Reproducibility of endothelial microparticles in children and adolescents

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Aim: We assessed reproducibility of endothelial microparticles (EMP) enumeration among youth. **Methods & results:** Four microparticle (MP) indices – total MP per microliter platelet free plasma (PFP), total EMPs per microliter PFP, percent activated EMPs and percent lactadherin positive (LACT[+]) of total EMPs – were measured at two visits (baseline and 7 ± 3 days follow-up) to determine reproducibility overall and by obesity status. We examined CD31⁺ or CD144⁺ with CD41-EMP events of size 0.3–1.0 µm. No statistically significant differences were observed between visits for any of the four MP indices. The within-participant and between-participant coefficient of variation was acceptable (range: 1.13–2.37) with good intraclass-correlation coefficient for all indices except total MP per microliter (range: 0.10–1.00). **Conclusion:** Total EMPs per microliter PFP, percent-activated EMPs and percent LACT(+) of total EMPs are reproducible among youth.

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Cell-derived endothelial microparticles (EMP) via endothelial blebbing can be detected in the plasma and may be an early biomarker of adverse endothelial activation, damage or apoptosis [1–3]. Higher levels of EMPs have been associated with numerous cardiovascular risk factors in adults, including acute coronary syndromes [4], endothelial dysfunction [5], increased arterial stiffness [6], adverse carotid artery remodeling [7], higher levels of inflammatory cytokines [8] and metabolic syndrome [9,10]. Furthermore, studies have shown adults who are overweight or with obesity have higher EMP levels and that EMPs may predict cardiovascular disease (CVD) mortality better than traditional CVD markers and risk scores [5,9–11]. A limited amount of data exist in the pediatric population. Existing EMP studies in children and adolescents have shown increased EMPs in Kawasaki disease [12], familial Mediterranean fever [13], Type 1 diabetes [14] and dialysis or predialysis patients [15]. Similar to data from adult studies, children with overweight and obesity have significantly higher EMP levels [16].

Based upon the available adult data and emerging pediatric evidence, EMPs may have utility as an early biomarker of CVD risk. In order for a biomarker for overt or subclinical CVD risk to be useful, the measurement must have evidence of predicting vascular events from cohort studies [17]. An additional crucial step in establishing biomarker utility is having low variability and high reproducibility between similar measurement time points [17]. Moreover, a biomarker should have similar variability among different metabolic phenotypes (e.g., obesity, diabetes, hypertension) at similar stages of senescence. Due to obesity's influence on CVD risk factors [18], establishing reproducibility of EMPs within different adiposity groups of children is critical for any potential biomarker.

As an initial step toward establishing the utility of EMPs as a biomarker, we designed the following study to examine the reproducibility of EMP enumeration in a pediatric population with a large range of BMI values. We

collected total microparticles (MPs) by the size of particles ranging from 0.3 to 1.0 μm with or without positive expression of FITC-lactadherin (LACT[+] or LACT[-]). We then examined four different MP measurements to assess reproducibility: total MP per microliter platelet-free plasma (PFP), total EMPs per microliter PFP, percent active EMPs of total EMPs and percent LACT (+) of total EMPs. MPs were measured at two time points, approximately 1 week apart, in youth with a range of adiposities. We *a priori* hypothesized that EMPs and MP would have high reproducibility defined by an intraclass-correlation coefficient (ICC) >0.75 [19]. We assessed reproducibility across the entire cohort and within obesity classification groups.

Materials & methods

A total of 107 children and adolescents from a larger cross-sectional study were recruited for this short-term reproducibility assessment. Participants with obesity ($n = 25$) and severe obesity ($n = 40$) were primarily recruited from the University of Minnesota Masonic Children's Hospital Pediatric Weight Management Clinic. Participants with normal weight ($n = 32$) and overweight ($n = 10$) were recruited from the surrounding community. The larger study stratified recruitment by adiposity with overweight and obesity combined as one stratum. Participants using various medications, including vasodilators, were excluded and no pharmacotherapy intervention was administered. Using this approach, participants with overweight and obesity were combined when determining reproducibility.

To characterize short-term variability of EMPs and MP, each participant had two visits: baseline and another visit 1 week later (7 ± 3 days). At each visit, a fasting (>8 h) blood sample was taken and measured for EMPs. BMI category was determined using sex, age, height and weight by the US CDC guidelines. Normal weight is $<85^{\text{th}}$ BMI percentile, overweight is $\geq 85^{\text{th}}$ and $<95^{\text{th}}$ percentile, obesity is $\geq 95^{\text{th}}$ percentile and severe obesity is $>1.2 \times 95^{\text{th}}$ percentile or BMI ≥ 35 kg/m² [2]. Height was measured using a wall-mounted stadiometer and weight was determined via an electronic scale. Other demographic variables including age, race and tanner stage (determined by a trained physician or registered nurse) were recorded. All demographic and BMI-related measurements were taken at baseline, but not at follow-up.

Endothelial microparticle measurement & characterization

Whole venous blood was collected and processed for PFP collection less than 3 h after being drawn. PFP samples were prepared using serial centrifugations and then frozen as 100 μl aliquots at -80°C . The measurement of MP was performed by using the flow cytometric method described by Nielsen *et al.* [20] on a BD FACSAria™ III High Speed Cell sorter (BD Biosciences, CA, USA). To quantitate MPs, the TruCount™ tube (BD Bioscience) was used for each sample for fluorescent labeling and flow cytometry analysis. FITC-conjugated lactadherin (Hematologic Technologies Inc., VT, USA) was used to identify phosphatidylserine on outer membrane of MP (LACT+). Antibodies to identify endothelial cell-origin MP were (AF647)-conjugated mouse anti-human CD31, (PerCP-Cy5.5)-conjugated mouse anti-human CD144. Antibodies to identify activated endothelial cell-origin MP was (PE)-conjugated mouse anti-human CD106. Antibody to identify platelet-origin MP was (APC-H7)-conjugated mouse anti-human CD41a. All the fluorescence-conjugated mouse anti-human antibodies were purchased from BD Bioscience. Before running the PFP samples, MP-size gate was established according to Neilsen's protocol by using a mixture of size-calibrated fluorescent beads with the sizes of 0.1 μm (Invitrogen, CA, USA), 0.2 μm (Invitrogen), and Megamix beads (0.5, 0.9 and 3.0 μm ; Biocytex, Marseille, France). MPs size ranging from 0.3 to 1.0 μm with or without staining with lactadherin were collected for analysis. We defined EMP as MP with CD31(+)/CD41(-) plus MP with CD 144(+)/CD31(-). Active EMP was identified as EMP with CD 106 (+). We then calculated the total MP per microliter PFP, total EMPs per microliter PFP, percent active EMPs of total EMPs and percent LACT (+) of total EMPs. A single-flow cytometry technician analyzed all samples from both time points while being blinded to the adiposity group of each participant which precludes the need to assess intertester reproducibility.

Statistical methods

Descriptive statistics were calculated by BMI category with mean and standard deviation (SD) along with median and interquartile range (IQR) for continuous variables or n and percentage for categorical variables. One outlier was omitted for total EMPs for an extreme follow-up value of 19.3 thousand/ μl PFP (17.2 SD above the mean). We used a paired t test to compare average difference in measurements between baseline and follow-up. ICC were determined using generalized linear mixed-effects Poisson models with log link on original scales, which accounts for overdispersion [21]. CV% for between-subject and within-subject ratios, defined by SD over the mean, were

Table 1. Baseline participant characteristics for each obesity group.

Characteristics	Overall (n = 107)	Normal weight (n = 32)	Overweight/obese (n = 35)	Severe obesity (n = 40)
Male	53 (49.5%)	20 (62.5%)	17 (48.6%)	16 (40.0%)
Tanner stage:				
– 1	26 (24.3%)	12 (37.5%)	9 (25.7%)	5 (12.5%)
– 2	21 (19.6%)	5 (15.6%)	4 (11.4%)	12 (30.0%)
– 3	19 (17.8%)	4 (12.5%)	8 (22.9%)	7 (17.5%)
– 4	28 (26.2%)	8 (25.0%)	8 (22.9%)	12 (30.0%)
– 5	13 (12.1%)	3 (9.4%)	6 (17.1%)	4 (10.0%)
Race:				
– Asian	5 (4.7%)	1 (3.1%)	3 (8.6%)	1 (2.5%)
– African American	13 (12.1%)	3 (9.4%)	3 (8.6%)	7 (17.5%)
– White	78 (72.9%)	27 (84.4%)	25 (71.4%)	26 (65.0%)
– Multiple races selected	11 (10.3%)	1 (3.1%)	4 (11.4%)	6 (15.0%)
Age (years)	12.7 (2.53)	12.8 (2.48)	12.5 (2.39)	12.6 (2.73)
BMI	27.4 (8.1)	18.7 (2.12)	26.3 (3.41)	35.4 (6.0)

Data are presented as n (%) or mean (SD) where appropriate.

calculated for each outcome to examine the variability of the measurements. Bland Altman plots were created to assess reproducibility across EMP values. Scatterplots stratified by obesity status were created to visually present the distribution and spread of values within each group. All analyses were conducted using R version 3.4.0

Results

MP and EMPs levels were examined in 107 children and adolescents (mean age = 12.8 ± 2.5; female n = 54; mean BMI 27.4 ± 8.1 kg/m²) at both time points. Tanner stage and race were reasonably similar across all obesity groupings, with majority of participants being white (Table 1). The variability of total MP was lowest for the normal weight group (baseline SD = 69.1/μl PFP in thousands; baseline IQR = 12.1/μl PFP in thousands) compared with the overweight/obesity (baseline SD = 92.3/μl PFP in thousands; baseline IQR = 60.8/μl PFP in thousands) and severe obesity groups (baseline SD = 205.2/μl PFP in thousands; baseline IQR = 150.1/μl PFP in thousands) participants. For percent LACT (+) of total EMPs, participants with severe obesity displayed the least variability (baseline SD = 19.2; baseline IQR = 9.9) as compared with participants in the overweight/obesity (baseline SD = 27.4; baseline IQR = 9.3) and normal weight groups (baseline SD = 38.2; baseline IQR = 64.1), though IQR was comparable between severe obesity and overweight/obesity. While baseline variability of total EMPs was relatively constant across groups (SD range: 870–1.079/μl PFP), the median and mean level of total EMPs was much lower in the normal weight group (baseline mean = 617/μl PFP; median = 32/μl PFP) as compared with the overweight/obesity (baseline mean = 1483/μl PFP; median = 1536/μl PFP) and severe obesity groups (baseline mean = 1424/μl PFP; median = 1475/μl PFP). Similarly, percent active EMPs of total EMPs demonstrated consistent variability across adiposity groups (SD range: 40.0–42.9%), but participants with normal weight had much lower mean and median values (baseline mean = 26.1%; median = 2.6%) as compared with those with overweight/obesity (baseline mean = 68.2%; median = 91.0%) and severe obesity (baseline mean = 60.9%; median = 86.9%; Table 2).

The between-participant CV% was similar for the overweight/obesity group and the severe obesity group for all outcomes (total MP = 1.52 and 1.51/μl PFP; total EMPs = 0.69 and 0.60/μl PFP; percent active EMPs of total EMPs = 0.63 and 0.64; and percent LACT [+] of total EMPs = 1.39 and 1.44 for overweight/obesity and severe obesity respectively). Between-participant CV% in the normal weight group was higher than both other groups for total MP, total EMPs and percent activated EMPs and lower for percent LACT (+) of total. Within-participant CV% showed less discrepancies between obesity groups, except for percent activated EMPs of total EMPs where the normal weight within-participant CV% (CV% = 0.66) was much higher than that of overweight/obesity (CV% = 0.13) or severe obesity (CV% = 0.16). As for measuring ICC, total MP had the lowest ICC at 0.42, while all other indices had ICC values of 0.84, 0.95 and 0.87 for total EMPs, percent active EMPs of total EMPs and percent LACT (+) of total EMPs, respectively (Table 3). A similar analysis stratified by sex found that the ICC for total MP was higher for males than females (0.534 and 0.248, respectively) and that females have a higher

Table 2. Mean (SD) followed by median [IQR] for each endothelial microparticle measurement at baseline and follow-up visit along with the mean difference and 95% CI and corresponding p-value.

Outcome	n (in thousands)	Baseline	Week 1	Difference of means, baseline-week 1 (95% CI)	p-value
Total MP/μl PFP					
Overall	107	76.8 (148.2) 17.0 [70.4]	76.6 (121.9) 30.7 [99.7]	0.2 (-28.0–28.4)	0.990
Normal weight	32	24.7 (69.1) 2.0 [12.1]	26.7 (52.5) 4.1 [14.9]	-2.0 (-26.1–22.2)	0.869
Overweight/obese	35	55.5 (92.3) 29.1 [60.8]	102.5 (139.3) 73.6 [135.8]	-47.0 (-100.6–6.6)	0.083
Severe obesity	40	137.1 (205.2) 52.7 [150.1]	94.0 (135.3) 45.5 [100.1]	43.2 (-12.5–98.8)	0.125
EMPs/μl PFP					
Overall	106	1200 (1051) 1236 [2067]	1183 (1038) 1254 [2085]	17 (-99–132)	0.773
Normal weight	32	617 (1079) 32 [365]	482 (922) 41 [186]	135 (-78–349)	0.206
Overweight/obese	35	1483 (1020) 1536 [1755]	1571 (1086) 1619 [2141]	-88 (-289.1–113)	0.380
Severe obesity	39	1424 (870) 1475 [1796]	1410 (788) 1414 [989]	14 (-186–214)	0.889
% Active EMPs of total EMPs					
Overall	107	52.9 (44.6) 84.1 [88.2]	53.6 (45.3) 87.4 [91.4]	-0.8 (-3.5–1.9)	0.565
Normal weight	32	26.1 (41.1) 2.6 [32.3]	23.7 (39.4) 2.9 [914.6]	2.4 (-4.1–8.9)	0.462
Overweight/obese	35	68.2 (42.9) 91.0 [93.6]	68.1 (43.0) 91.1 [94.60]	0.1 (-1.3–1.4)	0.901
Severe obesity	40	60.9 (40.0) 86.9 [85.3]	64.9 (41.0) 89.4 [86.6]	-4.1 (-9.1–0.9)	0.108
% LACT (+) of total EMPs					
Overall	106	31.5 (36.5) 11.0 [51.7]	29.8 (36.4) 10.0 [34.5]	1.7 (-2.0–5.3)	0.361
Normal weight	32	65.8 (38.2) 89.5 [64.1]	63.5 (39.1) 88.6 [71.5]	2.3 (-4.5–9.2)	0.495
Overweight/obese	35	20.2 (27.4) 8.2 [9.3]	20.3 (29.2) 7.1 [11.10]	-0.1 (-8.7–8.5)	0.982
Severe obesity	39	13.5 (19.2) 8.2 [9.9]	10.8 (15.4) 6.0 [8.3]	2.8 (-0.9–6.4)	0.139

Data are mean (SD) followed by median [IQR]. p-value was determined by a paired t-test.
EMP: Endothelial microparticle; IQR: Interquartile range; MP: Microparticle; PFP: Platelet-free plasma.

between-participant CV% for percent LACT (+) of total (CV% = 1.322) compared with males (CV% = 1.076). All other CV% and ICC results were comparable between males and females.

For total MP, 86 participants (80.4%) had differences less than 100,000 events (Figure 1A). The difference in EMPs was less than 100 for about half of the participants with a cluster of high mean counts exhibiting little to no change between visits (Figure 1B). All percent active EMPs of total EMPs measurements were <20% or >70% at both time points. Most participants had little change between visits with the exception of two participants who switched between near 0 and near 100% at baseline and follow-up, resulting in absolute differences near 100% (Figure 1C). Percent LACT (+) of total EMPs were also typically near 0 or 100%, but resulted in 13 participants (12.1%) with mean percentage between 30 and 70 (Figure 1D).

The range and mean of total MP increased with obesity status (Figure 2A). Youth with normal weight were more likely to have low values of EMPs compared with those with overweight/obesity and severe obesity. However, all three groups displayed similar maximum values of EMPs around 3000 (Figure 2B). For percent activated EMPs of total EMPs, about 75% of those with normal weight had values near 0%, unlike those with overweight/obesity or severe obesity for whom less than 50% of measurements were near 0% (Figure 2C). Measurements for percent LACT

Table 3. Intraclass correlation coefficient and between- and within-participant coefficient of variation for endothelial microparticle outcomes across obesity categories.

Outcome	Overall	Normal weight	Overweight/obese	Severe obesity
Total MP/μl PFP				
ICC	0.42	0.42	0.10	0.49
Within-participant CV%	0.55	0.55	0.53	0.58
Between-participant CV%	1.77	2.37	1.52	1.51
EMPs/μl PFP				
ICC	0.84	0.82	0.85	0.74
Within-participant CV%	0.30	0.41	0.26	0.26
Between-participant CV%	0.88	1.82	0.69	0.60
% Active EMPs of total EMPs				
ICC	0.95	0.90	1.00	0.92
Within-participant CV%	0.29	0.66	0.13	0.16
Between-participant CV%	0.84	1.61	0.63	0.64
% LACT (+) of total EMPs				
ICC	0.87	0.88	0.62	0.78
Within-participant CV%	0.29	0.21	0.35	0.31
Between-participant CV%	1.19	0.59	1.39	1.44

EMP: Endothelial microparticle; ICC: Intraclass-correlation coefficient; MP: Microparticle; PFP: Platelet-free plasma.

(+) of total EMPs tended to be higher for in the group with normal weight as compared with the other groups. Over half of all normal weight observations were above 80%, while less than 25% of those with overweight/obesity or severe obesity were above 80% (Figure 2D).

Discussion

Our results support that three of the four indices – total EMPs, percent activated EMPs and percent LACT (+) of total EMPs, but not total MPs – are reproducible in children and adolescents. This finding was consistent across obesity groups with acceptable or high ICCs in each group. Total MP did not meet reproducibility standards due to high within-participant and between-participant CV% among the overall group, suggesting that total EMPs are more reliable than total MP. These data support the potential utility of total EMPs as a biomarker of CVD risk in the pediatric population as total EMPs appear to be reproducible and may reflect early CVD-related pathophysiological changes.

Our ICC analysis supported that total EMPs, percent activated EMPs and percent LACT (+) of total EMPs have overall ICC values >0.75 , suggesting at least good reliability [19]. Percent active EMPs of total EMPs has excellent reliability with an overall ICC value of 0.95 and an ICC value for each BMI category >0.9 [19]. Previously, we observed that circulating endothelial cells were reasonably reproducible with an overall ICC of 0.70. However, the circulating endothelial cell reproducibility estimate was inferior to all three reproducibility estimates for EMPs indices, suggesting EMPs may have more utility as a clinical biomarker [22]. All three MP outcomes showed similar reproducibility compared to common measures such as fasting insulin (ICC = 0.9). Percent active EMPs of total EMPs displayed the highest reproducibility, similar to highly reproducible clinical measures such as fasting glucose (ICC = 0.95) [23].

Perhaps the most unique measures were percent active EMPs of total EMPs and percent LACT (+) of total EMPs, which had dichotomized values either near 0% (range = 0.0–20.6%) or near 100% (range = 77.0–99.7%) for all participants at each time point. Moreover, percent activated EMPs showed remarkable consistency between measurement time points with only two out of 107 observations differing substantially between time points. This suggests that the percent active EMPs tend to be relatively constant and highly reproducible both within- and between-participants over a 1-week follow-up. Whether the lower or higher percent active EMPs confers higher or lower CVD risk requires further evaluation. LACT-positive MPs had shown to be involved in the venous thromboembolism [24]. Recently, Kou's study [25] also suggested that phosphatidylserine positive MP (LACT [+]) may play a critical role in hypercoagulability state in heart failure. Perhaps the level of LACT (+) of EMPs may indicate the degree of CVD risk in the pediatric population. Additionally, more research is needed to understand

Figure 1. Bland Altman plots for endothelial microparticle outcomes. Bland Altman plot showing the reproducibility of 1-week apart total MP per microliter PFP (A), total EMPs per microliter PFP (B), percent active EMPs (C) and, percent LACT (+) of EMPs (D). Mean difference between baseline and follow-up is indicated by the dotted line and obesity status is indicated as labeled.

EMP: Endothelial microparticle; MP: Microparticle; PFP: Platelet-free plasma.

what causes this ‘all-or-nothing’ type of active EMPs or LACT (+) presence, as well as the implications for inference from having a high or low percentage.

When compared with existing adult studies, our EMPs values differed, but possibly due to difference in methodology. Adult studies have identified a trend of increased EMPs as adiposity increases [5,9,10]. Figure 2B shows a similar result, with normal weights having lower EMPs counts than higher adiposity groups. Our results had increased variability, but similar means compared with some adult studies [6,8]. In others that examined EMPs per microliter, our results were much higher [26,27]. When comparing methodologies across EMPs studies, methods to measure EMPs have varied by antigenic profiles and platelet poor or free plasma among others. This may be due to advancement in cytometers which has allowed for increased accuracy and detection of MPs and EMPs [28]. To ensure EMPs reproducibility, a consensus on how to define EMPs and what method to use to measure needs to be established.

Strengths of our study include a clinically-relevant design, utilization of a cohort with a large range of BMI values and a relatively large sample size at multiple time points. We had participants from a variety of ages, pubertal maturation and race. However, we arbitrarily decided on two time points 1-week apart, which is a potential

Figure 2. Distribution of endothelial microparticle outcomes. Displays individual responses for each adiposity group of total MP per microliter PFP (A), total EMPs per microliter PFP (B), percent active EMPs (C) and, percent LACT (+) of EMPs (D). Summary measures for each variable and group are depicted as indicated. EMP: Endothelial microparticle; MP: Microparticle; PFP: Platelet-free plasma.

limitation to our study. Further research is needed to determine EMPs stability across the lifespan and to compare pediatric and adult EMPs associations with various conditions and diseases.

Conclusion

In conclusion, our results support that on a cohort basis, total EMPs, percent activated EMPs and percent LACT (+) of EMPs are reproducible in children and adolescents. Reproducibility is consistent across obesity groups with low and acceptable within- and between-participant variability. Total MP failed to meet reproducibility guidelines. Further evaluation is needed to determine whether total EMPs, percent active EMPs or percent LACT (+) of EMPs confer greater CVD risk in the pediatric population.

Author contributions

All authors have seen, approved, and take full responsibility for the manuscript. JR Ryder, KD Rudser and AS Kelly developed the aims and hypothesis. AS Kelly provided the funding. KD Rudser, AM Kaizer and EF Northrop performed the statistical analysis. EF Northrop wrote the first draft. LC Milbauer, KD Rudser, CK Fox, AN Solovey, AM Kaizer, RP Hebbel, AS Kelly and JR Ryder provided subject matter expertise and critical review of the manuscript.

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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, informed consent has been obtained from the participants involved.

Summary points

Introduction

- Endothelial microparticles (EMP) are a potential cardiovascular disease biomarker in adults with little research conducted in youth.
- Reproducibility of EMPs will need to be established as part of the determining validity in the pediatric population.

Methods

- A total of 107 children and adolescents from a larger study were recruited for baseline and 1-week follow-up to assess reproducibility.
- Total microparticles (MP) per μ l platelet-free plasma (PFP), EMPs per microliter PFP, percent active EMPs of total EMPs and percent lactoferrin-positive (LACT [+]) of total EMPs were measured.

Results

- Between visits there was limited change in each of the four MP indices overall or within any obesity status.
- Low and acceptable variability was observed with ICC >0.75 for all MP indices except total MP per microliter.

Discussion

- EMPs per microliter PFP, percent activated EMPs of total EMPs and percent LACT(+) of total EMPs were found to be reproducible in a cohort setting among children and adolescents and consistent across obesity groups.
- Total MP per microliter PFP failed to meet reproducibility guidelines due to high within- and between-participant variability.

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