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Proliferative potential and angiogenic characteristics of blood outgrowth endothelial cells derived from middle-aged and older adults

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ABSTRACT

Objectives Autologous blood outgrowth endothelial cells (BOECs) have been proposed to induce therapeutic angiogenesis for treating cardiovascular diseases (CVDs). The aim of the present study was to investigate the proliferative potential and angiogenic characteristics of BOECs among middle-aged and older adults, the population particularly susceptible to CVDs.

Methods BOECs were isolated from 48 peripheral blood samples of subjects aged 56 ± 4 years. The cells were then distinguished based on their proliferative abilities, and their phenotype, tube formation capacity, and migratory activity were compared using immunofluorescence staining, flow cytometry, tube formation assay, and wound healing assay, respectively. Correlations between demographic, clinical, and dietary parameters with the number of BOECs were also assessed.

Results A total of 132 BOEC colonies with different proliferative potentials were obtained, including colonies lost proliferative ability before passage 3 (named LPA), stopped proliferating during passage 3–8 (HPA (3–8)), and proliferated after passage 8 (HPA (> 8)). LPA cells appeared later and displayed abnormal morphology, while HPA (3–8) cells exhibited alterations in von Willebrand factor morphology and lower KDR expression. HPA (> 8) cells obtained higher branching intervals and individual cell migration velocity compared with those of HPA (3–8) cells. Correlation analysis showed that the number of both LPA and HPA colonies were positively associated with several CVD risk factors. Additionally, the number of LPA colonies was positively associated with servings of meats and alternatives, fruits, fruits and vegetables, as well as the protein intake.

Conclusions Our findings provide evidence that the middle-aged and older populations possess BOECs with different proliferative and angiogenic potentials, exhibiting distinctions in cell morphology, appearance dates, VWF morphology, and KDR expression. Strikingly, a higher number of BOECs is likely associated with an increased risk of CVDs, while the number of BOECs with low proliferative ability may be regulated by diet.

Cardiovascular diseases (CVDs) are a group of disorders affecting the heart and blood vessels, and they have been identified as the leading cause of death by the World Health Organization.[1,2] Age is a significant risk factor for CVDs as it contributes to the decline of the vascular system's function, leading to an increased incidence of CVD in aging adults.[3] Moreover, population aging is expected to continue, even accelerate, due to declining fertility rates and improved living standards.[4] Consequently, CVDs pose a significant global economic and social burden.[5] Surgeries, medicines, and non-surgical procedures like angioplasty, stenting, and vein ablation are the most commonly used treatments for CVDs.[2,6] However, novel approaches to promote neovascularization are urgently needed because not all patients are suitable for these therapeutic options. Additionally, pharmacological treatment can only be used as adjuvant therapy.[7]

Cell-based therapy has emerged as a promising strategy for vascular regeneration, attracting considerable interest.[8] Multiple cell types have been proposed to reconstruct the damaged vascular network, among

which blood outgrowth endothelial cells (BOECs) are excellent candidates.[7,9] BOECs, regarded as "true" endothelial progenitor cells (EPCs), are also called endothelial colony-forming cells, outgrowth endothelial cells, or late-outgrowth endothelial cells. BOECs represent a culture-expandable cell source and can be derived from a culture of human peripheral or cord blood mononuclear cells.[10,11] BOECs exhibit endothelial characteristics, such as cobblestone-like morphology, expression of endothelial cell markers, and the absence of hematopoietic cell-specific antigens.[7] Moreover, BOECs demonstrate high clonogenic potential, tube formation capacity, and wound healing ability *in-vitro*, and have been shown to originate blood vessels *in-vivo*.[12] Therefore, BOECs represent a highly promising candidate for angiogenesis, offering a vital therapeutic option for people suffering from CVDs.[9]

Autologous transplantation of BOECs to induce therapeutic angiogenesis has been suggested by several studies.[7] However, understanding the angiogenic capacity of BOECs in aging adults is crucial for improving the efficacy of autologous therapy. Proliferative ability is a hallmark of BOECs, and studies have reported that blood can give rise to BOECs with different proliferative potentials.[13] Ingram, *et al.* [11] isolated BOECs from adult (age 20–50 years) peripheral and umbilical cord blood and found that BOECs display a hierarchy of proliferative potentials, and different types of BOEC-derived colonies can be distinguished based on their proliferative and clonogenic potential. Minami, *et al.* [14] classified BOECs derived from peripheral blood of healthy volunteers based on the time of their emergence in culture and reported that BOECs with different emergence times exhibited varied proliferative and angiogenic potential. However, the angiogenic capacity of BOECs in aging adults has not been well studied. To address this gap, we isolated BOECs from middle-aged and older adults, distinguishing them based on their proliferative abilities. We then examined the phenotypic, tube-formation, and migration characteristics of BOECs. Additionally, we collected demographic, CVD-related clinical, and dietary parameters of subjects and analyzed their correlations with the number of BOECs exhibiting different proliferative abilities. Our results provide more detailed information about the number, proliferation, and function of BOECs from middle-aged and older populations. This could serve as a theoretical foundation for the autologous transplantation of BOECs in this demographic.

METHODS

Demographic, Clinical, and Dietary Characteristics of Subjects

Subjects aged 50–75 years with no acute illness were recruited from a Singapore community, and their demographic, CVD-related clinical, and dietary characteristics were collected (as shown in Table 1). The study was conducted ethically following the Declaration of Helsinki, with written informed consent obtained from all subjects. The study protocol was approved by the National Healthcare Group's Domain Specific Review Board (NHG DSRB; Protocol 2018/0311).

After an overnight fast, participants' weight and height were measured using a Seca stadiometer (Hamburg, Germany) to calculate body mass index, which was calculated as weight divided by the height squared. Waist circumference was measured with a flexible measuring tape according to the World Health Organization guidelines. Blood pressure was measured using a BP7100 blood pressure monitor (Omron Healthcare Inc., Illinois, USA). Blood samples were collected by a trained phlebotomist via intravenous cannulation, and the serum lipid-lipoprotein concentrations (triglyceride, total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C)) were analyzed by Quest Laboratories Pte., Ltd. (Singapore). Plasma von Willebrand factor (VWF), angiopoietin-1, and angiopoietin-2 levels were determined using commercial human ELISA kits (Abcam Singapore Pte., Ltd., Singapore). We also calculated the atherogenic index using the formula (total cholesterol − HDL-C)/HDL-C).[15] Brachial artery flow-mediated dilatation and carotid intima-media thickness were measured using a high-resolution ultrasound machine (Prosound Alpha 10, Hitachi-Aloka Medical Ltd., Tokyo, Japan) per Toh, *et al.* [16]

The participants' diets were evaluated through 3-day food records and analyzed using Dietplan 7 software (Forestfield Software Ltd). Nutritional information was primarily sourced from the US Department of Agriculture (USDA) database, supplemented by data from the Singapore Health Promotion Board database and nutritional information panels on commercially available food products.[17] The serving sizes of whole grains, meats and alternatives, fruits, vegetables, and fruits and vegetables, as well as the intakes of micronutrients including protein, carbohydrates, and fat, were recorded.

Table 1 Demographic, clinical, and dietary characteristics of the study subjects.

Parameter	Range	Mean ± SD
Age, yrs	50–63	56 ± 4
Body mass index, kg/m^2	18.6–30.4	23.2 ± 3.2
Waist circumference, cm	66.3–96.5	80.8 ± 8.1
Systolic blood pressure, mmHg	79–155	110 ± 15
Diastolic blood pressure, mmHg	50–100	72 ± 11
Triglyceride, mmol/L	0.4–2.9	1.2 ± 0.5
Total cholesterol, mmol/L	3.1–8.1	5.3 ± 1.1
LDL-C, mmol/L	1.2–6.0	3.2 ± 1.0
HDL-C, mmol/L	0.7–2.2	1.6 ± 0.4
Atherogenic index	0.9–5.1	2.6 ± 1.1
Flow-mediated dilatation	0.2%–15.4%	4.6 ± 3.2
Intima-media thickness, mm	0.5–1.0	0.7 ± 0.1
Von Willebrand factor, IU/mL	1.0–4.5	2.3 ± 0.8
Angiopoietin-1, ng/mL	0.0–3.9	1.6 ± 1.1
Angiopoietin-2, ng/mL	0.4–2.3	1.1 ± 0.4
Dietary characteristics		
Whole grains (servings)	0.0–7.6	1.2 ± 1.3
Meats and alternatives (servings)	0.5–6.0	2.8 ± 1.1
Fruits (servings)	0.0–6.3	2.7 ± 1.3
Vegetables (servings)	0.4–7.6	2.7 ± 1.5
Fruits and vegetables (servings)	1.4–10.9	5.4 ± 2.3
Protein, g	36.7–309.6	90.7 ± 44
Carbohydrates, g	113.2–375.4	210 ± 62.6
Fat, g	24.6–186.5	67.6 ± 29.3

The female percentage is 79%. Among the subjects, 96% were Chinese, and the remaining 4% were Malay. Statistical comparisons were assessed with paired *t*-test for normally distributed data or Wilcoxon matched pairs test for non-normally distributed data. HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol.

Isolation and Culture of BOECs from Peripheral Blood

A total of forty-eight 27 mL peripheral blood samples were collected. Mononuclear cells were isolated and seeded onto 24-well tissue culture plates pre-coated with rat tail collagen type I (Life Technologies Corporation, Nebraska, USA) following previously described methods.[9] The culture medium used was EGM-2 (Lonza Bioscience Singapore Pte., Ltd., Singapore), which was supplemented with 20% fetal bovine serum and a cocktail of growth factors (EGM-2 BulletKit, Lonza, Singapore). After 48 h, non-adherent cells were removed, and fresh medium was added. The medium was then changed every 2 days. BOEC colonies with cobblestone morphology appeared in culture between 9 and 29 days. Cells enumeration was conducted by visual inspection using 4× and 10× objective lenses (EVOS XL Core Cell Imaging System, Life Technologies, USA). Cells derived from the colonies were passaged after they grown to confluence. Verificatin of BOECs was performed by checking their VWF expression using immunofluorescence staining (Eclipse Ti-E inverted microscope, Nikon Instruments Inc., Tokyo, Japan). Monoclonal antibody rabbit antihuman VWF conjugated to Alexa 488 (Abcam, Singapore) and 4',6-diamidino-2-phenylindole (Invitrogen, USA) were used to visualize VWF and nuclei, respectively.

Cells were enumerated using a trypan blue exclusion assay (Sigma-Aldrich, Missouri, USA) before seeding and after harvesting at each passage to monitor the retention of their proliferative ability. Phenotyping and functional evaluations of BOECs were conducted between passages 3 and 8.[12,18] Colonies that lost their

proliferative capacity before passage 3 were only recorded for their colony number, appearance date, and morphology. Colonies from the same sample that retained their proliferative ability were combined at passage 3 and differentiated based on their proliferative capacity after passage 8 for phenotype and functional assessment.

Cell Markers Expression Analysis

The surface marker expression levels, including KDR, CD34, and CD31, were determined on BOECs using fluorescence-activated cell sorter (CytoFLEX LX, Beckman Coulter, Indiana, USA). Mouse monoclonal antibodies against human KDR conjugated to phycoerythrin (BD Biosciences, California, USA), human CD34 conjugated to allophycocyanin (Invitrogen, Thermo Fisher Scientific, Massachusetts, USA), and human CD31 conjugated to Alexa Fluor 488 (BioLegend, Genomax Technologies Pte., Ltd., Singapore) were utilized. Each sample was analyzed in duplicate, and the data were analyzed using FlowJo software (Version 10, FlowJo LLC, Oregon, USA).

Matrigel Assay

To investigate the tube formation ability of BOECs, *in-vitro* Matrigel assays were conducted with minor modifications based on previous reports.[18–20] In brief, 1×10^4 cells/well in 100 μL of complete EGM-2 medium were seeded onto a 96-well tissue culture plate preloaded with 50 μL/well of growth factor-reduced Matrigel (Corning Inc., New York, USA). After 6 h incubation in a humidified atmosphere with 5% CO_2 at 37 °C, images were captured using an inverted microscope (Eclipse TS100, Nikon, Japan) with a digital sight camera. Experiments were performed in triplicate for each sample, and the acquired images were analyzed using the Angiogenesis Analyzer module in the ImageJ toolkit (National Institutes of Health, Maryland, USA) to quantify the results.[21]

Wound Healing Assay

BOECs were cultured in a 6-well plate coated with collagen in a complete EGM-2 medium until a confluent monolayer was formed. A thin "wound" was created using a pipette tip, and the cells were washed twice with PBS. Then, serum-starved EGM-2 medium plus 2 ng/mL actinomycin D (Thermo, USA) was added to observe further migration.[12,22] Cells were plated onto a temperature-controlled stage mounted atop an inverted optical microscope (Zeiss AxioObserver Z1; Carl Zeiss AG, Jena, Germany), and wound images were captured every 20 min for 6–7 h until the gap was at least half closed. The acquired images were then analyzed using ImageJ. Experiments were performed in triplicate. The collective cell migration rate (μm/min) and the time it took to close half of the wound (min) were calculated according to Jonkman *et al.* [22] Additionally, the average individual cell migration velocity (μm/min) and directionality were calculated by tracking thirty individual cells for each sample using the Manual Tracking plugin for ImageJ.[12]

Statistical Analysis

The mean values and standard deviation (SD) were used to express the data, and statistical analysis was performed using GraphPad Prism for Windows (Version 8.01, GraphPad Software Inc., California, USA). The normality assumption was tested using either the D'Agostino-Pearson test or Shapiro-Wilk normality test. Statistical comparisons of subject characteristics were assessed using a paired *t*-test for normally distributed data or Wilcoxon matched pairs test for non-normally distributed data. Comparisons of BOECs data were evaluated with an unpaired *t*-test with Welch's correction for normally distributed data or Mann–Whitney test for non-normally distributed data. To assess the relationship between variables, Spearman's coefficients correlation was used. All statistical tests were two-tailed, and $P < 0.05$ was considered statistically significant.

RESULTS

Obtaining BOECs with Different Proliferative Abilities

Out of 48 blood samples, 7 did not show any colony formation, and 132 colonies were obtained from the remaining 41 blood samples. Of these, 44 colonies lost proliferative ability before passage 3 (LPA-cols), while the remaining 88 colonies retained proliferative capacity after passage 3 (HPA-cols). It was observed that a blood sample could give rise to both LPA-cols and HPA-cols simultaneously, and the number of HPA-cols was significantly higher ($P = 0.014$) than the number of LPA-cols (Figure 1A). Moreover, the LPA-cols appeared significantly later ($P = 0.009$) than the HPA-cols when comparing their appearance dates (Figure 1B). Within the HPA-cols, BOECs were further classified as HPA (3–8) cells if they stopped proliferating during passages 3–8, while BOECs that continued to increase after passage 8 were classified as HPA (> 8) cells.

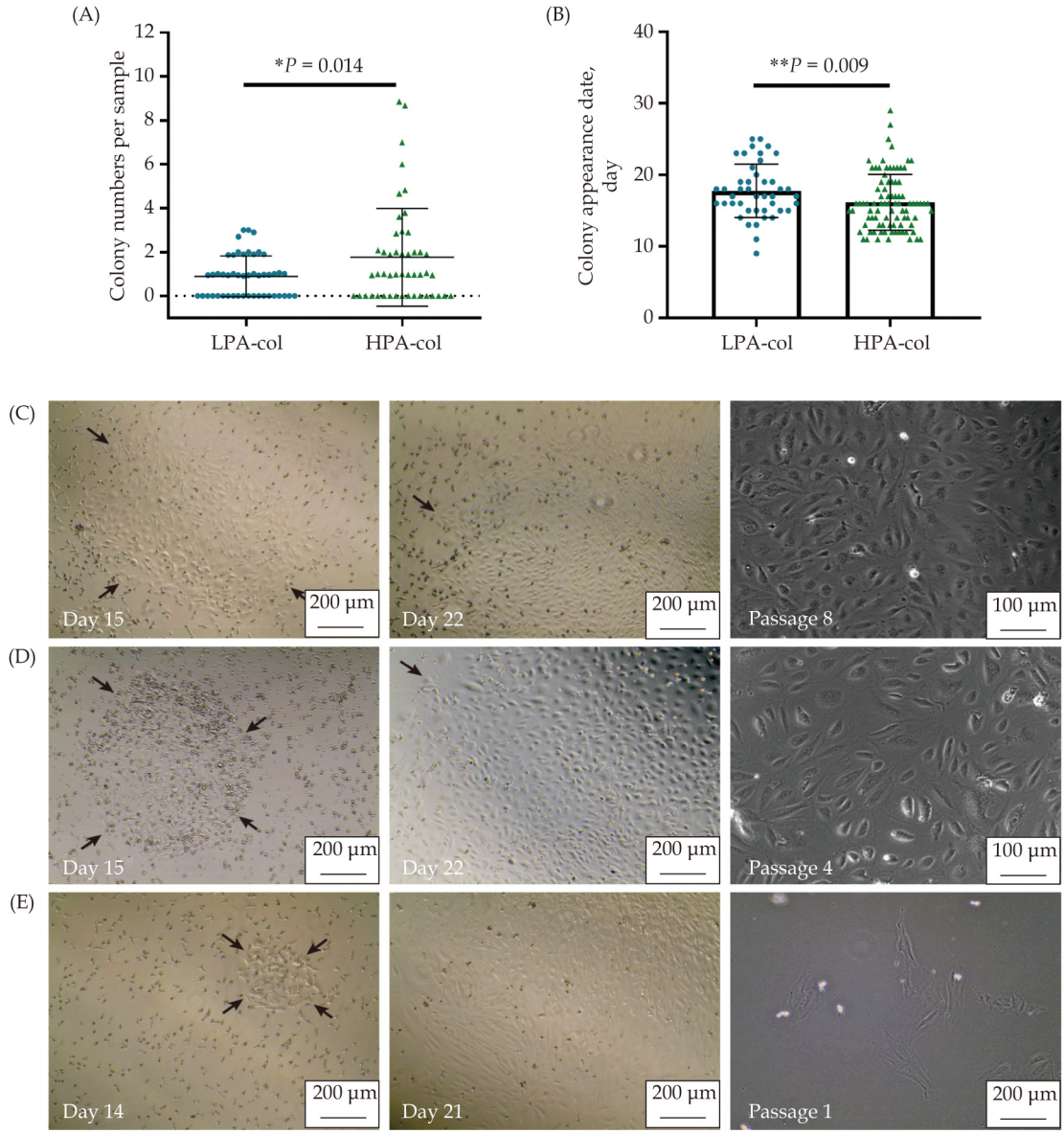

Figure 1 BOEC colonies derived from middle-aged and older adults. (A): The number of LPA-col and HPA-col in blood samples (n = 48), with each point representing the BOEC colony number in each blood sample. A Wilcoxon matched pairs test was performed for statistical comparison. (B): The appearance dates of LPA-col and HPA-col. Each point corresponds to the appearance date of a BOEC colony, with a total of 44 LPA-cols and 88 HPA-cols obtained. A Mann–Whitney test was performed for statistical comparison. (C): Representative BOECs that proliferated after passage 8. (D): Representative BOECs that stopped proliferating during passages 3–8 (passage 6). (E): Representative BOECs that lost proliferation ability before passage 3 (passage 1). The left and middle panels illustrate the BOEC colony morphology in a 24-well plate under 10 × objective at different time points, with black arrows indicating the edge of the colonies. The right panels show the cell morphology of BOECs after subculture. Data are presented as mean ± SD. *P < 0.05 and **P < 0.01. BOEC: blood outgrowth endothelial cell; HPA-col: colonies had proliferative ability after passage 3; LPA-col: colonies lost proliferative ability before passage 3.

Morphology of BOECs with Different Proliferative Abilities

Figure 1C-1D illustrates the representative appearance of BOECs during culture. Both HPA (> 8) and HPA (3–8) cells exhibited a typical cobblestone-like endothelial appearance. Whereas LPA cells (cells from LPA-cols) displayed an atypical shape and lacked the typical

cobblestone-like morphology. Due to insufficient cell numbers from LPA-cols, only HPA (> 8) and HPA (3–8) cells were used for further phenotypic and functional analyses. VWF immunofluorescence staining for HPA (> 8) and HPA (3–8) cells is presented in Figure 2A and 2B, respectively. Both HPA (> 8) and HPA (3–8) cells expressed VWF. While HPA (> 8) cells demonstrated intact VWF and nuclei with clear borders, morphological changes were observed in VWF of HPA (3–8) cells.

Cell Markers Expression of BOECs with Different Proliferative Abilities

The fluorescence-activated cell sorting analysis was used to evaluate the expression of endothelial markers (KDR and CD31) and progenitor markers (CD34) on BOECs (Figure 2C and 2D). Further analysis revealed that HPA (> 8) cells had significantly higher expression of KDR (99.8% ± 0.5%, $P = 0.0005$) compared to HPA (3–8) cells (79.9% ± 12.9%; Supplementary Table 1). Although BOECs expressed relatively high levels of CD31 and varied levels of CD34, no significant differences were found between HPA (> 8) and HPA (3–8) cells.

Tube Formation Capacity of BOECs with Different Proliferative Abilities

Representative capillary-like structures formed by HPA (> 8) and HPA (3–8) cells are presented in Figure 3A and 3B, respectively. The morphological features of these structures are quantified in Figure 3C–3E. Total branching length, number of segments, branches, extremities, junctions, meshes, and total mesh area were compared between HPA (> 8) and HPA (3–8) cells. The sample sizes for HPA (> 8) and HPA (3–8) cells are 24 and 5, respectively. The results showed that HPA (> 8) cells had significantly higher values for total branching length (16.4 ± 3.7 *vs.* 11.5 ± 5.5 mm) and the number of segments (73.7 ± 26.9 *vs.* 52.4 ± 36.9), branches (53.9 ± 10.2 *vs.* 57.1 ± 17.9), extremities (73.7 ± 13.5 *vs.* 84.0 ± 16.9), junctions (67.1 ± 20.2 *vs.* 54.2 ± 30.6), meshes (12.3 ± 6.5 *vs.* 7.0 ± 5.7), and total mesh area (0.6 ± 0.4 mm^2 *vs.* 0.2 ± 0.3 mm^2) than HPA (3–8) cells. Furthermore, the branching intervals of HPA (3–8) cells were significantly lower than those of HPA (> 8) cells. The branching intervals refer to the mean distance between two branches in the analyzed area and were measured to be 88.9 ± 39.9 μm for HPA (3–8) cells, compared to 181.2 ± 61.2 μm for HPA (> 8) cells ($P = 0.0025$).

Migration Activity of BOECs with Different Proliferative Abilities

Figure 4A and 4B display representative wound images of HPA (> 8) and HPA (3–8) cells at different time points. The sample numbers for HPA (> 8) and HPA (3–8) cells are 24 and 4, respectively. The quantification of collective (Figure 4C) and individual (Figure 4D) cell migration activities are presented. HPA (> 8) cells demonstrated significantly faster individual cell migration velocity than HPA (3–8) cells (0.44 ± 0.09 μm/min *vs.* 0.35 ± 0.05 μm/min; $P = 0.0201$). However, there was no significant difference in cell migration rate, the time it took to close half the original area, and individual cell directionality between HPA (> 8) and HPA (3–8) cells.

Correlations Between Demographic, Clinical, And Dietary Parameters with Number of BOECs

We investigated the correlations between demographic, clinical, and dietary parameters with BOEC colony numbers (Table 2). The total-col number was positively associated with waist circumference ($r = 0.294$, $P = 0.043$), systolic blood pressure ($r = 0.393$, $P = 0.006$), diastolic blood pressure ($r = 0.385$, $P = 0.007$), and atherogenic index ($r = 0.396$, $P = 0.005$). Moreover, the LPA-col number was positively associated with waist circumference ($r = 0.297$, $P = 0.041$) and serum triglyceride ($r = 0.361$, $P = 0.012$). Similarly, HPA-col number was positively associated with systolic blood pressure ($r = 0.367$, $P = 0.010$), diastolic blood pressure ($r = 0.421$, $P = 0.003$), LDL-C ($r = 0.291$, $P = 0.045$), and atherogenic index ($r = 0.420$, $P = 0.003$), but negatively associated with HDL-C ($r = -0.361$, $P = 0.012$). For dietary parameters, we observed positive associations between LPA-col number with servings of meats and alternatives ($r = 0.338$, $P = 0.019$), fruits ($r = 0.343$, $P = 0.017$), fruits and vegetables ($r = 0.347$, $P = 0.016$), as well as protein consumption ($r = 0.289$, $P = 0.047$). No other significant correlations were observed.

DISCUSSION

"EPCs" refer to a heterogeneous population of bone marrow-derived progenitor cells involved in neovascularization and vessel repair.[23–25] However, the term has been interpreted broadly in different studies, resulting in a non-uniform definition. This variation in definition stems from the fact that the types of cells considered as EPCs differ among studies, including circulating EPCs,

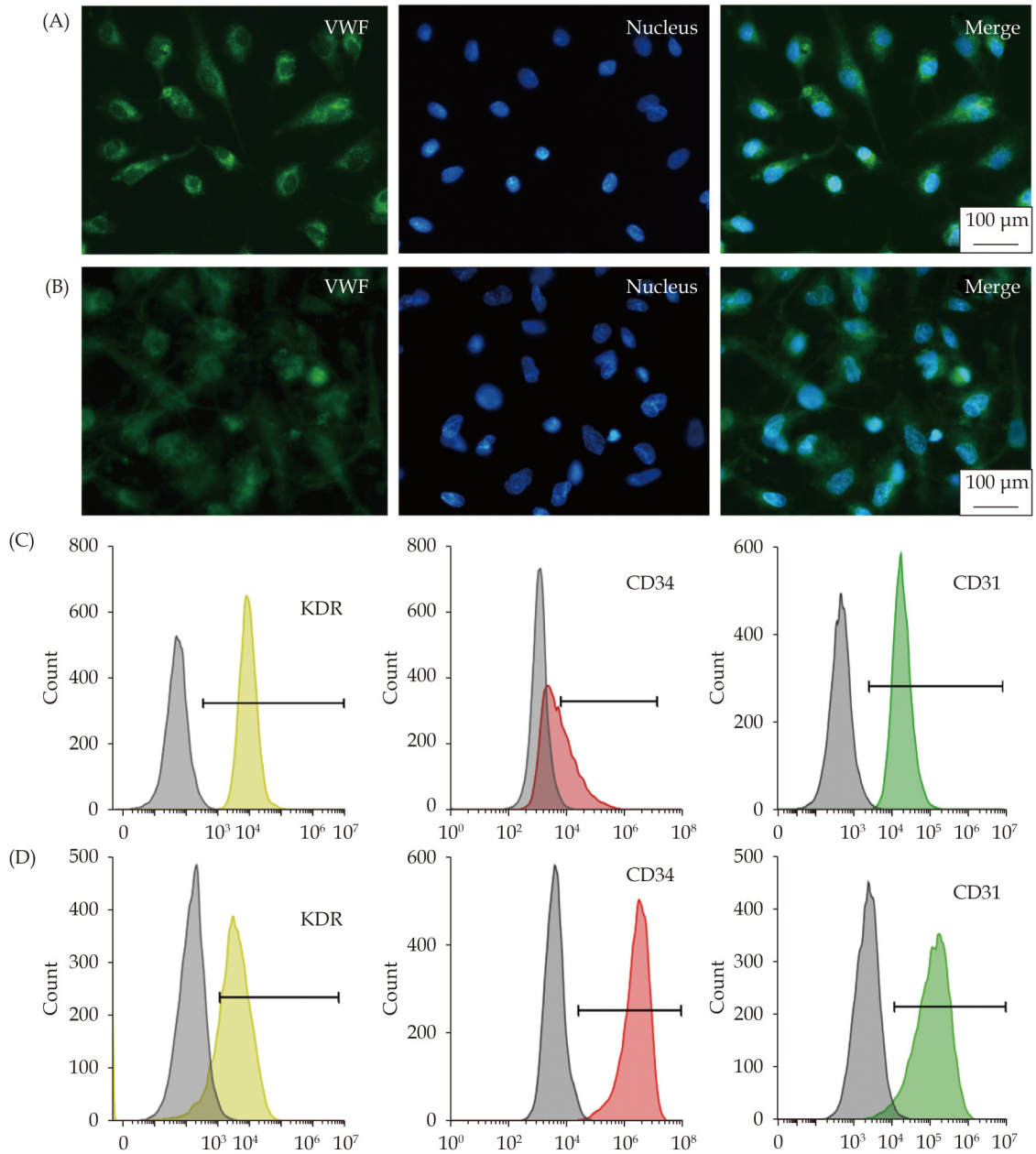

Figure 2 Immunofluorescence staining (A & B) and fluorescence-activated cell sorting analysis (C & D) of BOECs with different proliferation abilities. (A & C): Representative BOECs that proliferated after passage 8. (B & D): Representative BOECs that stopped proliferating during passages 3–8 (passage 6). The left panels in (A) and (B) display staining for VWF (green), while the middle panels illustrate staining for the nucleus (blue). The right panels exhibit co-staining for VWF (green) and nucleus (blue). A scale bar of 50 μm is shown. For panels (C) and (D), negative controls are overlaid as areas filled with gray on each histogram. BOECs: blood outgrowth endothelial cells; VWF: von Willebrand factor.

colony-forming unit-endothelial cells (CFU-ECs), circulating angiogenic cells (CACs), and blood outgrowth endothelial cells (BOECs).[8] In a strict sense, "true" human EPCs are cells with postnatal vasculogenic potential. Specifically, these cells proliferate at a clonal level and give rise to endothelial progeny, with the capacity to form endothelial tubes *in-vitro* and contribute to *de-novo* vessel formation, as well as repair injured vessel walls when implanted *in-vivo*.[8,24] Currently, the only cells that display these activities are BOECs.[8,10,26] BOECs have recently garnered attention from researchers due to their potential role in the pathogenesis of atherosclerotic dis-

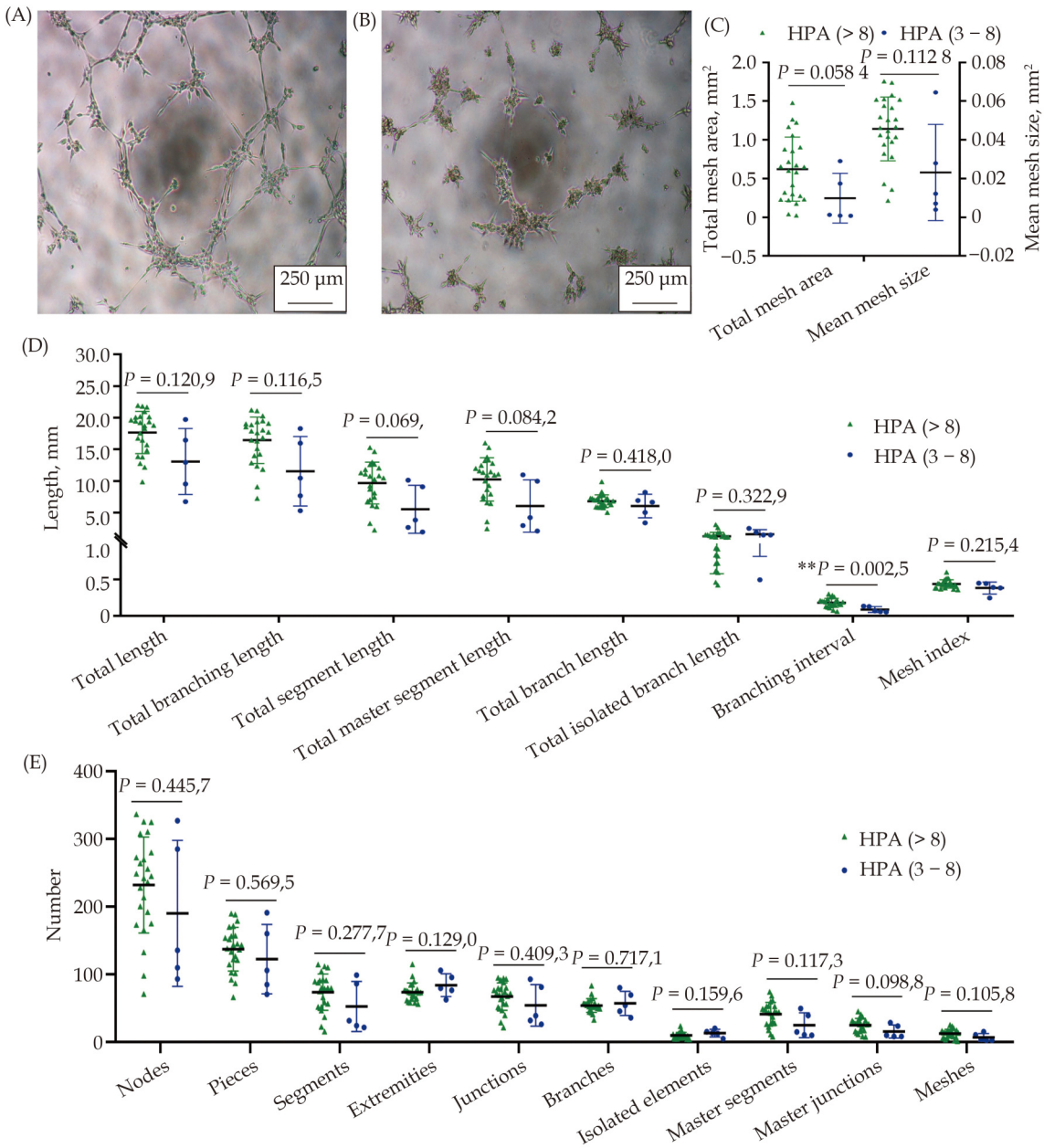

Figure 3 Characterization of capillary network formation of BOECs with different proliferation abilities. (A & B): Representative capillary-like structures of BOECs that proliferated after passage 8 and stopped proliferating at passage 6, respectively. (C-E): Different parameters that quantify morphological features of capillary-like structures. The image analysis area is 3.50 mm^2. Graphs are plotted as means with SD. The sample numbers for HPA (> 8) and HPA (3–8) cells are 24 and 5, respectively. Statistical comparison was performed by unpaired *t*-test with Welch's correction or Mann–Whitney test. $^{**}P < 0.01$. BOECs: blood outgrowth endothelial cells; HPA (>8): BOECs proliferated after passage 8; HPA (3–8): BOECs stopped proliferating during passages 3–8.

eases. Autologous BOECs have been studied as a cell-based therapy for human cardiovascular disorders.[7,8,10] In the present study, we derived peripheral BOECs with varying proliferative abilities from middle-aged and older adults and investigated their morphology, appearance dates, and angiogenic characteristics, as well as their relationship with subjects' demographic, CVD-related clinical, and dietary parameters.

A total of 132 colonies were obtained, appearing between 9–29 days, specifically 44 LPA-cols and 88 HPA-cols. Martin-Ramirez, *et al*.[9] stated that most BOEC colonies appear between days 14 and 28. Nevertheless,

Table 2 Correlations between demographic, clinical, and dietary parameters with BOEC colony numbers.

Parameters	LPA-col		HPA-col		Total-col	
	r	*P*	*r*	*P*	*r*	*P*
Age	-0.052	0.724	-0.089	0.549	-0.084	0.572
Gender	-0.181	0.218	-0.239	0.102	-0.222	0.130
Race	0.080	0.589	-0.136	0.356	-0.061	0.680
Body mass index	0.209	0.155	0.101	0.496	0.218	0.136
Waist circumference	0.297	0.041	0.165	0.263	0.294	0.043
Systolic blood pressure	0.271	0.063	0.367	0.010	0.393	0.006
Diastolic blood pressure	0.196	0.182	0.421	0.003	0.385	0.007
Triglyceride	0.361	0.012	-0.037	0.804	0.160	0.278
Total cholesterol	0.101	0.495	0.057	0.699	0.132	0.371
LDL-C	-0.027	0.853	0.291	0.045	0.235	0.108
HDL-C	0.033	0.824	-0.361	0.012	-0.259	0.075
Atherogenic index	0.110	0.457	0.420	0.003	0.396	0.005
Flow-mediated dilatation	-0.148	0.316	-0.021	0.889	-0.175	0.233
Intima-media thickness	0.167	0.257	0.220	0.133	0.245	0.093
Von Willebrand factor	-0.194	0.187	-0.220	0.133	-0.250	0.087
Angiopoietin-1	0.078	0.597	-0.161	0.274	-0.100	0.498
Angiopoietin-2	0.095	0.522	-0.217	0.138	-0.140	0.342
Dietary parameters						
Whole grains	0.063	0.671	0.137	0.352	0.190	0.197
Meats and alternatives	0.338	0.019	0.019	0.899	0.156	0.289
Fruits	0.343	0.017	0.002	0.988	0.168	0.254
Vegetables	0.241	0.099	0.135	0.362	0.247	0.091
Fruits and vegetables	0.347	0.016	0.094	0.527	0.249	0.088
Protein	0.289	0.047	-0.055	0.709	0.053	0.723
Carbohydrates	0.161	0.276	-0.251	0.085	-0.169	0.252
Fat	0.220	0.133	-0.228	0.119	-0.081	0.583

Spearman nonparametric correlation was conducted. LPA-col: colony lost proliferative ability before passage 3; HDL-C: high-density lipoprotein cholesterol; HPA-col: the colony had proliferative ability after passage 3; LDL-C: low-density lipoprotein cholesterol; Total-col: total colony.

earlier and later growth has also been observed, such as 5–22 days reported by Yoder *et al.*,[27] 7–22 days reported by Paschalaki, *et al.*[13] and 10–30 days reported by Minami, *et al.*[14] Comparing the appearance date of the colonies, LPA-cols were found to appear later than the HPA-cols. Similarly, Martin-Ramirez, *et al.*[9] reported that colonies that occur at a late stage generally cannot be expanded. Furthermore, Minami, *et al.*[14] found that BOECs emerging on days 17–23 showed higher proliferation than cells emerging on days 10–16 or 24–30. These findings collectively suggest avoiding colonies that appear during later stages of culture to obtain BOECs with high proliferative abilities.

Groeneveld *et al.* [12] reported a gradual loss of activity in BOECs at passage 8. Likewise, Wang *et al.* [18] used BOECs in passages 3 to 8 to investigate their angiogenic function. In this study, we divided HPA cells into two groups based on their proliferation capacity: HPA (3–8) and HPA (> 8). Both HPA (3–8) and HPA (> 8) cells displayed cobblestone-like appearances, while LPA cells did not exhibit typical endothelial morphology. However, we cannot confirm that LPA cells are BOECs since we did not verify their phenotype and functionality due to insufficient cell numbers. Further investigations are necessary to understand this subpopulation. Immunofluorescence staining results showed that HPA (3–8) cells exhibit altered VWF morphology, which was not observed in HPA (> 8) cells. VWF is a large multimeric glycoprotein that participates in multiple vascular processes and is best known for its hemostatic functions.[28,29] Addi-

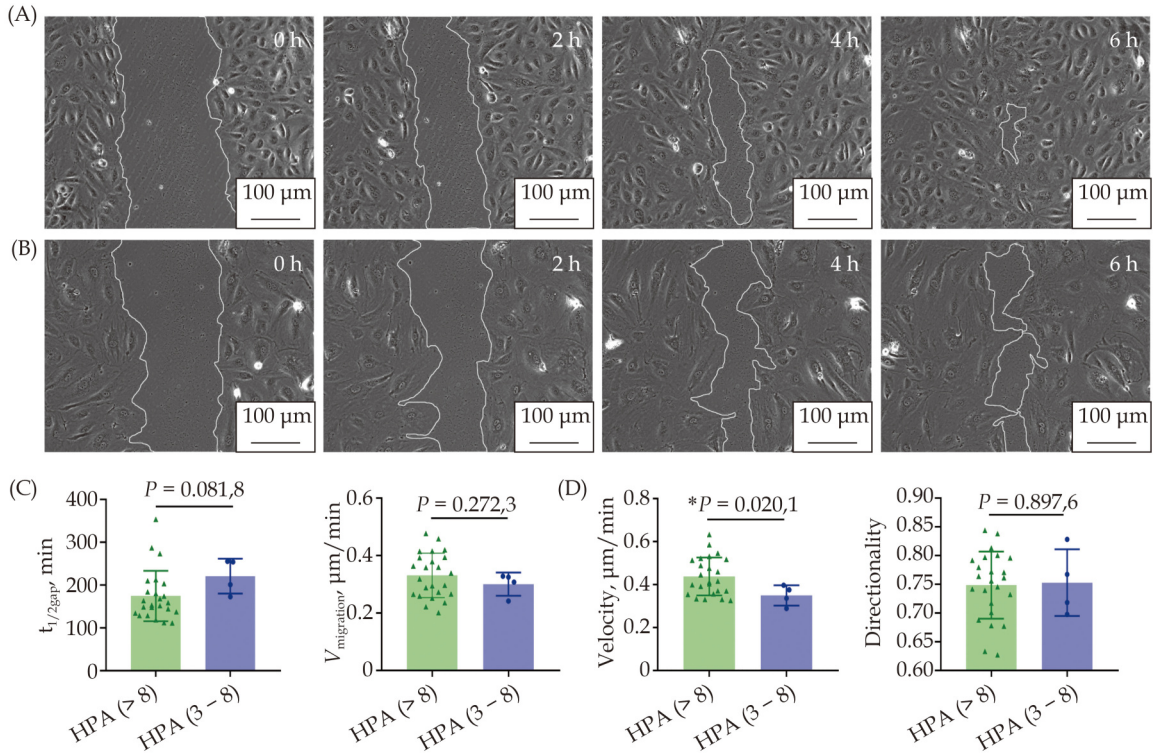

Figure 4 Migration activity of BOECs with different proliferation abilities. (A & B): Representative images of BOECs proliferated after passage 8 and stopped proliferating at passage 6, respectively, from the wound healing assay at different time points. (C): Collective migration activity of BOECs was demonstrated by cell migration rate ($v_{migration}$) and the time it took to close to half the original area ($t_{1/2gap}$). (D): Individual cell migration activity of BOECs was demonstrated by average individual cell migration velocity and directionality. Graphs are plotted as means with SD. Statistical comparison was performed using unpaired *t*-test with Welch's correction or Mann–Whitney test. *$P < 0.05$. The sample numbers for HPA (> 8) and HPA (3–8) cells are 24 and 4, respectively. BOEC: blood outgrowth endothelial cell; HPA (> 8): BOECs proliferated after passage 8; HPA (3–8): BOECs stopped proliferating during passage3–8.

tionally, we found that HPA (3–8) cells exhibited significantly lower KDR expression. KDR, also known as vascular endothelial growth factor receptor 2 or CD309, is critical for vascular development and adult angiogenesis.[28] VWF and KDR are primarily produced by endothelial cells.[29] The morphological changes in VWF and the decreased expression of KDR in HPA (3–8) cells may suggest a decline in their VWF production and angiogenic capacity.

Angiogenesis, the process of developing new blood vessels, plays an essential role in physiological processes such as vascular repair and pathological situations such as the development of atherosclerotic plaques.[30,31] The ability to form tubes in the Matrigel demonstrates the formation of new vessels by vascular endothelial cells.[32] Migration is one of the hallmarks of angiogenesis, and individual cell migration enables cell sheets to move toward induced "wound".[33,34] In a study by Hur, *et al.*,[35] the capillary network formation of BOECs isolated from donors aged 56.3 ± 5.6 was tested. Their total mesh area was found to be smaller than that of HPA (> 8) cells but larger than that of HPA (3–8) cells in the present study. The number of branches of both HPA (> 8) and HPA (3–8) cells was higher than that of patients with the end-stage renal disease described by Huuskes, *et al.*[36] Furthermore, individual cell migration activities of BOECs derived by Groeneveld, *et al.*[12] from healthy individuals or patients with von Willebrand disease were similar to those of HPA (> 8) cells, but higher than those of HPA (3–8) cells. Our result also demonstrated that HPA (3–8) cells possessed weaker tube formation capacity and slower individual cell migration velocity compared to HPA (> 8) cells. Our findings indicate that BOECs with high proliferative and angiogenic potential can be derived from middle-aged and older populations. Moreover, BOECs with lower proliferative potential possessed diminished angiogenic and migratory function, possibly due to altered VWF morphology and decreased KDR expression. Previous studies have demonstrated that VWF plays multiple roles in blood vessel

formation and is a regulator of angiogenesis. Similarly, besides contributing to angiogenesis, KDR is also responsible for endothelial cell migration.[28,37,38] Consistent with our results, Minami, *et al.* [14] reported that BOECs that emerged on days 17–23 had the highest proliferative potential and KDR expression while also exhibiting the highest tube formation capabilities *in-vitro* compared with cells occurring earlier or later. Furthermore, it was also revealed that only this subpopulation could significantly augment capillary formation *in-vivo*.

Previous research suggests an inverse relationship between the number of circulating EPCs and several cardiovascular risk factors, such as body mass index, waist circumference, carotid intima-media thickness, total serum cholesterol, and LDL-C levels. Moreover, a positive correlation has been found between the circulating EPCs and flow-mediated dilatation.[39–42] Hill, *et al.*[43] also observed a positive correlation between CFU-EC numbers and flow-mediated dilatation, as well as an inverse correlation with the Framingham risk score. In a similar vein, Vasa, *et al.*[44] demonstrated a significant correlation between the number of risk factors for coronary artery disease and the reduction of circulating angiogenic cell levels. In contrast to other EPC types, our correlation analysis revealed a positive association between the number of BOEC colonies and several CVD risk factors, indicating that individuals with higher CVD risks tended to have more BOECs. This observation aligns with the findings of Guven *et al.*,[26] who observed a positive correlation between the number of BOECs and the presence and severity of coronary artery disease. The divergent correlations between BOECs and other EPC types with CVD risks suggest that they may play distinct roles in CVD progression. Limited evidence exists on the association between various proliferative BOECs and clinical parameters related to CVDs. Our study showed that the LPA-col number was linked to waist circumference and serum triglyceride, while a higher HPA-col number was associated with elevated blood pressure, LDL-C, atherogenic index, and reduced HDL-C levels. While the mechanisms are yet to be fully understood, a plausible explanation is that a greater burden of CVD risk factors triggers the mobilization of BOECs with higher proliferative potential to counteract the detected cardiovascular deteriorations.[26] The quantity and function of BOECs may be related to age.[9] In the experiment, we found that the number of BOECs in the blood of elderly individuals is lower, and their growth rate is slower compared to the BOECs from the younger individuals that we used in the pilot experiment (data not published). However, based on current data, we did not observe a significant correlation between age and the number of BOECs. This may be due to the small age range (50–63 years) of our subjects.

Since dietary modifications can help prevent many CVD events,[45] there has been increasing focus on food and nutritional interventions in terms of EPC quantity and function. We previously reviewed the latest research progress on the impact of food and nutrients on EPCs.[46] Studies generally show that a healthy diet, such as the Mediterranean diet, specific foods like fruits and vegetables, and certain nutrients such as polyphenols, unsaturated fats, inorganic nitrate, and vitamins, generally promote higher EPC numbers and enhance EPC function. However, most studies have focused on circulating EPCs, CFU-ECs, or CACs. Only two articles have investigated the effect of diet or food on BOECs. Sun, *et al.*[47] assessed the effects of fasting therapy on the function of BOECs in overweight and obese individuals, reporting improved migration, adhesion, and tube formation capacity. The other study was conducted by our group, where we found that consuming a healthy dietary pattern improved BOECs' colony growth rate, migration activity, and tube formation capacity.[48] In addition, several *in-vitro* studies have reported functional improvements of BOECs with treatments with fermented rice extract, n-3 PUFAs, red wine, epigallocatechin gallate, and vitamin D.[49–52] In the present study, we found a positive association between the number of LPA-cols and the consumption of meats and alternatives, fruits, fruits and vegetables, as well as protein. These results regarding fruits and/or vegetables are consistent with the previous studies mentioned above, as fruits and vegetables are abundant in the healthy diet, and they are rich in polyphenols, inorganic nitrate, and vitamins.[46] However, regarding meats and alternatives, which are primarily sources of protein, there is currently a lack of research focusing on the dietary impact of meat or protein intake on BOECs. Unexpectedly, no correlation was found between HPA-cols or total-cols. We speculate that HPA-cols in individuals are relatively stable, and only LPA-cols are regulated by diet. Nevertheless, further research should be conducted to verify this speculation.

By examining a relatively large sample size, we differentiated BOECs with varying proliferative potentials and analyzed their angiogenesis and migration efficacy. Furthermore, we evaluated the correlations between CVD-

related clinical parameters and the number of BOECs, providing important insights into their contribution to CVDs. However, this study has certain limitations. The relatively small sample size of HPA (3–8) cells in comparison to tube formation capacity and migration activity might result in statistically underpowered analyses. Moreover, the underlying mechanisms of different proliferative potentials of BOECs remain unclear, and it may be necessary to examine senescence markers at the gene or protein level, as senescence could play a role in the narrow passage. Additionally, reviewing other monocyte/macrophage/endothelial features could provide a more comprehensive understanding of BOECs with varying proliferative potentials in the aging population. Furthermore, although evidence suggests that BOECs with higher *in-vitro* functions have enhanced *in-vivo* functions,[14] confirming their therapeutic potential will require further *in-vivo* studies.

In summary, the present study demonstrate that middle-aged and older individuals possess BOECs with varying proliferative potentials. Morphology monitoring and appearance dates will provide quick identification for cells that have lost proliferative ability before reaching passage 3. Cells that cease proliferation after passage 3, exhibiting typical cobblestone morphology, could be recognized by checking their VWF and KDR expression. Aging individuals with higher CVD risks may have an increased number of BOECs, while the number of low proliferative BOECs may be regulated by diet. Further clinical trials and molecular experiments are needed to explore the underlying mechanisms and confirm the association between BOEC quantity with clinical and dietary characteristics.

DECLARATIONS

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Competing interests

None.

Ethics approval and consent to participate

The research was conducted ethically following the Declaration of Helsinki. All subjects provided written informed consent, and the study protocol was approved by the National Healthcare Group's Domain Specific Review Board (NHG DSRB; Protocol 2018/0311).

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