Quantification and Identity of Neoantigens in Cutaneous T-cell Lymphoma

by

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Abstract

Background

Mycosis fungoides (MF) is the most common cutaneous T-cell lymphoma (CTCL). Early stage disease, limited to skin patches & plaques, is indolent. However, progression to tumour stage disease marks a significant drop in survival to less than 4 years. Treatments for advanced disease are palliative and MF remains incurable.

Given evidence that CTCL is immunogenic, immunotherapies are a promising avenue for newer treatments. However, trials of immune checkpoint inhibitors in MF have shown low to moderate response rates. The prevailing understanding of MF is that it is a highly mutated tumour demonstrating suboptimal response to immunotherapies. Neoantigens are predictors of immunotherapy response, but they have never been studied in CTCL. There is also no knowledge of neoantigen clonality in MF. Clonal tumours, comprised of genetically identical cells expressing the same neoantigen, are more susceptible to immunotherapies compared to subclonal tumours. There is an urgency in identifying neoantigens in MF given the mortality associated with advanced disease and the lack of significant advances in treatment.

Objective

The objective of this thesis was to gain insight into the immunogenicity of MF by characterizing the tumour mutation burden, identifying neoantigens for the first time, comparing neoantigens between disease stages and determining neoantigen clonality.

Our hypothesis is that a high tumour mutation burden in MF will result in a large number of neoantigens. However, these neoantigens are likely to be mostly subclonal, rendering them less responsive to immunotherapies.

Methods

We tested our hypothesis through a translational study utilizing bioinformatics. We obtained whole exome and whole transcriptome sequences from 24 MF samples (16 plaque, 8 tumour) from 13 patients. We used bioinformatics software (Mutect2, OptiType, MuPeXi) for mutation calling, HLA typing, and neoantigen prediction respectively. Using PhyloWGS, we subdivided malignant cells into stem and clades, to which neoantigens were matched to determine their clonality.

Results

We demonstrated that MF has a much higher tumour mutation burden than previously described (median 3217 nonsynonymous mutations). We found that MF expressed a significant number of total neoantigens (median 1309 per sample) including a significant number of neoantigens with high binding strength to MHC (median 328). In early disease most neoantigens were clonal but

with stage progression, 75% of lesions had >50% subclonal neoantigens. There was very little overlap in neoantigens across patients or between different lesions on the same patient, indicating a high degree of heterogeneity.

Conclusions

The neoantigen landscape of MF is characterized by a high neoantigen load. Disease progression is associated with an increase in the number of neoantigens and an increase in neoantigen subclonality. Neoantigen subclonality may be the critical factor limiting the efficacy of immunotherapy in patients with advanced disease. Stratification of patients by neoantigen load and clonality may be useful to select suitable candidates for immune checkpoint inhibitor trials.

Preface

This thesis is an original work by Arunima Sivanand. The research project, of which this thesis is a part, received research ethics approval from the Health Research Ethics Board of Alberta, Cancer Committee under the application HREBA.CC-16-0820-REN1.

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supervisory author and was involved in designing the experiments, supervising data analysis and editing the manuscript.

Dedication

This thesis is dedicated to my parents whom I cannot thank enough.

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List of Key Abbreviations

CTCL - Cutaneous T-Cell Lymphoma

ICI - Immune Checkpoint Inhibitor

MF - Mycosis Fungoides

SS - Sézary Syndrome

WES - Whole Exome Sequencing

WTS - Whole Transcriptome Sequencing

Glossary of Terms

Tumour mutation burden - the total number of non-synonymous, somatic, mutations in a tumour. In Chapter 3 (main study), the term is used to refer specifically to the non-synonymous somatic mutations that produce neoantigens.

Neoantigen - an altered peptide produced by tumour mutations, that is recognized as non-native and hence capable of eliciting an immune response.

Clone - genetically identical. In this thesis, clone may refer to a group of cancer cells that have identical mutations or that express identical neoantigens.

Subclone - subset of cells that are genetically identical. In this thesis, subclone may refer to a subset of cancer cells that have identical mutations or that express identical neoantigens. Subclonal tumours are overall genetically heterogenous and thus produce different neoantigens.

Chapter 1: Introduction

Note: Introductory material in this thesis is divided between Chapter 1 (basic definitions) and Chapter 2 (published review on immunogenicity and treatments).

1.1 Overview of cutaneous T-cell lymphoma

Primary cutaneous T-cell lymphomas (CTCL) are a heterogenous group of non-Hodgkin's lymphomas presenting in the skin (1). CTCL is overall uncommon, with an annual incidence of 11.32 cases per million people in Canada (2). The most common types of CTCL are mycosis fungoides (MF) and Sézary Syndrome (SS).

1.1.1 Mycosis fungoides

MF is the most common type of CTCL, comprising up to 60 % of cases (1). MF lesions are erythematous and scaly, and may appear as patches (flat lesions), plaques (raised lesions) or tumours (deep lesions). Early disease comprises patches and plaques, corresponding to T1 and T2 (tumour classification) or stages IA-IIA. Therapies in the early stage are skin-directed, and the disease is mostly indolent with patients surviving up to 12-20 years or more. The progression to tumour stage (T3 or stage IIB) marks advanced disease (stages IIB-IVB, also includes T4/erythroderma) and is associated with a precipitous drop in survival to less than 4 years.

Advanced disease necessitates systemic treatments (reviewed in Chapter 2), which are mostly palliative as there is no cure for MF (3).

1.1.2 Sézary Syndrome

SS is a rare leukemic variant of CTCL defined by circulating neoplastic T-cells (Sézary cells) which infiltrate the skin and lymph nodes. Clinically, SS presents with pruritic erythroderma (widespread redness of the skin) and generalized lymphadenopathy (1). SS is always considered advanced disease and corresponds to CTCL stages IVA1-IVB (3).

1.2 Current treatments and the role of immunotherapy

Advanced MF has been a major therapeutic challenge in dermatology with no significant increases in prognosis made despite decades of research (reviewed in Chapter 2). Given evidence MF is immunogenic (reviewed in Chapter 2), immunotherapies seem a promising avenue for further research. Immune checkpoint inhibitors (ICI), in particular, have revolutionized the treatment of advanced malignancies. Immune checkpoints are part of inhibitory pathways that limit and self-regulate immune responses. Immune checkpoints can be inhibited by drug antibodies, to enhance the host immune response against cancers (4). This technique has been used successfully in the programmed cell death protein (PD)-1/PD-L1 and cytotoxic T lymphocyte-associated antigen (CTLA)-4 inhibitors to treat malignancies such as melanoma and non-small cell lung cancer with dramatic increases in survival (5). However small trials of ICI in CTCL have only shown response rates of 9% to 56% (6–10).

This current study aimed to address the knowledge gap of why, despite evidence that MF is immunogenic, it responds poorly to immune checkpoint inhibitors. We also aimed to further elucidate immune markers in MF.

1.3 Tumour mutation burden

In cancers, the tumour mutation burden (TMB) refers to the number of non-synonymous somatic mutations (11). These mutations include single nucleotide polymorphisms (SNPs), insertions and deletions (indels), gene fusions and frameshift mutations (12). When translated, these mutations result in altered peptides called neoantigens. After processing, some of these neoantigens are presented by the major histocompatibility complex (MHC) on tumour cells which can then evoke an immune response (11). Accordingly, generally the higher the TMB, the higher the number of neoantigens produced and the greater the likelihood of eliciting an immune response (13). Hence for patients treated with immune checkpoint inhibitors, TMB has been a predictor of overall survival (14).

There are exceptions to the positive relationship between TMB and anti-tumour response in cancers such as acute lymphoblastic leukaemia and pediatric medulloblastoma. Despite having a low TMB, strong anti-tumour responses have been evoked in vitro as the resulting neoantigens were highly immunogenic (13,15). This suggests that the identity and immunogenicity of neoantigens may be a more important predictor of immune response than TMB alone.

In mycosis fungoides the TMB has previously been reported to be around 42-102 mutations (16–18), though our group has more recently estimated it to be higher, around 500-4500 per genome

(19). While other highly mutagenic cancers like melanoma and non-small cell lung cancer have demonstrated significant responses to immune checkpoint inhibitors and improvements in survival (5), what is unanswered is why, despite its high TMB, MF has not shown similar robust responses.

1.4 Neoantigens

Neoantigens are mutant peptides produced by tumour specific mutations. As these peptides are not present in normal human tissue, they are recognized as 'foreign' by cytotoxic (CD8+) T cells (12). Hence neoantigens may be highly immunogenic and are considered ideal targets for the patient's intrinsic anti-tumour, T-cell mediated, immune response (20). Immune checkpoint inhibitors enhance the anti-tumour response (21). Hence the neoantigen load, or number of neoantigens expressed by a tumour, positively correlates with response to immune checkpoint inhibitor therapy in highly mutagenic cancers such as melanoma and non-small cell lung cancer (22–24).

Neoantigens have previously never been identified, quantified or characterized in MF. Closing this knowledge gap is crucial to answer the question of why MF demonstrates a suboptimal response to immunotherapies. It is also important to elucidate the identity of these peptides to determine the feasibility of designing personalized immunotherapies to target these specific neoantigens.

1.5 Intratumour heterogeneity

The response to immune checkpoint inhibitors is not only influenced by neoantigen load, but also neoantigen clonality. Mutations in a tumour, and thus neoantigens, may be clonal (present in all cells) or subclonal (present only in a subset of cells). Subclonal mutations may be induced by alkylating agents, such as the cytotoxic agents used in chemotherapy. Tumours that have a high number of clonal neoantigens, and thus lower intratumour heterogeneity (ITH), demonstrate significantly better responses to immune checkpoint inhibitors (25). This is because the neoantigens recognized by CD8+ cytotoxic T-cells are present on a greater proportion of tumour cells.

MF was long thought to develop from a single T-cell and thus be a clonal malignancy. Our group recently demonstrated that MF is in fact a highly genetically heterogenous malignancy comprised of multiple T-cell clones (19). One possible explanation for MF's suboptimal response to ICI is that although its high TMB produces a high neoantigen load, these neoantigens are mostly subclonal, thus making ICI less effective.

1.6 Objective of the current study

Currently, it is known that MF has a poorer response to immunotherapies compared to other malignancies. Tumour mutation burden is one of the markers used to predict response to immune checkpoint inhibitors. What is unexplained is why MF has a high tumour burden but still responds poorly to ICI. Furthermore, there is a substantial knowledge gap in MF whereby

markers of ICI response such as neoantigen burden are unknown. There is an urgency in identifying these markers given the precipitous drop in survival with the progression to tumour stage MF.

Therefore, the objectives of our study is as follows:

1. To accurately quantify the tumour mutation burden and types of mutations in MF
2. To quantify and identify the neoantigen load, and to compare these between disease stages in MF
3. To determine the clonality of neoantigens in MF

1.7 Hypothesis

The current understanding of MF is that it is a highly mutated tumour demonstrating suboptimal response to immunotherapies. There is no knowledge about neoantigens in MF.

Our hypothesis is that a high tumour mutation burden in MF will produce a large number of neoantigens. However, these neoantigens are likely to mostly be subclonal, rendering them less responsive to immunotherapies.

Chapter 2: Immunotherapy for Cutaneous T-Cell Lymphoma: Current Landscape and Future Developments*

Note: Introductory material in this thesis is divided between Chapter 1 (basic definitions) and Chapter 2 (published review on immunogenicity and treatments).

2. 1 Abstract

Mycosis fungoides (MF) and Sézary syndrome (SS) are chronic, progressive primary cutaneous T-cell lymphomas (CTCL) for which there are no curative treatments. Skin-directed therapies, such as phototherapy, radiation therapy or topical nitrogen mustard provide only short-term remissions. Numerous attempts with different chemotherapeutic regimes failed to achieve meaningful clinical responses. Immunotherapy seems to be a promising avenue to achieve long-term disease control in CTCL. There is compelling evidence indicating that MF and SS are immunogenic lymphomas, which can be recognized by the patient's immune system. However, CTCL uses different strategies to impair host's immunity, for example via re-polarizing T-cell differentiation from type I to type II, recruiting immunosuppressive regulatory T-cells (Tregs) and by limiting the repertoire of lymphocytes in the circulation. Many currently used therapies, such as interferon-α, imiquimod, extracorporeal phototherapy and allogeneic bone marrow transplant seem to exert their therapeutic effect via activation of the anti-tumour cytotoxic

response and reconstitution of the host's immune system. It is likely that novel immunotherapies, such as immune checkpoint inhibitors, cancer vaccines and chimeric antigen receptor T-cells (CAR T-cells) will help to manage CTCL more efficiently. We also discuss how current genomic techniques, such as estimating the tumour mutation burden by whole genome sequencing, and identifying neoantigens, are likely to provide clinically useful information facilitating personalized immunotherapy of CTCL.

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2.2 Overview of cutaneous T-cell lymphomas

Primary cutaneous T-cell lymphomas (CTCL) are a rare and heterogenous group of extra nodal non-Hodgkin lymphomas (26). Mycosis fungoides (MF) and the leukemic form Sézary syndrome (SS) are the most common subtypes and account for approximately 60% of all CTCL cases (27). MF and SS remain one of the greatest therapeutic challenges in dermatology. The prognosis of MF is highly dependent on clinical stage. Early stage disease (IA) has a 5-year disease-specific survival of 98%, which precipitously drops in stage IIB disease (tumour) to 56% with a median survival of 4.7 years. Further advanced disease, stages IVA and IVB, have survival rates of approximately 40% and 18%, respectively, with a median survival of 1.4 to 3.8 years (27,28). Due to the inability of most treatments to induce long-term remissions, and the refractory nature of advanced disease, MF is generally considered incurable.

2.3 Objective of current literature review

The unprecedented success of immunotherapies, in particular immune checkpoint inhibitors (ICI) in solid tumours and chimeric antigen receptor (CAR)-T cells in B-cell leukemias, has rekindled interest in cancer immunotherapies for the treatment of lymphomas. Herein, we summarize the evidence indicating that CTCL is immunogenic, review the literature on current immunotherapies, and outline perspectives for immune-based treatments in these diseases.

2.4 CTCL is an area of medical need

T-cell lymphomas represent a major area of unmet medical need and challenge us as “the next, and largely unexplored frontier in lymphoma management” (29). Patients are diagnosed later and

have a poorer response to therapy and shorter survival (5-year overall survival at 10-30%) than comparable patients with aggressive B-cell lymphomas (29,30). Thus, the National Cancer Institute has identified clinical trials specifically targeting T-cell lymphomas as a priority (31).

2.5 Overview of current therapies

Despite decades of intensive research, advancements in CTCL therapy have been few and slow. Only seven randomized clinical trials have been completed in CTCL to date (32–38). None showed advantages for patient survival, and only one (38) was sufficiently powered to demonstrate benefit in progression-free survival. Treatment recommendations have not changed significantly over the last decade. Early disease has an excellent prognosis and is managed expectantly or with skin-directed treatments including topical corticosteroids (such as clobetasol), topical carmustine, topical mechlorethamine, bexarotene gel, psoralen plus ultraviolet A (PUVA), ultraviolet B (UVB), total skin electron beam therapy, and superficial x-irradiation (27,39). In advanced stages, various combinations of systemic therapies are used including interferons, retinoids, rexinoids and single agent chemotherapies. Brentuximab (an immunotoxin targeting CD30) and mogamulizumab (an anti-CCR4 antibody) are recent additions, but neither agent has been shown capable of inducing long-term and sustainable remissions in MF or SS (38,40,41).

2.6 Impaired host immunity in CTCL

One of the important causes of mortality and morbidity, especially in low-grade T-cell lymphomas, is progressive immune system exhaustion (42,43). In advanced CTCL, opportunistic

infections represent the leading cause of disease-related mortality (44). Bacterial colonization of the compromised skin barrier can also contribute to disease progression, due to byproducts such as staphyloccocal enterotoxin A activating oncogenic pathways (45). Impairment of immune functions occurs gradually during disease progression. Early MF is accompanied by slightly elevated counts of cytotoxic $CD8^+$ T-cells and natural killer (NK) cells, both of which are capable of mounting robust cell-mediated anti-tumour responses (46–48). The CD4+, CD8+, and NK cells isolated from CTCL patients have been shown capable of killing autologous malignant T-cells in vitro, and are therefore likely important components of the host's protective immunity that limit early stage CTCL development (48–50). Iatrogenic immunosuppression in early stage CTCL leads to rapid and dramatic disease exacerbation, underscoring the importance of an intact adaptive immune system for controlling this lymphoma (51–53).

2.6.1 Shift from the protective T_H1 response to the inflammatory T_H2 response

Progression of CTCL is associated with an immunosuppressive, inflammatory tumour microenvironment, and a loss in T-cell diversity (47,54–56). Malignant $CD4^+$ T cells play a role in reorchestrating the immune system from the protective T_H1 response to the T_H2 response characterized by excessive production of inflammatory cytokines including interleukin(IL)-4 IL-5, and IL-10 (57,58), as well as suppression of IL-2 and interferon(IFN)-γ (59). Chronic T_H2 biased inflammation favors an immunosuppressive state and ultimately facilitates tumour-survival (54,55,60,61).

2.6.2 Regulatory T cells

Early CTCL is associated with the infiltration of benign T regulatory (Treg) cells into the epidermis which is hypothesized to counteract the malignant expansion (62,63). The correlation

between the number of Treg cells and prognosis remains unclear. Although some studies have shown positive correlations between Treg cell activity and patient survival in CTCL (63), data from other centers demonstrate the opposite (64). However, as MF progresses into a high-grade lymphoma, there is a decrease in tumour infiltrating Treg cells (62,63,65) and a shift towards Treg phenotype (e.g. $FOXP3^+$ expression) in malignant T-cells, which is hypothesized to further suppress anti-tumour immunity (62,65,66).

2.7 Immunostimulation and immunomodulation as therapeutic mechanisms in CTCL

Many therapies currently used in CTCL exert their effects through immunomodulation.

2.7.1 Interferons

Named for their ability to impair viral replication, interferons are signaling proteins of the innate immune system (67). Interferons also exert antitumour, cytostatic, and, of particular interest for MF, immunomodulatory effects (68–73). Recombinant DNA technologies have generated three therapeutic groups of interferons: interferon-α, -β (type I interferons) and interferon-γ, that have comparable efficacies in vitro (72,73). Most relevant for the treatment of MF is interferon alfa (IFN-α) which is believed to bolster anti-tumour toxicity by increasing CD8+ and NK cell activation, balancing the T_H2 biased cytokine pattern, and impairing tumour cell proliferation (68,69,73–76). The efficacy of IFN-α does not depend on the clinical stage of CTCL (71,77) with one study demonstrating 53% partial responses (PR) and 27% complete responses (CR)(78). IFN-α augments the efficacy of PUVA (36) and total skin electron beam therapy (TSEBT) (63% CR compared to 36% CR with TSEBT alone (79)). Other cytokines, such as IL-

2, IL-12, and IFN-γ have also shown efficacy for MF treatment, though these are not routinely used in clinical practice (80).

2.7.2 Toll-like receptor agonists

Toll-like receptor (TLR) agonists, such as imiquimod (TLR7 agonist), function by inducing massive local cytokine release, including type I interferons, against MF. The response rate is 50%-100%, however the published literature on this topic is limited to mostly case reports and small case series (81). Other TLR agonists, such as topical resiquimod (TLR7/8 agonist) have produced high response rates (9 PR and 2 CR in 12 patients)(82). TLR agonists are also promising vaccine adjuvants for CTCL (see below).

2.7.3 Extracorporeal photopheresis

Extracorporeal photopheresis (ECP) is another treatment option that acts predominantly through immunomodulation. In ECP, T-lymphocyte-rich plasma obtained through apheresis is exposed to 8-methoxypsoralen (a photosensitizer), irradiated by a UVA source, and subsequently infused back into the patient (83,84). ECP induces apoptosis of the circulating malignant CTCL cells, which are then phagocytosed by dendritic cells, and in turn presented to activate anti-tumour $CD8^+$ cells (83,85,86). Furthermore, ECP has been observed to shift the cytokine pattern from T_H2 to T_H1 (87). Since ECP targets circulating malignant T-cells, it is most effective in SS and in erythrodermic CTCL with blood involvement. The efficacy of ECP in advanced MF has been confirmed in 19 trials of over 400 patients (86). The combined overall response rate (ORR) for all disease stages was 55.7%, with 17.6% of patients experiencing complete remission. Importantly, the combined ORR for stage IV MF was 57.6% with 15.3% experiencing complete response (86). ECP is also a first-line therapy for advanced (stage IIIa and IIB) MF, and SS (27).

2.7.4 Hematopoietic stem cell transplant

Allogeneic hematopoietic stem cell transplant (AHSCT) has proven to be the most potent, and potentially curative, approach to CTCL (27). Progression-free survival varies between 26%-32% (over 4-5 years)(88,89). However, almost half the cohort in one study experienced relapses, with the primary cause of death in the treatment group being disease progression (88,89) AHSCT is currently recommended as a treatment for stage IVA and IVB MF, and a second-line treatment for SS and stage IIB, IIIA, and IIIB MF (27). The mechanism of action of AHSCT is mainly through the graft-versus-lymphoma effect exerted by donor cells. In line with this mechanism, it was shown that autologous stem cell transplant does not provide benefit in CTCL (89,90) and that donor lymphocyte infusion of allogeneic, active CD8+ cells increases antitumour activity, albeit at a greater risk of severe graft-versus-host disease (90).

2.8 New immunotherapy approaches: immune checkpoint inhibitors, cancer vaccines and CAR T-cells

2.8.1 Immune checkpoint inhibitors

Immune checkpoint ligands, such as programmed death-ligand 1 (PD-L1) and B7, on antigen presenting cells, bind to their receptors, PD-1 and CTLA-4 respectively, on T cells, to downregulate immune responses. Since tumour-infiltrating lymphocytes in CTCL and other neoplasms are functionally and metabolically inactive (or even senescent)(91–93), inhibition of these checkpoint molecules restores an anti-tumour response (94). ICI has demonstrated marked

benefit in treating advanced malignancies, though as monotherapy it provides sustained benefit in only a subset of patients (14).

Limited clinical studies have examined PD-1 blocking antibodies, such as nivolumab and pembrolizumab, in CTCL. A phase 1 study of nivolumab in disease refractory to multiple prior treatments demonstrated a 15% ORR in 13 MF patients but no response in 3 patients with other forms of CTCL (6). A phase II study of pembrolizumab in advanced stage CTCL refractory to previous treatments demonstrated a 56% ORR among 9 MF patients and 27% ORR among 15 SS patients (95). Data on CTLA-4 inhibiting antibodies such as ipilimumab is also limited. A case report of ipilimumab to treat melanoma in a patient with concurrent MF resulted in complete remission of MF (7). Furthermore, a SS patient treated with ipilimumab experienced a rapid response that was attributed to a rare gene fusion between the extracellular/transmembrane domain of CTLA-4 (which has a high affinity for binding ligands) and the intracytoplasmic domain of PD-1 (8). The combination of ipilimumab with nivolumab showed comparable efficacy to nivolumab monotherapy in a phase I study of 11 T-cell non-Hodgkin's lymphoma patients. The ORR was 9% with 1 PR observed, however rates were not reported specifically for the subset of 7 CTCL patients (96). MF, as a tumour of T-cells, poses unique challenges for ICI. Malignant T-cells may express PD-1 and be under inhibitory control (97,98). Hence PD-1 blocking drugs could theoretically promote tumour growth (99), which complicates interpretation of the responses in small patient cohorts.

2.8.2 Cancer vaccines

Unlike prophylactic vaccines given to healthy individuals, therapeutic vaccines are administered in the presence of existing disease to sensitize the immune system to tumour neoantigens (100).

Despite an attractive mechanism of action, therapeutic cancer vaccines have been less successful than nonspecific augmentation of antitumour immunity by ICI (101). Clinical experience in cancer vaccines in CTCL is very limited. In a pilot study of 10 CTCL patients refractory to previous therapies, treated with a vaccine of tumour antigen-specific dendritic cells (generated by pulsing the cells in autologous tumour lysate), an ORR of 50% was observed (102).

Another principle of anticancer vaccines is the use of attenuated viruses. This technique takes advantage of the fact that malignant T cells are deficient in interferon signaling which renders them incapable of generating antiviral molecules. Patients are pretreated with IFN-α before administering an attenuated measles vaccine. The virus will only be able to replicate in interferon resistant CTCL cells which will render them susceptible to immune attack. In a phase I study, 5 CTCL patients were pretreated with IFN-α and anti-measles serum antibodies and before receiving 16 live intratumoural injections of the measles virus over 28 days. This resulted in 5 of the 6 treated sites showing an observable regression. The vaccine had an excellent safety profile and patients demonstrated measles antibody titres despite disease-induced immunosuppression (103). Furthermore, oncolytic viruses have the potential to be engineered to specific malignancies if tumour-specific surface proteins are known (103).

As mentioned previously, TLR agonists may augment the efficacy of vaccinations. A phase I/II study of 14 MF patients refractory to previous treatments injected intratumourally with a TLR9 agonist (PF-3512676) combined with local radiotherapy demonstrated an ORR of 35.7%. The TLR agonist was used for local stimulation of the antitumour immunity following radiation. Of 6 patients given a single immunization, 2 (33.3%) experienced a PR. A second dose was given at the 4-week mark to 8 patients whose lesions did not initially improve, 3 (37.5%) of whom proceeded to experience a PR. While side-effects were limited to injection site reactions and flu-

like symptoms, the observed responses lasted only a median of 7 weeks with the second vaccine dose not significantly improving the antitumour response (104).

Insufficient immune responses to vaccines may result from the use of tumour-associated self-antigens that do not elicit T cell responses due to immune tolerance (105). Tolerance may result from poorly immunogenic antigen delivery methods and from immunosuppressive tumour microenvironments (92,106). Nonetheless, the moderate to low response rates of the vaccines reviewed here are comparable to that of standard therapies such as interferons and retinoids (104).

2.8.3 Chimeric antigen receptor T-cells

Chimeric antigen receptors (CAR) can be engineered to target specific antigens and inserted into T-cells to eliminate cells expressing those antigens. CD19 directed CAR T-cells have demonstrated high response rates and been approved as treatments for B-cell malignancies, including relapsed/refractory B-cell acute lymphoblastic leukemia and diffuse large B-cell lymphoma (DLBCL). No studies on humans on CAR T-cells in CTCL exists, however one study was conducted in another T-cell based non-Hodgkin's lymphoma. 2 patients with anaplastic large-cell lymphoma (ALCL) were infused with autologous CAR T-cells (107) directed to CD30, a protein overexpressed in ALCL and under expressed in normal tissue (108). While 1 patient experienced no response, the other experienced a CR lasting 9 months after the fourth infusion (107).

In designing CAR T-cells for T-cell malignancies, challenges arise with distinguishing CAR, normal and tumour T-cells. Each group of T-cells may be killed if CAR T-cells are directed

towards shared antigens. Firstly, CAR T-cells recognizing other CAR T-cells can result in mutual killing, termed fratricide, that prevents the expansion of CAR T-cells required for therapeutic response (109). However, a preclinical study in mice showed that CD5 directed CAR T-cells eliminated T cell lymphoma cell lines in vitro with limited fratricide (110). Another study targeted CD2 which is expressed ubiquitously on SS cells. Engineering anti-CD2 CAR T-cells, with their own CD2 deleted, was found to prevent fratricide (111). Furthermore, the destruction of normal T- cells can lead to T-cell aplasia, which confers a significant risk of opportunistic infections, and is not well managed, unlike B-cell aplasia (109,112,113). In a preclinical study, deleting T-cell receptor α from anti CD2 CAR T-cells prevented alloreactivity (114). Additionally, there is a risk of contamination with circulating tumour T-cells when autologous T-cells are harvested to develop CAR T-cells. If there is subsequent generation of CAR-T tumour cells, these will escape recognition by therapeutic CAR T-cells (109). Finally, generating tumour-specific CAR T-cells requires the identification of target antigens expressed highly or exclusively on tumour cells compared to normal cells (109), and there is a paucity of data on tumour specific antigens in MF.

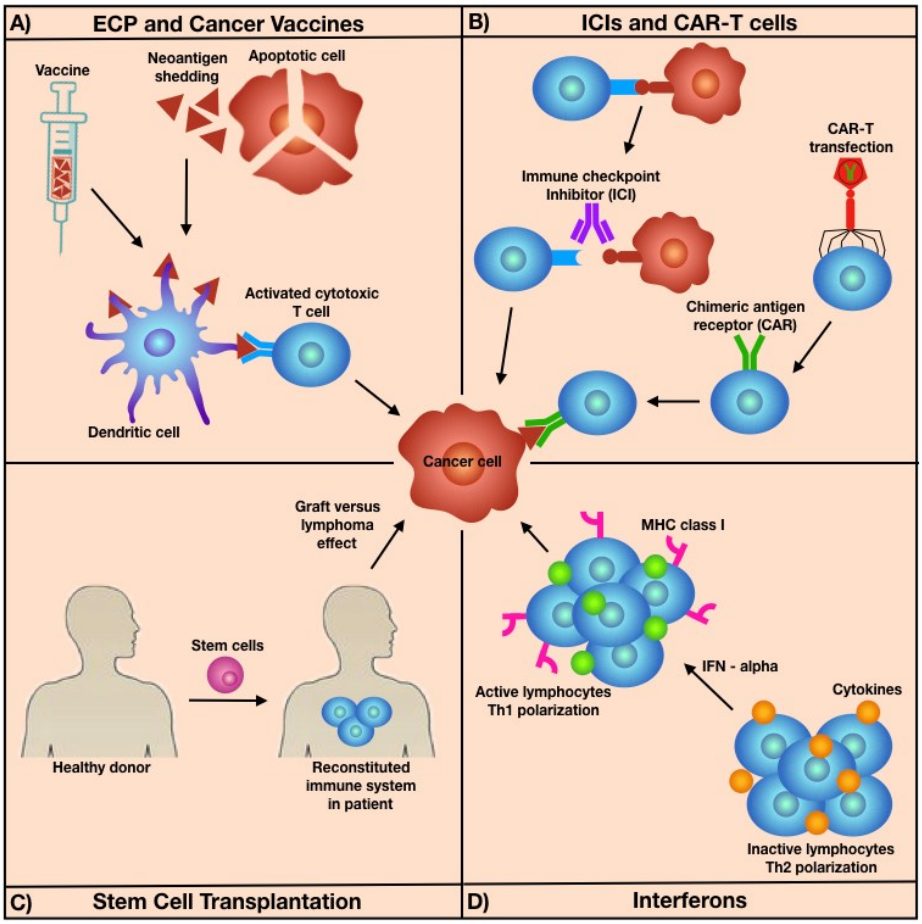


Figure 2.1: Summary of immunotherapy mechanisms

(A) Tumour specific antigens, or neoantigens, can be presented by dendritic cells (and other antigen presenting cells) to activate cytotoxic T-cells to eliminate cancer cells. Cancer vaccines may contain neoantigens, or dendritic cells specific to neoantigens. ECP works through a similar mechanism by causing apoptosis of malignant cells, which releases neoantigens. Not shown - Cancer vaccines may also contain foreign bodies such as oncolytic viruses and TLR agonists which induce a similar innate immune reaction.

(B) Immune checkpoint ligands on antigen presenting cells (such as malignant T-cells) bind to receptors (such as on tumour infiltrating T-cells) to limit the immune response. ICI drugs prevent this binding, thereby restoring the ability of tumour infiltrating T-cells to mount an anti-tumour response. Viruses can be used to insert chimeric antigen receptors (CAR) that recognize tumour specific proteins, into T-cells. The resulting CAR-T cells, once infused into the patient, can mount an immune response against tumour cells.

(C): CTCL is associated with immunocompromise and a loss in T-cell diversity. Allogeneic stem cell transplantation involves the transfer of donor stem cells, which can differentiate into cytotoxic T-cells in patients. This restores the ability of the immune system to mount an anti-tumour response.

(D): CTCL is associated with a Th2 biased state, characterized by the excessive production of inflammatory cytokines. This results in a chronic immunosuppressive state with inactive T-cells. Interferons can revert this to a protective Th1 state, in which active T cells are capable of mounting a downstream anti-tumour response.

Abbreviations: ECP: Extracorporeal photopheresis; ICI: Immune checkpoint inhibitor; Th1: T helper cell Type I; Th1: T helper cell Type II; TLR: Toll-like receptor

2.9 Genomics as a tool for discovering immunotherapy targets and biomarkers

Whole genome sequencing (WGS) and whole exome sequencing (WES) of tumour cells have made possible the discovery of tumour specific antigens as targets and biomarkers of immunotherapy (115). Most of these insights have come from studies in ICI. A range of predictive biomarkers for immunotherapies such as ICI exist, including expression levels of checkpoint molecules, immune cell infiltration and immune gene signatures (116) For instance, a high tumour mutation burden has been associated with improved response to ICI and longer overall survival (14). This is thought to be due to a high number of neoantigens which confers more opportunities for the immune system to recognize the tumour as foreign, immunogenic material (25). Indeed, a high neoantigen burden in cancer has been associated with significantly higher overall survival in patients treated with ICI (25,117). Unfortunately, no studies to date have described neoantigens in MF.

Neoantigen burden does not always correlate with prognosis. Tumours may utilize mechanisms to escape immune surveillance including losing mutations coding for neoantigens (118) and downregulating HLA class I genes that present neoantigens (119,120). Tumours may also select for mutations favouring ICI resistance, for instance by reducing expression of checkpoint

inhibition molecules (119,120). Prior treatment with alkylating chemotherapeutic agents can also induce a high number of subclonal neoantigens, that as a consequence of only being present on a subset of tumour cells, are harder to eliminate (25). This mechanism may be operating in CTCL because it has been shown that patients who fail aggressive chemotherapy have poorer outcomes than patients who are managed more conservatively (121).

Moreover, a proportion of neoantigens are not clonal (i.e. present in all tumour cells) but subclonal (present only in a subset of cells). Increased subclonal heterogeneity of neoantigens negatively correlates with ICI responses (25) CTCL is likely to represent a tumour of high level of heterogeneity (122,123) which may translate into neoantigen heterogeneity as well. MF has also been demonstrated to be heterogenous between patients, and over time in a given patient, highlighting the potential for a personalized medicine approach to immunotherapy (124).

2.10 Conclusion

Though MF mostly has a chronic, indolent course, the prognosis for advanced and relapsed disease is very poor. We present evidence that MF and SS are immunogenic, and that immunomodulation and immunostimulation are promising therapeutic options. However, CTCL being a T-cell derived disease presents unique challenges for ICI, CAR-T or tumour vaccine development. It is likely that genomic techniques facilitating the identification of tumour neoantigens and the monitoring of intratumoural T-cell activity will provide predictive biomarkers for personalized immunotherapy.

Chapter 3: The neoantigen landscape of mycosis fungoides*

3.1 Abstract

Background

Mycosis fungoides (MF) is the most common cutaneous T-cell lymphoma, for which there is no cure. Immune checkpoint inhibitors have been trialed in MF but the results have been inconsistent. To gain insight into the immunogenicity of MF we characterized the neoantigen landscape of this lymphoma, focusing on the known predictors of responses to immunotherapy: the quantity, HLA-binding strength and subclonality of neoantigens.

Methods

Whole exome and whole transcriptome sequences were obtained from 24 MF samples (16 plaque, 8 tumour) from 13 patients. Bioinformatic pipelines (Mutect2, OptiType, MuPeXi) were used for mutation calling, HLA typing, and neoantigen prediction. PhyloWGS was used to subdivide malignant cells into stem and clades, to which neoantigens were matched to determine their clonality.

Results

MF has a high mutational load (median 3217 non synonymous mutations), resulting in a significant number of total neoantigens (median 1309 per sample) and high-affinity neoantigens

(median 328). In stage I disease most neoantigens were clonal but with stage progression, 75% of lesions had >50% subclonal antigens and 53% lesions had CSiN scores <1. There was very little overlap in neoantigens across patients or between different lesions on the same patient, indicating a high degree of heterogeneity.

Conclusions

The neoantigen landscape of MF is characterized by high neoantigen load and significant subclonality which could indicate potential challenges for, and which might limit the efficacy of, immunotherapy in patients with advanced disease.

* A version of this chapter has been published as A Sivanand, D Hennessey, A Iyer, S O'Keefe, P Surmanowicz, G Vaid, Z Xiao and R Gniadecki, "The neoantigen landscape of mycosis fungoides", Frontiers in Immunology, 2020 November 23, doi:https://doi.org/10.3389/fimmu.2020.561234.

3.2 Introduction

3.2.1 Overview of MF

Mycosis fungoides (MF) is the most common type of cutaneous T-cell lymphoma (CTCL), and it develops from clonotypically diverse malignant T-cell precursors seeding the skin (122,125). Prognosis in the early stages (T1-T2, patches and plaques) is excellent, however the development of tumours (T3) or erythroderma (T4) is associated with a significant decrease in survival (27,28). Despite intensive research, MF remains incurable and treatments for advanced disease are mostly palliative (27).

3.2.2 Immunogenicity and current therapies

There is robust evidence that MF is an immunogenic tumour and that the immune system is an essential factor limiting its progression (126). It has been well documented that iatrogenic immunosuppression causes a catastrophic dissemination of MF (52,127). Many current therapies (interferons, imiquimod, extracorporeal photopheresis and allogeneic stem cell transplant) are considered to act primarily via stimulation of the antitumour immunity (85,90,128,129). However, the experience with immune checkpoint inhibitors has been disappointing in MF (126). The literature comprising approximately 50 cases of MF treated with various immune checkpoint inhibitors reports response rates ranging from 9% to 56% with only a few documented complete remissions (7–10,130). Of the few anticancer vaccine studies in CTCL, response rates have ranged from 33% to 50% (102–104). Those rather discouraging results are surprising in view of the fact that MF is a mutationally rich tumour with a mutation load in the range of 500-4,500 somatic mutations/genome (131). The number of mutations is usually

correlated with the number of neoantigens and consequently the immunogenicity of the cancer, which is predictive for immune checkpoint inhibitor efficacy (22,24,25).

3.2.3 Intratumour heterogeneity

It has recently been suggested that in addition to mutational load and the number of neoantigens, tumour heterogeneity has a major impact on the ability of the host immune system to mount an effective antitumour defense. Neoantigens can be classified as clonal (present on all cancer cells) or subclonal (present only on a subset (subclones) of cancer cells)(25). A high clonal neoantigen burden, for instance in malignant melanoma, favours effective immune surveillance, response to immunotherapy and significantly prolonged survival (25). In contrast, a tumour with a branched subclonal structure will be poorly recognized by the immune system, even if the mutation load is high, as documented for some immunotherapy-resistant tumours such as glioblastomas (132).

3.2.4 Objective of current study

To better understand the potential for immunotherapeutic approaches in MF we studied the landscape of neoantigen expression in this malignancy. Using whole transcriptome and whole exome sequencing, we determined the pattern of neoantigens in early lesions of patches and plaques and compared them to those of clinically advanced disease. We show that disease progression is correlated with an increase in mutational load and the number of neoantigens. However, advanced lesions of MF exhibit a high proportion of subclonal neoantigens which may limit the efficacy of immunotherapies.

3.3 Materials & Methods

3.3.1 Materials, sequencing, datasets

Institutional ethics approval was obtained under the application HREBA.CC-16-0820-REN1. We performed whole exome sequencing (WES) and whole transcriptome sequencing (WTS) of 24 MF samples (16 plaque, 8 tumour) and matched peripheral blood mononuclear cell (PBMC) in 13 patients (patient characteristics in Appendix Table A1). At the time of biopsy, none of the patients had received immune modulating therapies. DNA and RNA sequencing libraries were prepared from tumour cell clusters microdissected from skin biopsies using laser capture microdissection and sequenced as described previously (131,133) (**Figure 3.1**). The mean sequencing depth across samples was 162.62x (individual sequencing depths in Appendix Table A2). Additional datasets comprised sequencing data (study characteristics in Appendix Table A3) published by McGirt et al. (5 whole genome sequences (WGS) from 5 patients with MF)(16) and by Choi et al. (31 WES from 31 patients with Sézary syndrome)(134).

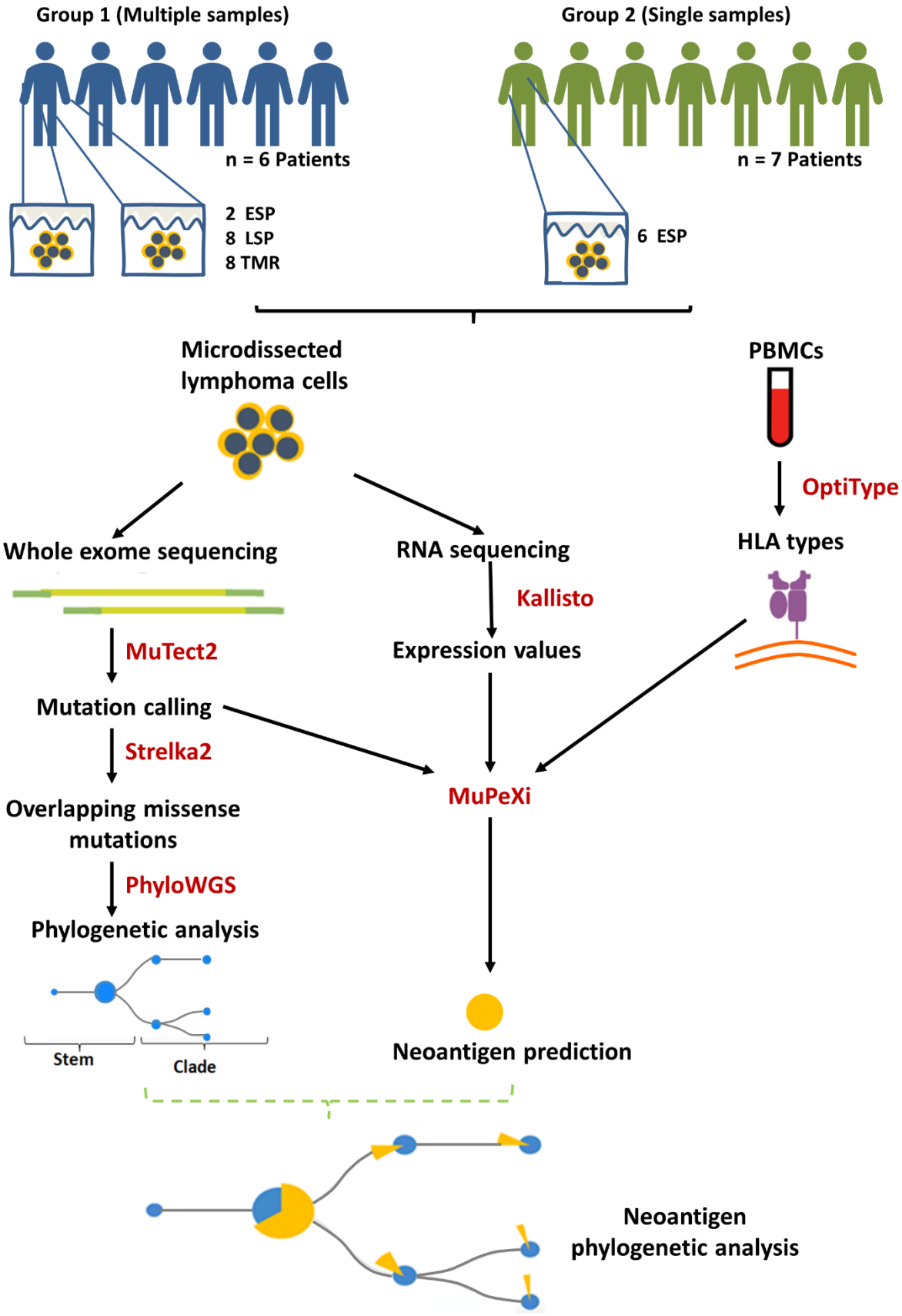

Group 1 (Multiple samples)
n = 6 Patients
2 ESP
8 LSP
8 TMR
Group 2 (Single samples)
n = 7 Patients
6 ESP
Microdissected lymphoma cells
PBMCs
OptiType
HLA types
Whole exome sequencing
RNA sequencing
Kallisto
Expression values
MuTect2
Mutation calling
Strelka2
Overlapping missense mutations
MuPeXi
PhyloWGS
Phylogenetic analysis
Stem
Clade
Neoantigen prediction
Neoantigen phylogenetic analysis

Figure 3.1: Summary of methods and study design. Biopsies of lesional skin and blood were obtained. 13 MF patients were divided into group 1 (multiple samples) and 2 (single samples) according to the number of biopsies contributed. The lesions were categorized according to the clinical stage and the morphology of the lesion: ESP (early stage plaques, i.e. MF plaques in stage I), LSP and TMR (respectively, late stage plaques and tumours biopsied from patients in stage ≥IIB). MuPeXi was used to predict neoantigens. For clonality analysis we used mutation data obtained from MuTect2 and Strelka2, as described previously (131). Predicted neoantigens were mapped to the clades and stems of the phylogenetic trees constructed using PhyloWGS (131).

3.3.2 Identification of neoantigens

Bioinformatics analysis involved a series of pipelines shown in **Figure 3.1**. GATK (v4.0.10) best practices guidelines (135) were used to process the initial WES fastq files. Reads were aligned to the hg38 reference genome. MuTect2 (v2.1) was used for variant calling to identify missense and indel mutations. OptiType (v1.3.1)(136) was used with default settings to predict class I human leukocyte antigen (HLA) types from WES of PBMC for the corresponding samples. Kallisto (v0.45.0)(137) was used to process the raw RNA fastq files with the bootstrapping function set to 500 to obtain the variance and expression level. The outputs of these pipelines (.vcf files from MuTect2, HLA types from Optitype and .tsv files from Kallisto) were imported into MuPeXi (v1.2)(138) to predict neoantigenic peptides (8-11 amino acids long). Of note, MuPeXi penalizes neopeptides that are identical to their wildtype, as these are likely not immunogenic due to central tolerance. Neopeptides are prioritized based on their dissimilarity to their unmutated form. NetMHCpan 4.0 (139) (incorporated in MuPeXi pipeline) was used to predict peptide binding affinities to up to 6 patient-specific HLA types.

3.3.3 Neoantigen filtering

We will refer to the raw output of prediction software as 'putative neoantigens' and the result once filtering criteria is applied as 'filtered neoantigens'. Our filtering criteria included: *(1)*

Mutant peptide binding strength, defined as eluted ligand (EL) likelihood percentile rank ≦0.5% , *(2)* RNA expression level >0.1 transcripts per million (TPM)(140), *(3)* Top peptide, applied last to group all predictions arising from the same mutation (chromosome and genomic position) and select the peptide with the lowest binding strength. While all peptides <0.5% rank are generally considered to be strong binders (141), we further divided these into high strength binders (<0.05%rank), intermediate strength binders (0.05≧%rank<0.15) and low strength binders (0.15≧%rank≦0.5).

3.3.4 Mutant peptide characterization

To further characterize mutant peptides, we identified the most frequently overlapping peptides between samples. We then used the mutant peptide sequence to search the IEDB database (142) for homologous peptides that were known immune epitopes. We searched for exact matches and if none were found, we reduced the threshold to blast >90%. If a known epitope was found, we further searched the Uniprot database (143) for details of the gene encoding the protein, and the protein function.

3.3.5 Neoantigen clonality analysis

For phylogenetic analysis, Strelka2 (v2.9.10)(144) was used for mutation calling to identify missense mutations that overlapped with those called by MuTect2. TitanCNA (145) was used to predict copy number aberrations (CNA). Default parameters were used except for alphaK which was changed to 2,500 as recommended for WES data. PhyloWGS (v1.0-rc2)(146) was used to build phylogenetic trees by clustering missense mutations using CNA. The stem and clade mutations producing neoantigens were then highlighted on the phylogenetic trees to determine the clonality of the neoantigens.

3.3.6 Cauchy-Schwarz index of Neoantigens (CSiN)

CSiN combines neoantigen load, neoantigen clonality and immunogenicity in a single score and is believed to reflect the sensitivity of the tumour to immunotherapy (147). We used transcripts per million (TPM) counts calculated by Kallisto (137) for RNA expression, and neoantigen binding strengths calculated by MuPeXI (138). We used seven binding strength thresholds (%rank) of 0.375, 0.5, 0.625, 0.75, 1.25, 1.75, and 2. Only neoantigens that had an expression ≥ 1 TPM and were also produced by the top 500 most common variants (variants with the highest variant allele frequency) were included in the CSiN score calculation.

3.3.7 Data visualization

Visual data representations were created using the R package beeswarm (www.cbs.dtu.dk/~eklund/beeswarm/), GraphPadPrism (v8.3.0, www.graphpad.com) , jvenn (148), Venn Diagram Tool (http://bioinformatics.psb.ugent.be/webtools/Venn/), PhyloWGS (146) and Microsoft Excel.

3.4 Results

3.4.1 Tumour mutation burden in MF is dominated by frameshift mutations

Early stage MF (IA-IIA) is characterized by thin cutaneous lesions of patches and plaques (T1-T2). The emergence of tumours (T3) heralds progression to the advanced stage IIB. It is important to note that most advanced stage patients may exhibit plaques persisting from the early stages in addition to the stage-defining tumours. To capture the impact of disease stage on mutation burden and neoantigen expression we classified biopsies into the following categories:

early stage plaques (ESP), i.e. lesions T1 and T2 (patches or plaques) obtained from patients in stage IA-IB, late stage plaques (LSP) and matched tumours (TMR) from patients in a clinical stage ≥ IIB **(Figure 3.1)**. In those lesions, we determined tumour mutation burden (TMB) defined as the number of non-synonymous mutations producing neoantigens. The median TMB was 3,217 mutations per sample, or 35 mutations/kB consisting primarily of frameshift mutations (70.3%), in-frame missense mutations (28.4%), insertions (1.1%) and deletions (0.2%) **(Figure 3.2)**. The median TMB in ESP was 2,455 (range 1,440-7,198), and its upper range increased in LSP (median 5014, range 890-8,697) and in TMR (median 2,697; range 1306-8,722).

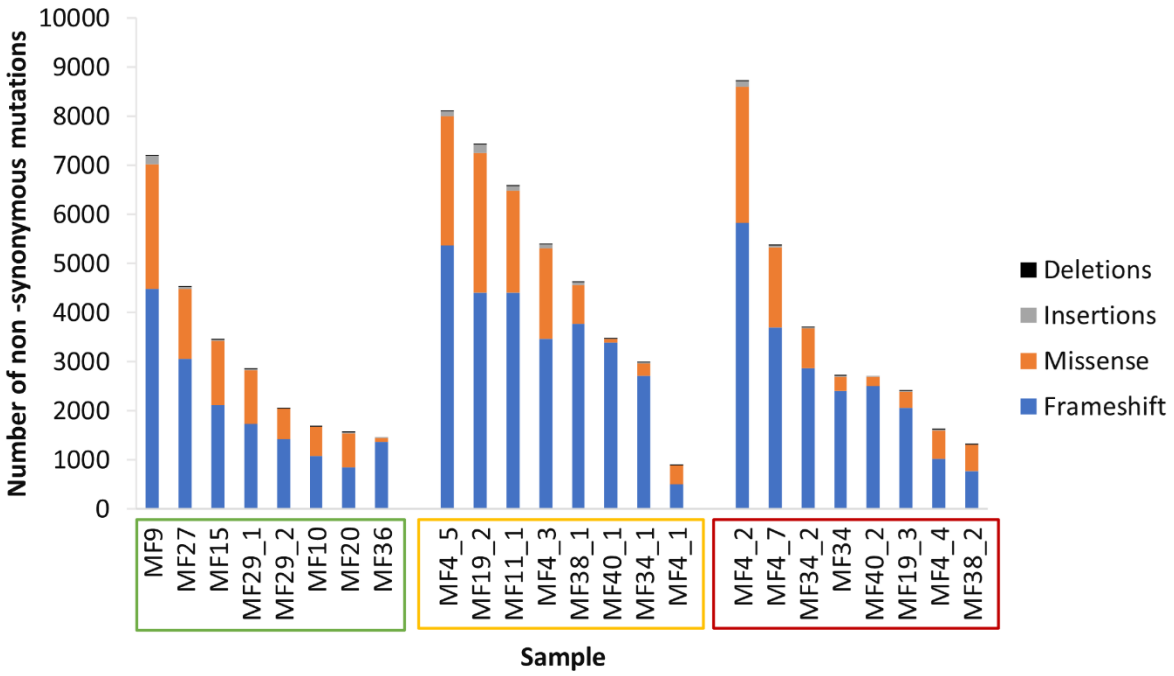


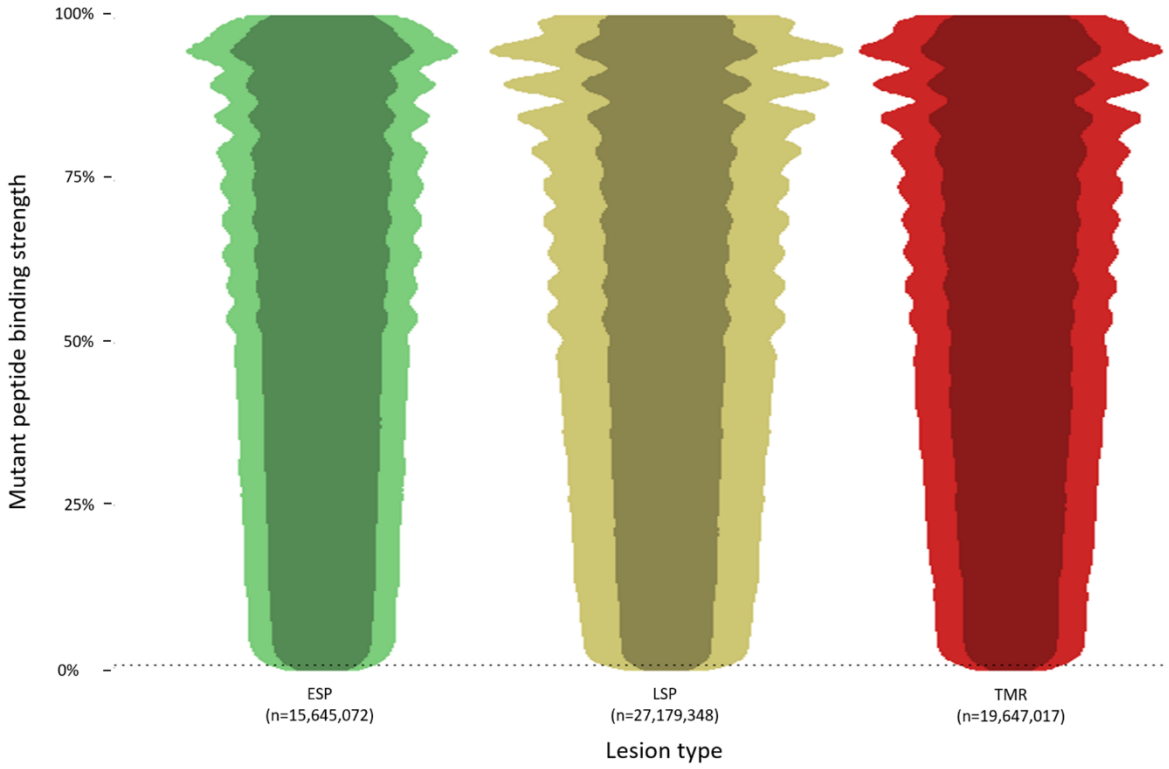
Figure 3.2: Tumour mutation burden. Samples are arranged in descending order of TMB and sample names enclosed in boxes with colours corresponding to the lesion type - early stage plaque (green), late stage plaque (yellow) and tumour (red). Samples are arranged in descending order of TMB. Frameshift mutations comprise the majority of non-synonymous mutations. Sample names are enclosed in boxes with colours corresponding to the lesion type – early stage plaque (green), late stage plaque (yellow) and tumour (red).

3.4.2 Increase in neoantigen load during disease progression

When examined by lesion type, patients with advanced disease had a greater number of putative neoantigens compared to early stage plaques (LSP - 27,179,348, TMR - 19,647,017 vs ESP - 15,645,072), though this was not statistically significant (P=0.368) (**Figure 3.3A**). There was no difference in median binding strength between ESP (median 56%), LSP (median 57%) and TMR (median 58%).

Filtering putative neoantigens is necessary to narrow down epitopes that are most likely expressed in patients. When we applied all filters ("RNA" column in **Figure 3.3B**), an average of 70% of predicted neoantigens were expressed at the RNA level (median neoantigens per sample was 1,309). A median of 328 were high strength binders (<0.05%rank), 376 were intermediate strength binders (0.05≧%rank<0.15) and 540 were low strength binders (0.15≧%rank≦0.5).

A


100%
75%
50%
25%
0%
Mutant peptide binding strength
ESP
(n=15,645,072)
LSP
(n=27,179,348)
TMR
(n=19,647,017)
Lesion type

B

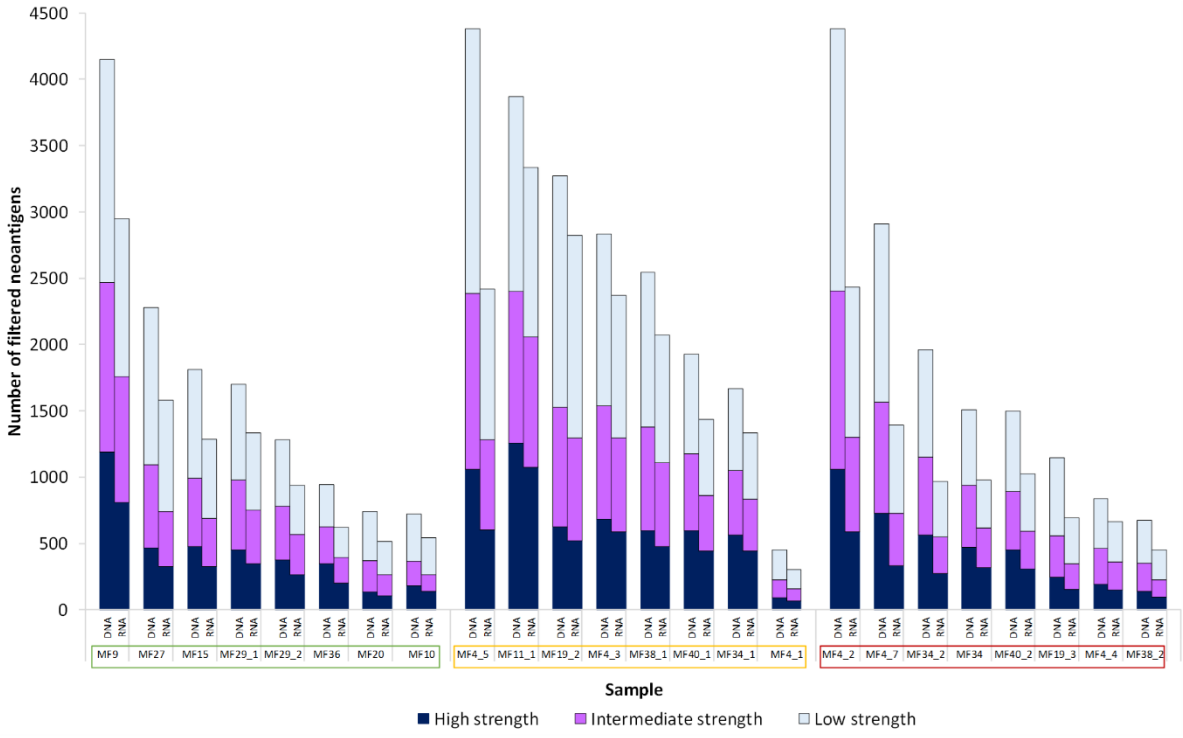
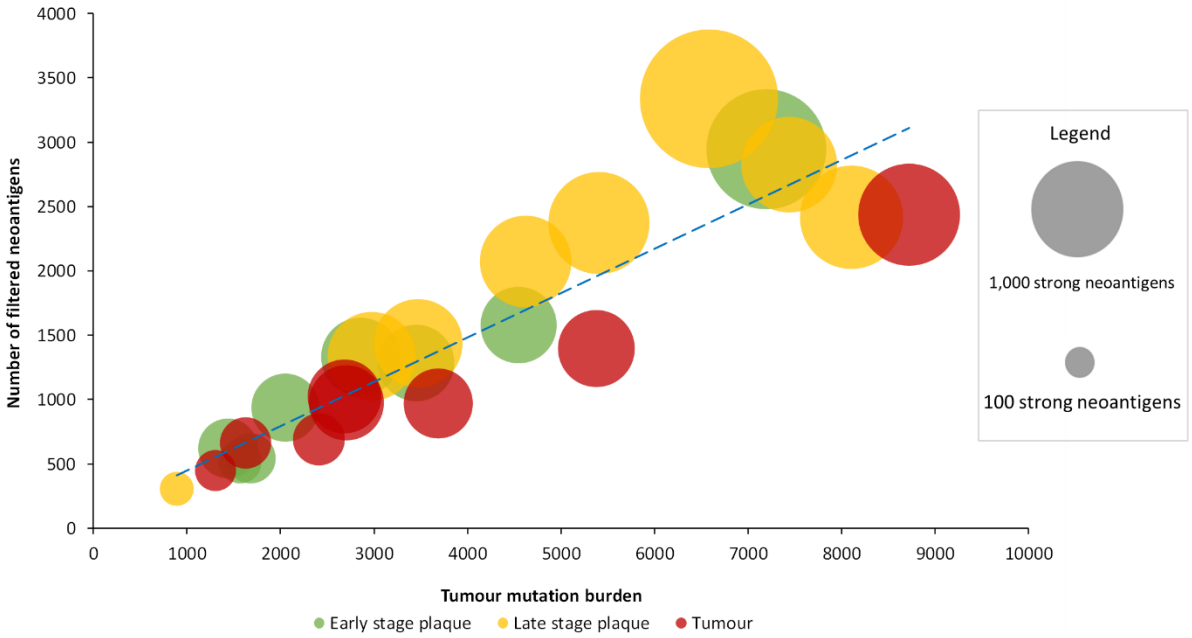

4500
4000
3500
3000
2500
2000
1500
1000
500
0
Number of filtered neoantigens
DNA
RNA
MF9
MF27
MF15
MF29_1
MF29_2
MF36
MF20
MF10
MF4_5
MF11_1
MF19_2
MF4_3
MF38_1
MF40_1
MF34_1
MF4_1
MF4_2
MF4_7
MF34_2
MF34
MF40_2
MF19_3
MF4_4
MF38_2
Sample
High strength
Intermediate strength
Low strength

Figure 3.3: Characterization of neoantigens in MF. A: Beeswarm plot representation of putative neoantigens prior to filtering. Due to the extensive size of the dataset, a random 1% of all data points were plotted to demonstrate the overall distribution and density of the data. The vertical axis shows mutant peptide binding strength as a percentile rank, with lower values representing increasingly strong binding peptides to HLA types. 0.5% rank (dashed line) represents the commonly used cutoff below which peptides are considered strong enough binders to be neoantigens. The width of each plot is proportional to the number of neoantigens at each binding strength. Overall, ESP lesions had fewer neoantigens compared to LSP and TMR. The darker shade within each plot represents the neoantigens expressed in RNA (TPM>0.1). B: Neoantigen load before and after applying the RNA filter. For each sample, the "DNA" column has all filters applied except for the RNA filter. The median number of filtered neoantigens per sample was 1,309. The "RNA" column has all filters including the RNA filter (expression >0.1 TPM) applied. On average 70% of predictions were expressed in RNA. Sample names enclosed in boxes with colours corresponding to the lesion type - early stage plaque (green), late stage plaque (yellow) and tumour (red).

We further compared the association between tumour mutation burden and the filtered neoantigen load (**Figure 3.4A**), which showed a strong positive linear relationship (r=0.92). The tumour mutation burden also demonstrated a positive linear relationship with the number of high strength neoantigens (r=0.81). The tumour mutation burden, filtered neoantigen load and number of high strength neoantigens are summarized in **Figure 3.4B**.

A



B

Lesion type	Tumour mutation burden (median (range))	Filtered neoantigen load (median (range))	High strength binding neoantigens (median (range))
Early stage plaque	2455 (1440-7198)	1111 (515-2947)	296 (103-809)
Late stage plaque	5014 (890-8107)	2223 (305-3336)	498 (65-1076)
Tumour	2697 (1306-8722)	974 (451-2435)	291 (95-588)

Figure 3.4: Relationship between tumour mutation burden and filtered neoantigen load in MF. A: A strong positive linear association (r=0.92, blue dashed trendline) was observed between tumour mutation burden and filtered neoantigen load. Each bubble represents a single sample, with its size proportional to the number of high strength neoantigens (<0.05%rank). A positive linear association was also observed between tumour mutation burden and the high strength neoantigen load (r=0.81). **B:** Mutations and neoantigen numbers by lesion type.

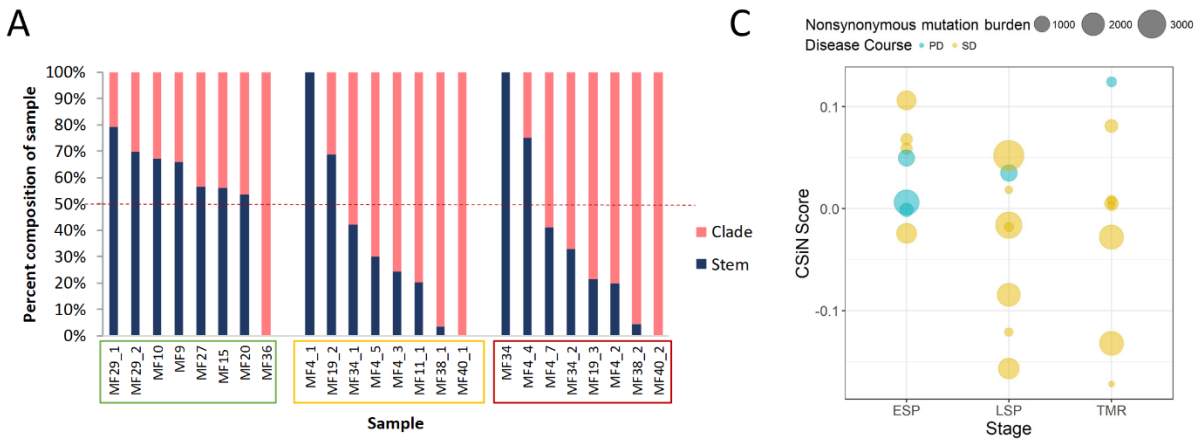
Comparing our data to the two previous CTCL studies of McGirt et al(16) and Choi et al(134) , we found that our dataset had a much higher neoantigen count (total 54,073,746 vs 615,761 in Choi et al. (134) or 135,042 in McGirt et al. (16), Appendix Figure A1). Tumour mutation burden and neoantigen count is influenced by various factors including CTCL subtype, methodology and sequencing depth. Choi's samples, as they were all Sézary Syndrome, permitted the use of cell sorting which improves tumour cell fraction (the percent of sample composed of tumour cells). While ours and McGirt's study comprised mycosis fungoides samples, our use of laser capture microdissection (instead of whole biopsies) increased tumour cell fraction. Additionally, the use of whole exome sequencing with greater sequencing depth in ours and Choi's studies increased sensitivity to mutations compared to McGirt's whole genome sequencing (which includes non-coding intronic regions) at lower sequencing depth. Details of the 3 studies are included in Appendix Tables A2&A3.

3.4.3 Increase in proportion of subclonal neoantigens in advanced MF

To determine the subclonality of the neoantigens we first constructed phylogenetic trees showing the subclonal architecture of MF, as described previously (131). Then we mapped the neoantigens to the stem and clades, with the latter representing the subclonal neoantigens (**Figure 3.5A**). This analysis demonstrated increasing branching with a higher proportion of clade neoantigens in advanced lesions, as demonstrated in LSP (median 62% clade neoantigens) and TMR (median 70%), compared to ESP (median 39%) (**Figures 5A & 5B**).

The Cauchy-Schwarz index of Neoantigens (CSiN) reduces the number of neoantigens, their clonality and immunogenicity in the sample to a single number (147). CSiN has been argued to out-perform existing metrics as a biomarker of tumour immunogenicity and response to immune

checkpoint inhibitors across different neoplasms (147). The CSiN scores of our samples are shown in **Figure 3.5C**. As expected, there was no significant correlation between CSiN and non-synonymous mutational burden (P = 0.637), however a greater proportion of early lesions (ESP) had the higher, advantageous CSiN scores >1 compared to the late stage lesions (LSP and TMR). However, higher CSiN scores did not predict more favorable prognosis (defined as lack of stage progression) in our cohort (**Figure 3.5C**, regression analysis, P = 0.142).


A
Percent composition of sample
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Clade
Stem
MF29_1
MF29_2
MF10
MF9
MF27
MF15
MF20
MF36
MF4_1
MF19_2
MF34_1
MF4_5
MF4_3
MF11_1
MF38_1
MF40_1
MF34
MF4_4
MF4_7
MF34_2
MF19_3
MF4_2
MF38_2
MF40_2
Sample
C
Nonsynonymous mutation burden
1000
2000
3000
Disease Course
PD
SD
CSiN Score
0.1
0.0
-0.1
ESP
LSP
TMR
Stage

B

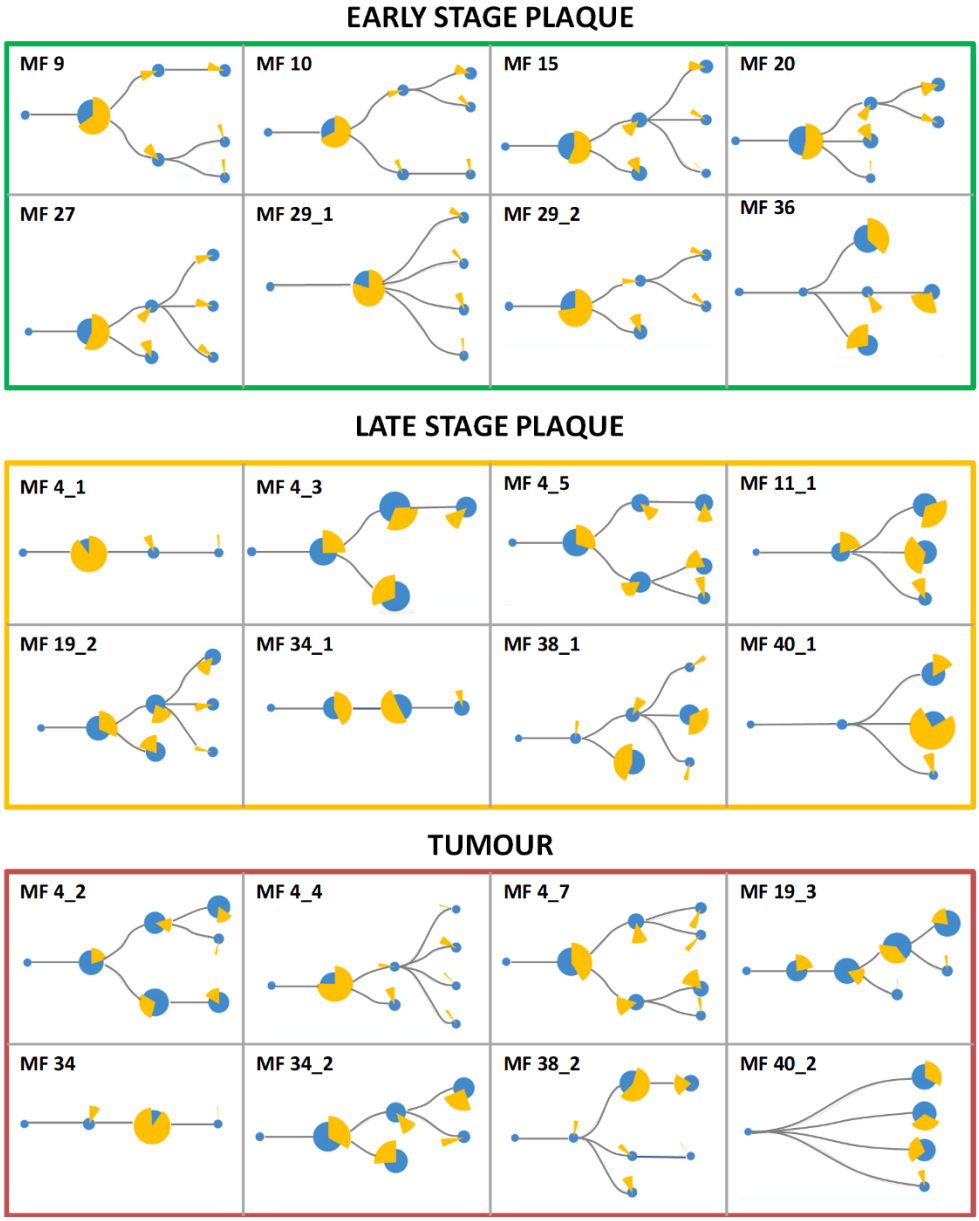
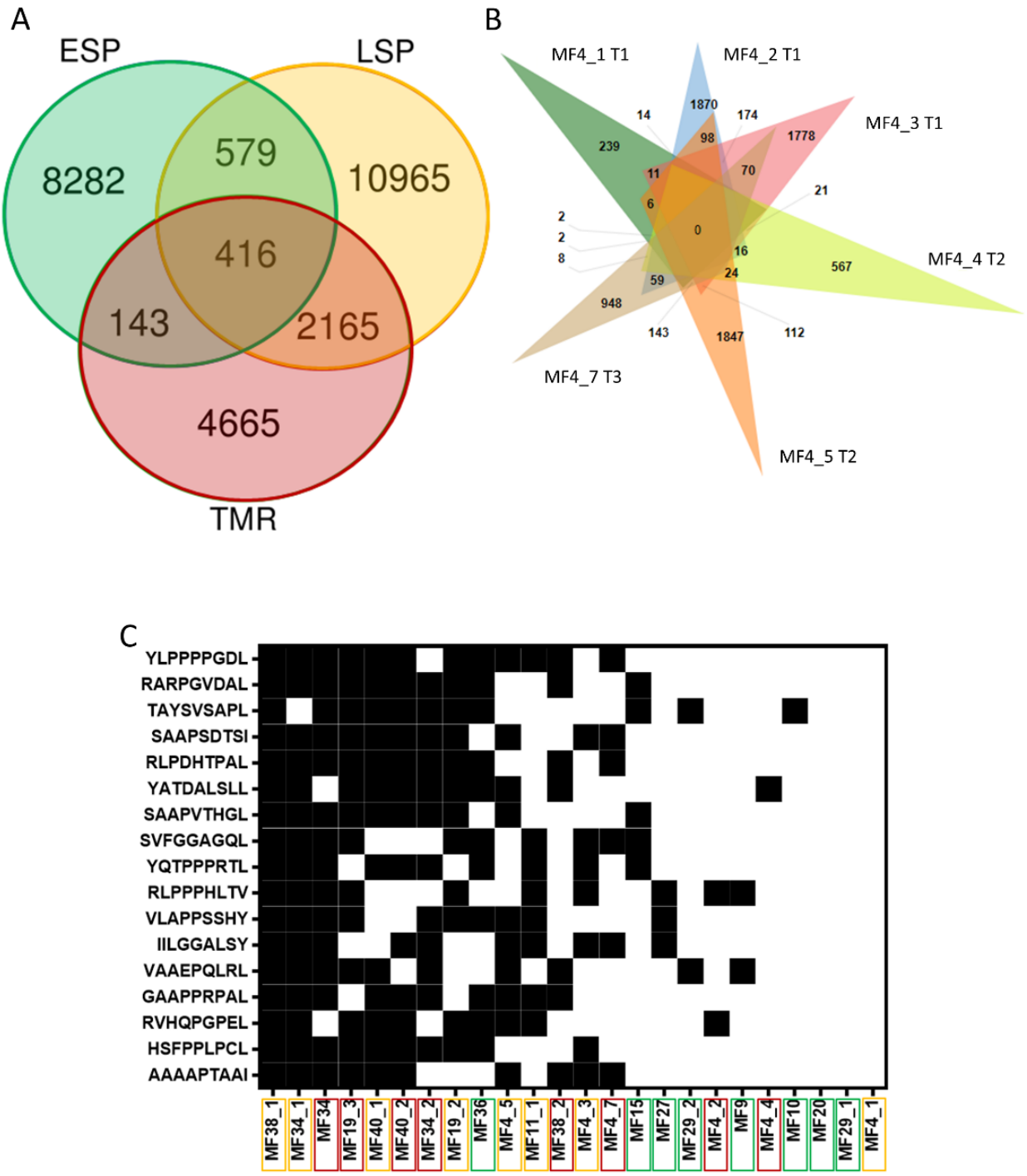

EARLY STAGE PLAQUE
MF 9
MF 10
MF 15
MF 20
MF 27
MF 29_1
MF 29_2
MF 36
LATE STAGE PLAQUE
MF 4_1
MF 4_3
MF 4_5
MF 11_1
MF 19_2
MF 34_1
MF 38_1
MF 40_1
TUMOUR
MF 4_2
MF 4_4
MF 4_7
MF 19_3
MF 34
MF 34_2
MF 38_2
MF 40_2

Figure 3.5: Clonality of neoantigens in MF. A: Proportion of stem and clade missense mutations producing putative neoantigens. Sample codes are enclosed in boxes with colours corresponding to the lesion type - early stage plaque (green), late stage plaque (yellow) and tumour (red). The dashed red line represents the 50% mark that distinguishes whether the majority of the sample is composed of stem or clade neoantigens. Early stage plaques have a greater proportion of stem mutations producing neoantigens compared to late stage plaques and tumours where more clade mutations produce neoantigens. **B:** Phylogenetic trees with putative neoantigen analysis. The size of the blue circles represents the proportion of missense mutations that comprise each node. 'Stem' nodes are those present prior to branching which then produces 'clade' nodes. The yellow pie chart in whole represents all neoantigens from the sample. Each slice of the pie chart represents the proportion of neoantigens originating from a node. With advancing disease stage, a greater proportion of neoantigens originate from clade mutations. **C:** CSiN scores of MF samples. The bubble plot shows individual CSiN scores and the number of non-synonymous mutations in ESP, LSP and TMR samples. Samples from patients who progressed in disease stage are colored in blue. Median CSiN scores are: ESP (0.027822, n=8), LSP (-0.01709, n=8) and TMR (0.004234, n=8).

3.4.4 Neoantigen overlap and peptide identity

We examined the overlap in filtered neoantigens by lesion type (**Figure 3.6A**) and within the same patient sampled longitudinally (**Figure 3.6B**). The overlap between late stage plaques (LSP) and tumours (TMR) was greater than between early stage plaques (ESP) and either lesion type. This was expected, as we separated advanced disease into LSP and TMR for analysis in our study. We also examined neoantigens from one patient from whom multiple, longitudinal samples were obtained. Among 6 samples (3 TMR, 3 LSP) obtained at 3 timepoints (0, 9 and 10 months respectively), we found no overlap in filtered neoantigens (**Figure 3.6B**). Most peptides were unique to each plaque or tumour site, further underscoring the predominantly subclonal structure of neoantigens in advanced disease. Finally, we examined the overlap of filtered neoantigens across samples (**Figure 3.6C**). No neoantigens were common to all samples, and the most common neoantigen was present in half of the 24 samples. Overlapping neoantigens were mostly present in late stage plaques and tumours. This is likely because advanced disease samples produced more neoantigens overall, increasing the likelihood of overlap.


A
ESP
LSP
TMR
8282
579
10965
416
143
2165
4665
B
MF4_1 T1
MF4_2 T1
MF4_3 T1
MF4_4 T2
MF4_5 T2
MF4_7 T3
1870
14
174
239
98
1778
11
70
21
6
2
2
8
0
16
567
59
24
948
143
1847
112
C
YLPPPPGDL
RARPGVDAL
TAYSVSAPL
SAAPSDTSI
RLPDHTPAL
YATDALSLL
SAAPVTHGL
SVFGGAGQL
YQTPPPRTL
RLPPPHLTV
VLAPPSSHY
IILGGALSY
VAAEPQLRL
GAAPPRPAL
RVHQPGPEL
HSFPPLPCL
AAAAPTAAI
MF38_1
MF34_1
MF34
MF19_3
MF40_1
MF40_2
MF34_2
MF19_2
MF36
MF4_5
MF11_1
MF38_2
MF4_3
MF4_7
MF15
MF27
MF29_2
MF4_2
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MF4_4
MF10
MF20
MF29_1
MF4_1

Figure 3.6: Intraindividual and interindividual overlap of neoantigens. A: Each lesion type comprises 8 samples, of which only unique peptides are included. The greatest overlap in filtered neoantigens is between plaques (LSP) and tumours (TMR). Early stage plaques (ESP) are also shown. **B:** Venn diagram of filtered neoantigens from 6 samples obtained from one patient. Each sample name is accompanied by the time point the biopsy was obtained (initial biopsy at T1, T2 at 9 months after T1 and T3 10 months after T1). There is no overlap in peptides between all lesions, and the predominant exclusivity of peptides to their individual sites indicates the highly branched nature of the tumour. **C:** Filtered neoantigens predicted in 10 or more samples out of the total 24 samples. Black indicates the presence of the peptide in the sample and white indicates the absence. Peptides are arranged from highest frequency (top) to lowest frequency (bottom). Sample names are arranged in order of those with the most overlapping neoantigens (left) to the least overlapping neoantigens (right). Sample names are enclosed in boxes with colours corresponding to the lesion type - ESP (green), LSP (yellow) and TMR (red). Overlapping neoantigens are mostly in the advanced stage disease samples (LSP and TMR) clustered on the left.

Using the neoantigens we found, we searched IEDB for closely related peptides from humans or human pathogens (Appendix Table A4). These known immune epitopes have been tested in experimental assays and are likely to elicit immunogenic responses in humans. We included epitopes tested in T-cell, B-cell and MHC ligand assays and did not require assays to be positive. Only 2 neoantigens were positive in T-cell assays.

3.4.5 Discussion

Our group has previously demonstrated that as MF progresses from early to advanced stages, the tumour accumulates somatic mutations and evolves to produce multiple genetic subclones (131). The impact of this genetic diversity on tumour immunogenicity is two-fold. An increase in mutation load would result in higher neoantigen expression and increased opportunities for the neoplasm to be recognized by the immune system. Conversely, the increasing subclonal distribution of neoantigens would direct the immune system to discrete subpopulations of the

most immunogenic tumour cells. This in turn would shield the less immunogenic subclones from the antitumour attack (149).

In this study, which to our knowledge is the first analysis of neoantigens in MF, we found that the neoantigen load mirrors the mutational load of MF and increases during disease progression. Our experimental approach using microdissected tumour tissue and deep exome sequencing allowed for identification of a markedly higher number of non-synonymous mutations (median 3,217) than previous MF studies (42-102 mutations)(16–18). The neoantigen load in our MF samples was also higher than other malignancies known to have a high neoantigen load such as malignant melanoma (median 121) and lung adenocarcinoma (median 335)(101). The differences are not only quantitative, as we were able to detect numerous frameshift mutations (median 2,604) which have hardly been captured in previous studies. Frameshift mutations are an essential source of neoantigens because they often produce highly immunogenic peptides due to global structural aberrations that render the peptide dissimilar from self (150,151). Overall CTCL is known to have a high number of chromosomal aberrations and protein fusion is likely an additional source of neoantigens (152), that should be studied in the future. Thus, MF can be viewed as a neoplasm of high immunogenic potential expressing a significant number (median 328) of high strength neoantigenic peptides.

Analysis of the subclonal heterogeneity of the neoantigens by bioinformatic deconvolution of phylogenetic trees and by multisampling distinct lesions of MF revealed a complex neoantigenic landscape. Our analysis demonstrated that different cutaneous lesions of MF exhibit highly diverse repertoires of non-overlapping neoantigens. It has been previously demonstrated that multiple longitudinal CTCL biopsies from a single patient show molecular heterogeneity (49). Likewise, the most informative was our analysis of six lesions from a single patient (**Figure**

3.6B) which did not demonstrate a single shared antigenic peptide. Similarly, the overlap between neoantigens in plaques and tumours from the same patient was poor. Thus, a single patient with MF presenting with numerous skin lesions may be considered as having a collection of multiple, immunologically different neoplasms.

Not only did different lesions vary by their neoantigens but significant neoantigenic heterogeneity was also detected in different lymphoma subclones. Using a bioinformatic approach we were able to show that a large proportion of neoantigens map to the subclones (clades) and that this proportion increased during stage progression. Although it is tempting to speculate that this high proportion of subclonal neoantigens will render advanced stage MF resistant to immunotherapy (25), we must acknowledge certain limitations of our computational approach. The phylogenetic trees were constructed by statistical modeling of point mutation distributions in the sample and were not verified by single-cell sequencing. Therefore, we cannot with certainty equate a branch of the phylogenic tree with a clone of tumour cells.

Although there was a clear increase in the number of neoantigens between early stage plaques and lesions in the late stage disease, it has not escaped our attention that the clinically more advanced lesions of tumours did not have a higher number of antigens (some even had a lower neoantigen load) compared to late stage plaques. This could not have been explained by a lower degree of genetic heterogeneity because the tumours had a highly branched subclonal architecture. We hypothesize that the reduction in neoantigen expression might be a result of immune editing, whereby the cells bearing the most immunogenic neoantigens are negatively selected by the immune system (153). To gain further insight into the significance of the neoantigen landscape as a biomarker of response to immunotherapies we calculated the CSiN indexes which provide a simple measure of cancer immunogenicity. Similar to what was shown

previously (147), the CSiN scores did not correlate with TMB and did not predict the risk of stage progression. We found however that a higher proportion of advanced lesions (LSP and TMR) have lower, unfavourable scores (CSiN<1) predictive of poor response to checkpoint inhibitor treatment. This may explain why a significant proportion of MF patients do not respond to immunotherapy (10). On the other hand, more CSiN scores >1 were found in the early MF lesions which makes those patients obvious candidates for target enrichment trials with immune checkpoint inhibitors.

Previous studies have reported that very few neoantigens are shared across patients in high mutation load malignancies (101) and as already mentioned, our cohort of MF patients did not share any neoantigenic peptides. However, several peptides were commonly found in some patients (**Figure 3.6C**) and these could represent potential therapeutic targets. We therefore searched for known homologous immune epitopes of the most frequently observed neoantigens (20). Although none of the homologous sequences were an exact match to our mutant peptides, there were numerous promising partial matches (90% sequence similarity) to immunogenic human sequences and the sequences of human pathogens such as *Mycobacterium tuberculosis* and protozoa (*Leishmania* and *Trypanosoma*) (Appendix Table A4). This observation was particularly interesting because neoantigenic peptides homologous to human pathogens are known to be robust activators of the immune response (20,141). It is important to clarify that this does not suggest our patients were infected with these organisms, but rather that they expressed peptides similar to those organisms, which can thus elicit an immune response. The overall relevance of these peptides is unclear as these organisms are uncommon to Canada and Denmark, from where patients were recruited. Other notable homologous epitopes included those from proteins implicated in other cancers, such as the ENA family (involved in cell

motility and adhesion) from breast cancer (154) , and baculoviral IAP repeat-containing protein 6 (involved in anti-apoptosis through caspase inhibition) from brain cancer (155). Future studies should validate candidate neoantigen expression at the protein level and their ability to elicit T-cell activation.

In conclusion, we have shown a bewildering degree of neoantigen heterogeneity in MF. Among hundreds of detected strong neoantigens there is little overlap between different individuals, between lesions in the same individual and between different subclones within the same lesion. We hypothesize that neoantigen heterogeneity may be an important factor limiting efficacy of immunotherapy in MF, and probably in other highly mutated, genetically heterogeneous cancers.

We show that disease progression is correlated with an increase in mutational load and the number of neoantigens. However, advanced lesions of MF exhibit a high proportion of subclonal neoantigens which may limit the efficacy of immunotherapies.

Chapter 4: Conclusion

4.1 Discussion

This thesis addresses the longstanding knowledge gap about why MF demonstrates suboptimal responses to immunotherapies. The major contributions of this thesis are as follows:

1) We found that MF has a much higher tumour mutation burden than previously described with a median 3,217 mutations per sample (35 mutations/kB). These are comprised primarily of frameshift mutations which were not previously identified as a major source of mutations in MF.
2) We quantified and characterized neoantigens in MF for the first time. We found that MF expressed a high number of total neoantigens (median 1309 per sample), with a significant number of neoantigens with high binding strength to MHC (median 328).
3) We demonstrated that the neoantigens in MF are highly subclonal. The neoantigens are also heterogeneous between lesions in a patient, and between different patients.
4) We demonstrated that an increase in disease stage is associated with a higher number of neoantigens and with more subclonal neoantigens.

4.1.1 Limitations

We acknowledge certain limitations of our work. Firstly, we predicted neoantigens based on genomic data and confirmed their expression on the transcriptome level but not on the protein

level. Future studies building on our work should confirm neoantigen expression at the protein level and test their ability to activate T-cells. Secondly, our construction of phylogenetic trees was based on statistical modelling. Future studies based on single-cell sequencing can definitively identify clones and subclones.

4.2 Conclusion

MF is a highly immunogenic neoplasm expressing a large number of high strength neoantigens. Disease progression is associated with an increase in the number of neoantigens and an increase in neoantigen subclonality. These neoantigens are also highly heterogeneous within and between patients. Hence MF may be considered a collection of immunologically varied neoplasms. Neoantigen subclonality may be a crucial factor limiting the efficacy of immunotherapy in MF in advanced disease. Stratification of patients by neoantigen load and clonality may be useful to select suitable candidates for immune checkpoint inhibitor trials. Given non-overlapping neoantigens between patients, identification of an individual patient's neoantigens may be ideal for personalized immunotherapies.

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Appendices

Appendix Table A1: Characteristics of patients and samples used in the study

Patient ID (age, sex, race)	Sample ID	Lesion type	Location	Diagnosis & stage
MF 4 (69, M, Caucasian)	MF4_1P	Plaque (Late stage)	Forearm	Mycosis fungoides IIB
	MF4_2T	Tumour	Flank	
	MF4_3P	Plaque (Late stage)	Shin/leg	
	MF4_4T	Tumour	Trunk	
	MF4_5P	Plaque (Late stage)	Leg	
	MF4_7T	Tumour	Neck	
MF9 (42, F, Caucasian)	MF9P	Plaque (Early stage)	Trunk	Mycosis fungoides IA

MF10 (56, M, Caucasian)	MF10P	Plaque (Early stage)	Triceps region/arm	Mycosis fungoides IB
MF11 (56, M, Caucasian)	MF11_1P	Plaque (Late stage)	Leg	Mycosis fungoides IIB
MF15 (65, M, Caucasian)	MF15P	Plaque (Early stage)	Leg	Mycosis fungoides IB
MF19 (74, M, Caucasian)	MF19_2P	Plaque (Late stage)	Arm	Mycosis fungoides IIB
	MF 19_3T	Tumour	Trunk	
MF20 (70, M, Caucasian)	MF20	Plaque (Early stage)	Trunk	Mycosis fungoides IB

MF27 (71, M, Caucasian)	MF27P	Plaque (Early stage)	Buttock	Mycosis fungoides IA
MF29 (87, F, Caucasian)	MF29_1P	Plaque (Early stage)	Neck	Mycosis fungoides IA
	MF29_2P	Plaque (Early stage)	Foot	
MF34 (65, M, Caucasian)	MF34T	Tumour	Abdomen	Mycosis fungoides IIB
	MF34_1P	Plaque (Late stage)	Abdomen	
	MF34_2T	Tumour	Arm	
MF36 (64, M, Caucasian)	MF36P	Plaque (Early stage)	Thigh	Mycosis fungoides IA

MF38 (76, M, Caucasian)	MF38_1P	Plaque (Late stage)	Abdomen	Mycosis fungoides IIB
	MF38_2T	Tumour	Chest	
MF40 (59, F, Caucasian)	MF40_1P	Plaque (Late stage)	Axilla	Mycosis fungoides IIB
	MF40_2T	Tumour	Axilla	

Appendix Table A2: Sequencing depth of individual samples

Sample ID	Lesion type	Sequencing depth (x)
MF4_1P	Plaque (Late stage)	140.5
MF4_2T	Tumour	192.0
MF4_3P	Plaque (Late stage)	183.8
MF4_4T	Tumour	190.0
MF4_5P	Plaque (Late stage)	111.2
MF4_7T	Tumour	155.8
MF9P	Plaque (Early stage)	146.9
MF10P	Plaque (Early stage)	122.1
MF11_1P	Plaque (Late stage)	180.7
MF15P	Plaque (Early stage)	199.5

MF19_2P	Plaque (Late stage)	186.3
MF 19_3T	Tumour	197.4
MF20	Plaque (Early stage)	138.6
MF27P	Plaque (Early stage)	179.1
MF29_1P	Plaque (Early stage)	112.3
MF29_2P	Plaque (Early stage)	100.1
MF34T	Tumour	171.3
MF34_1P	Plaque (Late stage)	149.8
MF34_2T	Tumour	203.7
MF36P	Plaque (Early stage)	145.3
MF38_1P	Plaque (Late stage)	170.8
MF38_2T	Tumour	168.5

MF40_1P	Plaque (Late stage)	196.9
MF40_2T	Tumour	184.3

Appendix Table A3: Characteristics of CTCL studies used in meta-analysis

Study	**Sample type**	**Sequencing method**	**Sequencing depth (x)**	**Number of samples**
Choi et al.	Sézary Syndrome	Whole exome sequencing	Range 142.219-333.623	31[g]
McGirt et al.	Mycosis fungoides	Whole genome sequencing	Range 32.04-44.24	5

Appendix Table A4: Homologous immune epitopes of filtered neoantigens. Only epitopes from humans or human pathogens were included. Included are epitopes tested in T-cell, B-cell and MHC ligand assays. There was no requirement that assays be positive. Peptides highlighted in green were positive in T-cell assays.

Mutant peptide	Number of samples present	IEDB search criteria	Homologous immune epitope				
			Sequence	Protein	Gene	Species	Protein function
YLPPPPGDL	12	Blast >90%	LPPPPGPPPPPPL	Protein enabled homolog (Ena/VASP family)	ENAH	*Homo sapiens (Human)*	Cytoskeleton remodeling for cell motility and adhesion
			LPPPPGSPL	Protein Wiz	WIZ	*Homo sapiens (Human)*	Zinc finger containing protein, forms and stabilizes heterodimers from histone methyltransferases.
RARPGVDAL	11	Blast >90%	VLPGVDALSNI[1]	Phosphoglycerate kinase 1	PGK1	*Homo sapiens (Human)*	Catalyzes ATP producing reactions in glycolytic pathway.
TAYSVSAPL	11	Blast >90%	AYSVSASSL	Other Leishmania major protein	Not specified[5]	*Leishmania major*	Not specified[5]
YQTPPPRTL	10	Blast >90%	KPQPPPRTL[4]	Homeobox protein HMX3	HMX3	*Homo sapiens (Human)*	Transcription factor involved in differentiation of neuronal cells
VLAPPSSHY	10	Blast >90%	SPAPPSSHSL[4]	Protein Daple	CCDC88C	*Homo sapiens (Human)*	Downregulates Wnt signalling pathway, may activate JNK signalling pathway
GAAPPRPAL	10	Blast >90%	APTPPRPAL[4]	Transcription factor IIIA	GTF3A	*Homo sapiens (Human)*	Involved in ribosomal large subunit formation and binding, may regulate transcription of other genes
AAAAPTAAI		Blast >90%	AEAAAAPTV	Numb-like protein	NUMBL	*Homo sapiens (Human)*	Required for neurogenesis
			AAAPTANLL	Baculoviral IAP repeat-containing protein 6	BIRC6	*Homo sapiens (Human)*	Anti-apoptotic protein that inhibits caspases and is an E3 ubiquitin-protein ligase
			MRFAQPSALSRFSALTRDWFTSTFAAPTAAQA[1]	ATP-dependent helicase	Not specified[5]	*Mycobacterium tuberculosis*	DNA dependent ATPase and helicase
			AAAAAPTLPTGGQDN AAAAPTLPTGGQDNG AGAPAAAAAPTLPTG APAAAAAPTLPTGGQ ASPGAGAPAAAAAPT GAGAPAAAAAPTLPT GAPAAAAAPTLPTGG PAAAAAPTLPTGGQD PGAGAPAAAAAPTLP SPGAGAPAAAAAPTL	Mucin-associated surface protein (MASP)	Not specified[5]	*Trypanosoma cruzi*	Transmembrane component
	10		ENVKNCSSAAAPTAT SENVKNCSSAAAPTA	Other Trypanosoma cruzi protein	Not specified[5]	*Trypanosoma cruzi*	Not specified[5]

The following peptides did not yield any relevant search results: SAAPSDTSI, RLPDHTPAL[2], YATDALSLL, SAAPVTHGL[3], SVFGGAGQL, RLPPPHLTV[3], IILGGALSY[2], VAAEPQLRL, RVHQPGPEL and HSFPPLPCL[3].

[1] Multiple homologous peptide sequences were found from the same protein or organism. The sequence tested in the most assays is listed here.

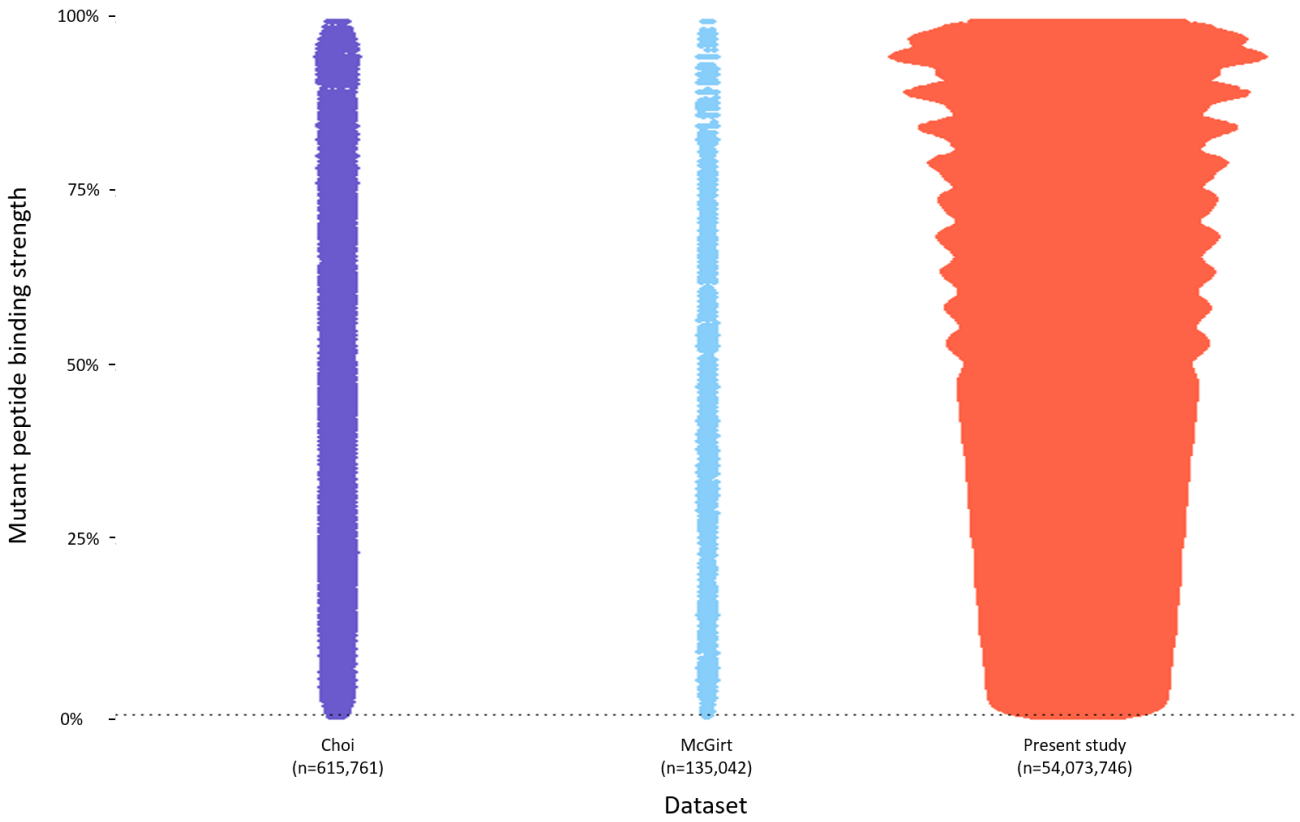
[2] The homologous sequence found had incomplete information and was excluded.

[3] The homologous sequence was from an organism that is not a typical human pathogen.

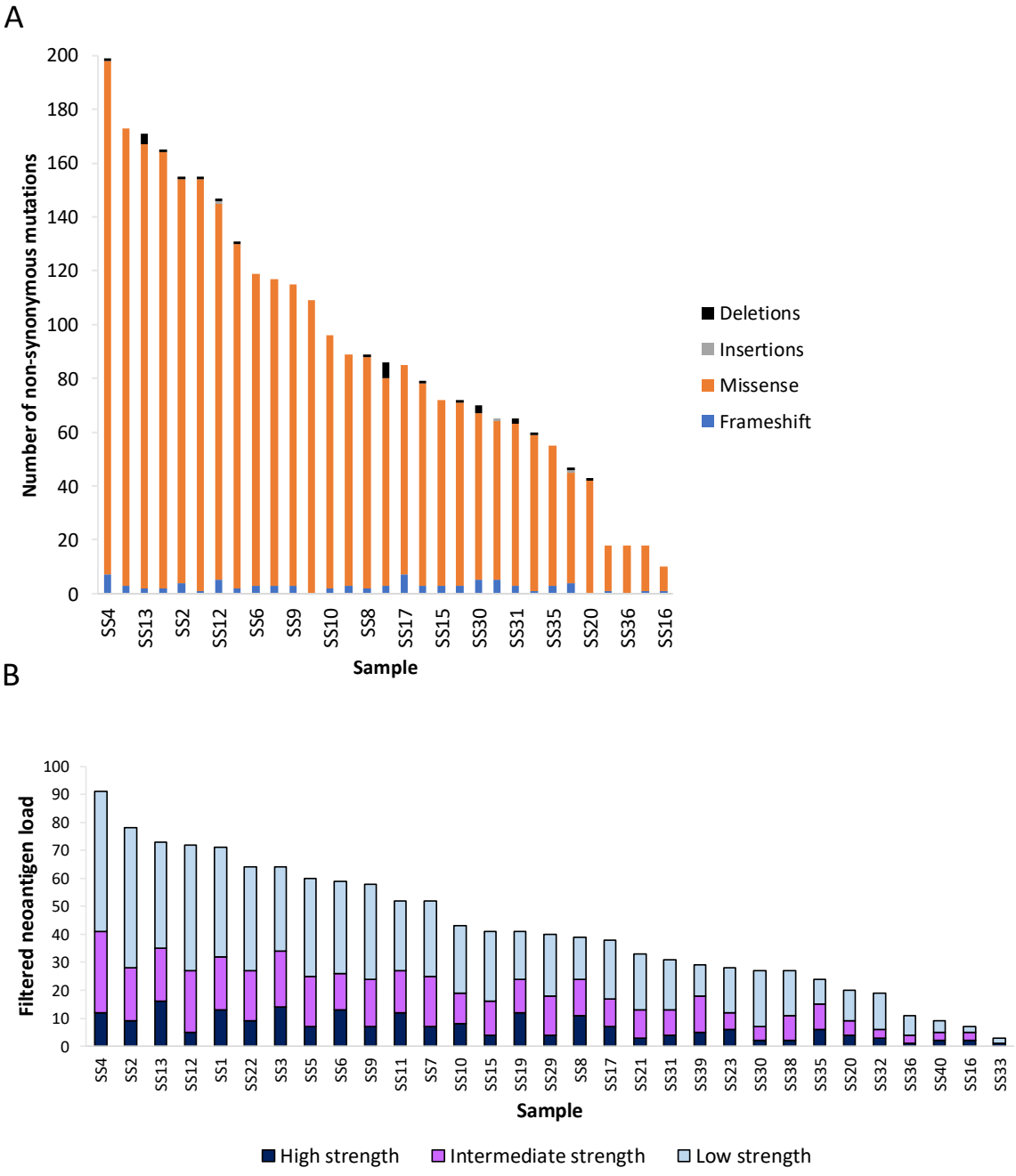
[4] Another homologous protein was excluded due to being from an organism that is not a typical human pathogen.

[5] "Not specified" indicates not enough distinguishing information was provided in the IEDB database to search peptide features.

Appendix Figure A1 (Next page): Comparisons of neoantigens from our datasets with those of Choi at al. (134) and McGirt et al. (16) This beeswarm plot shows putative neoantigens prior to filtering. Due to the extensive size of the dataset, a random 1% of all data points were plotted to demonstrate the overall distribution and density of the data. The vertical axis shows mutant peptide binding strength as a percentile rank, with lower values representing increasingly strong binding peptides to HLA types. 0.5% rank (dashed line) represents the commonly used cutoff below which peptides are considered strong enough binders to be neoantigens. The horizontal axis shows the three studies with the number of total putative neoantigens in each dataset specified in brackets. The width of each plot represents the quantity of neoantigens at each binding strength. Overall, our dataset had a many fold greater number of putative neoantigens compared to the Choi and McGirt datasets. The slight difference in median binding strength is likely due to the vastly greater size of our dataset (57% rank) compared to the Choi dataset (52% rank) and the McGirt dataset (52% rank). For the McGirt and Choi datasets, there was no RNA data or separation by lesion stages. Consequently, the median filtered neoantigen load was 40-46 per sample (Appendix Figure A2&A3)


100%
75%
50%
25%
0%
Mutant peptide binding strength
Choi
(n=615,761)
McGirt
(n=135,042)
Present study
(n=54,073,746)
Dataset

Appendix Figure A2: Characteristics of the dataset from Choi et al. (134). A:Tumour mutation burden. Samples are arranged in descending order of TMB. Missense mutations comprise 96% of the non-synonymous mutations. **B: Filtered neoantigen load.** All filters were applied with the exception of the RNA filter as expression data was not available. The median number of filtered neoantigens per sample was 40.



Appendix Figure A3: Characteristics of the dataset from McGirt et al. (16). A:Tumour mutation burden. Samples are arranged in descending order of TMB. Missense mutations comprise 98% of the non-synonymous mutations. **B: Filtered neoantigen load.** All filters were applied with the exception of the RNA filter as expression data was not available. The median number of filtered neoantigens per sample was 46.