

# Endothelial Colony-Forming Cells in Young Adults Born Preterm: A Novel Link Between Neonatal Complications and Adult Risks for Cardiovascular Disease

Mariane Bertagnolli, PhD; Li Feng Xie, MSc; Katryn Paquette, MD; Ying He, MSc; Anik Cloutier, MSc; Rafael Oliveira Fernandes, PhD; Chanel Béland, MD; Megan R. Sutherland, PhD; Jacques Delfrate, MSc; Daniel Curnier, PhD; Jean-Luc Bigras, MD; Alain Rivard, MD; Bernard Thébaud, MD, PhD; Thuy Mai Luu, MD, MSc; Anne Monique Nuyt, MD

**Background**—Preterm birth is linked to cardiovascular risks and diseases. Endothelial progenitor cells play a critical role in vascular development and repair. Cord blood endothelial progenitor cells of preterm-born infants, especially endothelial colony-forming cells (ECFC), show enhanced susceptibility to prematurity-related pro-oxidant stress. Whether ECFC dysfunction is present in adulthood following preterm birth is unknown.

**Methods and Results**—This cross-sectional observational study includes 55 preterm-born ( $\leq 29$  gestational weeks) young adults (18–29 years old, 38% male) and 55 sex- and age-matched full-term controls. ECFC were isolated from peripheral blood; cell proliferative and vascular cord formation capacities were assessed in vitro. Daytime systolic blood pressure was higher, whereas glucose tolerance and body mass index were lower in preterm-born subjects. ECFC colonies grew in culture for 62% of full-term- and 58% of preterm-born participants. Preterm-born participants have formed ECFC colonies later in culture and have reduced proliferation compared with controls. Only in preterm-born individuals, we observed that the later the ECFC colony grows in culture, the worse was overall ECFC function. In addition, in preterms, elevated systolic blood pressure significantly correlated with reduced ECFC proliferation ( $r_s = -0.463$ ;  $P = 0.030$ ) and numbers of branches formed on matrigel ( $r_s = -0.443$ ;  $P = 0.039$ ). In preterm-born subjects, bronchopulmonary dysplasia was associated with impaired ECFC function, whereas exposure to antenatal steroids related to better ECFC function.

**Conclusions**—This study is the first to examine ECFC in preterm-born adults and to demonstrate ECFC dysfunction compared with full-term controls. In the preterm-born group, ECFC dysfunction was associated with bronchopulmonary dysplasia, the major prematurity-related neonatal morbidity, and with increased systolic blood pressure into adulthood. (*J Am Heart Assoc.* 2018;7:e009720. DOI: 10.1161/JAHA.118.009720.)

**Key Words:** bronchopulmonary dysplasia • cardiovascular disease risk factors • endothelial progenitor cells • hypertension • pregnancy and postpartum • preterm birth

Ten percent of current-day children and young adults were born preterm (<37 weeks gestational age) (March of Dimes <http://www.marchofdimes.com/mission/globalpreterm.html>). Mounting evidence supports the adverse effect of

preterm birth, and more so very preterm birth (<32 weeks gestational age), on cardiovascular health.<sup>1,2</sup> Individuals born preterm display both structural and functional cardiovascular alterations, which include higher blood pressure,<sup>2</sup>

From the Sainte-Justine University Hospital Research Center (M.B., L.F.X., K.P., Y.H., A.C., R.O.F., C.B., M.R.S., J.D., D.C., J.-L.B., T.M.L., A.M.N.), Centre Intégré Universitaire de Santé et de Services Sociaux du Nord-de-l'Île-de-Montréal, Hôpital du Sacré-Cœur de Montréal Research Center (M.B.), Department of Kinesiology (M.B., J.D., D.C.), Division of Neonatology, Department of Pediatrics, Sainte-Justine University Hospital (K.P., A.M.N.), Division of Cardiology, Department of Pediatrics, Sainte-Justine University Hospital (J.L.B.), and Division of General Pediatrics, Department of Pediatrics, Sainte-Justine University Hospital and Research Center (T.M.L.), Université de Montréal, Quebec, Canada; Division of Cardiology, Department of Medicine, CHUM Research Center, Montréal, Canada (A.R.); Department of Pediatrics, Ottawa Hospital Research Institute, University of Ottawa, Ontario, Canada (B.T.).

Accompanying Tables S1 and S2 are available at <http://jaha.ahajournals.org/content/7/14/e009720/DC1/embed/inline-supplementary-material-1.pdf>

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**Correspondence to:** Anne Monique Nuyt, MD, Division of Neonatology, Department of Pediatrics, Sainte-Justine University Hospital and Research Center, 3175, Chemin de la Côte-Sainte-Catherine, H3T 1C5, Montréal, Québec, Canada. E-mail: [anne-monique.nuyt@recherche-ste-justine.qc.ca](mailto:anne-monique.nuyt@recherche-ste-justine.qc.ca)

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## Clinical Perspective

### What Is New?

- This study is the first to examine endothelial colony-forming cells (ECFC) in preterm-born adults and to demonstrate ECFC dysfunction in preterm-born subjects compared with full-term adults.

### What Are the Clinical Implications?

- This study demonstrates that preterm-born adults have dysfunctional ECFC compared with full-term controls.
- ECFC dysfunction in preterm-born subjects was linked to elevated systolic blood pressure and major prematurity-related morbidity as newborns such as bronchopulmonary dysplasia.
- The first generations of adults born very preterm are reaching an age at which the incidence of cardiovascular diseases rises; it is therefore important to determine whether ECFC can serve as a biomarker for cardiovascular risk and to monitor novel therapies, including for vascular repair.

microvasculature rarefaction,<sup>3,4</sup> increased vascular resistance,<sup>5</sup> as well as changes in heart shape and function,<sup>6–8</sup> which are independent harbingers of hypertension and cardiovascular disease.<sup>9,10</sup> Given that rates of preterm birth have increased along with improved survival, it is expected that the absolute number of adults born preterm, and therefore at risk of developing cardiovascular disease, will escalate in the future. Understanding the mechanisms underlying long-term cardiovascular changes after preterm birth is crucial to allow for intervention before, or early in the process of, overt cardiovascular disease with therapies targeted to their specific physiology.

Endothelial progenitor cells (EPC) are key to cardiovascular development and integrity.<sup>11</sup> These bone marrow-derived cells proliferate and differentiate into mature endothelial cells that contribute to blood vessel formation and growth.<sup>11</sup> They also play an important role during vascular repair and regeneration by homing to sites of tissue injury and activating the migration and proliferation of in situ endothelial cells.<sup>12,13</sup> These processes are critical during both organogenesis and postnatal growth and development. However, preterm birth is associated with perinatal conditions that are deleterious for organ development, and often inherently prooxidative (including the major rise in  $P_{O_2}$  occurring during the transition from fetal to extrauterine life) and proinflammatory in nature.<sup>14,15</sup> Importantly, studies have shown that EPC of preterm infants, more specifically the subpopulation of endothelial colony-forming cells (ECFC), have an increased vulnerability to hyperoxia-induced oxidative stress resulting in cell

dysfunction.<sup>16,17</sup> ECFC, although highly present in the cord blood of newborn infants, are only found in very low quantities in the peripheral circulation of adults.<sup>12,18,19</sup> When primary ECFC are successfully expanded from cord or peripheral blood mononuclear cells (PBMC), they form cobblestone-patterned colonies that are visible after 7 to 25 days in culture; accordingly ECFC are also named late-outgrowth EPC.<sup>12,13</sup> These cells are capable of self-renewal, high proliferation rates, as well as in vitro and in vivo angiogenesis.<sup>13,19</sup> To our knowledge, no study has examined ECFC from preterm-born subjects beyond infancy.

We therefore hypothesized that very preterm birth ( $\leq 29$  weeks of gestation) is associated with peripheral blood ECFC dysfunction in early adulthood. This study specifically aimed to (1) compare ECFC function of young adults born very preterm with full-term controls; (2) examine the relationship between ECFC function and adult cardiovascular measures; and (3) examine ECFC function in the preterm group in relation to perinatal factors.

## Methods

The data, analytic methods, and study materials will be made available to other researchers for purposes of reproducing the results or replicating the procedure after other ongoing analyses of this cohort will be completed.

## Study Design and Population

This cross-sectional observational study enrolled 55 young adults aged 18 to 29 years that were born preterm at  $\leq 29$  gestational weeks between 1987 and 1997, and 55 controls born at term (37–42 gestational weeks) matched for age and sex ( $23 \pm 2$  years, 38% male in both groups). Former preterm infants admitted to the neonatal intensive care unit of Sainte-Justine University Hospital (Centre Hospitalier Universitaire Sainte-Justine, Montreal, Quebec, Canada) were traced from lists of patients from the neonatal follow-up clinic, and 42 participants were recruited. An additional 13 preterm participants were recruited from advertisement targeted to patients born prematurely at any neonatal intensive care units in the province of Quebec. Gestational age was calculated from the date of last menstrual period or by obstetric ultrasound at  $< 24$  weeks. We excluded participants currently pregnant or with severe neurosensory deficit preventing test completion. Compared with all eligible preterms born at Centre Hospitalier Universitaire Sainte-Justine, the participants of the current study were similar in gestational age, birth weight, sex, incidence of small for gestational age, bronchopulmonary dysplasia (BPD), severe brain injury, and retinopathy of prematurity. This study was approved by the institutional human research ethics review board (Comité d'Éthique de la

Recherche du Centre Hospitalier Universitaire Sainte-Justine) and informed written consent was obtained from all participants.

### Perinatal Data

Perinatal data were collected from medical charts for all preterm-born participants. BPD, the most frequent major neonatal morbidity associated with very preterm birth, was defined as supplemental oxygen requirements at 36 weeks of postmenstrual age.<sup>20</sup> For full-term controls, we used medical charts, their immunization booklet (which contains birth information), or maternal recall in cases where no records were available. Participants completed questionnaires on lifestyle habits including smoking and medical history.

### Blood Sampling, Cell Isolation, and Culture

ECFC were isolated from venous peripheral blood following a protocol adapted from Martin-Ramirez et al.<sup>21</sup> A total volume of 24 mL was collected per individual, and PBMC were separated using Ficoll-Paque PLUS (GE Healthcare Life Sciences, Pittsburgh, PA) density gradient following 30 minutes 300g centrifugation at room temperature. PBMC were further washed twice with Dulbecco's phosphate-buffered saline (Gibco by Life Technologies, Burlington, ON, Canada) and subsequently plated on collagen I-coated (Corning, Corning, NY) 25 cm<sup>2</sup> tissue culture-treated Falcon flasks (Thermo Fisher Scientific, Waltham, MA) at a density of  $5.0 \times 10^6$  cells/flask. Cultured cells were maintained at 37°C, 21% O<sub>2</sub> and 5% CO<sub>2</sub>, using complete endothelial cell growth medium-2 (Lonza, Basel, Switzerland) supplemented with 1% penicillin/streptomycin (Gibco by Life Technologies) and 10% fetal bovine serum. Media was changed every 2 or 3 days, and cells were maintained for up to 30 days in culture. PBMC cultures were observed daily from days 7 to 30 to determine the first day of cobblestone-patterned ECFC colony formation. ECFC colonies were then passaged and further expanded under similar conditions. ECFC function was assessed using cells from the second passage.

### ECFC Phenotype and Functional Assessments

ECFC phenotype was examined in 3 samples to confirm ECFC clonogenic potential and the expression of classic ECFC surface markers including CD31 and CD34 (Figure). Paraformaldehyde-fixed cells were co-immunostained with anti-CD31 (Abcam, ab76533), anti-CD34 (Abcam, ab185732), and 4',6-diamidino-2-phenylindole (DAPI) to mark cell nuclei, and imaged using a fluorescence microscope (Leica), as shown in Figure A. In addition, ECFC clonogenic capacity was tested by diluting  $10^4$  ECFC to obtain 5 to 10 individual cells

per 1 mL of endothelial cell growth medium-2. Cells were plated in collagen-coated (Corning) 24-wells plates (Thermo Fisher Scientific) and observed using a phase contrast microscope (Leica) after 6, 48, and 96 hours for ECFC colony growth.

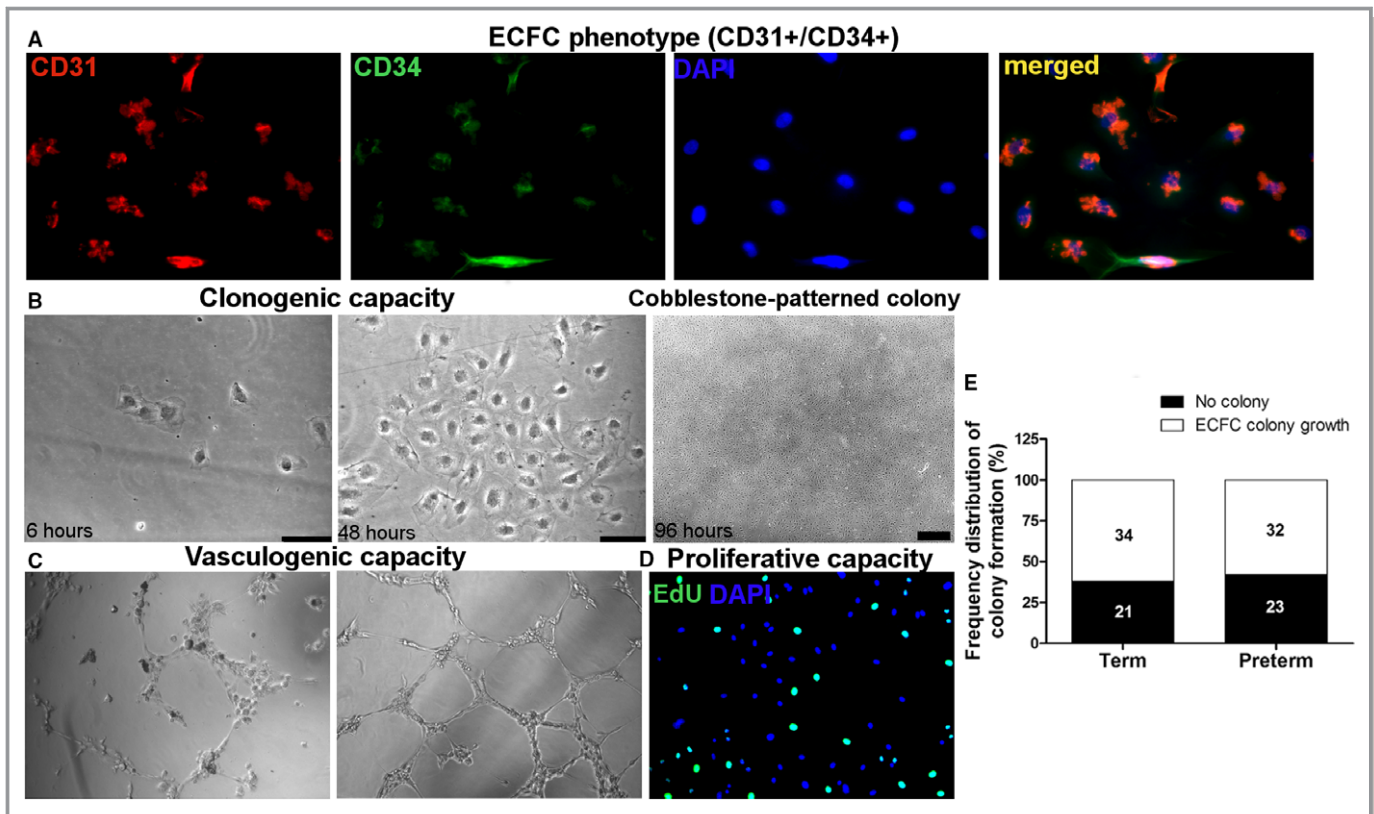
In all subjects for whom ECFC colonies grew, ECFC function was determined by cellular proliferation rate, as well as by the in vitro capacity to form branches or closed tubes on Matrigel (Corning), which are hallmarks of in vitro vascular cord formation and vasculogenic capacity.

Proliferation rate was assessed through the quantification of 5-ethynyl-2'-deoxyuridine (EdU) cellular incorporation using the Click-iT EdU Alexa Fluor 488 Imaging kit (Thermo Fisher Scientific). ECFCs ( $4.0 \times 10^4$ ) were plated on collagen-coated flasks of 1.86 cm<sup>2</sup> surface area, maintained with complete endothelial cell growth medium-2 and grown for 24 hours. Cells were then incubated with 10 μmol/L EdU in complete endothelial cell growth medium-2 (Lonza) for 4.5 hours at 37°C, then fixed with 3.7% formaldehyde in PBS, permeabilized with 0.5% Triton X-100 in PBS, and stained according to kit instructions. Assays were performed in triplicates and cell images were obtained using a fluorescence microscope (Leica) and a 10× objective. The number of cells under proliferation and incorporating EdU into their DNA, stained positive for EdU (AlexaFluor 488), were counted in 3 to 5 full pictures per assay, as well as the total number of cells with nuclei stained with DAPI; the percentage of proliferating cells (a measure of proliferation rate) was then calculated.

In vitro vascular cord formation was assessed by plating  $1.5 \times 10^4$  cells on 50 μL of growth factor reduced basement membrane matrix (Matrigel) in a 96-well plate. Cells were imaged using a phase contrast microscope (Leica) and 10× objective after 6 hours of incubation at 37°C. Assays were performed in triplicates, and the number of closed tubes and branches formed (not necessarily forming closed tubes) were quantified in 3 to 6 random images per participant using ImageJ software (National Institutes of Health). All ECFC functional assessments were performed blinded to the participants' status.

### Clinical Assessment of Cardiovascular Risk Factors

Anthropometric measures included weight, height, and calculation of body mass index. Blood glucose levels were measured after 12-hour fasting, and again at 2 hours following a 75-g oral glucose tolerance test. Peak aerobic exercise capacity (peak VO<sub>2</sub>) was assessed in 43 subjects per group on a cycle ergometer (Corrival, Lode, Netherlands) using a ramped exercise protocol at room air with patients breathing through a facemask connected to a gas analysis system (Jaeger CareFusion, Yorba Linda, CA). Peak VO<sub>2</sub> was



**Figure.** Endothelial colony-forming cell (ECFC) phenotype, functional characteristics, and frequency distribution of time until ECFC colony formation in full-term and preterm groups. A, ECFC phenotype shown by cells expressing CD31 (AlexaFluor 555, red), CD34 (AlexaFluor 488, green), DAPI (blue), and all merged. B, Representative images of ECFC clonogenic capacity and cobblestone-patterned colony formation after 6 and 48 hours, and later cobblestone-patterned colony formation while in culture; phase contrast imaging using 20× (50- $\mu$ m scale) and 5× objectives. C, ECFC vasculogenic capacity demonstrated by branches and tube formation on Matrigel, phase contrast imaging using 10× objective. D, ECFC proliferation by DNA EdU incorporation (AlexaFluor 488, green) and nonproliferative ECFCs marked with DAPI (blue), 10× objective. E, Frequency distribution of ECFC colony growth grouped as ECFC colony growth (from 7 to 30 days after plating PBMC) and no colony growth observed, in full-term controls and preterm-born subjects. The number of subjects in each category is shown in the histogram bars. DAPI indicates 4',6-diamidino-2-phenylindole; EdU, 5-ethynyl-2'-deoxyuridine; PBMC, peripheral blood mononuclear cells.

indexed to lean body mass determined by dual-energy X-ray absorptiometry (GE Healthcare, Chicago, IL).

### Blood Pressure Measurements

Brachial blood pressure was measured with subjects in a sitting position using an automated oscillometric device (DINAMAP model DPC300M-CF; GE Medical Systems Information Technologies Inc., Milwaukee, WI) in both arms and the average value was recorded. Participants also received an appropriately sized (nondominant arm) 24-hour ambulatory blood pressure measurement (ABPM) device (model 902007; Spacelabs Medical Inc., Redmond, WA) that recorded blood pressure every 15 minutes for a 24-hour period. For this analysis we only considered awake (daytime) blood pressure recordings since many participants removed the device, especially to sleep. Thirty-six preterm and 33 full-term had daytime ABPM recordings available for analyses. There was no difference in the clinical characteristics of study participants

born full-term and preterm with and without daytime ABPM (Table S1).

### Echocardiography

Echocardiographic imaging was performed by trained technicians blinded to prematurity status using a GE Vivid E9 ultrasound system, and the images were analyzed offline to measure left ventricular mass index and ejection fraction<sup>22,23</sup> with EchoPac PC software (both GE Healthcare, Horten, Norway). The mean of triplicate measurements were utilized for analysis.

### Statistical Analyses

Baseline characteristics of the term and preterm groups were compared using independent t test for continuous variables and a  $\chi^2$  test or Fisher exact tests for categorical variables. Between-group comparisons for ECFC functional data were

**Table 1.** Perinatal and Adult Characteristics of Young Adults Born Full-Term and Preterm

Clinical Characteristics	Term (n=55)	Preterm (n=55)	P Value
<b>Perinatal characteristics</b>			
Gestational age, wks	39.4±1.3	27.2±1.3	
Birth weight, g	3384±385	993±226	
Small for gestational age, n (%) <sup>*</sup>	3 (6)	2 (4)	0.647
Hypertensive complications of pregnancy, n (%)	3 (6)	8 (15)	0.112
Antenatal corticosteroids, n/N (%)	N/A	21/52 (40)	
Bronchopulmonary dysplasia, n/N (%) <sup>†</sup>	N/A	15/54 (28)	
<b>Adult characteristics</b>			
Age, y	23±2	23±2	0.389
Male sex, n (%)	21 (38)	21 (38)	1.00
Height, cm	169±8	166±9	0.059
Weight, kg	69±15	61±12	0.001
BMI, kg/m <sup>2</sup>	24±4	22±3	0.006
Peak VO <sub>2</sub> , mL/min per kg of lean body mass <sup>‡</sup>	33±9	32±8	0.660
Smoking, n (%)	9 (16)	12 (22)	0.471
2-h glucose, mmol/L	6.3±1.3	7.0±1.4	0.006
Resting SBP, mm Hg	115±10	118±10	0.199
Resting DBP, mm Hg	66±8	68±7	0.104
Daytime ABPM SBP, mm Hg <sup>§</sup>	120±7	124±10	0.049
Daytime ABPM DBP, mm Hg <sup>§</sup>	72±5	74±6	0.098
Left ventricular mass index, g/m <sup>2</sup>	63±13	62±13	0.509
Left ventricular ejection fraction, %	62±5	64±6	0.130

Data are mean±SD unless stated otherwise. Independent t test or  $\chi^2$  test. BMI indicates body mass index; DBP, diastolic blood pressure; N/A, not applicable; SBP, systolic blood pressure.

<sup>\*</sup>Birth weight <10th percentile for gestational age.

<sup>†</sup>Supplemental oxygen requirement at 36 wks post menstrual age.

<sup>‡</sup>VO<sub>2</sub>, peak oxygen consumption measured in a subgroup of 43 participants per group.

<sup>§</sup>ABPM (ambulatory blood pressure monitoring) determined in a subgroup of 33 term and 36 preterm subjects.

performed using independent-samples Mann–Whitney *U* test, given the lack of correlation within pairs despite study design (Cohen's kappa for ECFC growth within pairs=0.017,  $P=0.902$ ). Rho Spearman correlation analyses were conducted to assess the relationship between ECFC function and cardiovascular parameters with 95% confidence intervals estimated by Bootstrap analysis. Finally, independent-samples Mann–Whitney *U* test was performed to examine ECFC function among participants born preterm in relation to perinatal characteristics. All analyses were conducted using SPSS 21 (IBM, North Castle, NY).  $P<0.05$  were considered statistically significant.

## Results

### Population Description

Perinatal characteristics, anthropometric measures, and cardiovascular risk factors for the preterm- and full-term-born

participants are presented in Table 1. Daytime ABPM systolic blood pressures and 2-hour glycemia were significantly higher, whereas body mass index was significantly lower in the preterm group.

### In Vitro ECFC Characteristics in Young Adults Born Preterm Versus Full Term

Peripheral blood ECFC phenotype and functional characteristics, including in vitro clonogenic, vasculogenic, and proliferative capacities, are illustrated in Figure. Cobblestone-patterned ECFC colonies grew in 34/55 (62%) of full-term- and 32/55 (58%) of preterm-born participants' PBMC after a maximum of 30 days in culture (Figure E).

Perinatal and adult characteristics from individuals for whom ECFC colonies grew are shown in Table S2.

Table 2 shows comparisons of all ECFC functional parameters between term and preterm groups. The time for ECFC growth in culture before the first colony was

**Table 2.** ECFC Characteristics in Young Adults Born Full-Term Versus Preterm

ECFC Characteristics	Term (N)		Preterm (N)		P Value
Time to first ECFC colony formation, days, median (IQR)	34	12 (11–16)	32	15 (12–19)	0.028*
Proliferation rate, % of total cells, median (IQR)	31	27 (17–35)	31	20 (15–27)	0.042*
Closed tubes formed, n per $1.5 \times 10^4$ ECFC, median (IQR)	31	8 (1–15)	31	5 (1–10)	0.126
Branches formed, n per $1.5 \times 10^4$ ECFC, median (IQR)	31	32 (16–38)	31	25 (14–35)	0.310

Data assessed by independent samples Mann–Whitney *U* test. ECFC indicates endothelial colony-forming cells; IQR, interquartile range.

\* $P < 0.05$ .

observed was significantly longer in the preterm subjects, and the proliferation rate was significantly reduced in young adults born preterm compared with term controls (Table 2). However, significant between-group differences in number of closed tubes and number of branches formed were not identified.

In the preterm group, we observed a correlation between the number of days to form the first ECFC colony and ECFC proliferative and vascular cord formation capacity (Table 3). The longer the onset of first ECFC colony formation, the lower the proliferation rate and the number of closed tube and branch formation were, which was not changed when stratifying samples by sex. This relationship was not observed in the term group (Table 3).

### Relationship Between ECFC Function and Cardiovascular Risk Factors in Young Adults Born Preterm Versus Full-Term

We further investigated whether ECFC characteristics correlated with adult cardiovascular risk factors known to be associated with very preterm birth, in particular, blood pressure<sup>2,24</sup> and left ventricular mass index.<sup>6</sup> Table 4 shows the relationship between ECFC function and daytime ABPM systolic blood pressure and left ventricular mass index in preterm and term groups. In preterm participants, there was an inverse association between the proliferation rate and the number of branches formed on matrigel with systolic blood

pressure values. In the full-term participants, no significant correlation was detected between ECFC functional characteristics and systolic blood pressure.

ECFC functional characteristics did not correlate with left ventricular mass index in the preterm group (Table 4). However, only in term-born participants, enhanced ECFC proliferation rate was associated with higher left ventricular mass index. After additional analyses performed a posteriori to explore whether the result could be confounded by ejection fraction and maximum oxygen consumption ( $VO_2$ ), as proxy measures of physical fitness, this relationship was no longer significant ( $r_s = 0.325$ ,  $P = 0.140$ ).

### Relationship Between ECFC Function and Perinatal Factors in Young Adults Born Preterm

Colonies grew in 6/15 (40%) preterm individuals with BPD versus 25/39 (64%) individuals without, which was not statistically significantly different. Similarly, colonies grew in 13/21 (62%) preterm-born participants exposed to antenatal steroids versus 17/31 (55%) individuals without, also not significantly different.

Young adults with BPD had overall impaired ECFC function as shown by a lower ECFC proliferation rate, lower number of closed tubes and of branches formed (Table 5). Moreover, exposure to antenatal steroids was associated with a shorter time to grow the first ECFC colony, and an increase in the number of branches formed (Table 5).

**Table 3.** Association Between the Number of Days to First ECFC Colony Growth and ECFC Proliferative and Vasculogenic Capacity in Young Adults Born Full-Term and Preterm

	Rho Spearman Correlations (95% Confidence Interval)		
	Proliferation Rate*	Closed Tubes Formed <sup>†</sup>	Branches Formed <sup>†</sup>
Days to grow the first ECFC colony			
Term	−0.24 (−0.55, 0.13)	−0.24 (−0.60, 0.18)	−0.19 (−0.53, 0.19)
Preterm	−0.53 (−0.81, −0.17) <sup>‡</sup>	−0.45 (−0.80, −0.06) <sup>‡</sup>	−0.40 (−0.71, −0.05) <sup>‡</sup>

ECFC indicates endothelial colony-forming cells.

\*% of total cells.

<sup>†</sup>n per  $1.5 \times 10^4$  ECFC. Rho Spearman correlations.

<sup>‡</sup> $P < 0.05$ .

**Table 4.** Correlational Analyses of ECFC Characteristics and Cardiovascular Risk Factors in Young Adults Born Full-Term and Preterm

ECFC Characteristics	Rho Spearman Correlation Coefficients (95% Confidence Interval)			
	Daytime ABPM SBP, mm Hg		LV Mass Index, g/m <sup>2</sup>	
Term	n		n	
Days to first ECFC colony	20	−0.25 (−0.68, 0.18)	30	−0.34 (−0.66, 0.07)
Proliferation rate*	20	0.30 (−0.25, 0.71)	30	0.41 (0.05, 0.67) <sup>†,‡</sup>
Closed tubes formed <sup>§</sup>	20	0.19 (−0.29, 0.57)	30	0.11 (−0.18, 0.39)
Branches formed <sup>§</sup>	20	0.24 (−0.18, 0.59)	30	0.26 (0.01, 0.49)
Preterm				
Days to first ECFC colony	22	0.248 (−0.219, 0.622)	30	0.198 (−0.165, 0.536)
Proliferation rate*	22	−0.46 (−0.76, −0.04) <sup>†</sup>	30	−0.202 (−0.583, 0.193)
Closed tubes formed <sup>§</sup>	22	−0.28 (−0.65, 0.25)	30	−0.05 (−0.42, 0.30)
Branches formed <sup>§</sup>	22	−0.44 (−0.77, 0.04) <sup>†</sup>	30	−0.16 (−0.49, 0.22)

ABPM indicates ambulatory blood pressure monitoring; ECFC, endothelial colony-forming cells; LV, left ventricular; SBP, systolic blood pressure.

\*% of total cells.

<sup>†</sup> $P < 0.05$ .

<sup>§</sup>n per  $1.5 \times 10^4$  ECFC. Rho Spearman correlations.

## Discussion

This study showed that ECFC from young adults born very preterm were less proliferative and grew colonies significantly more slowly compared with controls born full term. More specifically, in preterm subjects only, the later the time to grow an ECFC colony at first isolation step, the worse the overall ECFC function. Additionally, in preterm, but not in term subjects, impaired ECFC proliferation and vasculogenic capacity, indicated by reduction of the numbers of branches formed on matrigel, were associated with higher systolic blood pressure into adulthood. This study also finds that young adults born preterm who had BPD, the most common

major complication of preterm birth characterized by impaired/interrupted pulmonary tissue and vascular development,<sup>25,26</sup> displayed poorer ECFC function. Conversely, exposure to antenatal steroids, which are administered to enhance fetal lung maturation and are associated with reduced neonatal morbidity and mortality,<sup>27</sup> was associated with faster ECFC growth and enhanced in vitro vasculogenic capacity in early adulthood.

Reduced EPC counts and/or impaired ECFC function have been proposed as markers of cardiovascular and metabolic disease risks.<sup>28,29</sup> Studies have reported an inverse correlation between the number of other types of EPC such as the colony-forming units (or myeloid angiogenic cells) or overall EPC counts

**Table 5.** ECFC Function Among Participants Born Preterm in Relation to Perinatal Factors

Neonatal Characteristics	ECFC Function, Median (IQR)				
	N	Days to First ECFC Colony	Proliferation Rate*	Closed Tubes Formed <sup>†</sup>	Branches Formed <sup>†</sup>
Male	13	17.0 (12.0; 19.5)	16.5 (9.2; 26.0)	2.8 (0.1; 6.5)	22.0 (8.5; 30.5)
Female	18	14.5 (11.8; 16.8)	22.5 (16.3; 27.4)	5.4 (3.0; 15.0)	27.3 (19.1; 35.5)
Antenatal steroids	12	12.5 (10.2; 15.5)	23.8 (16.1; 32.8)	6.4 (3.6; 15.7)	33.8 (23.8; 37.0)
No antenatal steroids	17	16.0 (13.0; 19.5) <sup>§</sup>	18.0 (9.2; 24.6)	4.4 (0.1; 9.0)	20.7 (5.3; 28.2) <sup>§</sup>
BPD	6	19.5 (13.5; 21.2)	12.0 (0.0; 16.8)	2.0 (0.0; 4.7) <sup>§</sup>	12.8 (2.4; 25.5) <sup>§</sup>
No BPD	24	14.0 (11.2; 16.8)	22.5 (16.1; 28.0) <sup>  </sup>	5.2 (2.2; 13.7) <sup>  </sup>	27.3 (19.6; 35.7) <sup>  </sup>

Data assessed by independent samples Mann–Whitney *U* test. BPD indicates bronchopulmonary dysplasia; ECFC, endothelial colony-forming cells; IQR, interquartile range.

\*% of total cells.

<sup>†</sup>n per  $1.5 \times 10^4$  ECFC.

<sup>§</sup> $P < 0.05$  vs antenatal steroids group and <sup>||</sup> $P < 0.05$  vs BPD group.

(circulating CD34+KDR+ cells), and hypertension, diabetes mellitus, combined Framingham risk factor score, as well as cardiovascular events and death.<sup>30–32</sup> A significant relationship between colony-forming units and EPC counts (CD133(+) or CD34(+)/KDR(+)) and function, and vascular endothelial function was also reported in patients with cardiovascular diseases.<sup>30,31,33</sup> The literature presents different EPC subtypes classified according to specific cellular characteristics and function. The myeloid-like colony-forming units type of EPC, also called early outgrowth EPC, can form colonies within 4 days after PBMC culture and last no longer than 7 to 9 days in culture.<sup>12,13,34</sup> These cells and colonies cannot be expanded and have no specific growing pattern, which also clearly differentiate them from the ECFC subtype.<sup>12,13</sup> ECFC, also called late outgrowth EPC, can form well-defined cobblestone-patterned colonies from days 7 to 30 in culture.<sup>13,19</sup> Unlike hematopoietic progenitor cells, ECFC do not express CD133 and CD45.<sup>19,35</sup> However, the presence of CD34 and CD31 markers added to their clonogenic and vasculogenic potential confers to these cells a progenitor and endothelial phenotype.<sup>19</sup> They additionally present remarkable self-renewable and expansion capacities *in vitro*, which also confer to this subtype of EPC higher angiogenic and regenerative potentials.<sup>12,13,36</sup>

Prematurity is now a well-recognized risk factor for hypertension and cardiovascular disease.<sup>2,37,38</sup> The differences we observed in blood pressure values between preterm and term participants are consistent with reported data.<sup>2,39</sup> To our knowledge, no previous study has examined any sort of EPC in adults born preterm. Our findings show that the *in vitro* angiogenic function of the ECFC subtype was impaired in preterm versus full-term subjects, suggesting a long-term impact of preterm birth on this cell function. During fetal and neonatal development, ECFC actively participate in vasculogenesis and angiogenesis.<sup>12,13,18</sup> The function of circulating ECFC into adulthood is still not fully understood in humans. Experimental studies have indicated that circulating or tissue resident ECFC may contribute to maintaining the vascular tree or to actively form new local microvessels postnatally or during regenerative processes.<sup>36,40,41</sup> These findings suggest that ECFC may play a role in the maintenance of microvasculature and potentially stimulate angiogenesis postnatally. Interestingly, microvasculature rarefaction, considered as an important element in the pathogenesis of increased peripheral resistance and hypertension, was previously reported in young adults born prematurely.<sup>3,4</sup> This phenomenon was more marked among those with higher blood pressure and an enhanced antiangiogenic state. Additionally, in a ventilatory assessment study, Lovering et al<sup>42</sup> made the interesting observation that leg fatigue significantly contributed to the limited exercise capacity of young adults born preterm. While leg discomfort was unexplained in Lovering's study, reduced microvascular growth was postulated,<sup>43</sup> considering that skeletal muscle capillary

rarefaction is present in rats exposed to neonatal hyperoxia.<sup>44</sup> Microvascular density was not assessed in our study.

In the current study, we also found that ECFC dysfunction was related to higher blood pressure in preterm subjects. The relationship between hypertension and EPC in general, and ECFC in particular, was previously reported, although results are still controversial in showing a positive correlation between blood pressure and ECFC dysfunction.<sup>45</sup> In addition, the mechanisms linking hypertension and ECFC dysfunction are still unknown. Beyond the hypothesis of a cause/effect relationship between ECFC dysfunction and elevated blood pressure, ECFC dysfunction and hypertension may result from common physiopathological pathways, such as the activation of the autonomic nervous system, renin–angiotensin system, and inflammation,<sup>45</sup> which in turn were all previously shown to be altered in preterm-born subjects or in experimental models of preterm birth.<sup>46–53</sup> These observations suggest that both ECFC dysfunction and elevated blood pressure could be long-term consequences of very preterm birth. Whether ECFC dysfunction is a cause or a consequence, or alternatively a co-occurrence of high blood pressure remains to be determined.

Furthermore, the adverse and premature exposure of preterm neonates to the extrauterine environment, resultant morbidities, and/or an intrinsic susceptibility to those neonatal exposures are all possible mechanistic avenues that can contribute to the observed ECFC dysfunction in adulthood. In the cord blood of preterm infants, EPC counts have been shown to be either similar to or increased versus full-term infants, but studies of ECFC subtype proliferation and vessel formation have yielded contradictory results.<sup>54</sup> Cord blood ECFC from preterm infants were shown to be particularly vulnerable to oxidant conditions (such as hyperoxia) with drastically impaired growth potential, disrupted nitric oxide/vascular endothelial growth factor signaling, accelerated cell senescence, as well as increased oxidative stress status.<sup>16,17,55</sup> In addition, human fetal lung ECFC and newborn rat ECFC function are also impaired after high oxygen exposure (mimicking preterm birth conditions).<sup>36</sup> EPC dysfunction in preterm cord blood, particularly the ECFC, has been associated with the development of BPD.<sup>56,57</sup> BPD is characterized by disrupted alveolar and lung vasculogenesis, and is considered an early manifestation of an impaired angiogenic capacity in preterm newborns.<sup>26</sup> Adult mice exposed to neonatal hyperoxia show reduced bone marrow–derived proangiogenic cell numbers and vasculogenic function, and reduced postischemic muscle neovascularization.<sup>58</sup> Interestingly, our findings show that individuals born preterm and who developed BPD are also those with more significant alterations in ECFC function into adulthood. On the other hand, exposure to antenatal steroids in preterm-born subjects had a positive impact on ECFC function into adulthood. This positive relationship can in turn reflect a beneficial effect of accelerated intrauterine maturation of preterm babies



stimulated by steroidal treatment or to the prevention of more severe neonatal complications in newborns exposed to this treatment.<sup>27</sup> Alternatively, one can also postulate that epigenetic changes could result from perinatal stressors associated with very preterm birth and may contribute to the establishment of ECFC dysfunction. Key pro-angiogenic pathways including vascular endothelial growth factor receptors and Notch expression in ECFC are susceptible to epigenetic histone modifications, which were shown to remarkably blunt in vitro ECFC vasculogenic capacity.<sup>59,60</sup> Together, our current study and others suggest that the function of adult EPCs, particularly ECFCs, may be determined by perinatal factors; however, the mechanisms by which such ECFC dysfunction can persist beyond birth and into adulthood remain to be studied.

Importantly, treatment with ECFC from cord blood of human full-term infants promoted lung vascular growth and reversed experimental BPD in rodents.<sup>36</sup> Thus, as cell therapy and regenerative medicine studies are being designed for prematurely born neonates and for adults with cardiovascular diseases, it is important to determine the mechanisms of inherent or programmed impaired function of ECFC for preterm-born subjects in order to determine the potential cellular role during cardiovascular disease development or in vascular regenerative therapies.

The main strengths of this study are the relatively large sample size, the homogeneity of the study groups, including the narrow gestational age in the preterm group, detailed functional characterization of proliferative and angiogenic cellular properties, and the extensive perinatal and adult clinical data. However, the evaluation of the impact of individual perinatal factors such as being small for gestational age and pregnancy-induced hypertension, which have been previously linked to both adult cardiovascular health and EPCs, was limited because of the small number of preterm individuals with these conditions and the numbers with ECFC growth. Our incidence of ECFC colony growth was  $\approx 60\%$ , which is consistent with values reported.<sup>61</sup> In addition, classical phenotypic characteristics of ECFC including clonogenic potential and formation of cobblestone-patterned colonies, as well as in vitro vascular cord formation capacity and expression of CD31 and CD34, were observed and confirm the progenitor and endothelial nature of ECFC isolated from peripheral blood. Finally, only a subgroup of our participants completed the 24-hour ABPM assessment; however, this noncompletion was not related to ECFC characteristics, therefore limiting the risk of selection bias.

## Perspectives

We have shown that adults born very preterm have impaired ECFC compared with term controls. Furthermore, in preterm

adults, ECFC dysfunction was associated with elevated systolic blood pressure beyond classical cardiovascular risk factors known to be associated with preterm birth. ECFC dysfunction was more pronounced in preterm adults who had BPD as neonates and was relatively prevented in preterm subjects exposed to antenatal steroids. The pathways linking deleterious conditions associated with preterm birth and impaired ECFC function in adults and whether ECFC dysfunction contributes to vascular alterations in preterm-born subjects, particularly in those with severe neonatal morbidities, remain to be explored. It also remains to be established whether ECFC function could be considered as a potential biomarker to identify among young adults born very preterm those carrying higher risk to develop cardiovascular diseases including hypertension, stroke, and heart failure. This is important to determine because the first generations of young adults born very preterm are now reaching an age at which the incidence of cardiovascular diseases rises, and there is a definite need to accelerate the search for vascular repair therapies.

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## Disclosures

None.

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# **SUPPLEMENTAL MATERIAL**

**Table S1. Clinical characteristics of study participants born full-term and preterm with and without day-time (awake) ambulatory blood pressure monitoring (ABPM).**

Clinical Characteristics	Term		Preterm		P		
	ABPM (n=33)	No ABPM (n=22)	ABPM (n=36)	No ABPM (n=19)	ABPM	Term	Interaction
Gestational age, weeks	39 ± 1	40 ± 1	27 ± 1	27 ± 2	.903	<.0001	.357
Birth weight, g	3346 ± 339	3546 ± 448	995 ± 216	988 ± 251	.134	<.0001	.107
Age, years	23 ± 2	23 ± 2	24 ± 2	23 ± 3	.059	.564	.534
Male sex, %	33%	46%	33%	47%	.180	.922	.922
Height, cm	169 ± 8	171 ± 7	167 ± 9	166 ± 10	.628	.045	.382
Weight, Kg	67 ± 15	72 ± 13	61 ± 12	59 ± 12	.545	.001	.145
BMI, Kg/m <sup>2</sup>	23 ± 4	25 ± 5	22 ± 3	21 ± 4	.596	.003	.183
Peak VO <sub>2</sub> , ml/min/Kg	35 ± 9	31 ± 9	33 ± 9	31 ± 6	.140	.686	.629
Smoking, %	15%	18%	19%	26%	.531	.432	.808
2-hours glycemia, mmol/L	6.3 ± 1.3	6.3 ± 1.2	7.0 ± 1.5	7.2 ± 1.2	.532	.005	.730
Resting SBP, mm Hg	114 ± 9	117 ± 10	119 ± 11	116 ± 7	.894	.384	.190
Resting DBP, mm Hg	65 ± 7	67 ± 9	69 ± 7	67 ± 6	.868	.496	.829
Left ventricular mass index, g/m <sup>2</sup>	63 ± 15	64 ± 12	62 ± 11	62 ± 17	.867	.496	.829
Left ventricular ejection fraction, %	63 ± 5	61 ± 5	63 ± 6	65 ± 6	.598	.040	.043
Days to 1 <sup>st</sup> colony, days	15 ± 6	14 ± 4	15 ± 4	16 ± 4	.959	.246	.538

ABPM, ambulatory blood pressure monitoring; BMI, body mass index; Peak VO<sub>2</sub>, peak oxygen consumption; SBP, systolic blood pressure; DBP, diastolic blood pressure. Within each study group (term and preterm), subjects with vs. without ABMP were compared using two-way ANOVA and Bonferroni *post-hoc* test. No statistically significant differences were noted.

**Table S2. Clinical and perinatal characteristics of study participants born full-term and preterm with ECFC colony growth.**

	Term n=34	Preterm n=32	<i>P</i>
<b>Clinical characteristics</b>			
<b>Perinatal characteristics</b>			
Gestational age, weeks,	39.6 ± 1.1	27.1 ± 1.3	<.001
Birth weight, g	3363 ± 299	978 ± 218	<.001
Small for gestational age, n (%) <sup>(†)</sup>	2 (7)	2 (6)	.392
Hypertensive complications of pregnancy, n (%)	2 (6)	5 (16)	.251
Antenatal corticosteroids, n/N (%)	N/A	13/30 (43)	
Bronchopulmonary dysplasia, n/N (%) <sup>(§)</sup>	N/A	6/31 (19)	
<b>Adult characteristics</b>			
Age, years	23 ± 2	23 ± 2	.329
Male sex, n (%)	13 (38)	13 (41)	1.00
Height, cm	169 ± 7	167 ± 10	.483
Weight, kg	68 ± 15	60 ± 12	.028
BMI, kg/m <sup>2</sup>	24 ± 4	21 ± 3	.016
Peak VO <sub>2</sub> , ml/min/kg of lean body mass <sup>(∞)</sup>	33 ± 9	33 ± 9	.862
Smoking, n (%)	4 (12)	5 (16)	.730
2-hours glucose, mmol/L	6.4 ± 1.3	7.0 ± 1.4	.093
Resting SBP, mm Hg	116 ± 11	117 ± 8	.612
Resting DBP, mm Hg	66 ± 8	67 ± 6	.401
Day-time ABPM SBP, mm Hg <sup>(¥)</sup>	119 ± 8	124 ± 10	.073
Day-time ABPM DBP, mm Hg <sup>(¥)</sup>	71 ± 5	75 ± 6	.049
Left ventricular mass index, g/m <sup>2</sup>	64 ± 12	63 ± 14	.758
Left ventricular ejection fraction, %	62 ± 5	64 ± 6	.435

<sup>(†)</sup>Birth weight < 10th percentile for gestational age; <sup>(§)</sup>supplemental oxygen requirement at 36 weeks post menstrual age; BMI, body mass index; <sup>(∞)</sup>VO<sub>2</sub>, peak oxygen consumption measured in a subgroup of 25 participants per group; SBP, systolic blood pressure; DBP, diastolic blood pressure; <sup>(¥)</sup> ABPM, ambulatory blood pressure monitoring determined in a subgroup of 23 term and 22 preterm subjects. N/A, not applicable. Data are mean ± standard deviation (SD) unless stated otherwise. Independent t-test or  $\chi^2$ -test.