A Pilot Study of Circulating Endothelial and Hematopoietic Progenitor Cells in Children with Sarcomas

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Abstract

Utilizing a multi-parametric flow cytometry (MPFC) protocol, we assessed various cell-types implicated in tumor angiogenesis that were found circulating in the peripheral blood of children with sarcomas (cases) based on their cell surface antigen expression. Circulating endothelial cells (CECs), endothelial colony forming cells (ECFCs) and the ratio of two distinct populations of circulating hematopoietic stem and progenitor cells (CHSPCs), the pro-angiogenic CHSPCs (pCHSPCs) and non-angiogenic CHSPCs (nCHSPCs) were enumerated. MPFC was analyzed in cases at baseline and at 4 additional time-points until the end of treatment and levels compared with each other and with healthy controls. At all time-points, cases had significantly lower levels of CECs, but elevated ECFCs and a pCHSPC:nCHSPC ratio compared to controls (all p values <0.05). There was no significant difference in any of the cell types analyzed based on tumor-histology, stage (localized v/s metastatic) or tumor-size. After treatment, only the CECs among the complete responders were significantly lower at end of therapy (p<0.01) compared to non-responders, whereas the ECFCs among all cases significantly increased (p<0.05)) compared to baseline. No decline in the pCHSPC:nCHSPC ratio was observed despite tumor response. Based on these results, a validation of CECs as prognostic biomarker is now warranted.

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Introduction

All solid tumors, including pediatric malignancies, depend on angiogenesis for tumor growth, invasion and metastases. Therefore, targeted anti-angiogenic therapies are promising novel strategies that have recently emerged in the treatment of pediatric solid tumor patients with the goal of improving survival, particularly for those with metastatic or relapsed disease. However, lack of predictive biomarkers for gauging tumor response makes it difficult to ascertain which patients receiving these anti-angiogenic agents would respond and thereby benefit. This deficit makes it difficult to individualize therapies and exposes non-responders to treatments that are ineffective, and furthermore, may cause harm.

To address this issue, several groups have identified circulating endothelial progenitor cells (EPCs) as potential predictive biomarkers of response to anti-angiogenic agents. EPCs were first shown to contribute to vasculogenesis and subsequently to tumor induced angiogenesis. However, there has been a lack of consensus in defining EPCs leading to grouping various cell types under the umbrella of “EPC” and thus an inability to compare study results. Utilizing a novel multi-parametric flow cytometry (MPFC) protocol, our group has identified and defined the bona fide EPC, namely the endothelial colony forming cells (ECFCs) that are identified as CD31+CD34brightCD45-AC133-CD14-LIVE/DEAD- cells, that have high proliferative potential, and give rise to perfused blood vessels in vivo. In addition, we have also enumerated circulating endothelial cells (CECs) that are identified as CD31brightCD45+CD34dimAC133- cells and are mature, apoptotic endothelial cells sloughed off from the vessel wall during vascular remodeling and do not form perfused blood vessels in vivo. Finally, using MPFC we have identified two unique circulating hematopoietic stem and progenitor cell (CHSPC) populations in both peripheral blood and umbilical cord blood, which are phenotypically and functionally distinct from EPCs but are still actively involved in angiogenesis. These CHSPCs are vital for blood vessel formation in physiological and pathological states through their interactions with mature endothelial cells and ECFCs, and are further classified into 2 distinct cellular subsets based on both cell surface antigen expression and function. The parent CHSPC population which contains both fractions is defined by the expression of CD31+CD34brightCD45dimCD14-LIVE/DEAD- with the expression of AC133 the hallmark between the two subsets. The pro-angiogenic CHSPCs (pCHSPCs) express AC133, while the non-angiogenic CHSPCs (nCHSPCs) do not. The ratio of the pCHSPCs to nCHSPCs is a way to normalize the wide variability observed in the total number of CHSPCs, pCHSPCs, or nCHSPCs, with normal healthy ratios between 1.2-1.8. An increased pCHSPC:nCHSPC ratio (>2.0) is associated with tumor induced angiogenesis.

To validate CECs, ECFCs, and the pCHSPC:nCHSPC ratio as biomarkers of tumor induced angiogenesis, prognostic biomarkers to current treatment strategies and predictive biomarkers of targeted anti-angiogenic agents further studies are essential. The purpose of
our current pilot study, an initial step in that direction was to evaluate longitudinal measurements of the CECs, ECFCs, and the pCHSPC:nCHSPC ratio in pediatric sarcoma patients. In the development of the MPFC protocol, we determined that both fresh blood and blood processed and stained within 24 hours (utilizing BD Vacutainer CPT Cell preparation tubes) were similar in terms of cellular populations.\textsuperscript{17,19,22} Our first aim was to demonstrate the feasibility of performing the real time MPFC protocol in a multi-institutional setting. This step was necessary for planning future multi-institutional studies since reliability of this protocol depends upon timely analysis of the peripheral blood within 24 hours after collection, and involves the processing and shipping of peripheral blood samples to a centralized core facility to perform the MPFC staining, collection, and analysis. Our next objectives were to examine the relationship and differences between baseline tumor characteristics and CECs, ECFCs, and the pCHSPC:nCHSPC ratio. Finally, we evaluated the impact of cancer-directed therapies on CECs, ECFCs, and the pCHSPC:nCHSPC ratio in a prospective setting.

**Materials and Methods**

**Peripheral Blood Collection**

We conducted a longitudinal study at three academic children's hospitals in the United States (James Whitcomb Riley Hospital for Children [Indianapolis, IN], Ann and Robert H. Lurie Children's Hospital [Chicago, IL], and the University of Kentucky Children's Hospital [Lexington, KY]). This study was approved by the institutional review boards at each collaborative site. Children (1-21 years of age) with a diagnosis of Osteosarcoma (OS), Ewing's sarcoma (EWS), Rhabdomyosarcoma (RMS) or Undifferentiated sarcoma (US) were eligible for this study provided they had not received any prior cancer directed therapy other than a diagnostic biopsy. Patients with localized disease that was completely resected with no other metastatic lesions were not eligible for the study. Age-matched, healthy controls were enrolled for comparison.

Informed parental consents, and when appropriate, children's assents, were obtained prior to enrollment. Enrolled patients had placement of a vascular access through which baseline peripheral blood was obtained and sent for enumeration of CECs, ECFCs, and CHSPCs using MPFC as previously described.\textsuperscript{17,19,22} The CECs and ECFCs were expressed as percentage of the total circulating mononuclear cell (MNC) population. The pCHSPC:nCHSPC ratio was determined by the percentage of live MNCs that were pro-angiogenic CHSPCs divided by the percentage of live MNCs that were non-angiogenic CHSPCs.

Since the MPFC assay was stained in real-time within 24 hours after peripheral blood collection at the Angio BioCore located at Indiana University, peripheral blood shipped from other clinics were processed by a defined standard operating procedure at site of collection and sent overnight via Federal Express for next morning delivery.
Antibodies and Staining Reagents

The following primary conjugated monoclonal antibodies were used: anti-human CD31 fluoroscein isothyocyanate (FITC, BD Pharmingen), anti-human CD34 phycoerythrin (PE, BD Pharmingen), anti-human AC133 allophycocyanin (APC, Miltenyi Biotec), anti-human CD14 PECy5.5 (Abcam), anti-human CD45 APC-AlexaFluor (AF) 750 (Invitrogen), and the fixable amine reactive viability dye, LiveDead (Violet, Invitrogen).

In order to resolve the rare and/or dim populations of interest, specific antigen and fluorochrome conjugate coupling was optimized for the five-antibody plus viability marker staining panel as previously described.\textsuperscript{17,19,22}

Multi-Parametric Flow Cytometry Immunostaining, Acquisition and Analysis

Peripheral blood MNCs were incubated with Fc blocking reagent (Miltenyi Biotec) and stained as previously described.\textsuperscript{17,19,22} “Fluorescent minus one” (FMO) gating controls were also used to ensure proper gating of positive events.\textsuperscript{17,19} Briefly, cells were incubated with titrated antibodies for 30 minutes at 4°C, washed twice in PBS with 2% fetal bovine serum (FBS), fixed in 1% paraformaldehyde (Tousimis), and run within 72 hours of fixation on a BD LSRII flow cytometer (BD, Franklin Lakes, NJ, USA) equipped with a 405nm violet laser, 488nm blue laser and 633nm red laser. Data were acquired uncompensated and exported as FCS 3.0 files, and analyzed utilizing FlowJo software, version 9.7.2 (Tree Star, Inc).

Tumor Characteristics, Cancer-therapies and Response Assessment

Baseline tumor characteristics evaluated were tumor histology, size, and presence or absence of metastases as shown in Table 1. All patients were enrolled either on a therapeutic clinical protocol or a standard clinical treatment plan with cancer-directed therapies summarized in Table 2. CECs, ECFCs and CHSPCs were enumerated at diagnosis; after the first cycle of chemotherapy at least 24 hours after ending granulocyte colony stimulating factor (G-CSF); up to 24 hours before initiating treatment of the primary tumor site with surgery or radiation therapy (local control); after local control of the primary tumor site prior to initiation of adjuvant chemotherapy; and finally at the end of all planned treatment (after count recovery). The only significant difference between those who received G-CSF vs. those who did not was with respect to the underlying tumor-pathology (p=0.0006) as shown in Table-2. Response evaluations were recorded by radiographic (measurements in 2 dimensions using the product of width and length per the WHO criteria), clinical, and staging evaluations at the end of all planned therapies.

Statistical Analysis

Categorical variables were summarized by frequencies and percentages. Pearson's chi-square tests were used for comparison between groups. Continuous variables were summarized by means and standard deviations (SDs). Normality was checked for each variable. When the normality assumption held, Student's t-tests were adopted for comparisons and Pearson's correlation coefficients were generated. When the normality assumption failed, Wilcoxon's signed rank tests and rank sum tests were used for
comparisons and Spearman’s correlation coefficients were generated. Complete case approach was adopted for all the analyses.

## Results

There were 31 Sarcoma patients enrolled as shown in Table 1 with 9 females and 22 males. The mean age was 11 years (SD=4.3). There was no significant difference of age and gender between cases and healthy controls. The control group had more females, however, the p-value was not significant because of the small sample size. There were an equal number of cases with EWS and OS (n=12 each) and the rest were RMS or US. Twenty-one patients had metastatic disease of which 2 patients had complete resection of the primary tumor at diagnosis and 10 patients had localized disease at baseline staging work-up. The average tumor size was 52.72 cms² (SD=52.99).

We next examined the tumor characteristics of size, stage and histology at baseline. As shown in Table 3, no significant correlations between tumor size and CECs, ECFCs, and the pCHSPC:nCHSPC ratio were found. In addition, there was no difference in CECs, ECFCs, and the pCHSPC:nCHSPC ratio based on tumor stage or histology (data shown for EWS and OS tumors only due to small sample size in RMS/US category).

The treatment summaries and response data for all evaluable cases is shown in Table 2. Two patients had progressive disease and were unable to complete their upfront planned treatment. Baseline CECs, ECFCs, and pCHSPC: nCHSPC ratio did not significantly correlate with response at the end of treatment. As shown in Figure 1 and Table 4, we examined the effects of therapy on CECs, ECFCs, and the pCHSPC:nCHSPC ratio.

The CECs were significantly lower at diagnosis and all subsequent treatment stages compared to healthy controls (Figure 1A; *p<0.001). There was no difference in CECs before and after local control. However, at the end of therapy all patients with a complete response had significantly lower levels of CECs (p=0.01) compared to those without a complete response. Lower levels of CECs in complete responders continued to be significantly lower than healthy controls (p<0.0001)

On the other hand, ECFCs were significantly elevated at diagnosis in all patients with tumors compared to the healthy controls (Figure 1B; *p=0.0021). After cycle-1 of chemotherapy the ECFCs significantly increased compared to diagnosis (p=0.036). There was no difference in the ECFCs before and after local control. All cases at end of therapy had significantly higher levels of ECFCs compared to levels at diagnosis (p=0.01) and cases who had a complete response at end of therapy also had significantly higher levels of ECFCs compared to levels at diagnosis (p=0.035). In addition, at the end of therapy all patients (responders and non-responders) had significantly elevated levels of ECFCs (Figure 1B; **p=0.0001) compared to healthy controls.

Finally, the pCHSPC:nCHSPC ratio was elevated at the time of diagnosis and at all treatment stages in all cases compared to healthy controls (Figure 1C; *all p values <0.05). There was no difference in the pCHSPC:nCHSPC ratio at any time-points during therapy, even when a therapeutic response was observed.
Discussion

This is the first report demonstrating decreased CECs and increased ECFCs and a pCHSPC:nCHSPC ratio among pediatric sarcoma patients compared to age matched healthy controls. This study demonstrated the feasibility of conducting MPFC analysis in real-time. We found that the CECs, ECFCs, and the pCHSPC:nCHSPC ratio were not associated with tumor size, tumor stage, or histology. Furthermore, we found that the CECs were the only cell type that was significantly lower among patients with a complete response compared to those without. Interestingly, there were significantly higher levels of ECFCs among all patients at the end of therapy compared to diagnosis.

The pCHSPC:nCHSPC ratio was elevated in all patients regardless of therapeutic outcome. In patients with a complete response, we would have expected the pCHSPC:nCHSPC ratio to normalize at the end of treatment. However, there was no significant difference in the pCHSPC:nCHSPC ratio at the time of diagnosis versus at the end of therapy even in the complete responders. It is possible that this ratio takes more time to normalize to that observed in healthy controls. It is also possible that the use of G-CSF, a known hematopoietic cell mobilizer, increased the level of pCHSPCs in the peripheral circulation.

Our previous observations of an increased pCHSPC:nCHSPC ratio in pediatric solid tumor patients compared to healthy controls was confirmed in this study. These results are similar to several adult studies with varying solid and hematopoietic malignancies that revealed elevated levels of circulating endothelial and progenitor cells. These progenitor cells, particularly the AC133⁺ pCHSPCs, secrete angiogenic factors such as VEGF and RANTES, and thereby stimulate the growth and viability of endothelial and cancer cells. We recently demonstrated in a novel dual humanized bone marrow melanoma xenograft model that an increase in the pCHSPC:nCHSPC ratio in the peripheral blood correlated with increased growth of human melanoma xenografts. Studies are currently in progress to fully define the homing and requirement of the pCHSPCs in solid tumor growth using additional dually humanized bone marrow tumor xenograft models including, glioblastoma in situ. Additionally, in vitro studies showed a pro-angiogenic cytokine profile from the conditioned media of pCHSPCs, and an increase in the tube formation of ECFCs when co-cultured, thus further indicating the pro-angiogenic capacity of the pCHSPCs. Therefore, the significantly elevated pCHSPC:nCHSPC ratio among our patient population compared to controls is consistent with the hypothesis that pediatric malignant tumors are nurtured by the pro-angiogenic effects of the pCHSPCs for tumor growth.

In contrast to Taylor et al., who showed higher levels of circulating vascular endothelial growth factor receptor 2⁺ (VEGFR-2⁺) bone marrow derived progenitor cells in the peripheral blood of pediatric solid tumor patients with metastatic disease, we did not find any significant differences in the levels of CECs, ECFCs or the pCHSPC:nCHSPC ratio based on tumor stage. This observation may be due to the differences in tumor types studied, but more importantly, it may be explained by the lack of consensus regarding the phenotypic definition of circulating progenitor cell subsets that are relevant in tumor induced angiogenesis. Most previous studies utilized CD34, AC133/CD133, and VEGFR-2/Kinase...
insert domain receptor (KDR) or any combinations of these cell surface antigens to quantify both hematopoietic and endothelial progenitor cells, therefore making comparisons between various clinical studies impossible. In addition to the lack of consensus on phenotypic definition and corresponding functional data to prove the identity of these cells, the inability to accurately titrate commercially available KDR antibodies has caused further confusion about the use of it as a cell-surface cytometry marker. Additionally, in a previously published study of OS patients, circulating endothelial cells and endothelial progenitor cells were not elevated and also did not correlate with OS tumor size, stage, or response to therapy compared to controls. The phenotypic enumeration of circulating endothelial progenitor cells in that study involved CD146⁺, CD31⁺, CD45⁻, and CD133⁺ cells. However, the true EPCs (i.e. ECFCs) are AC133⁻, so this difference in phenotypic expression may explain the difference in elevations of endothelial progenitor cells between our studies.

We did not find any significant correlations between baseline CECs, ECFCs, and the pCHSPC:nCHSPC ratio and tumor response. This may be due to small sample size in our pilot study. Interestingly, we encountered no decline in the pCHSPC:nCHSPC ratio following treatment compared to levels at the time of diagnosis. We also found a significant increase in ECFCs at the end of treatment when compared to baseline levels. Both the ECFCs and the pCHSPC:nCHSPC ratio continued to be significantly elevated at each time point when compared to controls. This persistent elevation may be attributed to rapid bone marrow mobilization of progenitor cells following chemotherapy and/or the use of G-CSF, as has been shown in pre-clinical studies. Additional studies have also confirmed bone marrow mobilization of hematopoietic stem and progenitor cells as a result of tissue injury and during tissue repair. This finding may explain the lack of difference in the ECFCs and the pCHSPC:nCHSPC ratio in our patients before and after local control since all patients had tissue injury following surgery and/or radiotherapy of their primary tumor site.

These findings have significant implications to change the paradigm of chemotherapy administration in sarcoma patients. Studies have shown that bone marrow mobilized cells can home towards viable tumor sites and promote angiogenesis off-setting the anti-tumor responses of conventional cancer therapies. Potentially, these stimulatory signals could be disrupted by anti-angiogenic agents, thereby sensitizing the anti-tumor effects of chemotherapeutic agents. Therefore, the timing of administration of anti-angiogenic agents to block the stimulatory signals of both the pCHSPCs and ECFCs released as a result of conventional cancer therapies might be as important as the choice of the agent. This strategy warrants additional pre-clinical investigations using humanized xenograft models. Measurement of the CECs, pCHSPCS:nCHSPC ratio, and ECFCs in real time could therefore aid in optimal administration of chemotherapy agents that can be personalized to individual patients. Although no novel anti-angiogenic agent was used in our study, future studies are being planned that will focus on measuring the CECs, ECFCs and the pCHSPC:nCHSPC ratio in patients getting only targeted anti-angiogenic agents such as in Phase-1 studies where mobilization effects on these progenitor cells as seen with conventional therapies would be minimal. The significant decrease in CECs, especially among patients with a complete response compared to those without a complete response,
needs further pre-clinical investigation to identify the role of the apoptotic mature endothelial cells in the successful remission of solid tumors.

In conclusion, this is the first multi-center pilot study highlighting the feasibility of a novel MPFC protocol to detect circulating cells involved in tumor angiogenesis in pediatric sarcoma patients. In this study we have confirmed our previous findings that sarcoma patients have elevated levels of endothelial and hematopoietic stem and progenitor cells associated with tumor angiogenesis. Although the study was not powered to validate these cells as prognostic biomarkers of tumor response, there was a significant decline in CECs among those with a complete response compared to non-responders. Validating the CECs, ECFCs and the pCHSPC:nCHSPC ratio as prognostic biomarkers will require statistically powered clinical trials and longer follow-up times.

Acknowledgments

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References


Figure 1. CECs, ECFCs and the pCHSPC:nCHSPC ratio for patients with cancer at different treatment stages and the control group

CECs in patients were significantly lower at baseline and all subsequent treatment stages compared to healthy controls (A; *p=0.01). ECFCs were significantly elevated at baseline in all patients compared to the controls (B; *p=0.010). At the end of therapy all patients (responders and non-responders) had significantly elevated ECFCs (B; **p<0.05)) compared to healthy controls, and baseline. The pCHSPC:nCHSPC ratio was elevated at baseline and at all treatment stages in all cases compared to controls (C; *p<0.01).
Table 1

Patient Characteristics at Baseline

<table>
<thead>
<tr>
<th></th>
<th>Cases N=31</th>
<th>Control Group N=21</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>9 (29.03%)</td>
<td>11 (52.38%)</td>
<td>0.09</td>
</tr>
<tr>
<td>Age (years)</td>
<td>11.23 (4.30)</td>
<td>11.24 (4.02)</td>
<td>0.99</td>
</tr>
<tr>
<td>Tumor Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ewing's Sarcoma</td>
<td>12 (38.71%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>12 (38.71%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhabdomyosarcoma</td>
<td>5 (16.13%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undifferentiated Sarcoma</td>
<td>2 (6.45%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Local</td>
<td>21 (67.74%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastatic</td>
<td>10 (32.25%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor Size (width × length) (cm²)</td>
<td>52.72 (52.99)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Categorical variables are displayed as frequency (%). Continuous variables are displayed as mean (SD)
Table 2
Treatment Summary and Response of all Evaluable Cases

<table>
<thead>
<tr>
<th>Cancer-directed Therapy and Response</th>
<th>EWS (n=12)</th>
<th>OS (n=12)</th>
<th>RMS/US (n=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemotherapy Regimens</td>
<td>VAdriaC; IE</td>
<td>Cisplat/Adria; HD-MTX</td>
<td>VAC; Ifos/Adria</td>
</tr>
<tr>
<td>Surgery for Local Control</td>
<td>4/12 = 33%</td>
<td>12/12 = 100%</td>
<td>1/5 = 20%</td>
</tr>
<tr>
<td>Radiation for Local Control</td>
<td>9/12 = 75%</td>
<td>1/12 = 8.3%</td>
<td>4/5 = 80%</td>
</tr>
<tr>
<td>G-CSF Support</td>
<td>12/12 = 100%</td>
<td>4/12 = 33%</td>
<td>2/5 = 40%</td>
</tr>
<tr>
<td>Complete Response</td>
<td>8/12 = 66%</td>
<td>9/12 = 75%</td>
<td>1/5 = 20%</td>
</tr>
</tbody>
</table>

EWS: Ewing's sarcoma; OS: Osteosarcoma; RMS/US: Rhabdomyosarcoma and Undifferentiated sarcoma; VAdriaC: Vincristine, Doxorubicin, Cyclophosphamide; IE: Ifosfamide and Etoposide; Cisplat/Adria: Cisplatin and Doxorubicin; HD-MTX: high-dose Methotrexate; VAC: Vincristine, Actinomycin-D and Cyclophosphamide; Ifos/Adria: Ifosfamide and Doxorubicin; G-CSF: Granulocyte Colony Stimulating Factor.
<table>
<thead>
<tr>
<th></th>
<th>CECs</th>
<th>ECFCs</th>
<th>pCHSPC:nCHSPC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Tumor size (n=29)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spearman correlation</td>
<td>-0.22</td>
<td>-0.22</td>
<td>0.13</td>
</tr>
<tr>
<td>p value *</td>
<td>0.27</td>
<td>0.27</td>
<td>0.53</td>
</tr>
<tr>
<td><strong>Metastatic vs Local</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD) difference</td>
<td>-0.0003 (0.0022)</td>
<td>-0.0042 (0.0232)</td>
<td>-0.31 (1.19)</td>
</tr>
<tr>
<td>p value *</td>
<td>0.60</td>
<td>0.46</td>
<td>0.76</td>
</tr>
<tr>
<td><strong>EWS vs. OS</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD) difference</td>
<td>-0.0004 (0.0020)</td>
<td>0.0027 (0.0069)</td>
<td>0.47 (1.26)</td>
</tr>
<tr>
<td>p value *</td>
<td>0.06</td>
<td>0.25</td>
<td>0.65</td>
</tr>
</tbody>
</table>

EWS: Ewing’s sarcoma; OS: Osteosarcoma; CECs: Circulating Endothelial Cells; ECFCs: Endothelial Colony Forming Cells; pCHSPC:nCHSPC ratio: Ratio of Pro-angiogenic Circulating Hematopoietic Stem and Progenitor Cells to Non-angiogenic Circulating Hematopoietic Stem and Progenitor Cells.

* p values are from Wilcoxon rank sum tests, for the normality assumptions do not hold.
Table 4
Comparisons of CECs, ECFCs and the pCHSPC:nCHSPC ratio for the cases with cancer at different treatment time points and with the healthy control group

<table>
<thead>
<tr>
<th>Group</th>
<th>CECs</th>
<th>ECFCs</th>
<th>pCHSPC:nCHSPC ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy Control</td>
<td>0.16 (0.25)</td>
<td>0.00096 (0.00097)</td>
<td>1.25 (0.20)</td>
</tr>
<tr>
<td>Cases, Baseline (n=29)</td>
<td>0.00099 (0.0022)</td>
<td>0.0090 (0.023)</td>
<td>2.13 (1.18)</td>
</tr>
<tr>
<td>Cases, After Cycle 1 (n=26)</td>
<td>0.0020 (0.0040)</td>
<td>0.0084 (0.0088)</td>
<td>3.40 (4.99)</td>
</tr>
<tr>
<td>Cases, Pre-local Control (n=22)</td>
<td>0.0014 (0.0026)</td>
<td>0.0057 (0.0065)</td>
<td>3.14 (5.56)</td>
</tr>
<tr>
<td>Cases, Post-local Control (n=27)</td>
<td>0.0029 (0.00057)</td>
<td>0.0076 (0.0073)</td>
<td>1.95 (1.35)</td>
</tr>
<tr>
<td>Cases, End of Therapy (n=20)</td>
<td>0.00049 (0.0010)</td>
<td>0.031 (0.076)</td>
<td>2.10 (1.51)</td>
</tr>
<tr>
<td>Cases, End of Therapy Complete Response (n=15)</td>
<td>0.00015 (0.00043)</td>
<td>0.035 (0.087)</td>
<td>2.16 (1.65)</td>
</tr>
</tbody>
</table>

CECs: Circulating Endothelial Cells; ECFCs: Endothelial Colony Forming Cells; pCHSPC:nCHSPC ratio: Ratio of Pro-angiogenic Circulating Hematopoietic Stem and Progenitor Cells to Non-angiogenic Circulating Hematopoietic Stem and Progenitor Cells.

Results presented as mean (SD). For the cases, all biomarkers at all treatment stages are significantly different from the healthy control group. (p<0.05)

* Significantly different from those without a complete-response (p=0.01)

\( \dagger \) Significantly different from the baseline values after Cycle-1 (p=0.036) and at end of treatment (p=0.010)