



# Phase I Study and Cell-Free DNA Analysis of T-DM1 and Metronomic Temozolomide for Secondary Prevention of HER2-Positive Breast Cancer Brain Metastases

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## ABSTRACT

**Purpose:** Preclinical data showed that prophylactic, low-dose temozolomide (TMZ) significantly prevented breast cancer brain metastasis. We present results of a phase I trial combining T-DM1 with TMZ for the prevention of additional brain metastases after previous occurrence and local treatment in patients with HER2<sup>+</sup> breast cancer.

**Patients and Methods:** Eligible patients had HER2<sup>+</sup> breast cancer with brain metastases and were within 12 weeks of whole brain radiation therapy (WBRT), stereotactic radiosurgery, and/or surgery. Standard doses of T-DM1 were administered intravenously every 21 days (3.6 mg/kg) and TMZ was given orally daily in a 3+3 phase I dose escalation design at 30, 40, or 50 mg/m<sup>2</sup>, continuously. DLT period was one 21-day cycle. Primary endpoint was safety and recommended phase II dose. Symptom questionnaires, brain MRI, and systemic CT

scans were performed every 6 weeks. Cell-free DNA sequencing was performed on patients' plasma and CSF.

**Results:** Twelve women enrolled, nine (75%) with prior SRS therapy and three (25%) with prior WBRT. Grade 3 or 4 AEs included thrombocytopenia (1/12), neutropenia (1/12), lymphopenia (6/12), and decreased CD4 (6/12), requiring pentamidine for *Pneumocystis jirovecii* pneumonia prophylaxis. No DLT was observed. Four patients on the highest TMZ dose underwent dose reductions. At trial entry, 6 of 12 patients had tumor mutations in CSF, indicating ongoing metastatic colonization despite a clear MRI. Median follow-up on study was 9.6 m (2.8–33.9); only 2 patients developed new parenchymal brain metastases. Tumor mutations varied with patient outcome.

**Conclusions:** Metronomic TMZ in combination with standard dose T-DM1 shows low-grade toxicity and potential activity in secondary prevention of HER2<sup>+</sup> brain metastases.

## Introduction

Breast cancer is the most common cancer in women, and the second most common cause of brain metastases (1). Brain metastases of breast and other cancers are a debilitating site of progression with both the

lesions and their treatment causing physical and neurocognitive impairment. In the HER2<sup>+</sup> subtype of breast cancer 25% to 50% of women with advanced disease develop brain metastases (2–4). The incidence of breast cancer brain metastases is rising, contributors include longer overall survival due to improvements in systemic therapy, limitation of therapeutic efficacy by the blood–brain barrier (BBB), and development of novel mutations in brain lesions. Most brain metastases are treated with stereotactic radiosurgery (SRS) and though follow-up data are scarce, up to half of patients will develop new brain metastases within 1 year (5). New combination therapies using HER2 kinase inhibitors with some brain permeability have increased progression-free survival (PFS) from 5.5 to 9.9 months (6–8) but further improvements are acutely needed.

A source of new therapeutic leads for brain metastases is the translational literature. Most of these preclinical experiments treat animals early and continuously, with the development of brain metastases as an endpoint, hence the therapies have a potential brain metastasis preventive effect. Where tested, preventive leads often do not shrink established lesions, likely because of drastically higher tumor burden and edema. Brain metastasis prevention was first reported using prophylactic cranial irradiation in small cell lung cancer (9), but the neurocognitive toxicity of this regimen precludes its widespread use across histologies (10, 11). Primary brain metastasis prevention trials have not been attempted in breast cancer; barriers include identification of a patient population at sufficient risk, time for completion, and cost. We and others have advocated for secondary brain metastasis trials to provide an indication of potential preventive benefit (12). Patients treated for limited brain metastases, and

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## Translational Relevance

Brain metastases are frequent in patients with metastatic HER2<sup>+</sup> breast cancer. We previously showed that prophylactic temozolomide could prevent brain metastases and prolong survival in a murine model of breast cancer metastasis. This phase I clinical trial translates those findings into a cohort of women with metastatic HER2<sup>+</sup> breast cancer to the brain after treatment with SRS or WBRT. Patients then received appropriate HER2-targeted systemic agent, T-DM1, in combination with low-dose, metronomic temozolomide for secondary prevention of brain metastases. Most toxicities were low grade. With a 9.6-month average follow-up, only two of 12 patients developed new parenchymal brain metastases. Sequencing of cell-free DNA from patients' CSF demonstrated cancer-related mutations in CSF at trial entry, despite recent local therapy, indicating ongoing brain metastatic colonization. Mutations in CSF or plasma varied with outcome. This is the first trial reporting secondary prevention of breast cancer brain metastases.

therefore at high risk for additional lesions, would receive an investigational compound with a primary endpoint of time to development of new brain metastases.

A nontraditional example of translational brain metastasis research identified temozolomide (TMZ) as a potential preventive agent. TMZ is a cytotoxic alkylating agent, has almost 100% oral bioavailability, is known to penetrate the BBB and is approved for treatment of glioblastoma multiforme (GBM) and recurrent anaplastic astrocytoma. Although not proven effective as a single agent to treat established metastatic breast cancer (13), TMZ has been extensively evaluated in the treatment of established brain metastases in combination with other agents and/or radiation therapy, showing mixed responses and good tolerability (refs. 14–18, as examples). In murine models of breast cancer, we demonstrated that TMZ significantly prevented development of brain metastases and extended survival (19). Prevention was achieved using a low TMZ dose administered in a prophylactic, metronomic fashion, distinct from the short duration, higher dose regimen used for GBM, but described in smaller clinical studies (20, 21). Consistent with the clinical literature, no effect was seen in established brain metastases preclinically.

We hypothesized that low dose, metronomic TMZ would prevent the outgrowth of brain lesions in HER2<sup>+</sup> patients, when added to a systemically active anti-HER2 treatment (22). The chosen anti-HER2 therapy was ado-trastuzumab emtansine (T-DM1), the standard second-line agent for treatment of metastatic HER2<sup>+</sup> breast cancer at the time this trial was started. Herein, we present the results of the phase I trial combining T-DM1 with TMZ for the prevention of additional brain metastases after their initial occurrence and local treatment, and an analysis of mutations in patients' plasma and cerebrospinal fluid (CSF).

## Patients and Methods

### Study design

This trial was approved by the Institutional Review Board of the Center for Cancer Research, NCI (ClinicalTrials.gov identifier: NCT03190967). It is an open-label phase I clinical trial evaluating TMZ in combination with T-DM1 for the secondary prevention of brain metastases in patients with HER2<sup>+</sup> breast cancer. It was per-

formed following a traditional 3+3 dose escalation design with standard dose T-DM1 (3.6 mg/kg intravenously every 21 days) in combination with three escalating dose levels of TMZ (30, 40, or 50 mg/m<sup>2</sup> orally daily). Treatment continued until progression of disease, development of unacceptable toxicity, or withdrawal of patient consent. The primary objective was to evaluate safety, tolerability, and identify the MTD and recommended phase II dose (RP2D) of TMZ when used in combination with T-DM1. Secondary endpoints include time to new parenchymal brain metastases development, time to whole brain radiation, and overall survival. Exploratory objectives include assessment of pharmacokinetics, neurotoxicity and neurocognitive effects, symptom burden, and quality of life. Blood and CSF were collected for correlative studies. The study was conducted in accordance with the Declaration of Helsinki, Belmont Report, and US Common Rule.

### Patient population

Informed written consent was obtained from each subject. Eligible patients were 18 years of age or older with an ECOG performance status ≤2, histologically confirmed HER2<sup>+</sup> metastatic breast cancer and adequate organ and marrow function. These patients had brain metastases that had been treated with SRS, surgical resection, or whole brain radiation therapy (WBRT) within 12 weeks of study enrollment. Patients with known leptomeningeal metastatic disease were excluded. Previous treatment with T-DM1 was allowed if patient did not have systemic progression while on it. Patients with chronic viral infections, impaired cardiac function, recent pulmonary embolism, significant peripheral neuropathy, or recent cerebral vascular accident or transient ischemic attack were not enrolled.

### Assessments

Toxicity was evaluated every 3 weeks by Common Terminology Criteria for Adverse Events (CTCAE) v4.0. Dose-limiting toxicity (DLT) was defined as grade 3 or 4 nonhematologic and grade 4 hematologic adverse events (AE) related to study medications occurring during the first cycle (21 days). Both systemic and CNS-specific effects were evaluated every 6 weeks per the RECIST v1.1 and The Response Assessment in Neuro-Oncology Brain Metastases (RANO-BM), respectively. Echocardiogram or MUGA scans were required every 3 months. CSF samples were collected at baseline and on the first day of cycle three. Blood for correlative studies was collected on the first day of each cycle. As recommended in glioblastoma treatment protocols with TMZ, CD4 counts were checked every 3 weeks and patients with counts <200/mm<sup>3</sup> received prophylactic pentamidine to prevent *Pneumocystis jirovecii* pneumonia (PJP). Other supportive care, including use of antiemetics and bisphosphonates, were allowed.

MRI images were evaluated at each cycle by an oncologist and a radiologist. After the trial was closed for analysis, all scans were evaluated blind by a single radiologist. All scans at trial enrollment were clear of parenchymal metastases; patients were also clear of leptomeningeal metastases by scans and, as needed, CSF cytology.

### Pharmacokinetics

Blood for pharmacokinetics was obtained prior to dosing and at 1-, 2-, 3-, 4-, and 8-hours after temozolomide in a subgroup of patients (*n* = 3), and plasma concentrations measured with a validated uHPLC/MS-MS method (range 5–1,000 ng/mL; ref. 23). Further, total trastuzumab serum concentrations were measured in available serum samples for comparison with literature using a commercially available

sandwich ELISA (Eagle Biosciences; sensitivity 10 ng/mL; See Supplementary Materials and Methods).

### Quality of life evaluation

The MD Anderson symptom inventory for brain tumor (MDASI-BT) and NeuroQOL Cognition Function were used in this study. Evaluations were completed by patients at baseline and at the first day of each odd-numbered cycle. An overall symptom burden and interference scores were calculated from the MDASI-BT representing global measure of symptom burden and its impact on functioning, respectively (see Supplementary Materials and Methods).

### Whole-exome sequencing and bioinformatics analysis

Cell-free DNA (cfDNA) was isolated from 2 mL of plasma and 4 mL of CSF, and whole-exome sequencing performed as described in Supplementary Materials and Methods. Briefly, all samples were mapped to the hg38 reference genome and variants were called Dragen (v4.0.3) using the tumor-only variant calling mode (24). Variants were filtered removing variants with tumor read depths <5 and an alternate allele count <2, annotated using Clin Var and common polymorphisms removed (See Supplementary Materials and Methods).

### Statistical methods

The safety evaluation was performed using a standard 3+3 design and three dose levels for a maximum of 18 total patients. The MTD was identified on the basis of the dose level at which 0 or 1 patients in 6 has a DLT. Exploratory endpoints, such as measures of neurotoxicity and neurocognitive function and symptom burden are reported at baseline and with treatment.

### Data availability

Data generated in the authors' labs are available upon request. DNA sequence data are deposited in dbGaP (<https://www.ncbi.nlm.nih.gov/gap>) under accession number phs003165.v1.p1.

## Results

### Patient characteristics

Patient characteristics are summarized in **Table 1**. Twelve patients were enrolled between April 18, 2018, and June 8, 2021. All patients were women with a median age 55.5 years (44–67). All had HER2<sup>+</sup> breast cancer, only 3 (25%) patients had tumors that were also hormone receptor positive (HR<sup>+</sup>) at initial breast cancer diagnosis. Most patients (9/12) were initially diagnosed with early-stage breast cancer, stages II and III and 8 of 12 underwent neoadjuvant therapy. In agreement with observational studies, 8 of 12 patients presented with brain metastases at the time of first recurrence diagnosis, concomitant, or not with systemic metastases. At least 5 (41%) received a previous line of systemic therapy for HER2<sup>+</sup> metastatic breast cancer, including 1 patient who received T-DM1 starting 18 months before trial enrollment.

All patients received local treatment for brain metastases. Nine (75%) patients received SRS therapy and 3 (25%) received WBRT prior to trial enrollment. Five (41%) patients also underwent brain lesion surgical resection before radiation. Four patients (33%) presented two or more episodes of brain metastases recurrence before enrolling in the current clinical trial. Two patients (16%) were receiving oral corticosteroids at trial enrollment but stopped before the second cycle of therapy.

**Table 1.** Baseline patient characteristics.

Characteristics:	Patients: (n = 12)
Median age, years (range)	55 (44–67)
Women, No. (%)	12 (100)
Race, No. (%)	
White	10 (84)
African American	2 (16)
ECOG PS, No. (%)	
0	5 (42)
1	7 (58)
Stage breast cancer at diagnosis, No. (%)	
II	5 (42)
III	4 (33)
IV	3 (25)
Brain metastases present at initial recurrence	8 (66)
Extracranial disease, No. (%)	
Measurable	5 (42)
Non-measurable	7 (58)
Prior CNS therapy No. (%)	
WBRT	3 (25)
SRS	9 (75)
Prior craniotomy/ biopsy	5/1 (42/8)
Median time since previous radiation therapy (weeks)	6 (3–12)
Episodes of prior CNS therapy	
1	8 (66)
2	2 (16)
>2	2 (16)
Prior neo/adjuvant treatment, No. (%)	
Neoadjuvant TCHP	9 (75)
Neoadjuvant T-DM1/Pertuzumab + ddAC	1 (8)
Prior treatment MBC, No. (%)	
None	6 (50)
THP	2 (16)
TCHP	1 (8)
T-DM1	1 (8)
HP	2 (16)
Median number of previous lines of treatment in metastatic setting (range)	1 (1–4)

Abbreviations: ddAC, dose dense doxorubicin and cyclophosphamide; HP, trastuzumab and pertuzumab; MBC, metastatic breast cancer; No., number; PS, performance status; TCHP, docetaxel, carboplatin, trastuzumab and pertuzumab; T-DM1, ado-trastuzumab emtansine; THP, paclitaxel, trastuzumab, and pertuzumab.

### Toxicities and dose optimization

The AEs recorded among 12 patients are listed in **Table 2**. Most AEs were grade 1 or 2 and related to gastrointestinal or laboratory findings and responded well to clinical management. Grade 3 or 4 AEs included thrombocytopenia (1/12–8.3%), neutropenia (1/12–8.3%), lymphopenia (7/12–58.3%), and decreased CD4 counts (6/12–50%). Four (33%) patients underwent dose reductions, all of them enrolled on TMZ dose level 3 (50 mg/m<sup>2</sup>); 1 patient needed two dose reductions for thrombocytopenia (one in cycle 22 and another in cycle 25), 1 patient underwent one dose level reduction on TMZ for persistent grade 1 fatigue on cycle 18, a third patient required a dose reduction in T-DM1 for thrombocytopenia on cycle 10, and the fourth patient had a dose reduction in T-DM1 on cycle 6 for Grade 2 peripheral neuropathy.

No DLT was identified. However, 4 of the 6 patients on dose level 3 of TMZ developed grade 3 CD4 decrease and required

**Table 2.** Treatment-related adverse events by maximum grade per patient.

Adverse event	Grade 1	Grade 2	Grade 3	Grade 4
<b>Hematologic</b>				
Lymphopenia	2	2	5	2
Thrombocytopenia	8	1	1	0
Leukopenia	2	5	1	0
CD4 count decreased	0	1	5	1
Anemia	2	3	1	0
Neutropenia	0	3	1	0
<b>Gastrointestinal</b>				
Nausea	10	0	0	0
Vomiting	8	0	0	0
Weight loss	1	7	0	0
Anorexia	4	0	0	0
Oral mucositis	4	0	0	0
Abdominal pain	4	0	0	0
Dry mouth	4	0	0	0
<b>Endocrinology and chemistry</b>				
AST increased	7	1	0	0
Alkaline phosphatase increased	9	0	0	0
ALT increased	7	1	0	0
GGT increased	5	1	0	0
Hypokalemia	4	0	1	0
Hyponatremia	5	0	0	0
Creatinine increased	5	0	0	0
<b>Other</b>				
Fatigue	5	1	0	0
Peripheral sensory neuropathy	2	4	0	0
Arthralgia	5	0	0	0
Headache	5	0	0	0

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transferase.

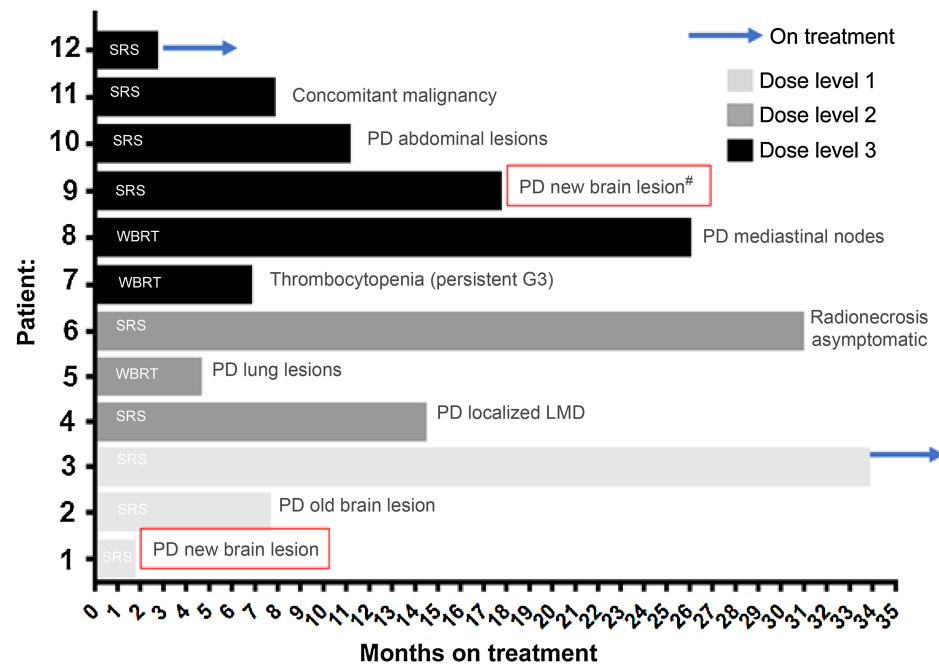
pentamidine for PJP prophylaxis (starting on C3, C5, C6, and C13). Notably those patients requiring PJP prophylaxis on C3, C5, and C6 had received either WBRT or SRS to an extensive number of lesions. One patient on dose level 2 and one patient on dose level 1 also required pentamidine intermittently at later cycles, C23 and C28, respectively. Considering that preclinical data showed a potential preventive effect using very low doses of TMZ, the potential need for continuous and extended use, and the profile seen in our phase I (potential effectiveness in all three levels, but clear early and important CD4 decreases), we opted to continue with a lower dose. The RP2D of TMZ is 40 mg/m<sup>2</sup> when combined with T-DM1.

#### Clinical activity

**Figure 1** depicts time on study and main outcomes. The median time on study was 9.6 months, ranging from 2.8 to 33.9 months at the time of these analyses. Four patients completed 1 year on study treatment. Ten patients without new parenchymal brain metastases are no longer on study: 1 for an asymptomatic new brain lesion on MRI, subsequently determined to be radionecrosis; 1 due to progressive growth at a previously irradiated site; 1 for focal leptomeningeal disease after neurosurgical reintervention; 1 for a new cancer (chronic myeloid leukemia, CML) unrelated to treatment; 3 for progression of systemic metastases (mediastinum, lung, and abdomen); and 1 for persistent thrombocytopenia. Two patients presented with new parenchymal brain metastases lesions, one of them at 1.8 months in the study, and the other at 17.8 months. The patient who had CNS progression at 17.8 months had more than 20 brain lesions and several rounds of SRS therapy before enrolling in this study. Two patients remained on study for 33.9 and 2.8 months at the cutoff date for analysis on September 1, 2021. Patient outcomes did not correlate with brain metastasis development as first metastatic recurrence or later.

**Figure 1.**

Time on study and outcomes. Patients received a standard regimen on 3.6 mg/m<sup>2</sup> of T-DM1 intravenously every 21 days and one of three indicated doses of oral TMZ (30, 40, or 50 mg/m<sup>2</sup>, dose levels 1–3, respectively), daily for each 21-day cycle. Patient number is indicated to the left. Initial local treatment and status as of 10 of 21 data cutoff is shown. Only 2 patients developed new parenchymal brain metastases over a median of 9.6 months. # >20 lesions at baseline. \*, Radionecrosis confirmed after patient off study for suspected PD that was not confirmed.



## PK studies

Metronomic oral temozolomide PK was assessed in 3 patients given doses ranging from 30 to 50 mg/m<sup>2</sup>. The dose normalized  $C_{max}$  and AUC were lower than prior reports of similar oral doses (25, 26). Accompanying the lower-than-expected exposure were a slightly faster apparent oral clearance (140 mL/min/m<sup>2</sup>, compared with 92–122 mL/min/m<sup>2</sup>, and a larger distribution volume (58 L/m<sup>2</sup> vs. 14.5 L/m<sup>2</sup>; refs. 25, 26). Surprisingly, the half-life in these patients was longer (5.8 hours vs. 1.8 hours); it is unclear why this may be, unless an interaction with emtansine (DM-1) occurred. Total trastuzumab serum trough concentrations averaged 12.8 µg/mL, which is higher than literature values (range 1–4 µg/mL), possibly due to the assay method which measures fully, partially, and un-conjugated T-DM1 in serum samples (27).

## Symptom burden, and perceived cognitive function

All 12 patients completed the MDASI-BT and NeuroQOL Cognition Function at baseline and at Day 1 of each odd-numbered cycle. Completion rates for the MDASI-BT were 99% by Cycle 15 and 90% by Cycle 41. Because of the limited sample size, no significance testing was performed but descriptive examination of longitudinal changes in MDASI-BT symptom factors and interference showed distinct patterns (Supplementary Fig. S1). Patients whose disease progressed by Cycle 15 ( $n = 5$ ) reported more severe symptom factors and greater interference compared with patients who did not progress ( $n = 7$ ). Similarly, patients whose disease progressed by Cycle 15 had lower NeuroQOL Cognition Function  $T$  scores compared with the general population whereas patients with no disease progression had higher NeuroQOL Cognition Function  $T$  scores above the normal population.

## cfDNA sequencing (cfDNA-seq)

We asked if mutation patterns in plasma or CSF provided additional information relevant to brain metastasis progression generally or the potential preventive effect of TMZ +TDM1. Plasma was collected at every treatment cycle and a lumbar puncture was performed at trial entry and after the end of cycle 2 of treatment (Fig. 2A). We also included the plasma sample from each patient's last treatment cycle, which varied from 2 to 31 months, as well as one cycle prior. cfDNA was extracted from plasma and CSF samples, whole-exome sequencing performed, and two published panels and one published analysis were used for identification of mutations: (i) MSKCC-Impact panel containing 342 key cancer genes (28); (ii) ClinVar, containing medically important variants in disease (29), (iii) OncodriveCLUST analysis of driver mutations (ref. 30; Fig. 2B–D). Total mutational burden was comparable within samples from a patient, and between patients (Supplementary Fig. S2). Supplementary Figs. 3 to 4 list the HER2 (ERBB2) variant/mutation status and copy numbers, respectively; germline ERBB2 was not sequenced for comparison.

Each patient's plasma and CSF samples exhibited a list of mutations distinct from that of other patients, in agreement with previous reports (31). In the CSF samples collected at trial entry and after C2, 77 unique mutations were identified. Of these, 11 (14%) were unique to the CSF, suggesting clonal diversion in brain metastasis development.

Two subgroups of the patients were of high interest: Patients 1 and 9 developed new parenchymal brain metastases at 2 and 18 months on trial, respectively. Four patients were designated long-term responders: Patients 3, 6, and 8 experienced  $\geq 1$  year free from a new parenchymal brain metastasis. Patient 3 remained on trial for 34 months as of January 09, 2021, patient 6 left after developing a suspected progression (unconfirmed after a diagnosis of radionecrosis

in subsequent evaluations) at 31 months on trial, and patient 8 left the trial at 26 months for systemic progression in a mediastinal node. Patient 4 also experienced over a year free from a new parenchymal brain metastasis but developed leptomeningeal metastasis (which was not included in the primary endpoint) 14.5 months from entry into the trial. Figure 3 shows mutation patterns of potential interest in the plasma or CSF of these two groups. Patients 1 and 9 both exhibited mutations in ARID1 gene: patient 1 had a missense mutation in ARID1B in plasma at the time of new brain metastasis development; patient 9 had a missense mutation in ARID1A in all samples. ARID1A mutations were not present in any of the four long-term responders. In contrast, three of the four long-term responders exhibited a DNMT3A mutation. Missense mutations in DNMT3A were apparent in all plasma samples of patients 3 and 6, whereas patient 8 exhibited a pathogenic missense mutation in all plasma samples and the entry CSF sample. DNMT3A mutations were not found in patients 1 and 9 who developed new parenchymal brain metastases. Additional mutations present in at least two of the four long-term survivors include TP53, AR, and ACTN2, and were not present in samples from the 2 patients who developed new brain metastases. Although based on a relatively small number of patients, the data are hypothesis generating, suggesting a correlation of epigenetic programs and other gene mutations with outcome.

Table 3 lists mutations identified in the patients' CSF at trial entry. At that time the patients' MRIs were clear of any new parenchymal or leptomeningeal brain metastases. Only one patient underwent a recent neurosurgic procedure, 11 weeks prior to trial entry. Of the 12 patients, 10 evidenced mutations in their CSF. Four patients exhibited a mutation in the potassium channel gene KCNJ18, identified on an analysis of all disease mutations. The remaining 6 patients exhibited multiple cancer-associated mutations identified on the MSKCC Impact panel, including the 2 patients who developed new parenchymal brain metastases on trial. Well known, mutated cancer genes identified in patient CSF at trial entry included ERCC4, NOTCH4, RPTOR, ALK, FANCA, MYCN, ERBB2, and AR, among others. These data provide molecular evidence of ongoing brain metastatic colonization in patients at trial entry.

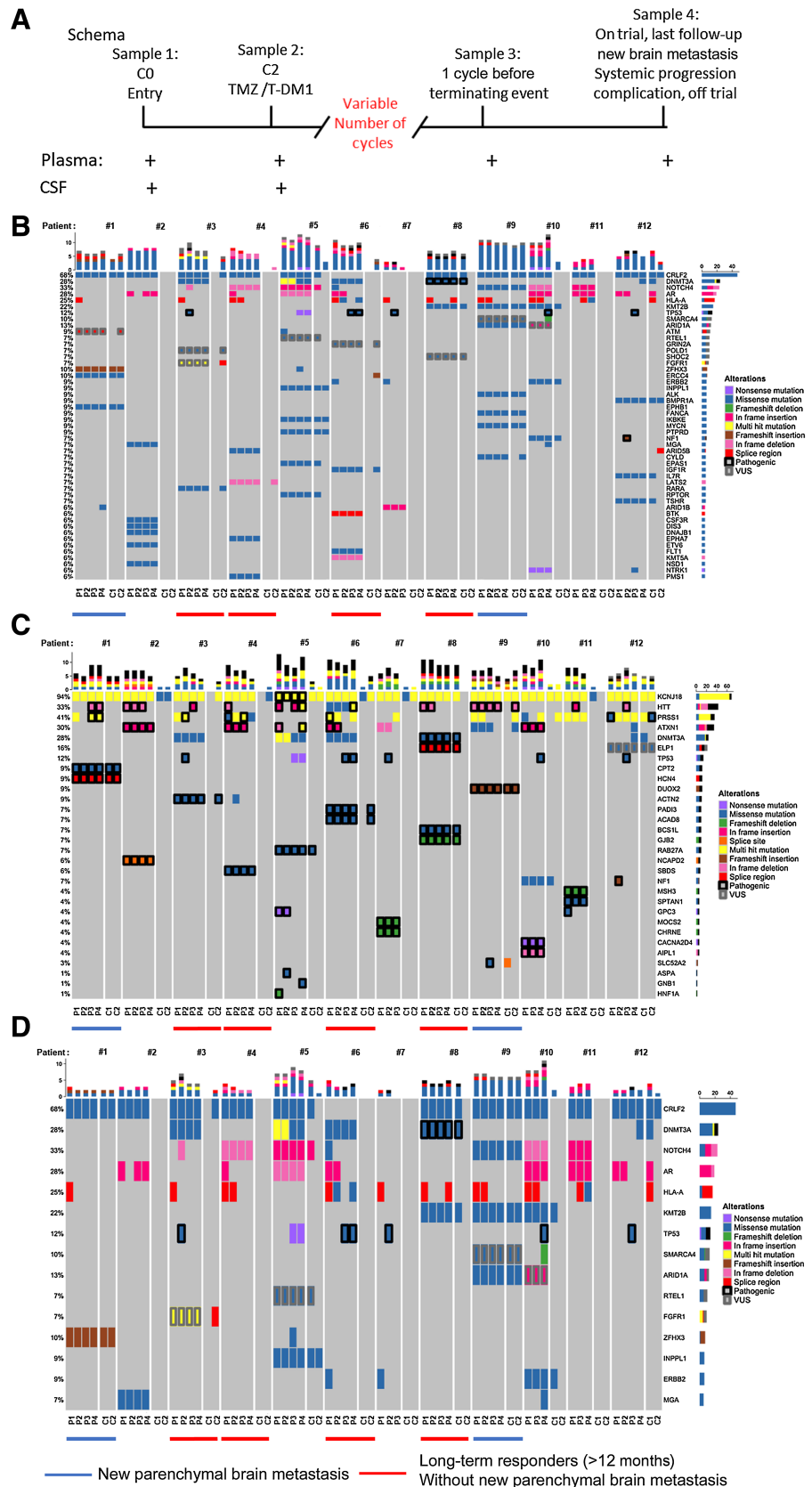
## Discussion

This is the first reported trial on secondary prevention of breast cancer brain metastases. On the basis of preclinical data that found an unexpected brain metastasis preventive activity for low dose, metronomic TMZ (19) and demonstrated activity of T-DM1 in the treatment of brain metastases (32), this phase I trial tested the combination of T-DM1 and three different dose levels of daily TMZ in a series of patients previously treated for brain metastases of HER2<sup>+</sup> breast cancer. This study established the safety and the RP2D of 40 mg/m<sup>2</sup> of TMZ for the combination of T-DM1 and TMZ. It also indicated preliminary evidence of potential activity in the prevention of secondary brain metastases in patients with HER2<sup>+</sup> breast cancer. The analysis of patient plasma and CSF samples advances our molecular understanding of brain metastatic colonization, a definition of high risk, and potential correlates of efficacy.

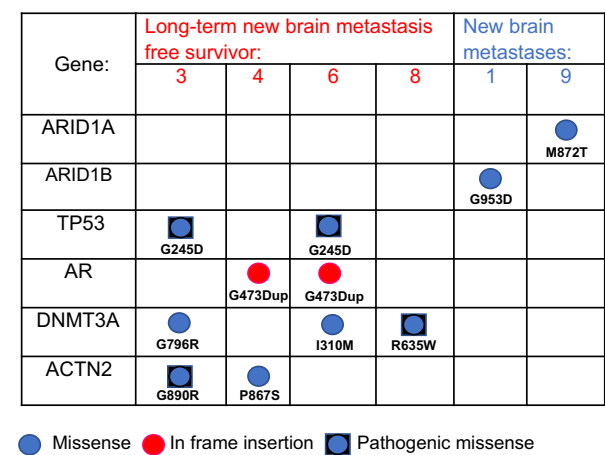
The combination TMZ and T-DM1 was well tolerated with the majority of toxicities related to hematologic and liver function laboratory abnormalities. The 2 patients requiring dose reductions due to thrombocytopenia were enrolled 6 weeks after WBRT. One of the patients who went off treatment for thrombocytopenia, underwent investigation with bone marrow biopsy and no marrow infiltration or

**Figure 2.**

Whole-exome sequencing of plasma and CSF cfDNA samples from patients with HER2<sup>+</sup> breast cancer. **A**, Schematic of phase I study with regard to samples collected. The sample collection time points of four plasma samples and two CSF samples are labeled as “+.” **B-D**, Identification of genomic alterations in the plasma and CSF cfDNA samples by Patient Number (**Fig. 1**). For each sample, genomic alterations were compared with known variants for cancer-related genes in the MSK-IMPACT panel (**B**), and main pathogenic/likely pathogenic for all diseases in the ClinVar database (**C**). The potential driver mutations were identified using the OncoPrintCLUST method (**D**). **P**, plasma. The bar graph above each panel totals the number of mutations detected in each cfDNA sample. On the left, the percentage of cfDNA reads with mutations in the genes is listed. On the right, the distribution of mutation type per gene is indicated. On the bottom of each panel, blue lines identify 2 patients who developed new parenchymal brain metastases during this trial, whereas red lines identify four long-term survivors (>12 months without a new parenchymal brain metastasis).







**Figure 3.** Mutation patterns for patients with new brain metastases versus long-term responders. Potential relationship between mutations in plasma and CSF cfDNA throughout the study, and the patients’ clinical outcome. Two patients who developed new brain metastases during this trial are listed as “New Brain Metastasis,” whereas 4 patients who remained free of a new parenchymal brain metastasis for at least 12 months were identified as “Long-term survivors” (see Fig. 1). The type of mutations is indicated by a colored circle.

myelodysplasia was demonstrated. This patient had already presented with grade 1 thrombocytopenia at enrollment, and we concluded it continued to worsen due to the cumulative toxicity from recent and previous cytotoxic therapies, including TMZ and T-DM1. One patient was diagnosed with CML without additional karyotypic abnormalities while on C11 of treatment in the trial. Careful hematologic evaluation deemed the diagnosis unrelated to trial treatment, which would be expected to present with a more aggressive phenotype and with a complex karyotype.

Overall, no strong scientific basis exists to raise concerns about TMZ and T-DM1 drug interactions. From a drug metabolism perspective, TMZ does not utilize the common pathways used by other enzymatically metabolized agents (CYPs, GSTs, UGTs, etc.) and is largely catabolically degraded into various species (33). Regarding T-DM1, trastuzumab is not enzymatically metabolized, while emtansine (DM-1) is metabolized by CYP3A4/5. TMZ does not utilize those pathways. Our PK analysis was limited to sampling a group of patients. Possible interactions extending the half-life of TMZ may have occurred and will prompt further evaluation, with a larger number of patients.

The main goal of this phase I trial is to evaluate safety and any consideration regarding clinical activity is exploratory. Considering the use of TMZ and T-DM1 as a preventive agent for new brain metastases for patients with HER2<sup>+</sup> metastatic breast cancer, only 2 (16%) patients presented a clear new metastatic lesion in the brain parenchyma, one of them beyond 12 months on trial. One of these 2 patients had a new brain lesion only 1.8 months after starting the trial, raising questions regarding the potential presence of subclinical metastases already at enrollment. The second patient was enrolled with more than 20 brain lesions, after multiple repeated recurrences in the brain and several SRS procedures, suggesting a higher chance of local recurrence. Historic estimates for the development of new brain metastases after an initial occurrence are few. Kased and colleagues estimated that 50% of patients experience an additional brain metastasis within 1 year, although this estimate is

not recent or limited to HER2<sup>+</sup> disease (5). Radiosurgical practices continue to evolve, and it would be helpful to obtain additional actuarial data on brain metastasis-free survival post-SRS. Alternatively, a randomized phase II trial comparing the efficacy of T-DM1/TMZ versus T-DM1 alone or standard of care therapy is needed. Given the preclinical data on TMZ prevention of brain metastases (19) and trends for T-DM1 in trials (32), it is possible that both drugs provided preventive activity.

In addition to brain metastases, progression in the leptomeninges was noted. One patient had a negative biopsy of a previously treated brain metastases to rule out recurrence during C13 and presented with two leptomeningeal areas of progression in the cycles after that. TMZ distribution in the CSF is reported to be limited (34).

Another component of this trial is the incorporation of patient reported outcomes. We used the MDASI-BT patient reported outcome tools, which have been used in recent brain metastases studies exploring the impact of hippocampal avoidance with whole brain radiotherapy as well as the Neuro-QOL Cognition Function reporting instrument (35) and obtained excellent survey completion rates. The preliminary analysis of neurocognitive symptoms and overall symptom burden in this small group of patients demonstrated that progression of disease at any site is potentially one of the most important factors impacting this aspect of a patient’s life.

Although in a small number of patients, our results are very encouraging in terms of safety, potential efficacy, and quality of life in patients currently expected to have rapid and continuous progression of their brain metastases. A phase II secondary prevention trial in the HER2<sup>+</sup> subgroup is warranted. Overall, 6 (50%) patients went off trial treatment before the 1-year mark, only 1 of them with new brain metastasis, exposing the importance of evaluating and increasing options of anti-HER2 specific therapy that has better control of systemic disease, to give in combination with TMZ. Although we

**Table 3.** DNA mutations in patients’ CSF at trial entry.<sup>a</sup>

Patient	Subgroup	Mutations identified in CSF
1	New brain metastasis	CRLF2, ZFXH3, ERCC4, EPHB1, KCNJ18, CPT2, HCN4
2		KCNJ18
3	Long-term survivor	
4	Long-term survivor	
5		CRLF2, NOTCH4, RTEL1, INPPL1, IKBKE, PTPRD, EPAS1, RPTOR, KCNJ18, RAB27A
6	Long-term survivor	KCNJ18
7		KCNJ18
8	Long-term survivor	CRLF2, DNMT3A, KMT2B, SHOC2, ELP1, BCS1L, GJB2, KCNJ18
9	New brain metastasis	CRLF2, NOTCH4, KMT2B, SMARCA4, ARID1A, ALK, FANCA, MYCN, KCNJ18, DUOX2, SLC52A2
10		KMT2B, ERBB2, NF1, KCNJ18
11		KCNJ18
12		CRLF2, DNMT3A, AR, HLA-A, BMPR1A, IL7R, TSHR, KCNJ18, PRSS1, ELP1

<sup>a</sup>Whole-exome sequencing of CSF samples from 12 patients at trial entry. New brain metastasis patients developed a new parenchymal brain metastasis while on trial. Long-term survivors were free of new parenchymal brain metastases for at least 12 months on trial.

originally considered 1-year freedom from a new brain metastasis to be a primary endpoint for a phase II trial, it is apparent in this population that systemic progression and CNS relapse at previously treated sites are rapid and frequent. A phase II trial that combines TMZ with physician's choice of multiple HER2-directed drugs with systemic activity may represent an improved design, so that systemic progression on one combination can move to another within the trial, and thereby complete 1 year of enrollment. Other aspects of clinical trial design that merit refinement include: a clear definition of new brain metastases versus confounding diagnoses (such as radionecrosis); ideal frequency of brain images; optimal number of previous brain lesions; and ideal number of previous recurrences.

Analysis of patient plasma and CSF by whole-exome sequencing of cfDNA mutations provided a window into facets of brain metastatic progression and prevention not previously investigated. As examples, prior efforts have examined CSF and plasma from limited patients with brain and/or leptomeningeal metastasis to demonstrate that mutations in parenchymal brain metastases were present in CSF (36) and a correlation of mutations in CSF with clinical course in a melanoma leptomeningeal metastasis patient (37). Both CSF and plasma samples were collected at trial entry and after C2, with CSF providing a potential window into CNS-specific mutational events. Mutations in 14% of the CSF samples were distinct from those of the matched plasma, indicating ongoing clonal evolution in the CNS.

We separated out two subgroups of patients with clear outcomes, 2 patients who developed new parenchymal brain metastases compared with 4 patients with >12-month new parenchymal brain metastasis-free survival for further analysis. Although limited by small patient numbers, across all samples, DNA mutational events separated these groups. Interestingly, both patients with new brain metastases showed cfDNA mutations in the ARID1 gene. ARID1 is a member of the SWI/SNF chromosome remodeling complex which, in its wild-type state, is regarded as a tumor suppressor involved in maintaining a luminal phenotype in breast cells. In metastatic breast cancer, ARID1A mutation is the most prevalent mutation of the SWI/SNF chromatin remodeling complex (38), whereas ARID1B is involved in DNA repair and contributes to several cancer phenotypes (39). Evidence suggests that ARID1B rescues loss of ARID1A (40, 41). In contrast, three of four long-term responders showed mutations in DNMT3A. DNMT3A is a member of a DNA methyltransferase family involved in epigenetic gene regulation; the 3A member acts as a *de novo* DNA methyltransferase in embryogenesis including CNS maturation. In cancer, DNMT3A is often mutated with pro- and anti-tumor effects (42). Other mutations identified in at least two of the long-term responders included AR, TP53, and ACTN2. These data are preliminary due to the small number of patients analyzed but suggest the hypothesis that mutations may be identified that predict brain metastasis development under this regimen. Also, the data suggest the intriguing hypothesis that chromatin modifiers play a fundamental role in this process, consistent with data from other types of brain metastases (e.g., refs. 43, 44).

Finally, 6 of 12 patients had cancer-linked DNA mutations, identified by the MSKCC Impact panel, in their CSF at study entry despite local therapy to prior brain metastases and a concurrent brain MRI without new lesions. These included well-studied genes such as NOTCH4, FANCA, MYCN, ALK, IL7R, and ERCC4 involved in DNA repair, oncogenesis, and immune function. The most likely cause for the presence of cfDNA mutations is ongoing brain metastatic colonization. Other potential contributors include recent surgical

intervention, leptomeningeal disease, and possibly radiation therapy. Only 1 patient had undergone recent CNS surgery (11 weeks prior to enrollment), and the median interval since local radiation was 6 weeks for this cohort. Given estimates of the half-life of cfDNA *in vivo* at under 2 hours (45), contributions from these sources are unlikely. All patient MRIs were re-reviewed by a single radiologist to confirm the absence of parenchymal or leptomeningeal disease. The data indicate that brain metastatic colonization could be ongoing in this population with no traditional evidence of active CNS disease and reinforce the validity of the enrollment criteria designed to test a population at high risk for the development of additional brain metastases.

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