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SUPPLEMENTARY INFORMATION

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# Molecular analysis of primary melanoma T cells identifies patients at risk for metastatic recurrence

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**Suppl. Table 1. Selected Clinicopathological Patient Characteristics**

| Characteristic | All cases | | Training Group | | Test Group | | p-value |
|---|---|---|---|---|---|---|---|
| | No. | % | No. | % | No. | % | |
| Total number of patients | 199 | 100.0 | 100 | 50.3 | 99 | 49.7 | NA |
| Sex | | | | | | | 0.0639[$] |
| Female | 74 | 37.2 | 44 | 44.0 | 30 | 30.3 | |
| Male | 125 | 68.2 | 56 | 56.0 | 69 | 69.7 | |
| Age, years | | | | | | | 0.7210* |
| Median (IQR) | 66.0 (55.0 -74.0) | | 65.0 (54.8 -74.0) | | 7.0 (55.0 -74.0) | | |
| Range | 28 – 92 | | 28 - 92 | | 30 - 90 | | |
| Location | | | | | | | 0.4865[$] |
| Head and neck | 57 | 28.7 | 30 | 30.0 | 27 | 27.3 | |
| Trunk | 56 | 28.1 | 26 | 26.0 | 30 | 30.3 | |
| Extremities | 84 | 42.2 | 42 | 42.0 | 42 | 42.4 | |
| Other | 2 | 1.0 | 2 | 2.0 | 0 | 0.0 | |
| Thickness, mm | | | | | | | 0.9693[$] |
| >1.0-2.0 | 50 | 25.1 | 25 | 25.0 | 25 | 25.2 | |
| >2.0-4.0 | 75 | 37.7 | 37 | 37.0 | 38 | 38.4 | |
| >4.0 | 74 | 37.2 | 38 | 38.0 | 36 | 36.4 | |
| Mitoses per mm2 | | | | | | | 0.9877[$] |
| <1 | 9 | 4.5 | 4 | 4.0 | 5 | 5.1 | |
| ≥1 (range 1-40) | 190 | 95.5 | 96 | 96.0 | 94 | 94.9 | |
| Ulceration | | | | | | | 0.7325[$] |
| Absent | 102 | 51.3 | 54 | 54.0 | 48 | 48.5 | |
| Present | 91 | 45.7 | 43 | 43.0 | 48 | 48.5 | |
| Not documented | 6 | 3.0 | 3 | 3.0 | 3 | 3.0 | |
| Clark level | | | | | | | 0.3994[$] |
| II | 1 | 0.5 | 0 | 0.0 | 1 | 1.0 | |
| III | 25 | 12.6 | 11 | 11.0 | 14 | 14.2 | |
| IV | 115 | 57.8 | 64 | 64.0 | 51 | 51.5 | |
| V | 55 | 27.6 | 24 | 24.0 | 31 | 31.3 | |
| Not documented | 3 | 1.5 | 1 | 1.0 | 2 | 2.0 | |
| Disease manifestation at time of diagnosis | | | | | | | 0.2251[$] |
| Local | 118 | 59.3 | 64 | 64.0 | 54 | 54.4 | |
| Local + Regional LN | 81 | 40.7 | 36 | 36.0 | 45 | 45.5 | |
| Sentinel node | | | | | | | 0.2657[$] |
| Negative | 79 | 39.7 | 43 | 43.0 | 36 | 36.4 | |
| Positive | 56 | 28.1 | 23 | 23.0 | 33 | 33.3 | |
| Not performed | 64 | 32.2 | 34 | 34.0 | 30 | 30.3 | |
| Stage | | | | | | | 0.3182[$] |
| I | 21 | 10.6 | 10 | 10.0 | 11 | 11.1 | |
| II | 97 | 48.7 | 54 | 54.0 | 43 | 43.4 | |
| III | 81 | 40.7 | 36 | 36.0 | 45 | 45.5 | |
| Total follow up, months | | | | | | | 0.8074* |
| Median (IQR) | 54.0 (23.5 - 74.0) | | 46.5 (23.8 - 76.3) | | 57.0 (24.0 - 67.5) | | |
| Progression-free survival[#], months | | | | | | | 0.9186* |
| Median (IQR) | 38.0 (10.5 - 71.0) | | 36.0 (9.0 - 76.3) | | 38.0 (11.5 - 63.5) | | |
| Disease recurrence | | | | | | | 1[$] |
| No | 107 | 53.8 | 54 | 54.0 | 53 | 53.5 | |
| Yes | 92 | 46.2 | 46 | 46.0 | 46 | 46.5 | |
| Survival status | | | | | | | 0.1649[$] |
| Alive | 109 | 54.8 | 54 | 54.0 | 55 | 55.6 | |
| Dead, melanoma | 75 | 37.7 | 35 | 35.0 | 40 | 40.4 | |
| Dead, cause unknown | 15 | 7.5 | 11 | 11.0 | 4 | 4.0 | |

Abbreviations: IQR, interquartile range; NA, not applicable. $ p-value calculated with Pearson's chi-square test. * p-value calculated with two-sided Wilcoxon-Mann-Whitney test. # Progression-free survival is defined as the time from surgical resection of the primary melanoma and any possible lymph node metastases, during which all patients of this study were classified as disease free, to disease progression or death from any cause.

## Suppl. Table 2a. 5-year PFS Hazard Ratios

| Variable | HR | Lower CI | Upper CI | Per Unit Increase | P-value | LRT P-value |
|---|---|---|---|---|---|---|
| Thickness | 1.08 | 1.04 | 1.13 | 1 mm | **0.000162** | **0.00133** |
| Age | 1.02 | 1.01 | 1.03 | 1 year | **0.00541** | **0.00444** |
| Sex: M (vs F) | 0.87 | 0.61 | 1.26 | - | 0.46 | 0.462 |
| Nodal Disease: Present (vs Absent) | 2.44 | 1.70 | 3.49 | - | **1.07E-06** | **1.39E-06** |
| Ulceration: Present (vs Absent) | 1.69 | 1.18 | 2.43 | - | **0.00433** | **0.00435** |
| Mitotic Rate | 1.03 | 1.01 | 1.05 | 1 mitosis/mm2 | **0.00167** | **0.00413** |
| Location: Extremities (vs Head and Neck) | 1.07 | 0.71 | 1.62 | - | 0.72112 | 0.03451 |
| Location: Other (vs Head and Neck) | 0.73 | 0.10 | 5.36 | - | 0.76287 | 0.03451 |
| Location: Trunk (vs Head and Neck) | 0.57 | 0.34 | 0.93 | - | **0.02584** | **0.03451** |
| Simpson's Clonality | 0.96 | 0.76 | 1.21 | 0.1 | 0.751 | 0.748 |
| T-cell Fraction (continuous) | 0.81 | 0.72 | 0.91 | 0.1 | **0.000207** | **2.33E-05** |
| T-cell Fraction: ≥ 20% (vs < 20%) | 0.39 | 0.25 | 0.59 | - | **1.28E-05** | **3.00E-06** |

Abbreviations: HR – Hazard Ration, CI – 95% Confidence Interval, p-value by two-sided Z-Test, LRT – Likelihood-ratio test. N=199.

## Suppl. Table 2b. 5-year OS Hazard Ratios

| Variable | HR | Lower CI | Upper CI | Per Unit Increase | P-value | LRT P-value |
|---|---|---|---|---|---|---|
| Thickness | 1.05 | 1.00 | 1.10 | 1 mm | **0.0357** | 0.0603 |
| Age | 1.02 | 1.00 | 1.03 | 1 year | **0.0168** | **0.0147** |
| Sex: M (vs F) | 1.06 | 0.72 | 1.57 | - | 0.755 | 0.754 |
| Nodal Disease: Present (vs Absent) | 1.92 | 1.32 | 2.80 | - | **0.000663** | **0.000746** |
| Ulceration: Present (vs Absent) | 1.57 | 1.07 | 2.30 | - | **0.0214** | **0.0212** |
| Mitotic Rate | 1.02 | 0.99 | 1.04 | 1 mitosis/mm2 | 0.142 | 0.162 |
| Location: Extremities (vs Head and Neck) | 0.85 | 0.55 | 1.31 | - | 0.4614205 | 0.151801 |
| Location: Other (vs Head and Neck) | 0.73 | 0.10 | 5.34 | - | 0.7583885 | 0.151801 |
| Location: Trunk (vs Head and Neck) | 0.56 | 0.34 | 0.93 | - | **0.0276621** | 0.151801 |
| Simpson's Clonality | 0.92 | 0.71 | 1.19 | 0.1 | 0.522 | 0.511 |
| T-cell Fraction (continuous) | 0.80 | 0.71 | 0.90 | 0.1 | **0.000183** | **1.61E-05** |
| T-cell Fraction: ≥ 20% (vs < 20%) | 0.37 | 0.24 | 0.59 | - | **2.04E-05** | **4.00E-06** |

Abbreviations: HR – Hazard Ration, CI – 95% Confidence Interval, p-value by two-sided Z-Test, LRT – Likelihood-ratio test. N=199.

**Suppl. Table 3a. True positive and True Negative Rates – Training group**

| | true pos | true neg | false pos | false neg | n TCFr ≥ 20% | n | true pos rate | true neg rate |
|---|---|---|---|---|---|---|---|---|
| **All** | 47 | 20 | 18 | 15 | 35 | 100 | 0.76 | 0.53 |
| **Breslow Group** | | | | | | | | |
| 1-2mm | 11 | 6 | 5 | 3 | 9 | 25 | 0.79 | 0.55 |
| 2-4mm | 18 | 10 | 5 | 4 | 14 | 37 | 0.82 | 0.67 |
| >4mm | 18 | 4 | 8 | 8 | 12 | 38 | 0.69 | 0.33 |
| **Stage** | | | | | | | | |
| I | 6 | 2 | 2 | 0 | 2 | 10 | 1.00 | 0.50 |
| II | 19 | 14 | 11 | 10 | 24 | 54 | 0.66 | 0.56 |
| III | 19 | 4 | 5 | 5 | 9 | 33 | 0.79 | 0.44 |
| **Sentinel Lymph Node** | | | | | | | | |
| NA | 21 | 6 | 5 | 2 | 8 | 34 | 0.91 | 0.55 |
| Negative | 15 | 11 | 8 | 9 | 20 | 43 | 0.63 | 0.58 |
| Positive | 11 | 3 | 5 | 4 | 7 | 23 | 0.73 | 0.38 |

**Suppl. Table 3b. True positive and True Negative Rates – Testing group**

| | true pos | true neg | false pos | false neg | n TCFr ≥ 20% | n | true pos rate | true neg rate |
|---|---|---|---|---|---|---|---|---|
| **All** | 47 | 20 | 19 | 13 | 33 | 99 | 0.78 | 0.51 |
| **Breslow Group** | | | | | | | | |
| 1-2mm | 7 | 8 | 4 | 6 | 14 | 25 | 0.54 | 0.67 |
| 2-4mm | 17 | 8 | 9 | 4 | 12 | 38 | 0.81 | 0.47 |
| >4mm | 23 | 4 | 6 | 3 | 7 | 36 | 0.88 | 0.40 |
| **Stage** | | | | | | | | |
| I | 4 | 5 | 2 | 0 | 5 | 11 | 1.00 | 0.71 |
| II | 14 | 10 | 13 | 6 | 16 | 43 | 0.70 | 0.43 |
| III | 28 | 5 | 4 | 7 | 12 | 44 | 0.80 | 0.56 |
| **Sentinel Lymph Node** | | | | | | | | |
| NA | 22 | 4 | 2 | 2 | 6 | 30 | 0.92 | 0.67 |
| Negative | 6 | 12 | 13 | 5 | 17 | 36 | 0.55 | 0.48 |
| Positive | 19 | 4 | 4 | 6 | 10 | 33 | 0.76 | 0.50 |

**Suppl. Table 3c. True positive and True Negative Rates – Complete cohort**

| | true pos | true neg | false pos | false neg | n TCFr ≥ 20% | n | true pos rate | true neg rate |
|---|---|---|---|---|---|---|---|---|
| **All** | 94 | 40 | 37 | 28 | 68 | 199 | 0.77 | 0.52 |
| **Breslow Group** | | | | | | | | |
| 1-2mm | 18 | 14 | 9 | 9 | 23 | 50 | 0.67 | 0.61 |
| 2-4mm | 35 | 18 | 14 | 8 | 26 | 75 | 0.81 | 0.56 |
| >4mm | 41 | 8 | 14 | 11 | 19 | 74 | 0.79 | 0.36 |
| **Stage** | | | | | | | | |
| I | 10 | 7 | 4 | 0 | 7 | 21 | 1.00 | 0.64 |
| II | 33 | 24 | 24 | 16 | 40 | 97 | 0.67 | 0.50 |
| III | 47 | 9 | 9 | 12 | 21 | 77 | 0.80 | 0.50 |
| **Sentinel Lymph Node** | | | | | | | | |
| NA | 43 | 10 | 7 | 4 | 14 | 64 | 0.91 | 0.59 |
| Negative | 21 | 23 | 21 | 14 | 37 | 79 | 0.60 | 0.52 |
| Positive | 30 | 7 | 9 | 10 | 17 | 56 | 0.75 | 0.44 |

Abbreviations: TCFr – T-cell fraction, true pos rate = true pos / (true pos + false neg), true neg rate = true neg / (true neg + false pos)

## Suppl. Table 4. Immunohistochemistry Antibody Panel

| Order | Antibody | Source | Clone | Manufacturer | Dilution | Diluent | Opal | AR pH | Detection Kit |
|---|---|---|---|---|---|---|---|---|---|
| **1** | FoxP3 | Mouse | 236A1E7 | Abcam | 1:1000 | Da Vinci Green (Biocare Medical) | 540 | 9 | Opal Polymer HRP Ms + Rb (Perkin Elmer) |
| **2** | CD103 | Rabbit | EPR4166(2) | Abcam | 1:800 | Antibody Diluent/Block (Perkin Elmer) | 520 | 9 | Opal Polymer HRP Ms + Rb |
| **3** | CD8 | Rabbit | C8/144B | Dako | 1:1000 | Antibody Diluent/Block | 570 | 9 | Opal Polymer HRP Ms + Rb |
| **4** | CD4 | Mouse | 4B12 | Biocare Medical | 1:400 | Van Gough Yellow (Biocare Medical) | 650 | 9 | Mach 3 Mouse HRP-polymer (Biocare Medical) |
| **5** | CD3 | Rabbit | MRQ-39 | Cell Marque | 1:2000 | Antibody Diluent/Block | 620 | 9 | Opal Polymer HRP Ms + Rb |
| **6** | CD39 | Rabbit | EPR20627 | Abcam | 1:2000 | Antibody Diluent/Block | 690 | 9 | Opal Polymer HRP Ms + Rb |

## Suppl. Table 5. Cell phenotyping by multiplex immunohistochemistry

| Cell type | Phenotype |
|---|---|
| **$CD4^+$ T cells** | DAPI+ CD3+ CD4+ CD8- |
| **$CD8^+$ T cells** | DAPI+ CD3+ CD8+ CD4- |
| **T cells** | $CD4^+$ T cells & $CD8^+$ T cells<br>(DAPI+ CD3+ CD4+ CD8-) + (DAPI+ CD3+ CD8+ CD4-) |
| **Tregs** | DAPI+ CD3+ CD4+ CD8- FoxP3+ |
| **$CD39^+$ $CD8^+$ T cells** | DAPI+ CD3+ CD8+ CD4- CD39+ |
| **$CD103^+$ $CD8^+$ T cells** | DAPI+ CD3+ CD8+ CD4- CD103+ |
| **$CD39^+$ $CD103^+$ $CD8^+$ T cells** | DAPI+ CD3+ CD8+ CD4- CD39+ CD103+ |