**Supplementary Material**

**Table S1:** Comparison of the patients’ characteristics between the discovery cohort (*N*=59) and the validation cohort (*N*=128).

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ***First-line ICI*** | | | ***P-value*** | ***Second-line ICI*** | | | ***P-value*** | ***ICI after Targeted Therapy*** | | | ***P-value*** |
|  | ***Discovery*** | ***Validation*** | ***Total*** | ***Discovery*** | ***Validation*** | ***Total*** | ***Discovery*** | ***Validation*** | ***Total*** |
| ***Variable*** | *N=32 (%)* | *N=77 (%)* | *N=109 (%)* | *N=27 (%)* | *N=51 (%)* | *N=78 (%)* | *N=19 (%)* | *N=36 (%)* | *N=55 (%)* |
| ***Age*** |  |  |  |  |  |  |  |  |  |  |  |  |
| ≤65 | *12 (38)* | *38 (49)* | *50 (46)* | *0.296* | *14 (52)* | *30 (59)* | *44 (56)* | *0.634* | *8 (42)* | *21 (58)* | *29 (53)* | *0.273* |
| >65 | *20 (62)* | *39 (51)* | *59 (54)* | *13 (48)* | *21 (41)* | *34 (44)* | *11 (58)* | *15 (42)* | *26 (47)* |
| ***Sex*** |  |  |  |  |  |  |  |  |  |  |  |  |
| Female | *7 (22)* | *25 (32)* | *32 (29)* | *0.357* | *7 (26)* | *20 (39)* | *27 (35)* | *0.319* | *5 (26)* | *15 (42)* | *20 (36)* | *0.378* |
| Male | *25 (78)* | *52 (68)* | *77 (71)* | *20 (74)* | *31 (61)* | *51 (65)* | *14 (74)* | *21 (58)* | *35 (64)* |
| ***AJCC Stage/M Classification*** |  |  |  |  |  |  |  |  |  |  |  |  |
| IIIc/M1a/M1b | *10 (32)* | *35 (45)* | *45 (41)* | *0.203* | *8 (30)* | *12 (24)* | *20 (26)* | *0.593* | *6 (32)* | *7 (19)* | *13 (24)* | *0.336* |
| M1c/M1d | *22 (68)* | *42 (55)* | *64 (59)* | *19 (70)* | *39 (76)* | *58 (74)* | *13 (68)* | *29 (81)* | *42 (76)* |
| ***Brain metastasis*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Yes* | *8 (25)* | *29 (38)* | *37 (34)* | *0.268* | *8 (30)* | *22 (43)* | *30 (38)* | *0.758* | *7 (37)* | *21 (58)* | *28 (51)* | *0.163* |
| *No* | *24 (75)* | *48 (62)* | *72 (66)* | *19 (70)* | *29 (57)* | *48 (62)* | *12 (63)* | *15 (42)* | *27 (49)* |
| ***Brain only metastasis*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Yes* | *2 (6)* | *1 (1)* | *3 (3)* | *0.206* | *5 (19)* | *8 (16)* | *13 (17)* | *0.758* | *5 (26)* | *8 (22)* | *13 (24)* | *0.749* |
| *No* | *30 (94)* | *76 (99)* | *106 (97)* | *22 (81)* | *43 (84)* | *65 (83)* | *14 (74)* | *28 (78)* | *42 (76)* |
| ***LDH levels*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Normal* | *8 (25)* | *55 (71)* | *63 (58)* | *-* | *10 (37)* | *35 (69)* | *45 (58)* | *-* | *6 (32)* | *24 (67)* | *30 (54)* | *-* |
| *Elevated* | *6 (19)* | *19 (25)* | *25 (23)* | *9 (33)* | *16 (31)* | *25 (32)* | *6 (32)* | *12 (33)* | *18 (33)* |
| *N/A* | *18 (56)* | *3 (4)* | *21 (19)* | *8 (30)* |  | *8 (10)* |  | *7 (34)* |  | *7 (13)* |
| ***Treatment*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Nivolumab* |  | *8 (10)* | *8 (7)* | ***\*0.048*** | *1 (3)* | *13 (25)* | *14 (18)* | *\*0.424* |  | *8 (22)* | *8 (14)* | *\*0.999* |
| *Pembrolizumab* | *22 (69)* | *29 (38)* | *51 (47)* | *17 (64)* | *26 (51)* | *43 (55)* | *12 (63)* | *16 (44)* | *28 (51)* |
| *Ipilimumab/Nivolumab* | *10 (31)* | *15 (20)* | *25 (23)* | *9 (33)* | *8 (16)* | *17 (22)* | *7 (37)* | *5 (14)* | *12 (22)* |
| *Ipilimumab/Pembrolizumab* |  | *25 (32)* | *25 (23)* |  | *4 (8)* | *4 (5)* |  | *7 (20)* | *7 (13)* |
| ***First-line therapy*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Vemurafenib* |  |  |  |  |  | *1 (2)* | *1 (1)* | *-* |  | *1 (3)* | *1 (2)* | *-* |
| *Encorafenib/Binimetinib* |  |  |  |  |  | *1 (2)* | *1 (1)* |  | *1 (3)* | *1 (2)* |
| *Dabrafenib/Trametinib* |  |  |  |  | *19 (70)* | *34 (67)* | *53 (68)* | *19 (100)* | *34 (94)* | *53 (96)* |
| *Ipilimumab* |  |  |  |  | *6 (22)* | *14 (27)* | *20 (26)* |  |  |  |  |
| *Pembrolizumab* |  |  |  |  | *2 (8)* |  | *2 (3)* |  |  |  |  |
| *Ipilimumab/Nivolumab* |  |  |  |  |  | *1 (2)* | *1 (1)* |  |  |  |  |
| ***BRAF mutation status*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *BRAF* *V600* Mutant | *2 (6)* | *35 (45)* | *37 (34)* | ***<0.0001*** | *21 (78)* | *38 (75)* | *59 (76)* | *0.656* | *19 (100)* | *36 (100)* | *55 (100)* | *-* |
| *BRAF V600* WT | *30 (94)* | *42 (55)* | *72 (66)* | *6 (22)* | *13 (25)* | *19 (24)* |  |  |  |

\*Analysis performed comparing anti-PD-1 monotherapy and anti-CTLA-4 plus anti-PD-1 combination therapy. N/A – not available.

**Table S2:** Specificity of ddPCR assays.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Assay** | **Healthy controls** | | **Maximum false positive concentration (copies/mL)** | **Company** |
| *Positive* | *Negative* |
| ***BRAF V600E*** | *0* | *22* | *0* | *Life Technologies* |
| ***BRAF V600K*** | *0* | *23* | *0* | *IDT* |
| ***BRAF V600R*** | *0* | *24* | *0* | *IDT* |
| ***BRAF V600E2*** | *1* | *12* | *1* | *IDT* |
| ***BRAF K601E*** | *3* | *7* | *2* | *IDT* |
| ***BRAF L597Q*** | *0* | *16* | *0* | *Bio-Rad* |
| ***NRAS Q61K*** | *0* | *19* | *0* | *Bio-Rad* |
| ***NRAS Q61L*** | *3* | *9* | *7* | *Bio-Rad* |
| ***NRAS Q61R*** | *7* | *24* | *9* | *Bio-Rad* |
| ***NRAS G12D*** | *4* | *6* | *3* | *Bio-Rad* |
| ***NRAS G13D*** | *4* | *6* | *5* | *Bio-Rad* |
| ***TERT C228T/C250T Mult.*** | *6* | *50* | *10* | *IDT* |
| ***DPH3 C8T*** | *1* | *10* | *2* | *IDT* |
| ***GRM3 E538K*** | *0* | *10* | *0* | *Bio-Rad* |
| ***GRM3 S491L*** | *6* | *12* | *6* | *Bio-Rad* |
| ***FLT1 E011K*** | *3* | *7* | *9* | *Bio-Rad* |
| ***KIT L576P*** | *1* | *11* | *2* | *Bio-Rad* |
| ***NF1 P1851S*** | *3* | *11* | *3* | *Bio-Rad* |
| ***RAC1 P29S*** | *0* | *12* | *0* | *Bio-Rad* |
| ***TP53 R248W*** | *2* | *14* | *2* | *Bio-Rad* |
| ***TP53 R248Q*** | *6* | *8* | *2* | *Bio-Rad* |
| ***TP53 R158H*** | *1* | *9* | *2* | *Bio-Rad* |

**Table S3:** Mutation status of the melanoma patients included in the study.

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
|  | ***Discovery Cohort*** | | |  | ***Validation Cohort*** | |
|  | ***1st line ICI*** | ***2nd line ICI*** | ***1st line TT*** |  | ***1st line ICI*** | ***2nd line ICI*** |
| ***Mutation status*** | *N=32 (%)* | *N=27 (%)* | *N=66 (%)* |  | *N=77 (%)* | *N=51 (%)* |
| ***BRAF*** |  |  |  |  |  |  |
| *BRAF V600E* |  | *13 (48)* | *50 (76)* |  | *24 (31)* | *27 (53)* |
| *BRAF V600E2* |  |  | *1 (2)* |  |  |  |
| *BRAF V600K* |  | *5 (18)* | *12 (18)* |  | *11 (14)* | *9 (17)* |
| *BRAF V600R* | *1 (3)* | *2 (6)* | *3 (4)* |  |  | *2 (4)* |
| *BRAF K601E* | *1 (3)* | *1 (4)* |  |  |  |  |
| *BRAF L597Q* | *1 (3)* |  |  |  | *1 (1)* |  |
| *BRAF L597S* |  |  |  |  |  | *1 (2)* |
| *BRAF L597R* |  |  |  |  | *2 (3)* |  |
| *BRAF G466E* |  |  |  |  | *1 (1)* |  |
| ***NRAS*** |  |  |  |  |  |  |
| *NRAS Q61K* | *8 (25)* | *1 (4)* |  |  | *12 (16)* | *4 (8)* |
| *NRAS Q61L* | *4 (13)* |  |  |  | *5 (7)* | *2 (4)* |
| *NRAS Q61R* | *3 (9)* |  |  |  | *13 (18)* | *4 (8)* |
| *NRAS Q61H* |  |  |  |  | *1 (1)* | *1 (2)* |
| *NRAS G12D* | *1 (3)* |  |  |  |  |  |
| *NRAS G13D* | *1 (3)* |  |  |  |  |  |
|  |  |  |  |  |  |  |
| ***TERT*** |  |  |  |  |  |  |
| *TERT Mult.* | *1 (3)* |  |  |  |  |  |
| *TERT C228T* | *1 (3)* | *1 (4)* |  |  |  |  |
| *TERT C250T* | *1 (3)* | *1 (4)* |  |  |  |  |
| *TERT -125CC>TT* |  |  |  |  |  | *1 (2)* |
| ***GRM3*** |  |  |  |  |  |  |
| *GRM3 E538K* | *1 (3)* |  |  |  |  |  |
| *GRM3 S491L* | *1 (3)* |  |  |  |  |  |
| ***FLT1*** |  |  |  |  |  |  |
| *FLT1 E1011K* | *1 (3)* |  |  |  |  |  |
| ***KIT*** |  |  |  |  |  |  |
| *KIT L576P* | *2 (7)* |  |  |  | *1 (1)* |  |
| *KIT K642E* |  |  |  |  | *4 (5)* |  |
| ***NF1*** |  |  |  |  |  |  |
| *NF1 P185S* |  | *1 (4)* |  |  |  |  |
| ***RAC1*** |  |  |  |  |  |  |
| *RAC1 P29S* | *2 (7)* |  |  |  | *1 (1)* |  |
| ***TP53*** |  |  |  |  |  |  |
| *TP53 R158H* | *1 (3)* |  |  |  |  |  |
| *TP53 R248W* | *1 (3)* |  |  |  |  |  |
| *TP53 R248Q* |  | *1 (4)* |  |  |  |  |
| *TP53 Y220S* |  |  |  |  | *1 (1)* |  |
| ***DPH3*** |  |  |  |  |  |  |
| *DPH3 C8T* |  | *1 (4)* |  |  |  |  |

**Table S4:** Clinical characteristics at baseline of the melanoma patients included in the survival analysis of the discovery cohort.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ***First-line ICI*** | | | ***P-value*** | ***Second-line ICI*** | | | ***P-value*** | ***ICI after Targeted Therapy*** | | | ***P-value*** |
|  | ***Low ctDNA*** | ***High ctDNA*** | ***Total*** | ***Low ctDNA*** | ***High ctDNA*** | ***Total*** | ***Low ctDNA*** | ***High ctDNA*** | ***Total*** |
| ***Variable*** | *N=18 (%)* | *N=14 (%)* | *N=32 (%)* | *N=18 (%)* | *N=9 (%)* | *N=27 (%)* | *N=14 (%)* | *N=5 (%)* | *N=19 (%)* |
| ***Age*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *≤65* | *5 (28)* | *7 (50)* | *12 (38)* | *0.277* | *9 (50)* | *5 (56)* | *14 (52)* | *0.999* | *5 (36)* | *3 (60)* | *8 (42)* | *0.603* |
| *>65* | *13 (72)* | *7 (50)* | *20 (62)* | *9 (50)* | *4 (44)* | *13 (48)* | *9 (64)* | *2 (40)* | *11 (58)* |
| ***Sex*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Female* | *3 (17)* | *4 (29)* | *7 (22)* | *0.669* | *6 (33)* | *1 (12)* | *7 (26)* | *0.363* | *4 (29)* | *1 (20)* | *5 (26)* | *0.999* |
| *Male* | *15 (83)* | *10 (71)* | *25 (78)* | *12 (67)* | *8 (88)* | *20 (74)* | *10 (71)* | *4 (80)* | *14 (74)* |
| ***Melanoma Subtype*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Cutaneous* | *18 (100)* | *14 (100)* | *32 (100)* | *-* | *18 (100)* | *9 (100)* | *27 (100)* | *-* | *14 (100)* | *5 (100)* | *19 (100)* | *-* |
| ***AJCC Stage/M Classification*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *M1a/M1b* | *8 (44)* | *2 (14)* | *10 (32)* | *0.124* | *6 (33)* | *2 (24)* | *8 (30)* | *0.676* | *5 (36)* | *1 (20)* | *6 (32)* | *0.999* |
| *M1c/M1d* | *10 (56)* | *12 (86)* | *22 (68)* | *12 (67)* | *7 (76)* | *19 (70)* | *9 (64)* | *4 (80)* | *13 (68)* |
| ***ECOG*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *0* | *11 (61)* | *4 (29)* | *15 (46)* | *0.087* | *11 (61)* | *3 (33)* | *14 (52)* | *0.237* | *8 (57)* | *2 (40)* | *10 (53)* | *0.629* |
| *1-3* | *7 (39)* | *10 (71)* | *17 (54)* | *7 (39)* | *6 (67)* | *13 (48)* | *6 (43)* | *3 (60)* | *9 (47)* |
| ***Brain metastasis*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Yes* | *5 (28)* | *3 (21)* | *8 (25)* | *0.999* | *6 (33)* | *2 (24)* | *8 (30)* | *0.676* | *6 (43)* | *1 (20)* | *7 (37)* | *0.603* |
| *No* | *13 (72)* | *11 (79)* | *24 (75)* | *12 (67)* | *7 (76)* | *19 (70)* | *8 (57)* | *4 (80)* | *12 (63)* |
| ***Brain only metastasis*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Yes* | *2 (11)* |  | *2 (6)* | *0.492* | *5 (28)* |  | *5 (19)* | *0.136* | *5 (36)* |  | *5 (26)* | *0.257* |
| *No* | *16 (89)* | *14 (100)* | *30 (94)* | *13 (72)* | *9 (100)* | *22 (81)* | *9 (64)* | *5 (100)* | *14 (74)* |
| ***LDH levels*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Normal* | *6 (33)* | *2 (14)* | *8 (25)* | *-* | *10 (55)* |  | *10 (37)* | *-* | *6 (43)* |  | *6 (32)* | *-* |
| *Elevated* | *1 (6)* | *5 (36)* | *6 (19)* | *3 (17)* | *6 (67)* | *9 (33)* | *3 (21)* | *3 (60)* | *6 (32)* |
| *N/A* | *11 (61)* | *7 (50)* | *18 (56)* |  | *5 (28)* | *3 (33)* | *8 (30)* |  | *5 (36)* | *2 (40)* | *7 (34)* |  |
| ***Therapy*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Nivolumab* |  |  |  |  | *1 (6)* |  | *1 (3)* | *\*0.193* |  |  |  |  |
| *Pembrolizumab* | *12 (67)* | *10 (71)* | *22 (69)* | *0.999* | *9 (50)* | *8 (89)* | *17 (64)* | *7 (50)* | *5 (100)* | *12 (63)* | *0.106* |
| *Ipilimumab/Nivolumab* | *6 (33)* | *4 (29)* | *10 (31)* | *8 (44)* | *1 (11)* | *9 (33)* | *7 (50)* |  | *7 (37)* |
| ***Timing of blood draw*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *During 1st line Rx* |  |  |  |  |  |  |  |  | *8 (57)* | *2 (40)* | *10 (53)* | *0.629* |
| *After 1st line Rx* |  |  |  |  |  |  |  |  | *6 (43)* | *3 (60)* | *9 (47)* |
| ***BRAF mutation status*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *BRAF V600 Mutant* | *1 (6)* | *1 (7)* | *2 (6)* | *0.999* | *15 (83)* | *6 (67)* | *21 (78)* | *0.367* | *14 (100)* | *5 (100)* | *19 (100)* | *-* |
| *BRAF V600 WT* | *17 (94)* | *13 (93)* | *30 (94)* | *3 (17)* | *3 (33)* | *6 (22)* |  |  |  |

\*Analysis performed comparing anti-PD-1 monotherapy and anti-CTLA-4 plus anti-PD-1 combination therapy. N/A – not available.

**Table S5**: Multivariate Cox proportional-hazards regression analysis for associations between ctDNA levels and PFS in patients receiving first-line ICI in the discovery cohort (*N*=32).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | ***First-line ICIs*** | | | | |
| ***Variables*** | ***Univariate*** | |  | ***Multivariate*** | | |
| *HR (95% CI)* | *P-value* |  | *HR (95% CI)* | *P-value* | |
| Age (≤65 vs. >65) | 0.84 (0.34 - 2.09) | 0.705 | 1.06 (0.39 - 2.85) | 0.906 | |
| Sex (female vs. male) | 0.54 (0.20 - 1.42) | 0.209 |  | 0.65 (0.23 - 1.81) | 0.405 | |
| AJCC Stage (M1a/b vs. M1c/d) | 4.05 (1.17 - 14.02) | **0.027** |  | 2.59 (0.67 - 10.12) | 0.170 | |
| Brain metastasis (no vs. yes) | 2.31 (0.89 - 5.92) | 0.082 |  | 1.84 (0.61 - 5.58) | 0.283 | |
| *BRAF* mutation status (mut vs. WT) | 1.33 (0.18 - 10.02) | 0.779 |  | 1.23 (0.14 - 10.47) | 0.851 | |
| ctDNA levels (low vs. high) | 5.70 (2.11 - 15.38) | **<0.001** |  | 5.18 (1.88 - 14.31) | **0.001** | |

**Table S6**: Multivariate Cox proportional-hazards regression analysis for associations between ctDNA levels and PFS in patients receiving first-line ICIs in the validation cohort (*N*=77).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
|  | ***First-line ICI*** | | | | |
| ***Variables*** | ***Univariate*** | |  | ***Multivariate*** | | |
| *HR (95% CI)* | *P-value* |  | *HR (95% CI)* | *P-value* | |
| Age (≤65 vs. >65) | 1.08 (0.57 - 2.03) | 0.824 | 0.72 (0.33 - 1.59) | 0.419 | |
| Sex (female vs. male) | 1.27 (0.63 - 2.55) | 0.512 |  | 1.28 (0.58 - 2.81) | 0.545 | |
| AJCC Stage (IIIc/M1a/b vs. M1c/d) | 1.44 (0.75 - 2.76) | 0.272 |  | 1.58 (0.62 - 3.03) | 0.341 | |
| LDH levels (normal vs. elevated) | 1.13 (0.53 – 2.41) | 0.752 |  | 0.74 (0.32 – 1.71) | 0.484 | |
| Brain metastasis (no vs. yes) | 1.24 (0.65 - 2.38) | 0.514 |  | 0.94 (0.38 - 2.32) | 0.889 | |
| *BRAF* mutation status (mut vs. WT) | 1.33 (0.69 - 2.54) | 0.397 |  | 1.52 (0.72 - 3.23) | 0.273 | |
| ctDNA levels (low vs. high) | 2.39 (1.26 - 4.55) | **0.008** |  | 2.42 (1.17 - 5.02) | **0.017** | |

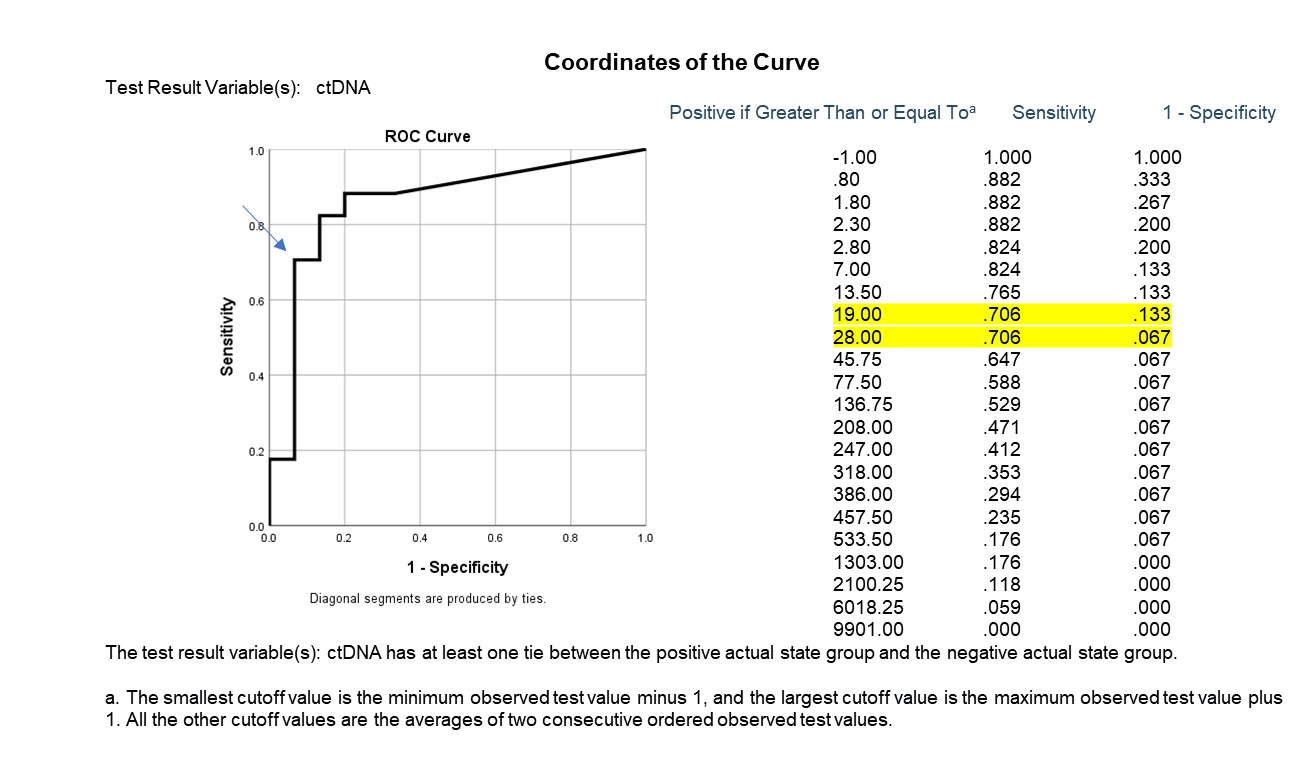
**Table S7:** Clinical characteristics at baseline of the melanoma patients included in the survival analysis of the validation cohort.

|  |  |  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ***First-line ICI*** | | | ***P-value*** | ***Second-line ICI*** | | | ***P-value*** | ***ICI after Targeted Therapy*** | | | ***P-value*** |
|  | ***Low ctDNA*** | ***High ctDNA*** | ***Total*** | ***Low ctDNA*** | ***High ctDNA*** | ***Total*** | ***Low ctDNA*** | ***High ctDNA*** | ***Total*** |
| ***Variable*** | *N=44 (%)* | *N=33 (%)* | *N=77 (%)* | *N=31 (%)* | *N=20 (%)* | *N=51 (%)* | *N=23 (%)* | *N=13 (%)* | *N=36 (%)* |
| ***Age*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *≤65* | *23 (51)* | *15 (45)* | *38 (49)* | *0.647* | *18 (58)* | *12 (60)* | *30 (59)* | *0.999* | *14 (61)* | *7 (54)* | *21 (58)* | *0.736* |
| *>65* | *21 (49)* | *18 (55)* | *39 (51)* | *13 (42)* | *8 (40)* | *21 (41)* | *9 (39)* | *6 (46)* | *15 (42)* |
| ***Sex*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Female* | *16 (36)* | *9 (27)* | *25 (32)* | *0.466* | *11 (35)* | *9 (45)* | *20 (39)* | *0.565* | *8 (35)* | *7 (54)* | *15 (42)* | *0.310* |
| *Male* | *28 (64)* | *24 (73)* | *52 (68)* | *20 (65)* | *11 (55)* | *31 (61)* | *15 (65)* | *6 (46)* | *21 (58)* |
| ***Melanoma Subtype*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Cutaneous* | *33 (76)* | *27 (82)* | *60 (78)* | *-* | *26 (84)* | *12 (60)* | *38 (75)* | *-* | *19 (82)* | *8 (62)* | *27 (74)* | *-* |
| *Acral* | *1 (2)* | *1 (3)* | *2 (3)* | *1 (3)* | *2 (10)* | *3 (6)* | *1 (5)* | *1 (7)* | *2 (6)* |
| *Mucosal* | *1 (2)* |  | *1 (1)* |  |  |  |  |  |  |
| *N/A* | *9 (20)* | *5 (15)* | *14 (18)* | *4 (13)* | *6 (30)* | *10 (19)* | *3 (13)* | *4 (31)* | *7 (20)* |
| ***AJCC Stage/M Classification*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *IIIc/M1a/M1b* | *24 (55)* | *11 (33)* | *35 (45)* | *0.071* | *8 (26)* | *4 (20)* | *12 (24)* | *0.743* | *5 (22)* | *2 (15)* | *7 (19)* | *0.999* |
| *M1c/M1d* | *20 (45)* | *22 (67)* | *42 (55)* | *23 (74)* | *16 (80)* | *39 (76)* | *18 (78)* | *11 (85)* | *29 (81)* |
| ***ECOG*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *0* | *26 (59)* | *16 (48)* | *42 (55)* | *-* | *16 (52)* | *4 (20)* | *20 (39)* | *-* | *10 (43)* | *1 (7)* | *11 (30)* | *-* |
| *1-3* | *11 (25)* | *14 (42)* | *25 (32)* | *9 (29)* | *10 (50)* | *19 (37)* | *8 (35)* | *7 (54)* | *15 (42)* |
| *N/A* | *7 (16)* | *3 (10)* | *10 (13)* |  | *6 (19)* | *6 (30)* | *12 (24)* |  | *5 (22)* | *5 (39)* | *10 (28)* |  |
| ***Brain metastasis*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Yes* | *13 (30)* | *16 (48)* | *29 (38)* | *0.102* | *16 (52)* | *6 (30)* | *22 (43)* | *0.157* | *16 (70)* | *5 (38)* | *21 (58)* | *0.089* |
| *No* | *31 (70)* | *17 (52)* | *48 (62)* | *15 (48)* | *14 (70)* | *29 (57)* | *7 (30)* | *8 (62)* | *15 (42)* |
| ***Brain only metastasis*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Yes* | *1 (2)* |  | *1 (1)* | *0.999* | *8 (26)* |  | *8 (16)* | ***0.016*** | *8 (35)* |  | *8 (22)* | ***0.032*** |
| *No* | *43 (98)* | *33 (100)* | *76 (99)* | *23 (74)* | *20 (100)* | *43 (84)* | *15 (65)* | *13 (100)* | *28 (78)* |
| ***LDH levels*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Normal* | *38 (86)* | *17 (52)* | *55 (71)* | ***0.002*** | *24 (77)* | *11 (55)* | *35 (69)* | *0.126* | *18 (78)* | *6 (46)* | *24 (67)* | *0.071* |
| *Elevated* | *5 (11)* | *14 (42)* | *19 (25)* | *7 (23)* | *9 (45)* | *16 (31)* | *5 (22)* | *7 (54)* | *12 (33)* |
| *N/A* | *1 (3)* | *2 (6)* | *3 (4)* |  |  |  |  |  |  |  |  |  |
| ***Therapy*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *Nivolumab* | *3 (7)* | *5 (15)* | *8 (10)* | *\*0.491* | *11 (35)* | *2 (10)* | *13 (25)* | *\*0.999* | *7 (30)* | *1 (7)* | *8 (22)* | *\*0.720* |
| *Pembrolizumab* | *20 (45)* | *9 (27)* | *29 (38)* | *13 (42)* | *13 (65)* | *26 (51)* | *9 (39)* | *7 (55)* | *16 (44)* |
| *Ipilimumab/Nivolumab* | *6 (14)* | *9 (27)* | *15 (20)* | *4 (13)* | *4 (20)* | *8 (16)* | *4 (17)* | *1 (7)* | *5 (14)* |
| *Ipilimumab/Pembrolizumab* | *15 (34)* | *10 (31)* | *25 (32)* | *3 (10)* | *1 (5)* | *4 (8)* | *3 (13)* | *4 (31)* | *7 (20)* |
| ***Timing of blood draw*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *During 1st line Rx* |  |  |  |  |  |  |  |  | *6 (26)* | *3 (23)* | *9 (25)* | *0.999* |
| *After 1st line Rx* |  |  |  |  |  |  |  |  | *17 (74)* | *10 (77)* | *27 (75)* |
| ***BRAF mutation status*** |  |  |  |  |  |  |  |  |  |  |  |  |
| *BRAF V600 Mutant* | *20 (45)* | *15 (45)* | *35 (45)* | *0.999* | *24 (77)* | *14 (70)* | *38 (75)* | *0.743* | *23 (100)* | *13 (100)* | *36 (100)* | *-* |
| *BRAF V600 WT* | *24 (55)* | *18 (55)* | *42 (55)* | *7 (23)* | *6 (30)* | *13 (25)* |  |  |  |

\*Analysis performed comparing anti-PD-1 monotherapy and anti-CTLA-4 plus anti-PD-1 combination therapy. N/A – not available.

**Table S8:** Clinical characteristics at baseline of the melanoma patients included in Figure 3.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | ***High ctDNA*** | | | ***P-value*** | ***Low ctDNA*** | | | ***P-value*** |
|  | ***Combination***  ***Immunotherapy*** | ***Anti-PD-1 monotherapy*** | ***Total*** | ***Combination***  ***Immunotherapy*** | ***Anti-PD-1 monotherapy*** | ***Total*** |
| ***Variable*** | *N=23 (%)* | *N=24 (%)* | *N=47 (%)* | *N=27 (%)* | *N=35 (%)* | *N=62 (%)* |
| ***Age*** |  |  |  |  |  |  |  |  |
| *≤65* | *10 (43)* | *9 (37.5)* | *19 (40)* | *0.770* | *16 (59)* | *12 (34)* | *28 (45)* | *0.721* |
| *>65* | *13 (57)* | *15 (62.5)* | *28 (60)* | *11 (41)* | *23 (66)* | *34 (55)* |
| ***Sex*** |  |  |  |  |  |  |  |  |
| *Female* | *9 (39)* | *4 (17)* | *13 (28)* | *0.110* | *11 (41)* | *8 (23)* | *19 (31)* | *0.169* |
| *Male* | *14 (61)* | *20 (83)* | *34 (72)* | *16 (59)* | *27 (77)* | *43 (69)* |
| ***AJCC Stage/M Classification*** |  |  |  |  |  |  |  |  |
| *IIIc/M1a/M1b* | *7 (30)* | *6 (25)* | *13 (28)* | *0.751* | *13 (48)* | *19 (54)* | *32 (52)* | *0.798* |
| *M1c/M1d* | *16 (70)* | *18 (75)* | *34 (72)* | *14 (52)* | *16 (46)* | *30 (48)* |
| ***Brain metastasis*** |  |  |  |  |  |  |  |  |
| *Yes* | *10 (43)* | *9 (37.5)* | *19 (40)* | *0.770* | *10 (37)* | *8 (23)* | *18 (29)* | *0.267* |
| *No* | *13 (57)* | *15 (62.5)* | *28 (60)* | *17 (63)* | *27 (77)* | *44 (71)* |
| ***Brain only metastasis*** |  |  |  |  |  |  |  |  |
| *Yes* |  |  |  | *-* | *2 (7)* | *1 (3)* | *3 (5)* | *0.575* |
| *No* | *23 (100)* | *24 (100)* | *47 (100)* | *25 (93)* | *34 (97)* | *59 (95)* |
| ***LDH levels*** |  |  |  |  |  |  |  |  |
| *Normal* | *10 (43)* | *9 (38)* | *19 (40)* | *-* | *20 (74)* | *24 (69)* | *44 (71)* | *-* |
| *Elevated* | *12 (52)* | *7 (29)* | *19 (40)* | *4 (15)* | *2 (5)* | *6 (10)* |
| *N/A* | *1 (5)* | *8 (33)* | *9 (20)* | *3 (11)* | *9 (26)* | *12 (19)* |
| ***BRAF mutation status*** |  |  |  |  |  |  |  |  |
| *BRAF V600 Mutant* | *11 (48)* | *5 (21)* | *16 (34)* | *0.068* | *15 (56)* | *6 (17)* | *21 (34)* | ***0.003*** |
| *BRAF V600 WT* | *12 (52)* | *19 (79)* | *31 (66)* | *12 (44)* | *29 (83)* | *41 (66)* |



**Figure S1: ROC curve for ctDNA values in the first-line ICI for the discovery cohort. Analysis** was performed using 6 months PFS as read out. The table indicates the sensitivity and the specificity associated to each cuttoff value. Higlight in yellow and indicated by the arrow is the cuttoff values surrounding 20 copies per mL. Data was analysed and plotted using SPSS.



**Figure S2: Baseline ctDNA levels relative to survival in first-line targeted therapy.** Progression-free survival (PFS) curve of melanoma patients with low and high ctDNA levels in the study cohort. Significant Cox regression *P-*values, Hazard Ratio (HR) and 95% confidence intervals (CI) are indicated in the plot.



**Figure S3: Cutoff optimisation by correlation with survival and ctDNA data. A-F**, Relation between various ctDNA cutoffs, progression-free survival (PFS) and hazard ratio (HR) value, including 95% CI (grey shade). Each dot represents a possible ctDNA cutoff and red dots denote non-predictive cutoffs (*P*>0.05), in the discovery (A-C) or validation cohorts (D-F). Each graph denotes survival HR for ctDNA cutoffs in patients treated with first-line ICI baselines (A, D), second-line ICI baselines (B, E) or only *BRAF* mutant patients receiving ICI that were treated with first-line targeted therapy (C, F).



**Figure S4:** **Kaplan-Meier curves for progression-free survival (PFS) of second-line melanoma patients without intracranial disease only treated with ICI.** Patients were stratified into those with low (green) or high (red) baseline ctDNA levels. Each graph denotes PFS outcomes in the discovery (A) or validation cohorts (B, C); for patients treated with ICI as second-line treatment (A, B); or for *BRAF* mutant patients receiving ICI after failing first-line targeted therapy (C). Log-rank *P-*values, Hazard Ratio (HR) and 95% confidence intervals (CI) are indicated for each plot.