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Immunologic phenotyping and S100 as biomarkers for response to anti-PD1 therapy in metastatic melanoma: A retrospective analysis

Degree Project in Medicine

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Programme in Medicine

Gothenburg, Sweden 2018

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1.Abbreviations

ACT	adoptive cellular therapy
AJCC	American Joint Committee on Cancer
APCs	antigen-presenting cells
BMI	body mass index
BOR	best overall response
CI	confidence interval
CTLA-4	cytotoxic T-lymphocyte–associated antigen 4
CR	complete response
CT	computed tomography
ECOG	Eastern Cooperative Oncology Group
FDA	Food and Drug Administration
HR	hazard ratio
ILP	isolated limb perfusion
IFN-α	interferon-alfa
LDH	lactate dehydrogenase
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
MM	malignant melanoma

OS	overall survival
P	p-value
PD	progressive disease
PD1	programmed cell-death protein 1
PDL1	programmed cell death 1 ligand
PFS	progression free survival
PR	partial response
PS	performance-status
RECIST	Response Evaluation Criteria In Solid Tumors
SD	stable disease
TCR	T cell receptor
Tx	treatment
ULN	upper limit of normal
UV	ultraviolet

2. Abstract

Degree Project, Programme in Medicine

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2018, Department of Oncology, Gothenburg, Sweden

2.1 Introduction

The prognosis of metastatic melanoma has historically been poor. New treatments have improved the prognosis including the immunotherapy anti-PD1 (programmed cell-death protein1) which activates anti-tumour T-cell immune-response. However, most patients do not show complete response to anti-PD1 and toxicities are common. Therefore, research to define biomarkers to predict response is needed. Potential biomarkers that requires further investigation are T-cells and S100.

2.2 Aim

To investigate different phenotypes of T-cells and S100 as predictive biomarkers for response to anti-PD1 therapy of metastatic melanoma.

2.3 Methods

The study was retrospective, variables of 116patients with metastatic melanoma, who initiated anti-PD1 therapy between 1st of September 2015 to 31st August 2017 at Jubileumskliniken, Sahlgrenska University Hospital, were collected anonymized from the patient's medical records. Variables of interest were best overall response (BOR), overall survival (OS), baseline immune panels, S100 baseline to 12weeks and S100 associated with progression.

2.4 Results

No significant difference in OS between the different baseline T cell-phenotypes-levels analysed was found.

Patients with elevated baseline S100 had a significant lower OS than patients with normal S100 (P=0.0038). 41.4% of patients had elevated baseline S100, the following analyses are done on these patients. Patients with progression had a significant different median change from baseline S100 of 143.8% compared to responding patients whose median change was -60.8% (P=0.0007). 68.8% of responding patients had decreasing values of S100 by ≥50% from baseline, 58.8% of patients with progress had increasing values of S100 by ≥50%. Patients with a >50% increase from baseline S100 had a significant lower OS than patients with a >50% decrease (P=0.0034).

2.5 Conclusions

Our findings suggest that, for a proportion of patients, S100 can be a useful biomarker for response to anti-PD1 therapy of metastatic melanoma and relevant for clinicians to predict response and survival.

2.6 Key Words

Metastatic melanoma, anti-PD1, biomarkers, T cells, S100

3. Introduction

3.1 Melanoma

Melanoma is an aggressive form of skin cancer that originates from pigment-producing cells of the skin called melanocytes. Due to various mutations of the genes the melanocytes become transformed and proliferate abnormally. The cells often begin to grow radially in the epidermis which can be followed by vertical growth, eventually leading to an invasion into the underlying dermis through the basement membrane and subsequent metastasis (1).

Melanoma occurs most frequently in the skin but can also appear in the eye (uvea, conjunctiva or orbita) or in the mucosa (for e.g. anus and vagina/vulva) (2).
A major risk factor for melanoma is Ultraviolet (UV) radiation from intermittent sun exposure (3). UVA and UVB radiation can cause DNA mutations, cellular growth, inflammation and a defect immune system with subsequent tumour development, other risk factors for the development of melanoma are, amongst others, a personal or a family history of melanoma, fair skin, light hair, freckles and multiple melanocytic nevi (4).

3.2 Epidemiology

Melanoma is the sixth most common cancer for men and the fifth most common for women in Sweden (5). The incidence of melanoma has increased in Sweden over the past 10 years with 5.3% per year for women and 5% per year for men and is currently 36.3/100 000 per year for women and 41.6/100 000 per year for men (6), making Sweden one of the countries with the largest incidence of melanoma skin cancer in Europe (4). The median age for diagnosis is 63.5 for women and 68 for men (7).

3.3 Diagnosis

Melanoma can develop de novo or from a pre-existing nevus that has begun to grow and changed size, form or colour. Other symptoms of melanoma can be pruritus or bleeding from the lesion but is most often asymptomatic. The diagnosis of melanoma is based on full body skin examination, dermoscopy and excision (8).

The American Joint Committee on Cancer (AJCC) staging system 8th version is used for the staging of cutaneous melanoma (9).

The excisional margins are 5 mm (5-10 mm if lentigo maligna), 10 mm if invasive with tumour thickness ≤1.0 mm and 20 mm if invasive and tumour thickness ≥1.0 mm. Sentinel node biopsy is recommended for melanoma >1.0 mm and melanoma ≤1.0 mm with ulceration. If metastasis >1.00 mm in Sentinel node, multidisciplinary board is recommended to evaluate whether regional lymph node dissection or clinical observation with ultra sound is required (4). However, the recently published MSLT trial showed no increased melanoma-specific survival in patients receiving lymph node dissection compared with clinical observation among patients with melanoma and sentinel-node metastases, thus questioning the use of sentinel node and lymph node dissection (10). Furthermore, lymph node metastasis indicates further investigation with FDG-PET-CT of the whole body or CT thorax/abdomen, CT head and neck and CT of the brain (4).

3.4 Treatment of metastatic melanoma

If metastatic melanoma is detected systemic therapy is indicated consisting of BRAF and MEK inhibitors or immunotherapy such as anti-PD1 (anti- programmed cell-death protein 1) therapy and anti-CTLA-4 (anti-cytotoxic T-lymphocyte–associated antigen 4) therapy or a combination of both. Patients with BRAF-mutations (for e.g. V600E or V600K mutations) can be treated with drugs called targeted therapies including the BRAF inhibitors vemurafenib

or dabrafenib normally in combination with the MEK inhibitors trametinib or cobimetinib (4) considering both groups being inhibitors of the MAPK (Mitogen-activated protein kinase) pathway in the melanoma-cell that activates transcription, cell growth, proliferation and survival of the melanoma-cells (11).

Furthermore, combination therapy with BRAF and MEK inhibitors compared with BRAF inhibitors alone has been shown to improve the rates of response, progression-free survival and overall survival in patients with metastatic melanoma and BRAF V600E or V600K mutations (12, 13).

The prognosis of metastatic melanoma, when chemotherapy and interferon-alfa (IFN-α) was the only treatment option, has historically been very poor but several new treatment options have improved the prognosis (8). The currently first-line treatment of metastatic melanoma is immunotherapy or targeted therapy (4). There are many phase III studies about these new treatment options. However, there is a lack of studies about patients receiving treatment in the everyday clinical situation and that may have lower performance-status (PS), brain metastasis as well as comorbidity and therefore would be excluded in a phase III study.

3.5 T cells

T cells or T lymphocytes are a part of the adaptive immune system together with B cells. Naive T cells are formed and receive their unique specificity in the bone marrow and thymus which are the primary lymphoid organs. Activation of the naive T cell depend upon antigen-presenting cells (APCs) that are present in the skin and in the mucosa. The APCs collect antigens that look different in comparison to the peptides on healthy cells for e.g. peptides that are expressed on cancer cells due to various mutations in the cell. The APCs present the antigen on MHC (Major histocompatibility complex) -molecules on the cell surface and migrate to the nearest lymph node via lymph vessels where also lymphocytes migrate.

Lymphocytes with the unique specificity against the antigen presented binds to the antigen on the APC which leads to activation, maturity and proliferation of the naive T cell resulting in a clonal expansion of the activated T cell that leaves the lymph node to defend the host against the antigen with some cells eventually developing to memory T cells (14).

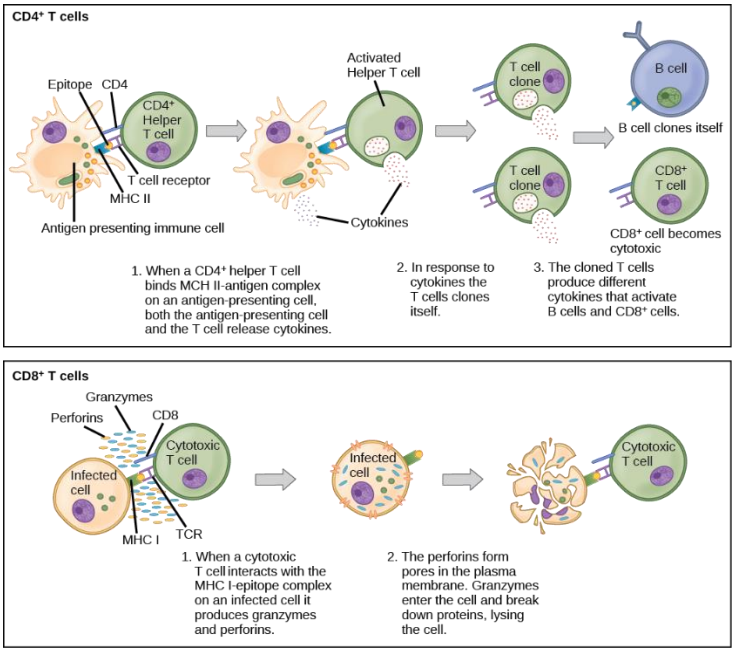

Figure 1. Activation of CD4+ T cells and CD8+ T cells by Antigen presenting immune cells. Abbreviations: MHC, major histocompatibility complex; TCR, T cell receptor.
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During antigen-mediated activation of a naive T cells several regulatory mechanisms are induced involving peptide-MHC engagement of the T cell receptor (TCR) and positive costimulatory signals, in this case interactions between CD28 on T cells and CD80 and/or CD86 on APCs (15). Furthermore, negative regulators are induced early in the process to regulate the activation such as CTLA-4 (cytotoxic T-lymphocyte–associated antigen 4) competing directly with CD28 for the ligands CD80 and CD86 (16). Likewise, PD1 (programmed cell-death protein 1) is expressed during T cell activation and counteracts positive signals through the TCR and CD28 by engaging its ligand PDL1 (programmed cell

death 1 ligand) expressed by many different cell types including cancer cells (17). Hence these inhibitory signals functions as breaks for the adaptive immune response and serves as immune checkpoints that effector T cells must pass to exert their full functions and maintains balance in the immune system. PD1 has a significant role in shaping the initial magnitude of the T cell response, in differentiation and in the development of immunological memory in CD4+ and CD8+ T cells. However, during cancer high and sustained expression of PD1 and its ligands such as PDL1 and PDL2 are common and can therefore limit protective immunity by potentially restrain trafficking to the tumour and effector functions in T cells that have recently been activated by tumour-antigen bearing APCs. On the other hand blockade of the PD1 pathway can improve T cell functions and therefore restore anti tumoral immunity (15).

3.6 Immunotherapy of metastatic melanoma

Immunotherapy has in the past decade been shown to improve the overall survival of patients with advanced stage cancer in phase III clinical trials, in particular Immune-cell-targeted monoclonal antibody (mAb) therapy (18) and adoptive cellular therapy (ACT) have appeared as effective (19). Immunotherapy generates or intensify an immune response against the cancer by stimulation of T-cell function with antibodies that block or activate regulatory receptors. T-cell co-stimulation is a method that activates T-cell function due to mAbs targeting their stimulatory receptors (for e.g. abatacept for the treatment of rheumatoid arthritis), checkpoint blockade on the contrary stimulates T-cell function with mAbs blocking their inhibitory receptors (for e.g. anti-PD1 and anti-CTLA4) for the treatment of metastatic melanoma). Immunomodulatory mAbs target immune cells and are therefore not specific to any cancer type (20). Anti-PD1 mAbs blocks PD1 on the T cell surface which inhibits the tumour cell ligands PDL1 and PDL2 from binding to PD1 and inhibiting T cell function, therefore blockade of PD1 increases T cell activity. Likewise, Anti-CTLA-4 mAbs binds to

the CTLA-4 receptor on the T cell surface and therefore blocks an inhibitory signal resulting in an increased cytotoxic T cell immune response (4) .

Metastatic melanoma was before 2011 considered a devastating disease and was almost uniformly fatal within 18 months of diagnosis. Over a brief period, the treatment landscape of metastatic melanoma has shifted drastically (21). This includes ipilimumab, an anti-CTLA-4 mAb, which was approved by the FDA (Food and Drug Administration) for the treatment of metastatic melanoma in 2011 (20). Ipilimumab has been shown to improve survival in patients with metastatic melanoma, nevertheless the response rates seem to be low and several adverse events have been noted with the majority being immune-related and potentially life-threatening (22). However, patients who respond to treatment with Ipilimumab have been shown to have a long-term survival effect of ipilimumab (23).

Furthermore, pembrolizumab and nivolumab two anti-PD1 mAbs were approved by the FDA in 2014 (20). Randomized, controlled, phase 3 studies have shown that pembrolizumab prolongs overall survival and progression-free survival and has less high-grade toxicity than ipilimumab in patients with advanced melanoma (24). Furthermore, nivolumab alone or combination therapy with nivolumab-ipilimumab has been shown to significantly longer survival among patients with advanced melanoma compared to ipilimumab alone (25). Moreover, nivolumab has an approximate 75% 1-year survival compared to the older treatment option chemotherapy that had an approximate 1-year survival rate of 30% (ref. personal communication, Henrik Jespersen).

Nonetheless, the majority of patients do not show complete response and long-lasting remission with PD1 pathway inhibitors (15). Furthermore, toxicity and immune-related adverse events have been observed, most commonly pruritus, rash, diarrhoea, nausea and thyroid disorders (although, adverse events seem to be associated with better treatment response) especially during combination therapy with PD1-targeted therapy and CTLA4-

targeted therapy (26, 27). Thus, indicating the relevance of a better mechanistic understanding of why modulation of the PD1 pathway leads to significant clinical benefit in some patients but temporary, partial or no clinical benefit among other patients. Hence several efforts are underway to define biomarkers to predict which patients will benefit from PD1 pathway blockade (15).

3.7 Biomarkers and S100

Tumour markers can be used for numerous purposes such as for screening, as diagnostic instruments, for staging, as a prognostic tool, to detect a recurrence and furthermore as a quality control assessment after therapy. Several patients may have a subclinical dissemination which can remain undetected by imaging methods, therefore biomarkers can be of help in staging and defining prognosis in patients with metastatic melanoma. Currently, the most important prognostic factors for mortality in melanoma are Breslow thickness, ulceration, mitosis and the presence of metastases (28). Furthermore, the most prominent tumour marker in melanoma is LDH (lactate dehydrogenase) which is used in the American Joint Committee on Cancer (AJCC) staging system for stage IV (9).

Table 1. Definition of Distant metastasis (M) according to the AJCC staging system.

	M Criteria	
M Category	**Anatomic site**	**LDH level**
M0	No evidence of distant metastasis	Not applicable
M1	Evidence of distant metastasis	See below
M1a	Distant metastasis to skin, soft tissue including muscle, and/or nonregional lymph node	Not recorded or unspecified
M1a(0)		Not elevated
M1a(1)		Elevated
M1b	Distant metastasis to lung with or without M1a sites of disease	Not recorded or unspecified
M1b(0)		Not elevated
M1b(1)		Elevated
M1c	Distant metastasis to non-CNS visceral sites with or without M1a or M1b sites of disease	Not recorded or unspecified
M1c(0)		Not elevated
M1c(1)		Elevated
M1d	Distant metastasis to CNS with or without M1a, M1b, or M1c sites of disease	Not recorded or unspecified
M1d(0)		Normal
M1d(1)		Elevated

S100 proteins are members of a multigene family of low-molecular-weight (9-13 kDa) calcium-modulated proteins (29). The S100 protein family includes 24 members (30) and was named after their solubility in 100% saturated ammonium sulphate at neutral pH by Moore who originally isolated the first member of the protein family as a protein fraction from bovine brain tissue in 1965 (31). Furthermore, it was discovered that melanoma cells are able to secrete a soluble form of S100 protein (32). Additionally, S100 in serum and cerebrospinal fluid has been shown to serve as a marker for acute damage of the nervous system e.g. in stroke patients (33). S100B (or S100β), a cytoplasmic protein and a member of the S100 family of calcium-binding proteins is expressed in elevated levels in the nervous system, melanocytes, dendritic cells and moreover, in various tumours including melanoma and schwannoma (34). S100B is also well expressed in adipocytes and chondrocytes. The intracellular functions of S100B includes involvement in Ca^{2+} homeostasis and regulation of enzyme activity. Through interactions with elements of the cytoplasmic cytoskeleton, S100B is also involved in the regulation of cell morphology. Moreover, S100 proteins seem to be involved in cell differentiation, cell motility, transcription and cell cycle progression. Calcium-dependent binding of target proteins is often required in the several cellular activities of the S-100 protein family. S100 proteins mostly exist as dimers and dimerization also seems to be crucial for the biological functions of S100 proteins (29).

Additionally, S100B is involved in signal transduction through the inhibition of protein phosphorylation such as the inhibition of calcium-dependent phosphorylation of p53 by protein kinase C which can lead to suppression of the p53 tumour suppressor mechanism and subsequent uncontrolled tumour growth (35).

Furthermore, numerous previous research about S100 role as a biomarker for melanoma-treatment has been done. Schultz *et al.* demonstrated a significant correlation between serum S-100B levels and clinical staging and survival where tumour progression was shown to be

accompanied by increasing S100B and the serum concentration of S100B also was shown to correlate with the number of affected organs in patients with distant metastases, suggesting S-100B as an interesting potential new tumour marker for clinical staging and monitoring of patients with metastatic melanoma (36). Later it was also shown by Hauschild *et al.* that 84% of patients with tumour progression during treatment had rising S100B levels, whereas 95% of patients with stable or regressing metastatic disease showed constant or declining S100B levels (37). Mocellin *et al.* suggested (based on data of a meta-analysis) that S100B haves a significant role in the therapeutic and follow-up management of patients with melanoma (34). Furthermore, it has been shown that an elevated serum S100B during follow-up has a 50% positive predictive value for recurrent disease in high risk-risk melanoma patients (38).

Barak *et al.* demonstrated that serum levels of S100B were significantly higher in all melanoma-patients before various therapies (surgery, chemotherapy, immunotherapy or their combinations) and decreased afterwards. Moreover, significantly higher levels of S100 were shown in advanced disease including metastasis in contrary to early disease (39). Notwithstanding, according to the National Swedish Guidelines of malignant melanoma, S100B in plasma is not validated as a marker for melanoma-diagnosis nor under follow-up (4). Nevertheless, S100 is frequently used as a marker for disease monitoring and response to treatment by the physicians in the Department of Oncology at the Sahlgrenska University Hospital in Gothenburg, Sweden. Also, no previous studies about the correlation between S100 and response to treatment with anti-PD1 therapy, to our knowledge, has been published. Furthermore, there is a lack of knowledge regarding T cell phenotypes roles as predictive biomarkers for response to treatment with anti-PD1 therapy. Thus, further research about S100 and T cell phenotypes roles as predictive biomarkers for response to treatment with anti-PD1 therapy in metastatic melanoma is required.

4. Aim

The overall aim is to investigate different phenotypes of T cells as predictive biomarkers for response to treatment with anti-PD1 therapy in metastatic melanoma, and S100 as a biomarker for response to treatment with anti-PD1 therapy in metastatic melanoma.

4.1 Specific objectives

1. Is there a correlation between T cells phenotypes and response to treatment with anti-PD1 therapy?

2. How does S100 levels correlate to response to treatment with anti-PD1 therapy?

5. Materials and Methods

5.1 Study population

The study population consisted of 116 adult patients with metastatic stage III-IV melanoma who initiated treatment with anti-PD1 therapy (nivolumab or pembrolizumab) in monotherapy between 1st September 2015 and 31st August 2017 at the Department of Oncology, Sahlgrenska University Hospital in Gothenburg. The cut-off for data-collection was set to 28th of February 2018 and thus the follow-up time for patients was at least 6 months. Anti-PD1 (nivolumab or pembrolizumab) therapy was administered intravenously. Nivolumab (3mg/kg) was given every second week whereas pembrolizumab (2mg/kg) was given every third week. The patients received anti-PD1 therapy for a maximum of 2 years.

5.2 Study design

The study was a retrospective analysis where all data was collected retrospectively from the patient's medical records as collected in the electronic journal system Melior and its supporting systems including laboratory analysis and radiology. A dataset in Microsoft Excel was constructed for the data collection with all patients receiving an anonymization code. Parts of the data collection had previously been done by another student one semester earlier who had another aim with her study. However, all the data had to be updated and a group of new patients and various new variables were added.

5.3 Collected variables

5.3.1 Clinically relevant variables

First, baseline characteristics about the patients were collected into the dataset consisting of gender, primary tumour resected (year), Breslow (mm), ulceration (yes/no), histotype, site,

age at tx start (treatment start), weight, length, BMI (body mass index), ECOG (Eastern Cooperative Oncology Group) PS, AJCC stage at tx start, lesions sites and number of lesion sites, LDH level, previous systemic treatment, mutational status and date of tx start. Second, data was collected about the following variables: diseased (yes/no), date of death (or censored), overall survival (OS) in months and best overall response (BOR) together with the response date. BOR was defined as complete response/CR, partial response/PR, progressive disease/PD or stable disease/SD and was collected from the physicians' dictum in the medical records about the clinical assessments of patients based on radiological evaluation. Time to response was calculated (time from tx start to BOR). Furthermore, data was collected about disease progression (or censored), anatomical site of progression, progression free survival (PFS) in months and duration of response was calculated, anti PD1 ongoing (yes/no), date for last treatment, time on treatment, reason for discontinuation, last follow up, follow up duration (months), pre-existing autoimmune condition and adverse events during treatment.

5.3.2 Immune panel

Lab-parameters consisting of baseline (baseline=within one month before tx start) T cells levels from flow cytometry with EDTA venous blood were collected in the form of naive CD3+ CD8+ 45RA+ (x10e9/L) T cells, memory CD3+ CD8+ 45RO+(x10e9/L) T cells and the CD3+8+45RA+/45RO+ quota (baseline) was calculated. Likewise, levels of naive CD3+ CD4+ 45RA+ (x10e9/L) T cells, memory CD3+ CD4+ 45RO+ (x10e9/L) T cells were collected and the CD3+4+45RA+/45RO+ quota (baseline) was calculated. CD3+DR+ (%) levels were also collected.

5.3.3 S100 analysis

The patients S100 baseline level and S100 levels within 12 weeks from treatment start were collected (and S100 change % was calculated) together with levels of S100 associated with disease progression and S100 levels within 3 months before radiological confirmed progression. Detection of S100 level were based on serum obtained from the patient's venous

blood where the test tube had been turned minimum five times and centrifuged 10 minutes after coagulation, levels <0.1µg/L are normal whereas levels ≥0.1 µg/L are pathological (40). The method of analysis is immunochemical and analyses all types of S100 proteins, the analysis range is 0.005-39 µg/L.

5.4 Statistical methods

The statistical analyses were performed in GraphPad Prism 7.0. Kaplan-Meier survival curves were used for the estimation of OS amongst all patients, OS depending on CD8+ RO/RA- CD4+ RO/RA- quota, OS and DR %, OS and CD8+RA+ level and OS depending on baseline S100-levels. Kaplan-Meier survival curves were also used to compare OS in patients with S100 >50% increase to OS in patients with >50% decrease from baseline. The OS was assessed using the date of start with anti-PD1 therapy and date of death or censored to the date for cut-off determined to 28th of February 2018. The hazard ratio (HR) and p-value were calculated by Log-rank test (Mantel-Cox).

Response was defined as CR or PR and No response was defined as SD or PD.

For proportions Clopper-Pearson confidence intervals (CI) were used. Fisher's exact test was performed for the correlation between S100- and LDH-level. Fisher's exact test were also used for the correlation between number of metastatic sites and S100 levels.

Contingency-tables were performed for S100 decrease (>/= 50%) vs response and S100 increase (>/= 50%) vs progress and correlation were estimated using Fisher's exact test, sensitivity and specificity was determined using Wilson-Brown method.

Box plots were used for the correlation between change in S100 (%) and response to treatment, the non-parametric Mann-Whitney U-test was used to compare the medians. Furthermore, boxplots were performed to visualize the correlation between S100 and number of metastatic sites, Mann-Whitney U-test was used to compare the medians. Spider plots were

performed to visualize 12 weeks change in S100 (%) and treatment response.

95% confidence intervals were used. $P<0.05$ was considered statistically significant.

6. Ethics

The study was retrospective and observational and hence non-interventional. Considering this, the analyses would not influence on the study population.

The head of the Department of Oncology approved the access to the patient journals for the student. The project was performed within a quality control of the patient group and did therefore not require ethical review initially. The collection of data was handled unidentified. All the patients received an anonymization code with only the student and the supervisor having the access for identification in a password protected excel-document.

7. Results

7.1 Baseline characteristic and OS for all patients

The study population included 116 patients.

The median age at tx start was 66 years. The most frequent metastatic stage was M1c (40.5% of the patients). 47.4% of the patients had a BRAF-mutated melanoma and 62.9% of the patients did not have a previous line of systemic therapy (Table 2).

The median OS was 27.9 months, the 1-year survival was 70.2% (Figure 2).

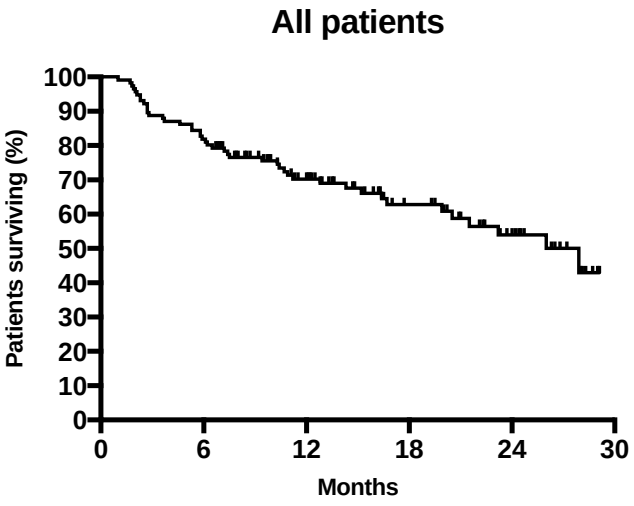


Figure 2. Kaplan-Meier survival estimate for Overall Survival (OS) in all patients. The median OS was 27.9 months. The 1-year survival was 70.2%

Table 1. Baseline characteristic of patients.

Characteristic	n=116	(%)
Age		
Median	66	
Range	27-98	
Age- no (%)		
<75	90	77.6
≥75	26	22.4
Sex – no (%)		
Male	62	53.4
Female	54	46.6
PS (ECOG)* - no (%)		
0-1	89	76.7
≥2	13	11.2
Unknown	14	12.1
Metastatic stage**-no. (%)		
M0	13	11.2
M1a	21	18.1
M1b	24	20.7
M1c	47	40.5
M1d	11	9.5
Number of lesion sites- no. (%)		
1	29	25.0
2	35	30.2
3	28	24.1
>3	24	20.7
LDH – no. (%)		
<ULN	71	61.2
>ULN	43	37.1
Unknown	2	1.7
BRAF mutation no. (%)***		
Yes	55	47.4
No	61	52.6
Previous lines of systemic therapy****-no. (%)		
0	73	62.9
≥1	43	37.1
Previous systemic treatment – no. (%)		
Chemotherapy	19	16.4
BRAF +/-MEK-inhibitor	31	26.7
Ipilimumab	8	6.9
Baseline S100 elevated		
No	58	50.0
Yes	48	41.4
Unknown	10	8.6

Abbreviations: PS (ECOG), performance-status (Eastern Cooperative Oncology Group); ULN, upper limit of normal.
*PS (ECOG) grade 0-5, higher grades associated with more severe disability.
**Metastatic stage according to TMN classification.
*** The analysis is done with PCR
****E.g. chemotherapy, targeted therapy, ipilimumab.

7.2 T cell phenotypes and response to treatment

T cells analyses were found in 48% of patients (56/116). No statistically significant difference in OS was shown in patients with baseline CD8+ RO/RA quota >1 compared to ≤1 (HR, 0.85; 95% CI of ratio; 0.38 to 1.89; P=0.68), or between CD4+ RO/RA quota <0.5 compared to ≥0.5 (HR, 0.90; 95% CI of ratio; 0.40 to 2.0; P=0.79). Moreover, no statistically significant difference in OS was shown among patients with baseline CD3+DR+ (%) ≤10% compared to >10% (HR, 1.33; 95% CI of ratio; 0.60 to 2.96; P=0.46), nor between CD8+RA+ ≥0.1 x 10e9/L compared to <0.1 x 10e9/L (HR, 0.76; 95% CI of ratio; 0.34 to 1.66; P= 0.48) (Figure 3).

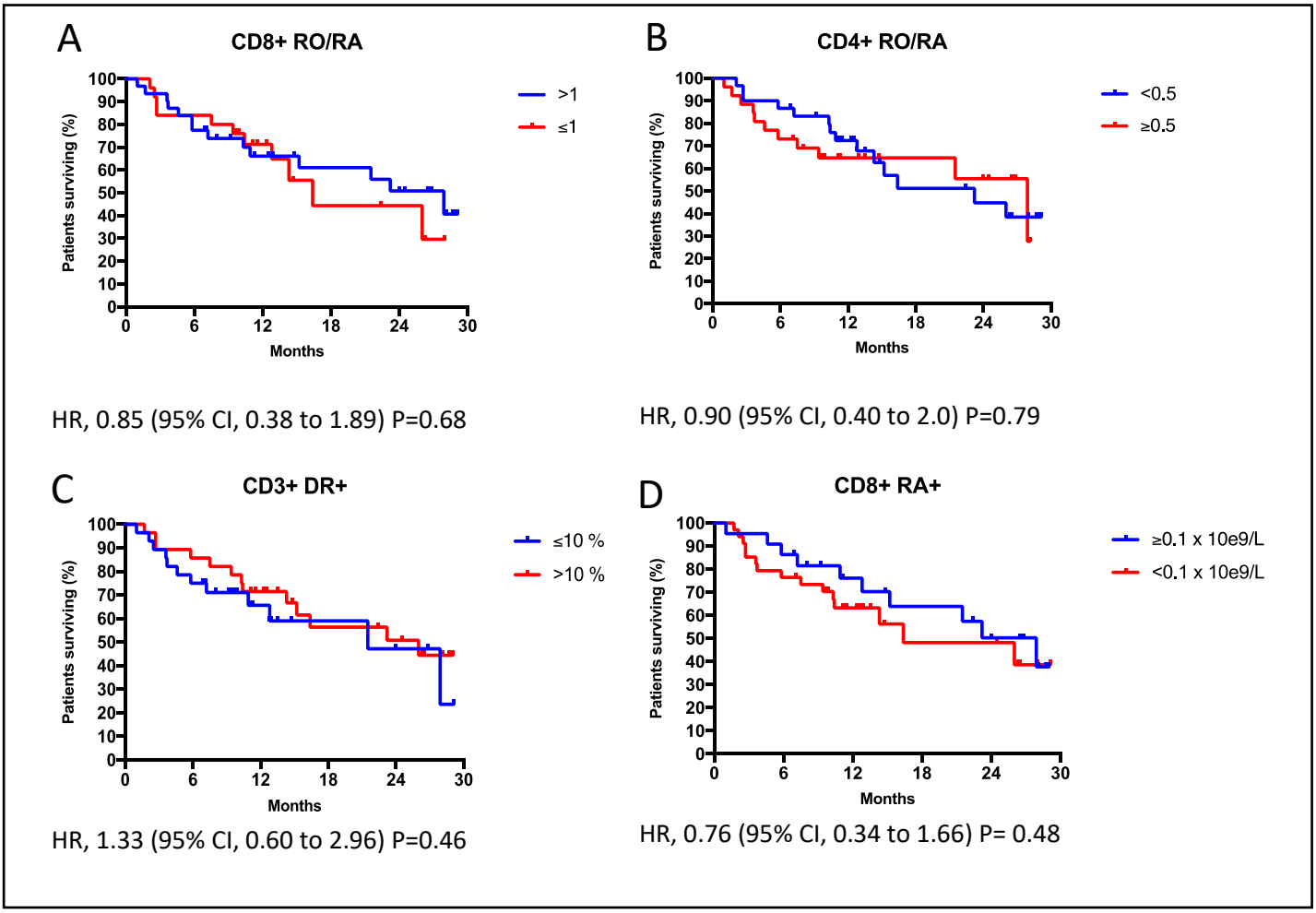
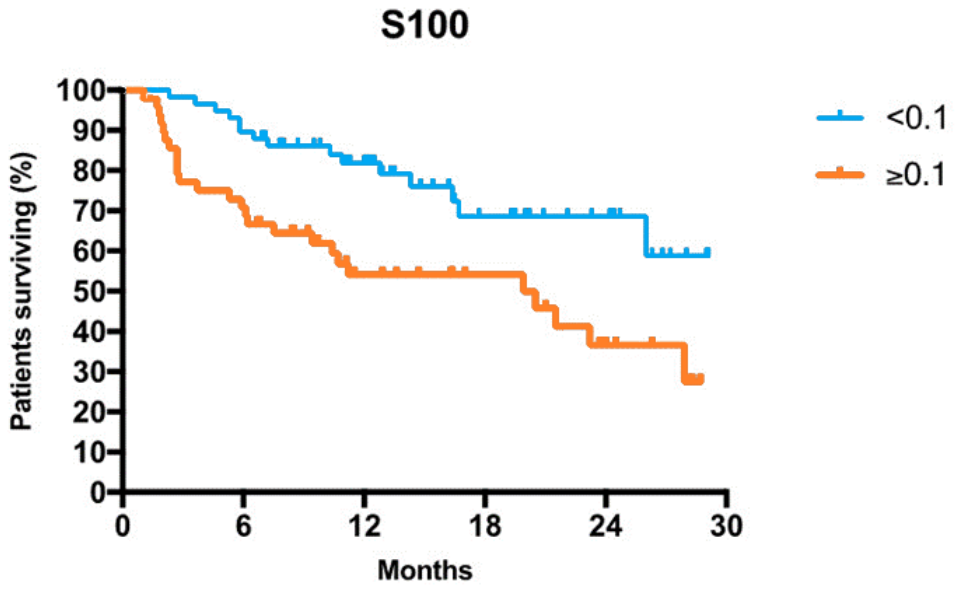


Figure 3. Kaplan-Meier survival estimates for Overall Survival (OS) in patients with (A) baseline CD8+ RO/RA quota >1 compared to ≤1, (B) baseline CD4+ RO/RA quota <0.5 compared to ≥0.5, (C) baseline CD3+DR+ (%) ≤10% compared to >10%, (D) baseline CD8+RA+ ≥0.1 x 10e9/L compared to <0.1 x 10e9/L. No statistically significant difference in OS was shown.
Abbreviations: HR, hazard ratio; CI, confidence interval; P, p- value.

7.3 S100 and response to treatment

S100 baseline was found in 91% of patients (106/116). 54.7% of the patients (58/106) had normal baseline S100 and 45.3 % (48/106) of the patients had elevated levels of S100 (95% CI; 35.6% to 55.3%). 37 patients with elevated baseline S100 had continuous S100 measurements prior to first radiological evaluation.

It was found that patients with elevated baseline S100 (≥0.1 µg/L) had a statistically significant lower OS than patients with normal baseline S100 (<0.1 µg/L) (Figure 4) (Median: <0.1: not reached, ≥0.1: 19.9 months; HR, 0.41; 95% CI of ratio; 0.22 to 0.75; P=0.0038).



HR, 0.41 (95% CI, 0.22 to 0.75) P=0.0038

Figure 4. Kaplan-Meier survival estimate for Overall Survival (OS) in patients with elevated baseline S100 ≥0.1 µg/L compared to normal baseline S100 <0.1 µg/L. Patients with elevated baseline S100 (≥0.1) had a statistically significant lower OS than patients with normal baseline S100 (<0.1) Median: <0.1: not reached, ≥0.1: 19.9 months.
Abbreviations: HR, hazard ratio; CI, confidence interval; P, p- value.

Disease progression was found in 74 patients and 44 of the patients had analysed S100 available at progression. 75% (33/44) of the patients with S100 available at progression had an elevated S100 (95% CI: 60% to 86.8%).

Among the patients with normal LDH level 74.2% (49/66) patients had normal S100 levels, 25.8% (17/66) had elevated S100 and among the patients with elevated LDH 22.5% (9/40) had normal S100 levels and 77.5% (31/40) had elevated S100 (fishers exact test P= <0.0001).

It was also found that 69% (40/58) of patients with 1-2 metastatic sites had normal S100 baseline levels and 62.5% (30/48) of patients with ≥3 metastatic sites had elevated baseline S100 (fishers exact test P=0.0017). Patients with ≥3 metastatic sites had statistically significant higher S100 baseline levels compared to patients with 1 metastatic site, the median baseline S100 for 1 metastatic site was 0.06 µg/L and the median baseline S100 for ≥3 metastatic sites was 0.15 µg/L (Mann-Whitney U; P=0.0049) (Figure 5A).

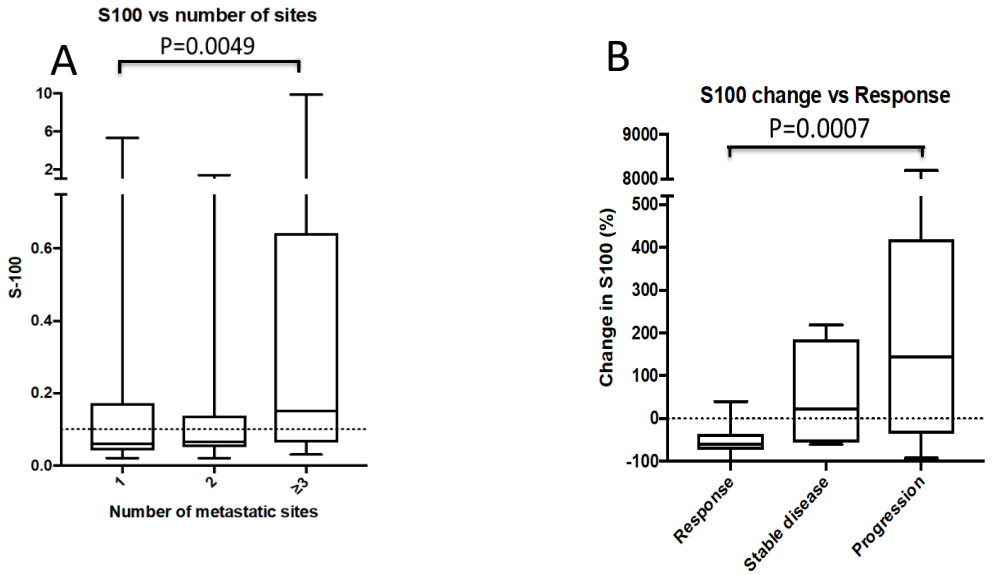


Figure 5. (A) Box plot for the correlation between baseline S100 level and number of metastatic sites. The median* baseline S100 for 1 metastatic site was 0.06 µg/L, the median baseline S100 for ≥3 metastatic sites was 0.15 µg/L. A statistically significant difference was found between median baseline S100 for 1 metastatic site compared to ≥3 metastatic sites (Mann Whitney U; P=0.0049)
(B) Box plot for the correlation between change in S100 (%) from baseline to 12 weeks and BOR (Response, Stable disease or Progression). The median* change for Response was -60.8%, for Stable disease 22.4% and for Progression 143.8%. A statistically significant difference of 204.6% was found between medians for S100 change (%) in patients with Response compared to Progression as BOR (Mann Whitney U; P=0.0007).
Abbreviations: P, p-value; BOR, best overall response.
*median= the band inside the box

The following analyses were done on patients with elevated baseline S100 (41.4% of patients). 68.8% (11/16) of responding patients had decreasing values of S100 by 50% or more from baseline. The sensitivity of a ≥50% decrease of S100 for predicting response was thus 68.8% (95% CI; 44.4% to 85.8%; P=0.009). The specificity of a ≥50% decrease of S100 from baseline for predicting response was 76.2% (95% CI; 54.9% to 89.4%; P=0.009). Several patients with response as BOR were seen having a 12 week decrease in S100 and a part of the patients had an initial rise in S100 before decrease (Figure 6A).

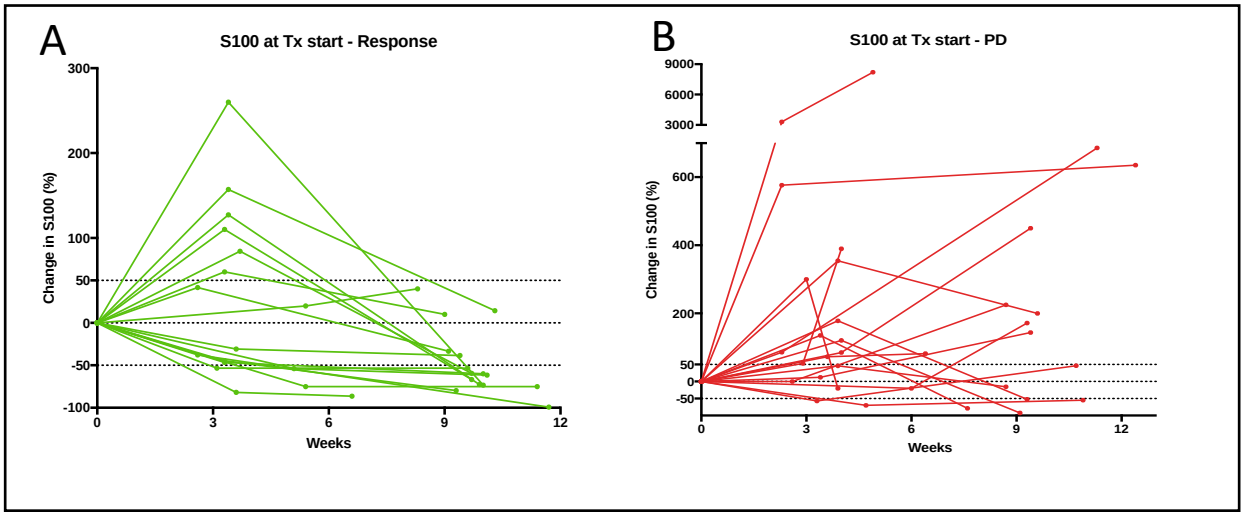


Figure 6. Spider plots for the visualisation of change in S100 (%) over 12 weeks for patients with (A) Response as BOR and (B) PD as BOR.
Abbreviations: BOR, best overall response; PD, progressive disease

58.8% (10/17) of patients with progress had increasing values of S100 by 50% or more from baseline. The sensitivity of a ≥50% increase of S100 for predicting progression was thus 58.8% (95% CI; 36.0% to 78.4%; P=0.0037). The specificity of a ≥50% increase of S100 from baseline for predicting progression was 90% (95% CI; 69.9% to 98.2%; P=0.0037). No patient with an increase from baseline S100 of > 50% had a response on first scan. Further, several patients with PD as BOR were seen having rising S100 levels 12 weeks from baseline (Figure 6B). It was also found that patients with a >50% increase from baseline S100 to 12 weeks had a significantly lower OS than patients with a >50% decrease (Figure 7),

the median OS for S100 >50% increase was 6.1 months (HR, 5.54; 95% CI of ratio; 1.69 to 18.1; P=0.0034).

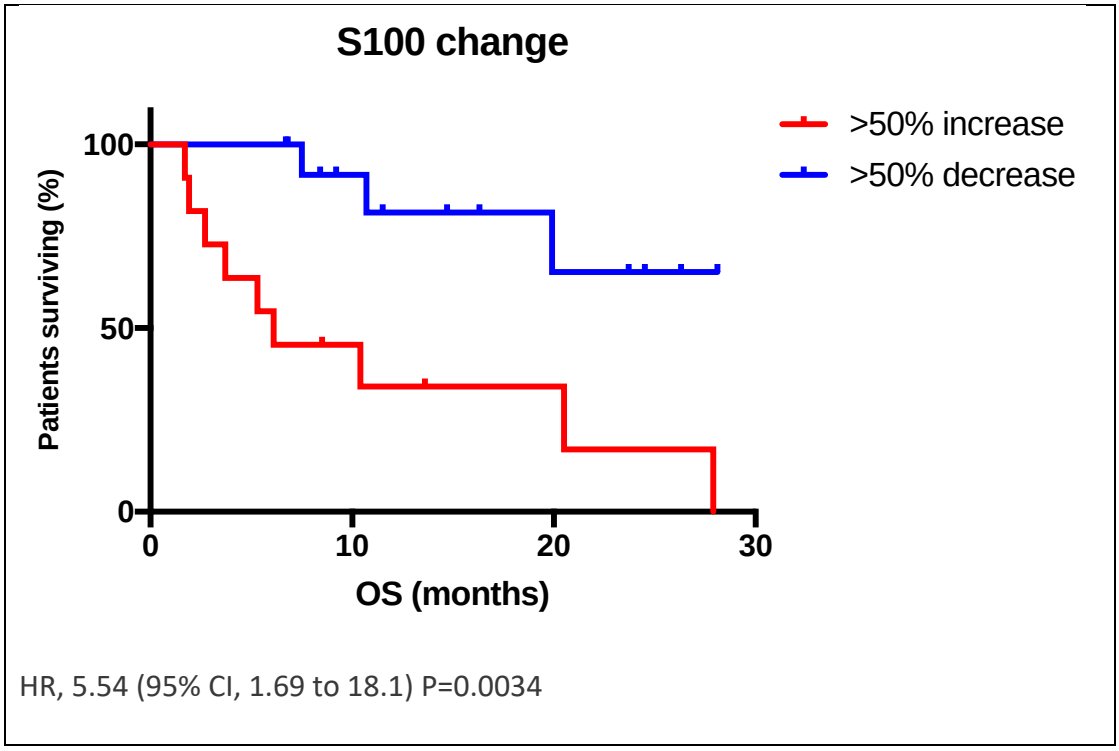


Figure 7. Kaplan-Meier survival estimate for change in S100 from baseline to 12 weeks and Overall Survival (OS). Patients with a >50% increase from baseline S100 to 12 weeks had a significantly lower OS than patients with a >50% decrease. The median OS for S100 >50% increase was 6.1 months (median OS for >50% S100 decrease undefined).
Abbreviations: HR, hazard ratio; CI, confidence interval; P, p- value.

Patients with progressive disease as BOR had a statistically significant different median change in S100 of 204.6% from baseline to 12 weeks compared to patients with response as BOR, the median change in S100 (%) for patients with progressive disease was 143.8%, whereas the median change in S100 for patients with response was -60.8% (Mann-Whitney U; P=0.0007). The median change for stable disease as BOR was 22.4%. (Figure 5B).

8. Discussion

8.1 S100 and response to treatment

An important aim of our study was to analyse S100 role as a biomarker and the correlation between S100 levels and response to treatment with anti-PD1 therapy of metastatic melanoma. No previous studies about S100 and response to treatment with anti-PD1 therapy, to our knowledge, has been published. However, numerous research has been done regarding S100 role as a biomarker in metastatic melanoma and the correlation of S100 levels to response to other treatments for metastatic melanoma.

First, our main findings were that patients with elevated baseline S100 had a statistically significant lower OS than patients with normal baseline S100), the median OS for patients with elevated S100 was 19.9 months (median for normal S100: not reached). Comparable results have been demonstrated by Bouwhuis et al. (41) who found lower OS among interferon-treated patients with S100 >0.2 µg/L compared to <0.2 µg/L and Buer et al. (42) who also demonstrated lower OS among patients with elevated S100 treated with chemoimmunotherapy. Hence, this study suggests that baseline S100 can be of importance to predict OS and that patients with elevated baseline S100 have worse prognosis which may be due to the correlation with greater disease burden as we showed that S100 correlated with the number of metastatic sites as well as LDH.

Second, it was found that 69% of patients with 1-2 metastatic sites had normal S100 baseline levels and 62.5% of patients with ≥3 metastatic sites had elevated baseline S100. Furthermore, patients with ≥3 metastatic sites were seen having statistically significant higher S100 baseline levels compared to patients with 1 metastatic site, this have previously been shown by Schultz et al. who observed that levels of S100 correlated well with the amount of

metastatic spread (36). Therefore, suggesting that baseline S100 can be of value for predicting metastatic spread.

Third, 75% of patients with an S100 measured at progression had an elevated S100. Furthermore, patients with progressive disease as BOR had a statistically significant greater median change from baseline S100 (%) of 143.8% compared to patients with response as BOR whose median change in S100 (%) was -60.8%. (the median change for stable disease as BOR was 22.4%). It was found that 68.8% of responding patients had decreasing values of S100 by 50% or more from baseline whereas 58.8% of patients with progress had increasing values of S100 by 50% or more from baseline and no patient with an increase from baseline S100 of > 50% had a response on first scan. Likewise, previous studies have shown increasing S100 levels associated to tumour progression (36, 37, 43), suggesting that increasing S100 might indicate progress, whereas decreasing S100 might indicate response. However, several patients with response as BOR were seen having an initial rise in S100 before decreasing and this remarkable observation can be of importance for the clinicians when evaluating the patients S100 levels. Additionally, patients with a >50% increase from baseline S100 to 12 weeks had a significantly lower OS than patients with a >50% decrease, the median OS for S100 >50% increase was 6.1 months (median for >50% decrease undefined). This data suggests that changes in S100 from baseline can be useful to estimate prognosis.

In summary, this study found that, for a proportion of patients, S100 can be a useful biomarker for response to treatment with anti-PD1 therapy of metastatic melanoma and relevant for clinicians for predicting survival and response. However, further research about S100 role as a biomarker is needed. For instance, it would be relevant to investigate S100 potential as a biomarker for other treatment options. Moreover, it can be of importance to study the correlation between S100 and AJCC-staging or S100 and adverse events.

8.2 Overall survival

The median OS for our patient group was 27.9 months, the 1-year survival was 70.2%, and can therefore be comparable to results found in previous studies, for instance Robert et al. (44) estimated the 1-year survival for patients treated with nivolumab to 72.9%.

8.3 T cell phenotypes and response to treatment

Furthermore, another aim of our study was to investigate different phenotypes of T cells roles as predictive biomarkers for response to treatment with anti-PD1 therapy of metastatic melanoma and if there was a correlation between T cells phenotypes and response to treatment. However, our study showed no statistically significant difference in overall survival between the different groups of baseline T cells phenotypes levels analysed. No literature about the correlation between baseline T cells phenotypes and OS for patients treated with anti-PD1 therapy was found, making these results difficult to compare with other studies. Nonetheless previous studies have investigated T cells phenotypes in metastatic melanoma, for instance Olofsson et al. (45) demonstrated an elevation of Melan-A+CD8+ T-cells in a subpopulation of patients after ILP (isolated limb perfusion) and Tumeh et al. (46) showed proliferation of intratumoral CD8+ T cells in patients responding to anti-PD1 therapy that correlated directly with radiological reduction in tumour size. Therefore, in future studies an analyse of changes of T cell phenotypes over time during treatment and correlation to response to treatment is required. However, T cells analyses were not found in the majority of patients and T cells analyses over time were even rarer making this analysis difficult to perform in the Department of Oncology at Sahlgrenska University Hospital.

We wanted to compare OS amongst patients that had baseline CD8+ RO/RA quota >1 with patients that had baseline CD8+RO/RA quota ≤1. Further, we also wanted to compare OS amongst patients that had baseline levels of CD8+RA+ ≥0.1 x 10e9/L with patients that had

baseline levels of CD8+RA+ <0.1 x 10e9/L. This seemed relevant because a previous study by Martner et al. (47), showed that baseline CD8+RO/RA quota >1 correlated significantly with complete response to treatment with ILP and that patients with complete response showed higher counts of baseline CD8+RA than patients without complete response. Nevertheless, our study did not find a statistically significant difference in OS between the different groups of baseline T cells phenotypes levels analysed. Perhaps another way to answer the question we asked would be to analyse the correlation between baseline T cell phenotypes and response/no response instead of OS alone or to analyse changes of T cell phenotypes over time during treatment and correlation to response to treatment. That we didn't find any significant differences could have been simply because we analysed T cell phenotypes at the wrong time. However, our findings suggest that baseline T cells phenotypes do not function as predictive biomarkers for response to treatment with anti-PD1 therapy.

Strengths and limitations

Strengths of our study include the large sample size (116 patients) and the long follow-up time. There were about as many men as women included and a wide range of ages. However, the study was retrospective and dependent on information from previously written medical records. Hence, there was a remarkable lack of information in the medical records especially regarding T cells analyses and S100 measurements making the subgroups for data-analysation smaller than they could have been if all the data was available, thus this is a limitation of the study. According to the clinicians, the patients who had continuous S100 measurements were most often the ones with baseline elevated S100 and therefore there is a lack of S100 measurements in order to optimize the data-analysis. Suggesting that this should be a work field for the clinicians at the Department of Oncology Sahlgrenska University Hospital. Nonetheless, another strength of this study is that we found statistically significant results

although the subgroups were small. Furthermore, another weakness is that there were many physicians involved in the patient care who might have done different assessments of patients. Also, there was a lack of RECIST (Response Evaluation Criteria In Solid Tumors) measurements in response evaluation. Furthermore, some of the patients had also previously been treated with other treatment options which might have affected clinical outcome.

9. Conclusions and Implications

This study found that elevated baseline S100 correlates with a lower OS than normal baseline S100. Furthermore, we found that baseline S100 correlates with the amount of metastatic spread. Moreover, we found that increasing S100 correlates with progress, whereas decreasing S100 correlates with response to anti-PD1 therapy. We also observed an initial rise in S100 before decrease in responding patients. Furthermore, we showed that changes in S100 from baseline can be of importance to estimate prognosis. Hence, our findings suggest that, for a proportion of patients, S100 can be a useful biomarker for response to anti-PD1 therapy of metastatic melanoma and relevant for clinicians to predict survival and response.

No statistically significant difference in overall survival was shown between the different groups of baseline T cells phenotypes levels analysed, suggesting that baseline T cells phenotypes do not function as predictive biomarkers for response to treatment with anti-PD1 therapy. However, there was a lack of lab parameters in many of the medical records suggesting that work should be done in this area to improve the situation of the patient group and the conditions for further research. Especially measurements of S100 can be of importance in the assessment of patients.

The excel-dataset used for data-collection will be used for data-collection in the future and the knowledge from the study will be of importance in future studies regarding predictive biomarkers to anti-PD1 treatment of the patients with metastatic melanoma in the Department of Oncology, Sahlgrenska University hospital.

10. Populärvetenskaplig sammanfattning

Immunologisk fenotypning och S100 som biomarkörer för respons till behandling med PD1-hämmare av metastaserat melanom: en retrospektiv analys

Malignt melanom är en cancertyp med ursprung från pigmentproducerande celler kallade melanocyter och uppkommer oftast i huden. Det är den femte vanligaste cancertypen hos kvinnor och den sjätte vanligaste för män i Sverige och förekomsten ökar. En stor riskfaktor för malignt melanom är solexponering. Behandlingen innebär borttagning av tumören med efterföljande vävnadsprov. Ifall tumören har spridit sig till andra delar av kroppen benämns sjukdomen som ”metastaserat melanom” och kräver andra behandlingsalternativ. Prognosen för metastaserat melanom har historiskt sett varit mycket dålig. De senaste åren har det dock tillkommit nya behandlingsprinciper som förbättrat prognosen avsevärt. Ett av dessa nya läkemedel är s.k. PD1-hämmare som tillhör gruppen immunterapi, behandlingsprincipen går ut på att aktivera T-celler (en typ av vita blodkroppar) vilket ger ett större immunförsvar mot cancern. Tyvärr svarar majoriteten av patienterna inte på behandlingen och biverkningar är vanligt förekommande. Därför är det viktigt med forskning för att hitta s.k. biomarkörer för att kunna förutsäga behandlingssvar med PD1-hämmare och därmed kunna avgöra vilka patienter som har nytta av behandlingen och vilka som inte har det.

Syftet med vår studie var att undersöka olika typer av T-celler och S100 (som är ett protein som utsöndras från tumören) som biomarkörer för behandlingssvar med PD1-hämmare hos patienter med metastaserat melanom. För att göra detta granskades 116 medicinska journaler tillhörande patienter behandlade med PD1-hämmare mot metastaserat melanom och information om bl.a. behandlingssvar samt labvärden av T-celler och S100 samlades in från journalerna i en databas i excel-format. Insamlingen av informationen hanterades avidentifierat. Informationen analyserades därefter i ett statistikprogram.

Vi fann att medianöverlevnaden för hela patientgruppen var 27,9 månader och att 1-årsöverlevnaden var 70,2%. Ingen signifikant (statistiskt säkerställd) skillnad i total överlevnad hittades hos de olika grupperna av T-cells typer som analyserades. Avseende S100 fann vi att patienter med förhöjt S100 innan behandling hade signifikant kortare total överlevnad än patienter med normalt S100 samt att S100-värdet var högre ju mer spridd sjukdom patienterna hade. Patienter med återfall som bästa behandlingssvar hade en signifikant större medianförändring i % av S100 från behandlingsstart jämfört med patienter som svarade på behandlingen (S100 ökade 143,8% för patienter med återfall och minskade 60,8% för patienter med behandlingssvar). 68,8% av patienterna som svarade på behandlingen hade minskande värden av S100 ≥50% från behandlingsstart och 58,8% av patienterna med återfall hade ökande värden med ≥50%. Patienter med en >50% ökning av S100 från behandlingsstart hade en signifikant kortare överlevnad än patienter med en >50% minskning.

Resultaten från denna studie visar att S100 kan vara en användbar biomarkör, för en del patienter, för behandlingssvar med PD1-hämmare och kunskapen kan komma till användning för läkare för att kunna förutsäga överlevnad och behandlingssvar.

11. Acknowledgements

I would like to thank Henrik Jespersen for guiding me through the project in a good and supportive way. Thank you for your kindness, patience and help with the million questions I asked.

I would also like to thank Lars Ny for the warm welcome into the world of Oncology Research, for guidance and support through the project and for making this project possible.

Further, I would like to thank the Department of Oncology/ Jubileumskliniken at Sahlgrenska University Hospital for the possibility of doing this study.

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