**Supplemental Digital Content (SDC)**

**Immune biomarkers in the peripheral blood and tumor microenvironment of classical Hodgkin lymphoma patients in relation to tumor burden and response to treatment**

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# SDC, Materials and Methods

***Contemporary Swedish national treatment guidelines***

Patients <70 years with limited-stage cHL (stage I-IIA) were treated with 2 or 4 cycles of ABVD followed by limited field RT at a dose of 20 Gy or 29,75 Gy, respectively, depending on the presence of ≥1 of the following risk factors: erythrocyte sedimentation rate ≥50 mm, bulky disease (defined as >10 cm tumor mass) and involvement >2 LN regions. In patients ≥70 years, bleomycin was omitted, and 30 Gy RT was given regardless of risk factors. In case the radiation field would be too extensive, 6 cycles of ABVD were given without RT.

Patients <60 years with advanced-stage cHL (stages IIB-IV) were treated with chemotherapy alone as follows: patients with ≤2 risk factors (male, age >45, stage IV, hemoglobin <10.5 g/dL, albumin <4 g/dL, white blood cell count >15x109 cells/L, lymphocytes <8% or 0,6x109 cells/L) were given 2 cycles of ABVD after which the response was assessed with an interim positron emission tomography (PET) scan. If the interim PET was negative, 4 more cycles of ABVD were given. Otherwise, 4 cycles of escalated BEACOPP were given. Patients with >2 risk factors were initially treated with escalated BEACOPP and if the interim PET was negative after two cycles, 4 cycles of ABVD were given. During the first 2 years of this study, 6 cycles of ABVD were also a treatment option for these patients. Patients with advanced-stage cHL aged 61-70 were treated with 4 cycles of ABVD followed by 2 cycles of doxorubicin-vinblastine-dacarbazine (AVD) and patients ≥70 years with 6 cycles of AVD, irrespective of risk factors.

***Flow cytometric analysis of immune cells in the peripheral blood***

Heparinized whole blood samples were stained with antibodies (see Supplementary Table S1), subsequently exposed to red blood cell lysis buffer (eBioscience, San Diego, CA, USA) and washed once in Cell Staining Buffer (CSB; BioLegend, San Diego, CA, USA) before flow cytometry. Peripheral blood mononuclear cells (PBMCs) were purified from heparinized whole blood by density gradient centrifugation using a Ficoll-Hypaque gradient (GE Healthcare, Uppsala, Sweden) and washed twice in Dulbecco’s Phosphate-Buffered Saline (Gibco, Life Technologies, Carlsbad, CA, USA). Following purification, PBMCs were immediately stained with antibodies (see Supplementary Table S2), then washed and resuspended in CSB before flow cytometry. See below, and Supplementary Table S3 and S4, for intracellular staining methods. Data were acquired on a 6-color FACSCanto II Flow Cytometer (BD Biosciences, San Diego, CA, USA). Analysis was performed using FACSDiva software version 6.1.3 (BD Biosciences). Absolute numbers of various cell subsets were calculated using a dual-platform method, combining the frequencies from the flow-cytometry analysis with the lymphocyte counts from the patients’ medical records.

***Flow cytometric analysis of diagnostic lymph node biopsies***

Both excisional and needle core biopsies from LNs were performed for routine diagnostic purposes. Standard protocols for clinical samples were followed at the Department of Clinical Pathology and Cancer Diagnostics, Karolinska University Hospital (Stockholm, Sweden). LN biopsies were disintegrated mechanically into single cell suspensions and stained with antibodies (see Supplementary Table S5). Flow-cytometry analysis was performed using an 8-color FACSCanto Clinical Flow Cytometry System (BD Biosciences).

***Intercellular staining methods***

PBMCs were stained with antibodies listed in Supplementary Table S3, then fixed and permeabilized using Fixation/Permeabilization Solution (eBioscience), washed in Permeabilization Buffer (eBioscience), stained with cytotoxic T-lymphocyte associated protein (CTLA)-4-phycoerythrin (PE) or the appropriate isotype control, then washed and resuspended in CSB before flow cytometry.

In parallel, PBMCs were stained with CD16-PE-cyanine (Cy)7 and CD56-PE, then fixed in 1.6% formaldehyde (Histolab Products, Askim, Sweden) and permeabilized in cold 80% methanol, washed, stained with the antibodies shown in Supplementary Table S4, then washed again and resuspended in CSB before flow cytometry.

***Enzyme-Linked ImmunoSorbent Assay of CCL17/TARC in plasma***

Plasma was isolated from whole heparinized blood by centrifugation and stored undiluted at −80°C until further use. CCL17/TARC concentrations were measured in triplicate in thawed plasma samples using the Human CCL17/TARC DuoSet enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s instructions. Sample concentrations were interpolated from a sigmoidal 4 parameter logistic curve fit through the duplicate set of serially diluted standard samples from the same plate.

# SDC, Table 1

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Antigen/Target | Clone | Fluorochrome | Manufacturer | Cat. nr. |
| Annexin V | n/a | FITC | MabTag | AnxF100 |
| CD3 | HIT3a | PE-Cy7 | BioLegend | 300316 |
| CD3 | UCHT1 | AF700 | BioLegend | 300424 |
| CD3 | UCHT1 | PerCp | BioLegend | 300428 |
| CD4 | RPA-T4 | PerCP | BioLegend | 300528 |
| CD4 | OKT-4 | FITC | BioLegend | 317408 |
| CD8 | HIT8a | AF700 | BioLegend | 300920 |
| CD8 | SK1 | APC | BioLegend | 344722 |
| CD16 | 3G8 | PE | BioLegend | 302008 |
| CD16 | 3G8 | PE-Cy7 | BioLegend | 302016 |
| CD19 | HIB19 | AF488 | BioLegend | 302219 |
| CD19 | HIB19 | APC | BioLegend | 302212 |
| CD19 | HIB19 | AF700 | BioLegend | 302226 |
| CD19 | HIB19 | PerCP | BioLegend | 302228 |
| CD45 | H130 | AF700 | BioLegend | 304024 |
| CD56 | HCD56 | PE | BioLegend | 318306 |
| CD62L | DREG-56 | APC | BioLegend | 304810 |
| CD69 | FN50 | AF488 | BioLegend | 310916 |
| CD226 | DX11 | BB515 | BD Biosciences | 565152 |
| CD314 | 1D11 | AF647 | BioLegend | 320825 |
| CD366 | 7D3 | BB515 | BD Biosciences | 565568 |
| CD279 | EH12.2H7 | APC | BioLegend | 329908 |
| HLA-DR | L243 | PerCP | BioLegend | 307628 |
| PI | n/a | n/a | BD Biosciences | 556463 |
| TIGIT | MBSA43 | APC | eBioscience | 17-9500-42 |

# SDC, Table 2

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Antigen/Target | Clone | Fluorochrome | Manufacturer | Cat. nr. |
| CD3 | HIT3a | PE-Cy7 | BioLegend | 300316 |
| CD3 | UCHT1 | AF700 | BioLegend | 300424 |
| CD3 | UCHT 1 | FITC | BioLegend | 300406 |
| CD4 | OKT-4 | AF700 | BioLegend | 317426 |
| CD4 | RPA-T4 | PerCP | BioLegend | 300528 |
| CD8 | HIT8a | AF700 | BioLegend | 300920 |
| CD8 | SK1 | APC | BioLegend | 344722 |
| CD11b | M1/70 | PE-Cy7 | BioLegend | 101216 |
| CD14 | HCD14 | AF700 | BioLegend | 325614 |
| CD15 | W6D3 | AF647 | BioLegend | 323012 |
| CD19 | HIB19 | PE-Cy7 | BioLegend | 302216 |
| CD19 | HIB19 | FITC | BioLegend | 302206 |
| CD25 | BC96 | APC | BioLegend | 302610 |
| CD25 | 2A3 | BB515 | BD Biosciences | 564467 |
| CD33 | WM53 | PerCP-Cy5.5 | BioLegend | 303414 |
| CD45RA | HI100 | AF488 | BioLegend | 304114 |
| CD45RO | UCHL1 | FITC | BioLegend | 304204 |
| CD56 | HCD56 | FITC | BioLegend | 318304 |
| CD69 | FN50 | AF488 | BioLegend | 310916 |
| CD127 | HIL-7R-M21 | PE-Cy7 | BD Biosciences | 560822 |
| CD127 | A019D5 | PE | BioLegend | 351304 |
| CD183 | 1C6/CXCR3 | APC | BD Biosciences | 550967 |
| CD194 | 1G1 | PE | BD Biosciences | 551120 |
| CD194 | L291H4 | PE-Cy7 | BioLegend | 359410 |
| CD196 | 11A9 | PE | BD Biosciences | 559562 |
| CD197 | 150503 | AF647 | BD Biosciences | 560816 |
| CD274 | MIH1 | PE | BD Biosciences | 557924 |
| Isotype | MOPC-21 | PE | BD Biosciences | 555749 |
| CD279 | EH12.2H7 | PE | BioLegend | 329906 |
| CD279 | EH12.2H7 | APC | BioLegend | 329908 |
| HLA-DR | L243 | PerCP | BioLegend | 307628 |
| HLA-DR | L243 | PE | BioLegend | 307606 |

# SDC, Table 3

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Antigen/Target | Clone | Fluorochrome | Manufacturer | Cat. nr. |
| CD3 | HIT3a | PE-Cy7 | BioLegend | 300316 |
| CD4 | RPA-T4 | PerCP | BioLegend | 300528 |
| CD8 | HIT8a | AF700 | BioLegend | 300920 |
| CD19 | HIB19 | APC | BioLegend | 302212 |

# SDC, Table 4

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Antigen/Target | Clone | Fluorochrome | Manufacturer | Cat. nr. |
| CD3 | UCHT1 | PerCP | BioLegend | 300428 |
| CD4 | OKT-4 | FITC | BioLegend | 317408 |
| CD8 | HIT8a | AF700 | BioLegend | 300920 |
| Ki-67 | Ki-67 | AF647 | BioLegend | 350510 |
| Isotype | MOPC-21 | AF647 | BioLegend | 400130 |

# SDC, Table 5

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| Antigen/Target | Clone | Fluorochrome | Manufacturer | Cat. nr. |
| CD3 | Sk7 | PerCP-Cy5.5 | BD Biosciences | 332771 |
| CD3 | Sk7 | PE-Cy7 | BD Biosciences | 341111 |
| CD3 | Sk7 | APC | BD Biosciences | 345767 |
| CD4 | SK3 | APC | BD Biosciences | 345771 |
| CD8 | Sk1 | PE-Cy7 | BD Biosciences | 335822 |
| CD19 | SJ25C1 | PE-Cy7 | BD Biosciences | 341113 |
| CD19 | SJ25C1 | APC | BD Biosciences | 345791 |
| CD19 | J3,119 | PE-Cy5 | Beckman Coulter | AO7771 |
| CD20 | L27 | PE | BD Biosciences | 345793 |
| CD20 | L27 | PerCP-Cy5.5 | BD Biosciences | 332781 |
| CD33 | P67,6 | PerCP-Cy5.5 | BD Biosciences | 333146 |
| CD33 | P67,6 | PE-Cy7 | BD Biosciences | 333952 |
| CD45 | HI30 | PerCP-Cy5.5 | BioLegend | 304028 |
| CD56 | NCAM16,2 | FITC | BD Biosciences | 345811 |
| CD56 | MY31 | PE | BD Biosciences | 345810 |
| CD56 | NCAM16,2 | PE-Cy7 | BD Biosciences | 335826 |

# SDC, Table 6

|  |  |
| --- | --- |
| Cell subsets | Markers |
| B cells | CD45+, CD3-, CD19+ |
| T cells | CD45+, CD19-, CD3+ |
| CD4+ T cells | CD45+, CD19-, CD3+, CD4+, CD8- |
| CD8+ T cells | CD45+, CD19-, CD3+, CD8+, CD4- |
| NK cells | CD45+, CD3-, CD56+, CD16+ |
| CD56bright | CD19-, CD3-, CD56++, CD16+/- |
| CD56 dim | CD19-, CD3-, CD56+, CD16+ |
| Tregs | CD3+, CD4+, CD25+, CD127-/low, CD194+ |
| Th1 | CD3+, CD8-, CD4+, CD183+, CD196- |
| Th2 | CD3+, CD8-, CD4+, CD183-, CD196- |
| Th17 | CD3+, CD8-, CD4+, CD183-, CD196+ |
|  | |
| *Teff/mem cells within CD4 and CD8 subpopulations* | |
| TN | CD3+, CD4+/-, CD8+/-, CD45RA+, CCR7+ |
| TCM | CD3+, CD4+/-, CD8+/-, CD45RA-, CCR7+ |
| TE | CD3+, CD4+/-, CD8+/-, CD45RA+, CCR7- |
| TEM | CD3+, CD4+/-, CD8+/-, CD45RA-, CCR7- |

# SDC, Table 7

|  |  |  |
| --- | --- | --- |
| Protein name abbreviation | Protein name | Uniprot ID |
| ADA | Adenosine deaminase | P00813 |
| ADGRG1 | Adhesion G-protein coupled receptor G1 | Q9Y653 |
| ANGPT1 | Angiopoietin-1 | Q15389 |
| ANGPT2 | Angiopoietin-2 | O15123 |
| ARG1 | Arginase-1 | P05089 |
| CAIX | Carbonic anhydrase 9 | Q16790 |
| CASP-8 | Caspase-8 | Q14790 |
| CCL17 | C-C motif chemokine 17 | Q92583 |
| CCL19 | C-C motif chemokine 19 | Q99731 |
| CCL20 | C-C motif chemokine 20 | P78556 |
| CCL23 | C-C motif chemokine 23 | P55773 |
| CCL3 | C-C motif chemokine 3 | P10147 |
| CCL4 | C-C motif chemokine 4 | P13236 |
| CD244 | Natural killer cell receptor 2B4 | Q9BZW8 |
| CD27 | Tumor necrosis factor receptor superfamily member 7 | P26842 |
| CD28 | T-cell-specific surface glycoprotein CD28 | P10747 |
| CD4 | T-cell surface glycoprotein CD4 | P01730 |
| CD40 | Tumor necrosis factor receptor superfamily member 5 | P25942 |
| CD40-L | Tumor necrosis factor ligand superfamily member 5 | P29965 |
| CD5 | T-cell surface glycoprotein CD5 | P06127 |
| CD70 | Tumor necrosis factor ligand superfamily member 7 | P32970 |
| CD83 | CD83 antigen | Q01151 |
| CD8A | T-cell surface glycoprotein CD8 alpha chain | P01732 |
| CRTAM | Cytotoxic and regulatory T-cell molecule | O95727 |
| CSF-1 | Macrophage colony-stimulating factor 1 | P09603 |
| CX3CL1 | C-X3-C motif chemokine 1, Fractalkine | P78423 |
| CXCL1 | Growth-regulated alpha protein | P09341 |
| CXCL10 | C-X-C motif chemokine 10 | P02778 |
| CXCL11 | C-X-C motif chemokine 11 | O14625 |
| CXCL12 | Stromal cell-derived factor 1 | P48061 |
| CXCL13 | C-X-C motif chemokine 13 | O43927 |
| CXCL5 | C-X-C motif chemokine 5 | P42830 |
| CXCL9 | C-X-C motif chemokine 9 | Q07325 |
| DCN | Decorin | P07585 |
| EGF | Pro-epidermal growth factor | P01133 |
| FASLG | Tumor necrosis factor ligand superfamily member 6 | P48023 |
| FGF2 | Fibroblast growth factor 2 | P09038 |
| Gal-1 | Galectin-1 | P09382 |
| Gal-9 | Galectin-9 | O00182 |
| GZMA | Granzyme A | P12544 |
| GZMB | Granzyme B | P10144 |
| GZMH | Granzyme H | P20718 |
| HGF | Hepatocyte growth factor | P14210 |
| HO-1 | Heme oxygenase 1 | P09601 |
| ICOSLG | Inducible T cell costimulator ligand | O75144 |
| IFN-gamma | Interferon gamma | P01579 |
| IL-1 alpha | Interleukin-1 alpha | P01583 |
| IL10 | Interleukin-10 | P22301 |
| IL12 | Interleukin-12 | P29459,P29460 |
| IL12RB1 | Interleukin-12 receptor subunit beta-1 | P42701 |
| IL13 | Interleukin-13 | P35225 |
| IL15 | Interleukin-15 | P40933 |
| IL18 | Interleukin-18 | Q14116 |
| IL2 | Interleukin-2 | P60568 |
| IL33 | Interleukin-33 | O95760 |
| IL4 | Interleukin-4 | P05112 |
| IL5 | Interleukin-5 | P05113 |
| IL6 | Interleukin-6 | P05231 |
| IL7 | Interleukin-7 | P13232 |
| IL8 | Interleukin-8 | P10145 |
| KIR3DL1 | Killer cell immunoglobulin-like receptor 3DL1 | P43629 |
| KLRD1 | Natural killer cells antigen CD94 | Q13241 |
| LAG3 | Lymphocyte activation gene 3 protein | P18627 |
| LAMP3 | Lysosome-associated membrane glycoprotein 3 | Q9UQV4 |
| LAP,  TGF-beta-1 | Latency-associated peptide,  Transforming growth factor beta-1 proprotein | P01137 |
| MCP-1, CCL2 | Monocyte chemotactic protein 1, C-C motif chemokine 2 | P13500 |
| MCP-2, CCL8 | Monocyte chemotactic protein 2, C-C motif chemokine 8 | P80075 |
| MCP-3, CCL7 | Monocyte chemotactic protein 3, C-C motif chemokine 7 | P80098 |
| MCP-4, CCL13 | Monocyte chemotactic protein 4, C-C motif chemokine 13 | Q99616 |
| MIC-A/B | MHC class I polypeptide-related sequence A/B | Q29983,Q29980 |
| MMP12 | Macrophage metalloelastase | P39900 |
| MMP7 | Matrilysin | P09237 |
| MUC-16 | Mucin-16 | Q8WXI7 |
| NCR1 | Natural cytotoxicity triggering receptor 1 | O76036 |
| NOS3 | Nitric oxide synthase, endothelial | P29474 |
| PD-L1 | Programmed cell death 1 ligand 1 | Q9NZQ7 |
| PD-L2 | Programmed cell death 1 ligand 2 | Q9BQ51 |
| PDCD1 | Programmed cell death protein 1 | Q15116 |
| PDGF subunit B | Platelet-derived growth factor subunit B | P01127 |
| PGF | Placenta growth factor | P49763 |
| PTN | Pleiotrophin | P21246 |
| TIE2 | Angiopoietin-1 receptor | Q02763 |
| TNF | Tumor necrosis factor | P01375 |
| TNFRSF12A | Tumor necrosis factor receptor superfamily member 12A | Q9NP84 |
| TNFRSF21 | Tumor necrosis factor receptor superfamily member 21 | O75509 |
| TNFRSF4 | Tumor necrosis factor receptor superfamily member 4 | P43489 |
| TNFRSF9 | Tumor necrosis factor receptor superfamily member 9 | Q07011 |
| TNFSF14 | Tumor necrosis factor ligand superfamily member 14 | O43557 |
| TRAIL | Tumor necrosis factor ligand superfamily member 10 | P50591 |
| TWEAK | Tumor necrosis factor ligand superfamily member 12 | O43508 |
| VEGFA | Vascular endothelial growth factor A | P15692 |
| VEGFR-2 | Vascular endothelial growth factor receptor 2 | P35968 |

# SDC, Figure 1



**SDC, Figure 1. Absolute numbers and percentages of exhausted T cells in the PB of limited-stage and advanced-stage cHL patients before primary treatment.** Percentages (left) and absolute numbers (right) of T cells that are double-positive for PD-1 and TIM-3 in the PB of healthy controls (n=7), LIM cHL patients (n=13), and ADV cHL patients (n=6). Data are depicted as violin plots with a thick line at the median and thin lines at the quartiles. Stars indicate the P value of a Mann Whitney test comparing the indicated groups. \*: P<0.05; \*\*: P<0.01; \*\*\*: P<0.001; \*\*\*\*: P<0.0001. LIM: limited-stage; cHL: classical Hodgkin lymphoma; ADV: advanced-stage.

# SDC, Figure 2

**SDC,Figure 2. Heatmap of disease-related plasma protein biomarkers.** The left column shows the results of multiple Kruskal-Wallis tests with Dun’s *post-hoc* test. The middle column depicts the median row-normalized normalized protein expression (NPX) values of the respective groups. The right column shows the individual row-normalized NPX values of the healthy individuals and patients clustered by group. Since MIC-A and MIC-B were measured with the same probe it was regarded as one unique protein. ADV cHL: advanced-stage classical Hodgkin lymphoma; Healthy: healthy individuals; LIM cHL: limited-stage classical Hodgkin lymphoma.

# SDC, Figure 3

**SDC, Figure 3. Immune profiles of cHL patients with combinations of high ESR and high tumor burden before primary treatment.** High erythrocyte sedimentation rate (ESR) was defined as ≥50 mm. High tumor burden (TB) was defined as any of the following: bulky disease, >2 nodal sites and stage IV. Multiple comparisons between patients with A) the combination of low ESR and low TB (n=11) vs. low ESR and high TB (n=18), B) the combination of low ESR and low TB (n=11) vs. high ESR and high TB (n=19), and C) the combination of low ESR and high TB (n=18) vs. high ESR and high TB (n=19). None of the patients had a combination of high ESR and low TB. Data are depicted as volcano plots of multiple Mann-Whitney tests with a P-value threshold of 0.05 and without correction for multiple comparisons. Y-axes depict the negative log10-transformed P values, so dots that are plotted above the horizontal line have a P value that is lower than 0.05. X-axes show the mean rank difference between the two indicated groups. A positive X-value means that the difference is in favor of the latter group in the graph title.

# SDC, Figure 4

**SDC, Figure 4. Heatmap of plasma protein biomarkers that changed after treatment.** The left column shows the results of multiple Wilcoxon signed-rank tests, which are corrected for multiple testing using the Benjamini-Hochberg procedure, between the respective timepoints. The middle column depicts the median row-normalized normalized protein expression (NPX) values of the healthy individuals and the patients at the respective timepoints. The right column shows the individual row-normalized NPX values of the healthy individuals and patients clustered by timepoint. Since MIC-A and MIC-B were measured with the same probe it was regarded as one unique protein. BL: baseline; EoT: end of treatment; FU: follow up 6 months after EoT; Healthy: healthy individuals.

# SDC, Figure 5



**SDC, Figure 5. Heatmap of plasma protein biomarkers that had significantly different levels between patients after treatment with or without radiotherapy.** The left column shows the results of multiple Mann-Whitney tests, which are corrected for multiple testing using the Benjamini-Hochberg procedure, between the respective groups. The middle column depicts the median row-normalized normalized protein expression (NPX) values of the respective groups. The right column shows the individual row-normalized NPX values of the patients clustered by group. EoT: end of treatment; FU: follow up 6 months after EoT; RT: radiotherapy.