

**Supplementary information to “Tumor DNA methylation profiles correlate with response to anti-PD-1 immune checkpoint inhibitor monotherapy in sarcoma patients” by Starzer et al.**

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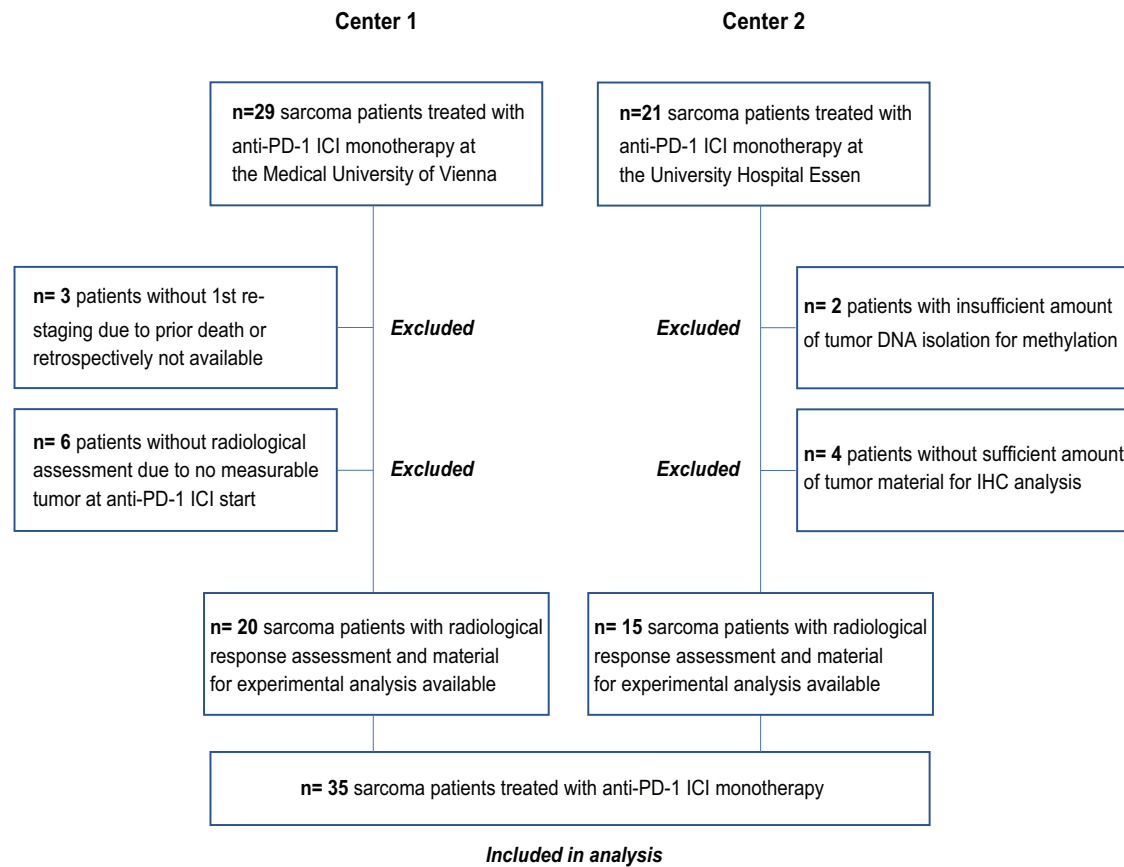
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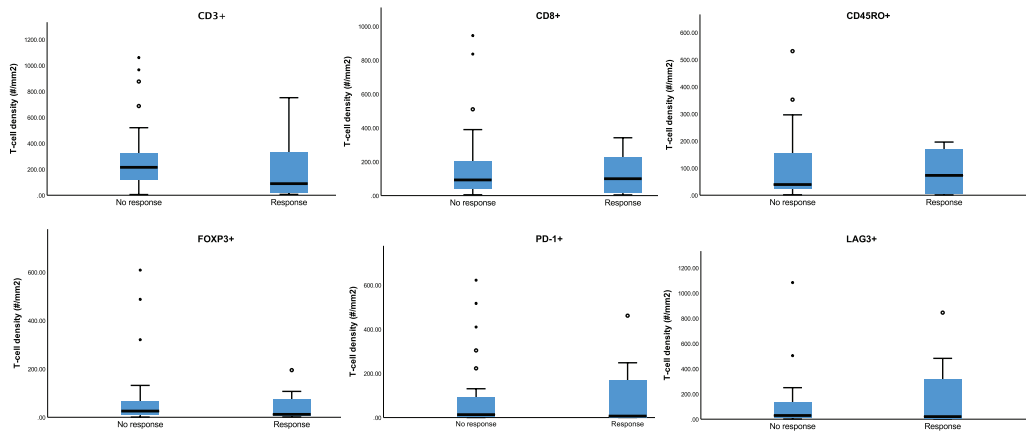
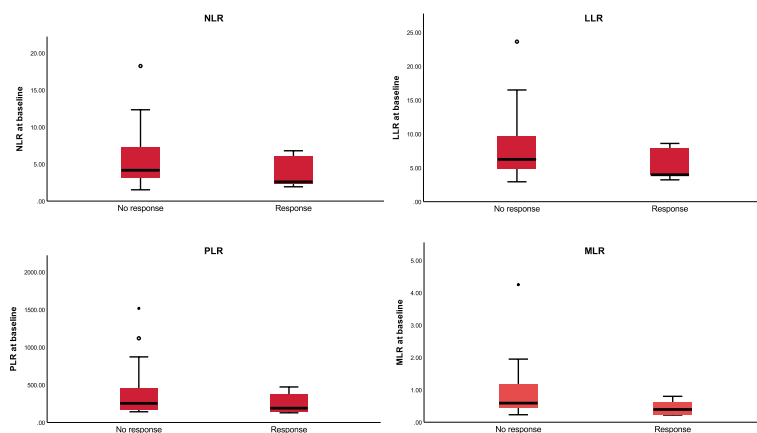
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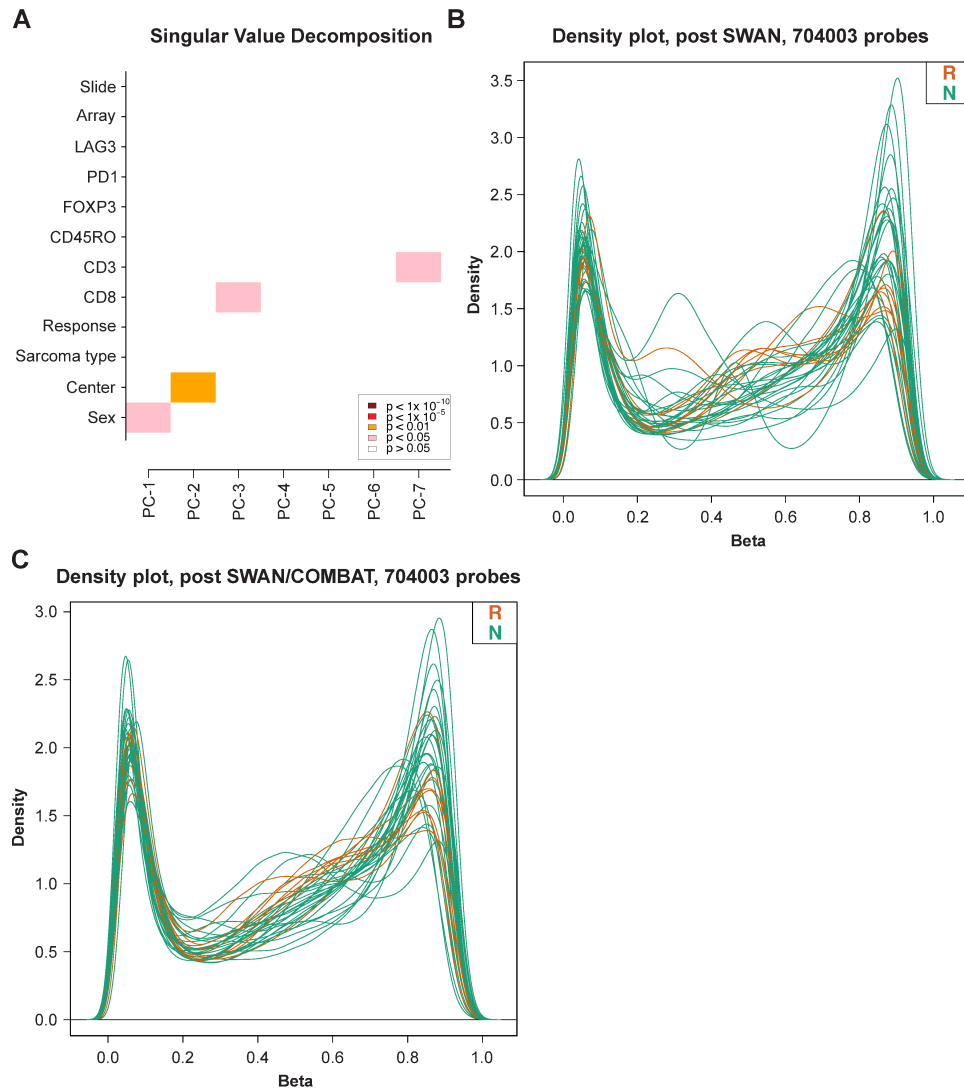
**Running title:** Methylation correlates with response to anti-PD-1 ICI in sarcoma patients



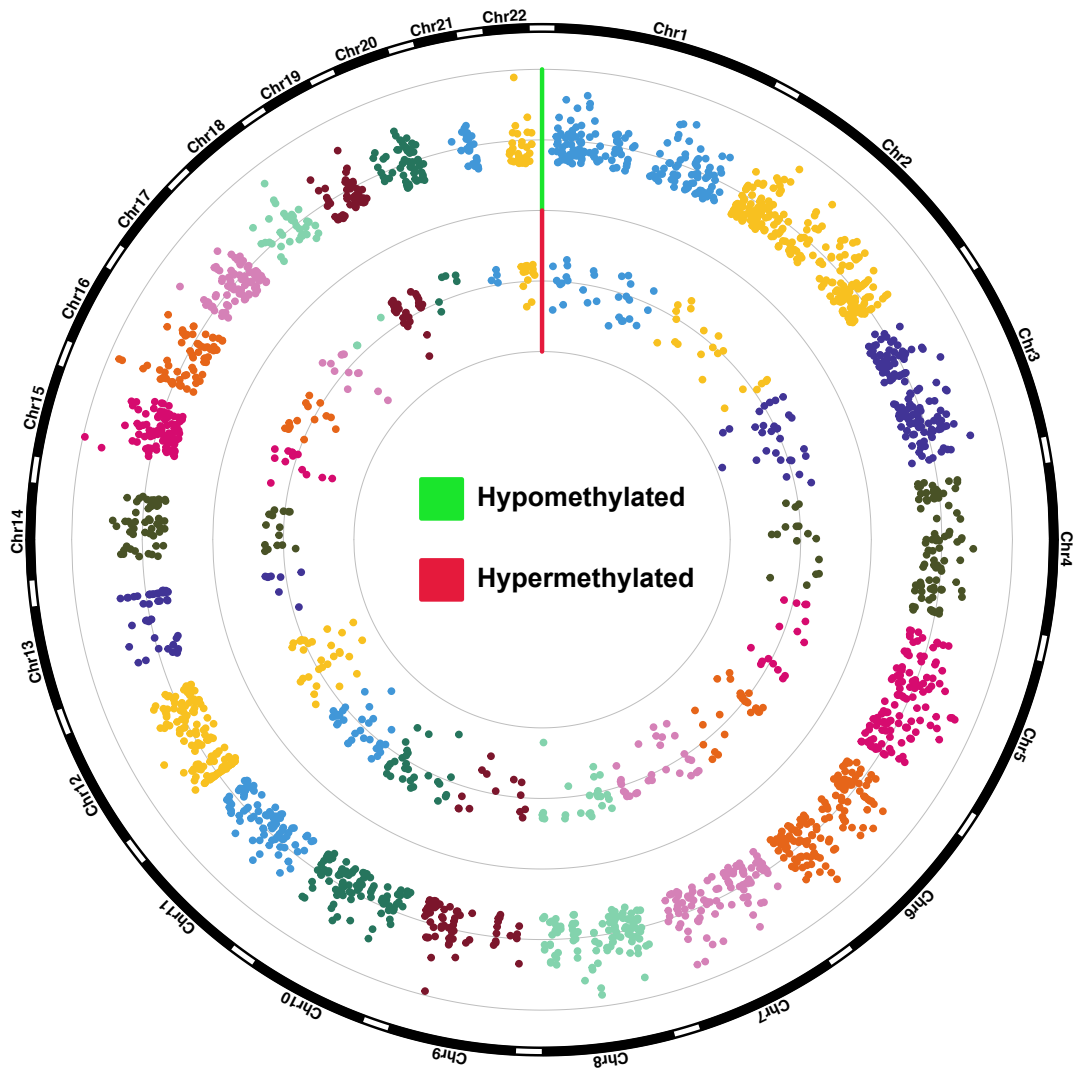
**Supplementary Figure S1.** Consort diagram of patient allocation

**A Immune cell density****B Systemic inflammation scores**

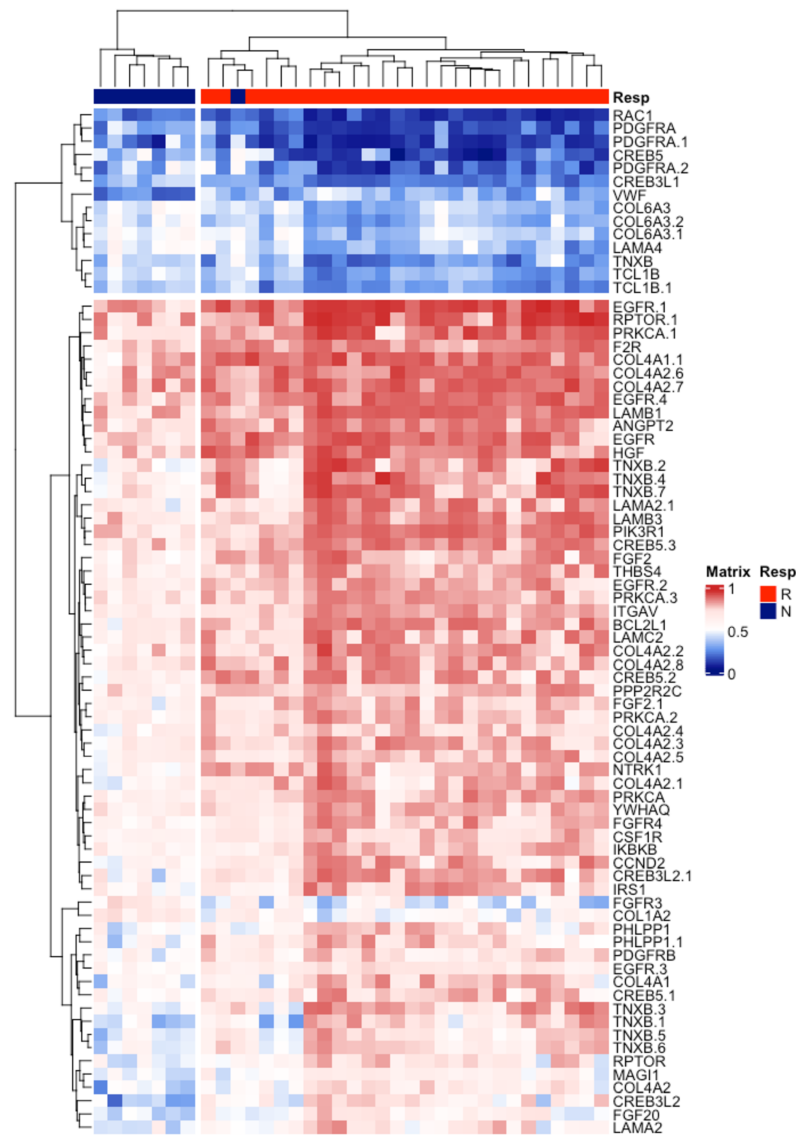
**Supplementary Figure S2. A** Median immune cell density and **B** systemic inflammation scores according to response or no response to anti-PD-1 ICI therapy. NLR, neutrophil-to-lymphocyte ratio; LLR, leucocyte-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; PLR, platelet-to-lymphocyte ratio



**Supplementary Figure S3. (A)** Singular Value Decomposition plot showing the influence of immune cell infiltration, center and sex on methylation of the study cohort of 35 sarcoma patients. Pink and orange boxes indicate statistical significance ( $P < 0.05$  and  $p < 0.01$ , respectively). **(B)** Density plot of SWAN normalized beta values of 8 responders (R) and 27 non-responders (NR) to anti-PD-1 ICI before and **(C)** after adjustment.

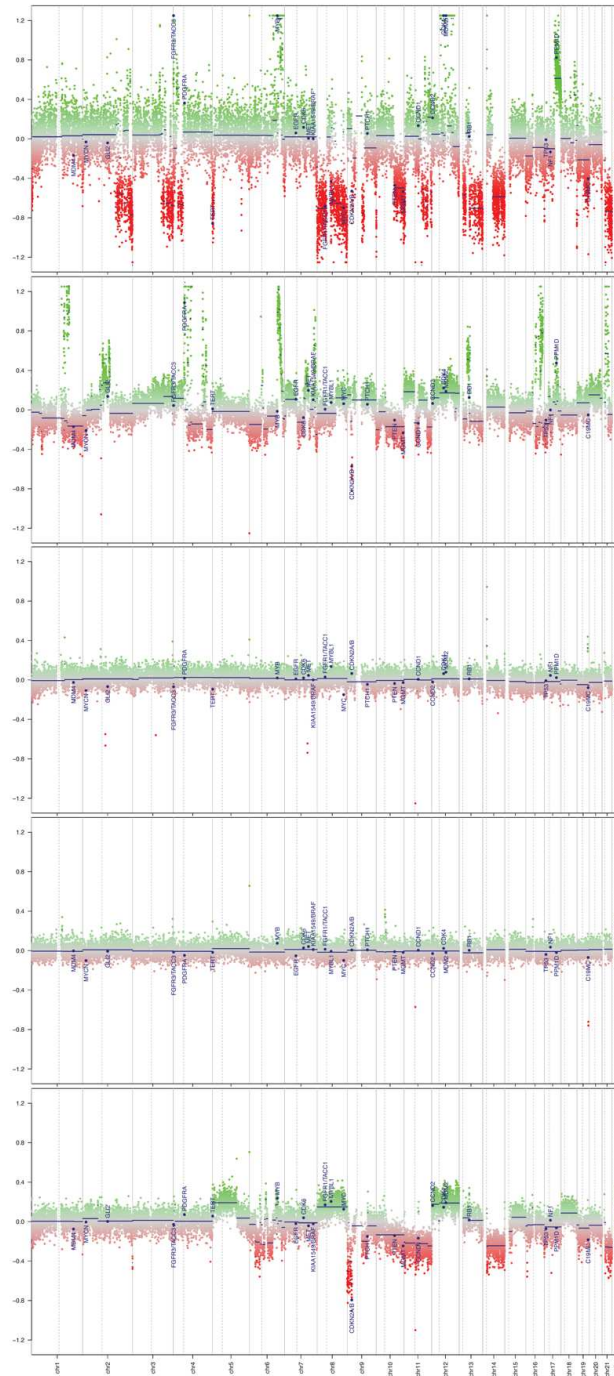


**Supplementary Figure S4.** Circular Manhattan plot showing chromosomal distributions of 2,043 hypomethylated (green; outer circle) and 410 hypermethylated (red; inner circle) DMPs between responders and non-responders to anti-PD-1 ICI.

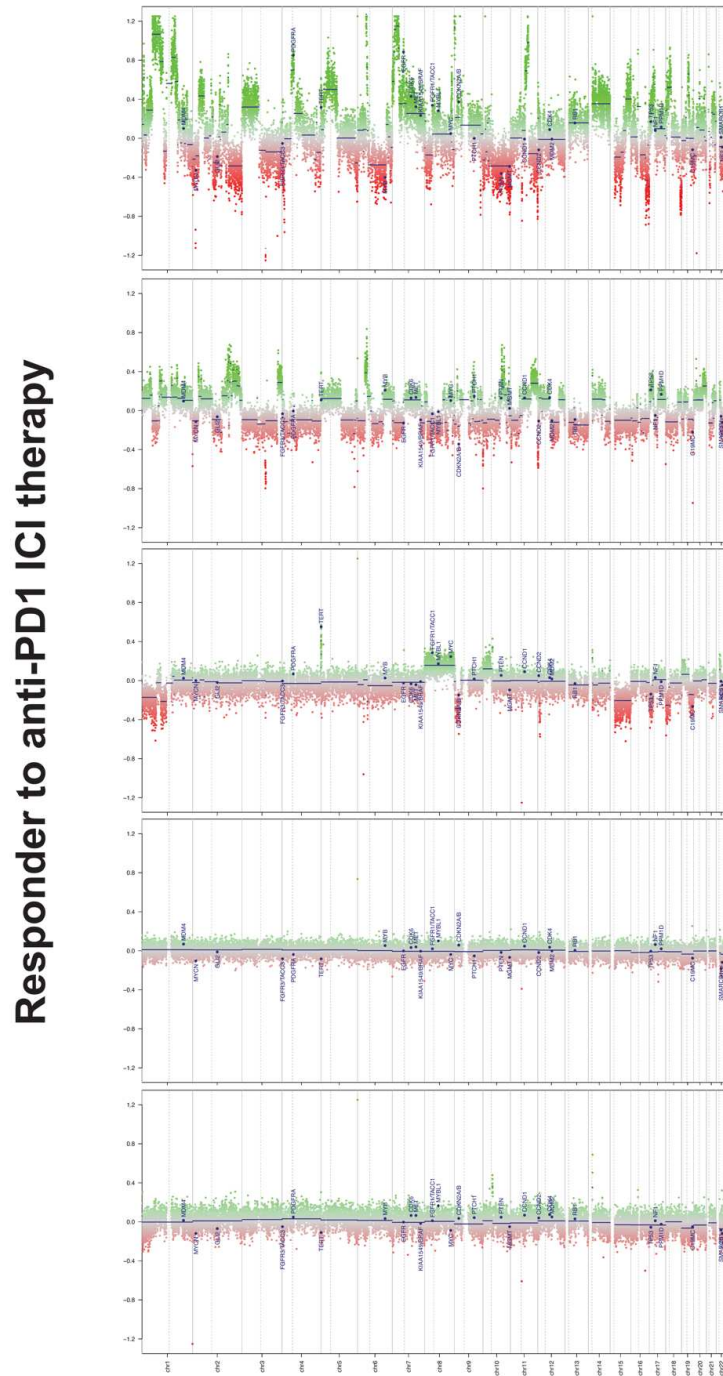


**Supplementary Figure S5.** Heatmap summarizing methylation of DMPs associated with PI3K/AKT signaling in 35 primary sarcoma samples (red, non-responder; blue, responder). Rows represent CpG sites and columns represent patient samples. Heatmap colors reflect beta values representing the degree of methylation from low (blue) to high (red). No centering/scaling of beta values was performed.

## Non-responder to anti-PD1 ICI therapy



**Supplementary Figure S6.** Copy number variations in 5 sarcoma patients non-responding to anti-PD-1 ICI.



**Supplementary Figure S7.** Copy number variations in 5 sarcoma patients responding to anti-PD-1 ICI.



**Supplementary Table S1.** Detailed patient listing for Center 1

ID	Sex	Age in years*	Histologic sarcoma type, localization	Sites of metastases*	Prior therapy lines*	Previous relapse free survival in months	Anti-PD-1 ICI specimen	PFS* (in months)	Response to anti-PD-1 ICI (iRECIST)
1	m	34	Ossifying fibromyxoid tumor, upper thigh	Lung, bone, soft tissue, lymph nodes	Anthracycline, ifosfamide, pazopanib, regorafenib, gemcitabine/dacarbazine, trabectedin, anthracycline, taxane, temsirolimus	2.5	Pembrolizumab	1.9	PD
2	f	50	Myxofibrosarcoma, cervical	Soft tissue	Anthracycline, pazopanib, gemcitabine/dacarbazine	2.5	Pembrolizumab	7.3	SD
3	f	50	Myxofibrosarcoma, gluteal	Lung, soft tissue	Anthracycline, ifosfamide, gemcitabine/dacarbazine, trabectedine, pazopanib	0.7	Pembrolizumab	0.8	PD
4	f	59	Dedifferentiated liposarcoma, retroperitoneal	Local recurrence	Taxane	2.9	Pembrolizumab	8.2	SD
5	m	65	Myxoid chondrosarcoma, ribs	Lung, soft tissue, lymph nodes	Sunitinib, anthracycline, ifosfamide	8.2	Pembrolizumab	5.9	PD
6	f	43	Myxoid/round cell liposarcoma, retroperitoneal	Soft tissue, lymph nodes	Anthracycline, trabectedin, taxane, gemcitabine/dacarbazine	5.3	Pembrolizumab	10.7	SD
7	m	54	Extra skeletal myxoid chondrosarcoma, hip	Lung, soft tissue	Anthracycline	25.8	Pembrolizumab	3	PD
8	m	50	Chondrosarcoma, hip	Bone	Denosumab	2.9	Pembrolizumab	9.4	SD
9	f	40	Angiosarcoma, breast	Lung, liver, soft tissue, bone	Taxane/pazopanib, trabectedin, anthracycline	5.2	Pembrolizumab	0.4	PD
10	m	53	Dedifferentiated liposarcoma, retroperitoneal	Lung, soft tissue	Anthracycline/ ifosfamide, trabectedin, taxane	2.6	Pembrolizumab	8.8	SD
11	f	26	Synovial sarcoma, upper thigh	Lung, soft tissue	Anthracycline, trabectedin, ifosfamide	2.6	Pembrolizumab	1.9	SD

12	m	52	Epithelioid sarcoma, knee	Lung, bone	Olaratumumab/placebo/anthracycline, gemcitabine/dacarbazine, trabectedin	1.6	Pembrolizumab	13.5	PR
13	f	41	Osteoblastic osteosarcoma, jaw	Lung, liver, bone, lymph nodes, soft tissue	Methotrexate/cisplatin/anthracycline, gemcitabine/dacarbazine, trabectedin	15.6	Pembrolizumab	30.1	PR
14	f	60	Extra skeletal osteosarcoma, uterus	Soft tissue	Anthracycline/ifosfamide	3.7	Pembrolizumab	16.5	CR
15	m	49	Chondrosarcoma, hip	Lung, bone	Methotrexate/cisplatin/anthracycline	1.3	Pembrolizumab	1.8	PD
16	m	55	Dedifferentiated liposarcoma, retroperitoneal	Soft tissue, bone	Olaratumumab/placebo/anthracycline, trabectedin, taxane, gemcitabine/dacarbazine	2.5	Pembrolizumab	1.8	PD
17	m	56	Chordoma, hip	Soft tissue, lymph nodes	Sunitinib	1.4	Pembrolizumab	7.5	SD
18	m	27	Osteosarcoma, upper arm	Lung, soft tissue	Methotrexate/cisplatin/anthracycline, chemotherapy	2.1	Pembrolizumab	32.6	CR
19	f	23	Osteosarcoma, upper thigh	Lymph nodes	Methotrexate/cisplatin/anthracycline	7.2	Pembrolizumab	17.9	CR
20	f	25	Alveolar soft part sarcoma, upper thigh	Lung, liver, bone, lymph nodes	Sunitinib	47.8	Pembrolizumab	1.3	PD

\*at/from anti-PD-1 ICI therapy start

Abbreviations: f female, m male, PD-1 programmed cell death 1, ICI immune checkpoint inhibitor, CR complete response, PR partial response, SD stable disease, PD progressive disease

**Supplementary Table S2.** Detailed patient listing for Center 2

ID	Sex	Age in years*	Histologic sarcoma type, localization	Number of metastatic organ sites*	Number of prior therapy lines*	Anti-PD-1 ICI specimen	PFS in months*	Response to anti-PD-1 ICI (iRECIST)
1	f	81	Myxofibrosarcoma, lower limb	1	0	Pembrolizumab	0.3	PD
2	f	61	Uterine leiomyosarcoma, abdomen and retroperitoneal	4	7	Nivolumab	1.9	PD
3	m	27	Alveolar soft part sarcoma, lower limb	2	1	Pembrolizumab	19.7	PR
4	f	60	Angiosarcoma, chest wall	3	4	Pembrolizumab	0.6	PD
5	m	36	Alveolar soft part sarcoma, lower limb	4	4	Pembrolizumab	9.3	PR
6	f	56	Myxofibrosarcoma, lower limb	4	4	Pembrolizumab	2.5	PD
7	f	23	Alveolar soft part sarcoma, lower limb	3	2	Pembrolizumab	11.2	SD
8	f	26	Epitheloide sarcoma, upper limb	2	4	Nivolumab	2.6	PD
9	m	23	Spindel cell rhabdomyosarcoma, abdomen and retroperitoneal	4	4	Pembrolizumab	1.0	PD
10	m	75	Dedifferentiated liposarcoma, abdomen and retroperitoneal	1	4	Pembrolizumab	0.6	PD
11	m	39	Chordoma, spine	1	0	Nivolumab	24.3	PR
12	m	56	Dedifferentiated chondrosarcoma, upper limb	2	1	Nivolumab	1.2	PD
13	f	69	Histiocytic sarcoma, head and neck	2	2	Nivolumab	1.6	PD
14	f	49	Spindel cell sarcoma, NOS, upper limb	4	5	Pembrolizumab	2.5	PD

15	m	49	Dedifferentiated liposarcoma, abdomen and retroperitoneal	4	3	Pembrolizumab	3.9	PD
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\*at/from anti-PD-1 ICI therapy start

Abbreviations: f female, m male, PD-1 programmed cell death 1, ICI immune checkpoint inhibitor, NOS not otherwise specified, PR partial response, SD stable disease, PD progressive disease

**Supplementary Table S3.** Antibodies and dilution protocols used for immunohistochemical analyses

<b>Marker</b>	<b>Antibody Clone</b>	<b>LOT number</b>	<b>Company</b>	<b>Dilution</b>
<b>CD3</b>	rabbit mAb, Clone SP7	910751903 B	Thermo Fisher Scientific, Cheshire, UK	1:200
<b>CD8</b>	mouse mAb, Clone C8/144B/M7103	20066516	DakoCytomation, Glostrup, Denmark	1:100
<b>CD45RO</b>	mouse mAb, Clone UCHL1	20059854	DakoCytomation, Glostrup, Denmark	1:400
<b>FOXP3</b>	mouse mAb, Clone 206D	B231983	BioLegend, San Diego, CA, USA	1:25
<b>PD-L1</b>	mouse mAb, Clone 5H1 (kindly provided by Dr. Lieping Chen)	BSH 100-6cd	BioSite	1:50
<b>PD-1</b>	mouse mAb, ab52587	GR3174613-8	Abcam, Cambridge, UK	1:750
<b>LAG3</b>	LAG3/C18692	132277	BioScience	1:100

Abbreviations: CD3, cluster of differentiation 3; CD8, cluster of differentiation 8; FOXP3, Forkhead Box Protein P3; PD-1, Programmed cell death 1; PD-L1, Programmed cell death ligand 1; mAb, monoclonal antibody; CD20, Cluster of differentiation 20; Lag3, Lymphocyte-activation gene 3.