Integrating Glycan Profiling into Multiplex Immunofluorescent Histology Staining to Improve Analysis and Diagnosis of Non-Small Cell Lung Cancer

Public Summary

Non-small cell lung cancer (NSCLC) is the most common type of lung cancer, and sadly, it is also the deadliest. One of the biggest challenges in treating this cancer is that many tumors do not respond to current treatments. Scientists are looking for better ways to help patients, and one of the newer approaches involves using drugs that help the immune system recognize and attack cancer cells, called immune checkpoint inhibitors. However, this treatment doesn't always work well because tumors are very complex and different in every patient - a single tumor can have many different types of cells inside it. Some of these cells may react to treatment, while others may resist it, making it hard to predict whether a treatment will be successful. One key factor that influences how a tumor behaves is the presence of glycans—tiny sugar molecules found on the surface of cells. These sugar molecules help cancer cells survive, grow, and even hide from the immune system. They play an important role in cell communication and can make cancer much harder to treat. The problem is that glycans are difficult to study because their structure is very complicated, and they are constantly changing. Scientists need better tools to understand them and Dr. Capicciotti and her team are leading the way by developing a new way to study glycans using special enzymes. These enzymes can "tag" glycans, allowing scientists to see them more clearly and understand how they influence cancer. At the same time, Dr. Cottrell's team is working on a technique called multiplex immunofluorescence (mIF). This advanced imaging method allows scientists to look at different proteins inside a tumor all at once. It also helps them see exactly where these proteins are located, which can reveal patterns that explain why some patients respond to treatment while others do not. By combining these two approaches—glycan labeling and advanced imaging—we hope to learn more about how tumors grow and resist treatment. This research could lead to better ways of predicting how a patient's tumor will behave, making it possible to create treatments that are personalized for each patient. In the future, this could improve survival rates and make lung cancer treatments much more effective.

Rationale

Non-Small Cell Lung Cancer (NSCLC) is a leading cause of cancer mortality worldwide (1). While immunotherapies targeting immune checkpoint molecules like PD-1,CTLA-4, TIM-3, LAG-3, and TIGIT, B7-H3 have revolutionized cancer treatments the response rate of these therapies is often incredibly low (2). The immune system is endowed with anti-cancer potential, but the selective pressure of an ongoing immune response can result in tumor expression of membrane-bound and soluble immunosuppressive factors during adaptive immune resistance. So, even with the adoption of ICB-based treatments, 50% of patients with advanced NSCLC die within one year with approximately 20% response to single agent therapy (3). This phenomenon arises from intra tumoural heterogeneity; the varying spatial arrangements and cellular composition within and between tumours resulting in a unique tumor metabolism that dictates

response to anti-tumoral interventions (4). Thus, phenotypical patterns indicative of pathophysiological landscapes must be uncovered to better diagnose tumour immune microenvironment (TIME) subtypes and predict patient response to immunotherapy (5).

Multiplex immunohistochemistry and immunofluorescent (IF) assays profile multiple proteins on a single side while preserving the tumoral spatial distribution, resulting in a highly sensitive, specific and reproducible study of the tumour immune microenvironment (TIME) (5). This approach provides critical information on interconnected multidimensional variables including the co-expression of key immunomodulatory molecules on cellular subsets, quantitation of marker expression intensity, and the spatial relationships between diverse cell types that are linked to antitumor immune response. As a result, cellular niches colocalizing with features of response have been identified and characterized to understand and predict patterns related to patient outcome to immunotherapy. The statistical power behind mIF is pivotal as it allows scoring algorithms to analyze phenotype data to make consistent and quantifiable conclusions about marker co-expression that were previously made by pathologists with low interobserver concordance (6). Further, cellular segmentation can be mathematically related by proximity within the tumour landscape to highest degree of resolution available to date. Novel biomarkers are being rapidly explored by immunohistochemistry to quantitively map the lineage-specific expression of targetable molecules and phenotypic determinants of the TIME. However, this time sensitive snapshot excludes the role of a crucial class of biomolecules, glycans, which are increasingly being recognized for their importance in cancer diagnosis, prognosis and immune evasion.

Every cell surface is coated in a glycocalyx composed of glycans, which are complex, branched structures comprising various cores and glyco-epitopes displayed on termini (7). Aberrant glycan expression profiles are closely correlated to tumorigenesis, metastasis, and therapeutic resistance (8). In addition, overexpression of sialylated glycan ligands has been implicated with cancer immunoevasion by engagement of inhibitory Siglec immune receptors (9). Cell membrane bound receptors and their ligands, which are responsible for normal physiological or disease state function, are also heavily glycosylated (10). Modifications to native glycans like core fucosylation, sialylation, and branching greatly impact ligand affinity, and therefore gatekeep protein-protein interactions. N-glycans in particular are overexpressed on tumour cells and within the TIME, and modulate the expression, stability and function of extracellular proteins that are heavily responsible for the evolution of the TIME including PD-L and PD-1L (11). Thus, cell-surface glycans are excellent biomarker candidates for early cancer detection, prognosis and treatment. However, glycans are not routinely profiled as biomarkers because reliable methods to efficiently image changes in the glycome with disease progression are lacking (12). There are few highly specific antibodies available to profile distinct specific glycan epitopes, and staining with lectins provides limited information as their low affinity and lack of specificity fail to distinguish between important cancer associate glycan subgroups. Given the current lack of powerful glycan profiling tools, there is an urgent need to develop new strategies to visualize spatially resolve glycans along with other biomarkers and immune checkpoint molecules across cellular subsets in the TIME to understand their role in the differentiation to NSCLC subtypes,

methods behind progression aggression, immune evasion mechanisms, and to better inform ICI treatments including PD-1 PD-L1 in a patient dependent manner.

With the goal to characterize the glycan microenvironment of NSCLC tumour tissue, a selective exo-enzymatic glycan labeling strategy will be integrated with a multiplex immunofluorescent workflow to efficiently and selectively profile the co-expression of biomarkers and glycans with spatial relativity. Expertise between two PIs, Dr. Tricia Cottrell and Dr. Chantelle Capicciotti, will be bridged to develop an efficient mIF workflow. Dr. Cottrell's team are leaders in the development of multiplex immunofluorescent staining and subsequent quantitative validation to address the urgent need for new biomarker-driven treatment options for patients with lung cancer. Dr. Capicciotti's team is at the forefront of developing novel glycan labeling strategies for studying the changes and the functional roles of glycans upon disease progression.

Project Proposal

High-level pathological analysis of the spatial arrangement of cells and co-expression patterns of markers in tissues is a novel avenue for the determination of predictive biomarkers for early disease diagnosis of Non-Small Lung Cancer, developing personalized and targeted therapies, and for immune profiling in precision immune-oncology (6). However, insight on the expression of a ligand is futile if its affinity and steric capacity for interaction is regulated by something else entirely: glycans. Glycans have yet to be well characterized in the context of the TIME of patient tissue samples. Current methods of glycan analysis lack adequate resolution to determine co-expressive relationships with biomarkers to benchmark tissue specimens in terms of relative immunological impact, and spatial visualization for studying glycosylation heterogeneity within phenotypically distinct tissue types and region. And yet, the unique glycosylation status of a cell-surface modulates cellular differentiation, signaling, and in cancer stimulates oncogenic signaling pathways, aids in immune evasion, and is implicated in metastasis (8). Thus, this project aims to integrate selective exoenzymatic glycol-engineering by sialyltransferases ST6/ST3Gal1 with CMPNeu5Biotin into a multiplex immunofluorescent staining panel to study the glycans in an immune-oncological context of NSCLC tumour tissue.

Glycoconjugate glycosylation patterns can be studied by exploiting catalytic activity of glycosyltransferases (GTs) to examine labeled substrates through the use of reporter molecules (13–15). GTs are responsible for the biosynthesis of glycoconjugates by forming highly regio- and stereo-specific glycosidic linkages between two monosaccharides. GT's can install chemoenzymatically synthesized nucleotide sugar derivatives functionalized with chemical reporters (azides, alkynes), biotin, fluorophores, or other functional groups to their tolerated acceptor sites. ST6Gal1 is an a2,6-Sialyltransferase with an acceptor specificity for the terminal Galactose of a N-Acetyllactosamine motif on N-glycans, with no tolerance for other enzyme classes. ST3Gal1 is an a2,3-SiaT with strict acceptor specificity for the terminal Galactose residue of Core-1 O-glycans. Thus, N- and O-linked glycans can be labeled and detected by harnessing the specificity and glycan-subclass selectivity of these GTs to profile cancer associated glycan changes; characteristic overexpression of N-linked glycans on tumour cells can be labeled via SEEL with ST6Gal1, while cancer associated overexpression of Core-1 O-glycans can be efficiently detected with ST3Gal1 labeling (Fig 1). The overexpression of Sialic Acid is

also common in the TIME, and is concurrent with cancer progression, immune evasion and modulation, and mediation of cell-cell communication but is still yet to be well characterized with context to key immune-oncological properties/mechanisms/molecules (16). Sialyation decorations can be characterized by labeling with and without sialidase treatment to compare native sialylated/non-sialylated glycans and their relationship to immune-oncological landscapes. While there has been a report of enzymatic glyco-engineering of FFPE tissue and detection by monoplex IF histological staining, the integration of glycan detection into multiplex IF has yet to be explored (17).

Preliminary Results: To perform multiplex IF staining, FFPE tissue samples must undergo a preservation reversal to remove paraffin and retrieval process to ensure epitopes are accessible by antibodies (1). Initial assessment of SK-BR-3 breast cancer cells as a model cancer line treated concurrently with a sialidase (NanH), ST6Gal1 and CMP-Neu5biotin demonstrated that detection of selectively biotinylated N-glycans by streptavidin after formalin fixation, or after FFPE and de-paraffinization, was similar to labeling on live cells, showing that preservation did not alter labeling (Fig 2). Preliminary labeling of lung adenocarcinoma tissues shows that Neu5biotin additions with ST6Gal1 and ST3Gal1with NanH, can successfully select biotinylated galactose residues on N- and O-glycans respectively (Fig 3).

Aim 1: Optimize Glyco-Engineering for IF and Assess Tolerance to a Multiplex Workflow: Enzymatic glycol-engineering on FFPE lung cancer tissue slides will first be optimized by examining selective labeling of N-glycans with ST6Gal1, O-glycans with ST3Gal1, both with or without NanH to analyze the sialylated and unsialylated glycans present in each subclass. Glyco-engineered samples will then be treated with antigen retrieval conditions used in mIF protocols to assess if glycan labeling is retained or lost in this step of the workflow.

Aim 2: Develop Hybrid Multiplex Immunofluorescent Staining Panel: Multiplex immunofluorescent staining will be conducted on archival tissue specimens from NSCLC subtypes at various states of progression, and treatment status. A 6-marker panel will quantify the expression of glycans and match expression status with known cell lineage markers: tumour cells (AE1AE3), cytotoxic T-cells (CD8), macrophages (CD68), T-cells (CD3), MPO (neutrophils), and a pan membrane stain (CD44/CD45/ATPase) with potential inclusion of targetable IC molecules like PD-L1, PD-1, VISTA, COX2, FoxP3, TiGIT, or TIM3. Initially, each IF antibody stain is optimized against gold standard IHC to ensure the accuracy and specificity. Glyco-engineering with CMP-Neu5Btn with ST6Gal1/ST3Gal1 +/- NanH will be merged with sequential single antibody staining to assess the tolerance to a multiplex IF protocol, then be integrated into sequential multiplex staining. Clinical NSCLC samples will then be stained, and whole-slide scans will be taken with the Vectra 3.0 Automated Quantitative Pathology Imaging System (Akoya Biosciences), followed by image processing using the digital image analysis software inform (Ver 2.3, Akoya Biosciences). Single-cell expression patterns will be calculated using methods described in Berry et al. to examine and determine glycosylation states related to predictive patient response, and early modulation of the TIME in non-small cell lung carcinoma clinical specimens, comparing NSCLC subtypes and progression (11).

Statement of Impact

This project will result in a novel method of detecting and identifying glycans in situ to analyze the spatial co-expression of glycans and glycosylated biomarkers across cellular subsets in the tissue microenvironment, to gather insights on tumour heterogeneity for application in guiding clinical decisions. Currently, most clinically applicable biomarkers in lung cancer are only valuable in advanced stages (III+IV) and are poorly sensitive in early stage (I) of lung cancer (18). The overexpression of particular glycans found in the TIME is a result of the activation of hundreds of glycosyltransferases, transcription factors, protein backbones, and glycosidases, thus reflect the immune-oncological status of a tumour and its cells (19). Most studies investigating the use of glycans as biomarkers examine digested serum and tissues: mass-spectrometry analysis of lung adenocarcinoma in patients at different stages of diseases show elevated levels of N-glycan compositions, with 42 species found exclusively in cancerous tissue and structural heterogeneity increasing with disease progression (20). This shows promise for the application of N-linked glycans as biomarkers but is limited in depth of relation to cellular subtypes and their features comprising the TIME. Thus, to truly maximize the potential of glycans as a biomarker they must be analyzed at a high resolution to decipher patterns in expression and localization related to other tumorigenic elements. Glycans coating cell surface proteins alter their interactive properties including targetability by antibody-based assays; non-native glycosylation can disrupt binding capacity (21). Treatment with peptide-N-glycosidase F to remove global N-linked glycosylation of PD-L1 was found to enhance patient histoscore after chromogenic staining of lung cancer tissue, indicating that N-linked glycosylation of PD-L1 impedes its detection by anti-PD-L1 antibodies. Since tumour proportion scores used to determine ICI eligibility are based on the percentage of anti-PD-L1 membrane staining, the impediment of non-native glycosylation on biomarker staining resulted in an exclusion of 16.4-24.5% of patients that could have otherwise received anti-PD-1/PD-L1 therapy. This highlights the drastic need to understand the glycosylation status of biomarker biology in the TME, as standard clinical practices surrounding diagnostics and therapeutic decisions adjusted to reflect the significance of glycans. Further, most ICI treatment approaches rely on direct protein interactions and therefore depend on the accessibility of targeted molecules; N-glycosylation of the N58 site of PD-1 promotes the binding of ICI antibodies directed against PD-1 on T-cells (11). Thus, the expression of biomarkers is not enough to adequately predict therapeutic success, but the glycosylation status must also be considered; abnormal glycosylation profiles may sterically hinder targeted therapies. Understanding glycosylation modifications of predictive biomarkers uncovers a new layer of insight into the state of the tumour microenvironment at the time of harvest, previously inaccessible by traditional analytical techniques, and will aid in guiding patient-specific treatment plans.

Budget Justification

Financial Request:

A total of $49,998.4 is requested to cover consumable costs ($40,380), research staff ($8,618.4), and costs of knowledge translation ($1000). An estimated 150 multiplex slides will be stained, including tumour microarrays of NSCLC samples by subtype, treatment status, and diagnosis.

Budget Justification (Total Cost Estimate $49,998.4)

1. Research Staff (total $8,618.4)

Given the irreplaceable value of these clinical trial tissues, highly qualified research staff will be involved to ensure optimal protocol development, technical execution, project management, and support of research associate Katherine Brewer. The currently available digital image analysis software requires time-intensive training and quality review of machine learning based algorithms. Algorithm performance is visually inspected for every marker on every slide to ensure the highest quality data generation.

Research Associate Katherine Brewer will perform slide staining and scanning, including IHC, mIF, and mIHC, train and review machine learning based algorithms for biomarker detection, and make biological observations related to glycan expression in relation to other phenotypic variables. Research associate will have the opportunity to learn cross-disciplinary skills in pathology, immunology, glycobiology, chemistry, and bioinformatics. Associate will be paid $15.39/h for 20h/week for 28 weeks.

Research Technician As needed will assist with slide staining and scanning, purchasing, specimen management, manage equipment maintenance and development of standard operating procedures (e.g., for slide scanning, image storage and analysis, and laboratory safety).

Department of Pathology, Chemistry, and Computing PhD and MS students will assist with digital image analysis and processing of single cell data, panel design and protocol development, and biochemical interpretation of results.

Co-Supervision by Dr. Chantelle Capicciotti, Associate Professor in the department of Chemistry *blurb??*

Co-Supervision by Dr. Tricia Cottrell, Assistant Professor in the department of Pathology and Molecular Medicine

2. Consumables ($40,380)

	Per 10 Slides Multiplex Slides	Per 120 Multiplex Slides
Antibody and Opal Reagents	2740	32880
Glycan Reagents	200	2400
Other reagents, consumables	375	4500

Histology Services	50	600
Total	3365	40380

Histology services (slide sectioning, H&E staining, control tissue TMA construction) and IF/IHC staining reagents + consumables for an estimated n=120 slides from archival tissues (panel development, validation, TMA slides).

3. Knowledge Translation ($1000)

Conference attendance for research associate. Estimated costs include conference registration ($520), poster production costs ($80), and travel and accommodation ($400).

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Figures

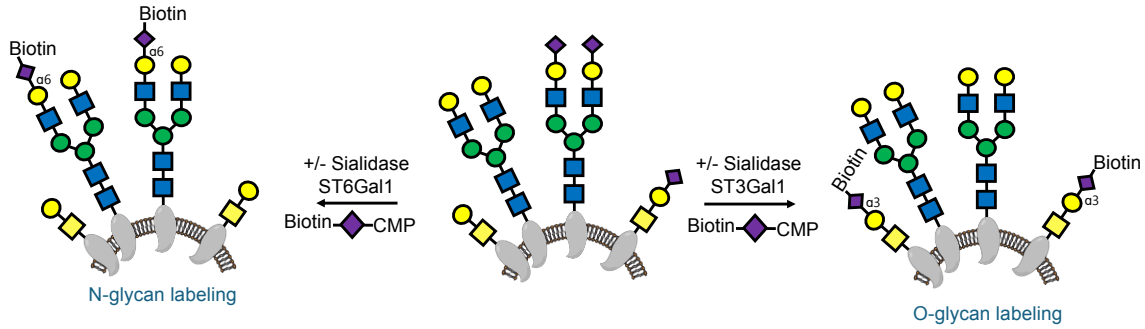

Fig 1. Schematic of glycan labelling by selective enzymatic glyco-engineering of cell-surface glycans. The sialyltransferase ST6Gal1 can be used to selectively label N-glycans with CMP-Neu5Biotin (left), whereas ST3Gal1 can be used to selectively label Core-1 O-Glycans with CMP-Neu5Biotin (right). Concurrent labelling with sialidase (ex. NanH) improves labelling by creating additional acceptor sites for enzymatic transfer, whereas labeling without NanH provides insight on free acceptor galactose units natively present on the cancer cell or tissue of the respective glycan subclass.

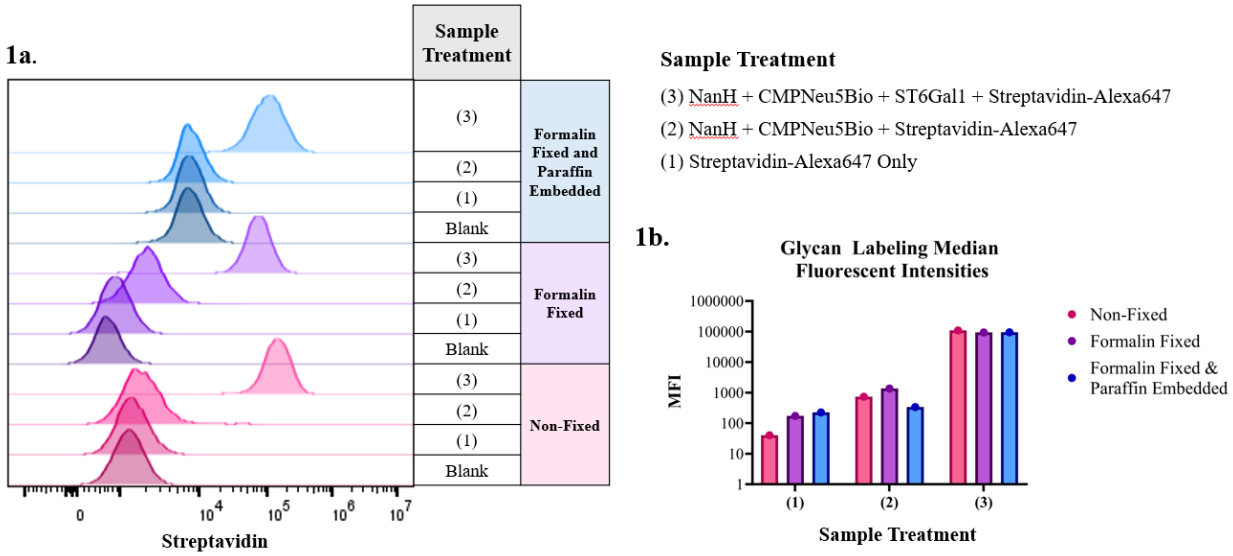

Fig 2: Flow cytometry analysis of selective enzymatic glycan labeling of SK-BR-3 N-glycans. N-linked glycan labeling was achieved with CMPNeu5Bio, ST6Gal1 and NanH. Biotin installation was analyzed by streptavidin-alexa647 staining. Flow cytometry data gated for single cells on each control for non-fixed live cells, formalin fixed cells or FFPE/De-paraffinized cells prior to glycan labelling. a) Flow cytometry streptavidin staining histograms; b) median fluorescent intensity quantification.

3. Lung Cancer Tissue Slices Treated with CMP-Neu5Biotin + ST6/ST3Gal1 + NanH

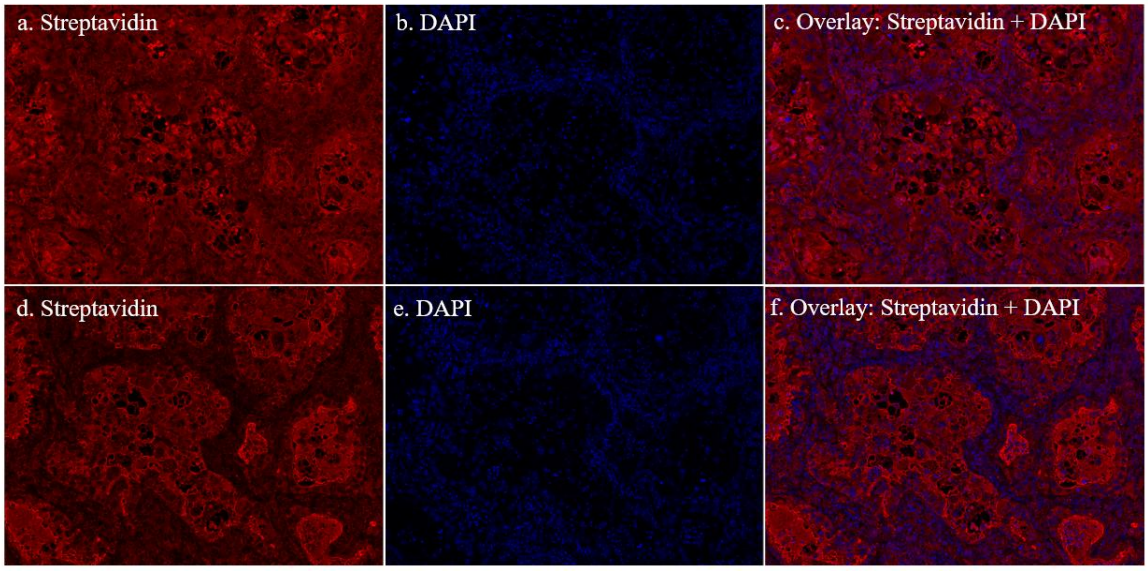

Fig 3: Microscopy analysis of immunofluorescent and selective exoenzymatic glycan labeling demonstrating success of staining on FFPE lung tissue. Preserved FFPE breast tissues underwent selective exo-enzymatic labeling of N-linked glycans with CMPNeu5Bio by ST6Gal1 stained with: a) streptavidin; b) DAPI nuclear stain; c) overlay of a,b. and by ST3Gal1 stained with d) streptavidin; e) DAPI nuclear stain; f) overlay of d,e.

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Feb. 7, 2025

Ambition Awards Committee
Lung Cancer Canada

RE: Dr Cottrell's grant application

Dear Committee members:

I am writing in strong support of Dr. Tricia Cottrell's application to the Ambition Awards competition entitled "Integrating Glycan Detection into Multiplex Immunofluorescent Histology Staining by Enzymatic Glyco-Engineering". This project will address the priority for enhancing early detection.

Since her recruitment from Johns Hopkins University in 2019, Dr. Cottrell has built a rapidly growing research program, with strong mentorship, and high level of engagement with local, national and international collaborators. Dr. Cottrell's success as an early career researcher is particularly impressive given that her first years on faculty coincided with the global pandemic – a testament to her determination as well as the support of deeply invested mentors at Queen's University and Johns Hopkins.

As a fellow cancer immunology investigator and the Director of Sinclair Cancer Research Institute (SCRI), I have had the privilege of supporting the development of Dr. Cottrell's research program. For example, I assisted in the internal peer review process for her CIHR grant application in 2020, which was funded on the first submission. I observed that while Dr. Cottrell is a gifted scientist and writer, she is eager to leverage the expertise of those around her in pursuit of excellence.

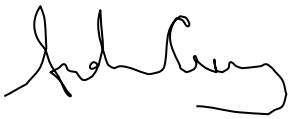
Dr. Cottrell's innovative research focuses on mapping the tumor microenvironment (TME) to identify biomarkers associated with response to immune checkpoint blockade in lung cancer. Since coming to Queen's, Dr. Cottrell has acquired two state-of-the-art multispectral imaging systems (Akoya Polaris/PhenoImager) and a Leica Bond autostainer through individual and collaborative CFI funding awards (JELF and ExCELLirate). Dr. Cottrell has attracted numerous undergraduate and graduate students to her lab with her boundless enthusiasm and dedicated mentoring, including graduating four master's students this year. Through her ongoing collaboration with Johns Hopkins University, Dr. Cottrell is able to leverage hardware and software infrastructure comprising the AstroPath Platform for whole slide multiplex immunofluorescence. This highly optimized and validated image processing and database capacity, along with expertise in spatial analyses provided by her Astronomy collaborators at Johns Hopkins, provides a unique competitive advantage for Dr. Cottrell's research. Continued support from OICR will allow Dr. Cottrell to build more local computational capacity, and to be the first and only researcher in Canada to implement the AstroPath Platform.

Another distinguishing feature of Dr. Cottrell's research program is her focus on translation of her biomarker research into clinical tests that will improve patient care such as early detection biomarkers for lung cancer. Her project will advance this work beyond protein biomarkers to glycobiomarkers. Her role as a physician scientist and practicing pathologist provides a valuable perspective on the types of assays that can be supported clinically. Moreover, Dr. Cottrell's position as a Senior Investigator in the Canadian

Cancer Trials Group (CCTG) affords her a unique perspective and access to optimal clinical trial designs needed to gain regulatory approval for novel biomarkers. As the Deputy Director of the CCTG biobank, Dr. Cottrell also has unique access to highly valuable clinical trial biospecimens needed for cutting edge biomarker research in the broader research community.

I am highly supportive of this research project and can attest to the feasibility of the project with the research facilities available to the Cottrell lab.

Sincerely yours,



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