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Multi-platform analysis of the heterogeneity of circulating melanoma cells and tumor DNA as useful tool to track disease evolution and targeted therapy response

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Background

Melanoma heterogeneity is one the main obstacles for the management of metastatic melanoma. Although the advent of targeted therapy has significantly improved patient outcome, the occurrence of resistance makes the monitoring of tumor genetic landscape mandatory. Liquid biopsy, being a sum of the systemic disease, is currently evaluated as an important biomarker for the real-time tracing of disease evolution.

Methods

In this pilot study, 17 stage IV melanoma patients, treated with BRAF/MEK inhibitors, have been enrolled, and followed for up to 24 months. A longitudinal screening at different time points (52 samples) has been applied to identify liquid biopsy dynamics during response to treatment and progression. Considering that resistance develops at a median time of 11-12 months, blood has been collected before starting the therapy, after 6 and 10 months to test the ability of our approach to detect early signs of tumor escape, and at relapse. We devised a multi-platform approach exploiting high-sensitivity techniques (NGS, ddPCR) and an FDA-cleared platform (CellSearch) to analyze circulating tumor DNA (ctDNA) trend, circulating melanoma cell (CMC) count, together with their customized genetic analysis and copy number variation assessment.

Results

BRAF mutant ctDNA was detected by ddPCR in 82% of patients, and its amount prior to the beginning of therapy was significantly correlated with response to treatment; a cut-off was also identified for a fast translation to the clinic. Moreover, when considering on-treatment changes, patients without ctDNA clearance up to the first 6 months had a significant correlation with early progression/no response, suggesting a further endpoint for this biomarker. In addition, single nucleotide variants (SNVs) known, or suspected, to confer resistance (involving, among others, MEK1, PTEN, NRAS genes) were identified by NGS in ctDNA and/or CMC DNA in 60% of patients. Finally, CMC number was confirmed to be a prognostic biomarker as a significant correlation between CMC count >0 at baseline and worse overall survival/progression free survival was identified.

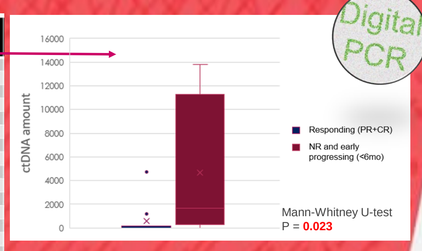
Conclusions

This study provides the proof-of-principle of the power of this multi-platform analysis. Indeed, it can provide ctDNA tracking and profiling, together with CMC count variation, and genetic landscape, useful for capturing tumor evolution. Although a validation of this data in a lager cohort is mandatory, this kind of strategy opens new scenarios for the management and real time monitoring of melanoma patients.

ctDNA ANALYSIS, TRACKING & TREND

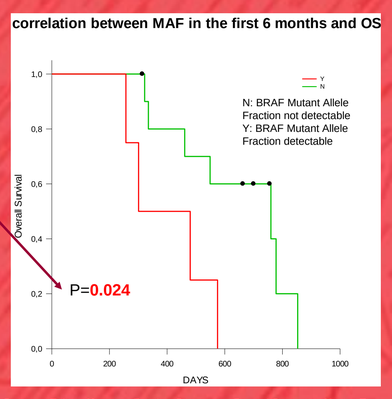
*BRAF mutated copies; NA: not applicable; ND: not done; § below quantitative limit; Undetectable: LOD (0,06-0,5% depending on sample concentration and availability for multiple replicates)

case	patient ID	baseline ctDNA copies/ml*	ctDNA at ≤ 6 mo follow -up copies/ml*	ctDNA at progression copies/ml*
1	#1	7.6	undetectable	8,6
2	#3	13768.3	17.4	4365,2
3	#6	1.7 §	undetectable	42,5
4	#8	1190.1	undetectable	ND
5	#9	128.2	undetectable	74,2
6	#10	Undetectable	undetectable	undetectable
7	#12	167.7	undetectable	NA
8	#13	10432.1	8469.4	8469,4
9	#15	Undetectable	undetectable	NA
10	#17	399.4	undetectable	undetectable
11	#19	894.4	2241.1	2241,1
12	#24	Undetectable	undetectable	NA
13	#27	99.9	791.4	48633,11
14	#28	4711.5	undetectable	141008,7
15	#34	2.1 §	ND	ND
16	#48	17.8	undetectable	5,54
17	#49	2673.9	ND	ND



Association **between baseline ctDNA amount** (BRAF mutated copies/ml plasma) and **response to treatment**

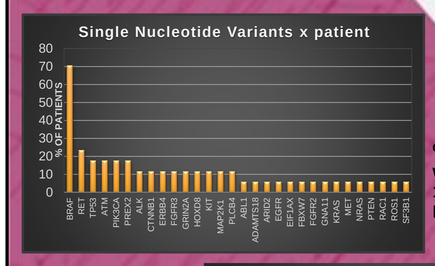
Significant correlation between a detectable BRAF-mutated fraction during the first 6 months of treatment and overall survival



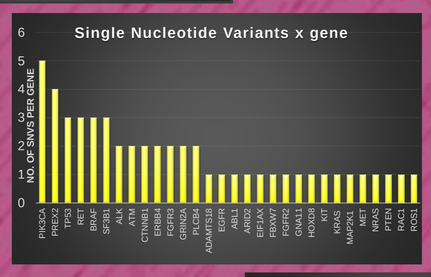
Group	Total	Missing	Events	Censored	Percent Censored	Median Time
Y	11	0	7	4	36	760,000
N	4	0	4	0	0	301,000
Overall	15	0	11	4	27	

GENETIC LANDSCAPE ANALYSIS

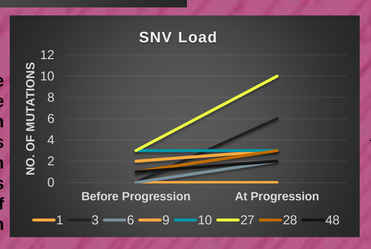
NGS custom panel
- 52 genes for SNVs/INDEL
- 11 genes for CNV



% Patients with at least 1 SNV in at least 1 sample



Number of SNVs identified for every gene, among the cohort of 52 samples



Comparison between the number of SNVs before and after progression in 8 patients. It is noteworthy how much the number of mutations increases at the time of progression

SNVs with clinical significance found at progression

PATIENT NO.	BRAF	KRAS	NRAS	MEK1	TP53	PTEN	PIK3CA	RAC1	FBXW7	PPP6C
3	p.Val600Lys					p.Leu247*			p.Arg465Cys	
6	p.Val600Glu									
9	p.Val600Glu		p.Gln61Leu							
13	p.Val600Glu			p.Pro124Leu						
19	p.Val600Glu			p.Pro124Leu						
27	p.Val600Lys	p.Gly12Ala						p.Pro29Ser		
28	p.Val600Glu						p.Glu542Lys			
48	p.Thr599dup									p.His151Tyr
49	p.Val600Glu				p.Ser241Phe					

Acquired resistance

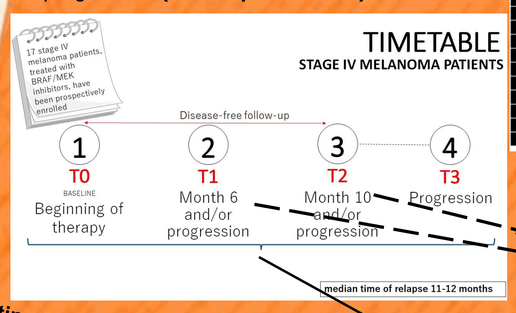
Intrinsic resistance

Patients in which a BRAF mutant allele fraction was detected at T1

The rise of **new SNVs putatively conferring resistance** to treatment has been observed in 3 patients at recurrence, while the presence **of SNVs that could confer intrinsic resistance** was observed in 6 cases

PATIENT RECRUITMENT AND CHARACTERISTICS

A **longitudinal screening** at different time points has been applied to **identify the dynamic changes in the liquid biopsy** during response to treatment and/or at progression **(52 samples in total).**



case	patient_ID	sex	age	Mutation	stage	treatment	response	type of response	PFS >6mo	survival status	Number of plasma tested in longitudinal follow-up
1	#1	F	75	V600E	M1d	Dabrafenib/trametinib	Y	PR	Y	Dead	2
2	#3	F	81	V600K	M1c	Dabrafenib/trametinib	N	SD	Y	Dead	2
3	#6	F	42	V600E	M1c	Dabrafenib/trametinib	Y	PR	Y	Dead	3
4	#8	M	85	V600E	M1d	Dabrafenib/trametinib	Y	PR-SR	Y	Dead	2
5	#9	F	37	V600E	M1c	Dabrafenib/trametinib	Y	PR	Y	Dead	3
6	#10	F	65	V600K*	M1d	Dabrafenib/trametinib	Y	PR	Y	Dead	4
7	#12	M	59	V600E	M1a	Dabrafenib/trametinib	Y	CR (SR)	Y	Alive	2
8	#13	F	42	V600E	M1d	Dabrafenib/trametinib	Y	PR	N	Dead	3
9	#15	F	46	V600E*	M1c	Dabrafenib/trametinib	Y	PR (SR)	Y	Alive	2
10	#17	M	48	V600E	M1c	Dabrafenib/trametinib	Y	CR	N	Dead	1
11	#19	M	64	V600E	M1b	Dabrafenib/trametinib	N	PD	N	Dead	1
12	#24	M	51	V600E*	M1c	Dabrafenib/trametinib	Y	CR (SR)	Y	Alive	2
13	#27	M	85	V600K	M1c	Dabrafenib/trametinib	Y	PR	Y	Dead	3
14	#28	M	84	V600E	M1c	Dabrafenib/trametinib	Y	PR	Y	Dead	2
15	#34	F	75	V600K	M1b	Dabrafenib/trametinib	N	PD	N	Dead	-
16	#48	M	59	T599dup	M1b	Dabrafenib/trametinib	Y	PR	Y	Alive	2
17	#49	M	64	V600E	M1d	Dabrafenib/trametinib	N	PD	N	Dead	-

SD: stable disease; PR: partial response; CR: complete response; SR: still responding; PD: progressive disease
***determined from FFPE sample analysis**

Timepoints set **before the median time** of relapse trying to understand if we are able to detect **early signs of progression**

52 samples analysed

Starting from a single blood draw

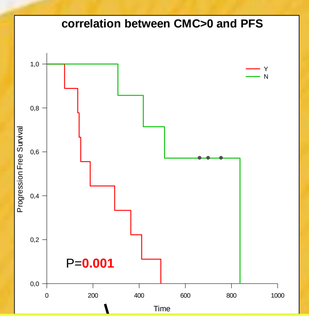
a **multi-platform** approach was designed
- **to track ctDNA trend**
- **to count CMCs**
- **to customize ctDNA/CMC genetic analysis**
- **to analyse copy number variation**

CellSearch platform

CMC COUNT

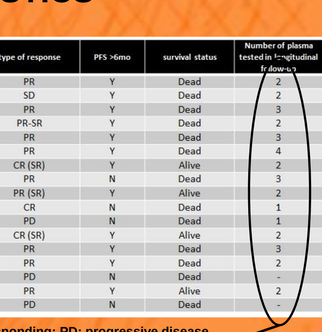
case	patient ID	CMC/7,5 ml at baseline	γH2AX+/ sample	CMC/7,5 ml at progression	γH2AX+/ sample
1	#1	0	0	0	0
2	#3	5	1	1	0
3	#6	1	0	0	0
4	#8	0	0	ND	ND
5	#9	12	1	3	1
6	#10	0	0	0	0
7	#12	0	0	NA	NA
8	#13	5	3	1	1
9	#15	0	0	NA	NA
10	#17	1	0	ND	ND
11	#19	2	0	0	0
12	#24	0	0	NA	NA
13	#27	0	0	0	0
14	#28	9	3	1	1
15	#34	2	2	ND	ND
16	#48	ND	ND	1	1
17	#49	1	1	ND	ND

NA: not applicable; ND: not done;



Group	Total	Missing	Events	Censored	Percent Censored	Median Time
Y	9	0	9	0	0	188,000
N	7	0	4	3	43	837,000
Overall	16	0	13	3	19	

Significant correlation between a **CMC count>0** at baseline and **worse progression free survival**



Group	Total	Missing	Events	Censored	Percent Censored	Median Time
Y	9	0	9	0	0	322,000
N	7	0	4	3	43	778,000
Overall	16	0	13	3	19	

Significant correlation between a **CMC count>0** at baseline and **worse overall survival**

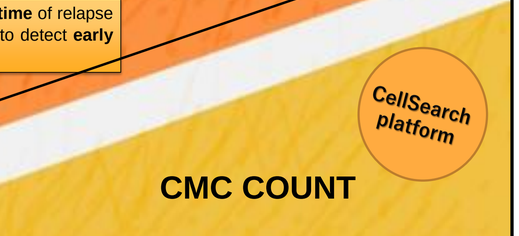
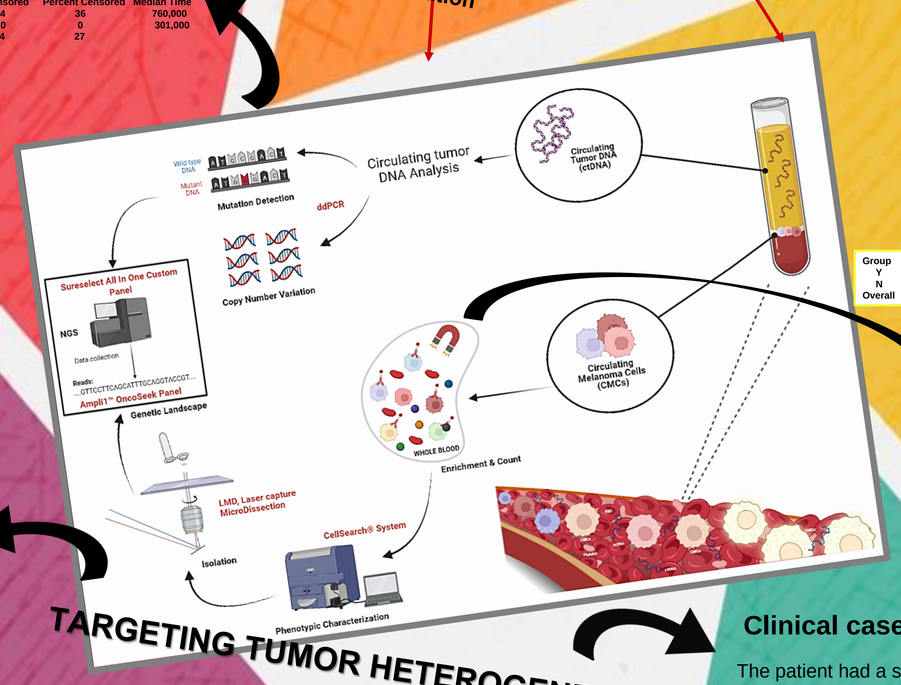


Image gallery of γH2AX+/- CMCs detected in clinical case 8 by the CellSearch platform. γH2AX is an early marker of DNA damage. An event is classified as a CMC when its morphological features are consistent with those of a tumor cell, and it exhibits the phenotype:
CD146+,
HMW-MAA+ (MEL-PE),
DAPI+,
CD34/45- (APC)



Clinical case 8

Catoni et al., *Cancers* 2022

The patient had a stage IV melanoma harbouring the mutation **BRAF p.V600E**

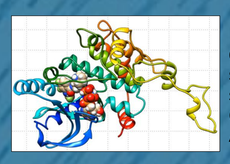
The patient **relapsed after only 4 months** of **Dabrafenib+Trametinib** therapy

By NGS + ddPCR analysis, we found:
- **BRAF V600E**
- **BRAF gain** → Resistance to Dabrafenib
- **MAP2K1 P124L** → Resistance to Trametinib

We set up a **molecular dynamics simulation** to understand if the mutation could inhibit at the same way a different MEKi, **Cobimetinib**

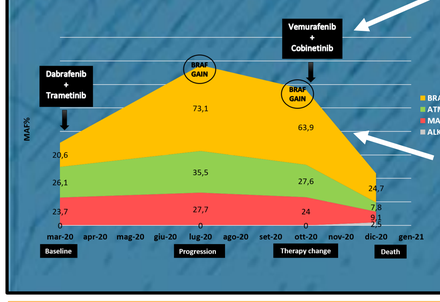
The binding is stable and the mutation does not modify the interaction

We suggested **a change in therapy** from Dabrafenib+Trametinib to **Vemurafenib+Cobimetinib**

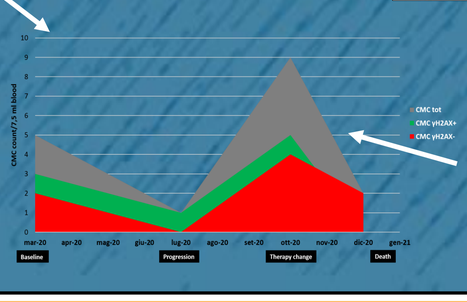


Molecular dynamics simulation showing MEK1, Cobimetinib and ATP interaction.

Trend of the ctDNA abundance/CMC number, suggestive of a strong response



dramatic decrease of BRAF/MEK mutated ctDNA fractional abundance



γH2AX+/- CMC stratification: CMC count decreased; unfortunately, the disease was still progressing, and the patient eventually died.

Clinical case 16

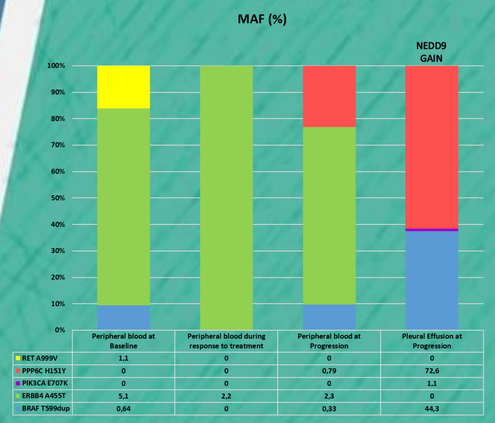
The patient had a stage IV melanoma harbouring the rare mutation **BRAF p.T599dup**

The patient was treated with BRAFi and MEKi (**Dabrafenib+Trametinib**) and a **partial response** occured

The patient **relapsed after 9 months**

At progression the **BRAF mutation** rebounded, together with the **PPP6C p.H151Y**

The prevalence of BRAF- and PPP6C-mutated fractions was even more evident from cfDNA derived from the **pleural effusion**, where a **NEDD9 gain** was also detected



	Peripheral blood at Baseline	Peripheral blood during response to treatment	Peripheral blood at Progression	Pleural Effusion at Progression
RET A999V	1,1	0	0	0
PPP6C H151Y	0	0	0,79	72,6
PIK3CA E707K	0	0	0	1,1
ERBB4 A455T	5,1	2,2	2,3	0
BRAF T599dup	0,64	0	0,33	44,3

Contribute of the MAF (%) of each SNV over the total MAF (%) detected in each sample

PPP6C loss promotes signaling through the ERK pathway and **decreases sensitivity to MEKi**

PPP6C p.H151Y renders the PP6 catalytic subunit unable to interact with the regulatory subunits and therefore leads to a PP6 null state in which **AURKA activity is increased**

NEDD9 stabilizes AURKA protein expression and **increases AURKA activity** by protecting AURKA from binding cdh1 and stimulating AURKA autophosphorylation

AURKA inhibitors could provide a putative treatment alternative

CONCLUSIONS

This study provides the **proof-of-principle of the power of a multiparameter liquid biopsy analysis**, revealing that
- it could **provide cfDNA and CMC profiling**, useful for capturing tumor heterogeneity/evolution, while assessing sensitivity to specific drugs.
- Overall, our liquid biopsy analysis showed **informative SNV/CNV profiles**, useful for **early detection** of relapse and **therapy resistance**.
- Relevant information can be obtained for most patients, putatively **spendable in the clinical setting.**
- The strength of this approach stands in the exploitation of different sources of information in order to face the absence of data from CMC with those recovered from ctDNA, and vice versa. When available, the use of information coming from both the analytes represents, obviously, the ideal situation, as the two sources of information have to be considered **complementary rather than overlapping**.

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