STATISTICAL ANALYSIS PLAN

Pre-Treatment of Highly Suspicious Pigmented Skin Lesions with Interleukin-2

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Nova Scotia Health Authority
Halifax
Canada

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1. INTRODUCTION

Skin cancer is by far the world's most common form of cancer – with more diagnoses annually than breast, prostate, lung, and colon cancers combined.[1] Every year in Canada over 80,000 cases of skin cancer are diagnosed, over 6,800 of which are Melanoma, the most deadly form of skin cancer.[2] It is estimated that in 2016, over 1200 Canadians have died of Melanoma.[2]

The incidence of melanoma in Canada is on the rise in both men and women. In women the incidence of melanoma has increased by 2.8% between 2001 and 2010, an annual percentage change surpassed only by thyroid cancer.[2] As mortality rates for almost all other forms of cancer decrease – the age-standardized mortality rate of melanoma is on the rise. Melanoma is the number one cancer killer of women aged 25 to 30.[1]

As a whole, 5-year age-standardized survival rates for melanoma have been reported as high as 88%.[2] When discovered early, melanoma can usually be cured with surgery alone, but once it metastasizes therapeutic options are limited. 5-15% of patients with melanoma will progress to stage IV MM.[3,4] MM is one of the most aggressive malignancies, with 5-year survival rates of advanced disease between 5-23%, depending on the location of tumor dissemination.[5,6] Although several new therapeutic interventions for metastatic melanoma are undergoing clinical trials, for the foreseeable future MM is a disease characterized by a worrying rate of mortality, and treatments of questionable efficacy.[5,7]

Cure rates for melanoma are high when the disease is discovered before it has spread from its primary location; however, metastasis frequently occurs, presenting a clinical challenge that has frustrated physicians and researchers alike for decades. Up until 2011, only two therapies were approved by the US food and drug administration (FDA) for the treatment of advanced (stage III or stage IV) MM. DTIC, the only chemotherapeutic licensed to treat MM was approved in 1975, has limited therapeutic utility. Patients on DTIC can expect a one-in-eight chance of having tumors shrink.[8] Systemic therapy utilizing high-dose IL-2 was approved by the FDA in 1998. IL-2 is a naturally occurring glycoprotein secreted by T cells to mediate cellular immune response, and has been used as a cancer immunotherapy for almost 40 years.[9] IL-2 mediates bystander activation and proliferation of CD4+ T-cell, CD8+ T-cell lymphocytes and to a lesser extent, NK cells.[9] Although 4% of patients were cured using this immunological therapy, the side effects of systemic administration are significant and are fatal in 2% of patients.[10] Even with these treatments, the outcome for patients with distant metastases is bleak, with median survival of 6 to 10 months and less than 5% of patients surviving for more than 5 years.[6]

Indeed, immune control of melanoma is achievable in specific circumstances – elucidation of the molecular identity of several antigens that are recognized by the immune system of melanoma patients has led to the discovery of pathways affecting tumor immunity at a cellular and molecular level.[11–13] Still MM has proven to be excessively difficult to treat, and

the development of novel immunologically-mediated therapeutics has been slow. Many adjuvant vaccine trials in melanoma have been conducted including GMK vaccine and granulocyte-monocyte colony-stimulating factor (GM-CSF), but these have been largely unsuccesful.[14,15] Similarly, trials assessing the treatment of MM with immune stimulants such as Bacillus Calmette–Guerin (BCG), Corynebacterium parvum and levamisole have yielded mixed inconsistent responses or been ineffective and even harmful to patients.[16]

Significant inroads have been made in recent years with the development of novel immunotherapies for the treatment of MM. In 2011 the FDA approved the use of monoclonal antibody ipilimumab as a checkpoint immunotherapy for the treatment of advanced MM.[17] Ongoing studies are assessing whether adjuvant therapies such as chemotherapy or various immunologic therapies may improve on the antitumor effects achieved with ipilimumab.[7]

Multiple systemic antibody-mediated immunotherapies for advanced MM – including nivolumab and pembrolizumab – are current under development, and have shown significant advances;[18] However response to these novel therapeutics have been tempered as these therapies are associated with serious (sometimes fatal) immune-mediated side effects and prohibitive pricing.[19]

The serious toxicity profile associated with systemic immunotherapies can be altogether avoided by treating instead with intralesional injections. Studies have shown lower rates of toxicity are associated with intralesional injections when compared with systemic administration; furthermore, by delivering an increased concentration of the drug at the site of action, increased rates of efficacy are observed.[20] Intralesional IL-2 is associated with modest flulike symptoms alone – a vast improvement on the toxicity profile associated with systemic administration of IL-2.[10,21] Intralesional injections of IL-2 have an added bonus of causing a so-called “bystander effect”, whereby cancerous cells that are not immediately adjacent to the local injection site, also are effectively treated through development of an adaptive regional immune response.[20]

IL-2 has been effectively used to treat MM when administered intralesionally.[21] This mode of treatment using IL-2 is being effectively delivered by Dr. Carman Giacomantonio to treat patients with advanced cutaneous MM at the QEII HSC, NSHA. IL-2 used in this capacity is a “reactive” treatment to MM. Herein we propose the use of IL-2 as a “proactive” treatment to pre-metastatic melanoma.

We plan to treat patients with highly suspicious lesions – as diagnosed by academic dermatologists (Drs. R. Langley, K. Purdy and P. Green) – with intralesional IL-2 in an effort to generate an adaptive immune response with activation and proliferation of CD8+ T-cell effector lymphocytes and immune sensitized CD8+ T memory cells to address the potential risk of subsequent melanoma metastasis.[9] Moreover, after treatment with IL-2, the proliferation of

CD8+ T-memory lymphocytes may allow cells of immune surveillance to mount an immune response to new, *de novo* pre-cancerous and/or cancerous melanoma cells.

Given the paucity of effective therapeutic interventions for MM, successful strategies in treating melanoma will include therapies or therapeutic adjuvants which limit, or altogether prevent metastases. With over 1200 deaths in Canada in 2016 and even more expected in 2017 due to metastatic melanoma, finding preventative treatments is crucial. We believe that a "Proactive" pre-treatment with IL-2 is the future of melanoma therapy.

1.1 Study Objectives

1.1.1 Primary Objectives

- To assess the efficacy of intralesional IL-2 vs a placebo in activating adaptive immunity in cutaneous lesions suspicious of melanoma or melanoma in situ as diagnosed by a dermatologist before histological confirmation. Adaptive immunity will be assessed by determining the number of tumor-infiltrating lymphocytes (TILs) in formalin-fixed, paraffin-embedded (FFPE), sections of patient biopsies used to confirm the diagnosis of melanoma. Immunohistochemical (IHC) staining of sections will be used to assess several components of the adaptive immune system, including:
    - Natural killer (NK) cells
    - Dendritic cells (DC)
    - T-cell lymphocytes (CD3+, CD4+ and CD8+)
    - Macrophages (Mac-1+ and Mac-2+)
    - Immunosuppressive cells (FoxP3+, PD-1+, and PD-L1+)

1.1.2 Secondary Objectives

- To assess the efficacy of intralesional IL-2 vs a placebo in preventing disease metastasis or new disease formation in patients with suspected melanoma or melanoma in situ as diagnosed by a dermatologist before histological confirmation.

- To assess the effect of intralesional IL-2 vs a placebo in modulating systemic immune response using proteomic and metabolomic markers in patients suspected of melanoma or melanoma in situ as diagnosed by a dermatologist before histological confirmation.

1.2 Study Design

This is a single-centre, double-blind, placebo-controlled, randomised clinical trial designed to evaluate intralesional IL-2 versus placebo as a means of activating adaptive immunity and preventing disease metastasis in those with cutaneous lesions suspected to be melanoma or melanoma in situ from the maritime provinces of Canada.

The study will be conducted at one centre in Halifax, Nova Scotia, Canada at the surgery clinic (4th floor Dickson Center) QEII HSC, NSHA. The study will enroll 20-60 patients with approximately equal distribution between treatment arms. The study period will be 260 weeks (5 years); 52 weeks of patient recruitment and initiation of the double-blind treatment followed by 208 weeks (4 years) of patient follow-up to assess disease metastasis and new disease formation.

Participants are otherwise healthy, aged 16-75, who have been recently diagnoses with pigmented lesions highly suspicious of melanoma or melanoma in situ who are not currently immunocompromised, on immuno-therapy for other diagnosis, have known inflammatory or autoimmune diseases.

In total, up to 60 participants will be randomly allocated in a 1:1 ration to be treated with IL-2 or placebo. Randomization will be achieved through the pharmacy preparing treatment and placebo solutions. At the onset of the study, 20 opaque envelopes will be prepared by the investigators, randomized, and given to the pharmacy. Ten of these envelopes will contain instruction to prepare treatment (IL-2), and the other 10 will contain directions to prepare the injectable control (saline); in this way randomization of treatment and control groups will occur in the pharmacy. Once the randomization envelopes are depleted they will be replaced 10 at a time (5 treatment, 5 control) until study conclusion. At the time of treatment randomization, the patient will be assigned a codified number (contained within the randomization envelope) to be used to track samples which will be collected and analyzed as a part of this study. The Principal Investigator will also have a randomisation envelope for each serial number, containing the treatment information. The randomisation envelope should be opened only if it is deemed necessary to prematurely unblind a subject.

Treatments and incisional and fine needle biopsy will be conducted at the surgery clinic (4th floor Dickson Center) QEII HSC, NSHA. Treatment group patients will be treated intralesionally with IL-2 (Aldesleukin, Novartis Pharmaceuticals Canada Inc.) at a dose of 500,000 IU in 0.1 ml for 2 treatment cycles (1 treatment per week) prior to excisional biopsy; control group patients will be treated intralesionally with sterile saline (0.9% m/v) of the same volume for the same treatment cycles. Patient response to treatment will be monitored at each visit.

Prior to the first treatment and after excisional biopsy of the lesion, all patients will have blood (4 vials) and urine (25-50 ml) samples taken for proteomic and metabolomics analysis (Figure 1). Before delivery of the lesion to pathology, the lesion will be subjected to two fine needle aspiration biopsies: one directly into the lesion to be used for RNA analysis to assess the genetic profile of the suspected melanoma, and one in the clear margins of patient tissue obtained during the excisional biopsy. All samples obtained from pathology will be returned to pathology within 60 days of receipt, with the exception of the core needle biopsies which are destroyed in the process of analysis.

All blood, urine, and lesion biopsy samples will be labeled with a codified number (will not contain any patient identifying information) and will be immediately transported to Dr. Carman Giacomantonio laboratory (Sir Charles Tupper Medical Building, 11F11) at Dalhousie University for storage. After histological diagnosis and staging, cells obtained as FFPE sections from pathology will be obtained directly from pathology; these too will be labeled using the patients designated codifier.

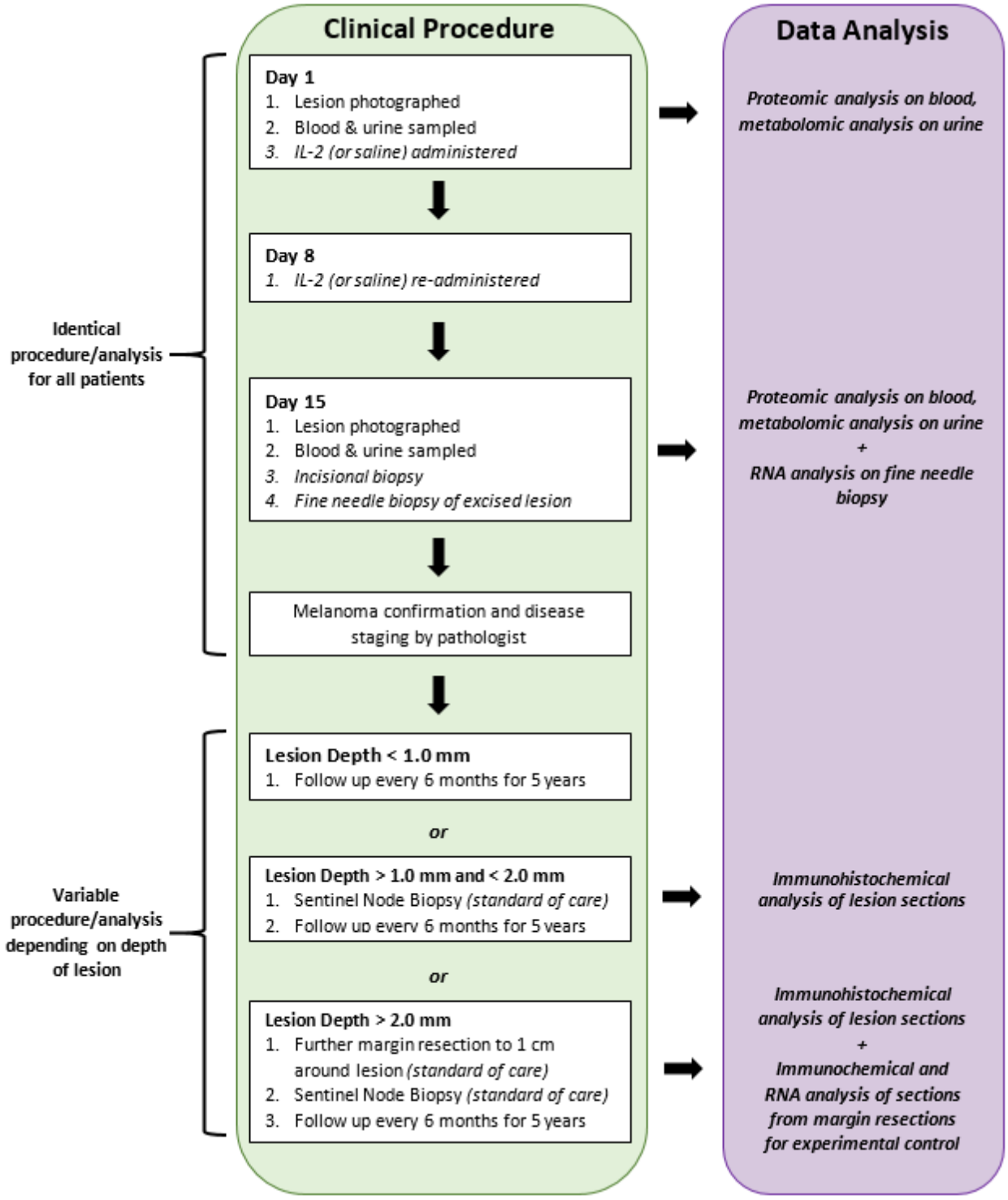

Figure 1 *– Clinical procedure and data collection/analysis algorithm for study participants.*

Sections from pathological analysis of the lesions will be analyzed using IHC staining techniques analysis to look for levels of TILs. Fine needle aspiration samples will be assessed for tumor genetic and epigenetic profile. Blood and urine will be assessed for proteomic and metabolomic profiles, respectively.

A database containing patient IDs and codified numbers will be restricted to the office of Dr. Giacomantonio (11th floor, VG). This is a locked office, and the data will only be accessible to the investigators named on this application. This database for will contain patient IDs, and info on disease status, treatment/control, follow up visits, and sample analysis – only info pertinent to the outcome measures. All sample data will be assessed using codified descriptors and will contain no patient ID info. Once the RNA and immunohistochemical data has been analyzed, unmasking treatment and control groups will be conducted at the VG office of Dr. Giacomantonio, by the named investigators. If the data reaches statistically significance, the study will not accept any more patients, if not, a further 10 patients will be incorporated – to a maximum of 60 patients. Any publications of study results will be completed devoid of any information that could be used to identify patients included in the study.

All study participants will receive biannual assessments for 5 years after the initial intervention to assess disease progression, or the development of new melanoma, to compare between both treatment and control groups. This aspect of the study is identical to the patient assessment conducted as per normal standard of care for melanoma patients. Again, any publications of study results will be completed devoid of any information that could be used to identify patients included in the study.

Further details of the schedule of assessments recorded at each visit are reported below.

Treatment

Baseline – Clinical Visit

- Review of inclusion and exclusion criteria
- Informed consent obtained and subject number assigned
- Demography
- Family history of skin cancer
- Medical history
- Prior and concomitant medications
- Vital signs
- Lesion Characteristics
    - Localization (‘axial’ or ‘extremity’)
    - Histology (‘nodular’ or ‘nonnodular’)
    - Ulceration (‘yes’ or ‘no’)

Week 1 – Clinical Visit

- Randomisation
    - Blinded IL-2 or plaebo saline
- Lesion photographed

- Blood sampled
- Urine Sampled
- Treatment administered
- Vital signs
- Adverse events

Week 2 – Clinical Visit

- Treatment administered
- Vital signs
- Adverse events

Week 3 – Clinical Visit

- Vital signs
- Adverse effects
- Lesion photographed
- Blood sampled
- Urine Sampled
- Incisional biopsy of lesion
- Needle aspiration biopsy of excised lesion
    - Lesion
    - Clear margins for control
- Sample to pathology for histological confirmation

Follow-Up Visits

Week 4 – Clinical visit

- Vital signs
- Adverse effect
- Histological confirmation of diagnosis given to patient
    - Other histological information
        - Breslow thickness (‘< 2mm’ or ‘>2 mm’)

Week 26 – Clinical Visit

- Assessment of area around biopsy
- Assessment of disease metastasis and new disease
- Blood tests

Week 52 – Clinical Visit

- Assessment of area around biopsy
- Assessment of disease metastasis and new disease
- Blood tests

Week 78 – Clinical Visit

- Assessment of area around biopsy
- Assessment of disease metastasis and new disease
- Blood tests

Week 104 – Clinical Visit

- Assessment of area around biopsy
- Assessment of disease metastasis and new disease
- Blood tests

Week 130 – Clinical Visit

- Assessment of area around biopsy
- Assessment of disease metastasis and new disease
- Blood tests

Week 156 – Clinical Visit

- Assessment of area around biopsy
- Assessment of disease metastasis and new disease
- Blood tests

Week 182 – Clinical Visit

- Assessment of area around biopsy
- Assessment of disease metastasis and new disease
- Blood tests

Week 208 – Clinical Visit

- Assessment of area around biopsy
- Assessment of disease metastasis and new disease
- Blood tests

Week 234 – Clinical Visit

- Assessment of area around biopsy
- Assessment of disease metastasis and new disease
- Blood tests

Week 260 – Clinical Visit

- Assessment of area around biopsy
- Assessment of disease metastasis and new disease
- Blood tests

1.3 Study Timepoints

Participating patients will be treated over the course of 4 weeks (including biopsy and histological confirmation), and will participate in this study for a maximum of 260 weeks. Patients will attend the study site, at the surgery clinic (4th floor Dickson Center) QEII HSC, NSHA for informed consent and confirmation of eligibility.

Primary Objective – TILs

The primary efficacy endpoint for assessment of TILs will be completed on biopsies obtained from patients in week 3; once 20 patients have been enrolled in the study (10 treatment, 10 control) – within the first 12 months of study initiation – IHC of the FFPE sections will be completed.

Secondary Objective – Disease Metastasis/New Disease Formation

The primary efficacy endpoint for assessment of disease metastasis/new disease is assessed at week 130 (2.5 years), the secondary efficacy endpoint will be assessed up to week 260 (5 years).

Secondary Objective – Proteomic and Metabolomic Analysis

The primary efficacy endpoint for assessment of patient metabolomic/proteomic profile will be completed after all participants are enrolled (within 2 years of study initiation).

Visit Number	Study Phase	Visit Label for Statistical Output
1	Treatment Phase	Week 1
2		Week 2
3		Week 3
4		Week 4
5	Follow-Up Phase	Week 26
6		Week 52
7		Week 78
8		Week 104
9		Week 130
10		Week 156
11		Week 182
12		Week 208
13		Week 234
14		Week 260

Should it be necessary to conduct an unscheduled visit for any reason, the date, week and reason for unscheduled visit will be presented in a data listing only. The study does not record any study-specific measurements on unscheduled visits.

2. STUDY POPULATIONS

Two study populations will be used for all summaries and analyses. Subjects who have satisfied the population criteria will be classified in the designated populations and will only be included in analysis for which they have available data.

Intent-to-Treat (ITT) Population

The ITT population is defined as all randomised subjects, regardless of when they withdrew from the study that have received 2 courses of treatment (therapy or placebo). The ITT populations will be used to present efficacy data by randomised treatment group. Subjects will be summarised according the treatment which they received.

Safety Population

The safety population is defined as all randomised subjects who received at least one dose of study treatment. The Safety population will therefore be identical to the ITT population if all randomised subjects receive both doses of the study treatment. The Safety population will be used to present the safety summaries by actual treatment received.

3. DEFINITIONS AND DERIVED VARIABLES

3.1 TILs

TILs are to be determined using IHC staining of FFPE tissue sections prepared form incisional biopsy samples used for histopathological diagnosis and staging of disease which are collected Week 3 of the study.

IHC scoring is undertaken by using a microscope eyepiece grid to standardize the assessed area. In brief, duplicate cores of each immunostained tumor are reviewed at low magnification, and the core exhibiting a tumor/stroma ratio closest to 50:50 and the highest density of positive cells will be selected. This core will then be assessed at higher magnification (×20 objective) with a grid overlaid on the center of the core. Under a ×20 objective magnification, this grid defined an area of 0.56 mm2. The number of positive intraepithelial lymphocytes will be quantified within the area of the grid (intraepithelial localization was defined as lymphocytes within tumor cell nests or in direct contact with tumor cells, consistent with the method used by Denkert et al..[22] To account for variation in epithelial-stromal proportions between different samples, intraepithelial TIL levels will be calculated by dividing the number of observed intraepithelial TIL by the fraction of grid area occupied by epithelium.

3.2 New Disease Formation/Metastasis

New disease formation and assessment of metastasis will be conducted during the follow-up period of the study (Weeks 26, 52, 78, 104, 130, 156, 182, 208, 234 and 260). Melanoma staging will be assessed using the clinical staging criteria of the American Joint Committee on Cancer (AJCC):

Stage	Brief Description
0	Melanoma *in situ*.
I	Thin melanoma (confined to the epidermis).
II	Thick melanoma (extends to the dermis, but no metastasis).
III	Metastasis confined to the lymphatic system.
IV	Distant metastasis to organs and deep tissue.

At each follow up appointment the investigator will assess whether new lesions are identified. The number of new lesions, if any, during a given follow-up appointment will be recorded as an integer value. Distance from the primary lesion will be recorded. Any new lesions will lead to disease staging via standard clinical and histopathological methodologies. After each follow up appointment the following parameters will be recorded:

- New lesions ('Yes' or 'No')
- Number of new lesions
- Distance of lesion(s) from original (cm)
- Staging of disease (Stage 0, Stage I, Stage II, Stage III, or Stage IV)

3.3 Proteomic and Metabolomic Profile

Patient serum and urine samples are to be analysed via ultra-pressure liquid chromatography-Tandem mass spectrometry (UPLC-MS). Chromatograms will be built with peaks recognized using the local minimum search function, and the ion intensities, matching m/z, and retention time was grouped into peak lists; peak lists will then me exported individually for analysis. Chromatograms will contain the following information:

- Peak elution time
- Peak area
- Ion intensity
- m/z

4. EFFICACY PARAMETERS

4.1 Primary Efficacy Endpoint

The primary efficacy endpoint is the achievement of an increase in TILs at Week 3 after 2 intralesional treatments. The primary efficacy endpoint will be analysed using an unpaired T-test.

4.2 Secondary Efficacy Endpoints

The secondary efficacy endpoints are:

4.2.1 Disease Metastasis

4.2.1.1 Decreased Metastases at 130 Weeks

The decreased in metastases at 130 weeks (2.5 years) after treatment initiation will assess both number of metastases and stage of progression as defined by the AJCC. Number of metastases will be assessed as an integer value and stage of progression will be analysed as a categorical variable (Stage 0, Stage I, Stage II, Stage III, or Stage IV).

4.2.1.2 Decreased Metastases at 260 Weeks

The decreased in metastases at 260 weeks (5 years) after treatment initiation will assess both number of metastases and stage of progression as defined by the AJCC. Number of metastases will be assessed as an integer value and stage of progression will be analysed as a categorical variable (Stage 0, Stage I, Stage II, Stage III, or Stage IV).

4.2.1.3 TILs and Metastases at 260 Weeks

The effect on TILs on future metastases will assessed for all immune cells assessed (NK, DC, CD4+, CD8+, M1+, M2+, FoxP3+, PD-1+, PD-L1+). The median infiltration will be determined for each cell type, and rate of metastases and stage of progression will be compared as such by comparing two categories of patients for each cell type:

- Patients with infiltration > median infiltration for each cell type
- Patients with infiltration ≤ median infiltration for each cell type
- *For example; when comparing TIL of CD3+ cells we will be comparing two patient populations in each arm, those with CD3+ > median infiltration and those with CD3+ ≤ median infiltration)*

4.2.1.4 Increased Survival at 260 Weeks

Survival after 260 weeks (5 years) after treatment initiation will be assessed using Kaplan-Meier methods and compared using Log-rank tests.

4.2.1.5 TILs and Survival at 260 Weeks

The effect on TILs on survival will assessed for all immune cells assessed (NK, DC, CD4+, CD8+, M1+, M2+, FoxP3+, PD-1+, PD-L1+). The median infiltration will be determined for each cell type, and rate of metastases and stage of progression will be compared as such by comparing two categories of patients for each cell type:

- Patients with infiltration > median infiltration for each cell type
- Patients with infiltration ≤ median infiltration for each cell type
- *For example; when comparing TIL of CD3+ cells we will be comparing two patient populations in each arm, those with CD3+ > median infiltration and those with CD3+ ≤ median infiltration)*

4.2.2 Systemic Immune Response

Systemic immune response will be assessed by means of comparison of metabolic/proteomic profiles.

5. SAFETY PARAMETERS

The safety data will be summarised for all subjects in the Safety population.

Safety will be assessed through summary of adverse events and compliance with study treatment.

Adverse events will be coded using Medical Dictionary for Regulatory Activities (MedDRA) version 10.1. Events will be summarised by system organ class and preferred term for active IL-2 and placebo for the following:

- All adverse events
- All serious adverse events
- Events judged to be related to study treatment
- Events leading to discontinuation of study treatment

These parameters will be summarised appropriately by treatment group and visit.

6. STATISTICAL METHODOLOGY

6.1 Statistical and Analytical Issues

6.1.1 Statistical Methods

All study practices and statistical methods are based on the International Conference on Harmonization (IHC) document “Statistical Principles for Clinical Trials.”

Data will be summarised by treatment group. Baseline characteristics, and safety outputs total overall columns will be included to summarise all subjects.

For all baseline, demographic, safety and efficacy outputs data will be summarised by treatment group.

In summary tables of continuous variables, the minimum and maximum statistics, the arithmetic mean and median, the 95% confidence interval, standard deviation, and standard error will be presented will to the same number of decimal places as the original data.

In summary tables of categorical variables, counts and percentages will be used. The denominator for each percentage will be the number of subjects within the population treatment group unless otherwise specified.

All hypothesis testing will be carried out at the 5% (2-sided) significance level unless otherwise specified.

P-values will be rounded to three decimal places. P-values less than 0.001 will be reported as <0.001 in tables.

The treatment label for all tables, listings and figures will be:

Treatment	Label
2 treatment cycles of 500,000 IU of IL-2 in 0.1 mL	IL-2 Treatment
0.1 mL of sterile saline (0.9% m/v)	Placebo
All Treatments	Total

Where that any of the statistical methods described herein prove unsuitable during analysis, more appropriate methods will be used. All changes in methodology will be documented in the clinical study report.

Additional ad-hoc analyses may be conducted as deemed suitable.

6.1.2 Dropouts and Missing Data

Subject inclusion/exclusion criteria will be determined at baseline visit, and subjects who do not meet all criteria will not be entered into the study. Those subjects deemed eligible to participate will be allocated a 3-digit number at randomization prior to the initial treatment.

If a subject is discontinued at any time after entering the study, the Investigator will ensure this does not affect the patient's standard of care. At the patients request all unused biological samples (blood, urine, and core biopsies) will be immediately destroyed. The reasons for withdrawal will be recorded on the CRF and will be included in the final report. Failure to complete both (2) treatment cycles will result in patient removal from the trial.

6.1.3 Determination of Sample Size

The primary objective of this study is to assess TILs in IL-2 treated patients. To date, trials assessing intralesional therapies for in-transit MM have been small with studies conducted by Eberhard-Karls-University and the University of Western Ontario having patient cohorts numbering 24 and 39, respectively.[21,23] Both of these trials saw excellent response rates with intralesional IL-2 treatment (51-62%) which translated into statistically significant survival rates; however, neither trail looked systematically assessed TILs. Because no study to our knowledge has systematically assessed TIL response to intralesional IL-2 therapy, there is no data available for a statistical power calculation. However TILs were assessed sporadically in one study and showed a good response, albeit with no placebo control.[23] Taken together, with excellent treatment response rates and evidence of TILs, it is our opinion that 60 participants will be sufficient to assess whether intralesional IL-2 therapy will increase TILs.

6.2 Subject Characteristics

6.2.1 Subject disposition

The subject disposition table will summarise the following and will be presented for all subjects by treatment group and overall.

- The number (%) of subjects entered into the study after Baseline visit
- The number (%) of subjects withdrawn before treatment completion (2 cycles)
- The number (%) of subject withdrawn before week 130
- The number (%) of subject withdrawn before week 260
- The number (%) of subjects who complete the study

The number (%) of subjects who complete and withdraw from the study and the primary reason for withdrawal will be summarised by treatment group and overall for all subjects.

6.2.2 Background and Demographic Characteristics

Demographic data presented will be age and gender. Family history of skin cancer is also collected at Baseline visit. Demographic and background data will be summarised using summary statistic for continuous variables (number of subjects, mean, standard deviation, median, minimum, and maximum).

6.2.3 Prior and Concomitant Medications

Prior and concomitant medications taken by or administered to a subject will be recorded. Prior medications are defined as the medication that started and stopped before Baseline. Concomitant medications are defined as the medications that started before Baseline and continued into the study.

6.2.4 Medical History

Investigators should document all significant immunological or dermatological conditions, or cancers, that the subject has experienced in the past. Any medical condition present at the time informed consent is obtained is to be regarded as concomitant and will result in the subject being ineligible for the study.

6.3 Efficacy Analyses

6.3.1 Primary Efficacy Variable

The primary efficacy endpoint is the achievement of an increase in TILs at Week 3 after 2 cycles of the intralesional treatments.

The comparison of TILs in the two treatment arms will be reported as mean values along with standard error. Means will be reported individually for the following cells:

- Natural killer (NK) cells

- Dendritic cells (DC)
- T-cell lymphocytes (CD3+, CD4+ and CD8+)
- Macrophages (Mac-1+ and Mac-2+)
- Immunosuppressive cells (FoxP3+, PD-1+, and PD-L1+)

6.3.2 Secondary Efficacy Variables

Decreased metastases after 130 weeks

The comparison of metastases at 130 weeks (2.5 years) after treatment initiation will assess in both treatment arms through both number of metastases and stage of progression as defined by the AJCC. Number of metastases will be reported as mean values along with standard errors and compared using an unpaired t-test. Statistical significance will be assessed using two-sided t-tests with significance established with P values less than 0.05. Stage of progression will be analysed as a categorical variable (Stage 0, Stage I, Stage II, Stage III, or Stage IV).

Decreased metastases after 260 weeks

The comparison of metastases at 260 weeks (5 years) after treatment initiation will assess in both treatment arms through both number of metastases and stage of progression as defined by the AJCC. Number of metastases will be reported as mean values along with standard errors and compared using an unpaired t-test. Statistical significance will be assessed using two-sided t-tests with significance established with P values less than 0.05. Stage of progression will be analysed as a categorical variable (Stage 0, Stage I, Stage II, Stage III, or Stage IV).

TILs and metastases at 260 weeks

The effect on TILs on future metastases will assessed for all immune cells assessed (NK, DC, CD4+, CD8+, M1+, M2+, FoxP3+, PD-1+, PD-L1+). The median infiltration will be determined for each cell type, and rate of metastases and stage of progression will be compared as such by comparing two categories of patients for each cell type:

- Patients with infiltration > median infiltration for each cell type
- Patients with infiltration ≤ median infiltration for each cell type

The number of metastases observed for both groups (1: cell infiltration > median infiltration, and 2: cell infiltration ≤ median infiltration) will be reported as means with standard errors and statistical significance will be assessed using two-sided t-tests with significance established with P values less than 0.05.

Increased survival at 260 weeks

Survival after 260 weeks (5 years) after treatment initiation will be assessed using Kaplan-Meier methods and compared using Log-rank tests. Statistical significance will be assessed using two-sided t-tests with significance established with P values less than 0.05.

TILs and survival at 260 weeks

The effect on TILs on survival will assessed for all immune cells assessed (NK, DC, CD3+, CD4+, CD8+, M1+, M2+, FoxP3+, PD-1+, PD-L1+). The median infiltration will be determined for each cell type, and rate of metastases and stage of progression will be compared as such by comparing two categories of patients for each cell type:

- Patients with infiltration > median infiltration for each cell type
- Patients with infiltration ≤ median infiltration for each cell type

The survival observed for both groups (1: cell infiltration > median infiltration, and 2: cell infiltration ≤ median infiltration) will be reported as means (months) with standard errors, and statistical significance will be assessed using two-sided t-tests with significance established with P values less than 0.05.

Systemic Immune Response

Systemic immune response will be assessed by means of comparison of metabolic/proteomic profiles. To distinguish IL-2 treatment from the controls, orthogonal partial least squares discriminant analysis (OPLS‑DA) will be performed. Based on the OPLS‑DA model, specific metabolites will be determined by applying Mann–Whitney U‑test with P value threshold of 0.05. For each biomarker, a receiver operating characteristic (ROC) curve will be generated. The area under curve (AUC) value and 95% confidence interval (CI) will be calculated to determine the specificity and sensitivity of IL-2.

6.4 Safety Analyses

Safety analysis will be performed using the Safety population. All outputs will be summarised by actual treatment received.

6.4.1 Adverse Events

Adverse events may be volunteered by the patient or discovered because of general questioning by the investigator. All adverse events for a subject are recorded as separate events. The following is a list of known adverse events with systemic IL-2 administration that the investigator will inquire directly to the patient about upon follow up:

- Local erythema
- Mild swelling
- Fever
- Flu-like symptoms
- Pain
- Fatigue
- Nausea/vomiting
- Stomach pain
- Diarrhoea
- Headache
- Muscle cramp
- Tachykardia

Any adverse events not listed here will be added to the final listing of adverse events. The number of subjects having at least one adverse event will be tabulated using counts and percentages, and the number of each will be tabulated. All serious adverse events will be listed separately. Any adverse events leading to discontinuation of study treatment will be recorded as "discontinued," and the number (%) of discontinuations will be reported. Adverse events leading to discontinuation of study treatment will be listed.

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