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# A multiparameter liquid biopsy-based approach allows longitudinal tracking of cutaneous melanoma dynamics and early resistance to treatment

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**Background**: Melanoma heterogeneity is an obstacle in metastatic disease management. Although the advent of targeted therapy has significantly improved patient outcome, the occurrence of resistance makes monitoring of the tumor genetic landscape mandatory. Liquid biopsy could represent an important biomarker to track the evolution of the disease in real time. Thus, we aimed to correlate liquid biopsy dynamics with treatment response/progression by devising a multiplatform approach applied to longitudinal monitoring.

**Methods**: We exploited NGS, digital PCR, and CellSearch platforms to analyze circulating tumor DNA (ctDNA) trend and circulating melanoma cell (CMC) count, together with their customized genetic and CNV analysis. The approach was applied to 50 samples from 17 stage IV melanoma patients treated with BRAF/MEK inhibitors, followed up to 24 months.

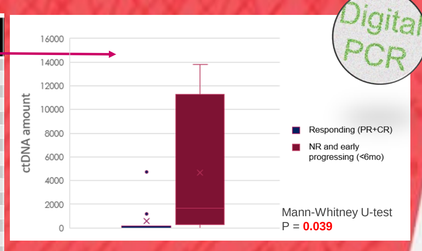
**Results***:* BRAF mutations were detected in the plasma of 82% of patients. There was a significant difference in ctDNA amount at baseline in responders versus non-responders/early progressing patients (p=0.039). Moreover, a cut-off able to discriminate responders from non-responders was identified. Undetectable BRAF-mutant ctDNA at the first treatment observational point correlated with best overall survival (OS) (p=0.024), and lack of BRAF-mutant ctDNA clearance up to the first 6 months of treatment correlated with non-response or early progression (p=0.015). Single nucleotide variants (SNVs) known or suspected to confer resistance were identified in 60% of patients. Moreover, the number of baseline SNVs correlated with progression free survival (PFS) (p=0.041). Finally, CMCs confirmed to be a prognostic biomarker, as the presence of 1 or more CMCs correlated with worse PFS (p=0.001) and OS (p=0.003).

**Conclusions**: This work provides proof-of-principle of the power of this approach and paves the way for a validation study to evaluate early ctDNA-guided treatment decisions in stage IV melanoma. The molecular profile complemented the analysis of ctDNA trend and, together with CMC analysis, revealed to be useful in capturing tumor evolution.

## ctDNA ANALYSIS, TRACKING & TREND

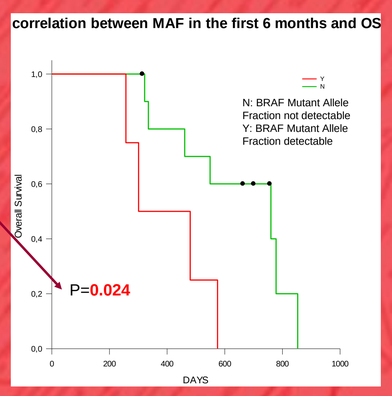
*BRAF mutated copies; NA: not applicable; ND: not done; § below quantitative limit; Undetectable: LOD (0,06-0,5% depending on sample concentration and availability for multiple replicates)

| 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 | 694.4 | 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**

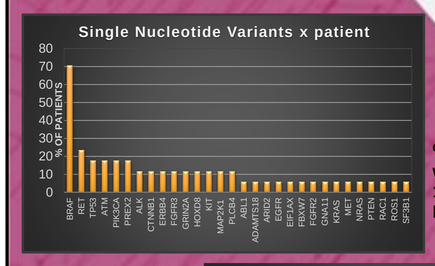
**Significant correlation between a detectable BRAF-mutated fraction during the first 6 months of treatment and overall survival**



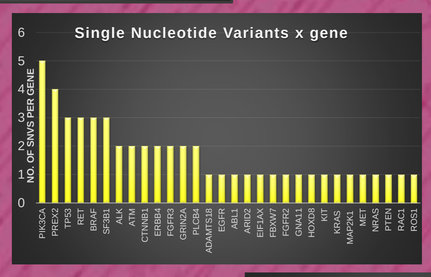

| Group | Total | Missing | Events | Censored | Percent Censored | Median Time |
|---|---|---|---|---|---|---|
| Y | 11 | 0 | 7 | 4 | 36 | 760,000 |
| N | 4 | 0 | 4 | 0 | 0 | 301,000 |
| Overall | 15 | 0 | 11 | 4 | 27 | |

## GENETIC LANDSCAPE ANALYSIS

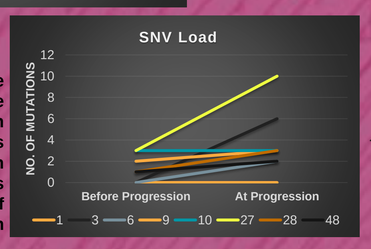
NGS custom panel
- 52 genes for SNVs/INDEL
- 11 genes for CNV




**% 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

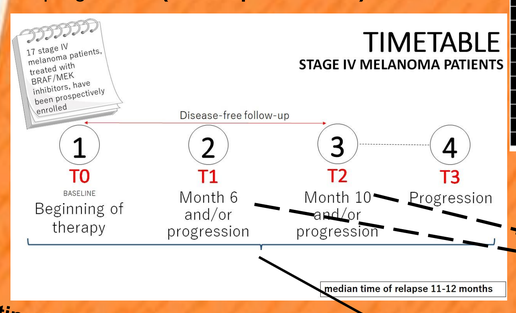
Intrinsic resistance

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**

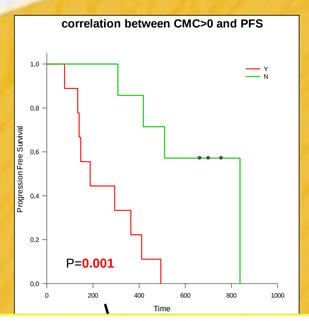
a **multi-platform** approach was designed
- **to track ctDNA trend**
- **to count CMCs**
- **to customize ctDNA/CMC genetic analysis**
- **to analyse copy number variation**

CellSearch platform

## CMC COUNT

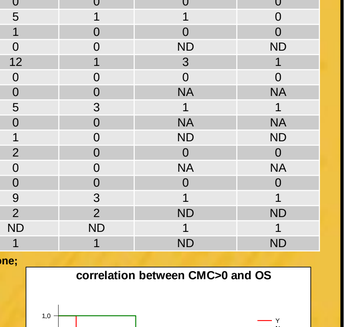
| case | patient ID | CMC/7,5 ml at baseline | γH2AX+/ sample | CMC/7,5 ml at progression | γH2AX+/ sample |
|---|---|---|---|---|---|
| 1 | #1 | 0 | 0 | 0 | 0 |
| 2 | #3 | 5 | 1 | 1 | 0 |
| 3 | #6 | 1 | 0 | 0 | 0 |
| 4 | #8 | 0 | 0 | ND | ND |
| 5 | #9 | 12 | 1 | 3 | 1 |
| 6 | #10 | 0 | 0 | 0 | 0 |
| 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**

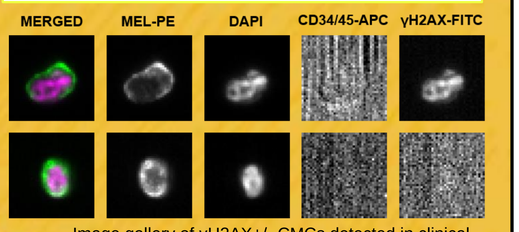
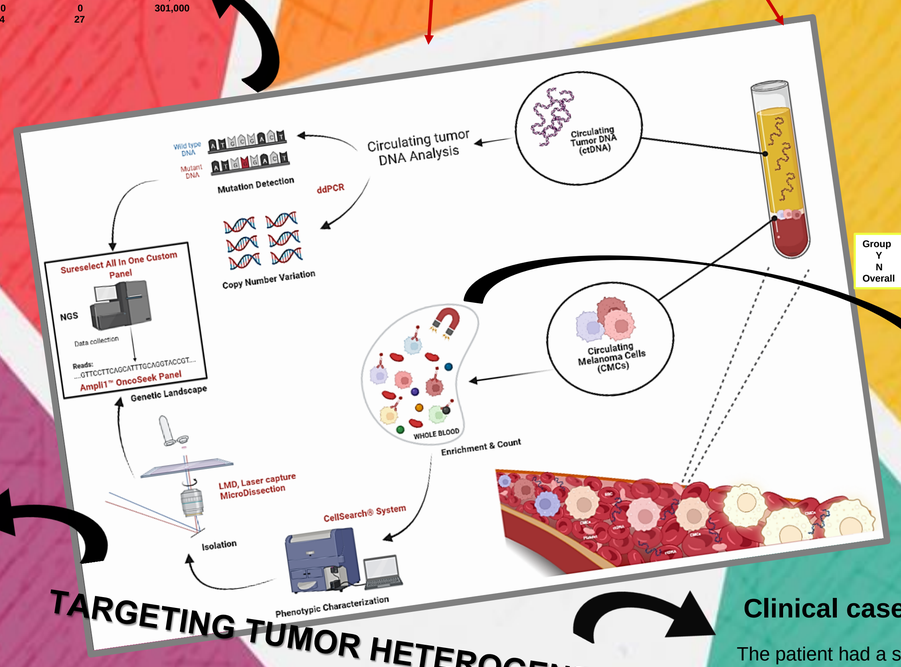



Image gallery of γH2AX+/- CMCs detected in clinical case 8 by the CellSearch platform. γH2AX is an early marker of DNA damage. An event is classified as a CMC when its morphological features are consistent with those of a tumor cell, and it exhibits the phenotype:
CD146+,
HMW-MAA+ (MEL-PE),
DAPI+,
CD34/45-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




## Clinical case 16

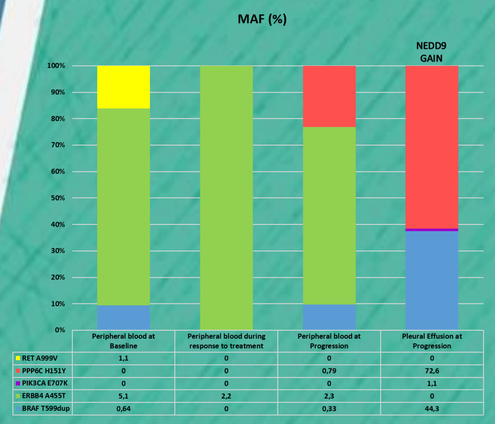
The patient had a stage IV melanoma harbouring the rare mutation **BRAF p.T599dup**

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




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

## 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

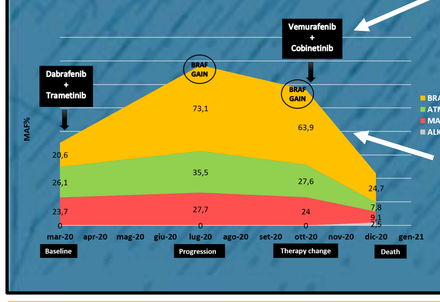
We set up a **molecular dynamics simulation** to understand if the mutation could inhibit at the same way a different MEKi, **Cobimetinib**

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

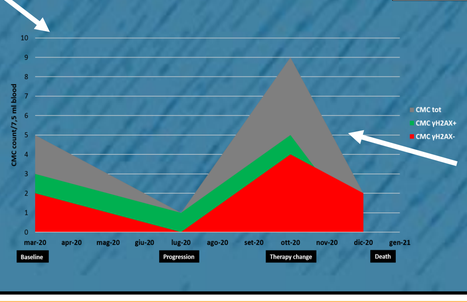
We suggested **a change in therapy** from Dabrafenib+Trametinib to **Vemurafenib+Cobimetinib**

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.

## 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**.

SCAN ME

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