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# Circulating endothelial cells and endothelial-derived vesicles identification in patients with infantile hemangioma: preliminary results of a prospective cohort study

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**Abstract**. Circulating Endothelial Cells (CEC) and Circulating Extracellular Vesicles (cEVs) are biological markers of endothelial activity/disfunction and may play a pivotal role in the evolution of IH. Our aims were to detect variations in CEC and cEVs concentration: 1. in IH versus healthy controls; 2. in IH before and after propranolol administration. Twenty-two children (15 cases; 7 controls) from our Department underwent peripheral blood sampling to study CECs, EPCs and EVs via cytofluorimetry. Statistical analyses were performed using XLSTAT ver. 2014.5.03 and GraphPad Prism ver. 8.0. Data were expressed as mean ± standard deviation, median, 5-95$^{th}$ percentile. The number of CEC and HSC was higher among cases than controls, even if not statistically significant. The number of CEC decreased with the evolution of the hemangioma; number of HSC decreased proportionally with the age of the patienst. Number of EVs tended to increase when the CECs decrease and vice-versa in the group of cases but not among controls. Number of CEC and HSC was significantly reduced after oral propranolol administration (p: <0,0001). In conclusion CECs may represent a marker of progression of disease. Propranolol seems to affect the number of CECs and HSCs.

**Keywords:** circulating endothelial cells, extracellular vesicles, children, infantile hemangioma, propranolol.

## INTRODUCTION

Infantile Hemangioma (IH) is the most common vascular tumor of infancy, affecting up to 10% of children, especially premature with low birth weight, with a female predominance [Mattassi R et al., Darrow DH et al. 2015, Huang J et al. 2019, Satterfield KR 2019, Bauland GC et al. 2006]. They are characterized by an abnormal proliferation of endothelial cells that

express the Glucose Transporter-1 protein (GLUT1), which is not usually expressed by normal endothelial cells, and that do not show a regular vascular architecture [Huang J et al. 2019, Bauland GC et al. 2006].

Oral propranolol, a non-selective beta-blocker, is the therapy of choice in the treatment of "complicated" IH [Huang J et al. 2019, Satterfield KR 2019, Bauland GC et al. 2006].

It is well known that the endothelial homeostasis is highly influenced by the evolution of the tumors and Circulating Endothelial Cells (CEC), Endothelial Progenitor Cells (EPC) and Circulating Extracellular Vesicles (cEV) are recognized biological markers used for monitoring the endothelial activity/disfunction [Lanuti P et al. 2012].

Therefore, it is likely that CEC, EPC and cEV may be used as markers of progression of the disease.

The aims of our study were to detect:

1 variations in CEC and cEVs concentration and in the CEC/EPCs ratio in children with IH versus healthy controls;
2. changes in concentration of CEC and cEVs after oral propranolol administration.

## MATERIALS AND METHODS

### *Patients*

After local ethical committee approval (V 1.0, 4 February 2016), 22 children (15 cases and 7 controls) were enrolled in the study from the "Department of Pediatric Surgery", "Santo Spirito" Hospital of Pescara, Italy.

After written informed consent was obtained from the parents or guardians, a peripheral blood sample was withdrawn from each child and repeated in cases after 1 month of oral propranolol therapy.

### *Data acquisition*

10 × 106 events/sample were acquired by flow cytometry (FACSCanto II, BD Biosciences) at "medium" flow rate mode.

CEC and cEV were identified as previously reported [Lanuti P et al. 2018, Marchisio M et al. 2016, Lanuti P et al. 2020].

### *Statistical analysis*

Statistical analyses were performed using XLSTAT ver. 2014.5.03 (Addinsoft, Paris, France) and GraphPad Prism ver. 8.0 (GraphPad Software Inc., La Jolla, Ca, USA).

Parametric or non-parametric tests were used as appropriate.

Data were expressed as mean ± standard deviation (SD), median, 5-95$^{th}$ percentile.

## RESULTS

Twenty-two infants and children were included in the study, 15 cases and 7 controls.

We identified the Circulating Endothelial Cells (CEC) as CD45-/ CD34bright/ CD146+ events by flow cytometry, while Hematopoietic Stem Cells (HSC) as CD45dim/ CD34+ events. We also analyzed the extracellular vesicles (EV) whose origin was endothelial (CD31+) or staminal (CD133+).

Among the whole population, mean CEC number was 29,55 ± 39,12 (range 0-159,8), while mean HSC number was 4015 ± 4865 (range 79,39-18983). Mean number of EV CD31+ was 114,6 ± 202,6/ml (range 0-700,00) and mean number of EV CD133+ was 66,89 ± 103,9/ml (range 0-355,6).

When comparing cases versus controls, we found a higher number of CEC and HSC among cases than controls, even if not statistically significant (table 1).

We did not find any statistical significance also when comparing the number of EV CD31+ and CD133+ in cases versus controls (table 1). However, the number of EV CD 133+ was higher in cases than controls, even if not statistically significant.

We then analyzed the number of CEC and HSC in cases in relation to the phase of the evolution of the infantile hemangioma (IH). We found that the number of CEC and HSC decreased proportionally with the evo-

**Table 1.** Comparison between cases and controls. p significative when <0.05.

| | Cases (n=15) | Controls (n=6) | p *value* |
|---|---|---|---|
| CEC (mean ± SD) (range) | 36,5 ± 43,55 (1,96-159,8) | 8,732 ± 8,783 (0-20,17) | 0.1444 |
| HSC (mean ± SD) (range) | 4866 ± 5359 (647,4-18983) | 1503 ± 1713 (79,39-45,46) | 0.1549 |
| EV CD31+/ml (mean ± SD) (range) | 117,7 ± 211,8 (0-700,00) | 121,8 ± 206,9 (0-617,4) | 0.9642 |
| EV CD133+/ml (mean ± SD) (range) | 86,05 ± 115,9 (2,8-355,6) | 36,93 ± 75,67 (0-222,6) | 0.2907 |

CEC: circulating endothelial cells, HSC: hematopoietic stem cells, EV: extracellular vesicles. Mann–Whitney U test.

**Table 2.** Number of CEC and HSC according to the stage of the infantile hemangioma. The patient HRA was analyzed before (HRA_16) and after (HRA_18) oral propranolol administration.

| ID | DOB | Gender | Phase of IH | CEC (n) | HSC (n) |
|---|---|---|---|---|---|
| HRA_16 | 01/01/20 | F | proliferation | 87,43 | 3906,91 |
| CM_22 | 08/07/20 | F | proliferation | 32,6 | 3495,79 |
| RR_9 | 16/06/19 | M | proliferation | 65,73 | 18982,97 |
| DMA_12 | 20/01/19 | F | proliferation | 50,61 | 3197,01 |
| GD_11 | 31/07/19 | F | proliferation | 13,48 | 4210,24 |
| TM_23 | 14/05/20 | F | proliferation | 29,44 | 2637,97 |
| AI_15 | 19/05/19 | F | quiescence | 7,20 | 2800,75 |
| MD_24 | 25/11/19 | M | quiescence | 9,02 | 1399,82 |
| PC_20 | 16/07/19 | F | involution | 159,83 | 14478,02 |
| VE_06 | 06/10/12 | M | involution | 25,86 | 2218,27 |
| HRA_18 | 01/01/20 | F | involution | 14,28 | 2066,56 |
| DCS_13 | 13/07/17 | F | involution | 9,91 | 647,43 |
| DBA_8 | 18/07/19 | M | involution | 3,67 | 7236,06 |

DOB: date of birth, IH: infantile hemangioma; CEC: circulating endothelial cells, HSC: hematopoietic stem cells.

lution of the hemangioma and this association was clearer when eliminating patient nr.20 (PC_20; table 2).

Of note, the number of CEC and HSC was significantly reduced in the patient HRA, before (HRA_16: CEC 87,43; HSC 3906,91) and after oral propranolol administration (HRA_18: CEC 14,28; HSC 2066,56; p: <0,0001) [table 2, figure 1].

Moreover, we found that the number of HSC tends to decrease when the child becomes older, and this correlation appears to be clear also when analyzing cases and controls separately.

Among the HSC population, we found a subgroup of HSC CD117+ which was highly expressed in cases but not in controls (3477±4752, range 286,4-15043 in cases; 654,7±614,9, range 42,34-1428 in controls; p= 0.0003).

## DISCUSSION

It is well known that the endothelial homeostasis depends on the equilibrium between CECs and EPCs [Lanuti P et al. 2015, Sabatier F et al. 2009, Najjar F et al. 2018].

Any variation in their concentration may represent a marker of endothelial activity or damage: in fact, their changes have been studied in cardiovascular diseases and tumors [Bauland GC et al. 2006, Lanuti P et al. 2016, Najjar F et al 2018, Shantsila E et al. 2008].

However, there are no studies which focused their attention on the role of the CECs in the etiopathogenesis and development of IH.

In a recent study on healthy adults, the normal values of CECs were determined which were different in females compared to males (12,10 ±10,02 versus 13,69±9,19; p=0,0218) [Lanuti P et al. 2012].

However, there are no studies that define a normal range of CECs in children. In our study, we found that the mean CEC count in the whole population was slightly higher than the one found in adults (22,04 ± 25,78). Moreover, the number of CECs was higher in children affected by IH than in controls (p=ns).

The number of CECs in cases decreases proportionally with the evolution of the IH and this is maybe due to the reduction of the endothelial activity in the involutive and involute phases, when the vessels are replaced by fatty tissue. Therefore, the number of CECs may be identified as biomarker of evolution of the IH.

It has also been demonstrated that in peripheral blood of healthy adults the number of HSC is about 2663,3±1288,33 [Marchisio M et al. 2020]. We found a higher number of HSC in our population (4015 ± 4865) compared to adults, which is even higher when analyzing only the IH group (4866 ± 5359), thus reflecting a higher recruitment of the staminal compartment.

Moreover, we found a subgroup of CD117+ cells which are highly expressed only in cases than controls. This subpopulation needs to be further investigated.

Propranolol seems to influence cells viability and proliferation [Erdbruegger U et al. 2010]. We found it to affect the number of CECs and HSCs; however larger studies are needed to confirm this assumption.

The number of EVs itself does not seem to be a marker of the disease, but the study of their molecular cargo will help in elucidating the mechanisms at the basis of the evolution of the IH.

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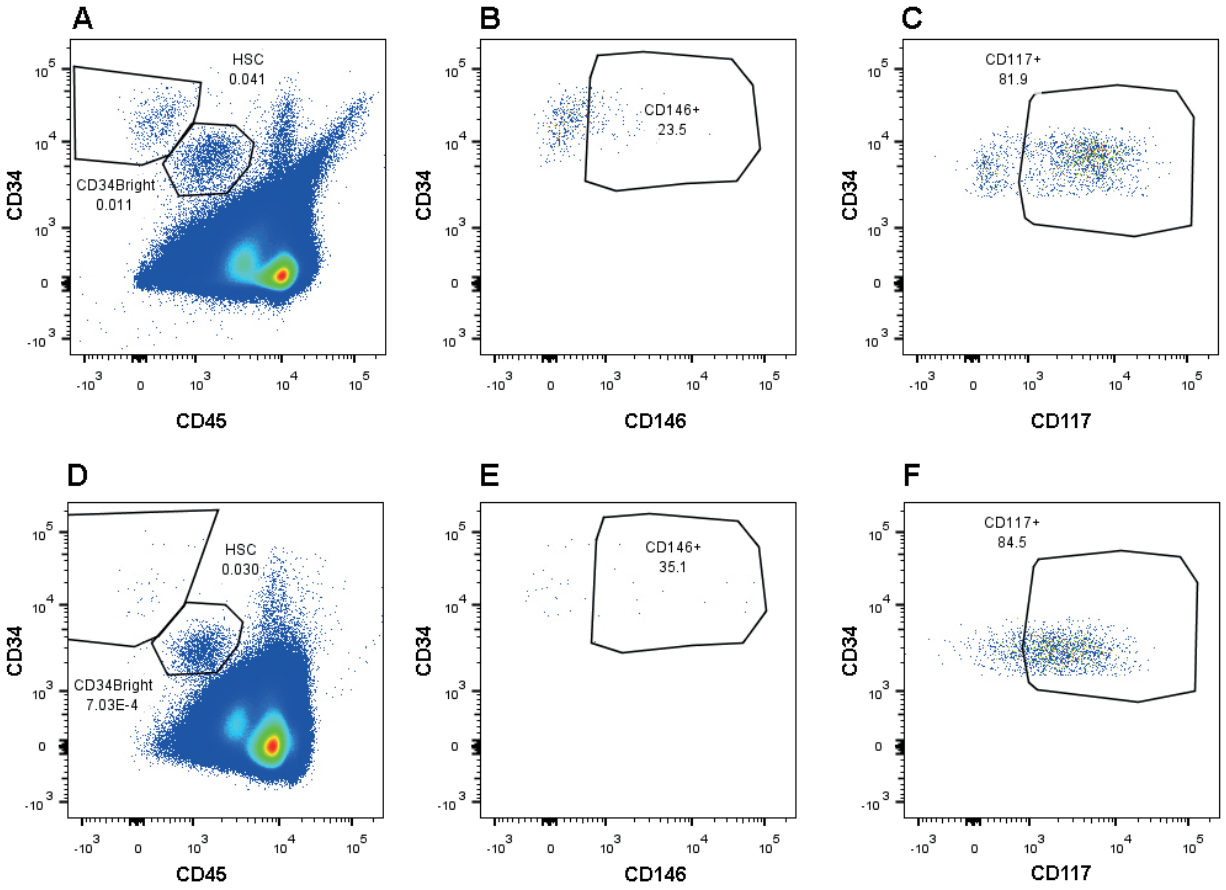


**Figure 1.** Flow cytometry identification of CEC and HSC in PB samples. **A, D** Cells displaying the lymph-monocyte morphology, alive and nucleated were analyzed for CD45 and CD34 expression (on a CD45/CD34 dot plot). The whole CD34pos cell compartment was identified and two subpopulations, displaying different levels of CD34 surface expression, were identified and gated separately: CD34 positive cells, which are CD45dim and represent the hematopoietic stem cell compartment (HSC), and a CD34 bright population, which resulted CD45 negative (CD34 bright). **B, E** CD34 bright/CD45neg cell populations were than analyzed for CD146 expression, on a CD146/CD34 dot plot and represent CEC compartment. **C, F** The hematopoietic stem cell compartment (HSC) was analyzed for CD117 expression, on a CD117/CD34 dot plot.

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