cancers

MDPI

Supplementary Materials

The Prognostic Impact of Circulating Tumour DNA in Melanoma Patients Treated with Systemic Therapies—Beyond *BRAF* Mutant Detection

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Additional Materials and Methods

Plasma Samples Preparation and cfDNA Extractions

Blood samples were collected prior to initiation of treatment and during subsequent follow-ups. For most patients, bloods were collected within a week prior treatment initiation (102/142, 72%), extending for some patients to up to -51 days, with no treatment received during this period. Blood was collected into EDTA vacutainer or Cell-Free DNA BCT® (Streck, La Vista, NE) tubes. Within 24 hours of blood collection, plasma was separated by centrifugation at 300 g for 20 minutes, followed by a second centrifugation at 4700 g for 10 minutes. All isolated plasma was stored at -80°C until extraction. Plasma cell-free DNA (cfDNA) was isolated from 1–5 mL of plasma using QIAamp Circulating Nucleic Acid Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The recovered cfDNA was eluted in 40 µL AVE buffer (Qiagen, Hilden, Germany) and stored at -80°C until ctDNA quantification by droplet digital polymerase chain reaction (ddPCR).

Tissue Analysis

Mutational profile of *BRAF* WT patients were identified from tissue biopsies as previously described by Calapre et al. [1]. A custom targeted next generation sequencing panel of 30 melanoma-associated genes (Illumina, San Diego, CA, USA) with 950 amplicons and an Illumina MiSeq instrument were used to identify mutational targets for circulating tumour DNA (ctDNA) analysis in *BRAF* WT patients. Genomic variants were annotated using the Illumina Variant Studio 3.0 software (Illumina). Mutational targets were selected based on the criteria previously described [1].

Plasma ctDNA Analysis

Commercially available and/or customised probes were used to analyse ctDNA by ddPCR. Droplets were generated using an Automatic Droplet generator QX200 AutoDG (Bio-Rad, Hercules, CA). Amplifications were performed in 40 µL reactions using cycling conditions previously described [2]. Twenty-six different mutation variants in 10 different genes were utilised. Customised primers and probes for *TERT* and *DPH3* promoter mutation analyses were performed as previously reported by McEvoy et al. [3] and Calapre et al. [1], respectively. Limit of blank for the ctDNA assays was determined using normal plasma samples from at least 10 healthy controls. Levels of ctDNA were defined based on the level of false positive droplets as previously specified in Calapre *et al.* [1] or are detailed in Table S2. Samples yielding copies/mL of plasma equal or below the maximum false positive concentration were deemed ctDNA negative.

Whole Exome Sequencing

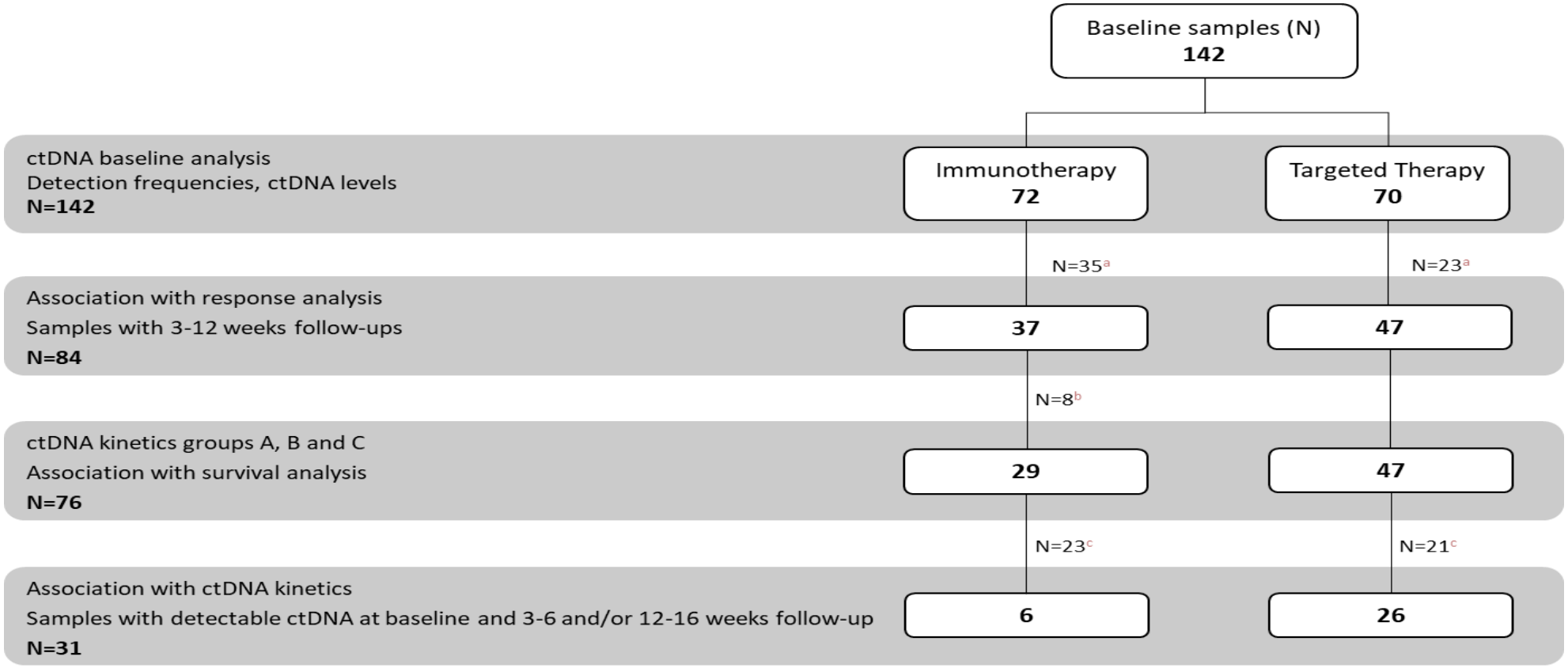
The concentrations of cfDNA used for WES ranged from 1 to 7 ng/uL of cfDNA, with ctDNA fraction >7% abundance. Whole exome sequencing (WES) was carried out using the Exome-seq

Agilent V6 capture Kit (Agilent) by Novogene (Hong Kong, China). Sequence reads were aligned against human reference genome (hg19) using the Burrows-Wheeler aligner (BWA). Duplicate reads were marked with Picard Tools, reads were realigned against known indels and base qualities recalibrated using Genome Analysis Toolkit. As BWA assumes a unimodal distribution of fragment size, we adjusted the Proper Pair bit in read pairs following the approach in BWA but the fragment sizes were fitted against a mixture of two Gaussian models.

Somatic variants were identified with an in-house tool using the statistical framework described by Li et al. [4]. We used a model assuming diploid germline and calculated the phred-scaled likelihoods for possible genotypes. The tumour sample was modelled as a mixture of tumour and normal cell DNA and likelihoods were calculated for an array of different variant allele frequencies. The constrained log-likelihood ratio (CLR) was calculated and variants with CLR score of < 70 were excluded from further analysis. Identified variants were further annotated using ANNOVAR.

Neoepitope Load Prediction

To predict neoantigens formed by the somatic variants, we used pVACseq v4.0.9 [5] with epitope lengths 8-11 and NetMHCpan binding predictions [6]



[a] Exclusion of samples without follow-up within 12 weeks of therapy. [b] Exclusion of ipilimumab cases. [c] Exclusion of samples without detectable ctDNA at baseline and assessable 3-6 and/or 12-16 week follow-ups.

Figure S1. Flow chart showing group of samples included in the analyses.

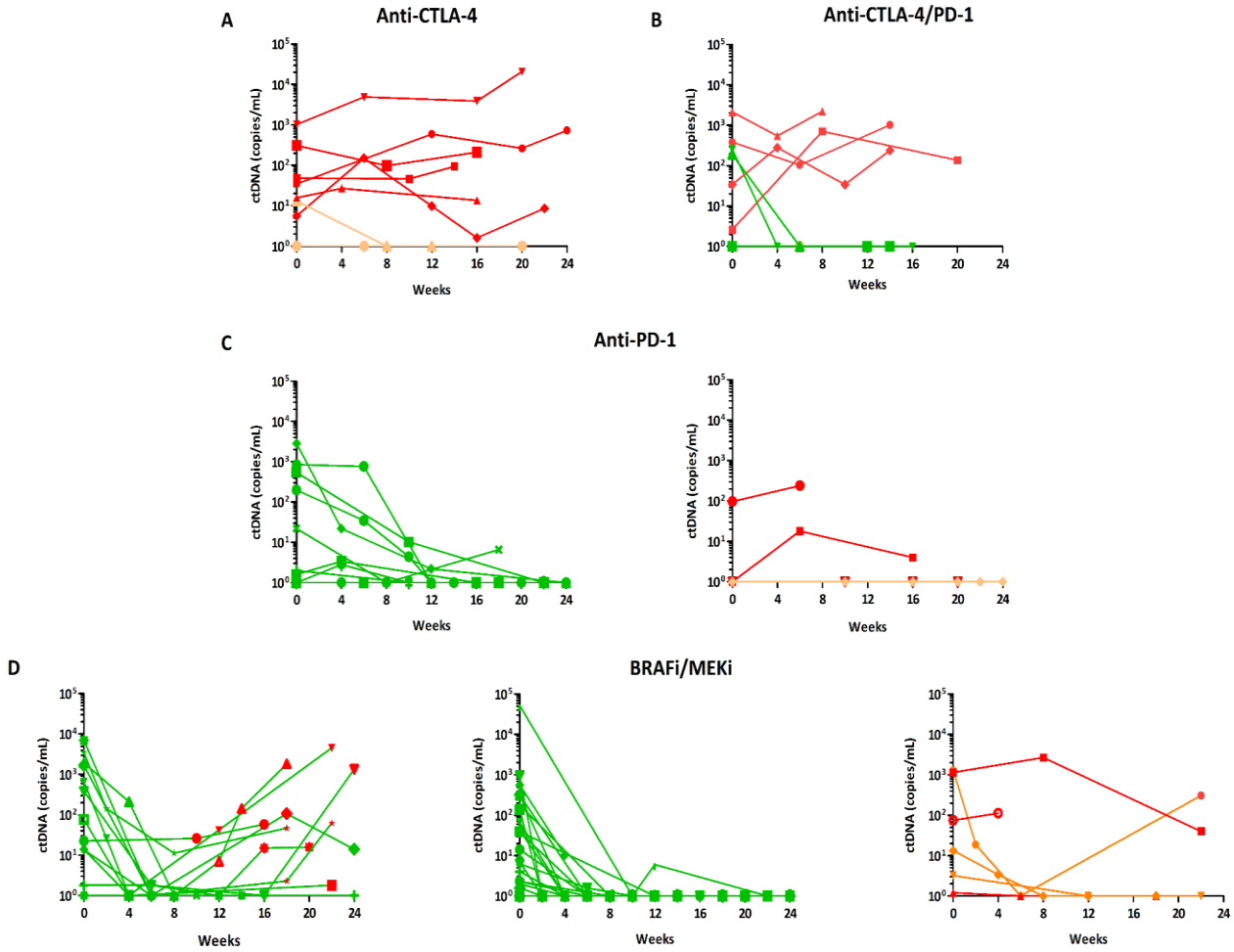


Figure S2. Kinetics of ctDNA decay. Time course of biological response for patients undergoing first or second-line treatment with (**A**) anti-CTLA-4 (N = 8), (**B**) anti-CTLA-4 plus anti-PD-1 (N = 8), (**C**) anti-PD-1 (N = 21) or (**D**) targeted therapy (BRAF/MEKi) (N = 47). Solid lines in green, orange and red denotes treatment responders, stable disease and non-responders, respectively. Solid lines in green with red symbol represents patients that developed resistance to targeted therapy.

Table S1. Demographic, clinicopathologic and treatment characteristics of included samples.

Variable	All samples N = 142	Immunotherapy N = 72 (%)	Targeted Therapy N = 70 (%)
Age			
20-49		11 (15)	20 (29)
50-69		38 (53)	31 (44)
70-99		23 (32)	19 (27)
Sex			
Female		18 (25)	25 (36)
Male		54 (75)	45 (64)
M Classification			
M1a		16 (22)	15 (21)
M1b		7 (10)	6 (9)
M1c		31 (43)	32 (46)
M1d		18 (25)	17 (24)
Mutational Status			
BRAF Mutant		30 (42)	70 (100)
NRAS Mutant		20 (28)	
Others		22 (30)	
Treatment			
Anti-PD1 inhibitor			
Pembrolizumab		40 (56)	
Nivolumab		1 (1)	
Anti-CTLA-4 inhibitor			
Ipilimumab		12 (17)	
Anti-PD-1 plus anti-CTLA-4 inhibitor			
Ipilimumab/Nivolumab		19 (26)	
BRAFi			
Vemurafenib			4 (6)
Dabrafenib			1 (1.5)
BRAFi plus MEKi			
Dabrafenib/Trametinib			64 (91)
Vemurafenib/Cobimetinib			1 (1.5)

10 **Table S2.** Specificity of droplet digital polymerase chain reaction (ddPCR) assays.

Assay	Healthy controls		Maximum false positive concentration (copies/mL)
	Positive	Negative	
BRAF V600E	0	22	0
BRAF V600K	0	23	0
BRAF V600R	0	24	0
BRAF V600E2	1	12	1
BRAF K601E	3	7	2
BRAF L597Q	0	16	0
NRAS Q61K	0	19	0
NRAS Q61L	3	9	7
NRAS Q61P	1	9	2
NRAS Q61R	7	24	9
NRAS G12D	4	6	3
NRAS G13D	4	6	5
TERT C228T	*1*	29	*2*
TERT C250T	2	18	4
DPH3 C8T	1	10	2
FLT1 T543I	1	17	1
FLT1 E011K	3	7	9
NF1 P1851S	3	11	3
KIT L576P	1	11	2
KIT V559A	0	12	0
KIT W557R	0	13	0
RAC1 P29S	0	12	0
TP53 R248Q	6	8	2
TP53 R248W	2	14	2
TP53 R158H	1	9	2
GRM3 E538K	0	10	0
GRM3 S491L	6	12	6

Table S3. Clinical characteristics at baseline of the melanoma patients categorised in Groups A, B and C included in the survival analysis (N = 76).

	Immunotherapy					Targeted Therapy				
	Group A	**Group B**	**Group C**	**Total**	***P*-value* (A/B/C)**	**Group A**	**Group B**	**Group C**	**Total**	***P*-value* (A vs B)**
Variable	N=14 (%)	N=8 (%)	N=7 (%)	N=29 (%)		N=11 (%)	N=33 (%)	N=3 (%)	N=47 (%)	
Age										
≤65	5 (36)	6 (75)	3 (43)	14 (48)	0.196	5 (45)	18 (55)	2 (67)	25 (53)	0.601
>65	9 (64)	2 (25)	4 (57)	15 (52)		6 (55)	15 (45)	1 (33)	22 (47)	
Gender										
Female	3 (21)	1 (12)	4 (57)	8 (28)	0.120	5 (45)	10 (30)	1 (33)	16 (34)	0.359
Male	11 (79)	7 (88)	3 (43)	21 (72)		6 (55)	23 (70)	2 (67)	37 (66)	
M Classification										
M1a/M1b	9 (67)	2 (25)	1 (14)	12 (41)	**0.049**	2 (18)	9 (27)	1 (33)	12 (26)	0.546
M1c/M1d	5 (36)	6 (75)	6 (86)	17 (59)		9 (82)	24 (73)	2 (67)	35 (74)	
Brain metastasis										
Yes	3 (21)	2 (25)	1 (14)	6 (21)	0.873	6 (55)	5 (15)	1 (33)	12 (26)	**0.009**
No	11 (79)	6 (75)	6 (86)	23 (79)		5 (45)	28 (85)	2 (67)	36 (74)	
Brain only metastasis										
Yes	2 (14)			2 (7)	-	2 (18)			2 (4)	**0.012**
No	12 (86)	8 (100)	7 (100)	27 (93)		9 (82)	33 (100)	3 (100)	45 (96)	
ECOG status										
0	10 (71)	4 (50)	3 (43)	17 (59)	0.385	9 (82)	21 (64)	2 (67)	32 (68)	0.262
1-3	4 (29)	4 (50)	4 (57)	12 (41)		2 (18)	12 (36)	1 (33)	15 (32)	
LDH levels										
Normal	7 (50)	2 (25)	1 (14)	10 (34)	-	8 (73)	13 (39)		21 (44)	-
Abnormal	1 (7)	4 (50)	3 (43)	8 (28)			13 (39)	1 (33)	14 (30)	
Not available	6 (43)	2 (25)	3 (43)	11 (38)		3 (27)	7 (22)	2 (67)	12 (26)	
Prior lines of therapy										
Yes	6 (43)	4 (50)	1 (14)	11 (38)	0.316	1 (6)	2 (6)		3 (6)	0.729
No	8 (57)	4 (50)	6 (86)	18 (62)		10 (94)	31 (94)	3 (100)	44 (94)	
BRAF mutational status										
BRAF Mutant	6 (43)	2 (25)	1 (14)	9 (31)	0.373	11 (100)	33 (100)	3 (100)	47 (100)	-
BRAF WT	8 (57)	6 (75)	6 (86)	20 (69)						

*The *P*-value was calculated using the Chi-square test

Table S4. Univariate and multivariate Cox proportional-hazards regression analysis for associations between ctDNA levels and survival.

	Group A/B/C Immunotherapy				**Group A/B Targeted Therapy**			
UNIVARIATE								
Variables	**Progression free survival**		**Overall survival**		**Progression free survival**		**Overall survival**	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Age (≤65 vs. >65)	0.46 (0.15 - 1.40)	0.174	0.83 (0.25 - 2.73)	0.761	1.18 (0.60 - 2.34)	0.625	1.06 (0.45 - 2.50)	0.894
Gender (female vs. male)	0.58 (0.19 - 1.75)	0.333	1.24 (0.31 - 5.03)	0.758	1.49 (0.71 - 3.12)	0.294	1.17 (0.47 - 2.90)	0.738
M Classification (M1a/b vs. M1c/d)	1.93 (0.64 - 5.83)	0.242	3.81 (0.80 - 18.08)	0.092	0.97 (0.44 - 2.15)	0.947	1.54 (0.52 - 4.60)	0.436
Brain metastasis (no vs. yes)	1.98 (0.60 - 6.50)	0.262	1.795 (0.46 - 7.01)	0.404	1.57 (0.74 - 3.34)	0.240	1.765 (0.71 - 4.39)	0.223
Brain only metastasis (no vs. yes)	-	-	-	-	2.76 (0.63 - 12.18)	0.179	4.19 (0.92 - 19.00)	0.063
ECOG (0 vs. 1-3)	1.52 (0.53 - 4.34)	0.437	1.82 (0.55 - 6.01)	0.323	**2.34 (1.16 – 4.75)**	**0.018**	**4.56 (1.88 – 11.06)**	**0.001**
Prior lines of therapy (no vs. yes)	1.00 (0.35 - 2.89)	0.998	0.63 (0.18 - 2.20)	0.464	1.12 (0.34 - 3.67)	0.852	0.57 (0.07 - 4.24)	0.580
BRAF mutational status (WT vs. mut)	0.77 (0.35 - 2.31)	0.636	0.72 (0.20 - 2.53)	0.607	-	-	-	-
ctDNA levels								
Group A vs Group B	2.07 (0.51 - 8.36)	0.305	1.61 (0.32 - 8.06)	0.563	2.590 (0.99 - 6.77)	0.052	1.73 (0.57 - 5.18)	0.329
Group A vs Group C	**9.16 (2.47 - 33.93)**	**0.001**	**7.46 (1.70 - 32.76)**	**0.008**				
MULTIVARIATE								
Variables	**Progression free survival**		**Overall survival**		**Progression free survival**		**Overall survival**	
	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	P-value	HR (95% CI)	*P*-value
Age (≤65 vs. >65)	0.32 (0.09 - 1.15)	0.081	**7.33 (1.01 - 53.25)**	**0.049**				
Gender (female vs. male)								
M Classification (M1a/b vs. M1c/d)			**7.75 (1.02 - 58.76)**	**0.048**				
Brain metastasis (no vs. yes)	3.39 (0.92 - 12.54)	0.068						
Brain only metastasis (no vs. yes)					**13.27 (2.02 - 87.23)**	**0.007**	**8.77 (1.74 - 44.31)**	**0.009**
ECOG (0 vs. 1-3)					**2.28 (1.09 - 4.76)**	**0.029**	**5.69 (2.23 - 14.51)**	**0.000**
Prior lines of therapy (no vs. yes)								
BRAF mutational status (WT vs. mut)								
ctDNA levels								
Group A vs Group B	1.57 (0.37 - 6.61)	0.539	0.69 (0.12 - 3.86)	0.671	**3.45 (1.02 – 11.65)**	**0.046**		
Group A vs Group C	**15.11 (3.33 - 68.54)**	**0.000**	**16.01 (2.44 - 105.07)**	**0.004**				

Table S5. Clinical characteristics of melanoma patients in this pilot cohort.

Sample ID	BRAF Status	Treatment	Best Clinical Response	Previous Immunotherapy	Start of Treatment	Treatment Completion or Latest Clinic	Length of Treatment (wks)	Anatomy	Adrenal	Brain	Bone	Liver	Uropharynx	Lung	Lymph Node	Mesentery	Pancreas	Pelvic Region	Sub-cutaneous
MP0104	BRAF Mut	**Pembrolizumab**	**PR**	Yes (Ipilimumab)	25/08/2014	29/08/2016	105								**x**				
MP0105	BRAF WT	**Pembrolizumab**	**CR**	Yes (Ipilimumab)	10/02/2015	29/12/2017	150			**x**				**x**					**x**
MP0201	BRAF Mut	**Pembrolizumab**	**CR**	No (Radiation Therapy)	5/08/2016	27/02/2018	82				**x**	**x**		**x**					
MP0303	BRAF Mut	**Pembrolizumab**	**PR**	No	15/09/2017	Ongoing	90[a]				**x**	**x**							
MP0302	BRAF Mut	**Pembrolizumab**	**SD**	No	13/12/2017	31/05/2018	24		**x**					**x**	**x**				
MP0102	BRAF WT	**Pembrolizumab**	**PD**	No	10/12/2015	12/02/2016	9						**x**		**x**	**x**			
MP0103	BRAF Mut	**Pembrolizumab**	**PD**	No	13/05/2016	4/07/2016	7				**x**	**x**			**x**				
MP0301	BRAF WT	**Pembrolizumab**	**PD**	No	11/06/2015	18/09/2015	14												
MP0304	BRAF WT	**Pembrolizumab**	**PD**	No	03/04/2017	25/05/2017	7	**x**		**x**	**x**								

[a] Weeks in treatment as of 31/08/2019.

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