Is the ratio of flow-mediated dilation and shear rate a statistically sound approach to normalization in cross-sectional studies on endothelial function?

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[1]*Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, United Kingdom;* [2]*Department of Physiology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands;* [3]*School of Sport Science, Exercise and Health, The University of Western Australia, Crawley, Australia; and* [4]*Health and Social Care Institute, School of Health and Social Care, Teesside University, Middlesbrough, United Kingdom*

Atkinson G, Batterham AM, Black MA, Cable NT, Hopkins ND, Dawson EA, Thijssen DH, Jones H, Tinken TM, Green DJ. Is the ratio of flow-mediated dilation and shear rate a statistically sound approach to normalization in cross-sectional studies on endothelial function? *J Appl Physiol* 107: 1893–1899, 2009. First published October 15, 2009; doi:10.1152/japplphysiol.00779.2009.—It has been deemed important to normalize flow-mediated dilation (FMD), a marker of endothelial function, for between-subject differences in the eliciting shear rate (SR) stimulus. Conventionally, FMD is divided by the area under the curve of the SR stimulus. In the context of a cross-sectional comparison across different age cohorts, we examined whether this ratio approach adhered to established statistical assumptions necessary for reliable normalization. To quantify brachial artery FMD and area under the curve of SR, forearm cuff inflation to suprasystolic pressure was administered for 5 min to 16 boys aged 10.9 yr (SD 0.3), 48 young men aged 25.3 yr (SD 4.2), and 15 older men aged 57.5 yr (SD 4.3). Mean differences between age groups were statistically significant ($P < 0.001$) for nonnormalized FMD [children: 10.4% (SD 5.4), young adults: 7.5% (SD 2.9), older adults: 5.6% (SD 2.0)] but not for ratio-normalized FMD ($P = 0.10$). Moreover, all assumptions necessary for reliable use of ratio-normalization were violated, including regression slopes between SR and FMD that had *y*-intercepts greater than zero ($P < 0.05$), nonlinear and unstable relations between the normalized ratios and SR, skewed data distributions, and heteroscedastic variance. Logarithmic transformation of SR and FMD before ratio calculation improved adherence to these assumptions and resulted in age differences similar to the nonnormalized data ($P = 0.03$). In conclusion, although ratio normalization of FMD altered findings about age differences in endothelial function, this could be explained by violation of statistical assumptions. We recommend that exploration of these assumptions should be routine in future research. If the relationship between SR and FMD is generally found to be weak or nonlinear or variable between samples, then ratio normalization should not be applied.

ratios; statistical assumptions

IT IS NOW well-documented that the endothelium has a pivotal role in vascular health, via its influence on vasomotor, thrombotic, inflammatory, and cellular proliferation pathways (12, 35). Endothelial dysfunction has been reported to predict clinical outcomes among patients with mild coronary artery disease (32) and peripheral vascular disease (16). Importantly, endothelial dysfunction may develop decades before clinical presentation in patients with hypercholesterolemia (9), dia-

betes (10), and smoking (8). Recently, it was found that endothelial dysfunction is associated with the progression of carotid intima-media thickness in late-middle-aged individuals studied over a 6-yr period (18), supporting the notion that measurements related to the endothelium predict disease evolution and can aid in the evaluation of treatment strategies.

The flow-mediated dilation (FMD) technique provides valuable information about endothelial function of conduit arteries. It is desirable for efficient clinical practice that such information is gained from one measurement of FMD in each patient. When the appropriate methods are employed (17, 20, 21), the FMD response is predominantly mediated by nitric oxide (NO). Recent attention has focused on whether measurements of FMD should be normalized for the shear rate (SR) stimulus, given that physical pressure-related manipulations of SR appear to induce proportional within-subjects changes in FMD (29–31). Nevertheless, the presence of a within-subjects correlation between shear and FMD is not a necessary assumption at all for reliable statistical analyses in cross-sectional studies (13). If the influence of SR on FMD is substantial in a between-subjects context and not controlled for with appropriate "normalization" methods, then the utility of FMD for predicting clinical outcomes in cross-sectional studies could be compromised. Therefore, important questions have arisen recently regarding the most relevant indexes of SR and the most appropriate statistical approaches to adopt within this normalization process.

One question that has been explored with a within-subjects study design is whether the peak SR or the area under the curve of SR is the most influential stimulus for FMD (30). When FMD was divided by each of these indexes, the area under the curve of SR was found to provide the strongest correlation with changes in FMD. Consequently the ratio of FMD and area under the curve of SR has been the preferred normalization approach in recent studies (25, 26). Another question that has arisen, but not yet been examined, is whether other statistical approaches such as analysis of covariance (ANCOVA) are more appropriate for normalization of FMD than the simple ratio of FMD and SR (19). It has been advised that future researchers should check that different approaches to normalization do not alter data interpretation, and that underlying assumptions for accurate statistical analysis are upheld (34). To date, these important issues within this measurement dilemma have not been examined.

Violation of underlying statistical assumptions can lead to serious errors in data interpretation and study conclusions (5, 13). The relevant assumptions for the acceptable use of ratios

to normalize data in cross-sectional group comparisons have been presented (2), yet never applied to FMD data before now. According to Allison et al. (2), normalization via division of one variable (e.g., FMD) by another (e.g., SR) is valid only if *1*) the relationship between SR and FMD is linear, *2*) the intercept for the regression slope of this relationship between SR and FMD is zero, *3*) data (including the residuals from any inferential test) are normally distributed, *4*) variances are similar (homogeneous) between groups, and *5*) the ratio does not lead to spurious correlations with other variables. Violation of *assumptions 1* and *2* means that the normalization process would be biased, i.e., the ratio is not fully or consistently controlling for the effects of the denominator. Violation of *assumptions 3* and *4* could lead to false inferential claims, i.e., incorrect rejections (or nonrejections) of statistical null hypotheses and imprecise estimations of effect sizes. Violation of *assumption 5* leads to errors in interpreting relations between physiological outcomes and their proposed moderators (4). Our overall aim is to present the first formal exploration of the ratio approach to normalization of FMD data in the context of a between-subjects analysis. Our two objectives are to examine the degree of adherence to underlying statistical assumptions, including those relevant to comparing ratios between samples (2) and to examine how conclusions in a cross-sectional study are influenced by ratio normalization of FMD.

MATERIALS AND METHODS

Participants. The male participants involved in the present study were selected from the sample of male and female individuals studied previously for an exploration of the general magnitude of correlations between different indexes of SR and FMD (33). In brief, 79 healthy males were allocated to three samples of children (n = 16, 9–10 yr old), young adults (n = 48, 20–39 yr old), and older people (n = 15, 50–64 yr old). These datasets are particularly relevant to the present topic, since FMD normalization has been deemed especially important for between-subjects analyses (34), and the presence of group differences in sample size make adherence to assumptions underlying the use of ratios even more important (2). Participant characteristics are presented in Table 1. All participants were nonsmokers and were normotensive (<140/90 mmHg), and none had any history of diabetes, insulin resistance, or cardiovascular disease or were taking any cardiovascular-relevant medications. The study was approved by the local ethics committee and the procedures adhered to the Declaration of Helsinki. All adult participants provided written informed consent, and a parent or guardian provided written informed consent for each of the children who participated in the study.

Study design. This study was of the cross-sectional and correlational descriptive type (33). After reporting to the laboratory between 0900 and 1600, participants rested for at least 20 min and then the brachial artery FMD response was measured. Although FMD has been found to be attenuated immediately after waking compared with other times of day, it is relatively stable within the data collection period adopted in our study (24, 25) when controls over prior posture and diet are sound. All measurements were obtained under standardized conditions in a quiet, temperature-controlled room and after at least 6 h of fasting and at least 8 h of abstinence from caffeine or alcohol. No participant performed strenuous physical activity for at least 24 h before testing, since exercise can result in a transient change in FMD (11). In addition, the distribution of test times was random for participants within all three samples.

Brachial artery FMD. As detailed in full by Thijssen et al. (33), patients rested supine with the right arm extended and immobilized with foam, supported at an angle of ~80° from the torso. Heart rate and mean arterial pressure was determined from an automated sphygmomanometer (Dinamap; GE Pro 300V2, Tampa, FL) on the contralateral arm. For assessment of the FMD response, a rapid inflation/deflation pneumatic cuff was positioned on the imaged arm distal to the olecranon process to provide a stimulus to forearm ischemia. The brachial artery was imaged in the distal third of the upper arm. After a resting period of at least 20 min, the brachial artery diameter and velocity were measured at baseline for 1 min. Subsequently, the occlusion cuff was inflated to >200 mmHg for 5 min. Brachial artery diameter and velocity recordings were made at least 30 s before cuff deflation and continued for at least 3 min after deflation. Peak artery diameter and flow, and the time to reach peak diameter after cuff deflation, were recorded.

Brachial artery diameter and blood flow. Brachial artery diameters were analyzed using custom-designed edge detection and wall-tracking software, which is independent of investigator bias (37). From synchronized measurements of diameter and velocity data, blood flow [the product of cross-sectional area (CSA) and Doppler velocity (v)] and SR (4 times velocity divided by diameter) were calculated at 30 Hz. Reproducibility of the diameter measurements using the semiautomated software is significantly better than manual methods, reduces observer error significantly, and possesses an intraobserver coefficient of variation of 6.7% (37).

Table 1. *Subject characteristics of the participants (n = 79), who were allocated to groups of children, young men, and older men*

Measured Variable	Children (C) (n = 16)	Young Adults (Y) (n = 48)	Older Adults (O) (n = 15)	Statistical Significance and Multiple Contrast Results
Age, yr	10±1	25±4	58±4	$P < 0.0005$ C < Y < O
Systolic blood pressure, mmHg	107±10	116±10	121±16	$P = 0.002$ C < Y = O
Diastolic blood pressure, mmHg	64±6	66±8	72±10	$P = 0.01$ C = Y < O
Mean arterial pressure, mmHg	78±6	79±13	88±11	$P = 0.02$ C = Y < O
Height, cm	143±8	180±7	173±7	$P < 0.0005$ C < O < Y
Body mass, kg	40.4±10.4	78.0±9.3	80.9±17.1	$P < 0.0005$ C < Y = O
BMI, kg/m^2	19.5±3.7	24.2±2.6	26.9±3.9	$P < 0.0005$ C < Y < O

Data are presented as means ± SD. BMI, body mass index.

Data reduction. Baseline diameter and blood flow were determined during the 1 min before cuff inflation. Peak diameter following cuff deflation was automatically detected according to an algorithm that identified the maximum bracket of data subsequent to performance of a moving window smoothing function. The maximum value of all the calculated median values is automatically detected and chosen to represent the peak of the diameter curve. FMD was calculated as the percentage rise of this peak diameter from the preceding baseline diameter. SR was calculated at 30 Hz. The postdeflation SR area under the curve (SR_{AUC}) was calculated using the Riemann sum technique from deflation to the time of peak diameter for each individual (6).

Data analysis. Differences in FMD, SR, and FMD/SR between age groups were explored with one-factor (age) general linear models (13). Significant *F*-values were followed-up with multiple contrasts using the Games-Howell procedure, which is appropriate when sample sizes are different between groups (14). The FMD/SR data were examined for parity with the criteria for valid use of ratios (2) using exploratory analyses (13). These data explorations included examinations of zero regression *y*-intercept, no correlation between the ratio and its denominator, normal distribution (Kolmogorov-Smirnov test), and homoscedasticity of variances (F_{max} test). When these two latter assumptions were violated, an appropriate data transformation (e.g., logarithmic) was applied, and the data were reanalyzed. All statistical analyses were performed using SPSS 16.0 (SPSS, Chicago, IL) and Statistica (Statsoft, Tulsa, OK) software. Group comparison data are reported as means (SD). Ninety-five percent confidence limits (95% CL) are also cited where relevant.

RESULTS

Relationship between SR and FMD. Scatterplots of the relationship between SR and FMD in the three groups and the pooled sample are presented in Fig. 1, *A* and *B*, respectively. The relation between SR and FMD appeared linear for the pooled sample and was statistically significant, although only moderate ($r = 0.28$, $P = 0.01$). Correlations were very weak and not statistically significant for the children ($r = 0.02$, $P = 0.95$) and older people ($r = 0.03$, $P = 0.92$). The correlation between SR and FMD was $r = 0.31$ ($P = 0.04$) for the young adults. The assumption of a zero *y*-intercept was violated in all three samples as well as in the pooled sample (Fig. 1). The estimates of the *y*-intercepts (95% CL) for the children, young adults, and older people were 10.6% (3.3–17.9), 5.8% (4.0–7.5), and 5.7% (3.1–8.4), respectively ($P < 0.001$). The *y*-intercept for the pooled sample was 5.9% (95% CL = 4.3–7.5). In view of the potential influence of measurement error on least-squares regression parameters (3), we repeated the above analyses with regression methods that have no special assumptions regarding the distribution of the samples and measurement errors (28). This analysis did not alter our finding that all regression *y*-intercepts were greater than zero.

FMD and SR data were subsequently logarithmically transformed to explore whether there was improved adherence to these assumptions. Although none of the regression *y*-intercepts were found to be significantly different from zero after transformation ($P > 0.05$), none of the correlations between log SR and log FMD were actually statistically significant for the three age groups ($P > 0.05$). A significant, albeit weak, correlation between log SR and log FMD was found only for the pooled sample ($P = 0.03$).

Relation between ratio-normalized FMD and SR. A clear relationship existed between FMD/SR and SR (Fig. 2*A*), and there was some indication that this relationship was nonlinear
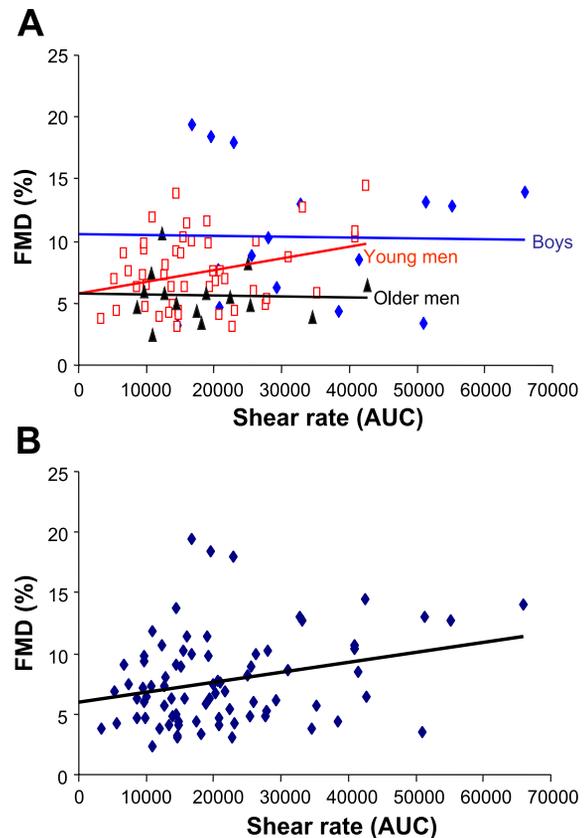

Fig. 1. Scatterplots showing the relationship between shear rate and flow-mediated dilation (FMD) for the 3 age groups (*A*) and the pooled sample (*B*). In *A*, data and fitted regression lines are shown for the boys (blue), young adults (red), and older adults (black). All correlation coefficients were weak to moderate in magnitude, and all regression *y*-intercepts were significantly greater than zero. AUC, area under the curve.

in nature. This nonlinear relationship is traditionally interpreted as evidence that the FMD/SR normalization is not fully controlling for SR in a uniform manner over the measurement range (1, 2). Following logarithmic transformation, no relationship between logFMD/logSR and SR was present (Fig. 2*B*).

Distribution assumptions. For the pooled sample, both the absolute FMD/SR data and the residuals from the between-groups ANOVA were not normally distributed, the distribution being skewed to the left in both cases (Fig. 3). A Kolmogorov-Smirnov test for a non-Gaussian distribution was statistically significant in the children ($P < 0.0005$) and young adults ($P = 0.001$), but not in the older participants ($P = 0.14$). Logarithmic transformation before calculation of the FMD/SR ratio brought the data closer to a normal distribution, the Kolmogorov-Smirnov test being not significant for all age groups and the pooled sample ($P > 0.20$). The relationship between SR and FMD was also clearly heteroscedastic (Fig. 1) with greater variance of data at the upper end of the measurement range. Hartley's F_{max} test was employed to explore whether variances could be assumed to be homogeneous between the three age groups (13). This assumption was upheld with FMD/SR ($P = 0.30$) and logFMD/logSR ($P = 0.11$).

Effects on inferential claims. A significant effect of age on nonnormalized FMD was found (Table 2). The nonnormalized mean FMD of the children and young adults was significantly higher than that of the older adults. However, normalization
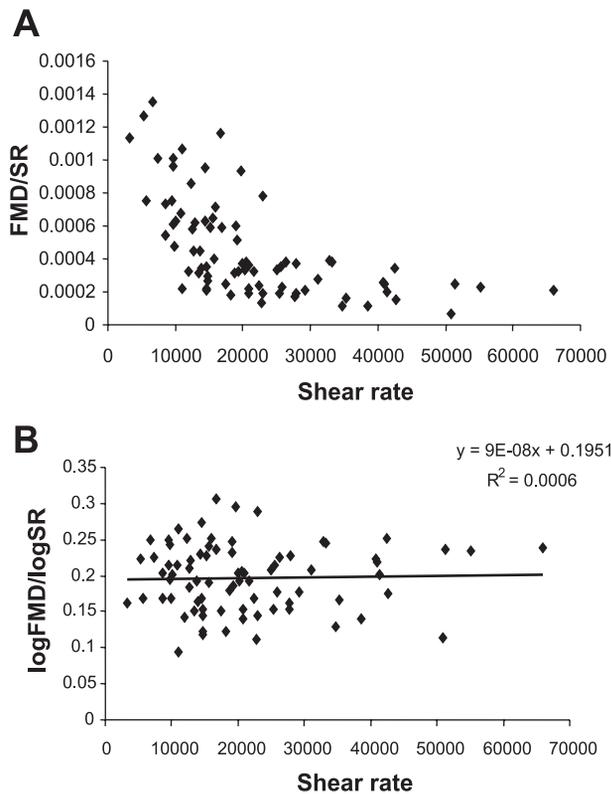
A

B

$y = 9E\text{-}08x + 0.1951$
$R^2 = 0.0006$

Fig. 2. Scatterplots of the relationship between shear rate (SR) and ratio normalized FMD (FMD/SR). The nonlinear relationship in *A* can be interpreted as an indication that the ratio normalization is not consistent for all values of shear rate. After logarithmic transformation (*B*), the lack of a relationship can be interpreted as an indication that the ratio normalization is consistent over the whole measurement range of shear rates.

using FMD/SR resulted in no statistically significant differences between age groups being observed ($P = 0.10$). Moreover, the trend in normalized FMD across age groups was different from that of the nonnormalized FMD, the young adults showing the highest normalized FMD and the children and older adults showing similar mean values. Nevertheless, following logarithmic transformation of data, a significant effect of age was again found for the log normalized FMD ($P = 0.03$), with a trend across age groups that corresponded to the normalized data. Similar results were obtained when the relationship between age (as a continuous variable) and FMD was examined with linear least-squares regression analysis. A significant negative correlation was found between age and nonnormalized FMD ($r = -0.34$, $P = 0.002$). Nevertheless, no significant correlation was found for the relationship between age and FMD/SR ($r = -0.07$, $P = 0.56$). When logarithmic transformation of FMD and SR were performed before calculation of their ratio, a significant negative correlation was again found between age and this log normalized FMD ($r = -0.27$, $P = 0.018$).

DISCUSSION

We have undertaken the first study of the statistical appropriateness of the FMD/SR ratio approach to normalization in the context of a between-subjects analysis. Such normalization has been deemed important for controlling for interindividual differences in SR (19, 34) and thereby improving the predictive

utility of FMD as a convenient marker of endothelial function. Our data from 79 healthy males aged 9–64 yr clearly indicate that the FMD/SR normalization approach is imprecise in two ways. First, the ratio did not fully control for differences in SR to a uniform degree over our measurement range of FMD. Second, the ratio did not adhere to assumptions necessary for reliable results from the parametric data analyses that have been routinely employed by previous researchers. Like other researchers (25, 26), we found that the magnitude and, in our case, even the direction of differences in FMD between samples were altered substantially when FMD was normalized via division by SR. However, in view of the statistical problems we have identified, these past analyses, and the conclusions derived from them, should be interpreted with caution until more precise information is gained about the nature (linear or nonlinear), magnitude, and stability (within and between subjects) of the relationship between SR and FMD.

Our specific research situation. It is important to note that we explored the appropriateness of the ratio normalization approach in the specific context of a between-subjects analysis of FMD in 79 males of varying age. Nevertheless, the characteristics of our dataset have helped to illustrate some statistical issues that are quite common in physiological research. Sample
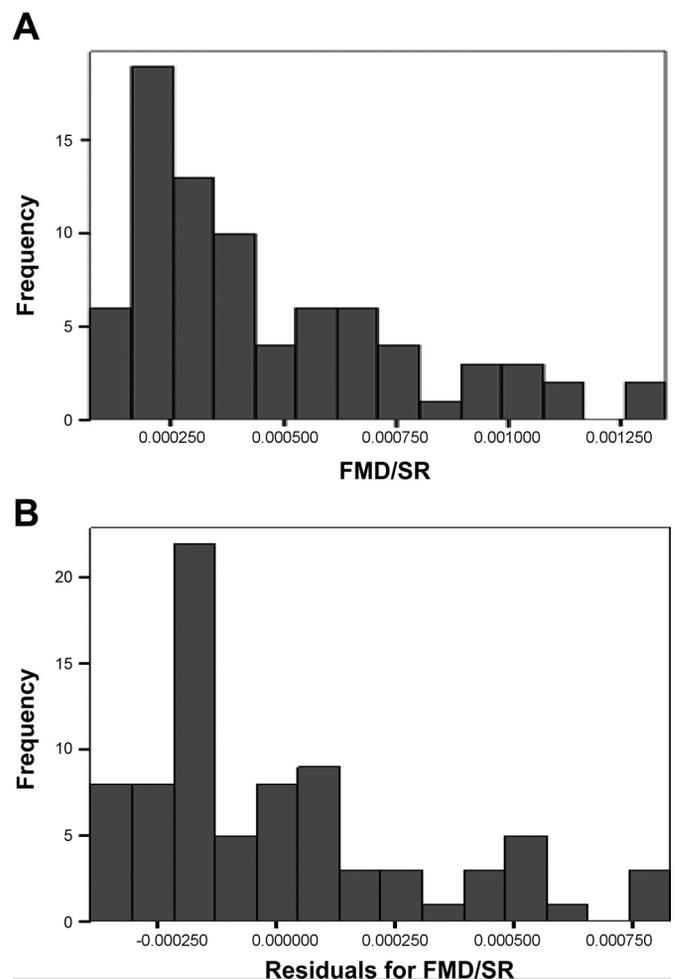
A

B

Fig. 3. Histograms showing the highly skewed distribution of FMD/SR data (*A*) and the skewed residuals resulting from the general linear model used to examine age differences in FMD/SR (*B*).

Table 2. *Brachial artery characteristics at baseline and during the FMD% response of the participants (n = 79), who were allocated to groups of children, young men and older men*

Measured Variable	Children (C) (*n* = 16)	Young Adults (Y) (*n* = 48)	Older Adults (O) (*n* = 15)	Statistical Significance and Multiple Contrast Results
Baseline diameter, mm	2.6±0.5	4.2±0.5	4.7±0.6	*P* < 0.0005 C < Y < O
Peak diameter, mm	2.9±0.6	4.5±0.5	4.9±0.6	*P* < 0.0005 C < Y < O
Change from baseline, mm	0.26±0.13	0.31±0.11	0.26±0.09	*P* = 0.22
Change from baseline, FMD%	10.4±5.4	7.5±2.9	5.6±2.0	*P* = 0.001 C = Y > O
Time to peak dilation, s	65±27	75±39	91±34	*P* = 0.13
SR_{AUC}, s^{-1} (in 10^3)	33.3±15.5	18.0±9.5	18.9±9.7	*P* < 0.0005 C > Y = O
FMD%/SR_{AUC}, %/s^{-1} (in 10^{-4})	3.8±3.1	5.2±3.1	3.6±2.2	*P* = 0.10
log FMD%/log SR_{AUC}	0.21±0.04	0.20±0.04	0.17±0.04	C = Y > O

Data are presented as means ± SD. FMD, flow-mediated dilation; SR, shear rate, AUC, area under the curve.

sizes and variances were not equal between our three age groups. There was also evidence that the data were not drawn from normally distributed populations. Depending on the overall number of participants recruited for the research, these characteristics can lead to specific implications for statistical power and the "robustness" of statistical tests, i.e., how prone tests are to violation of assumptions (36). Therefore, we maintain that the analyses undertaken on our dataset raise important issues that are common in research on endothelial function and, indeed, physiology in general. Another common research situation involves the examination of changes in FMD over time, or in response to certain crossover-administered interventions. It would be important for future researchers to explore the appropriateness of the ratio normalization approach in this repeated-measures situation.

Assumptions underlying the consistent control of SR. The FMD/SR approach did not adhere to any of the assumptions required for appropriate and accurate use of ratio normalization in between-subjects studies (1, 2). Violation of the zero intercept assumption means that the normalization of FMD will be biased at lower and higher ends of the measurement range. There is also evidence in Fig. 2 that not only is the control of SR inconsistent, but it also is nonlinear over the measurement range. The fact that this relationship was not apparent after logarithmic transformation of data leads us to question whether the overriding relationship between SR and FMD is in fact nonlinear. In their repeated-measures experimental manipulation of SR, Pyke and Tschakovsky (30) did not analyze whether the relationship between SR and FMD was better described with a nonlinear than a linear function. It was also unclear whether the lines drawn on their scatterplots were derived from least-squares linear regression or from some other regression or line-fitting approach. Recently, Padilla et al. (26) indicated that the average within-subjects regression slope for the SR-FMD relationship was not significantly different from zero. However, the between-subjects correlation was not isolated in their analysis. One important issue in past studies (26, 30) is that data were pooled across repeated trials for each subject. The validity of such data pooling has been questioned (7), since it inflates the degrees of freedom in the analysis, breaks the assumption of independence of cases, and can mask true relationships between variables derived from an intra- and

intersubject analysis. It would be important for future researchers to isolate and examine both the within- and between-subjects relationship between SR and FMD.

Normalization and study inferences. Ageing is a primary risk factor for cardiovascular disease, and a key component is reduced endothelial function (15). With increasing age, there is a decrease of nitric oxide contribution, an enhancement of vasoconstrictor prostanoids, and a modification of the action of either the endothelium-derived hyperpolarizing factor or the vasoconstrictor peptide endothelin (23). Intuitively, normalization of FMD for SR would help to unravel these mechanisms, since differences between populations in normalized FMD could represent differences in the intrinsic endothelial NO-vasodilator system. Our findings agree with those from others (25, 26) that ratio normalization of FMD can influence study conclusions. When the nonnormalized data were analyzed, there was a decrease in FMD across the age groups. Nevertheless, when the ratio normalization approach was applied, the mean FMD/SR of the children was similar to that of the older adults. This disparity between FMD/SR and the nonnormalized method of presenting differences in FMD can be explained by the violations of assumptions described earlier, and especially by the lack of stability in the relationship between SR and FMD, rather than any underlying biological age-related mechanism. Therefore, the statistical problems we have identified could have detrimentally affected physiological insight into aging and endothelial function.

The relationship between SR and FMD was actually not present in the samples of children and older adults. In the between-subjects context we explored, it is clearly erroneous to attempt to control for the effects of another variable when that variable is not related to the outcome variable under study. This point also relates to the question whether analysis of covariance (ANCOVA) is a valid alternative approach to normalization (19). ANCOVA is an approach that reduces between-subjects error variance in a cross-sectional comparison by partitioning some of the "unexplained" variance to another variable (a covariate) that is also observed to correlate with the outcome variable under study (13). ANCOVA is appropriate for group comparisons only if the proposed covariate is actually related to the outcome variable in the particular research situation (22). If this is not so, then ANCOVA can

actually be a less powerful analysis. It is also important for the relationship between covariate and outcome to be consistent between groups. This issue of "parallelism" was raised by Tschakovsky et al. (34). The data presented in Fig. 1*A* suggest that the relation between SR and FMD is actually very variable between samples. In addition, the results of ANCOVA can be misleading if the covariate is related not just to the outcome variable but also the independent variable under study (22). Our independent variable of age was related to that of SR, in that SR was significantly higher in the children compared with the other two age groups (Table 2). Therefore, our data do not offer support that ANCOVA is a more appropriate approach to normalization than FMD/SR. It would be valuable for future researchers to undertake similar comparative analyses.

The importance of an appropriate data transformation. One solution for severe violation of statistical assumptions is to transform the data before the application of parametric statistical tests (38). In view of the nonlinearity in the FMD-SR relationship as well as the skewed and heteroscedastic data distributions, we logarithmically transformed FMD and SR before calculating their ratio and exploring for age differences. In agreement with general statistical theory, this transformation improved the adherence to underlying statistical assumptions, and the age-related differences were found to be statistically significant and similar in nature to the analysis of nonnormalized data. It remains to be seen whether logarithmic transformation is appropriate for data collected in other related studies on endothelial function.

Although it is possible to normalize any dataset using a specific statistical model, it is desirable that a general normalization approach is appropriate in most studies. Transformation of data to linearize the normalization process is also inherent in the simple allometric approach to this general problem, most used in biological scaling research (1). With this allometric model, instead of a simple ratio, the most appropriate normalization approach is described by a power function equation such that:

$$\text{normalized FMD} = \text{FMD/SR}^k$$

where k is the coefficient of allometry (an exponential term related to the curvilinearity of the data). Again, it is crucial for the value of k to be relatively stable for such a normalization approach to be generally adopted.

Conclusions. Our data clearly indicate that the FMD/SR normalization approach did not adhere to assumptions necessary for reliable results from parametric data analyses in a cross-sectional context. In view of the statistical problems we have identified, the results of between-subjects analyses involving the ratio approach to normalization could be compromised. Logarithmic transformation of FMD and SR before division improved adherence to assumptions with our dataset. This approach could be extended to formal allometric scaling, as long as more precise information is gained about the nature (linear or nonlinear), magnitude, and stability (within and between subjects) of the relationship between SR and FMD. If this relationship is generally found to be weak or nonlinear or very variable between samples, then ratio normalization should not be applied.

While our results raise important questions regarding the validity of some normalization procedures as applied to between-subjects comparisons, we strongly believe that consideration of SR as a stimulus to FMD is important. Further research will be required to determine the optimal and most robust method of normalization in various populations and research situations. We recommend that, in the interim, researchers into FMD quantify SR and report it in their methods or results sections. It would also be informative if future researchers report the characteristics of the relationship between SR and FMD.

DISCLOSURES

No conflicts of interest are declared by the authors.

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