PEMBROLIZUMAB IN MMR-PROFICIENT METASTATIC COLORECTAL CANCER PHARMACOLOGICALLY PRIMED TO TRIGGER DYNAMIC HYPERMUTATION STATUS.

THE **ARETHUSA** TRIAL

IMP Identifiers: PEMBROLIZUMAB

TEMOZOLOMIDE

Protocol Number: IFOM-CPT002/2018/PO001

EudraCT Number: 2018-001441-14

Protocol Version (Date): V3.1, 20th Nov 2019

Sponsor IFOM – Istituto FIRC di Oncologia Molecolare

CONFIDENTIAL

SIGNATURE PAGE

Sponsor Signature

Dr.ssa Silvia Marsoni. (*Sponsor Representative*)

	28/01/2020
Signature	Date

Clinical Study Chair Signature

Prof. Salvatore Siena

	28/01/2020
Signature	Date

Translational Study Chair Signature

Prof. Alberto Bardelli

	27/01/2020
Signature	Date

Principal Investigator Agreement

I have read the Protocol entitled "**PEMBROLIZUMAB IN MMR-PROFICIENT METASTATIC COLORECTAL CANCER PHARMACOLOGICALLY PRIMED TO TRIGGER DYNAMIC HYPERMUTATION STATUS***"* and I agree to conduct the study as detailed herein and in compliance with ICH Guidelines for Good Clinical Practice and applicable regulatory requirements. I will provide all study personnel under my supervision with all information provided by the Sponsor and I will inform them about their responsibilities and obligations.

_____

_____

_____

Principal Investigator

(printed name, Institution, Department and location)

_____ _____

Signature Date

LIST OF ABBREVIATIONS

AE	Adverse Event
ALT	Alanine Aminotransferase (Serum Glutamic-pyruvic Transaminase)
ANC	Absolute Neutrophil Count
aPTT	Activated Partial Thromboplastin Time
AST	Aspartate Aminotransferase (Serum Glutamic-oxaloacetic Transaminase)
B-ML	Baseline Mutational Load
CI	Confidence Interval
CL	Clearance
CR	Complete Response
CRC	Colorectal Cancer
CRF	Case Report Form
CRO	Clinical Research Organization
CT	Computerized Tomography
CTCAE	Common Terminology Criteria for Adverse Events
ctDNA	Circulating tumor DNA
DCL	Data Clarification List
D-ML	Dynamic Mutational Load
eCRF	Electronic Case Report Form
ECG	Electrocardiogram
ECOG	Eastern Cooperative Oncology Group
EE	Efficacy Evaluable
iEE	Immunotherapy Efficacy Evaluable
EGFR	Epidermal Growth Factor Receptor
FACS	Fluorescence activated cell sorting
FFPE	Formalin Fixed Paraffin Embedded
FPI	First Patient In
FPFV	First Patient First Visit
GCP	Good Clinical Practice
G-CSF	Granulocyte Colony Stimulating Factor
IC	Informed Consent
IEC	Independent Ethics Committee
ICH	International Conference on Harmonization
IRB	Institutional Review Board
IV	Intravenous
LB-B-ML	Liquid Biopsy-Baseline-Mutational Load

LB-D-ML	Liquid Biopsy-Dynamic-Mutational Load
LB-TMZ-ML	Liquid Biopsy-Temozolomide-Mutational Load
LDH	Lactate Dehydrogenase
LPI	Last Patient In
LPLV	Last Patient Last Visit
LVEF	Left Ventricular Ejection Fraction
mAb	Monoclonal Antibody
mCRC	Metastatic Colorectal Carcinoma
MGMT	O6-methylguanine–DNA methyltransferase gene
MMR	Mismatch repair
MMR-D	Mismatch repair deficient
MMR-P	Mismatch repair proficient
MRI	Magnetic Resonance Imaging
MSI	microsatellite instable
MSS	microsatellite stable
NCI	National Cancer Institute
NGS	Next Generation Sequencing
ORR	Objective Response Rate
OS	Overall Survival
PET	Positron Emission Tomography
PD	Progressive Disease
PD-ML	Mutational Load assey performed at progression after pembrolizumab
PFS	Progression-Free Survival
PR	Partial Response
QW, Q2W, Q3W, Q8W, Q9W	Every Week, Every 2 Weeks, Every 3 Weeks, Every 8 Weeks, Every 9 Weeks
RAS mCRC	Extended RAS mutated CRC (Extended RAS= KRAS and NRAS)
RBC	Red Blood Cell
RECIST	Response Evaluation Criteria In Solid Tumors
iRECIST	Immune Response Evaluation Criteria In Solid Tumors
ROC	Receiver Operator Characteristic
SAE	Serious Adverse Event
SAS	Statistical Analysis System
SD	Stable Disease
SE	Safety Evaluable
SUSAR	Suspect Unexpected Serious Adverse Reaction
TMF	Trial Master File
TMZ	Temozolomide

ULN	Upper Limit of Normal
WBC	White Blood Count / White Blood Cells
WKS	Weeks
WT	Wild Type

1.0 STUDY PERSONNEL

STUDY CHAIRS
CLINICAL **Prof. Salvatore Siena** Niguarda Cancer Center, Grande Ospedale Metropolitano – MILANO e Dipartimento di Oncologia ed Onco-Ematologia Università degli Studi di Milano Telefono: 02 6444 2291; Email: salvatore.siena@ospedaleniguarda.it **TRANSLATIONAL** **Prof. Alberto Bardelli** Fondazione Piemontese per la Ricerca sul Cancro (FPRC) – CANDIOLO (TO) e Dipartimento di Oncologia Università degli Studi di Torino Telefono: 011 993 3548; Email: alberto.bardelli@ircc.it **OPERATIONAL** **Dr.ssa Silvia Marsoni** IFOM – Istituto FIRC di Oncologia Molecolare (MILANO) Telefono: 02 57430 3862; Email: silvia.marsoni@ifom.eu

PROJECT MANAGER
Dr. Paolo Luraghi IFOM – Istituto FIRC di Oncologia Molecolare (MILANO) Telefono: 02 57430 3799; Email: paolo.luraghi@ifom.eu

BIOSTATICIAN
Dr. Valter Torri Istituto Mario Negri – MILANO Telefono: 02 390 141; Email: valter.torri@marionegri.it

2.0 SUMMARY

Abbreviated Title	**THE ARETHUSA TRIAL**
Trial Phase	*2*
Clinical Indication	Metastatic colorectal cancer
Trial Type	Interventional
Type of control	NA
Route of administration	Intravenous Pembrolizumab and oral Temozolomide
Trial Blinding	NA
Treatment Groups	TMZ priming followed by Pembrolizumab
Number of trial participants	54 in TRIAL Phase | 67 PRIMING Phase | 670 SCREENING Phase
Estimated enrollment period	*24 months*
Estimated duration of trial	*36 months (40 months to final report)*
Duration of Participation	36 months + Follow Up
Estimated average length of treatment per patient	*6 months (Pembrolizumab); 3 months (Temozolomide)*

3.0 SYNOPSIS

Protocol Number	IFOM-CPT002/2018/PO001
Title	**PEMBROLIZUMAB IN MMR-PROFICIENT METASTATIC COLORECTAL CANCER PHARMACOLOGICALLY PRIMED TO TRIGGER DYNAMIC HYPERMUTATION STATUS**
Brief title	THE **ARETHUSA** TRIAL
Sponsor	IFOM - Istituto FIRC di Oncologia Molecolare
Clinical Phase	Phase II
Study Rationale	ARETHUSA has been designed on the basis of four translational findings, namely that: i) pembrolizumab is active only in MMR deficient mCRC patients; ii) the DNA alkylator temozolomide is active only in mCRC patients whose tumor harbours a silenced O-6-methylguanine-DNA methyltransferase (MGMT), a DNA repair enzyme that removes alkylated adducts from the DNA. MGMT is silenced by gene promoter hypermethylation, a favourable context of susceptibility to DNA damage (Amatu et al., 2016; Esteller and Herman, 2004); iii) in the tumor specimens of mCRC patients with MMR proficient tumors collected at treatment progression we observed that resistance to TMZ correlated with inactivation of MMR and increased mutational loads (Germano et al., 2017) iv) TMZ can cause inactivation of DNA repair, increased mutational burden and effective immune surveillance in preclinical models, *de facto* turning a MMR-P tumors into a 'MMR-D like' (Germano et al., 2017). Moreover, mutational load has been correlated with response to immune checkpoint blockade. In particular clinical evidence, mostly derived from melanoma patients treated with ipilimumab, suggests that the mutational burden associated with clinical benefit is between 10 and 20 mutations per MB (Lauss et al., 2017; Snyder et al., 2014; Van Allen et al., 2015). Indeed a recent study (Panda et al., 2017) in 224 colorectal cancer shows that 100% of 193 cases with < 20 mutations were MMR proficient and that 100% of 31 cases with > 20 mutations were either MMR-deficient (n 28) or POLE mutated (n 3). Thus, an absolute mutational load cutoff of 20 mutations per MB was able, in CRC patients, to detect with digital sensitivity and specificity the subset of patients most likely to achieve a clinical response from immunotherapy. Collectively these results suggest that an increase in mutational load (and the ensuing neo-antigen burden) induced by TMZ treatment, might sensitize to immune checkpoint blockade, and drive the rationale of the ARETHUSA trial. A 20

	mutations/MB has been defined as the absolute post-temozolomide cut-off value for patient eligibility on the basis of the studies reported above.
Primary Objective	To assess the objective response rate of pembrolizumab in metastatic MMR proficient, extended RAS mutated CRC patients who have failed standard therapies, with MGMT-negative tumors treated with TMZ, given with the intent of increasing the mutational load of their tumors (cohort P).
Secondary Objective(s)	• To assess the objective response rate in MMR deficient patients (cohort D) identified during the MMR screening. • To assess the Progression Free Survival (PFS) and Overall Survival (OS) both P and D cohorts patients. • To assess the safety and tolerability of pembrolizumab after TMZ-priming therapy (cohort P). • To assess the safety and tolerability of pembrolizumab (cohort D).
Translational Objective(s)	• To validate the assessment of mutational load in plasma using the tissue mutational load as 'gold' standard. • To assess the relationship between selected immune biomarkers in archival and/or fresh tumor tissue and blood with response to pembrolizumab. • To compare these immunomarkers profiles prior and after TMZ in cohort P patients, and prior and after pembrolizumab treatment in all patients (cohort D and P). • To assess the relationship of RAS status in MMR deficient patients (cohort D) with response to Pembrolizumab.
Primary Endpoint	Objective response rate (ORR) to pembrolizumab in cohort P according to RECIST (as specified in table 9.1.3).
Secondary Endpoint(s)	• ORR to pembrolizumab in cohort D according to RECIST (as specified in table 9.1.3). • PFS and OS in both cohorts P and D. • Safety and tolerability according to CTCAE version 4.03.
Translational endpoint(s)	• Validation of tumor mutational load assessment in plasma. • Expression of immune markers and their correlation with response to temozolomide and pembrolizumab. • ORR to pembrolizumab in RAS and non-RAS mutant stratified cohort D according to RECIST.
Trial Design	2-cohort, open label, transformative, precision medicine, phase II trial in extended RAS-mutated MMR-Proficient metastatic CRC (RAS mCRC) and unselected MMR-Deficient mCRC patients refractory to standard treatments. Extended RAS

	mutations include KRAS and NRAS mutations. The trial consists of 3 different phases: **Screening Phase**: MMR-Deficient (MMR-D) patients proceed directly to TRIAL Phase (cohort D) to be treated with pembrolizumab. RAS mutant MMR-Proficient (MMR-P) patients, instead, are further tested for O6-methylguanine–DNA methyltransferase gene expression (MGMT) status in tissue (MGMT protein IHC and MGMT promoter methylation). MGMT IHC negative and promoter methylated patients will proceed to PRIMING phase. **Priming Phase:** MMR-P patients showing negative MGMT protein and high levels of MGMT promoter methylation in tissues will receive TMZ therapy until progression. Two tumor biopsies will be taken prior to starting therapy and at progression to determine the mutational load. Patients with a mutational load < 20 mutations/megabase will go off-study. Patients with a mutational load ≥20 mutations/megabase, will proceed to trial phase no longer than week 5 post TMZ-ML. **Trial Phase:** Eligible patients, i.e. MMRD patients (cohort D) and patients with a TMZ-ML ≥ 20 mutations per megabase at TMZ-ML (cohort P), will be treated with pembrolizumab 200 mg administered as an intravenous infusion over 30 minutes every 3 weeks until disease progression, unacceptable toxicity, or up to 24 months in patients without disease progression.
Inclusion Criteria	Patient selection criteria are progressively restrictive according to the 3-step selection strategy of the trial and are reported separately for each phase. **SCREENING Phase** 1. Histologically confirmed diagnosis of metastatic colorectal cancer. 2. Documented RAS extended mutations in the archival sample (cohort P only). 3. ECOG performance status 0-1. 4. SCREENING phase informed consent signed. 5. Understanding and accepting the need for undergoing two tumor biopsies if eligible for PRIMING Phase. 6. Age ≥ 18 years. 7. Availability of all diagnostic FFPE blocks (primary tumor and or metastases), or at least 20 slides (primary tumor and/or metastases). Formalin-fixed, paraffin embedded (FFPE) tissue blocks are preferred to slides.

8. Normal organ functions.

PRIMING Phase

1. Fulfilment of all the SCREENING inclusion criteria;
2. PRIMING informed consent signed;
3. Confirming the willingness to undergo two tumor biopsies,
4. Acceptance that, if the mutational load determination is unfeasible for technical reasons (not enough tissue, substandard test performance, etc.), access to TRIAL phase will not be possible.
5. Imaging documented failure of previous standard CRC therapies including fluoropyrimidine, oxaliplatin, irinotecan plus or minus antiangiogenics agents (Bevacizumab, Aflibercept, Regorafenib, others).
6. At least one measurable tumor lesion as per RECIST v1.1. Lesions in previously irradiated areas or those that have received other loco-regional therapies (i.e. percutaneous ablation) should not be considered measurable unless there is clear documented evidence of progression of the lesion since therapy. Imaging must be performed maximum within 28 days prior to enrolment.
7. ECOG performance status 0 or 1;
8. Following results in the SCREENING Phase tests:

    - Proficient MMR status assessed by IHC or MSI-Low status defined by PCR (Bethesda panel);
    - Negative score for the MGMT protein expression IHC test;
    - Positive score for the MGMT promoter methylation performed on Tissue.

9. Women with childbearing potential should complete a pregnancy test and be willing to use highly effective contraceptive methods.
10. Normal organ functions.

TRIAL Phase

1. Fulfilment of all the SCREENING inclusion criteria and Deficient MMR status (IHC) or MSI-High status (PCR) (cohort D only).
2. Fulfilment of all the SCREENING and PRIMING inclusion criteria (cohort P only).
3. TRIAL Phase informed consent signed (both cohorts).
4. Imaging documented PD to TMZ (cohort P only).
5. A mutational load value $\geq$ 20 mutations/MB at TMZ-ML assay (cohort P only).

<table>
<tr><td></td><td>
6. Imaging documented failure of previous standard CRC therapies including fluoropyrimidine, oxaliplatin, irinotecan plus or minus targeted agents (Bevacizumab, Aflibercept, Regorafenib, Cetuximab, Panitumumab, others) (cohort D only).
7. At least one measurable tumor lesion as per RECIST v1.1. Lesions in previously irradiated areas or those that have received other loco-regional therapies (i.e. percutaneous ablation) should not be considered measurable unless there is clear documented evidence of progression of the lesion since therapy. Imaging must be performed maximum within 28 days prior to enrolment (both cohorts).
8. Woman with childbearing potential should complete a pregnancy test and be willing to use highly effective contraceptive methods (both cohorts).
9. Normal organ functions. Blood specimens must be collected within 10 days prior to the start of study treatment (both cohorts).

See exclusion criteria in the protocol
</td></tr>
<tr><td>Treatments</td><td>
<table>
<tr><th>Drug</th><th>Dose/Potency</th><th>Dose Frequency</th><th>Route of Administration</th><th>Regimen/Treatment Period</th><th>Use</th></tr>
<tr><td>Temozolomide</td><td>150 or 200 or 100 mg/ (7.3.1.2)</td><td>Q4W</td><td>P.O.</td><td>Days 1-5 every 28 days (PRIMING Phase)</td><td>Experimental</td></tr>
<tr><td>Pembrolizumab</td><td>200 mg</td><td>Q3W</td><td>IV infusion</td><td>Day 1 of each 3 week cycle (TRIAL Phase)</td><td>Experimental</td></tr>
</table>
</td></tr>
<tr><td>Sample Size</td><td>
A two-stage Green-Dahlberg design has been used to calculate the sample size for the P-cohort under the following assumptions:

• The null hypothesis that the true (H_0) response rate is 10% (not considered clinically compelling) will be tested against a one-sided alternative (H_1).

• In the first stage, 10 patients will be accrued. If no responses are observed, the study will be stopped. Otherwise, 10 additional patients will be accrued for a total of 20.

• The null hypothesis will be rejected if 5 or more responses are observed in 20 patients.

• This design yields a type I error rate of 0.05, and power of 90% when the true response rate is 40%.

• In order to recruit 20 patients in the P-cohort the number of MMR unselected patients to be pre-screened for MMR status MGMT promoter
</td></tr>
</table>

methylation and the number of MGMT-PM+ to be primed with TMZ are reported in the Table below

Number of Patients to be:	**N**	**Expected Percent**
RAS mutant MMR proficient cases to be screened	670	A
expected MGMT IHC negative	335	B = 50% of A
expected promoter methylated in tissue	67	C = 20% of B and 10% of A
expected with increase of mutational load ≥ 20 mutations/Mb (PRIMING Phase)	20	D= 30% of C

For cohort D a precise sample size has NOT been calculated since the ORR in deficient patients is a secondary objective of the trial. We will enroll up to 34 MMR-D cases according to an estimated prevalence of 4-5%. These patients will constitute the **D-cohort.** We expect a response rate between 40% (95% C.I .18-.71) and 33% (95% C.I .21-.46) as reported by Le et al (2015 & 2019) with 11-14 responses accordingly.

4.0 TRIAL DESIGN

4.1 Overall Trial Design

This is a 2-cohort, open label, transformative, precision medicine, phase II trial in extended RAS-mutated metastatic CRC (RAS mCRC) patients refractory to standard treatments. Extended RAS mutations include KRAS and NRAS mutations.

The design of Arethusa is based on two main observations (see 6.1.1). First that objective responses with pembrolizumab can only be achieved in MMR-deficient tumors with a high mutational load (and ensuing high neo-antigen burden)(Le et al., 2015). Second, that the low mutational load, typical of MMR-proficient tumors, can be increased *in vitro* and in *in-vivo* models (with an ensuing increase in the tumor's neo-antigen burden) by a pre-treatment with DNA-alkylating agents, thus sensitizing the tumor to the immune checkpoint blockade (Germano et al., 2017). We have designed Arethusa accordingly with the purpose of i) identifying upfront MMR-deficient patients (cohort D); ii) using TMZ to exploit the favorable context of susceptibility to DNA damage of MGMT methylated tumors as an enrichment strategy for MMR-proficient patients (cohort P), and iii) using cohort D patients as a 'natural positive comparator' for cohort P results.

On the basis of this translational rationale and due to the its proof of concept nature, Arethusa consists of three different phases - SCREENING Phase, PRIMING phase, and TRIAL Phase, as described below and reported in Trial Diagram (see 4.2).

4.1.1 SCREENING Phase

MMR-Deficient (MMR-D) patients proceed directly to TRIAL Phase (cohort D) to be treated with pembrolizumab. RAS mutant MMR-Proficient (MMR-P) patients, instead, are further tested for O6-methylguanine–DNA methyltransferase gene expression (MGMT) status in tissue (MGMT protein by IHC and MGMT promoter methylation). MGMT IHC negative and promoter methylated patients will proceed to PRIMING phase. MGMT IHC positive and/or MGM promoter non-methylated patients will go off-study.

4.1.2 PRIMING Phase

MMR-P patients who have failed standard therapies including fluoropyrimidines, oxaliplatin, irinotecan plus or minus antiangiogenic agents (Bevacizumab, Regorafenib, others), showing negative MGMT protein in tissues and high levels of MGMT promoter methylation in tissue, will undergo a tumor biopsy (non-mandatory for the enrollment) to retrospectively determine the mutational load of their tumor (Baseline Mutational Load = B-ML), and will then receive TMZ therapy (OS dx5,q28 days) until progression.

At PD patients will be re-biopsied (mandatory for the enrolment) to prospectively determine the tumor mutational load (Post Temozolomide Mutational Load= TMZ-ML). Patients with a mutational load < 20 mutations/megabase will go off-study. Patients with a mutational load ≥20 mutations/megabase, will proceed to trial phase no longer than week 5 post TMZ-ML. This time gap is related to the technical time requirements to perform and interpret the mutational load assay.

During the 5-week gap, standard palliative treatment with the exception of regorafenib, can be delivered, at physician discretion, for symptomatic disease control.

Two liquid biopsies for the experimental NGS determination of the mutational load in plasma will also be performed concomitantly with the tissue biopsies: before treatment with TMZ (LB-B-ML) and at PD following TMZ treatment (LB-TMZ-ML).

4.1.3 TRIAL Phase

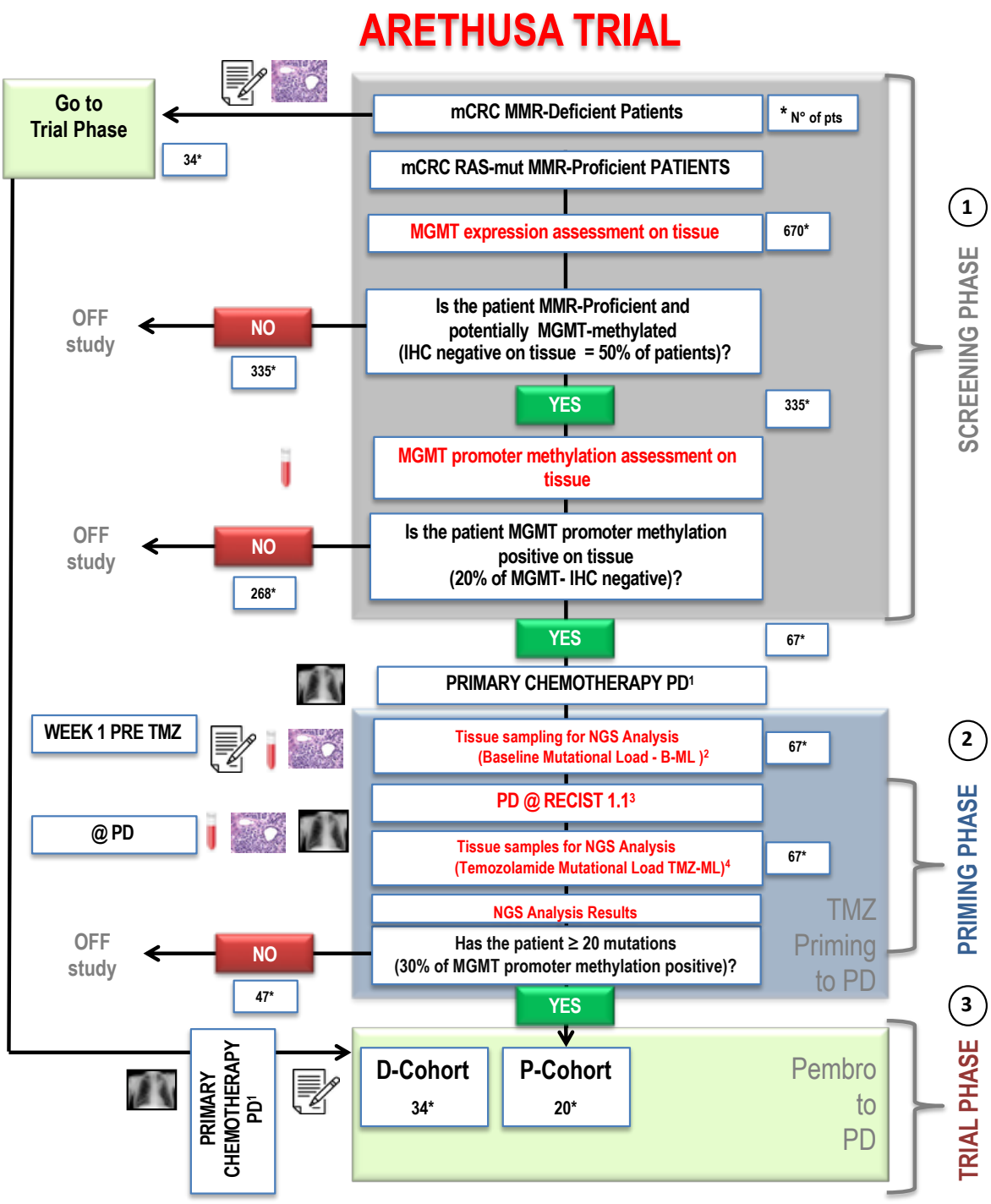
Eligible patients, i.e. MMRD patients (cohort D) who have failed standard therapies including fluoropyrimidines, oxaliplatin, irinotecan plus or minus targeted agents (Bevacizumab, Aflibercept, Regorafenib, Cetuximab, Panitumumab, others), and patients with a TMZ-ML $\geq$ 20 mutations per megabase at TMZ-ML (cohort P), will be treated with pembrolizumab 200 mg administered as an intravenous infusion over 30 minutes every 3 weeks until disease progression, unacceptable toxicity, or up to 24 months in patients without disease progression.

Two further tissue biopsies might be performed at each patient before (cohort D only, non-mandatory) and after Pembrolizumab treatment (both cohorts, non-mandatory) to perform mutational load test (Dynamic and PD Mutational Load = D-ML and PD-ML).

In addition, for the experimental NGS determination of mutational load in plasma, two liquid biopsies will also be performed concomitantly with expected D-ML and PD-ML tissue biopsies (LB-D-ML and LB-PD-ML). These will be done even in the case tissue biopsies will not be executed.

Results from D-ML and LB-D-ML (if the liquid biopsy assay will result reliable, see point 5.3) will be used post-hoc to stratify patients according to the ratio of predicted private/shared antigens. Private antigens are defined as predicted neo-antigens present at one specific time point, while shared antigens are present in all the 3 time points collected (LB-B-ML; D-ML; LB-PD-ML). PD-ML and LB-PD-ML data will be used for correlative studies on primary and secondary resistance to pembrolizumab.

4.2 Trial Diagram



1: 1st line FOLFOXIRI or 1st line FOLFOX→ 2nd line FOLFIRI or viceversa ; ± bevacizumab, aflbercept or regorafenib.
2: B-ML is non-mandatory.
3: Patients will enter the Pembrolizumab Phase once in PD (or if TMZ toxicity is unacceptable).
4: TMZ-ML is mandatory for prospective patients enrichment.

5.0 OBJECTIVES OF THE TRIAL

5.1 Primary Objectives

The primary objective of ARETHUSA is to assess the objective response rate of pembrolizumab in metastatic MMR proficient, extended RAS mutated CRC patients with MGMT positive tumor treated with TMZ, given with the intent of increasing the mutational load of their tumors (cohort P), who have failed standard therapies.

The primary hypothesis tested by ARETHUSA is whether immunoediting in MMR proficient cancers can be favourably disrupted by pharmacologically increasing the tumor mutational load with DNA alkylators, thus enabling the tumor to dynamically express proteins (neo-antigens) that can positively affect T cell functions under checkpoint inhibition.

5.2 Secondary Objectives

The secondary objectives of ARETHUSA are;

1. To assess the objective response rate in MMR deficient patients (cohort D) identified during the MMR screening.
2. To assess the Progression Free Survival (PFS) and Overall Survival (OS) both P and D cohorts patients.
3. To assess the safety and tolerability of pembrolizumab after TMZ-priming therapy (cohort P).
4. To assess the safety and tolerability of pembrolizumab (cohort D).

5.3 Exploratory Objectives

The exploratory objective of ARETHUSA are:

1. To validate the assessment of mutational load in plasma using the tissue mutational load as ‘gold’ standard.
2. To assess the relationship between selected immune biomarkers in archival and/or fresh tumor tissue and blood with response to pembrolizumab.
3. To compare these immunomarkers profiles prior and after TMZ in cohort P patients, and prior and after pembrolizumab treatment in all patients (cohort D and P).
4. To assess the relationship of RAS status in MMR deficient patients (cohort D) with response to Pembrolizumab.

5.4 Primary Endpoint

Objective response rate (ORR) to pembrolizumab in cohort P according to RECIST (as specified in table 9.1.3).

5.5 Secondary Endpoints

1. ORR to pembrolizumab in cohort D according to RECIST (as specified in table 9.1.3).
2. PFS and OS in both cohorts P and D.

3. Safety and tolerability according to CTCAE version 4.03.

5.6 Exploratory Endpoints

1. Validation of tumor mutational load assessment in plasma.
2. Expression of immune markers and their correlation with response to temozolomide and pembrolizumab.
3. ORR to pembrolizumab in RAS and non-RAS mutant stratified cohort D according to RECIST.

6.0 BACKGROUND & RATIONALE

6.1 Background

6.1.1 Translational background

In Western countries, colorectal cancer (CRC) is the third common tumor type and the second leading cause of cancer-related death (Le et al., 2015). Even with a survival exceeding two years in genetically selected patients (Siegel et al., 2013), metastatic CRC remains mostly not curable. While the molecular landscape of CRC is well described (Douillard et al., 2013; Network, 2012), the unmet need for therapies targeting new oncogenic drivers and overcoming resistance to EGFR antagonists still remains. Insights from genetic studies have provided the impetus for efforts to target key signaling pathways, but have also led researchers to revisit established targets, such as HER2 (Bertotti et al., 2015), or vulnerability molecular contexts such as mismatch-repair (MMR) deficiency (Sartore-Bianchi et al., 2016a).

CRCs with genetic defects in MMR pathways harbor hundreds to thousands of somatic mutations mostly in regions of repetitive DNA known as microsatellites (Boland and Shike, 2010; Lynch and de la Chapelle, 2003). The accumulation of mutations in these regions is termed microsatellite instability (MSI) and occurs in approximately 15% of CRC but only in less than 5% of metastatic tumors (Goldstein et al., 2014). It has been recently demonstrated that a high burden of mutations results in a greatly increased number of mutation-associated neoantigens which can be recognized by the patient autologous immune system (Segal et al., 2008). In this setting, treatment with the anti–programmed death ligand 1 (PD-1) immune checkpoint inhibitor pembrolizumab is effective in inducing durable objective responses in 40% of cases (Sartore-Bianchi et al., 2016a). These results should be regarded as quite remarkable, especially since clinical efficacy was independent from RAS mutations presence, a mutation greatly constraining the use of targeted treatment and negatively affecting prognosis in CRC (Sartore-Bianchi et al., 2016b).

Given the low incidence of MMR-deficient mCRC limited to less than 5% of cases, it is crucial to understand if selected subsets of MMR-proficient patients could still benefit from checkpoint inhibition (CPi). In other tumor types MMR-proficient cancers with high mutational burden are susceptible to CPi (Rizvi et al., 2015; Yadav et al., 2016). Whether a MMR-proficient phenotype can be turned into ‘MMR-deficient like’, is thus a key question in CRC.

We previously demonstrated that specific environmentally-derived carcinogens can cause genetic instability in CRC cell lines. Specifically, treatment with the alkylating agent N-methyl-N9-nitro-Nnitrosoguanidine (MNNG) can select for tumor cells with MMR-deficient status (Bardelli et al.,

2001). TMZ is known to induce loss of the mismatch repair protein MSH6 in human glioblastomas (Cahill et al., 2007) and an increase in G>A mutation incidence (Alexandrov et al., 2013). In parallel, other studies have demonstrated that TMZ side effects can influence the immune cell compartment by selectively depleting the immuno-suppressive T regulator lymphocytes (Tregs) (Coffield et al., 2004), and activating the immuno-active T cytotoxic lymphocytes (Tc) and natural killers (NK) (Hervieu et al., 2013).

While alkylating agents represent the backbone of systemic treatment for many solid tumors, this is not the case for CRC where this class of compounds is of limited use. Indeed, only few data are available regarding treatment of CRC with alkylating agents. Specifically their use has been mainly based on the presence of a deficiency of O(6)-methylguanine-DNA-methyltransferase (MGMT), that occurs through gene promoter hypermethylation, as a favorable context of susceptibility to DNA damage (Amatu et al., 2016; Esteller and Herman, 2004). MGMT is the DNA repair enzyme in charge of removing DNA alkylated adducts caused by carcinogens and certain cytotoxics. We have previously reported that MGMT is hypermethylated at high levels in 20% CRC samples (Barault et al., 2015). We have also described that alkylating agents, such as the iv dacarbazine or its oral form temozolomide (TMZ), have clinical efficacy in a subgroup of microsatellite stable (MSS) metastatic CRCs displaying MGMT promoter hypermethylation even though clinical benefit is limited in time (Amatu et al., 2013, 2016). Importantly, in these phase II studies, clinical specimens from five patients with MMR proficient tumors were collected and it was observed that resistance to TMZ correlated with inactivation of MMR and increased mutational loads in 2 of 5 cases. These clinical finding prompted a 'reverse translational' set of in vitro and in vivo experiments showing that TMZ, in syngeneic mouse MMR proficient colon cancer models, can indeed causes inactivation of DNA repair, increased mutational burden and effective immune surveillance followed by dynamic mutational profiles, resulting in persistent renewal of neo-antigens. TMZ untreated proficient models instead exhibit stable mutational loads and neo-antigen profiles over time. Similarly, MMR inactivation following TMZ resistance in human colorectal cancer cells also resulted in continuous renewal of neo-antigens (Germano et al., 2017).

Pembrolizumab is a potent humanized immunoglobulin G4 (IgG4) monoclonal antibody (mAb) with high specificity of binding to the programmed cell death 1 (PD-1) receptor, thus inhibiting its interaction with programmed cell death ligand 1 (PD-L1) and programmed cell death ligand 2 (PD-L2). Based on preclinical in vitro data, pembrolizumab has high affinity and potent receptor blocking activity for PD-1. Pembrolizumab has an acceptable preclinical safety profile and is in clinical development as an intravenous (IV) immunotherapy for advanced malignancies. Keytruda® (pembrolizumab) is indicated for the treatment of patients across a number of indications because of its mechanism of action to bind the PD-1 receptor on the T cell. For more details on specific indications refer to the Investigator brochure.

On May 23, 2017, pembrolizumab (KEYTRUDA, Merck & Co.) was granted a first world-wide tissue/site-agnostic accelerated approval by the U.S. Food and Drug Administration for adult and pediatric patients with unresectable or metastatic, microsatellite instability-high (MSI-H) or mismatch repair deficient (dMMR) solid tumors that have progressed following prior treatment and who have no satisfactory alternative treatment options including MSI-H or dMMR colorectal

cancer that has progressed following treatment with a fluoropyrimidine, oxaliplatin, and irinotecan (see https://www.fda.gov/drugs/informationondrugs/approveddrugs/ucm560040.htm.)

6.1.2 Pharmaceutical and Therapeutic Background

The importance of intact immune surveillance function in controlling outgrowth of neoplastic transformations has been known for decades (Disis, 2010). Accumulating evidence shows a correlation between tumor-infiltrating lymphocytes in cancer tissue and favorable prognosis in various malignancies. In particular, the presence of CD8+ T-cells and the ratio of CD8+ effector T-cells/FoxP3+ regulatory T-cells (T-regs) correlates with improved prognosis and long-term survival in solid malignancies, such as ovarian, colorectal, and pancreatic cancer; hepatocellular carcinoma; malignant melanoma; and renal cell carcinoma. Tumor-infiltrating lymphocytes can be expanded ex vivo and reinfused, inducing durable objective tumor responses in cancers such as melanoma (Dudley et al., 2005; Hunder et al., 2008).

The PD-1 receptor-ligand interaction is a major pathway hijacked by tumors to suppress immune control. The normal function of PD-1, expressed on the cell surface of activated T-cells under healthy conditions, is to down-modulate unwanted or excessive immune responses, including autoimmune reactions. PD-1 (encoded by the gene *Pdcd1*) is an immunoglobulin (Ig) superfamily member related to cluster of differentiation 28 (CD28) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) that has been shown to negatively regulate antigen receptor signaling upon engagement of its ligands (PD-L1 and/or PD-L2)(Greenwald et al., 2005; Okazaki et al., 2001).

The structure of murine PD-1 has been resolved (Zhang et al., 2004). PD-1 and its family members are type I transmembrane glycoproteins containing an Ig-variable–type (IgV-type) domain responsible for ligand binding and a cytoplasmic tail responsible for the binding of signaling molecules. The cytoplasmic tail of PD-1 contains 2 tyrosine-based signaling motifs, an immunoreceptor tyrosine-based inhibition motif, and an immunoreceptor tyrosine-based switch motif. Following T-cell stimulation, PD-1 recruits the tyrosine phosphatases, SHP-1 and SHP-2, to the immunoreceptor tyrosine-based switch motif within its cytoplasmic tail, leading to the dephosphorylation of effector molecules such as CD3 zeta (CD3ζ), protein kinase C-theta (PKCθ), and zeta-chain-associated protein kinase (ZAP70), which are involved in the CD3 T-cell signaling cascade(Chemnitz et al., 2004; Okazaki et al., 2001; Riley, 2009; Sheppard et al., 2004). The mechanism by which PD-1 down-modulates T-cell responses is similar to, but distinct from, that of CTLA-4, because both molecules regulate an overlapping set of signaling proteins (Francisco et al., 2010; Parry et al., 2005). As a consequence, the PD-1/PD-L1 pathway is an attractive target for therapeutic intervention in extended RAS mutated mCRC.

6.1.3 Preclinical and Clinical Trial Data

Refer to the Investigator's Brochure for Preclinical and Clinical data.

6.2 Rationale

6.2.1 Translational rationale for ARETHUSA enrichment strategy

ARETHUSA has been designed on the basis of four translational findings, as described in the background. Namely that: i) pembrolizumab is active only in MMR deficient mCRC patients; ii) the DNA alkylator temozolomide is active only in mCRC patients whose tumor harbor a silenced O-6-methylguanine-DNA methyltransferase (MGMT), a DNA repair enzyme that removes alkylated adducts from the DNA. MGMT is silenced by gene promoter hypermethylation, a favorable context of susceptibility to DNA damage (Amatu et al., 2016; Esteller and Herman, 2004); iii) in the tumor specimens of mCRC patients with MMR proficient tumors collected at treatment progression we observed that resistance to TMZ correlated with inactivation of MMR and increased mutational loads (Germano et al., 2017) iv) TMZ can cause inactivation of DNA repair, increased mutational burden and effective immune surveillance in preclinical models, *de facto* turning a MMR-P tumors into a 'MMR-D like' (Germano et al., 2017).

Moreover, mutational load has been correlated with response to immune checkpoint blockade. In particular clinical evidence, mostly derived from melanoma patients treated with ipilimumab, suggests that the mutational burden associated with clinical benefit is between 10 and 20 mutations per MB (Lauss et al., 2017; Snyder et al., 2014; Van Allen et al., 2015). Indeed a recent study (Panda et al., 2017)in 224 colorectal cancer shows that 100% of 193 cases with < 20 mutations were MMR proficient and that 100% of 31 cases with > 20 mutations were either MMR-deficient (n 28) or POLE mutated (n 3). Thus, an absolute mutational load cutoff of 20 mutations per MB was able, in CRC patients, to detect with digital sensitivity and specificity the subset of patients most likely to achieve a clinical response from immunotherapy.

Collectively these results suggest that an increase in mutational load (and the ensuing neo-antigen burden) induced by TMZ treatment, might sensitize to immune checkpoint blockade, and drive the rational of the ARETHUSA trial. A 20 mutations/MB has been defined as the absolute post-temozolomide cut-off value for patient eligibility on the basis of the studies reported above.

Accordingly, we have designed a three-phased enrichment strategy for the trial (see trials design 4.1) based on the following assumptions that have been adjusted according to the actual screening results (see Appendix 5 and Trial diagram, 4.2 for details);

- Within a population of extended RAS mutated MMR-P patients, 50% will score MGMT negative in tissue.
- Of these, 20% (10% of the initial population) will score MGMT promoter methylation positive (MGMT- PM+). Only these patients will be eligible for the TMZ priming phase.
- At progression during treatment with TMZ, it is expected that 30% of patients will have a tissue mutational load of $\geq$ 20 mutations/mb.
- Only patients showing a $\geq$ 20 mutations/mb mutational load might benefit from a therapy with pembrolizumab.

6.2.2 Justification for Dose

The dose-dense regimen for TMZ has been chosen to maximize the targeting on DNA repair proteins. The tolerability of the dose dense schedule has been proven by our own previous phase II trial in chemorefractory mCRC patients (Amatu et al., 2016; Barault et al., 2015). Pembrolizumab will be used at FDA approved dose for mCRC.

The planned dose of pembrolizumab for this study is 200 mg every 3 weeks (Q3W). Based on the totality of data generated in the Keytruda development program, 200 mg Q3W is the appropriate dose of pembrolizumab for adults across all indications and regardless of tumor type. As outlined below, this dose is justified by:

- Clinical data from 8 randomized studies demonstrating flat dose- and exposure-efficacy relationships from 2 mg/kg Q3W to 10 mg/kg every 2 weeks (Q2W),
- Clinical data showing meaningful improvement in benefit-risk including overall survival at 200 mg Q3W across multiple indications, and
- Pharmacology data showing full target saturation in both systemic circulation (inferred from pharmacokinetic [PK] data) and tumor (inferred from physiologically-based PK [PBPK] analysis) at 200 mg Q3W

Among the 8 randomized dose-comparison studies, a total of 2262 participants were enrolled with melanoma and non-small cell lung cancer (NSCLC), covering different disease settings (treatment naïve, previously treated, PD-L1 enriched, and all-comers) and different treatment settings (monotherapy and in combination with chemotherapy). Five studies compared 2 mg/kg Q3W versus 10 mg/kg Q2W (KN001 Cohort B2, KN001 Cohort D, KN002, KN010, and KN021), and 3 studies compared 10 mg/kg Q3W versus 10 mg/kg Q2W (KN001 Cohort B3, KN001 Cohort F2 and KN006). All of these studies demonstrated flat dose- and exposure-response relationships across the doses studied representing an approximate 5- to 7.5-fold difference in exposure. The 2 mg/kg (or 200 mg fixed-dose) Q3W provided similar responses to the highest doses studied. Subsequently, flat dose-exposure-response relationships were also observed in other tumor types including head and neck cancer, bladder cancer, gastric cancer and classical Hodgkin Lymphoma, confirming 200 mg Q3W as the appropriate dose independent of the tumor type. These findings are consistent with the mechanism of action of pembrolizumab, which acts by interaction with immune cells, and not via direct binding to cancer cells.

Additionally, pharmacology data clearly show target saturation at 200 mg Q3W. First, PK data in KN001 evaluating target-mediated drug disposition (TMDD) conclusively demonstrated saturation of PD-1 in systemic circulation at doses much lower than 200 mg Q3W. Second, a PBPK analysis was conducted to predict tumor PD-1 saturation over a wide range of tumor penetration and PD-1 expression. This evaluation concluded that pembrolizumab at 200 mg Q3W achieves full PD-1 saturation in both blood and tumor.

Finally, population PK analysis of pembrolizumab, which characterized the influence of body weight and other participant covariates on exposure, has shown that the fixed-dosing provides similar control of PK variability as weight-based dosing, with considerable overlap in the distribution of exposures from the 200 mg Q3W fixed dose and 2 mg/kg Q3W dose. Supported

by these PK characteristics, and given that fixed-dose has advantages of reduced dosing complexity and reduced potential of dosing errors, the 200 mg Q3W fixed-dose was selected for evaluation across all pembrolizumab protocols.

6.2.3 Rationale for Endpoints

6.2.3.1 Efficacy Endpoints

Objective response rate has been chosen as primary end-point for historical comparison with the seminal results of the first pembrolizumab monotherapy trial in MMR deficient mCRC patients (Le et al., 2015). On the same token we have chosen progression, death, and toxicity as secondary endpoints.

6.2.3.2 Biomarker Research

In addition to the MMR and MGMT markers, and the longitudinal mutational burden determination, which are an inherent component for the enrichment strategy of the trial, the following potential markers of pembrolizumab pharmacodynamics will be considered:

Analysis of PD-L1 and PD1 expression in TRIAL Phase eligible patients:

- PD-L1 will be measured on archival or newly obtained core or excisional biopsies by centralized IHC using QualTeck assay and 22C3 antibody clone (Merck). Positivity is defined as membranous PD-L1 expression in > 1% of cells in tumor and stroma.
- PD1 and other lymphocyte activation markers will be measured in peripheral blood lymphocyte (PBL) post-treatment, and correlated with mutational load. PBL will be collected at baseline and with each treatment. PBL are isolated and stored frozen until use.

Investigation of the immunomodulatory effect of TMZ:

We plan to explore also the association of PD-1 positivity, and tumor infiltrating lymphocyte characteristics with clinical responses to subsequent Pembrolizumab treatment. In such cases archived tumor tissues and tissue obtained at biopsy will be compared. Archival and post-TMZ tumor biopsies will be analyzed for PD-1/PD-L1 expression as well as for a variety of potential immune-markers as described in 9.1.4.2.

7.0 METHODOLOGY

7.1 Entry Criteria

Patient selection criteria are progressively restrictive according to the 3-step selection strategy of the trial and are reported separately for each phase.

7.1.1 Entry criteria for SCREENING Phase

1. Histologically confirmed diagnosis of metastatic colorectal cancer.

2. Documented RAS extended mutations in the archival sample (cohort P only).
3. ECOG performance status 0-1.
4. SCREENING phase informed consent signed.
5. Understanding and accepting the need for undergoing two tumor biopsies if eligible for PRIMING Phase.
6. Age ≥ 18 years.
7. Availability of all diagnostic FFPE blocks (primary tumor and or metastases), or at least 20 slides (primary tumor and/or metastases). Formalin-fixed, paraffin embedded (FFPE) tissue blocks are preferred to slides.
8. Normal organ functions (see Table 7.1.4 below).

7.1.2 Entry Criteria for PRIMING Phase

Participants are eligible to be included in the PRIMING Phase, only if all of the following criteria apply:

1. Fulfilment of all the SCREENING inclusion criteria;
2. PRIMING informed consent signed;
3. Confirming the willingness to undergo two tumor biopsies,
4. Acceptance that if the mutational load determination is unfeasible for technical reasons (not enough tissue, substandard test performance, etc.), access to TRIAL phase will not be possible.
5. Imaging documented failure of previous standard CRC therapies including fluoropyrimidine, oxaliplatin, irinotecan plus or minus antiangiogenics agents (Bevacizumab, Aflibercept, Regorafenib, others).
6. At least one measurable tumor lesion as per RECIST v1.1. Lesions in previously irradiated areas or those that have received other loco-regional therapies (i.e. percutaneous ablation) should not be considered measurable unless there is clear documented evidence of progression of the lesion since therapy. Imaging must be performed maximum within 28 days prior to enrolment.
7. ECOG performance status 0 or 1;
8. Following results in the SCREENING Phase tests:

    - Proficient MMR status assessed by IHC or MSI-low status defined by PCR (Bethesda panel);
    - Negative score for the MGMT protein expression IHC test;
    - Positive score for the MGMT promoter methylation performed on tissue.

9. Women with childbearing potential should complete a pregnancy test and be willing to use highly effective contraceptive methods.
10. Normal organ functions as defined in the Table 7.1.4 below.

7.1.3 Entry Criteria for TRIAL Phase

1. Fulfilment of all the SCREENING inclusion criteria and deficient MMR status (IHC) or MSI-High status (PCR) (cohort D only).
2. Fulfilment of all the SCREENING and PRIMING inclusion criteria (cohort P only).
3. TRIAL Phase informed consent signed (both cohorts).
4. Imaging documented PD to TMZ (cohort P only).
5. A mutational load value ≥ 20 mutations/MB at TMZ-ML assay (cohort P only).
6. Imaging documented failure of previous standard CRC therapies including fluoropyrimidine, oxaliplatin, irinotecan plus or minus targeted agents (Bevacizumab, Aflibercept, Regorafenib, Cetuximab, Panitumumab, others) (cohort D only).
7. At least one measurable tumor lesion as per RECIST v1.1. Lesions in previously irradiated areas or those that have received other loco-regional therapies (i.e. percutaneous ablation) should not be considered measurable unless there is clear documented evidence of progression of the lesion since therapy. Imaging must be performed maximum within 28 days prior to enrolment (both cohorts).
8. Women with childbearing potential should complete a pregnancy test and be willing to use highly effective contraceptive methods (both cohorts).
9. Normal organ functions as defined in the Table 7.1.4 below. Blood specimens must be collected within 10 days prior to the start of study treatment.

7.1.4 Adequate Organ Function Laboratory Values

System	Laboratory Value
Hematological	
Absolute neutrophil count (ANC)	≥1500/μL
Platelets	≥100.000/μL
Hemoglobin	≥9.0 g/dL or ≥5.6 mmol/L[a]
Renal	
Creatinine OR Measured or calculated[b] creatinine clearance (GFR can also be used in place of creatinine or CrCl)	≤1.5 × ULN OR ≥30 mL/min for participant with creatinine levels >1.5 × institutional ULN
Hepatic	
Total bilirubin	≤1.5 ×ULN OR direct bilirubin ≤ULN for participants with total bilirubin levels >1.5 × ULN
AST (SGOT) and ALT (SGPT)	≤2.5 × ULN (≤5 × ULN for participants with liver metastases)
Coagulation	
International normalized ratio (INR) OR prothrombin time (PT) Activated partial thromboplastin time (aPTT)	≤1.5 × ULN unless participant is receiving anticoagulant therapy as long as PT or aPTT is within therapeutic range of intended use of anticoagulants

ALT (SGPT)=alanine aminotransferase (serum glutamic pyruvic transaminase); AST (SGOT)=aspartate aminotransferase (serum glutamic oxaloacetic transaminase); GFR=glomerular filtration rate; ULN=upper limit of normal.

[a] Criteria must be met without erythropoietin dependency and without packed red blood cell (pRBC) transfusion within last 2 weeks.

[b] Creatinine clearance (CrCl) should be calculated per institutional standard.

Note: This table includes eligibility-defining laboratory value requirements for treatment; laboratory value requirements should be adapted according to local regulations and guidelines for the administration of specific therapies.

7.2 Exclusion Criteria

Participants are excluded from the study if any of the following criteria apply:

1. A woman of child bearing potential who has a positive serum pregnancy test within 72 hours prior to allocation (see Appendix 3).
2. Has received prior therapy with an anti-PD-1, anti-PD-L1, or anti-PD-L2 agent or with an agent directed to another stimulatory or co-inhibitory T-cell receptor (eg, CTLA-4, OX-40, CD137).
3. Has received prior systemic anti-cancer therapy including investigational agents within 4 weeks [could consider shorter interval for kinase inhibitors or other short half-life drugs] prior to enrollment.
    a. Note: Participants must have recovered from all AEs due to previous therapies to ≤Grade 1 or baseline. Participants with ≤Grade 2 neuropathy may be eligible.
    b. Note: If participant received major surgery, they must have recovered adequately from the toxicity and/or complications from the intervention prior to starting study treatment.
4. Has received prior radiotherapy within 2 weeks of start of study treatment (with Pembrolizumab). Participants must have recovered from all radiation-related toxicities, not require corticosteroids, and not have had radiation pneumonitis. A 1-week washout is permitted for palliative radiation (≤2 weeks of radiotherapy) to non-CNS disease.
5. Has received a live vaccine within 30 days prior to the first dose of study drug. Examples of live vaccines include, but are not limited to, the following: measles, mumps, rubella, varicella/zoster (chicken pox), yellow fever, rabies, and typhoid vaccine. Seasonal influenza vaccines for injection are generally killed virus vaccines and are allowed; however, intranasal influenza vaccines (eg, FluMist®) are live attenuated vaccines and are not allowed.
6. Is currently participating in or has participated in a study of an investigational agent or has used an investigational device within 4 weeks prior to the first dose of study treatment.

   a. Note: Participants who have entered the follow-up phase of an investigational study may participate as long as it has been 4 weeks after the last dose of the previous investigational agent.
7. Has a diagnosis of immunodeficiency or is receiving chronic systemic steroid therapy (in dosing exceeding 10 mg daily of prednisone equivalent) or any other form of immunosuppressive therapy within 7 days prior to the first dose of study drug.
8. Has a known additional malignancy that is progressing or has required active treatment within the past 3 years. Note: Participants with basal cell carcinoma of the skin, squamous cell carcinoma of the skin, or carcinoma in situ (e.g. breast carcinoma, cervical cancer in situ) that have undergone potentially curative therapy are not excluded.
9. Has known active CNS metastases and/or carcinomatous meningitis. Participants with previously treated brain metastases may participate provided they are radiologically stable, i.e. without evidence of progression for at least 4 weeks by repeat imaging (note that the repeat imaging should be performed during study screening), clinically stable and without requirement of steroid treatment for at least 14 days prior to first dose of study treatment.
10. Has severe hypersensitivity (≥Grade 3) to pembrolizumab and/or any of its excipients.
11. Has severe hypersensitivity (≥Grade 3) to temozolomide and/or any of its excipients.
12. Has active autoimmune disease that has required systemic treatment in the past 2 years (i.e. with use of disease modifying agents, corticosteroids or immunosuppressive drugs). Replacement therapy (eg., thyroxine, insulin, or physiologic corticosteroid replacement therapy for adrenal or pituitary insufficiency, etc.) is not considered a form of systemic treatment.
13. Has a history of (non-infectious) pneumonitis that required steroids or has current pneumonitis.
14. Has an active infection requiring systemic therapy.
15. Has a known history of Human Immunodeficiency Virus (HIV). Note: No HIV testing is required.
16. Has a known history of Hepatitis B (defined as Hepatitis B surface antigen [HBsAg] reactive) or known active Hepatitis C virus infection. Note: no testing for Hepatitis B and Hepatitis C is required unless mandated by local health authority.
17. Has a known history of active TB (Bacillus Tuberculosis).
18. Has a history or current evidence of any condition, therapy, or laboratory abnormality that might confound the results of the study, interfere with the subject's participation for the full duration of the study, or is not in the best interest of the subject to participate, in the opinion of the treating investigator.
19. Has known psychiatric or substance abuse disorders that would interfere with cooperation with the requirements of the trial
20. Is pregnant or breastfeeding, or expecting to conceive or father children within the projected duration of the study, starting with the screening visit through 120 days after the last dose of trial treatment.

7.2.1 Lifestyle Restrictions

7.2.1.1 Meals and Dietary Restrictions

Participants should maintain a normal diet unless modifications are required to manage an AE such as diarrhea, nausea or vomiting.

7.2.1.2 Contraception

ICH M3 guidance requires precautions to be taken to minimize risk to fetus or embryo when including women of childbearing potential in clinical studies.

Temozolomide and Pembrolizumab may have adverse effects on a fetus in utero. Refer to Appendix 3 for approved methods of contraception.

7.2.2 Pregnancy

If a participant inadvertently becomes pregnant while on treatment with Temozolomide or pembrolizumab, the participant will be immediately discontinued from study treatment. The site will contact the participant at least monthly and document the participant's status until the pregnancy has been completed or terminated. The outcome of the pregnancy will be reported to delegated pharmacovigilance and to Merck Global Safety according to timelines as defined in section 9.2.3 if the outcome is a serious adverse experience (e.g., death, abortion, congenital anomaly, or other disabling or life-threatening complication to the mother or newborn). The study Investigator will make every effort to obtain permission to follow the outcome of the pregnancy and report the condition of the fetus or newborn to Merck. If a male participant impregnates his female partner, the study personnel at the site must be informed immediately and the pregnancy must be reported to delegated pharmacovigilance and to Merck Global Safety followed as described in section 9.2.3.

7.2.3 Use in Nursing Women

It is unknown whether pembrolizumab is excreted in human milk. Since many drugs are excreted in human milk, and because of the potential for serious adverse reactions in the nursing infant, participants who are breast-feeding are not eligible for enrollment.

7.3 Trial Treatments

Only qualified personnel familiar with procedures that minimize undue exposure to themselves and to the environment should undertake the preparation, handling, and safe disposal of chemotherapeutic agents.

The treatment to be used in this trial is outlined below in Table 7.3.

Table 7.3 Trial Treatment

Drug	**Dose/Potency**	**Dose Frequency**	**Route of Administration**	**Regimen/Treatment Period**	**Use**
Temozolomide	150 or 200 or 100 mg/m^2 (7.3.1.2)	Q4W	P.O.	Days 1-5 every 28 days (PRIMING Phase)	Experimental
Pembrolizumab	200 mg	Q3W	IV infusion	Day 1 of each 3 week cycle (TRIAL Phase)	Experimental

Trial treatment should begin as close as possible to the date on which treatment is allocated/assigned.

7.3.1 Temozolomide - Dose Selection/Modification

7.3.1.1 Dose Selection

The rationale for selection of doses to be used in this trial is provided in section 6.2.2 Justification for Dose.

7.3.1.2 Temozolomide-Dose modification (Escalation/Titration/Other)

The starting dose of TMZ is 150 mg/sq/day. The dose can be modified at the start of cycle 2. During treatment, a complete blood count should be obtained on Day 22 (21 days after the first dose of TMZ). According to day 22 CBC results and non-haematological toxicity observed during cycle 1 the dose of TMZ remain 150 mg/sq/day, or is modified as reported below.

Table 7.3.1.2 ***Temozolomide - Dose modification***

	Temozolomide 2nd cycle dose		
Toxicity	**Increase dose to 200 mg/sq/day**	**Reduce dose to 100 mg/sq/day**	**Discontinue TMZ**
Absolute Neutrophil Count day 22 cycle 1	$\geq$ 1.5 x 10^9/L,	< 1.0 x 10^9/L	Na
Thrombocyte Count	$\geq$ 100 x 10^9/L.	< 50 x 10^9/L	Na
CTC Non-Hematologic toxicity (except for alopecia, nausea, vomiting)	Grade $\leq$ 2 (except for alopecia, nausea and vomiting),	CTC Grade 3	CTC Grade 4

In the event of multiple toxicities, dose modifications should be applied based upon the most severe toxicity observed.

7.3.1.3 Patients with hepatic or renal impairment

The pharmacokinetics of TMZ are comparable in patients with normal hepatic function and in those with mild or moderate hepatic impairment. No data are available on the administration of TMZ in patients with severe hepatic impairment (Child's Class C) or with renal impairment. Based on the pharmacokinetic properties of TMZ, it is unlikely that dose reductions are required in patients with severe hepatic impairment or any degree of renal impairment. However, caution should be exercised when TMZ is administered in these patients.

7.3.1.4 Elderly patients

Based on a population pharmacokinetic analysis in patients 19-78 years of age, clearance of TMZ is not affected by age. However, elderly patients (> 70 years of age) appear to be at increased risk of neutropenia and thrombocytopenia.

7.3.1.5 Contraindications to Temozolomide treatment

- Hypersensitivity to the active substance or to any of the excipients.
- Hypersensitivity to dacarbazine (DTIC).
- Severe myelosuppression.

Note: For warnings see the Temozolomide SmPC.

7.3.1.6 TMZ-Timing of dose administration

Temozolomide will be administered orally once daily at the dose of 150-200 mg/m^2/day for 5 consecutive days of each treatment cycle (see 7.3.1.2). A treatment cycle will comprise 5 days of Temozolomide administration (Day 1 to 5) followed by 23 days of rest for a total of 28 days (4 weeks) period (dose-schedule: 150 mg/m2 day 1-5 q28). After an overnight fasting, with free access to water, patients will take the study drug with a large glass of plain water without ice. A light breakfast can be served 1.5-2 hours after study drug intake.

Dose modification should be implemented according to recommendations detailed in section 7.3.1.2.

Each patient will remain on treatment until PD unless patient refusal, consent withdrawal, or the occurrence of unacceptable toxicity.

The site personnel handling the study medication to patients must ensure that they fully understand that the treatment period (cycle) comprises 28 days, consisting of 5 days of daily administration, followed by a 23-day resting period, from Day 6 to Day 28. During each treatment cycle, extension of treatment period to Days >5 is not allowed, even in case of missed doses.

Additionally, the patients should be informed:

- to take the study drug in fasting state.
- to swallow the capsules whole with a glass of plain water without ice; capsules must not be opened or chewed; water is allowed during fasting period.
- to take the study drug at the same time each day (1.5-2 hours before breakfast).
- if the patient forgets to take the study drug at the usual time, to take it between meals at any time during the same day and record the event.
- if the patient misses one day, to take the normal amount the next day, if scheduled. Not to double the daily prescribed dose of the study drug and to track the number of capsules missed and the reason for it.
- if vomiting occurs during or after having taken the daily dose, not to take extra capsules that day and to take the normal amount the next day, if scheduled. Not to double the daily prescribed dose of the study drug and record the event to adhere to the principles of safe handling and storage of the study medication.
- Breaking/crushing or opening the capsules must be avoided. In such cases, record of the broken capsules should be kept. In case of opening of the capsules, to avoid contact or inhalation. In case of skin contact, to wash the affected area with plenty of water or soap and water. In case of eye-contact, to rinse thoroughly with plenty of water and see medical advice as soon as possible.
- To return all packaging as well as not-taken capsules (if applicable) to the site personnel.

As much of this information as possible should first be given at the pre-treatment visit and reinforced on subsequent visits. A diary of TMZ assumption must be given to the patient every treatment cycle and the correct compilation must be revised by the investigator.

7.3.1 Pembrolizumab - Dose Selection/Modification

7.3.1.1 Dose Selection

The rationale for selection of doses to be used in this trial is provided in section 4.0– Background and Rationale.

7.3.2 Timing of Dose Administration

Trial treatment should be administered on Day 1 of each cycle after all procedures/assessments have been completed as detailed on the Trial Flow Chart (section 6.0).

- Pembrolizumab 200 mg will be administered on an outpatient basis as a 30 minute IV infusion every 3 weeks. Sites should make every effort to target infusion timing to be as close to 30 minutes as possible. However, given the variability of infusion pumps from site to site, a window of -5 minutes and +10 minutes is permitted (i.e., infusion time is 30 minutes: -5 min/+10 min).
- Pembrolizumab may be administered up to 3 days before or after the scheduled Day 1 of each cycle due to administrative reasons.

7.3.2 Dose Modification and toxicity management for immune-related AEs associated with pembrolizumab

AEs associated with pembrolizumab exposure may represent an immunologic etiology. These immune-related AEs (irAEs) may occur shortly after the first dose or several months after the last dose of pembrolizumab treatment and may affect more than on body system simultaneously. Therefore, early recognition and initiation of treatment is critical to reduce complications.

- Based on existing clinical study data, most irAEs are reversible and could be managed with interruptions of pembrolizumab, administration of corticosteroids and/or other supportive care.
- For suspected irAEs, ensure adequate evaluation to confirm etiology or exclude other causes. Additional procedures or tests such as bronchoscopy, endoscopy, skin biopsy may be included as part of the evaluation. Based on the severity of irAEs, withhold or permanently discontinue pembrolizumab and administer corticosteroids.
- Dose modification and toxicity management guidelines for irAEs associated with pembrolizumab are provided in Table 7.3.2.

Table 7.3.2 Dose modification and toxicity management guidelines for immune-related AEs associated with pembrolizumab

General instructions:

1. Corticosteroid taper should be initiated upon AE improving to Grade 1 or less and continue to taper over at least 4 weeks.
2. For situations where pembrolizumab has been withheld, pembrolizumab can be resumed after AE has been reduced to Grade 1 or 0 and corticosteroid has been tapered. Pembrolizumab should be permanently discontinued if AE does not resolve within 12 weeks of last dose or corticosteroids cannot be reduced to ≤10 mg prednisone or equivalent per day within 12 weeks.
3. For severe and life-threatening irAEs, IV corticosteroid should be initiated first followed by oral steroid. Other immunosuppressive treatment should be initiated if irAEs cannot be controlled by corticosteroids.

Immune-related AEs	**Toxicity grade or conditions (CTCAEv4.03)**	**Action taken to pembrolizumab**	**irAE management with corticosteroid and/or other therapies**	**Monitor and follow-up**
Pneumonitis	Grade 2	Withhold	• Administer corticosteroids (initial dose of 1-2 mg/kg prednisone or equivalent) followed by taper	• Monitor participants for signs and symptoms of pneumonitis • Evaluate participants with suspected pneumonitis with radiographic imaging and initiate corticosteroid treatment • Add prophylactic antibiotics for opportunistic infections
	Grade 3 or 4, or recurrent Grade 2	Permanently discontinue		
Diarrhea / Colitis	Grade 2 or 3	Withhold	• Administer corticosteroids (initial dose of 1-2 mg/kg prednisone or equivalent) followed by taper	• Monitor participants for signs and symptoms of enterocolitis (ie, diarrhea, abdominal pain, blood or mucus in stool with or without fever) and of bowel perforation (ie, peritoneal signs and ileus).

	Grade 4 or recurrent Grade 3 (for =Colitis)	Permanently discontinue		• Participants with ≥ Grade 2 diarrhea suspecting colitis should consider GI consultation and performing endoscopy to rule out colitis. • Participants with diarrhea/colitis should be advised to drink liberal quantities of clear fluids. If sufficient oral fluid intake is not feasible, fluid and electrolytes should be substituted via IV infusion.
AST / ALT elevation or Increased bilirubin	Grade 2	Withhold	• Administer corticosteroids (initial dose of 0.5- 1 mg/kg prednisone or equivalent) followed by taper	• Monitor with liver function tests (consider weekly or more frequently until liver enzyme value returned to baseline or is stable
	Grade 3 or 4	Permanently discontinue	• Administer corticosteroids (initial dose of 1-2 mg/kg prednisone or equivalent) followed by taper	
Type 1 diabetes mellitus (T1DM) or Hyperglycemia	Newly onset T1DM or Grade 3 or 4 hyperglycemia associated with evidence of β-cell failure	Withhold	• Initiate insulin replacement therapy for participants with T1DM • Administer anti-hyperglycemic in participants with hyperglycemia	• Monitor participants for hyperglycemia or other signs and symptoms of diabetes.
Hypophysitis	Grade 2	Withhold	• Administer corticosteroids and initiate hormonal replacements as clinically indicated.	• Monitor for signs and symptoms of hypophysitis (including hypopituitarism and adrenal insufficiency)
	Grade 3 or 4	Withhold or permanently discontinue[1]		
Hyperthyroidism	Grade 2	Continue	• Treat with non-selective beta-blockers (eg, propranolol) or thionamides as appropriate	• Monitor for signs and symptoms of thyroid disorders.
	Grade 3 or 4	Withhold or		

		permanently discontinue[1]		
Hypothyroidism	Grade 2-4	Continue	• Initiate thyroid replacement hormones (eg, levothyroxine or liothyroinine) per standard of care	• Monitor for signs and symptoms of thyroid disorders.
Nephritis and Renal dysfunction	Grade 2	Withhold	• Administer corticosteroids (prednisone 1-2 mg/kg or equivalent) followed by taper.	• Monitor changes of renal function
	Grade 3 or 4	Permanently discontinue		
Myocarditis	Grade 1 or 2	Withhold	• Based on severity of AE administer corticosteroids	• Ensure adequate evaluation to confirm etiology and/or exclude other causes
	Grade 3 or 4	Permanently discontinue		
All other immune-related AEs	Intolerable/ persistent Grade 2	Withhold	• Based on type and severity of AE administer corticosteroids	• Ensure adequate evaluation to confirm etiology and/or exclude other causes
	Grade 3	Withhold or discontinue based on the type of event. Events that require discontinuation include and not limited to: Gullain-Barre Syndrome, encephalitis		
	Grade 4 or recurrent Grade 3	Permanently discontinue		

1. Withhold or permanently discontinue pembrolizumab is at the discretion of the investigator or treating physician.

NOTE: For participants with Grade 3 or 4 immune-related endocrinopathy where withhold of pembrolizumab is required, pembrolizumab may be resumed when AE resolves to ≤ Grade 2 and is controlled with hormonal replacement therapy or achieved metabolic control (in case of T1DM).

7.3.3 Dose modification and toxicity management of infusion-reactions related to pembrolizumab

Pembrolizumab may cause severe or life-threatening infusion-reactions including severe hypersensitivity or anaphylaxis. Signs and symptoms usually develop during or shortly after drug infusion and generally resolve completely within 24 hours of completion of infusion. Dose modification and toxicity management guidelines on pembrolizumab associated infusion reaction are provided in Table 7.3.3.

Table 7.3.3 Pembrolizumab Infusion Reaction Dose modification and Treatment Guidelines

NCI CTCAE Grade	Treatment	Premedication at Subsequent Dosing
Grade 1 Mild reaction; infusion interruption not indicated; intervention not indicated	Increase monitoring of vital signs as medically indicated until the participant is deemed medically stable in the opinion of the investigator.	None
Grade 2 Requires therapy or infusion interruption but responds promptly to symptomatic treatment (e.g., antihistamines, NSAIDs, narcotics, IV fluids); prophylactic medications indicated for ≤24 hrs	**Stop Infusion.** Additional appropriate medical therapy may include but is not limited to: IV fluids Antihistamines NSAIDs Acetaminophen Narcotics Increase monitoring of vital signs as medically indicated until the participant is deemed medically stable in the opinion of the investigator. If symptoms resolve within 1 hour of stopping drug infusion, the infusion may be restarted at 50% of the original infusion rate (e.g. from 100 mL/hr to 50 mL/hr). Otherwise dosing will be held until symptoms resolve and the participant should be premedicated for the next scheduled dose. **Participants who develop Grade 2 toxicity despite adequate premedication should be permanently discontinued from further study drug treatment**	Participant may be premedicated 1.5h (± 30 minutes) prior to infusion of _____ with: Diphenhydramine 50 mg po (or equivalent dose of antihistamine). Acetaminophen 500-1000 mg po (or equivalent dose of analgesic).

Grades 3 or 4 Grade 3: Prolonged (i.e., not rapidly responsive to symptomatic medication and/or brief interruption of infusion); recurrence of symptoms following initial improvement; hospitalization indicated for other clinical sequelae (e.g., renal impairment, pulmonary infiltrates) Grade 4: Life-threatening; pressor or ventilatory support indicated	**Stop Infusion.** Additional appropriate medical therapy may include but is not limited to: Epinephrine** IV fluids Antihistamines NSAIDs Acetaminophen Narcotics Oxygen Pressors Corticosteroids Increase monitoring of vital signs as medically indicated until the participant is deemed medically stable in the opinion of the investigator. Hospitalization may be indicated. **In cases of anaphylaxis, epinephrine should be used immediately. **Participant is permanently discontinued from further study drug treatment.**	No subsequent dosing
Appropriate resuscitation equipment should be available at the bedside and a physician readily available during the period of drug administration. For further information, please refer to the Common Terminology Criteria for Adverse Events v4.03 (CTCAE) at http://ctep.cancer.gov		

7.3.4 Other allowed dose interruption for pembrolizumab

Pembrolizumab may be interrupted for situations other than treatment-related AEs such as medical / surgical events or logistical reasons not related to study therapy. Participants should be placed back on study therapy within 3 weeks of the scheduled interruption, unless otherwise discussed with the Sponsor. The reason for interruption should be documented in the patient's study record.

7.3.5 Second Course

All participants who stop study treatment with SD or better, may be eligible for up to an additional 17 cycles (approximately 1 year) of pembrolizumab treatment if they progress after stopping study treatment from the initial treatment phase. This retreatment is termed the Second Course Phase of this study and is only available if the study remains open and the participant meets the following conditions:

Either

- Stopped initial treatment with study treatment after attaining an investigator-determined confirmed CR based on RECIST 1.1, and
    - Was treated with at least 8 cycles of study treatment before discontinuing treatment, and
    - Received at least 2 treatments with pembrolizumab beyond the date when the initial CR was declared

OR

- Had SD, PR, or CR and stopped study treatment after completion of 35 administrations (approximately 2 years) of study treatment for reasons other than disease progression or intolerability

AND

- Experienced an investigator-determined radiographic disease progression by RECIST 1.1 after stopping initial treatment, and
    - No new anticancer treatment was administered after the last dose of study treatment, and
    - The participant meets all of the safety parameters listed in the inclusion criteria and none of the safety parameters listed in the exclusion criteria, and
    - The study is ongoing

An objective response or disease progression that occurs during the Second Course Phase for a participant will not be counted as an event for the primary analysis of either endpoint in this study.

7.4 Randomization or Treatment Allocation

MMR Deficient or MSI-H patients are allocated to Cohort D. Originally MMR Proficient or MSI-low patients at the end of the PRIMING phase either are allocated to Cohort P if TMZ-ML $\geq$ 20 mut/Megabase, or go off protocol if TMZ-ML $\leq$ 20 mut/Megabase.

7.5 Stratification

For exploratory analysis purpose only, patients will be stratified post-hoc according to selected immune biomarkers (see 9.1.4.2) or other putative predictive markers.

7.6 Concomitant Medications/Vaccinations (allowed & prohibited)

Medications or vaccinations specifically prohibited in the exclusion criteria (see 7.2) are not allowed during the ongoing trial. If there is a clinical indication for one of these or other medications or vaccinations specifically prohibited during the trial, discontinuation from trial therapy or vaccination may be required. The final decision on any supportive therapy or vaccination rests with the investigator and/or the participants's primary physician.

7.6.1 Acceptable Concomitant Medications

Throughout the study, investigators may prescribe any concomitant medications or treatments deemed necessary to provide adequate supportive care.

Anticancer therapy (chemotherapy, biologic or radiation therapy, palliative radiotherapy covering > 25% of the bone marrow reserve, and surgery) must not be given to patients during the study treatment, except to TMZ during TMZ Priming Phase.

Radiotherapy for palliation in non-target lesions with antalgic aim is allowed. Recombinant human granulocyte colony stimulating factor (G-CSF) or erythropoiesis stimulating agent use according to the current approved label or institutional guidelines is permitted.

All treatments that the investigator considers necessary for a participant’s welfare may be administered at the discretion of the investigator in keeping with the community standards of medical care including palliative treatment (with the exception of regorafenib), delivered for symptomatic disease during the 5-week gap between progression to TMZ and start of pembrolizumab treatment (see 4.1.2).. All concomitant medication will be recorded on the case report form (CRF) including all prescription, over-the-counter (OTC), herbal supplements, and IV medications and fluids. If changes occur during the trial period, documentation of drug dosage, frequency, route, and date may also be included on the CRF.

All concomitant medications received within 28 days before the first dose of trial treatment and 30 days after the last dose of trial treatment should be recorded. Concomitant medications administered after 30 days after the last dose of trial treatment should be recorded for SAEs and ECIs as defined in section 9.2.

7.6.2 Prohibited Concomitant Medications

Parcticipants are prohibited from receiving the following therapies during the Screening and Treatment Phase (including retreatment for post-complete response relapse) of this trial:

- Antineoplastic systemic chemotherapy or biological therapy (with the exception described in 5.1.2 and 8.2.1)
- Immunotherapy not specified in this protocol
- Chemotherapy not specified in this protocol
- Investigational agents other than TMZ (PRIMING Phase) and pembrolizumab (TRIAL Phase)
- Radiation therapy other than required for palliation in non-target lesions or to a symptomatic brain metastasis.
- Live vaccines within 30 days prior to the first dose of study treatment and while participating in the study. Examples of live vaccines include, but are not limited to, the following: measles, mumps, rubella, varicella/zoster, yellow fever, rabies, BCG, and typhoid vaccine. Seasonal influenza vaccines for injection are generally killed virus vaccines and are allowed; **however, intranasal influenza vaccines (eg, FluMist®) are live attenuated vaccines and are not allowed**.
- Systemic glucocorticoids for any purpose other than to modulate symptoms from an event of clinical interest of suspected immunologic etiology. The use of physiologic doses of corticosteroids may be approved after consultation with the Sponsor.

Participants who, in the assessment by the investigator, require the use of any of the aforementioned treatments for clinical management should be removed from the study. All treatments that the Investigator considers necessary for a participant's welfare may be administered at the discretion of the Investigator in keeping with the community standards of medical care.

Medications or vaccinations specifically prohibited in the exclusion criteria are not allowed during the ongoing study. If there is a clinical indication for any medication or vaccination specifically prohibited during the study, discontinuation from study therapy or vaccination may be required. The final decision on any supportive therapy or vaccination rests with the investigator and/or the participant's primary physician. However, the decision to continue the participant on study treatment requires the mutual agreement of the investigator, the Sponsor and the participant.

There are no prohibited therapies during the Post-Treatment Follow-up Phase (TRIAL phase).

7.6.3 Rescue Medications & Supportive Care

Participants should receive appropriate supportive care measures as deemed necessary by the treating investigator. Suggested supportive care measures for the management of AEs with potential immunologic etiology are outlined along with the dose modification guidelines in Table 7.3.3. Where appropriate, these guidelines include the use of oral or IV treatment with corticosteroids, as well as additional anti-inflammatory agents if symptoms do not improve with administration of corticosteroids. Note that several courses of steroid tapering may be necessary as symptoms may worsen when the steroid dose is decreased. For each disorder, attempts should be made to rule out other causes such as metastatic disease or bacterial or viral infection, which might require additional supportive care. The treatment guidelines are intended to be applied when the Investigator determines the events to be related to pembrolizumab.

Note: If after the evaluation of the event, it is determined not to be related to pembrolizumab, the Investigator does not need to follow the treatment guidance. Refer to Table 7.3.3 for guidelines regarding dose modification and supportive care.

It may be necessary to perform conditional procedures such as bronchoscopy, endoscopy, or skin photography as part of evaluation of the event.

7.7 Participant Withdrawal/Discontinuation Criteria

Participants may discontinue study treatment at any time for any reason or be dropped from the study treatment at the discretion of the investigator should any untoward effect occur. In addition, a participant may be discontinued from study treatment by the investigator or the Sponsor if study treatment is inappropriate, the trial plan is violated, or for administrative and/or other safety reasons. Specific details regarding procedures to be performed at study treatment discontinuation are provided in section 9.1.6 – Other Procedures.

A participant must be discontinued from study treatment but continue to be monitored in the study for any of the following reasons:

- The participant or participant's legally acceptable representative requests to discontinue study treatment
- Confirmed radiographic disease progression outlined in section 9.1.3.2.
- Any progression or recurrence of any malignancy, or any occurrence of another malignancy that requires active treatment
- Unacceptable adverse experiences as described in section 7.3.3.
- The participant has a medical condition or personal circumstance which, in the opinion of the investigator and/or sponsor, placed the participant at unnecessary risk from continued administration of study treatment.
- The participant has a confirmed positive serum pregnancy test
- Noncompliance with study treatment or procedure requirements

- Recurrent Grade 2 pneumonitis

- Discontinuation of treatment may be considered for participants who have attained a confirmed complete response (CR) and have been treated for at least 8 cycles (at least 24 weeks), receiving at least 2 doses of pembrolizumab; beyond the date when the initial CR was declared. These participants may be eligible for second course treatment described in section 7.3.5.
- The participant is lost to follow-up
- Completion of 35 treatments (approximately 2 years) with pembrolizumab

Note: The number of treatments is calculated starting with the first dose. Participants who stop the combination or pembrolizumab after receiving 35 doses may be eligible for retreatment if they progress after stopping study treatment provided they meet the requirements detailed in section 7.3.5. Participants may be retreated in the Second Course Phase (Retreatment) for up to an additional 17 cycles (approximately 1 year).

- Administrative reasons

7.8 Participant Replacement Strategy

The following patients will be substituted:

7.8.1 PRIMING Phase

Patients not receiving at least two courses of TMZ for undue toxicity or consent withdrawal or patients refusing or failing to have a mutational load determined at post-TMZ biopsy (PRIMING Phase only)

7.8.2 TRIAL Phase

Patients that cannot complete the first administration of pembrolizumab due to severe infusion reactions requiring treatment suspension.

7.9 Clinical Criteria for Early Trial Termination

Early trial termination will be the result of the criteria specified below:

1. Quality or quantity of data recording is inaccurate or incomplete
2. Poor adherence to protocol and regulatory requirements
3. Incidence or severity of adverse drug reaction in this or other studies indicates a potential health hazard to participants
4. Plans to modify or discontinue the development of the study drug

In the event of Merck decision to no longer supply study drug, ample notification will be provided so that appropriate adjustments to participant treatment can be made.

8.0 TRIAL FLOW CHART

Trial Period:	**SCREENING Phase**	**PRIMING Phase**		**TRIAL Phase**		**End of Treatment**	**Post-Treatment**		
Treatment Cycle/Title:	Baseline Screening Phase (Visit 1)	TMZ Cycle 1 Day 1 and 5	Cycle ≥ 2 Day 1 and 5	Pembro Cycle 1 Day 1 Q3	Cycle ≥ 2 Day 1 Q3 week	Discon-tinuation	Safety Follow-up Visit (7)	Follow Up Visits (8)	Survival Follow-Up
Scheduling Window (Days):	Up to 28 days before Start of priming phase	treatment day 1, before drug administration	treatment day 1, before drug administration	treatment day 1, before drug administration	treatment day 1, before drug administration	At time of Discon-tinuation	30 days post discon-tinuation	Every 8 weeks post discon-tinuation	Every 12 weeks
Administrative Procedures									
SCREENING Phase Informed Consent	X								
PRIMING Phase Informed Consent		X							
TRIAL Phase Informed Consent				X					
Inclusion/Exclusion Criteria	X	X		X					
Demographics and Medical History	X								
Clinical Procedures/Assessments									
Prior and Concomitant Medication Review		X	X	X	X	X	X	X	
Post-study anticancer therapy status							X	X	
Survival Status									X
Treatment Administration (Temozolomide)		X[1]	X[1]						
Treatment Administration (Pembrolizumab)				X	X				
Review Adverse Events		X	X		X	X	X		
Full Physical Examination		X	X	X	X	X	X	X	
Vital Signs, Height and Weight		X	X	X	X	X	X	X	
ECG		X		X					
ECOG Performance Status		X	X	X	X	X	X	X	

Trial Period:	**SCREENING Phase**	**PRIMING Phase**		**TRIAL Phase**		**End of Treatment**	**Post-Treatment**		
Treatment Cycle/Title:	Baseline Screening Phase (Visit 1)	TMZ Cycle 1 Day 1 and 5	Cycle ≥ 2 Day 1 and 5	Pembro Cycle 1 Day 1 Q3	Cycle ≥ 2 Day 1 Q3 week	Discon-tinuation	Safety Follow-up Visit (7)	Follow Up Visits (8)	Survival Follow-Up
Scheduling Window (Days):	Up to 28 days before Start of priming phase	treatment day 1, before drug administration	treatment day 1, before drug administration	treatment day 1, before drug administration	treatment day 1, before drug administration	At time of Discon-tinuation	30 days post discon-tinuation	Every 8 weeks post discon-tinuation	Every 12 weeks
Laboratory Procedures/Assessments: analysis performed by LOCAL laboratory									
Pregnancy Test – Serum β-HCG		X		X					
PT/INR and aPTT		X	X	X	X	X	X		
CBC with Differential		X	X	X	X	X	X		
Comprehensive Serum Chemistry Panel		X	X	X	X	X	X		
Urinalysis		X							
T3, FT4 and TSH				X	Q6 weeks or when clinically indicated				
Efficacy Measurements									
Tumor Imaging		X	Q8 weeks	X	Q9 weeks	X		X	
Archival Tissue Collection/Tumor Biopsies/Correlative Studies									
Archival Tumor FFPE	X								
Tumor Biopsies (2)		X	at PD3;4	X		X			
Correlative Studies - Blood Collection		X5	at PD	X	X	X			
Correlative Studies - Faeces Collection		X	at PD	X	X6	X			
Correlative Studies - Buccal Swabs Collection		X	at PD	X	X6	X			

1: TMZ diiary must be completed by patient and checked by the Investigator

2: tissue biopsies are not mandatory in MMR-D patients and might be performed at physiscian's discretion

3 : mandatory (MMR-P)

4 : within max. 7 days from imaging PD

5 : up to 28 days before TMZ administration on cycle 1, and then every 2 cycles

6 : at the 5th cycle of treatment

7 : Mandatory after the last dose of study treatment or before the initiation of a new anti-cancer treatment

8 : Participants who discontinue study treatment for a reason other than disease progression. Every 12-weeks after 1 year. Informations regarding disease status until the start of new anti-cancer therapy, disease progression, death, end of the study or if the participant begins retreatment with pembrolizumab (as detailed in Section 7.3.5).

9.0 TRIAL PROCEDURES

9.1 Trial Procedures

The Trial Flow Chart - section 8.0 summarizes the trial procedures to be performed at each visit. Individual trial procedures are described in detail below. It may be necessary to perform these procedures at unscheduled time points if deemed clinically necessary by the investigator.

Furthermore, additional evaluations/testing may be deemed necessary by the Sponsor and/or Merck for reasons related to participant safety. In some cases, such evaluation/testing may be potentially sensitive in nature (e.g., HIV, Hepatitis C, etc.), and thus local regulations may require that additional informed consent be obtained from the participant. In these cases, such evaluations/testing will be performed in accordance with those regulations.

9.1.1 Administrative Procedures

9.1.1.1 Informed Consent

The Investigator must obtain documented consent from each potential participant prior to participating in a clinical trial. Due to the complex trial design of Arethusa, three Informed Consent (IC) will be obtained by each patient, and documented prior the patient's entry to each subsequent phase of the trial (SCREENING, PRIMING and TRIAL), if appropriate. The specific set of conditions that have to be included in each IC form are defined by the eligibility criteria of the three phases (see 7.1 and 7.2)

Consent must be documented by the participant's dated signature or by the participant's legally acceptable representative's dated signature on a consent form along with the dated signature of the person conducting the consent discussion.

Copies of the signed and dated consent forms should be given to the participant before participation in each Phase of the trial.

The initial informed consent form, any subsequent revised written informed consent form and any written information provided to the participant must receive the IRB/ERC's approval/favorable opinion in advance of use. The participant or his/her legally acceptable representative should be informed in a timely manner if new information becomes available that may be relevant to the participant's willingness to continue participation in the trial. The communication of this information will be provided and documented via a revised consent form or addendum to the original consent form that captures the participant's dated signature or by the participant's legally acceptable representative's dated signature.

Specifics about a trial and the trial population will be added to the consent form template at the protocol level.

The informed consent will adhere to IRB/ERC requirements, applicable laws and regulations and Sponsor requirements.

9.1.1.2 Inclusion/Exclusion Criteria

All inclusion and exclusion criteria will be reviewed by the investigator or qualified designee to ensure that the participant qualifies for the trial.

9.1.1.3 Medical History

A medical history will be obtained by the investigator or qualified designee. Medical history will include all active conditions, and any condition diagnosed within the prior 10 years that are considered to be clinically significant by the Investigator. Details regarding the disease for which the participant has enrolled in this study will be recorded separately and not listed as medical history.

9.1.1.4 Prior and Concomitant Medications Review

9.1.1.4.1 Prior Medications

The investigator or qualified designee will review prior medication use, including any protocol-specified washout requirement, and record prior medication taken by the participant within 28 days before starting the trial. Treatment for the disease for which the participant has enrolled in this study will be recorded separately and not listed as a prior medication.

9.1.1.4.2 Concomitant Medications

The investigator or qualified designee will record medication, if any, taken by the participant during the trial. All medications related to reportable SAEs and ECIs should be recorded as defined in section 9.2.

All concomitant medications received within 30 days after the last dose of trial treatment should be recorded.

9.1.1.5 Disease Details and Treatments

9.1.1.5.1 Disease Details

The investigator or qualified designee will obtain prior and current details regarding disease status.

9.1.1.5.2 Prior Treatment Details

The investigator or qualified designee will review all prior cancer treatments including systemic treatments, radiation and surgeries.

9.1.1.5.3 Subsequent Anti-Cancer Therapy Status

The investigator or qualified designee will review all new anti-neoplastic therapy initiated after the last dose of trial treatment. If a participant initiates a new anti-cancer therapy within 30 days after the last dose of trial treatment, the 30 day Safety Follow-up visit must occur before

the first dose of the new therapy. Once new anti-cancer therapy has been initiated the participant will move into survival follow-up.

9.1.1.6 Assignment of Screening Number

Each patient enrolled in the SCREENING phase of the study will receive an univocal Subject code that will be assigned automatically by the eCRF. In the case that the patient will be enrolled in the PRIMING and TRIAL phases a Patient ID will be assigned by the Sponsor upon enrollment request of the participating centers.

9.1.2 Clinical Procedures/Assessments

9.1.2.1 Adverse Event (AE) Monitoring

The investigator or qualified designee will assess each participant to evaluate for potential new or worsening AEs as specified in the Trial Flow Chart and more frequently if clinically indicated. Adverse experiences will be graded and recorded throughout the study and during the follow-up period according to NCI CTCAE Version 4.03 (see Appendix 2). Toxicities will be characterized in terms regarding seriousness, causality, toxicity grading, and action taken with regard to trial treatment.

Please refer to section 9.2 for detailed information regarding the assessment and recording of AEs.

9.1.2.2 Full Physical Exam

The investigator or qualified designee will perform a complete physical exam during the screening period. Clinically significant abnormal findings should be recorded as medical history. A full physical exam should be performed during screening and during all on treatment and FU visit as reported in the Trial Flow Chart. For cycles that do not require a full physical exam per the Trial Flow Chart, the investigator or qualified designee will perform a directed physical exam as clinically indicated prior to trial treatment administration.

9.1.2.3 Vital Signs

The investigator or qualified designee will take vital signs at screening, prior to the administration of each dose of trial treatment and at treatment discontinuation as specified in the Trial Flow Chart (section 8.0). Vital signs should include temperature, pulse, respiratory rate, weight and blood pressure. Height will be measured at screening only.

9.1.2.4 ECG

The investigator or qualified designee will perform a 12-lead ECG.

9.1.2.5 Eastern Cooperative Oncology Group (ECOG) Performance Scale

The investigator or qualified designee will assess ECOG status (see Appendix 1) at screening, prior to the administration of each dose of trial treatment and discontinuation of trial treatment as specified in the Trial Flow Chart (section 8.0).

9.1.3 Tumor Imaging and Assessment of Disease

9.1.3.1 Tumor Imaging

Tumour imaging will be performed at each center by local radiologists according to RECIST and to iRECIST (see appendix 4). However CT-Scans will be cloud stored for post-trial centralized response revision.

Tumor imaging is strongly preferred to be acquired by computed tomography (CT). For the abdomen and pelvis, contrast-enhanced magnetic resonance imaging (MRI) may be used when CT with iodinated contrast is contraindicated, or when local practice mandates it. MRI is the strongly preferred modality for imaging the brain. The same imaging technique regarding modality, ideally the same scanner, and the use of contrast should be used in a participant throughout the study to optimize the reproducibility of the assessment of existing and new tumor burden and improve the accuracy of the assessment of response or progression based on imaging.

Imaging assessment will be as follow:

- **SCREENING Phase**: CT scans performed up to 28 days prior to screening eligibility check are considered valid.

- **PRIMING Phase**: RECIST 1.1 will be used to assess response to TMZ. Patients in CR/PR/SD are reassessed every 8 weeks. However, in order to fulfil RECIST, the FIRST occurrence of CR/PR MUST be reconfirmed not earlier than 4 and not later than 5 weeks later.

- **TRIAL Phase**: Tumor imaging must be performed within 0 to 28 days prior to starting treatment with pembrolizumab. Local reading (Investigator assessment with site radiology reading) will be used to determine eligibility.
  - Baseline for D-cohort patient; Imaging data from Screening phase can be used, provided timeframe is less than 28 days.
  - Baseline for P-cohort patients: the baseline correspond to the TMZ PD imaging assessment.
  - During trial for both cohorts: tumor assessment must be performed every 9 weeks. However in order to fulfil RECIST, the FIRST occurrence of CR/PR MUST be reconfirmed not earlier than 4 and not later than 5 weeks later.
  - At RECIST PD clinically stable patients should not undergo treatment discontinuation until progression is confirmed locally 4 to 8 weeks later according to iRECIST (see Table 9.1.3 and Figure 9.1.3).

Participants who have unconfirmed disease progression may continue on treatment at the discretion of the Investigator until progression is confirmed by the site provided they are clinically stable. Participants who receive confirmatory imaging do not need to undergo the next scheduled tumor imaging if it is less than 4 weeks later; tumor imaging may resume at the subsequent scheduled imaging time point, if clinically stable. Participants who have confirmed disease progression by iRECIST, as assessed by the site, will discontinue study treatment.

- In participants who discontinue study treatment, tumor imaging should be performed at the time of treatment discontinuation (±4-week window). If previous imaging was obtained within 4 weeks prior to the date of discontinuation, then imaging at treatment discontinuation is not mandatory. In participants who discontinue study treatment due to documented disease progression, this is the final required tumor imaging.
- In case of discontinuation of treatment for any reason other than disease progression, patients will still be re-evaluated every 8 weeks until disease progression or start of a new treatment or death or patients refusal, whichever comes first.
- After 1 year, the imaging time point will occur approximately every 12 weeks.
- At trial end, all in-Trial tumor assessments radioimaging data will be reviewed centrally by two radiologists who will read the CT/MRI scans blinded using the Quibim Precision® software to collect, store, and guide the revision of the imaging results. The imaging review protocol and tumor assessment re-conciliation report will be included in the final study report and or in the publication of the study.

9.1.3.2 Disease assessment

RECIST 1.1 (referencing a maximum of 5 target lesions in total and 2 per organ) will be used as a primary measure for assessment of tumor response and as date of disese progression.

The Sponsor might also require, for research purpose, to enable a broader sampling of tumor burden to be conducted centrally.

RECIST 1.1 Progression (PD) definition will be used for discontinuation of study treatment during the temozolomide PRIMING phase, and in TRIAL phase whenever the Investigator at site decide NOT to use iRECIST criteria, as described below. iRECIST is based on RECIST 1.1, but adapted to account for the unique tumor response seen with immunotherapeutic drugs.

Disease assessment in the TRIAL phase for both Cohorts takes into account the observation that some patients treated with immunotherapy can have a transient tumor flare, which might be misconstrued for PD by RECISTs, in the first few months after the start of pembrolizumab, and then experience subsequent disease response. Thus in the TRIAL phase, when tumor imaging shows PD by RECIST 1.1 the local investigator will re-assess the patient per iRECIST criteria according to the flow-chart in figure 9.1.3, table 9.1.3.

Per iRECIST, disease progression should be confirmed by the site 4 to 8 weeks after first radiologic evidence of RECIST 1.1 PD in clinically stable participants. Participants who have unconfirmed disease progression may continue on treatment at the discretion of the Investigator until progression is confirmed provided they have met the conditions detailed in Table 9.1.3 below.

Patients who receive confirmatory imaging do not need to undergo the next scheduled tumor imaging if it is less than 4 weeks later; tumor imaging may resume at the subsequent scheduled imaging time point, if clinically stable. Participants who have confirmed disease progression by iRECIST, as assessed by the site, will discontinue study treatment. Exceptions are detailed in table 9.1.3.

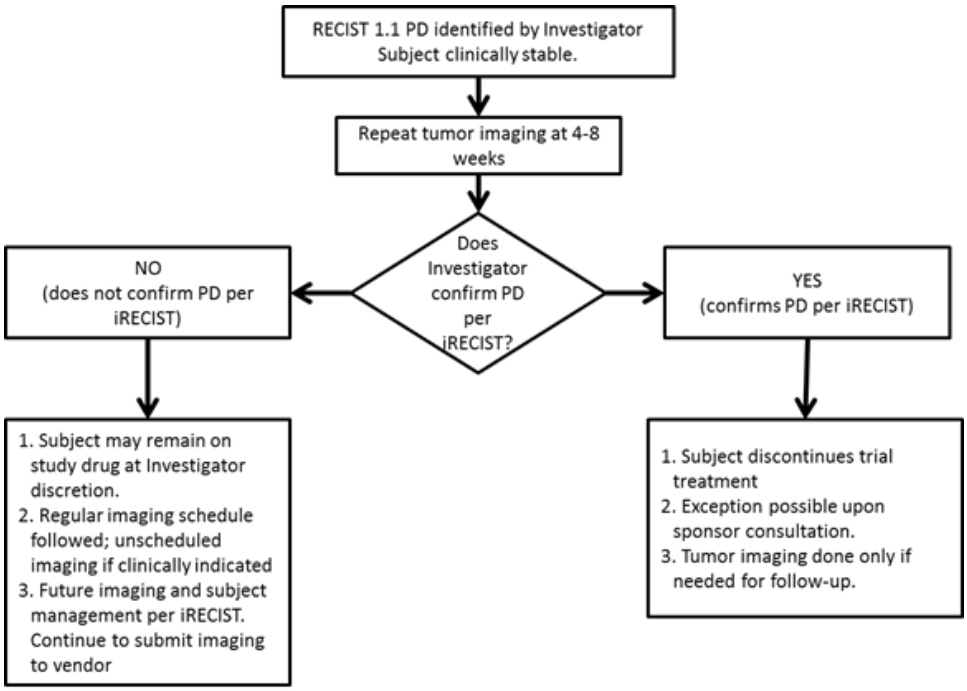
A description of the adaptations and iRECIST process is provided in Appendix 4, with additional detail in the iRECIST publication (Seymour et al., 2017).

Table 9.1.3: Imaging and Treatment after First Radiologic Evidence of Progressive Disease

	Clinically Stable		**Clinically Unstable**	
	Imaging	**Treatment**	**Imaging**	**Treatment**
Imaging and treatment options at first RECIST PD determination				
First radiologic evidence of PD by RECIST 1.1	Repeat imaging at 4 to 8 weeks to confirm PD.	May continue study treatment at the Investigator's discretion while awaiting confirmatory tumor imaging by site by iRECIST.	Repeat imaging at 4 to 8 weeks to confirm PD per Investigator's discretion only.	Discontinue treatment
Imaging and treatment options upon iRECIST determination				
Repeat tumor imaging confirms PD (iCPD) by iRECIST per Investigator assessment	No additional imaging required.	Discontinue treatment (exception is possible upon consultation with Sponsor).	No additional imaging required.	Not applicable
Repeat tumor imaging shows iUPD by iRECIST per Investigator assessment	Repeat imaging at 4 to 8 weeks to confirm PD. May occur at next regularly scheduled imaging visit.	Continue study treatment at the Investigator's discretion.	Repeat imaging at 4 to 8 weeks to confirm PD per Investigator's discretion only.	Discontinue treatment
Repeat tumor imaging shows iSD, iPR, or iCR by iRECIST per Investigator assessment.	Continue regularly scheduled imaging assessments.	Continue study treatment at the Investigator's discretion.	Continue regularly scheduled imaging assessments.	May restart study treatment if condition has improved and/or clinically stable per Investigator's discretion. Next tumor image should occur according to the regular imaging schedule.

iCPD = iRECIST confirmed progressive disease; iCR = iRECIST complete response; iRECIST = modified Response Evaluation Criteria in Solid Tumors 1.1 for immune-based therapeutics; iSD = iRECIST stable disease; iUPD = iRECIST unconfirmed progressive disease; PD = progressive disease; RECIST 1.1 = Response Evaluation Criteria in Solid Tumors 1.1..

Figure 9.1.3 : Imaging and Treatment for Clinically Stable Participants after First Radiologic Evidence of PD Assessed by the Investigator



9.1.4 Tumor Tissue Collection and Correlative Studies Blood Sampling

Specific Standard Operating Procedures for sample collection will be used according to our Internal Sample Collections Guidelines following the schedule reported in section 8.0.

Residual sample material available after completion of the designated analyses may be used in the future for identification of additional pharmacodynamic or predictive markers or to enhance understanding of disease biology. If biomarker samples are drawn but study drug(s) is not administered, samples will be retained.

9.1.4.1 Mandatory assays

- **Assessment of MMR/MSI status and MGMT expression**: during the screening phase all patients will be tested for the expression of MMR proteins by IHC on the diagnostic FFPE block or slides, or for microsatellite instability by PCR analysis of unstable microsatellite loci (Bethesda panel). All MMR proficient or MSI-low patients, will be assessed for expression of MGMT by IHC on diagnostic FFPE blocks or slides. MGMT-negative cases by IHC will be confirmed by MGMT promoter methylation analysis on tissue. Percent of methylation will be normalized on tumor content

evaluated by hematoxylin and eosin staining to correct for stromal infiltration present in the original slide used for DNA extraction.

- **Mutational Load determination**: the mutational load (ML) will be determined in tissue (TMZ-ML mandatory) and plasma (translational) by NGS on Illumina platform with a minimum depth of 10X and using a proprietary bioinformatic pipeline (IDEA) to estimate the mutational burden normalized per mega base (MB). Only alterations supported by 5% significance level are considered.

  ML will be assessed in MGMT-methylated, MMR-Proficient patients enrolled in the TMZ PRIMING Phase in samples collected at different time points, as follows:

  - *Baseline Mutational Load (B-ML):* Pre TMZ treatment, in tissue (non-mandatory) and plasma (translational) samples collected before TMZ administration.
  - *Priming Mutational Load (TMZ-ML):* at PD post TMZ treatment to declare patients eligible or not for the pembrolizumab TRIAL Phase, in tissue (mandatory) and plasma (translational) samples . NOTE: Patients with a TMZ-ML > 20/mB will be eligible for P- cohort of PEMBRO Trial Phase
  - *Dynamic Mutational Load (D-ML):* pre pembrolizumab first dose in either tissue (only D-cohort, whenever possible) or plasma (both cohorts, translational).
  - *Progression Mutational Load (PD-ML):* at PD during/after pembrolizumab treatment, in tissue (whenever possible) and plasma (translational) samples.

Timepoints for tissue biopsy and blood sampling are reported in trial diagram (Figure 4.2) and detailed in section 8.0 – Trial Flow Chart.

9.1.4.2 Companion translational assays.

The following samples, in addition to the mandatory tissue sample (see 9.1.4.1), are collected, stored and used to perform complementary translational assays:

- Diagnostic FFPE blocks which are collected for all patients and stored until the end of the trial.
- Peripheral blood samples will be also collected along trial treatments following the schedule reported in section 8.0: in particular every 2 cycles for TMZ and before every treatment for pembrolizumab, from subjects enrolled in both D- and P-cohorts.
- Vital tumor tissue from tumor biopsy which are collected, at baseline (B-ML) and at tumor progression after TMZ treatment (TMZ-ML), before (only Cohort-D) and at tumor progression (both Cohorts) after pembrolizumab treatment when possible (D-ML and PD-ML).

Complementary translational assays may include analysis on:

Serum. To identify potential new serum markers with prognostic and/or predictive value for clinical outcomes (response, progression-free survival and overall survival, toxicity), soluble factors, such as cytokines, chemokines, soluble receptors, and antibodies to tumor antigens will be characterized and quantified by immunoassays in serum. Analyses may include, but not necessarily be limited to: soluble PD-1, Interleukines (1β, **2** , **4, 6, 10, 12α**), **Tumor Necrosis Factor (TNF), TGFbeta, Interferon gamma** (IFNγ), monocyte chemotactic protein (MCP-1), and others;

Peripheral Blood Mononuclear Cells (PBMC). To characterize circulating immune cells specific lymphocyte subsets will be isolated from PBMC and expression levels of T cell co-stimulatory markers on PBMC will be quantified by flow cytometry. Analyses may also include, but not necessarily be limited to, the quantification of **T** (**CD3; CD4; CD8** and the proportion of memory and effector T cell subset), **B, NK** (CD3-; CD56+) and **TREG** (CD3+/CD4+; CD25+; FOXP3+), **MDSC** (CD33+;CD11b, CD14-; HLA-DR low) cell-subsets, and the evaluation of expression levels of **PD-1, PD-L1**, and others immune-regulatory molecules on the same subsets of cells. To identify potential neo-antigens, T cell receptor (TCR) re-arrangements will be analyzed by NGS analysis of PBMC or subsets of Lymphocytes isolated by FACS. Amplification and sequencing of CDR3 regions in rearranged TCR β-chains will be analyzed using the immunoSEQ Assay (Adaptive Biotechnologies). The assay combines multiplex PCR with high-throughput sequencing and a sophisticated bioinformatics pipeline for TCRβ CDR3 region analysis.

To identify potential predictive signature(s) of response to pembrolizumab gene expression profiling will be determined from PBMC or circulating immune cell-subsets isolated by FACS by RNASeq and/or Affymetrix gene array technology and/or quantitative real-time polymerase chain reaction (qPCR).

Tumor Samples. Biopsy samples may be used for the following analysis:

Characterization of tumor infiltrating lymphocytes (TILs) and tumor antigens. Immunohistochemistry (IHC) will be used to assess the number and composition of immune infiltrates in order to define the immune cell subsets present within formalin-fixed, paraffin embedded (FFPE) tumor tissue. These IHC analyses will include, but not necessarily be limited to, the following markers: **CD4, CD8, CD45RO, FOXp3, PD-1, PD-L1, PD-L2, myeloid cell markers (CD33, CD68, CD11b)** and others.

Characterization of T cell repertoire. TCR sequencing (immunoseq) will be performed on tumor biopsies in order to characterize T cell clonality, either on FFPE or RNA later-preserved samples or on subsets of immune cells isolated from fresh tumor tissue.

Gene expression profiling. Tumor biopsies that are collected in RNAlater or equivalent fixative or FACS-sorted cell population isolated from fresh tumor biopsies will also be examined for mRNA gene expression by RNASeq and/or Affymetrix gene array technology and/or quantitative real-time polymerase chain reaction (qPCR) to detect expression of genes with possible prognostic or predictive value.

Immune cells isolated form blood (fresh isolated or vitally DMSO-cryopreserved PBMC or immune cell subsets) and biopsies (tumor infiltrating immune cells) might undergo functional assays to assess specificneaontigen recognition and to investigate molecular markers of response.

Biopsies at confirmed progression are strongly encouraged for the purposes of understanding mechanisms of resistance to therapy.

Other translational studies.

Microbiome analysis. The composition of gut microbiome had been correlated with pathogenesis and progression of colorectal cancer through several mechanisms, including induction of chronic inflammation. In addition, the composition of microbiome had been suggested to influence the innate and adaptive immune system composition and function possibly influencing the activity of pembrolizumab in colorectal cancer.

Thus DNA and RNA may be extracted from fecal samples or buccal swabs taken prior to therapy and on-treatment, and microbiota might be isolated from the same samples for functional analysis, with the purpose of characterizing the gut microbiome and to correlate longitudinal changes in its composition with response to therapy. Patients will be provided with the kit and the instructions for the collection of stools, and they will be asked to fill a diary reporting diet information on the 3 days before collection.

The relative priority of each individual assays might change over time depending on the availability of material, and according to the results of the currently prioritized marker analyses.

9.1.5 Laboratory Procedures/Assessments

Details regarding specific laboratory procedures/assessments to be performed in this trial are provided below.

Laboratory tests for hematology, chemistry, urinalysis, and others are specified in Table 9.1.5

Table 9.1.5 Laboratory Tests

Hematology	**Chemistry**	**Urinalysis**	**Other**
Hematocrit	Albumin	Blood	Serum β-human chorionic gonadotropin†
Hemoglobin	Alkaline phosphatase	Glucose	(β-hCG)†
Platelet count	Alanine aminotransferase (ALT)	Protein	PT (INR)
WBC (total and differential)	Aspartate aminotransferase (AST)	Specific gravity	aPTT
Red Blood Cell Count	Lactate dehydrogenase (LDH)	Microscopic exam *(If abnormal)*	Total thriiodothyronine (T3)
Absolute Neutrophil Count	Carbon Dioxide ‡	results are noted	Free tyroxine (T4)
Absolute Lymphocyte Count	*(CO_2 or biocarbonate)*	Serum pregnancy test †	Thyroid stimulating hormone (TSH)
	Uric Acid		
	Calcium		
	Chloride		Blood for correlative studies
	Glucose		Stools
	Phosphorus		Buccal Swab
	Potassium		
	Sodium		
	Magnesium		
	Total Bilirubin		
	Direct Bilirubin *(If total bilirubin is elevated above the upper limit of normal)*		
	Total protein		
	Blood Urea Nitrogen		
	Creatinine/Creatinine Clearance		

† Perform on women of childbearing potential only.

‡ If considered standard of care in your region.

Laboratory tests for screening or entry into the Second Course Phase should be performed within 10 days prior to the first dose of treatment. After Cycle 1, pre-dose laboratory procedures can be conducted up to 72 hours prior to dosing. Results must be reviewed by the investigator or qualified designee and found to be acceptable prior to each dose of trial treatment.

9.1.6 Other Procedures

9.1.6.1 Withdrawal/Discontinuation

When a participant discontinues/withdraws prior to trial completion, all applicable activities scheduled for the final trial visit should be performed at the time of discontinuation. Any adverse events which are present at the time of discontinuation/withdrawal should be followed in accordance with the safety requirements outlined in section 9.2 - Assessing and Recording Adverse Events. Participants who a) attain a CR or b) complete 24 months of treatment with pembrolizumab may discontinue treatment with the option of restarting treatment if they meet the criteria specified in section 7.3.5. After discontinuing treatment following assessment of CR, these participants should return to the site for a Safety Follow-up Visit (described in section 9.1.7.4.1) and then proceed to the Follow-Up Period of the study (described in section 9.1.7.4.2).

9.1.6.2 Blinding/Unblinding

This is an open-label trial; therefore, the Sponsor, investigator and subject will know the treatment administered.

9.1.7 Visit Requirements

Visit requirements are outlined in section 8.0 - Trial Flow Chart. Specific procedure-related details are provided above in section 9.1 - Trial Procedures.

9.1.7.1 Screening Phase period

CT scans performed up to 28 days prior to screening eligibility check are considered valid. From -28 to -1 days before the TMZ priming baseline visit as reported in section 8.0 - Trial Flow Chart.

All patients at the baseline of the Screening Phase will be tested for MMR status on the diagnostic FFPE block or slides according to the IHC MSI test (Bethesda panel).

All MMR proficient patients, will be assessed for immunohistochemical expression of MGMT as previously described (9.1.4.1). IHC negative cases will be assessed for MGMT promoter methylation status on tissue as described previously (9.1.4.1). Percent of methylation will be normalized on tumor content evaluated by hematoxylin and eosin staining to correct for stromal infiltration present in the original slide used for DNA extraction. Tumors with values below 63% will be considered MB-low, while those with values above or equal to 63% will considered MB-high.

9.1.7.2 PRIMING Phase Period

Each patient will be treated until PD unless patient refusal, consent withdrawal, or the occurrence of unacceptable toxicity.

The treatment period (cycle) comprises 28 days, consisting of 5 days of daily administration, followed by a 23-day resting period, from Day 6 to Day 28. During each treatment cycle, extension of treatment period to Days > 5 is not allowed, even in case of missed doses. A diary of TMZ assumption must be given to the patient every treatment cycle and the correct compilation must be revised by the investigator.

9.1.7.3 TRIAL Phase Period

Patients eligible for the TRIAL phase will be treated with Pembrolizumab. The drug will be administered every 3 weeks unless earlier progression, patient refusal, consent withdrawal, or the occurrence of unacceptable toxicity (see section 7.3).

The treatment period (cycle) comprises 21 days, consisting of 1 day of daily administration, followed by a 20-day resting period, from Day 2 to Day 21.

9.1.7.4 Post-Treatment TRIAL Phase Visits

Patients who discontinue Pembrolizumab treatment should be scheduled for a safety follow-up visit within 30 days after the last dose of study or after the decision to discontinue study treatment.

9.1.7.4.1 Safety Follow-Up TRIAL Phase Visit

The mandatory Safety Follow-Up Visit should be conducted approximately 30 days after the last dose of study treatment or before the initiation of a new anti-cancer treatment, whichever comes first. All AEs that occur prior to the Safety Follow-Up Visit should be recorded. Participants with an AE of Grade > 1 will be followed until the resolution of the AE to Grade 0-1 or until the beginning of a new anti-cancer therapy, whichever occurs first. SAEs that occur within 90 days of the end of treatment or before initiation of a new anti-cancer treatment should also be followed and recorded. Participants who are eligible for retreatment with pembrolizumab (as described in 7.3.5) may have up to two safety follow-up visits, one after the Initial Treatment Period and one after the Second Course Treatment.

9.1.7.4.2 Follow-up Visits

Participants who discontinue study treatment for a reason other than disease progression will move into the Follow-Up Phase and should be assessed every 8 weeks (56 ± 7 days) by radiologic imaging to monitor disease status. After 1 year, the imaging time point will occur every 12 weeks (± 7 days). Every effort should be made to collect information regarding disease status until the start of new anti-cancer therapy, disease progression, death, end of the study or if the participant begins retreatment with pembrolizumab as detailed in section 7.3.5.

Information regarding post-study anti-cancer treatment will be collected if new treatment is initiated.

Participants who are eligible to receive retreatment with pembrolizumab according to the criteria in section 7.3.5 will move from the follow-up phase to the Second Course Phase when they experience disease progression.

9.1.7.4.3 Survival Follow-up

Participants who experience confirmed disease progression or start a new anticancer therapy, will move into the Survival Follow-Up Phase and should be contacted by telephone every 12 weeks to assess for survival status until death, withdrawal of consent, or the end of the trial, whichever occurs first.

9.2 Safety Assessment

All patients will be evaluable for safety and toxicity from the time of their first treatment with temozolomide and pembrolizumab. Toxicity will be assessed using NCI Clinical Trials Criteria for Adverse Events (CTCAE) Version 4.03 (see appendix 2). All adverse events (AEs) occurring after the last administration of study treatment will be recorded on the case report forms (CRF) as described below (section 9.2.6). The investigator will decide if those events are drug related (not related, not likely, possibly, probably, certainly) and this decision will be recorded on the forms for all AEs. Serious adverse events (SAEs), as defined by the GCP Guideline, must be immediately reported according to the procedure detailed below (section 9.2.1).

9.2.1 Definitions for Adverse Event and Serious Adverse Event

An Adverse Event (AE) is defined as any untoward medical occurrence in a patient or clinical investigation participant administered a medicinal product and which does not necessarily have to have a causal relationship with this treatment. An AE can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding, for example), symptom, or disease temporally associated with the use of a medicinal product or protocol-specified procedure, whether or not considered related to the medicinal product or protocol-specified procedure. Any worsening (i.e., any clinically significant adverse change in frequency and/or intensity) of a pre-existing condition that is temporally associated with the use of the investigational products, is also an AE.
AEs may occur during the course of the use of investigational product in clinical trials, or as prescribed in clinical practice, from overdose (whether accidental or intentional), from abuse and from withdrawal.

A Serious Adverse Event (SAE) is any adverse event that:

- Results in death;
- Is life threatening;
- Results in persistent or significant disability/incapacity;

- Results in or prolongs an existing inpatient hospitalization;
- Is a congenital anomaly/birth defect;
- Is another important medical event
- **Note:** In addition to the above criteria, adverse events meeting either of the below criteria, although not serious per ICH definition, are reportable to the delegated pharmacovigilance service and to Merck Global Safety (if concerning pembrolizumab, Attn: Worldwide Product Safety; FAX 215-661-6229) in the same timeframe as SAEs to meet certain local requirements. Therefore, these events are considered serious by Merck for collection purposes.
- Is a new cancer (that is not a condition of the study);
- Is associated with an overdose.

Refer to Table 9.2 for additional details regarding each of the above criteria.

9.2.2 Reporting of Adverse Events

AEs will be collected from the time the first dose of study medication (either pembrolizumab or temozolomide) is administered until 30 days following discontinuation of study treatment. SAEs will be collected over the same time period as stated above for AEs of temozolomide, or up to 90 days for pembrolizumab. In addition, any SAE assessed as related to study participation, study medication or concomitant medication must be recorded from the time a subject consents to participate in the study up to and including any follow-up contact. After discontinuation of study medication, the investigator will monitor all AEs/SAEs that are ongoing until resolution or stabilization of the event or until the subject is lost to follow-up.

Reporting criteria for AEs/SAEs:

- All AEs, SAEs and other reportable safety events that occur after the consent form is signed but before planned treatment initiation must be reported by the investigator if the event cause the participant to be excluded from the study.

- All AEs must be reported by the investigator from the time of treatment allocation through 30 days following cessation of study treatment.

- SAEs regarding pembrolizumab must be reported by the investigator from the time of treatment allocation through 90 days (pembrolizumab) or 30 days (temozolomide) following cessation of study treatment, or 30 days following cessation of study treatment if the participant initiates new anticancer therapy, whichever is earlier.

- All pregnancies must be reported by the investigator from the time of treatment allocation through 120 days (pembrolizumab) or 90 days (temozolomide) following cessation of study treatment, or 30 days following cessation of study treatment if the participant initiates new anticancer therapy, whichever is earlier.

- Any SAE brought to the attention of an investigator at any time outside of the time period specified above must be reported immediately by the investigator if the event is considered to be study drug-related.

- A death on study requires reporting regardless of causality and attribution to treatment or other cause must be provided. Death due to disease progression is not to be reported as an AE. Deaths that occur beyond 30 days after the end of study drug administration/initiation of an alternate therapy, do not qualify as SAEs.

Note: Adverse events will not be collected for subjects during the SCREENING Phase period (for determination of archival tissue status) as long as that subject has not undergone any protocol-specified procedure or intervention. Since we will use FFPE blocks and routine blood draws to determine both MMR and MGMT status the subject will first required to provide consent to the main study but AEs will NOT be captured.

9.2.3 Reporting of Serious Adverse Events

Each adverse event is to be classified by the investigator as SERIOUS or NON-SERIOUS. This classification of the seriousness of the event determines the reporting procedures to be followed.

In order to comply with SAEs reporting responsibilities, the Sponsor of the study will appoint a delegated CRO as "Third Party Pharmacovigilance Provider". If a SAE occurs, the Pharmacovigilance has to be notified by fax or by e-mail, using the designated form, within 24 hours of awareness of the event by the Investigator.

Moreover, in case of any SAE, or follow up to a SAE, including death due to any cause whether or not related to the Merck product (pembrolizumab), it must be reported also to Merck Global Safety within 2 working days. Additionally, any SAE considered by the investigator to be related to pembrolizumab, which is brought to the attention of the investigator either at any time between informed consent to the end of the specified safety follow-up period, or at any time outside of the follow-up time period, must also be reported immediately to Merck Global Safety (Attn: Worldwide Product Safety; FAX 215-661-6229).
Investigators are not obligated to actively seek AE or SAE or other reportable safety events in former study participants. However, if the investigator learns of any SAE, including a death, at any time after a participant has been discharged from the study, and he/she considers the event to be reasonably related to the study treatment or study participation, the investigator must promptly notify Merck Global Safety (Attn: Worldwide Product Safety; FAX 215-661-6229).

The initial report should be followed by submission of more detailed adverse event information within 5 calendar days after the Investigator first became aware of the serious adverse event. Reporting requirements for adverse events are summarized in the table below.

Gravity	**Reporting Time**	**Type of Report**

SERIOUS	Within 24 hours from awareness by the investigator	Initial report on SAE report form + case report form
	Within 5 calendar days from initial report	Follow-up/Final report on SAE report form
NON SERIOUS	Per case report form submission procedure	Case report form

If for any reason the SAE form transmission is not possible, Pharmacovigilance should be informed by phone of the occurrence of the event. In this exceptional case, Pharmacovigilance will complete a SAE form with information received, which will be sent to the investigator for confirmation, and in the meanwhile Pharmacovigilance procedures will be initiated.

SAEs should also be reported on the AE case report form (CRF). The form to be used for SAE expedited reporting is not the same as the AE CRF, but where the same data are collected, the forms must be completed in a consistent manner. For example, the same AE term should be used on both forms.

Pharmacovigilance assesses each SAE reported by the study Investigators to identify any suspected unexpected serious adverse reactions (SUSAR), i.e. serious adverse events considered at least possible associated to the study treatment by the Investigator and not listed in the IMP reference document(s) and provide this information to the sponsor for the final evaluation. If a SAE is assessed as a possible SUSAR, the sponsor, through delegated Pharmacovigilance, may urgently require further information to the Investigator. Pharmacovigilance will issue a SUSAR notification whenever appropriate, and submit it to all concerned recipients according to current law and following sponsor's indication.

Follow-up information is to be reported on a new SAE form and transmitted to the same fax number as the initial report. A follow-up report is to be filled in, not only to complete the information provided on the initial report but also to modify any incorrect data. The SAE fax delivery confirmation sheets must be retained at the study sites.
At the end of the study all original SAE report forms are to be collected in the Investigator File, while the corresponding copies must be retained by the CRO personnel and archived in the TMF.

For the expectedness assessment of each case with pembrolizumab, the Sponsor will use the Investigator Brochure as the Reference Safety Information. For the expectedness assessment of each case with temozolomide, the Sponsor will use the SmPC (Undesirable Effects, section 4.8) and the safety data of the studies published in colorectal cancer (Amatu et al., 2013, 2016).

9.2.4 Definition and Reporting of an Overdose for This Protocol

Temozolomide
Doses of 500, 750, 1,000, and 1,250 mg/m2 (total dose per cycle over 5 days) have been evaluated clinically in patients. Dose-limiting toxicity was haematological and was reported with any dose but is expected to be more severe at higher doses. An overdose of 10,000 mg (total dose in a single cycle, over 5 days) was taken by one patient and the adverse reactions

reported were pancytopenia, pyrexia, multiorgan failure and death. There are reports of patients who have taken the recommended dose for more than 5 days of treatment (up to 64 days) with adverse events reported including bone marrow suppression, with or without infection, in some cases severe and prolonged and resulting in death. In the event of an overdose, haematological evaluation is needed. Supportive measures should be provided as necessary.

Pembrolizumab

For purposes of this study, an overdose of pembrolizumab will be defined as any dose of 1,000 mg or greater (≥5 times the indicated dose). No specific information is available on the treatment of overdose of pembrolizumab. In the event of overdose, the participant should be observed closely for signs of toxicity. Appropriate supportive treatment should be provided if clinically indicated.

If an adverse event(s) is associated with ("results from") the overdose of a Merck product, the adverse event(s) is reported as a serious adverse event, even if no other seriousness criteria are met.

If a dose of Merck's product meeting the protocol definition of overdose is taken without any associated clinical symptoms or abnormal laboratory results, the overdose is reported as a non-serious Event of Clinical Interest (ECI), using the terminology "accidental or intentional overdose without adverse effect."

All reports of overdose with and without an adverse event must be reported within 24 hours to Pharmacovigilance and within 2 working days hours to Merck Global Safety (Attn: Worldwide Product Safety; FAX 215-661-6229).

9.2.5 Events of Clinical Interest (pembrolizumab)

Selected non-serious and serious adverse events are also known as Events of Clinical Interest (ECI) and must be reported within 2 working days to Merck Global Safety. (Attn: Worldwide Product Safety; FAX 215-661-6229).

For the time period beginning when the consent form is signed until treatment allocation/randomization, any ECI, or follow up to an ECI, that occurs to any participant must be reported within 2 working days to Merck Global Safety if it causes the participant to be excluded from the trial, or is the result of a protocol-specified intervention, including but not limited to washout or discontinuation of usual therapy, diet, placebo treatment or a procedure.

For the time period beginning at treatment allocation through 90 days following cessation of treatment, or 30 days following cessation of treatment if the participant initiates new anticancer therapy, whichever is earlier, any ECI, or follow up to an ECI, whether or not related to Merck product, must be reported within 2 working days to Merck Global Safety.

Events of clinical interest for this trial include:

1. an overdose of Merck product, as defined in section 9.2.4 - Definition of an Overdose for This Protocol and Reporting of Overdose to the Sponsor, that is not associated with clinical symptoms or abnormal laboratory results.
2. an elevated AST or ALT lab value that is greater than or equal to 3X the upper limit of normal and an elevated total bilirubin lab value that is greater than or equal to 2X the upper limit of normal and, at the same time, an alkaline phosphatase lab value that is less than 2X the upper limit of normal, as determined by way of protocol-specified laboratory testing or unscheduled laboratory testing.*

*Note: These criteria are based upon available regulatory guidance documents. The purpose of the criteria is to specify a threshold of abnormal hepatic tests that may require an additional evaluation for an underlying etiology.

9.2.6 Recording AEs/SAEs in the Case Report Form

AEs can be assessed directly by the Investigator during a clinical visit or based on laboratory/Instrumental examinations or can be referred by the patient.

Pre-existing Conditions
A pre-existing condition (i.e., a disorder starting before the adverse event reporting period) should not be reported as an adverse event unless the condition worsens during the adverse event reporting period.

Procedures
Diagnostic and therapeutic procedures, such as surgery, should not be reported as adverse events, while the medical condition for which the procedure was performed should be reported if it meets the definition of an adverse event. For example, an appendectomy performed for an acute appendicitis occurring during the adverse event reporting period should not be reported as adverse event; while "acute appendicitis" is to be reported as adverse event. If a patient undergoes a surgical procedure that was planned prior to entry into the trial, and surgery is not performed due to a worsening of a baseline condition, this baseline condition should not be reported as an adverse event.

Symptoms of Targeted Disease
Tumor-related signs and symptoms will be followed at each visit. Although a measure of efficacy, these will always be reported as pre-existing conditions at baseline and during treatment only if they meet the definition of adverse event.
For all adverse events the Investigator will be asked to assess its relationship with the study treatment.

Causality Assessment and Grading of Adverse Event Severity
The assessment of relationship to study drug will be done according to the following causality scale based on the WHO definitions:

- Certain: A clinical event, including laboratory test abnormality, occurring in a plausible time relationship to drug administration, and which cannot be explained by concurrent disease or other drugs or chemicals. The response to withdrawal of the drug (de-challenge) should be clinically plausible. The event must be definitive pharmacologically or phenomenologically, using a satisfactory re-challenge procedure if necessary
- Probable: A clinical event, including laboratory test abnormality, with a reasonable time sequence to administration of the drug, unlikely to be attributed to concurrent disease or other drugs or chemicals, and which follows a clinically reasonable response on withdrawal (rechallenge). Rechallenge information is not required to fulfil this definition.

- Possible: A clinical event, laboratory test abnormality, with a reasonable time sequence to administration of the drug, but which could also be explained by concurrent disease or other drugs or chemicals. Information on drug withdrawal may be lacking or unclear
- Unlikely: A clinical event, laboratory test abnormality, with a temporal relationship to drug administration which makes a causal relationship improbable, and in which other drugs, chemicals or underlying disease provide plausible explanations Severity grading of adverse events and pre-existing conditions will be done according to the National Cancer Institute (NCI) Common Toxicity Criteria (CTCAE) V. 4.03.

AEs that are not defined in the NCI CTCAE should be evaluated for severity according to the following scale:

- Grade 1 = Mild – transient or mild discomfort; no limitation in activity; no medical intervention/therapy required;
- Grade 2 = Moderate – mild to moderate limitation in activity, some assistance may be needed; no or minimal medical intervention/therapy required;
- Grade 3 = Severe – marked limitation in activity, some assistance usually required; medical intervention/therapy required, hospitalization is possible;
- Grade 4 = Life threatening – extreme limitation in activity, significant assistance required; significant medical intervention/therapy required, hospitalization or hospice care probable;
- Grade 5 = Death - the event results in death.

Note the distinction between the gravity and the severity of an adverse event. Severe is a measure of intensity; thus, a severe reaction is not necessarily a serious reaction. For example, a headache may be severe in intensity but would not be classified as serious unless it meets one of the criteria for serious events listed above.

Pregnancy Reporting

Although pregnancy and infant exposure during breast feeding are not considered adverse events, it is the responsibility of investigators or their designees to report any pregnancy or lactation in a participant (spontaneously reported to them) that occurs during the study using the "exposure in utero" form.

Pregnancies and infant exposures during breastfeeding that occur after the consent form is signed but before treatment allocation/randomization must be reported by the investigator if they cause the participant to be excluded from the trial, or are the result of a protocol-specified intervention, including but not limited to washout or discontinuation of usual therapy, diet, placebo treatment or a procedure.

Pregnancies and infant exposures during breastfeeding that occur from the time of treatment allocation/randomization through 120 days (pembrolizumab) or 90 days (temozolomide) following cessation of study product, or 30 days following cessation of treatment if the participant initiates new anticancer therapy, whichever is earlier, must be reported by the investigator to delegated Pharmacovigilance within 2 weeks of learning of its occurrence.. All reported pregnancies must be followed to the completion/termination of the pregnancy. Pregnancy outcomes of spontaneous abortion, missed abortion, benign hydatidiform mole, blighted ovum, fetal death, intrauterine death, miscarriage and stillbirth must be reported as serious events (Important Medical Events). If the pregnancy continues to term, the outcome (health of infant) must also be reported.

Such events must be reported within 24 hours to delegated Pharmacovigilance and within 2 working days hours to Merck Global Safety. (Attn: Worldwide Product Safety; FAX 215-661-6229).

Overdose

Reporting if any overdose (accidental or intentional) which results in serious adverse reactions is to be handled following the SAE procedures. This includes reports related to drug intake with suicidal intentions and consequent drug overdose.
Overdose reporting even not associated with adverse reactions shall be anyhow reported immediately to delegated Pharmacovigilance using the most rapid type of communication (phone, e-mail).

Follow-up of Unresolved Adverse Events

All adverse events should be followed at least until 30 days following the last dose of IMP. Drug-related and serious adverse events ongoing at the end of this observation period must be recorded until they are resolved or the investigator assesses them as chronic or the subject is lost to follow-up or starts a new anti-cancer treatment, whichever occurs earlier.

Table 9.2 Evaluating Adverse Events

An investigator who is a qualified physician, will evaluate all adverse events as to:

V4.03 CTCAE Grading	**Grade 1**	**Mild; asymptomatic or mid symptoms; clinical or diagnostic observations only; intervention not indicated.**
	Grade 2	**Moderate; minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental ADL.**
	Grade 3	**Severe or medically significant but not immediately life-threatening; hospitalization or prolongation or hospitalization indicated; disabling; limiting self-care ADL.**
	Grade 4	**Life threatening consequences; urgent intervention indicated.**
	Grade 5	**Death related to AE**
Seriousness	A serious adverse event is any adverse event occurring at any dose or during any use of Merck product that:	
	†**Results in death**; or	
	†**Is life threatening; or** places the participant, in the view of the investigator, at immediate risk of death from the event as it occurred (Note: This does not include an adverse event that, had it occurred in a more severe form, might have caused death.); or	
	†**Results in a persistent or significant disability/incapacity** (substantial disruption of one's ability to conduct normal life functions); or	
	†**Results in or prolongs an existing inpatient hospitalization** (hospitalization is defined as an inpatient admission, regardless of length of stay, even if the hospitalization is a precautionary measure for continued observation. (Note: Hospitalization for an elective procedure to treat a pre-existing condition that has not worsened is not a serious adverse event. A pre-existing condition is a clinical condition that is diagnosed prior to the use of a Merck product and is documented in the patient's medical history.); or	
	†**Is a congenital anomaly/birth defect** (in offspring of participant taking the product regardless of time to diagnosis);or	
	Is a new **cancer** (that is not a condition of the study) **(**although not serious per ICH definition, is reportable to the Sponsor within 24 hours and to Merck within 2 working days to meet certain local requirements); **or**	
	Is an overdose (whether accidental or intentional). Any adverse event associated with an overdose is considered a serious adverse event for collection purposes. An overdose that is not associated with an adverse event is considered a non-serious event of clinical interest and must be reported within 24 hours to the Sponsor and to Merck within 2 working days..	
	Other important medical events that may not result in death, not be life threatening, or not require hospitalization may be considered a serious adverse event when, based upon appropriate medical judgment, the event may jeopardize the participant and may require medical or surgical intervention to prevent one of the outcomes listed previously (designated above by a †).	
Duration	Record the start and stop dates of the adverse event. If less than 1 day, indicate the appropriate length of time and units	
Action taken	Did the adverse event cause Merck product to be discontinued?	
Relationship to Merck Product	Did Merck product cause the adverse event? The determination of the likelihood that Merck product caused the adverse event will be provided by an investigator who is a qualified physician. The investigator's signed/dated initials on the source document or worksheet that supports the causality noted on the AE form, ensures that a medically qualified assessment of causality was done. This initialed document must be retained for the required regulatory time frame. The criteria below are intended as reference guidelines to assist the investigator in assessing the likelihood of a relationship between the test drug and the adverse event based upon the available information. **The following components are to be used to assess the relationship between Merck product and the AE**; the greater the correlation with the components and their respective elements (in number and/or intensity), the more likely Merck product caused the adverse event (AE):	
	Exposure	Is there evidence that the participant was actually exposed to Merck product such as: reliable history, acceptable compliance assessment (pill count, diary, etc.), expected pharmacologic effect, or measurement of drug/metabolite in bodily specimen?
	Time Course	Did the AE follow in a reasonable temporal sequence from administration of Merck product? Is the time of onset of the AE compatible with a drug-induced effect (applies to trials with investigational medicinal product)?
	Likely Cause	Is the AE not reasonably explained by another etiology such as underlying disease, other drug(s)/vaccine(s), or other host or environmental factors

Relationship to Merck Product **(continued)**	**The following components are to be used to assess the relationship between the test drug and the AE: (continued)**	
	Dechallenge	Was Merck product discontinued or dose/exposure/frequency reduced? If yes, did the AE resolve or improve? If yes, this is a positive dechallenge. If no, this is a negative dechallenge. (Note: This criterion is not applicable if: (1) the AE resulted in death or permanent disability; (2) the AE resolved/improved despite continuation of the Sponsor's product; or (3) the trial is a single-dose drug trial); or (4) Sponsor's product(s) is/are only used one time.)
	Rechallenge	Was the participant re-exposed to Merck product in this study? If yes, did the AE recur or worsen? If yes, this is a positive rechallenge. If no, this is a negative rechallenge. (Note: This criterion is not applicable if: (1) the initial AE resulted in death or permanent disability, or (2) the trial is a single-dose drug trial); or (3) Sponsor's product(s) is/are used only one time). NOTE: IF A RECHALLENGE IS PLANNED FOR AN ADVERSE EVENT WHICH WAS SERIOUS AND WHICH MAY HAVE BEEN CAUSED BY MERCK PRODUCT, OR IF REEXPOSURE TO MERCK PRODUCT POSES ADDITIONAL POTENTIAL SIGNIFICANT RISK TO THE PARTICIPANT, THEN THE RECHALLENGE MUST BE APPROVED IN ADVANCE BY THE SPONSOR AS PER DOSE MODIFICATION GUIDELINES IN THE PROTOCOL.
	Consistency with Trial Treatment Profile	Is the clinical/pathological presentation of the AE consistent with previous knowledge regarding Merck product or drug class pharmacology or toxicology?
The assessment of relationship will be reported on the case report forms /worksheets by an investigator who is a qualified physician according to his/her best clinical judgment, including consideration of the above elements.		
Record one of the following		**Use the following scale of criteria as guidance (not all criteria must be present to be indicative of Merck product relationship).**
Yes, there is a reasonable possibility of Merck product relationship.		There is evidence of exposure to Merck product. The temporal sequence of the AE onset relative to the administration of Merck product is reasonable. The AE is more likely explained by Merck product than by another cause.
No, there is not a reasonable possibility of Merck product relationship		Participant did not receive the Merck product OR temporal sequence of the AE onset relative to administration of Merck product is not reasonable OR the AE is more likely explained by another cause than the Merck product. (Also entered for a participant with overdose without an associated AE.)

9.2.7 Sponsor Responsibility for Reporting Adverse Events

All Adverse Events will be reported to regulatory authorities, IRB/IECs and investigators in accordance with all applicable global laws and regulations.

10.0 STATISTICAL CONSIDERATIONS

10.1 General considerations

The hypothesis tested by ARETHUSA is whether immunoediting in MMR proficient cancers can be disrupted by increasing the tumor mutational load with TMZ, enabling the tumor to dynamically express neo-antigens that positively affect T cell functions under check-point inhibition.

The main analysis thus will concern the response to pembrolizumab in MMR proficient patients (cohort P) with measurable disease at baseline who have received at least 1 cycle of pembrolizumab (iEE population, see 10.3.1).

10.2 Sample size calculation

A two-stage Green-Dahlberg design has been used [Green SJ and Dahlberg S Statistics in Medicine 11:853-862,1992], to calculate the sample size for the **P-cohort** under the following assumptions:

- The null hypothesis that the true (H_0) response rate is 10% (not considered clinically compelling) will be tested against a one-sided alternative (H_1).
- In the first stage, 10 patients will be accrued. If no responses are observed, the study will be stopped. Otherwise, 10 additional patients will be accrued for a total of 20.
- The null hypothesis will be rejected if 5 or more responses are observed in 20 patients.
- This design yields a type I error rate of .05, and power of 90% when the true response rate is 40%.
- In order to recruit 20 patients in the P-cohort the number of MMR unselected patients to be pre-screened for MMR status MGMT promoter methylation and the number of MGMT-PM+ to be primed with TMZ are reported in Table 10.1. below as well as in Study Diagram, section 4.2.

Table 10.1 Number of patients required in each trial Phase.

Number of Patients to be:	**N**	**Expected Percent**
RAS mut MMR proficient cases to be screened	670	A
expected MGMT IHC negative	335	B = 50% of A
expected promoter methylated in tissue	67	C=20% of B and 10% of A
expected with increase of mutational load ≥ 20 mutations/Mb (PRIMING Phase)	20	D= 30% of C

For cohort D, a precise sample size has NOT been calculated since the ORR in deficient patients is a secondary objective of the trial We will enroll up to 34 MMR-D cases according to an estimated prevalence of 4-5 %. These patients will constitute the **D-cohort.** We expect a response rate between 40% (95% C.I .18-.71) and 33% (95% C.I .21-.46) as reported by Le et al., (2015 & 2019) with 11-14 responses accordingly.

10.3 Statistical Analysis Plan

10.3.1 Populations considered for the analysis

All patients signing the informed consent for the PRIMING and TRIAL Phases will be accounted for. The number of patients who will not result eligible, who died or withdrew before these phases, will be specified.

A patient will be considered to be PRIMING and TRIAL Phases eligible if he/she did not have any deviation from the patient entry criteria listed in section 7.1.2 (PRIMING) and 7.1.3 (TRIAL). Potential eligibility problems will be assessed by the Clinical Study Coordinator at time of medical review.

The final efficacy analysis will occur after 20 patients entered in the P-Cohort are evaluable for response according to RECIST 1.1. The distribution of follow-up time in both cohorts (P and D) will be described and the number of patients lost to follow-up will be reported.

Conclusions will be based on all eligible patients in each cohort. Further analysis may be performed excluding those patients for whom major protocol deviations have been identified (e.g., early death due to other reasons, early discontinuation of treatment, major protocol violations, etc.). However, these sub-analyses may not serve as the basis for drawing conclusions concerning treatment efficacy, and the reasons for excluding patients from the analysis should be clearly reported.

Three populations will be considered for the analysis, as follows:

• The Safety Evaluable (SE) population defined as all treated patients (i.e. eligible as decided at the time of registration that receives at least 1 dose of any study treatment i.e. TMZ and Pembrolizumab). An incorrect treatment schedule or drug administration or an early termination of treatment does not result in exclusion of patients from this population. This population will be the object of the Safety and Tolerability analysis. Patients with major deviations from the eligibility criteria affecting safety or from the treatment schedule at cycle 1 for reasons other than toxicity may be presented in separate tables/listings.

• The Efficacy Evaluable (EE) population defined as all patient treated with pembrolizumab in P-cohort, with no major deviations from the eligibility criteria affecting efficacy evaluation, for which the tumor response could be evaluated at least once while on treatment. These patients should have received at least 2 cycles after treatment starts, unless disease progression occurs within this period.

• The immunotherapy Efficacy Evaluable (iEE) population. Anti-tumor immune responses such as those associated with pembrolizumab may result in objective responses that are delayed and that can be preceded by initial apparent radiological progression. This initial apparent progression may occur as a result of either delayed anti-tumor activity and/or robust tumor immune cell infiltration with a concomitant increase in tumor size. In addition, lesions that might otherwise be undetectable with conventional imaging may increase in size as a result of these processes and be recorded as new lesions. Conventional RECIST 1.1 response criteria may not be thus adequate to capture and describe the anti-tumor activity of pembrolizumab. Therefore, modified response criteria have been developed that account for the possible appearance of new lesions and allow radiological progression to be confirmed at subsequent assessment. For these reason a second efficacy analysis will be conducted (iEE analysis) on the same population used for the EE analysis using the Modified RECIST criteria.

10.3.2 Primary analysis

The main analysis will focus on the objective response rate (ORR) of the P Cohort.

An objective response is defined as either a CR or PR assessed, as outlined in sections 9.1.3 and 10.3.1 (iEE population), first by RECIST 1.1 and then by iRECIST criteria at the discretion of the local Investigator.

Best objective response of either type will be centrally confirmed using the Quibim Precision® software (the cloud-based repository for radioimaging). In case of disagreement between local and central evaluation, the central response evaluation will be used for the analysis. The duration of response will be calculated accordingly.

An estimate of ORR and its 95% CIs will be calculated using the Clopper-Pearson method. Discrepant results, impinging in the acceptance of rejection of the null hypothesis, will be discussed and agreed upon between the Protocol Chair and the Sponsor.

10.3.3 Secondary analyses

A secondary analysis will also estimate the ORR, as defined above, in the D-Cohort and compare it to the ORR in the P-cohort. CIs for the difference in ORRs between the two cohorts

will be determined using the normal approximation to the binomial distribution. The ORRs will be compared between the two cohorts with use of the stratified Cochran-Mantel-Haenszel test.

PFS and OS, for both cohorts, will be estimated using the Kaplan-Meier product-limit method. Estimates at weekly intervals will be tabulated with their standard error; medians will be provided with 95% confidence interval.

DCR is defined as the rate of patients with complete or partial response as best response or stable disease maintained for ≥ 18 weeks per RECIST v1.1. The analysis methods for DCR will be the same as those for the analysis of ORR.

Duration of response, defined as the time between the date of the first occurrence of a complete or partial response and the date of first documented progressive disease or death is documented, will also be estimated using Kaplan-Meier methodology above. Patients who have not progressed or who have not died by the data cutoff date for analysis will be censored at the time of last tumor assessment date. If no tumor assessments were performed after date of the first occurrence of a complete or partial response, DOR will be censored at the date of the first occurrence of a complete or partial response plus 1 day.

Adverse events will reported as proportions.

10.3.4 Exploratory analysis

Exploratory biomarker analyses will be performed in order to understand the association of these markers with study drug response, including efficacy and/or adverse events.

Exploratory biomarker analyses will include assessments of biomarkers in both tumor tissue and blood. Changes in biomarkers will be listed by cohort and response status. The pharmacodynamic biomarker analyses will include patients with at least one predose and one postdose biomarker assessment.

11.0 LABELING, PACKAGING, STORAGE AND RETURN OF CLINICAL SUPPLIES

11.1 Investigational Product

The investigator shall take responsibility for and shall take all steps to maintain appropriate records and ensure appropriate supply, storage, handling, distribution and usage of investigational product in accordance with the protocol and any applicable laws and regulations.

Pembrolizumab will be provided by Merck and Temozolomide by the Sponsor through Niguarda Pharmacy, as summarized in Table 11.

Table 11. Product Descriptions

Product Name & Potency	Dosage Form
Pembrolizumab 100 mg/ 4mL	Solution for Injection
Temozolomide 5, 20, 100, 250 mg	Hard Capsules

11.2 Packaging and Labeling Information

Supplies will be labeled in accordance with regulatory requirements. Pembrolizumab will be labelled as MK-3475.

11.3 Clinical Supplies Disclosure

This trial is open-label; therefore, the participant, the trial site personnel, the Sponsor and/or designee are not blinded to treatment. Drug identity (name, strength) is included in the label text; random code/disclosure envelopes or lists are not provided.

11.4 Storage and Handling Requirements

Clinical supplies must be stored in a secure, limited-access location under the storage conditions specified on the label.

Receipt and dispensing of trial medication must be recorded by an authorized person at the trial site.

Clinical supplies may not be used for any purpose other than that stated in the protocol.

11.5 Returns and Reconciliation

The investigator is responsible for keeping accurate records of the clinical supplies received from Merck or designee, the amount dispensed to and returned by the participants and the amount remaining at the conclusion of the trial.

Upon completion or termination of the study, all unused and/or partially used investigational product will be destroyed at the site per institutional policy. It is the Investigator's responsibility to arrange for disposal of all empty containers, provided that procedures for proper disposal have been established according to applicable federal, state, local and institutional guidelines and procedures, and provided that appropriate records of disposal are kept.

12.0 ADMINISTRATIVE AND REGULATORY DETAILS

12.1 Confidentiality

Sponsor assures that the key design element of this protocol will be posted in a publicly accessible database such as EUDRACT and clinicaltrial.gov; in addition, upon study

completion, the results of this study will be submitted for publication and posted in publicly accessible database for clinical trial studies.

All information regarding study drug supplied by Sponsor to the investigator is privileged and confidential information. The investigator agrees to use this information to accomplish the study and will not use it for other purposes without consent from Sponsor.

It is understood that there is an obligation to provide Sponsor with complete data obtained during the study. The investigator agrees to keep in confidence all the results obtained from the study. Such information shall not be disclosed to third parties without prior written permission from Sponsor, except to regulatory authority(ies), when requested.

Individual investigators may present results of the study at scientific meetings. However prior to the submission, the Sponsor will have the opportunity to review and comment the abstracts for a period of up to 15 calendar days prior to the submission.

12.2 Data Management

Data Management will be carried out by a delegated CRO. Medical terms are coded according to the MedDRA dictionary. Data will be analyzed using SAS® System currently used at CRO. Data cleaning will include both visual and computer-driven procedures in order to minimize logical inconsistencies and errors within the collected data. The data are checked for completeness, accuracy and consistency. The errors detected will be rectified by means of Data Clarification List (DCL) that will be used by the monitor for resolution of queries.

12.2.1 Record Retention

To enable evaluation and/or audits and/or regulatory authorities inspections, the Investigator agrees to keep records, including the identity of all participating subjects ("Subject identification Log"), all original signed informed consent forms, copies of all case report forms, source documents, detailed records of treatment disposition as well as the documentation included in the Investigator File according to local regulations or as specified in the Clinical Trial Agreement.

If the Investigator relocates, retires, or for any reason withdraws from the study, Sponsor should be prospectively notified. The study records must be transferred to an acceptable designee, such as another investigator, another institution, or to delegated CRO. The investigator must obtain Sponsor's written permission before disposing of any records.

13.0 REFERENCES

Alexandrov, L.B., Nik-Zainal, S., Wedge, D.C., Aparicio, S.A.J.R., Behjati, S., Biankin, A.V., Bignell, G.R., Bolli, N., Borg, A., Børresen-Dale, A.-L., et al. (2013). Signatures of mutational processes in human cancer. Nature *500*, 415–421.

Amatu, A., Sartore-Bianchi, A., Moutinho, C., Belotti, A., Bencardino, K., Chirico, G., Cassingena, A., Rusconi, F., Esposito, A., Nichelatti, M., et al. (2013). Promoter CpG island hypermethylation of the DNA repair enzyme MGMT predicts clinical response to dacarbazine in a phase II study for metastatic colorectal cancer. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. *19*, 2265–2272.

Amatu, A., Barault, L., Moutinho, C., Cassingena, A., Bencardino, K., Ghezzi, S., Palmeri, L., Bonazzina, E., Tosi, F., Ricotta, R., et al. (2016). Tumor MGMT promoter hypermethylation changes over time limit temozolomide efficacy in a phase II trial for metastatic colorectal cancer. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. *27*, 1062–1067.

Barault, L., Amatu, A., Bleeker, F.E., Moutinho, C., Falcomatà, C., Fiano, V., Cassingena, A., Siravegna, G., Milione, M., Cassoni, P., et al. (2015). Digital PCR quantification of MGMT methylation refines prediction of clinical benefit from alkylating agents in glioblastoma and metastatic colorectal cancer. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. *26*, 1994–1999.

Bardelli, A., Cahill, D.P., Lederer, G., Speicher, M.R., Kinzler, K.W., Vogelstein, B., and Lengauer, C. (2001). Carcinogen-specific induction of genetic instability. Proc. Natl. Acad. Sci. U. S. A. *98*, 5770–5775.

Bertotti, A., Papp, E., Jones, S., Adleff, V., Anagnostou, V., Lupo, B., Sausen, M., Phallen, J., Hruban, C.A., Tokheim, C., et al. (2015). The genomic landscape of response to EGFR blockade in colorectal cancer. Nature *526*, 263–267.

Boland, C.R., and Shike, M. (2010). Report from the Jerusalem workshop on Lynch syndrome-hereditary nonpolyposis colorectal cancer. Gastroenterology *138*, 2197.e1-7.

Cahill, D.P., Levine, K.K., Betensky, R.A., Codd, P.J., Romany, C.A., Reavie, L.B., Batchelor, T.T., Futreal, P.A., Stratton, M.R., Curry, W.T., et al. (2007). Loss of the mismatch repair protein MSH6 in human glioblastomas is associated with tumor progression during temozolomide treatment. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. *13*, 2038–2045.

Chemnitz, J.M., Parry, R.V., Nichols, K.E., June, C.H., and Riley, J.L. (2004). SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation. J. Immunol. Baltim. Md 1950 *173*, 945–954.

Coffield, V.M., Helms, W.S., Jiang, Q., and Su, L. (2004). Galpha13 mediates a signal that is essential for proliferation and survival of thymocyte progenitors. J. Exp. Med. *200*, 1315–1324.

Disis, M.L. (2010). Immune regulation of cancer. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. *28*, 4531–4538.

Douillard, J.-Y., Oliner, K.S., Siena, S., Tabernero, J., Burkes, R., Barugel, M., Humblet, Y., Bodoky, G., Cunningham, D., Jassem, J., et al. (2013). Panitumumab–FOLFOX4 Treatment and RAS Mutations in Colorectal Cancer. N. Engl. J. Med. *369*, 1023–1034.

Dudley, M.E., Wunderlich, J.R., Yang, J.C., Sherry, R.M., Topalian, S.L., Restifo, N.P., Royal, R.E., Kammula, U., White, D.E., Mavroukakis, S.A., et al. (2005). Adoptive cell transfer therapy following non-myeloablative but lymphodepleting chemotherapy for the treatment of patients with refractory metastatic melanoma. J. Clin. Oncol. Off. J. Am. Soc. Clin. Oncol. *23*, 2346–2357.

Esteller, M., and Herman, J.G. (2004). Generating mutations but providing chemosensitivity: the role of O6-methylguanine DNA methyltransferase in human cancer. Oncogene *23*, 1–8.

Francisco, L.M., Sage, P.T., and Sharpe, A.H. (2010). The PD-1 pathway in tolerance and autoimmunity. Immunol. Rev. *236*, 219–242.

Germano, G., Lamba, S., Rospo, G., Barault, L., Magrì, A., Maione, F., Russo, M., Crisafulli, G., Bartolini, A., Lerda, G., et al. (2017). Inactivation of DNA repair triggers neoantigen generation and impairs tumour growth. Nature *552*, 116–120.

Goldstein, J., Tran, B., Ensor, J., Gibbs, P., Wong, H.L., Wong, S.F., Vilar, E., Tie, J., Broaddus, R., Kopetz, S., et al. (2014). Multicenter retrospective analysis of metastatic colorectal cancer (CRC) with high-level microsatellite instability (MSI-H). Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. *25*, 1032–1038.

Greenwald, R.J., Freeman, G.J., and Sharpe, A.H. (2005). The B7 Family Revisited. Annu. Rev. Immunol. *23*, 515–548.

Hervieu, A., Rébé, C., Végran, F., Chalmin, F., Bruchard, M., Vabres, P., Apetoh, L., Ghiringhelli, F., and Mignot, G. (2013). Dacarbazine-mediated upregulation of NKG2D ligands on tumor cells activates NK and CD8 T cells and restrains melanoma growth. J. Invest. Dermatol. *133*, 499–508.

Hunder, N.N., Wallen, H., Cao, J., Hendricks, D.W., Reilly, J.Z., Rodmyre, R., Jungbluth, A., Gnjatic, S., Thompson, J.A., and Yee, C. (2008). Treatment of metastatic melanoma with autologous CD4+ T cells against NY-ESO-1. N. Engl. J. Med. *358*, 2698–2703.

Lauss, M., Donia, M., Harbst, K., Andersen, R., Mitra, S., Rosengren, F., Salim, M., Vallon-Christersson, J., Törngren, T., Kvist, A., et al. (2017). Mutational and putative neoantigen load predict clinical benefit of adoptive T cell therapy in melanoma. Nat. Commun. *8*, 1738.

Le, D.T., Uram, J.N., Wang, H., Bartlett, B.R., Kemberling, H., Eyring, A.D., Skora, A.D., Luber, B.S., Azad, N.S., Laheru, D., et al. (2015). PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. N. Engl. J. Med. *372*, 2509–2520.

Le *et al.*, D.T. Phase II Open-Label Study of Pembrolizumab in Treatment-Refractory, Microsatellite Instability-High/Mismatch Repair-Deficient Metastatic Colorectal Cancer: KEYNOTE-164. *J Clin Oncol*, JCO1902107 (2019).

Lynch, H.T., and de la Chapelle, A. (2003). Hereditary Colorectal Cancer. N. Engl. J. Med. *348*, 919–932.

Network, T.C.G.A. (2012). Comprehensive molecular characterization of human colon and rectal cancer. Nature *487*, 330.

Okazaki, T., Maeda, A., Nishimura, H., Kurosaki, T., and Honjo, T. (2001). PD-1 immunoreceptor inhibits B cell receptor-mediated signaling by recruiting src homology 2-domain-containing tyrosine phosphatase 2 to phosphotyrosine. Proc. Natl. Acad. Sci. U. S. A. *98*, 13866–13871.

Panda, A., Betigeri, A., Subramanian, K., Ross, J.S., Pavlick, D.C., Ali, S., Markowski, P., Silk, A., Kaufman, H.L., Lattime, E., et al. (2017). Identifying a Clinically Applicable Mutational Burden Threshold as a Potential Biomarker of Response to Immune Checkpoint Therapy in Solid Tumors. JCO Precis. Oncol. 1–13.

Parry, R.V., Chemnitz, J.M., Frauwirth, K.A., Lanfranco, A.R., Braunstein, I., Kobayashi, S.V., Linsley, P.S., Thompson, C.B., and Riley, J.L. (2005). CTLA-4 and PD-1 receptors inhibit T-cell activation by distinct mechanisms. Mol. Cell. Biol. *25*, 9543–9553.

Riley, J.L. (2009). PD-1 signaling in primary T cells. Immunol. Rev. *229*, 114–125.

Rizvi, N.A., Hellmann, M.D., Snyder, A., Kvistborg, P., Makarov, V., Havel, J.J., Lee, W., Yuan, J., Wong, P., Ho, T.S., et al. (2015). Mutational landscape determines sensitivity to PD-1 blockade in non–small cell lung cancer. Science *348*, 124–128.

Sartore-Bianchi, A., Trusolino, L., Martino, C., Bencardino, K., Lonardi, S., Bergamo, F., Zagonel, V., Leone, F., Depetris, I., Martinelli, E., et al. (2016a). Dual-targeted therapy with trastuzumab and lapatinib in treatment-refractory, KRAS codon 12/13 wild-type, HER2-positive metastatic colorectal cancer (HERACLES): a proof-of-concept, multicentre, open-label, phase 2 trial. Lancet Oncol. *17*, 738–746.

Sartore-Bianchi, A., Loupakis, F., Argilés, G., and Prager, G.W. (2016b). Challenging chemoresistant metastatic colorectal cancer: therapeutic strategies from the clinic and from the laboratory. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. *27*, 1456–1466.

Segal, N.H., Parsons, D.W., Peggs, K.S., Velculescu, V., Kinzler, K.W., Vogelstein, B., and Allison, J.P. (2008). Epitope landscape in breast and colorectal cancer. Cancer Res. *68*, 889–892.

Seymour, L., Bogaerts, J., Perrone, A., Ford, R., Schwartz, L.H., Mandrekar, S., Lin, N.U., Litière, S., Dancey, J., Chen, A., et al. (2017). iRECIST: guidelines for response criteria for use in trials testing immunotherapeutics. Lancet Oncol. *18*, e143–e152.

Sheppard, K.-A., Fitz, L.J., Lee, J.M., Benander, C., George, J.A., Wooters, J., Qiu, Y., Jussif, J.M., Carter, L.L., Wood, C.R., et al. (2004). PD-1 inhibits T-cell receptor induced

phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta. FEBS Lett. *574*, 37–41.

Siegel, R., Naishadham, D., and Jemal, A. (2013). Cancer statistics, 2013. CA. Cancer J. Clin. *63*, 11–30.

Snyder, A., Makarov, V., Merghoub, T., Yuan, J., Zaretsky, J.M., Desrichard, A., Walsh, L.A., Postow, M.A., Wong, P., Ho, T.S., et al. (2014). Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. N. Engl. J. Med. *371*, 2189–2199.

Van Allen, E.M., Miao, D., Schilling, B., Shukla, S.A., Blank, C., Zimmer, L., Sucker, A., Hillen, U., Foppen, M.H.G., Goldinger, S.M., et al. (2015). Genomic correlates of response to CTLA-4 blockade in metastatic melanoma. Science *350*, 207–211.

Yadav, V.K., DeGregori, J., and De, S. (2016). The landscape of somatic mutations in protein coding genes in apparently benign human tissues carries signatures of relaxed purifying selection. Nucleic Acids Res. *44*, 2075–2084.

Zhang, X., Schwartz, J.-C.D., Guo, X., Bhatia, S., Cao, E., Lorenz, M., Cammer, M., Chen, L., Zhang, Z.-Y., Edidin, M.A., et al. (2004). Structural and functional analysis of the costimulatory receptor programmed death-1. Immunity *20*, 337–347.

APPENDICES

Appendix 1: ECOG Performance Status

Grade	Description
0	Normal activity. Fully active, able to carry on all pre-disease performance without restriction.
1	Symptoms, but ambulatory. Restricted in physically strenuous activity, but ambulatory and able to carry out work of a light or sedentary nature (e.g., light housework, office work).
2	In bed <50% of the time. Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	In bed >50% of the time. Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
4	100% bedridden. Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
5	Dead.
* As published in Am. J. Clin. Oncol.: *Oken, M.M., Creech, R.H., Tormey, D.C., Horton, J., Davis, T.E., McFadden, E.T., Carbone, P.P.: Toxicity And Response Criteria Of The Eastern Cooperative Oncology Group. Am J Clin Oncol 5:649-655, 1982.* The Eastern Cooperative Oncology Group, Robert Comis M.D., Group Chair.	

Appendix 2: Common Terminology Criteria for Adverse Events V4.03 (CTCAE)

The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.03 will be utilized for adverse event reporting. (https://evs.nci.nih.gov/ftp1/CTCAE/CTCAE_4.03/CTCAE_4.03_2010-06-14_QuickReference_5x7.pdf)

Appendix 3: Contraception Requirements

Female Participants:

Female participants of childbearing potential are eligible to participate if they agree to use one of the contraception methods described in Table A3 consistently and correctly during the protocol-defined time frame in section 8.0 and 9.0.

Table A3: Contraceptive Methods

Acceptable Contraceptive Methods *Failure rate of >1% per year when used consistently and correctly.*
● Male or female condom with or without spermicide ● Cervical cap, diaphragm or sponge with spermicide

Highly Effective Contraceptive Methods That Are User Dependent [a] *Failure rate of <1% per year when used consistently and correctly.*
● Combined (estrogen- and progestogen- containing) hormonal contraception [b] ○ Oral ○ Intravaginal ○ Transdermal ○ Injectable
● Progestogen-only hormonal contraception [b] ○ Oral ○ Injectable
Highly Effective Methods That Have Low User Dependency *Failure rate of <1% per year when used consistently and correctly.*
● Progestogen- only contraceptive implant [b, c] ● Intrauterine hormone-releasing system (IUS) [b] ● Intrauterine device (IUD) ● Bilateral tubal occlusion
● **Vasectomized partner** A vasectomized partner is a highly effective contraception method provided that the partner is the sole male sexual partner of the WOCBP and the absence of sperm has been confirmed. If not, an additional highly effective method of contraception should be used.
● **Sexual abstinence** Sexual abstinence is considered a highly effective method only if defined as refraining from heterosexual intercourse during the entire period of risk associated with the study treatment. The reliability of sexual abstinence needs to be evaluated in relation to the duration of the study and the preferred and usual lifestyle of the participant.)
Notes: Use should be consistent with local regulations regarding the use of contraceptive methods for participants of clinical studies. a) Typical use failure rates are lower than perfect-use failure rates (i.e. when used consistently and correctly). b) If hormonal contraception efficacy is potentially decreased due to interaction with study treatment, condoms must be used in addition to the hormonal contraception during the treatment period and for at least 60 days after the last dose of study treatment . c) If locally required, in accordance with Clinical Trial Facilitation Group (CTFG) guidelines, acceptable hormonal contraceptives are limited to those which inhibit ovulation.

Pregnancy Testing: WOCBP should only be included after a negative highly sensitive urine or serum pregnancy test.

Pregnancy testing will be performed whenever an expected menstrual cycle is missed or when pregnancy is otherwise suspected.

Appendix 4: Response assessement criteria

Response Evaluation Criteria in Solid Tumors (RECIST) 1.1

RECIST version 1.1* will be used in this study for assessment of tumor response. While either CT or MRI may be utilized, as per RECIST 1.1, CT is the preferred imaging technique in this study.

* As published in the European Journal of Cancer:

E.A. Eisenhauer, P. Therasse, J. Bogaerts, L.H. Schwartz, D. Sargent, R. Ford, J. Dancey, S. Arbuck, S. Gwyther, M. Mooney, L. Rubinstein, L. Shankar, L. Dodd, R. Kaplan, D. Lacombe, J. Verweij. New response evaluation criteria in solid tumors: Revised RECIST guideline (version 1.1). Eur J Cancer. 2009 Jan;45(2):228-47.

In addition, volumetric analysis will be explored by central review for response assessment.

Immune Response Evaluation Criteria in Solid Tumors (iRECIST) Criteria for Evaluating Response in Solid Tumors

iRECIST will be used in this study for assessment of tumor response. While either CT or MRI may be utilized, as per iRECIST, CT is the preferred imaging technique in this study.

In addition, volumetric analysis will be explored by central review for response assessment.

Assessment at Screening and Prior to RECIST 1.1 Progression

Until radiographic progression based on RECIST 1.1, there is no distinct iRECIST assessment.

Assessment and Decision at RECIST 1.1 Progression

In participants who show evidence of radiological PD by RECIST 1.1 the Investigator will decide whether to continue a participant on study treatment until repeat imaging is obtained. This decision by the Investigator should be based on the participant's overall clinical condition.

Clinical stability is defined as the following:

- Absence of symptoms and signs indicating clinically significant progression of disease
- No decline in ECOG performance status
- No requirements for intensified management, including increased analgesia, radiation, or other palliative care

Any participant deemed clinically unstable should be discontinued from study treatment at site-assessed first radiologic evidence of PD, and is not required to have repeat tumor imaging for confirmation of PD by iRECIST.

If the Investigator decides to continue treatment, the participant may continue to receive study treatment and the tumor assessment should be repeated 4 to 8 weeks later to confirm PD by iRECIST, per Investigator assessment. I

Tumor flare may manifest as any factor causing radiographic progression per RECIST 1.1, including:

- Increase in the sum of diameters of target lesion(s) identified at baseline to ≥ 20% and ≥ 5 mm from nadir
  - Please note: the iRECIST publication uses the terminology "sum of measurements", but "sum of diameters" will be used in this protocol, consistent with the original RECIST 1.1 terminology.
- Unequivocal progression of non-target lesion(s) identified at baseline
- Development of new lesion(s)

iRECIST defines new response categories, including iUPD (unconfirmed progressive disease) and iCPD (confirmed progressive disease). For purposes of iRECIST assessment, the first visit showing progression according to RECIST 1.1 will be assigned a visit (overall) response of iUPD, regardless of which factors caused the progression.

At this visit, target and non-target lesions identified at baseline by RECIST 1.1 will be assessed as usual.

New lesions will be classified as measurable or non-measurable, using the same size thresholds and rules as for baseline lesion assessment in RECIST 1.1. From measurable new lesions, up to 5 lesions total (up to 2 per organ), may be selected as New Lesions – Target. The sum of diameters of these lesions will be calculated, and kept distinct from the sum of diameters for target lesions at baseline. All other new lesions will be followed qualitatively as New Lesions – Non-target.

Assessment at the Confirmatory Imaging

On the confirmatory imaging, the participant will be classified as progression confirmed (with an overall response of iCPD), or as showing persistent unconfirmed progression (with an overall response of iUPD), or as showing disease stability or response (iSD/iPR/iCR).

Confirmation of Progression

Progression is considered confirmed, and the overall response will be iCPD, if ANY of the following occurs:

- Any of the factors that were the basis for the initial iUPD show worsening
  - For target lesions, worsening is a further increase in the sum of diameters of ≥ 5 mm, compared to any prior iUPD time point
  - For non-target lesions, worsening is any significant growth in lesions overall, compared to a prior iUPD time point; this does not have to meet the "unequivocal" standard of RECIST 1.1
  - For new lesions, worsening is any of these:
    - An increase in the new lesion sum of diameters by ≥ 5 mm from a prior iUPD time point
    - Visible growth of new non-target lesions
    - The appearance of additional new lesions
- Any new factor appears that would have triggered PD by RECIST 1.1

Persistent iUPD

Progression is considered not confirmed, and the overall response remains iUPD, if:

- None of the progression-confirming factors identified above occurs AND
- The target lesion sum of diameters (initial target lesions) remains above the initial PD threshold (by RECIST 1.1)

Additional imaging for confirmation should be scheduled 4 to 8 weeks from the scan on which iUPD is seen. This may correspond to the next visit in the original visit schedule. The assessment of the subsequent confirmation scan proceeds in an identical manner, with possible outcomes of iCPD, iUPD, and iSD/iPR/iCR.

Resolution of iUPD

Progression is considered not confirmed, and the overall response becomes iSD/iPR/iCR, if:

- None of the progression-confirming factors identified above occurs, AND
- The target lesion sum of diameters (initial target lesions) is not above the initial PD threshold.

The response is classified as iSD or iPR (depending on the sum of diameters of the target lesions), or iCR if all lesions resolve.

In this case, the initial iUPD is considered to be pseudo-progression, and the level of suspicion for progression is "reset". This means that the next visit that shows radiographic progression, whenever it occurs, is again classified as iUPD by iRECIST, and the confirmation process is repeated before a response of iCPD can be assigned.

Management Following the Confirmatory Imaging

If repeat imaging does not confirm PD per iRECIST, as assessed by the Investigator, and the participant continues to be clinically stable, study treatment may continue and follow the regular imaging schedule. If PD is confirmed, participants will be discontinued from study treatment.

NOTE: If a participant has confirmed radiographic progression (iCPD) as defined above, but the participant is achieving a clinically meaningful benefit, an exception to continue study treatment may be considered. In this case, if study treatment is continued, tumor imaging should continue to be performed following the intervals as outlined in section 8.0.

Detection of Progression at Visits After Pseudo-progression Resolves

After resolution of pseudo-progression (ie, achievement of iSD/iPR/iCR), iUPD is indicated by any of the following events:

- Target lesions
  - Sum of diameters reaches the PD threshold (≥ 20% and ≥ 5 mm increase from nadir) either for the first time, or after resolution of previous pseudo-progression. The nadir is always the smallest sum of diameters seen during the entire trial, either before or after an instance of pseudo-progression.
- Non-target lesions
  - If non-target lesions have never shown unequivocal progression, their doing so for the first time results in iUPD.
  - If non-target lesions had shown previous unequivocal progression, and this progression has not resolved, iUPD results from any significant further growth of non-target lesions, taken as a whole.
- New lesions
  - New lesions appear for the first time
  - Additional new lesions appear
  - Previously identified new target lesions show an increase of ≥ 5 mm in the new lesion sum of diameters, from the nadir value of that sum
  - Previously identified non-target lesions show any significant growth

If any of the events above occur, the overall response for that visit is iUPD, and the iUPD evaluation process (see Assessment at the Confirmatory Imaging above) is repeated. Progression must be confirmed before iCPD can occur.

The decision process is identical to the iUPD confirmation process for the initial PD, except in one respect. If new lesions occurred at a prior instance of iUPD, and at the confirmatory scan the burden of new lesions has increased from its smallest value (for new target lesions, their sum of diameters is ≥5 mm increased from its nadir), then iUPD cannot resolve to iSD or iPR. It will remain iUPD until either a decrease in the new lesion burden allows resolution to iSD or iPR, or until a confirmatory factor causes iCPD.

Additional details about iRECIST are provided in the iRECIST publication (Seymour et al., 2017).

Appendix 5 Results of MGMT analysis by IHC and methyl-Beaming

Results obtained from ARETHUSA data accrual at the 20th of November 2019 on MGMT screening: MGMT IHC results show a ~50% of negativity (77 negative over 160 tested); MGMT promoter methylation results (by methyl-Beaming) show a positivity of ~20% (15 positive over 73 tested).