516	565.3093	20	130	15.3
PD-L1	LQDAGVYR.medium	TRUE	464.7516	338.1823	20	130	15.3
PD-L1	LFNVTSTLR.light	FALSE	525.8007	790.4417	20	130	17.3
PD-L1	LFNVTSTLR.light	FALSE	525.8007	577.3304	20	130	17.3
PD-L1	LFNVTSTLR.light	FALSE	525.8007	375.2027	20	130	17.3
PD-L1	LFNVTSTLR.heavy	TRUE	530.8049	800.45	20	130	17.3
PD-L1	LFNVTSTLR.heavy	TRUE	530.8049	587.3387	20	130	17.3
PD-L1	LFNVTSTLR.heavy	TRUE	530.8049	375.2027	20	130	17.3
PD-L1	LFNVTSTLR.medium	TRUE	529.3093	797.4589	20	130	17.3
PD-L1	LFNVTSTLR.medium	TRUE	529.3093	584.3476	20	130	17.3
PD-L1	LFNVTSTLR.medium	TRUE	529.3093	375.2027	20	130	17.3
PD-L1	LFN[+0.984016]VTSTLR.light	FALSE	526.2928	791.4258	20	130	17.3
PD-L1	LFN[+0.984016]VTSTLR.light	FALSE	526.2928	577.3304	20	130	17.3
PD-L1	LFN[+0.984016]VTSTLR.light	FALSE	526.2928	376.1867	20	130	17.3
PD-L1	LFN[+0.984016]VTSTLR.heavy	TRUE	531.2969	801.434	20	130	17.3
PD-L1	LFN[+0.984016]VTSTLR.heavy	TRUE	531.2969	587.3387	20	130	17.3
PD-L1	LFN[+0.984016]VTSTLR.heavy	TRUE	531.2969	376.1867	20	130	17.3
PD-L1	LFN[+0.984016]VTSTLR.medium	TRUE	529.8013	798.4429	20	130	17.3
PD-L1	LFN[+0.984016]VTSTLR.medium	TRUE	529.8013	584.3476	20	130	17.3
PD-L1	LFN[+0.984016]VTSTLR.medium	TRUE	529.8013	376.1867	20	130	17.3
PD-L2	ATLLEEQLPLGK.light	FALSE	656.3796	913.4989	20	130	21.3
PD-L2	ATLLEEQLPLGK.light	FALSE	656.3796	784.4563	20	130	21.3

PD-L2	ATLLEEQLPLGK.light	FALSE	656.3796	414.2711	20	130	21.3
PD-L2	ATLLEEQLPLGK.heavy	TRUE	660.3867	921.5131	20	130	21.3
PD-L2	ATLLEEQLPLGK.heavy	TRUE	660.3867	792.4705	20	130	21.3
PD-L2	ATLLEEQLPLGK.heavy	TRUE	660.3867	422.2853	20	130	21.3
PD-L2	ATLLEEQLPLGK.medium	TRUE	663.3967	927.5333	20	130	21.3
PD-L2	ATLLEEQLPLGK.medium	TRUE	663.3967	798.4907	20	130	21.3
PD-L2	ATLLEEQLPLGK.medium	TRUE	663.3967	421.2883	20	130	21.3
NT5E	GPLASQISGLYLPYK.light	FALSE	803.9456	940.5138	20	130	25.9
NT5E	GPLASQISGLYLPYK.light	FALSE	803.9456	520.313	20	130	25.9
NT5E	GPLASQISGLYLPYK.light	FALSE	803.9456	407.2289	20	130	25.9
NT5E	GPLASQISGLYLPYK.heavy	TRUE	807.9527	948.528	20	130	25.9
NT5E	GPLASQISGLYLPYK.heavy	TRUE	807.9527	528.3272	20	130	25.9
NT5E	GPLASQISGLYLPYK.heavy	TRUE	807.9527	415.2431	20	130	25.9
NT5E	GPLASQISGLYLPYK.medium	TRUE	811.4613	955.5452	20	130	25.9
NT5E	GPLASQISGLYLPYK.medium	TRUE	811.4613	535.3443	20	130	25.9
NT5E	GPLASQISGLYLPYK.medium	TRUE	811.4613	415.2431	20	130	25.9
NT5E	GPLASQISGLYLPYK.light	FALSE	536.2995	853.4818	20	130	14.5
NT5E	GPLASQISGLYLPYK.light	FALSE	536.2995	683.3763	20	130	14.5
NT5E	GPLASQISGLYLPYK.light	FALSE	536.2995	407.2289	20	130	14.5
NT5E	GPLASQISGLYLPYK.heavy	TRUE	538.9709	861.496	20	130	14.5
NT5E	GPLASQISGLYLPYK.heavy	TRUE	538.9709	691.3905	20	130	14.5
NT5E	GPLASQISGLYLPYK.heavy	TRUE	538.9709	415.2431	20	130	14.5
NT5E	GPLASQISGLYLPYK.medium	TRUE	541.3099	868.5132	20	130	14.5
NT5E	GPLASQISGLYLPYK.medium	TRUE	541.3099	698.4077	20	130	14.5
NT5E	GPLASQISGLYLPYK.medium	TRUE	541.3099	415.2431	20	130	14.5
PD-1	LAAFPEDR.light	FALSE	459.7376	734.3468	20	130	15.3
PD-1	LAAFPEDR.light	FALSE	459.7376	516.2413	20	130	15.3
PD-1	LAAFPEDR.light	FALSE	459.7376	258.6243	20	130	15.3
PD-1	LAAFPEDR.heavy	TRUE	464.7418	744.355	20	130	15.3
PD-1	LAAFPEDR.heavy	TRUE	464.7418	526.2495	20	130	15.3
PD-1	LAAFPEDR.heavy	TRUE	464.7418	263.6284	20	130	15.3
PD-1	LAAFPEDR.medium	TRUE	463.2462	734.3468	20	130	15.3
PD-1	LAAFPEDR.medium	TRUE	463.2462	516.2413	20	130	15.3
PD-1	LAAFPEDR.medium	TRUE	463.2462	258.6243	20	130	15.3
LCK	ITFPGLHELVR.light	FALSE	427.9153	920.5312	20	130	10.6
LCK	ITFPGLHELVR.light	FALSE	427.9153	653.3729	20	130	10.6
LCK	ITFPGLHELVR.light	FALSE	427.9153	460.7693	20	130	10.6
LCK	ITFPGLHELVR.heavy	TRUE	431.2514	930.5395	20	130	10.6
LCK	ITFPGLHELVR.heavy	TRUE	431.2514	663.3812	20	130	10.6
LCK	ITFPGLHELVR.heavy	TRUE	431.2514	465.7734	20	130	10.6
LCK	ITFPGLHELVR.medium	TRUE	434.5938	930.5395	20	130	10.6
LCK	ITFPGLHELVR.medium	TRUE	434.5938	663.3812	20	130	10.6
LCK	ITFPGLHELVR.medium	TRUE	434.5938	465.7734	20	130	10.6

ZAP70	LIATTAHER.light	FALSE	506.2827	785.39	20	130	16.7
ZAP70	LIATTAHER.light	FALSE	506.2827	714.3529	20	130	16.7
ZAP70	LIATTAHER.light	FALSE	506.2827	393.1987	20	130	16.7
ZAP70	LIATTAHER.heavy	TRUE	511.2869	795.3983	20	130	16.7
ZAP70	LIATTAHER.heavy	TRUE	511.2869	724.3612	20	130	16.7
ZAP70	LIATTAHER.heavy	TRUE	511.2869	398.2028	20	130	16.7
ZAP70	LIATTAHER.medium	TRUE	509.7913	785.39	20	130	16.7
ZAP70	LIATTAHER.medium	TRUE	509.7913	714.3529	20	130	16.7
ZAP70	LIATTAHER.medium	TRUE	509.7913	393.1987	20	130	16.7
Cell accelerator voltage was always set to 4 and the polarity was always positive.							