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Original Article

Changes in endothelial progenitor cell subsets in normal pregnancy compared with preeclampsia

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Abstract

Background: The results of studies measuring the number of endothelial progenitor cells (EPCs) in normal pregnancies and in preeclampsia have been highly controversial or even contradictory because of cross-sectional designs and different methodologies enumerating three distinct subsets of EPCs: circulating angiogenic cells (CAC), colony-forming unit endothelial cell (CFU-ECs), and endothelial colony forming cells (ECFCs). To provide a clear explanation for these underlying controversies, we designed a prospective study to compare the number of all EPC subsets between three trimesters of normal gestation and a case–control study to compare these values as preeclampsia occurs with those from gestational age (GA) matched normal pregnancy.

Methods: Samples from peripheral blood of nine women were taken during their three consecutive trimesters of normal pregnancy, and from eight women with preeclampsia. To cover most of the reported phenotypes for CACs and ECFCs in the literature, we enumerated 13 cell populations by quantitative flow cytometry using various combinations of the markers CD34, CD133, CD309, and CD45. We used routine culturing techniques to enumerate CFU-ECs.

Results: The numbers of CACs and ECFCs were higher in women with preeclampsia ($p = 0.014$). By contrast, preeclampsia was associated with a reduced number of CFU-ECs ($p = 0.039$). The CAC number rose with the increase in GA ($p = 0.016$) during normal pregnancy, while the number of CFU-ECs and ECFCs did not differ during the trimesters.

Conclusion: Although we did demonstrate an increase in absolute counts of CACs and ECFCs in preeclampsia, fewer colony formation capacities indicated a loss in their functional capabilities. By contrast, the number of CACs increased without alterations in colony formation ability in normal pregnancy with the growth of the fetus. Here, by comparing different methodologies to calculate the number of EPC subsets, we could imitate the existing controversy in the literature for such calculations, which may help to elucidate clearer explanations.

Keywords: endothelial cell; hemangioblast; hypertension; preeclampsia; pregnancy

Conflicts of interest: The authors declare that there are no conflicts of interest related to the subject matter or materials discussed in this article.

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1. Introduction

Normal pregnancy is associated with enhanced endothelial function and formation of new blood vessels. Regulation of endothelial function requires an interaction between endothelial cells and subpopulations of circulating cells, termed *endothelial progenitor cells* (EPCs).[1] Since first explained by Asahara et al,[2] the definition of EPCs and techniques used for their assessment have gone through many changes. Hill and coworkers[3] introduced a colony-forming assay [colony forming unit (CFU)-Hill] in the field, and Peichev et al[4] suggested the combination of CD34, CD133, and CD309 as the flow cytometric identifying the phenotype for EPCs. However, more recent data are also persuasive, further suggesting that the two mentioned methods introduce cells that exhibit monocyte characteristics (such as expressing CD14 and CD45) in conjunction with markers from endothelial lineage (such as CD34 and CD309), which are from a hematopoietic hierarchy. These cells do not differentiate into endothelial cells or assemble into vascular networks *in vitro*, and thus cannot be true EPCs.[5] Eventually, Ingram et al[6] discovered cells with more appropriate characteristics as true EPCs, the so-called *endothelial colony-forming cells* (ECFCs). Based on that, ECFCs express CD34 and CD309 but lack CD14, CD45, and CD133.[6] Despite these, both CFU-Hills and circulating EPCs measured by flow cytometry remained under the classification umbrella of EPCs in the literature but they were renamed as *CFU-endothelial cells* (CFU-ECs) and *circulating angiogenic cells* (CACs), respectively.[5,7] Nevertheless, reduced CFU-EC and CAC numbers correlate with increased risk of vascular disease and the strong correlation between their numbers and abnormal function of endothelial cells cannot be ignored.[5]

In this regard, only a few studies have been performed regarding the role of EPCs in normal pregnancies and preeclampsia, as an example of gestational vascular disorder, and the results of those studies are highly controversial.[8] This may be due to the variety of surface markers, and functional assays have been used to assess EPC numbers that make the comparison between their results more difficult. Furthermore, all of these studies were cross-sectional and relatively few studies used both flow cytometry and culture techniques to compare EPC subsets. Therefore, using flow cytometry and culturing techniques in a prospective study, we compared the number of all EPC subsets including: CACs, CAC precursors ($CD34^{-}CD133^{+}CD309^{+}$ cells),[9] putative ECFCs, and CFU-ECs in three trimesters of normal pregnancy and in a case–control study the similar values from patients with preeclampsia were compared with the findings from gestational age-matched normal pregnancies.

2. Methods

2.1. Study population

Nine healthy women in their first trimester of pregnancy were enrolled in this study. The participants were followed-up during their total course of pregnancy from April 2011 to May 2012 and peripheral blood samples were taken sequentially in each trimester. The exclusion criteria consisted of affliction with diabetes mellitus, malignancies, autoimmune disease, hypertension, chest pain induced by physical activities, vascular claudication, current episode of infection, and a family history of premature cardiac events or severe abnormal lipid profiles. Eight pregnant women diagnosed with preeclampsia, defined as the new onset of hypertension (systolic blood pressure $\geq$ 140 mmHg and diastolic blood pressure $\geq$ 90 mmHg) and proteinurea ($\geq$ 0.3 g in a 24-hour urine specimen) after 20 weeks of gestation in a previously normotensive woman were recruited in the study.[7] All participants gave their written informed consent before enrolment in this study. This study was approved by the local Ethics Committee and conformed to the Declaration of Helsinki.

2.2. Preparation of mononuclear cells

Peripheral blood samples (20 mL) were taken, diluted using 15 mL of phosphate buffered saline (PBS), and brought onto the layer of Lymphosep (Biosera, Boussens, France). They were then centrifuged at 300*g* continuously for 25 minutes and finally the layer of mononuclear cells (MNCs) was isolated. The total number of isolated cells was determined using hemocytometer and the purified cells were divided into two tubes for the following steps.

2.3. CFU-EC assay

Half of the isolated MNCs were resuspended in 4 mL of Endocult medium (Stem Cell Technologies, Vancouver, BC, Canada) supplemented with 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco/Invitrogen, Carlsbad, CA, USA). Two-milliliter aliquots of this suspension was seeded per well of 6-well fibronectin-coated plates (BD Biosciences, San Jose, CA, USA) and incubated for 2 days at 37°C, 5% CO_2, and 95% humidity. Afterwards, the nonadherent cells were harvested by flushing prewarmed media and replated in 96-well fibronectin-coated plates (BD Biosciences). After 5 days, the culture medium was removed and the plates washed with PBS to remove nonadherent cells. To increase the accuracy, the tests were done in duplicate and the colonies were counted by two independent expert technicians.

2.4. Quantitative flow cytometry

Because of the low frequency of the target cells in the peripheral blood, our flow cytometry protocols followed rare cell analysis protocols.[10] Consequently, all the mononuclear cells purified from half of the blood sample (equivalent to 10 mL or at least 10^6 cells) were stained and analyzed through flow cytometry. Propidium iodide and dead cell removal kits (Miltenyi Biotec, Bergisch Gladbach, Germany) were used before the actual staining to exclude dead cells from the

harvested cells to decrease the chance of nonspecific cell bindings.[10] The isolated cells were stained with antibodies against CD34-fluorescein isothiocyanate, CD133- phycoerythrin, CD309-allophycocyanin, and CD45-peridinin chlorophyll protein while the cells were treated with Fc receptor blocking reagent (all obtained from Miltenyi Biotec). Cell suspensions were incubated in the dark at 4–8°C and washed twice; the cell pellets were resuspended in 520 μL of PBS-EDTA buffer supplemented with 2% fetal bovine serum. To enumerate cell population of interest, TruCOUNT kit (BD Biosciences) was used. Accordingly, stained cell suspensions were transferred to the TruCOUNT tubes and immediately analyzed by four-color FACS-Calibur instrument (BD Biosciences). Isotype matched antibodies (Miltenyi Biotec) were used to exclude nonspecific staining of the cells in every staining. CellQuest Pro software (BD Biosciences) was used for data acquisition and WinMDI software was used for data analysis and graphical presentation of data. Fig. 1 shows the way that cells were gated for analysis and enumeration of the desired cells. The absolute cell count per mL of blood samples was calculated by the formula provided in the TruCOUNT kit instructions:

Number of events in target region × number of beads in the tube/number of events in bead region × sample volume.

We considered $CD34^+CD133^+CD309^+CD45^+$ cells as CACs,[5] $CD34^+CD133^-$ $CD309^+CD45^-$ as ECFCs,[6] and $CD34^-CD133^+CD309^+$ cells as CAC precursors.[9] In addition, to cover other reported phenotypes for CACs and ECFCs in the literature,[11] we enumerated 10 other cell populations listed in Tables 1 and 2.[5,11,17,18,20–23]

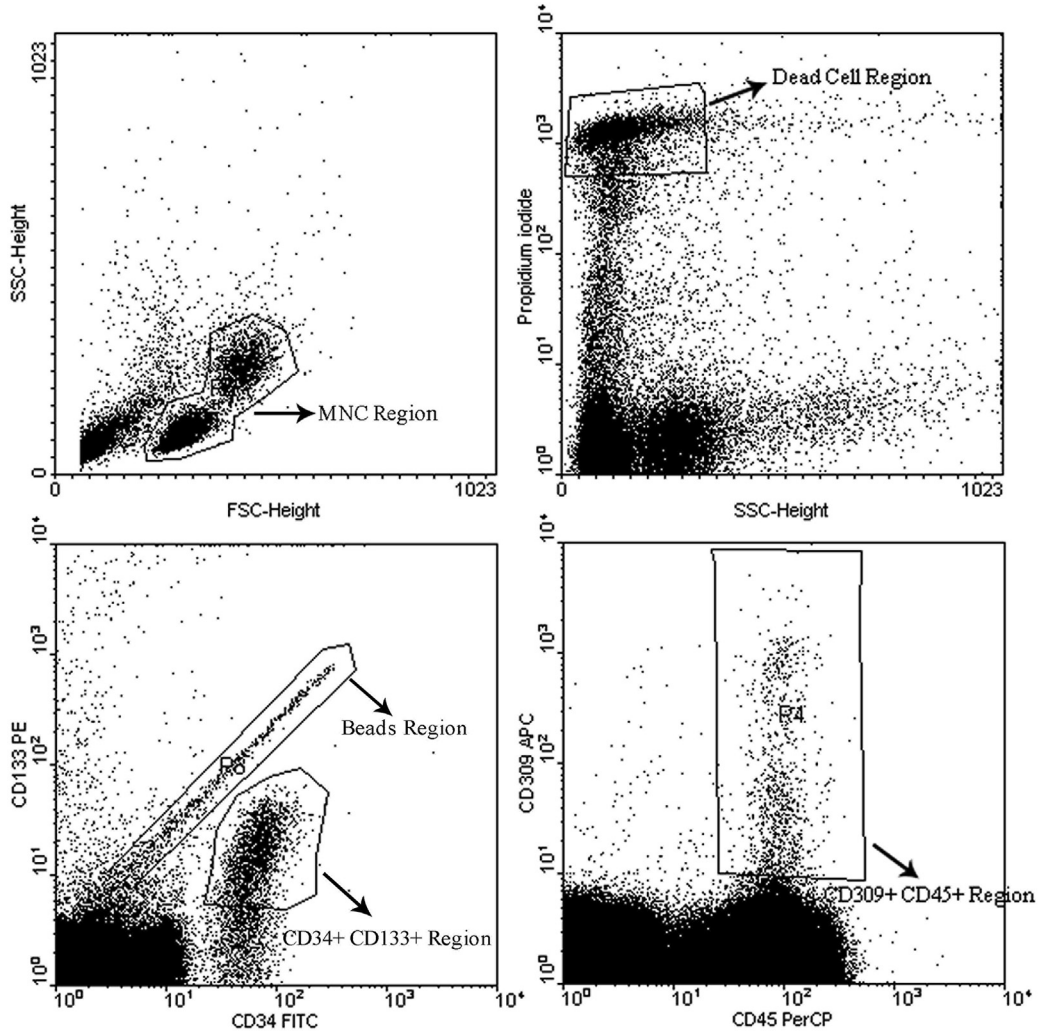

Fig. 1. Typical dot plot diagrams defining some of the variable regions used to enumerate absolute counts of the circulating angiogenic cells marked as $CD34^+CD133^+CD309^+CD45^+$ cells.

Table 1
Enumeration data for 10 putative endothelial progenitor cell populations during three gestational trimesters produced by flow cytometry analysis.

Groups	Used as alternative for	Reference	1st trimester	2nd trimester	3rd trimester	Statistical analysis (p)		
Variables			Median (range)	Median (range)	Median (range)	1st & 2nd	1st & 3rd	2nd & 3rd
$CD34^+$/mL	—	—	13,880 (3794–472,929)	6670 (2594–23,641)	17,004 (6222–29,187)	0.063	0.605	0.024
$CD309^+$/mL	—	—	8693 (2516–52,947)	3445 (1442–7383)	7284 (5489–10,598)	0.077	0.546	0.004*
$CD133^+$/mL	CAC	11	11,069 (3743–39,230)	6993 (2382–11,589)	11,774 (6555–20,566)	0.113	0.387	0.006*
$CD34^+$ 133^+/mL	CAC	20	4156 (1491–34,144)	4061 (1090–6045)	5606 (2976–8422)	0.436	0.190	0.031
$CD34^+$ 309^+/mL	CAC	21	359 (121–4481)	160 (58–503)	380 (59–691)	0.094	0.489	0.222
$CD133^+$ 45^-/mL	CAC	22	375 (30–2123)	192 (33–1490)	139 (34–449)	0.489	0.094	0.436
$CD133^+$ 309^+/mL	CAC	23	713 (465–1543)	493 (351–852)	1197 (681–2544)	0.050	0.063	< 0.001*
$CD34^+$ 45^-/mL	ECFC	17	1704 (12–87,502)	260 (62–4115)	124 (18–3984)	0.094	0.050	0.258
$CD309^+$ 45^-/mL	ECFC	18	623 (167–25,092)	614 (48–3310)	996 (277–4546)	0.605	0.387	0.258
$CD34^+$ 133^- 309^+/mL	ECFC	5	36 (7–255)	29 (5–157)	158 (11–292)	0.387	0.258	0.063
$CD34^+/10^6$ cells	—	—	5780 (1999–112,276)	3423 (1267–6285)	6996 (1153–10,258)	0.094	0.931	0.094
$CD309^+/10^6$ cells	—	—	2258 (566–19,327)	1536 (662–6956)	2286 (1134–3836)	0.546	0.796	0.190
$CD133^+/10^6$ cells	CAC	11	3278 (1695–9313)	2812 (1094–7339)	5074 (1271–7939)	0.387	0.863	0.258
$CD34^+$ $133^+/10^6$ cells	CAC	20	1252 (799–8106)	1216 (602–3826)	2263 (577–3663)	0.605	0.387	0.297
$CD34^+$ $309^+/10^6$ cells	CAC	21	169 (16–3169)	63 (32–189)	84 (11–303)	0.222	0.436	0.546
$CD133^+$ $45^-/10^6$ cells	CAC	22	74 (24–504)	118 (4–824)	55 (10–99)	0.863	0.077	0.258
$CD133^+$ $309^+/10^6$ cells	CAC	23	397 (64–653)	207 (89–427)	480 (172–669)	0.489	0.436	0.040
$CD34^+$ $45^-/10^6$ cells	ECFC	17	507 (6–13,720)	123 (37–999)	58 (8–751)	0.136	0.031	0.136
$CD309^+$ $45^-/10^6$ cells	ECFC	18	245 (23–9159)	191 (26–3119)	412 (54–842)	0.863	0.730	0.546
$CD34^+$ 133^- $309^+/10^6$ cells	ECFC	5	29 (1–104)	14 (3–59)	38 (2–134)	0.489	0.489	0.190

*$p < 0.017$ considered statistically significant.
1st, 2nd, 3rd = trimesters; CAC = circulating angiogenic cells; ECFC = endothelial colony forming cells; GA = gestational age.

2.5. Statistical analysis

The numbers of 13 distinct cell populations were measured with flowcytometry and those for CFU-EC derived from culturing techniques were calculated in 1 mL of whole blood or in 10^6 purified MNCs to address the controversies in the literature. Eventually, 28 parameters were gathered for comparison between the three trimesters or between preeclampsia with GA-matched normal pregnancies. The number of cells is reported as mean ± standard deviation. Normality of the distribution of continuous data was examined using the Kolmogorov–Smirnov test. Logarithmic transformation was

Table 2
Enumeration data for 10 putative endothelial progenitor cell populations in preeclamptic and GA matched normal pregnancies produced by flow cytometry analysis.

Groups	Used as alternative for	Reference	GA-matched normal pregnancy			Preeclampsia			p
Variable			Median	Min	Max	Median	Min	Max	
$CD34^+$/mL	—	—	11,964	4347	36,025	13813	4644	61155	0.622
$CD133^+$/mL	—	—	11589	3888	25693	11,458	5693	44,323	0.733
$CD309^+$/mL	CAC	11	7383	2871	17,920	11,233	5499	57,134	0.381
$CD34^+$ 133^+/mL	CAC	20	5172	2007	21,340	3573	1302	11,501	0.205
$CD34^+$ 309^+/mL	CAC	21	161	59	691	520.5	38	1589	0.154
$CD133^+$ 45^-/mL	CAC	22	181	34	488	449.5	90	1309	0.023*
$CD133^+$ 309^+/mL	CAC	23	1014	452	2544	1352.5	1072	4893	0.112
$CD34^+$ 45^-/mL	ECFC	17	192	18	4116	315	55	6795	0.791
$CD309^+$ 45^-/mL	ECFC	18	1324	272	4546	3880	1264	13309	0.014*
$CD34^+$ 133^- 309^+/mL	ECFC	5	47.5	6	292	139	8	748	0.267
$CD34^+/10^6$ cells	—	—	4823	1153	10,258	6155	1470	8621	0.267
$CD133^+/10^6$ cells	—	—	3455	1271	7939	4431	1802	6157	0.569
$CD309^+/10^6$ cell	CAC	11	2551	1134	6956	4181	1740	5522	0.066
$CD34^+$ $133^+/10^6$ cells	CAC	20	1783.5	577	3826	1189.5	412	2265	0.178
$CD34^+$ $309^+/10^6$ cells	CAC	21	80	11	303	212	12	267	0.132
$CD133^+$ $45^-/10^6$ cells	CAC	22	50	7	338	148.5	53	284	0.029*
$CD133^+$ $309^+/10^6$ cells	CAC	23	345	171	669	596	339	833	0.014*
$CD34^+$ $45^-/10^6$ cells	ECFC	17	68	8	999	103	32	657	0.733
$CD309^+$ $45^-/10^6$ cells	ECFC	18	334	54	3119	1129	680	1879	0.008*
$CD34^+$ 133^- $309^+/10^6$ cells	ECFC	5	14	2	134	71.5	2	82	0.178

*$p < 0.05$ considered statistically significant.
CAC = circulating angiogenic cells; ECFC = endothelial colony forming cells; GA = gestational age.

performed for non-normally distributed data. Data were analyzed using Friedman one-way analysis of variance with repeated measures and Bonferroni's post-tests for multiple comparisons. Additionally, Kruskal–Wallis and Mann–Whitney *U* tests were used where appropriate. The p value < 0.05 when comparing two groups, or $p < 0.017$ when comparing three groups, were considered as significant in analyses. SPSS software version 19.0 for windows (IBM, Armonk, NY, USA) was used for statistical analysis. Prism (GraphPad Software, San Diego, CA, USA) was used for graphical presentation of data.

3. Results

3.1. Samples and participants

In this study, we compared the number of EPC subsets in the peripheral blood samples from nine normal pregnant women and eight patients with preeclampsia. The mean gestational age of the patients with preeclampsia and gestational age matched normal pregnant women were 32.29 weeks and 31.8 weeks, respectively, which were not significantly different ($p = 0.641$). Some of the clinical characteristics of the patients with preeclampsia and of healthy pregnancies in subsequent trimesters are shown in Table 3.

3.2. Quantification of EPC subsets

Flow cytometry was used to enumerate CACs, precursors of CAC and putative populations of ECFCs, and culture techniques were used to enumerate the number of CFU-ECs, both in 1 mL of blood and in 10^6 purified MNCs.

There were higher numbers of CFU-ECs when comparing per mL of blood during the first trimester of the normal pregnancies (27.8 colonies/mL) than the second and third trimesters with slightly higher numbers in the third trimester compared to the second (16.72 colonies/mL and 24.02 colonies/mL, respectively; Fig. 2A). Nevertheless, neither of the observed variations during normal pregnancy was significant ($p = 0.322$). The number of CFU-ECs when determined in 10^6 MNCs showed no significant change either (Fig. 2B, $p = 0.181$). The frequency of CACs found in the unit of the peripheral blood was significantly elevated as gestational age increased in normal pregnancies. For example, CAC frequency was 106.25 cells/mL during the first trimester, and increased to 239 cells/mL in the third trimester (Fig. 2C; $p = 0.016$). However, the increase showed no significance when numbers of CACs were calculated among 10^6 MNCs (Fig. 2D, $p = 0.136$). The number of precursors of CACs in 1 mL of blood rose as gestational age increased (Fig. 2E, $p < 0.001$) and again this rise was insignificant when the numbers were calculated among 10^6 MNCs (Fig. 2F, $p = 0.019$). Measuring the frequencies of ECFCs by the mentioned phenotypes did not show a significant variation during different trimesters of normal pregnancies (Fig. 2G and H, $p = 0.031$). In addition to those populations, the actual frequencies of 10 other cell populations expressing various combinations of surface markers from the peripheral blood of women with normal pregnancy are listed in Table 1.

We found significantly higher numbers of CFU-EC per mL of blood in the gestational age matched normal pregnant women compared with preeclamptic patients (Fig. 3A, $p = 0.039$). When extrapolating the data in 10^6 MNCs, this significance was lost (Fig. 3B, $p = 0.078$). The counts of CACs per 1 mL of blood and in 10^6 MNCs had a difference that was not statistically significant (Fig. 3C and D, $p = 0.205$ and $p = 0.235$, respectively). The number of CAC precursors counted among 10^6 MNCs was also significantly higher in the preeclampsia group (Fig. 3F, $p = 0.029$). The analysis of the ECFC numbers showed no considerable difference between the two studied groups (Fig. 3G and H, $p = 0.677$). Absolute numbers from 10 other cell populations expressing various combinations of surface markers in normal and diseased states are shown in Table 2.

4. Discussion

In the present study, through flow cytometric studies we could show that higher numbers of CACs and ECFCs subsets

Table 3
Clinical profiles of the individuals with normal pregnancy or preeclampsia.

	General characteristics of normal pregnancies		
Maternal age (y)	29.33 ± 4.58 (21–36)		
Nuliparity (%)	55.6		
	1st trimester	2nd trimester	3rd trimester
Gestational age at the day of sampling (wk)	9.8 ± 1.7 (7–13)	24.7 ± 2.1 (22–27)	33.6 ± 2.6 (31–38)
Systolic blood pressure (mmHg)	108.8 ± 9.2 (100–120)	107.6 ± 11.2 (90–120)	106.8 ± 9.2 (90–120)
Diastolic blood pressure (mmHg)	67.7 ± 8.3 (60–80)	68.7 ± 7.3 (60–80)	67.9 ± 9.1 (60–80)
Urine protein (dipstick)	0+	0+	0+
	Gestational age matched normal pregnancies	Preeclamptic pregnancies	*p*
Number of samples	9	8	—
Maternal age (y)	29 ± 4.6 (21–36)	29.7 ± 7.1 (17–37)	0.782
Nuliparity (%)	53	14	0.036
Gestational age at the day of sampling (wk)	31.8 ± 3.3 (27–36)	32.29 ± 3.09 (26–35)	0.641
Systolic blood pressure (mmHg)	107.19 ± 10.64 (90–120)	160 ± 14.14 (150–180)	0.012
Diastolic blood pressure (mmHg)	63.13 ± 8.3 (60–80)	99.29 ± 8.3 (90–115)	0.032
Urine protein (dipstick)	0+	~2.5+	0.021

Data are presented as mean ± SD unless otherwise indicated.

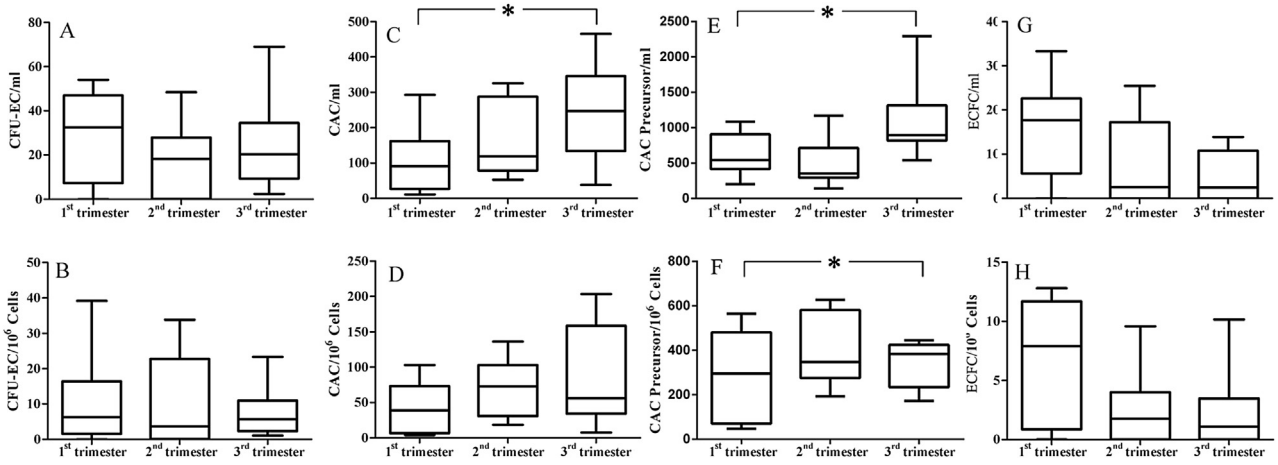

Fig. 2. Box plot defining median and range from 10 of the calculated values in three gestational trimesters (*$p < 0.017$). The number of circulating angiogenic cells (CACs) increases as gestational age advances, and reaches the significance level when reported as numbers in 1 mL of blood ($p = 0.016$). Number of CAC precursors increases as gestational age increases as well. Numbers of colony forming unit endothelial cells (CFU-ECs) and endothelial colony forming cells (ECFCs) did not vary significantly.

are mobilized in the peripheral blood of pregnant women with preeclampsia compared with normal pregnancies, but with lower colony-forming capacities (shown by CFU-EC assay). By contrast, the number of these cells increases as gestation goes ahead in a healthy pregnancy and their functional capacities remain intact in this setting. The pattern of these changes and their significance is open to comparison of these results with those from previous studies, as discussed below.

In this regard, some controversies have been previously reported about the frequency of EPCs in different trimesters of normal pregnancies as well as in preeclampsia. This might be related to several factors. First of all, there is a lack of consensus on the definitive EPC phenotype and a variety of surface markers, and functional assays have been used to enumerate these cells. Besides, the resultant data are reported in two ways: numbers in the volume of blood and frequencies in a defined number of MNCs, which makes the comparison of different clinical studies difficult. Furthermore, some technical issues were not taken into consideration. For example, in flow cytometric studies, Matsubara et al[12] did not perform an absolute count of the cells, while Buemi et al[13] did that only on the $CD34^+$ population, and none of the studies analyzed a sufficient number of cells as needed in the settings of rare cell analysis.[10] In addition, the absence of a prospective approach, at least in part, could explain some of the existing variations among reported results. Therefore, in this prospective study we analyzed all the EPC subsets by considering the suggested patterns in flow cytometry and culture techniques. Moreover, the counts were determined both by calculating the number of cells in the unit volume of blood and the number of cells in 10^6 purified MNCs. Comparison of these two methods of data calculation showed a significant difference in the final results which is discussed below in comparison with previous studies.

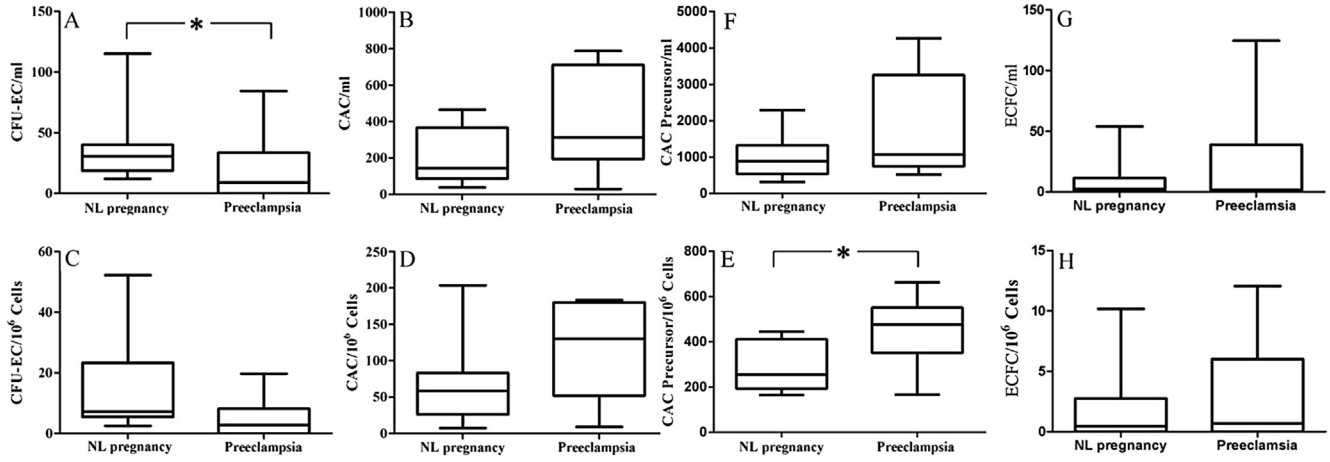

Fig. 3. Box plot defining median and range from 10 calculated values in the group with preeclampsia and gestational age matched the normal pregnancy group (*$p < 0.05$). The number of colony-forming unit endothelial cells (CFU-ECs) decreases as preeclampsia occurs and reaches the significance level when reported as numbers in 1 mL of blood ($p = 0.039$). The number of circulating angiogenic cell (CAC) precursors measured among 10^6 mononuclear cells increased in the preeclampsia group. Numbers of CACs and endothelial colony forming cells (ECFCs) increased without statistical significance. NL = normal.

Regarding healthy pregnancies, Sugawara et al[14] showed a significant increase in CFU-ECs when calculating the cells among 1.5×10^6 MNCs along the gestation time. By contrast, Savvidou et al[15] reported only minor decreases in CFU-ECs calculated among MNCs with progression of pregnancy. Our finding was more consistent with the results of Savvidou et al,[15] reporting EPCs as CFU-ECs counted among 10^6 MNCs. Similar to our findings, Matsubara et al[12] showed a decrease in CFU-ECs in the unit volume of blood as gestational age increased. However, we could see some increase afterwards during the third trimester. Regarding the number of CACs, our data were in accordance with the report by Buemi et al,[13] which showed an increase in the number of CACs in 1 mL of blood with progress in gestational ages. However, our data contradicted results found by Matsubara et al,[12] who concluded that the frequency of CACs in 10^5 MNCs decreased with gestational age. In our study, the number of $CD34^-CD133^+CD309^+$ cells that had been purposed to be precursors of CACs[9] increased significantly as gestational age increased.

Similar to the findings of Sugawara et al[16] and Lin et al,[7] we could also show the presence of reduced CFU-ECs among a defined number of MNCs in patients with preeclampsia compared with the gestational age-matched normal pregnancies. In the same way, we showed a lower frequency of CFU-ECs in 1 mL of blood of patients with preeclampsia although it was in contrast with the findings of Matsubara et al.[12] In our study, consistent with the report by Buemi et al,[13] the number of CACs in 1 mL of blood increased in preeclampsia. However, the calculated numbers in 10^6 purified MNCs were in disagreement with the results reported by Matsubara et al.[12] The number of precursors of CACs was also significantly higher in the preeclampsia group.

In our study, we used a flow cytometric technique to enumerate the possible ECFCs. We found a higher number of all possible combination markers as preeclampsia occurred, although only $CD309^+CD45^-$ profile reached statistical significance. This is in accordance with the findings in acute myocardial infarction; as endothelial damage occurs, the number of ECFCs rises in the peripheral blood.[17,18] In regards to the normal pregnancy, we did not find any significant differences in the number of possible ECFCs with changes in gestational age. Here, we dispensed with culturing techniques to enumerate ECFCs because the frequency of these cells in the peripheral blood is very low, and large volumes of blood samples are needed for accurate measurements. So, most of our participants refused to donate such volumes of blood in their pregnancy period. In addition, it is shown that the number of culture-derived ECFCs colonies strongly correlated with the number of $CD34^+CD45^-$ and $CD34^+CD309^+$ flow cytometric enumerated cells and without any correlation to the $CD34^+CD133^+$ as marker of CACs.[17,18]

Due to the biological functions of EPCs, the differences between the number of CACs and CFU-ECs in normal pregnancies and those with preeclampsia can be expected. It was shown that EPCs can be mobilized from the bone marrow during stress or endothelial injuries (as it happens in preeclampsia or acute myocardial infarction), which results in an increased number of cells in the peripheral blood.[19] In fact, this can be an attempt to promote re-endothelialization of the damaged vessels. However, the functionality of these cells can be affected by chronic inflammation in vascular disorders.[3] Accordingly, in our study there were increased numbers of CACs and ECFCs in the blood of patients with preeclampsia, possibly as a result of mobilization. However, due to inflammation, the cells were incapable of performing their normal function, which was demonstrated by lower abilities for colony formation. By contrast, among the higher gestational ages of a normal pregnancy, as the fetus grows the demand for EPC rises and the number of CACs increases with normal functional capability for colony formation.

These findings emphasize again the importance of EPCs as a target for biological therapies of preeclampsia and other vascular diseases. Moreover, by comparing different methodologies to calculate the number of EPC populations, or considering different populations as target, we could imitate the existing controversy in the literature for such calculations, which may help to extract more clear explanations. Prospective studies should be conducted to determine whether or not measuring the EPC numbers in early pregnancy can help predict the occurrence of preeclampsia.

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