Reproducibility of circulating endothelial cell enumeration and activation in children and adolescents

Introduction: We examined the reproducibility of circulating endothelial cells (CEC) enumeration and activation among youth. **Materials & methods:** CECs from 151 youth were measured at baseline and 1 week follow-up. Enumeration of CEC in fresh whole blood was determined by direct assessment of buffy coat smears (CD146+ nucleated cells) and activated CEC (%VCAM-1 expression) was determined after immunomagnetic enrichment and co-staining of nuclei, plus positivity for P1H12 and VCAM-1. **Results:** No statistically significant difference in CEC enumeration (1.2 ± 2.5 vs 1.3 ± 2.2 CEC/milliliter of whole blood, $p = 0.745$) or activated CEC (57.1 ± 24.4 vs 58.0 ± 21.3 %VCAM-1, $p = 0.592$) between baseline and 1 week follow-up. **Conclusion:** On a cohort basis, CEC enumeration and activation are reproducible in youth. Relatively high individual biological variability may limit its clinical utility.

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Keywords: biomarkers • cardiovascular disease risk • circulating endothelial cells • obesity • pediatric • reproducibility • youth

Endothelial dysfunction is one of the earliest manifestations of the atherosclerotic process [1]. Examining early perturbations in the vascular endothelium may provide clinicians and researchers with better biomarkers of subclinical cardiovascular disease (CVD) risk, especially in pediatric populations where clinically useful biomarkers are lacking [2]. Circulating endothelial cells (CEC) may be one such biomarker since they are thought to reflect structural damage and injury to the endothelial monolayer [3]. The total number of CECs within circulation (CEC enumeration) along with characterization of cell surface expression of vascular cell adhesion molecule-1 (VCAM-1), an indicator of CEC activation (activated CEC), provide a robust model for examining early stages of endothelial cell biology adaptations [3–5].

In order for biomarkers to be deemed as having clinical utility and worthy of measurement in the research setting they must be

reproducible, be proximal to the pathogenesis of a disease, provide prognostic value beyond currently established risk factors and biomarkers and be predictive of disease outcome [2,6]. Data from adult studies have demonstrated that CECs (enumeration and/or activation) are independently associated with numerous cardiovascular complications including peripheral artery atherosclerosis [7], endothelial dysfunction [8], systemic inflammation [9], sickle cell anemia [4], Kawasaki disease [10] and acute myocardial infarction [11]. Furthermore, CECs predict cardiovascular events in adults, independent of traditional CVD risk factors [12,13]. Therefore, in adults, CECs meet several of the requisite criteria characteristic of a useful biomarker as defined by the American Heart Association [6].

Despite documented utility among adults, CECs (enumeration and activation: %VCAM-1 expression) have yet to be established as a clinically useful biomarker of CVD

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risk in youth. In children, CECs have been shown to be elevated in youth with familial hypercholesterolemia [14], pulmonary arterial hypertension [15], and in youth with severe obesity [16]. Importantly, in youth, CECs have been shown to be permeable to change [15] and present with a dynamic range of values, particularly in the context of obesity [16]. However, before CECs can be viewed as a valid biomarker of CVD risk in the pediatric population, reproducibility must be established. Therefore, the purpose of this study was to examine the reproducibility of CEC enumeration and activation in a group of children and adolescents across a wide age-range and spectrum of body mass index (BMI) values.

Materials & methods

Data from 151 children and adolescents (mean age = 12.8 ± 2.6 ; males = 75 / females = 76) participating in larger cross-sectional study were used for this short-term longitudinal reproducibility study. Participants with obesity or severe obesity were predominantly recruited from the University of Minnesota Masonic Children's Hospital Pediatric Weight Management Clinic; participants with overweight or normal weight were recruited from the community.

Participants completed two visits at the University of Minnesota Clinical Translational Research Institute. Each visit was conducted after participants had been fasting for ≥ 12 h. Data obtained at the first visit included demographic and anthropometric characteristics along with CEC enumeration and expression. CEC enumeration and expression were again evaluated on a separate visit 7 ± 3 days later. Height and weight were determined using a wall-mounted stadiometer and an electronic scale, respectively. BMI was calculated as the body weight in kilograms divided by the height in meters squared. BMI percentiles were determined using age- and gender-based definitions from the Centers for Disease Control and Prevention. Normal weight was defined as BMI ≥ 5 th to < 85 th percentile, overweight/obesity was defined as BMI ≥ 85 th to < 120 % of the 95th percentile, and severe obesity was defined as BMI ≥ 120 % of the 95th percentile or an absolute BMI ≥ 35 kg/m² [17]. Tanner stage was determined by a trained pediatrician or nurse [18,19].

Circulating endothelial cell enumeration & phenotyping

CEC were defined as nucleated blood cells expressing CD146, as identified by a skilled immuno/histopathologist highly experienced with CEC; and our data are reported exactly as obtained, the CEC content directly counted within 1.0 ml blood (see below). CD146 positivity was detected using the monoclonal anti-

body (mAb), P1H12, originated by our group [4,20]. Importantly, the source of P1H12 was our own parent CHO^{P1H12} cell line, not subclones maintained by commercial sources over which we have no quality control. Our methods and procedures, previously described in much greater detail [4], were developed to maximize detection while minimizing nonspecific labeling.

We acknowledge that others, using CHO^{P1H12} subclone sources of P1H12, have described a small subset (< 5 %) of CD146⁺ activated CD4⁺ or CD8⁺ lymphocytes in normals, i.e., in absence of apparent inflammation [21]. Therefore, approaches to CEC enumeration subsequently developed by others often utilize antibody to CD45 to screen these out. However, we note that in both normals and the chronically inflamed (sickle cell anemia patients), our method previously found > 97 % of scored CEC to be positive for von Willebrand factor and thrombomodulin, a combination unique to endothelial cells [4]. Since the present study found modal values for CEC in the tested 1.0 ml blood to be 1 or 2, it is very unlikely that any given scored CEC here would have been a CD146⁺ non-endothelial cell.

Specific CEC enumeration method

Fresh blood collected in EDTA was processed within 3 h of collection. From 1.0 ml blood, mononuclear cells at a plasma-Ficoll interface were collected and divided for smearing upon four microscopic slides. Labeling utilized hematoxylin to visualize nuclei and P1H12 followed by a secondary, alkaline phosphatase-conjugated antibody. Using light microscopy we counted CEC present on the four slides prepared from the starting 1.0 ml blood. Thus, our enumeration method is direct and thus differs importantly from those subsequently developed by avoiding the more assertive sample manipulations they usually employ for needed cell enrichment.

CEC phenotype

Phenotyping of these rare CEC did require their enrichment, as a matter of practicality for the microscopist [4]. For this we used ferromagnetic Dynabeads carrying goat antimouse IgG (Invitrogen Dynal AS; cat# 110.33) that we coated with our P1H12. These were mixed with 1–5 ml fresh EDTA blood, as determined by size of the subjects, and a magnetic concentrator (Dynal) was used to capture cells carrying beads. These were cytospun onto slides and assessed for VCAM-1 and CD146 positivity by applying: a PE-conjugated rabbit polyclonal antibody to VCAM-1; and our P1H12 and a FITC-conjugated secondary antibody. The percentage of CEC (P1H12+) also positive for VCAM-1 was scored.

Reproducibility within the same samples was assessed for CEC enumeration and activation

	Overall n = 151	Normal weight n = 45	Overweight/obese n = 50	Severe obese n = 56
Covariate				
Age (years)	12.8 (2.6)	13.0 (2.6)	12.5 (2.5)	12.9 (2.7)
Sex (male)	75 (49.7%)	28 (62.2%)	24 (48.0%)	23 (41.1%)
Weight (kg)	69.8 (26.3)	46.9 (13.1)	66.2 (17.1)	91.5 (23.8)
Height (cm)	157 (13.6)	154 (15.2)	157 (13.9)	160 (11.4)
BMI (kg/m ²)	27.4 (7.8)	18.9 (2.3)	26.3 (3.4)	35.0 (5.7)
BMI Percentile (%)	83.7 (23.7)	52.3 (21.2)	95.0 (3.9)	98.8 (0.4)
Race				
– Asian	5 (3.3%)	1 (2.2%)	3 (6.0%)	1 (1.8%)
– African-American	19 (12.6%)	4 (8.9%)	5 (10.0%)	10 (17.9%)
– White	113 (74.8%)	39 (86.7%)	35 (70.0%)	39 (69.6%)
– Mixed	12 (7.9%)	1 (2.2%)	5 (10.0%)	6 (10.7%)
– Other	2 (1.3%)	0 (0.0%)	2 (4.0%)	0 (0.0%)
Ethnicity Latino/Hispanic	15 (9.9%)	3 (6.7%)	5 (10.0%)	7 (12.5%)
Tanner stage				
– I	35 (23.2%)	13 (28.9%)	14 (28.0%)	8 (14.3%)
– II	32 (21.2%)	9 (20.0%)	7 (14.0%)	16 (28.6%)
– III	26 (17.2%)	7 (15.6%)	10 (20.0%)	9 (16.1%)
– IV	39 (25.8%)	13 (28.9%)	11 (22.0%)	15 (26.8%)
– V	19 (12.6%)	3 (6.7%)	8 (16.0%)	8 (14.3%)
CEC enumeration (cell count)				
Mean (SD)	1.3 (2.4)	1.0 (1.5)	1.4 (2.7)	1.4 (2.7)
Median (25th, 75th Percentiles)	0 (0, 2)	0 (0, 2)	0 (0, 2)	0 (0, 2)
CV% (within-participant)	1.15	0.61	1.15	1.35
CV% (between-participant)	1.90	1.52	1.96	1.95
Activated CEC (%VCAM-1 expression)				
Mean (SD)	57.9 (22.4)	55.6 (25.2)	57.4 (22.5)	60.1 (19.8)
Median (25th, 75th percentiles)	60 (42.9, 75.0)	50 (37.2, 75.0)	57.1 (44.1, 71.4)	60.0 (50.0, 75.0)
CV% (within-participant)	0.32	0.26	0.43	0.36
CV% (between-participant)	0.39	0.45	0.39	0.33
Values presented are mean (SD) or N (%) where indicated. BMI: Body mass index; CEC: Circulating endothelial cells; CV%: Coefficient of variation.				

(%VCAM-1 expression) phenotyping in a subset (n = 10) of participants. The coefficient of variation for activated CEC (%VCAM-1 expression) was 16.9%. Of the ten samples, seven had the same CEC enumeration with the remaining three being within one CEC count of the other.

Statistical analysis

Descriptive statistics were calculated overall and by obesity groups which included means with standard deviations for continuous variables and frequencies

with percentages for categorical variables. Data for CEC enumeration and activated CEC (%VCAM-1 expression) were compared between visits using a paired t-test. Coefficient of variation (CV%) and interclass correlation coefficients (ICC) were calculated between visits. ICC was calculated for the original scale based on a multiplicative overdispersion model (generalized linear mixed-effects poisson model with log link) [22]. Bland-Altman plots were created to examine agreement and reproducibility of the data. All analyses were conducted using R v3.1.1 and the lme4 package v1.1–7.

Results

There was an even distribution of males and females overall, with the normal-weight group having a higher proportion of males and the severe obese group having a higher proportion of females (Table 1). By design, participants had a range of BMI values with a relatively even distribution of BMI classifications: normal weight (n = 45), overweight/obese (n = 50), and severe obesity (n = 56). Participants were mostly White with similar age and a relatively even distribution of Tanner Stage across obesity groups. CEC enumeration was similar across groups with CV% ranging from 0.61 to 1.35% within-participant and 1.52 to 1.96% between-participant. Activated CEC (%VCAM-1 expression) increased across adiposity groups with CV% ranging from 0.26 to 0.43% within-participant and 0.33 to 0.45% between-participant. In addition, we examined whether sex differences were present for CEC enumeration (1.21 ± 2.61 vs 1.23 ± 2.34 , male and female, respectively) and activated CEC (57.7 ± 23.9 vs 56.3 ± 24.9 , male and female, respectively) with no meaningful differences observed. The overall ICC for activated CEC was 0.695, while within normal weight, obese and severely obese the ICC was 0.643, 0.614 and 0.777, respectively.

Table 2 compares CEC enumeration and activated CEC between visits on the group as a whole and split by BMI category. No significant differences were observed for CEC enumeration or activated CEC overall or in any of the BMI categories (all $p > 0.05$).

Figure 1 displays modified Bland-Altman plots for CEC enumeration and activated CEC. The distribution of the difference between visits for CEC enumeration was as follows: 48 participants (34.8%) no difference, 37 (26.8%) had difference of ± 1 , 24 (17.4%) ± 2 , 10 (7.2%) ± 3 and 5 (3.6%) ± 4 . Of the 138 participants measured, 15 had a difference in CEC count ≥ 5 between visits. For activated CEC, three baseline entries (one from each BMI category) and three of the 1-week post-baseline entries (one normal-weight, two overweight/obese) were removed from the analysis due to small VCAM-1 denominator expression amounts (<3) within the sample. Overall activated CEC showed good agreement overall between visits; however, a large degree of individual heterogeneity was observed between visits. Thirteen (10.2%) participants had the same value at baseline and follow-up and 98 (76.6%) had values within $\pm 20\%$, with a wide degree of variation ranging from +80 to -60% between visits.

Figure 2 depicts individual responses in CEC enumeration and activated CEC split by BMI category. Similar variability was observed across BMI category with no distinct pattern of difference. Youth with severe obesity were more likely to have values >5 for CEC enumeration (n = 6 [6.0%]) than normal weight (n = 2 [2.4%]) and overweight/obese (n = 4 [4.3%]). Overall, activated CEC ranged from 0 to 100% with a full spectrum of values represented.

Table 2. Comparison of CEC enumeration and activated CEC (%VCAM-1 expression) between visits, split by adiposity groups.				
	Baseline	1 week post-baseline	Difference (95% CI)	p-value
CEC enumeration				
Normal weight (n = 42)	1.05 (1.48)	0.91 (1.54)	-0.095 (-0.67, 0.48)	0.740
Overweight/obese (n = 46)	1.37 (3.05)	1.40 (2.17)	0.065 (-1.12, 1.25)	0.912
Severe obesity (n = 50)	1.23 (2.56)	1.49 (2.70)	0.280 (-0.81, 1.37)	0.607
Overall (n = 138)	1.22 (2.47)	1.28 (2.22)	0.094 (-0.48, 0.67)	0.745
Activated CEC (%VCAM-1 expression)				
Normal weight (n = 38)	54.8 (27.7)	57.4 (22.5)	3.3 (-7.1, 13.6)	0.527
Overweight/obese (n = 42)	54.7 (23.1)	59.4 (24.0)	4.9 (-4.0, 13.8)	0.272
Severe obesity (n = 48)	61.0 (22.7)	57.4 (18.1)	-3.2 (-11.2, 4.8)	0.425
Overall (n = 128)	57.1 (24.4)	58.0 (21.3)	1.4 (-3.7, 6.5)	0.592
Data are presented as mean $\pm$ SD. p-values were determined using paired t-test.

Figure 1. Modified Bland-Altman plot of CEC enumeration (A) and activated CEC (B) between visits.

Discussion

This is the first study to examine the reproducibility of CEC enumeration and activated CEC in a pediatric

population. Our findings suggest that, on an overall cohort basis, both CEC enumeration and activated CEC are reproducible among children and adolescents.

Figure 2. CEC enumeration (A) and activated CEC (B) by BMI category.

Furthermore, values tended to be reproducible through a wide BMI range, which is important considering the high prevalence of obesity in youth [23]. We observed low CV% values but modest ICC values for both CEC

enumeration and activated CEC, suggesting reasonable consistency/stability over time. Therefore, CECs may serve as a useful biomarker within the context of the research setting. However, the generally low numbers of CECs found in our cohort and the high degree of individual variability between measurement time-points may limit its clinical utility as a diagnostic measure in youth.

Consistently elevated values for CEC enumeration, which might be indicative of subclinical CVD risk [3], were not present in this population of youth. We observed a relatively narrow range of values for CEC enumeration (0–20 cells; mean 1.25 ± 2.37), the maximum value recorded was only 20 cells. Data from adults using similar methodology (immunobeads) report ranges of CEC enumeration from 0 to 56 cells (mean 13.2 ± 11.8) for patients with sickle anemia [4] and from 2 to 72 cells (mean 27.2 ± 6.9) for patients with secondary pulmonary hypertension [24]. We did observe transient elevations in CEC levels; however, elevated baseline values ($n = 4$, CEC >5) were all within the 0–5 range at the 1-week follow-up time-point, conversely elevated values at 1-week follow-up ($n = 8$, CEC >5) were not observed at baseline with no participants showing elevated levels at both measurements. The transient nature of CEC enumeration has been documented in patients with acute coronary symptoms [11,13], thus potentially indicative of acute inflammation or vascular injury being responsible for the elevated levels rather than a chronic condition [3]. Therefore, the lack of consistently elevated levels for CEC enumeration suggest that even in youth with severe obesity, a disease associated with serious cardiovascular comorbidities [17], numbers are relatively low.

Our group previously reported that activated CEC are elevated in the context of pediatric severe obesity [16]. In the current study, on a cohort basis, we observed activated CEC to be consistent across time-points; however, similar %VCAM-1 expression values between baseline and follow-up were rarely observed. Only 13 (10.2%) participants had the same value at baseline and follow-up and only 98 (76.6%) had values within $\pm 20\%$ with a wide degree of variation ranging from +80 to -60% between visits. This high degree of individual variability could be explained by normal physiological fluctuations, acute inflammation, dietary changes, exercise, acute mental stress and/or assay limitations or variability with measuring activated CEC. Regarding the latter, the %VCAM-1 expression is calculated as a ratio of the number of CEC positive for VCAM-1 divided by the total population of CEC within a given sample of blood [4]. Therefore, the denominator used in this calculation dictates the number of possible values the %VCAM-1 expression can

be. To address this issue, we chose to exclude results derived from denominators of less than 3 ($n = 6$ [3 at baseline and 3 at follow-up]). However, our choice was admittedly arbitrary and places practical limits on the precision of our results (e.g., a denominator of 4 limits the possible values of %VCAM-1 expression to 0, 25, 50, 75 and 100%) increasing the error of measurement and decreasing the reliability of data. However, despite these constraints, our within-assay precision for activated CEC was 16.9% (CV%), similar to that of fasting insulin [25], which is commonly-used in the clinical setting [26].

Our study has many strengths including a clinically-relevant design, a population with a wide range of BMI values, age, and stages of pubertal maturation, and the largest sample size examining the reproducibility of these markers of endothelial cell biology to date. We are limited by the utilization of only 2 arbitrary time-points of measurements, inability to control for menstrual cycle variation, lack of inclusion of patients whom might have persistently elevated CEC (i.e., youth with Kawasaki's disease [10]), a lack of control or comparison group and inability to control for transient conditions which may influence endothelial cell biology (i.e., physical activity) [27]. Further research is needed in order to determine the impact of exercise and its timing on CEC stability. Future studies which compare pediatric and adult CEC values are needed for translation across the lifespan. Due to the limited samples size we were unable to determine racial or pubertal differences which should be considered for generalizability.

Conclusion

In conclusion, these data support that on a research cohort basis, CEC enumeration and activation are reproducible in children and adolescents, suggesting these biomarkers may be useful within the context of the research setting. However, on an individual basis, the high biological variability of CEC enumeration and activation, coupled with the methodological limitations in measurement, may limit the clinical utility of these biomarkers in youth.

Disclaimer

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Executive summary

Introduction

- Circulating endothelial cells (CEC) have potential as biomarkers of cardiovascular disease risk in children and adolescents.
- Reproducibility of CECs in youth is unknown and an important consideration for any potential biomarker.

Methods

- We examined 151 youth at two time-points separated by 7–10 days to examine consistency of measurements.
- CEC enumeration and activation (%VCAM-1) expression were measured.

Results

- No statistically significant differences between time points were observed on the group as a whole or within different obesity groups.
- High individual variations for elevated CEC levels was observed between time points.

Discussion

- On a research cohort basis, CEC enumeration and activation are reproducible in children and adolescents.
- High biological variability of CEC enumeration and activation, coupled with the methodological limitations in measurement, may limit the clinical utility.

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