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Methodological Approaches to Optimize Reproducibility and Power in Clinical Studies of Flow-Mediated Dilation

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Objectives Our aim was to determine reproducibility of the flow-mediated dilation (FMD) response profile, and discriminatory ability of the components.

Background Brachial FMD is widely used to study conduit artery endothelial function. Automated B-mode image edge detection (B-ED) provides a full response profile. Reproducibility and biological relevance of these additional components have not been fully explored.

Methods Forty-two healthy adults underwent FMD using B-ED repeated at fixed time intervals up to 3 months. The FMD profile was assessed for diameter changes, area under the curve, and time course. Measures were compared in 25 adults with hypercholesterolemia, 25 subjects with diabetes, and 50 matched control subjects.

Results The maximum change in FMD was the most reproducible (coefficient of variation = 9.8%, 10.6%, 6.6%, and 9.2% at 4 to 6 h, 1 week, 1 month, and 3 months, respectively). Most of the variability occurred between subjects rather than within. All FMD measures except time course were significantly reduced in hypercholesterolemia and diabetes. Power curves were generated to indicate the appropriate number of subjects for parallel and crossover study designs.

Conclusions Maximum FMD percentage change from baseline is the most reproducible of the response curve measures and best identifies those with risk factors. Flow-mediated dilation measured by B-ED is robust and practical to assess the effect of interventions on endothelial function in clinical trials. (J Am Coll Cardiol 2008;51:1959–64)

Atherosclerosis begins in early life. Strategies to study and manage 'lifetime' risk from atherosclerosis require robust intermediate phenotypes, on the causal pathway for disease. Endothelial function is linked to cardiovascular risk factors, provides prognostic information, and can be studied noninvasively by measurement of flow-mediated dilation (FMD) (1). This reflects local nitric oxide (NO) bioavailability and enables both examination of mechanisms involved in the initiation and progression of pre-clinical vascular disease and the impact of acute and long-term interventions (2).

While the principles of FMD have not changed, operator technique and analysis have been refined (3). We now report the reproducibility of components of the FMD response over time periods up to 3 months, and their ability to discriminate between healthy subjects and those with diabetes or hypercholesterolemia. Power curves have been generated to assist design of both crossover and parallel trials to evaluate the impact of interventions on endothelial function. We have also compared B-mode edge detection (B-ED) software (Medical Imaging Applications, Coralville, Iowa) to A-mode wall tracking (A-WT) (Vadirec, Medical Systems Arnhem, Oosterbeek, the Netherlands) an early semi-automatic method relying on M-mode vascular images (4,5).

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Methods

Study protocols. **STUDY 1: REPRODUCIBILITY OF FMD COMPONENTS BY B-ED.** We studied 42 healthy adults (19 men, mean age 43 years [range 20 to 75 years]) free from

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Abbreviations and Acronyms

A-WT = A-mode wall tracking

B-ED = B-mode edge detection

CV = coefficient of variation

FMD = flow-mediated dilation

FMDmax = maximum flow-mediated dilation percentage change from baseline

NO = nitric oxide

T2DM = type 2 diabetes mellitus

cardiovascular risk factors at 4- to 6-h, 1-week, 1-month, and 3-month intervals.

STUDY 2: FMD PROFILE IN SUBJECTS WITH TYPE 2 DIABETES MELLITUS (T2DM) OR HYPERCHOLESTEROLEMIA. Twenty-five patients (16 men, age 53 years [range 39 to 67 years]) with T2DM ≥2 years duration with fasting glucose ≥6.9 mmol/l (124 mg/dl) and/or on hypoglycemic treatment (16 sulphonylureas, 9 metformin, 7 combination, and 7 diet) were matched with 25 healthy control subjects recruited contemporaneously.

Twenty-five patients (18 men, age 50 years [range 19 to 67 years]) with total fasting cholesterol >6.0 mmol/l (232 mg/dl) (7 on statins) were compared with 25 healthy control subjects matched for age, gender, and vessel diameter.

STUDY 3: REPRODUCIBILITY OF FMD BY A-WT. We studied 36 healthy adults free of cardiovascular risks (14 men, 33 years [range 23 to 61 years]) on 2 occasions 1 month apart.

Assessment of endothelial function. All subjects gave informed written consent. Studies were approved by the local ethics committee and were carried out under standardized conditions by trained operators:

- Rest 10 min in a warm, quiet room (22°C to 26°C) after 4 h abstinence from food and caffeinated drinks.
- Right arm extended laterally and brachial artery imaged longitudinally 5 to 10 cm above antecubital fossa (image stability achieved by anterior plane, scanning through the biceps muscle with the probe in a stereotactic holder with micrometer movement).
- Pneumatic cuff (7 × 30 cm) (Scanmed, Moreton-in-Marsh, Gloucestershire, United Kingdom) placed immediately below the medial epicondyle. Inflation time 5 min at 300 mm Hg. The arm, hand, and head position noted, along with the distance between the pneumatic cuff and the probe. A thermal print of the arterial image was taken for matching at subsequent visits.
- B-mode ultrasound images were updated on the R-wave of the electrocardiogram, with Doppler flow and electrocardiogram displayed continuously.

B-ED methodology. A standard protocol was used as previously described (3). The lumen diameter was measured from within the same region-of-interest box for each image (Fig. 1A). Diameters were plotted for FMD and area under the curve measurement (Fig. 1B). Additionally, the incremental area under the diameter versus time curve was calculated, accounting for baseline diameter over intervals post-cuff release of 0 to 1 min, 0 to 3 min, and 0 to 5 min

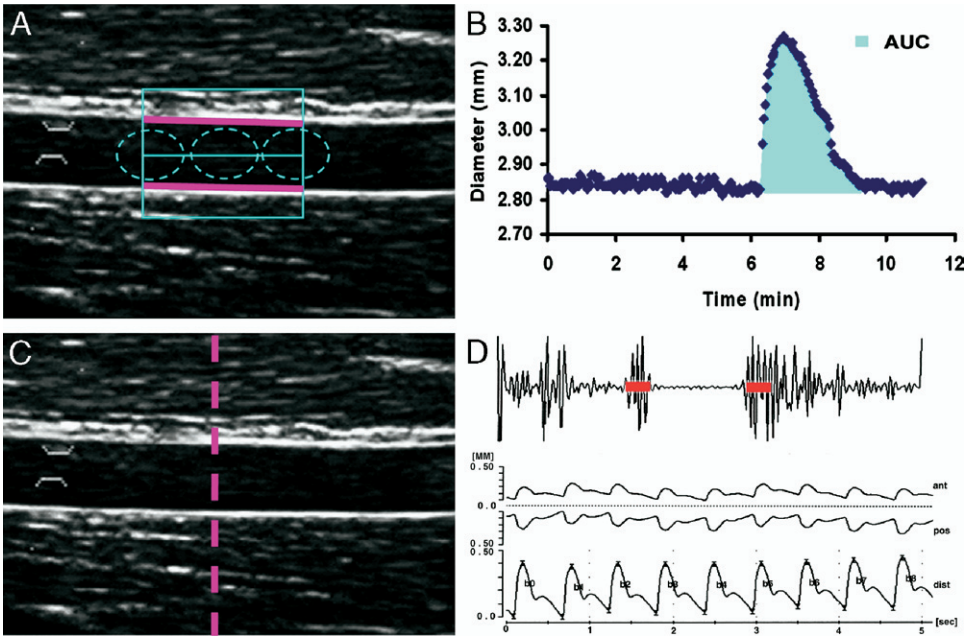

Figure 1 Image Acquisition and Readout for FMD Using B-ED and A-WT

(A) B-mode ultrasound image of the brachial artery, with region-of-interest box for edge detection diameter measurement. **(B)** Flow-mediated dilation (FMD) using B-mode edge detection (B-ED). **(C)** B-mode image with perpendicular M-line for A-mode wall tracking (A-WT). **(D)** A-mode wall tracking output. **(Upper trace)** A-mode radio-frequency signal. **(Middle trace)** Anterior and posterior wall motion. **(Lower trace)** Distension waveform generating end-diastolic diameter. AUC = area under the curve.

(0 = cuff deflation point). The times to reach maximum dilation and to recover to half the absolute change in diameter after cuff release were also assessed to examine the response time course after reactive hyperemia. All analyses were conducted by an experienced investigator (A.E.D.) blinded to subject identity and visit order.

A-WT methodology. A standard protocol was used as previously described (6). The cuff was placed 4 to 6 cm below the elbow crease to reduce image movement on cuff inflation/deflation. A single M-line was placed perpendicular to the vessel walls on the image to record the systolic to diastolic excursion of the brachial artery (Fig. 1C) providing the distension waveform and end-diastolic diameter (Fig. 1D).

Statistical analysis. All measures are expressed as mean ± standard deviation unless otherwise stated.

Reproducibility for each time interval between pairs of visits was expressed as the coefficient of variation (CV) of a single measurement, defined as the standard deviation of the difference between paired values divided by the mean and divided by $\sqrt{2}$, as previously described (3). Paired *t* tests were used to compare patients and control subjects. To assess the number of subjects required for crossover and parallel studies, for 80% statistical power and 5% significance, and to assess the effect of varying the number of readings pre- and post-treatment, power curves were constructed from the between- and within-subject variances as previously reported (7). Reproducibility of B-ED and A-WT over 1 month was compared using Bland-Altman plots. SPSS (version 12, SPSS Inc., Chicago, Illinois) was used for analyses.

Results

Study 1. Six of 144 scans were excluded (1 noncompliance with fasting protocol, 2 poor image quality, 2 subject movement, 1 equipment failure). Baseline vessel diameter, baseline blood flow, reactive hyperemia, blood pressure, and heart rate were similar for each interval. The maximum change in diameter expressed as a percentage and as an absolute value, maximum flow-mediated dilation percentage change from baseline (FMDmax), and absolute FMD change from baseline, respectively, were the most reproducible components of the FMD profile at each interval (Table 1). Maximum FMD percentage change from baseline correlated inversely with age ($p < 0.01$), female subjects had significantly smaller vessels than male subjects ($p < 0.01$), and vessel size correlated with age ($p < 0.01$). Between- and within-subject variance was similar at all visit intervals (13.3, 11.9, 14.3, and 11.1 and 1.3, 1.3, 0.6, and 1.1 for studies done 4 to 6 h, 1 week, 1 month, and 3 months apart, respectively). We constructed power curves from the data recorded 4 to 6 h and 3 months apart for both crossover and parallel studies for protocols using 1, 2, or 4 FMD measurements before and after an intervention (Fig. 2). Increasing the number of assessments in the protocol had minimal impact in a parallel study design. By contrast, in a crossover design, increasing the number of FMD scans reduced the number of subjects slightly, but at the cost of a more complex protocol.

Study 2. Disease groups and control groups had similar diameters and flows at baseline. All components of the FMD response except peak time and recovery half time were lower in subjects with T2DM or hypercholesterolemia, but did not further differentiate patients from control subjects compared with FMDmax. Absolute FMD change from baseline and FMDmax showed the greatest differences between patients and control subjects for both disease groups (Table 2).

Study 3. Comparing A-WT with B-ED over a 1-month interval, reproducibility of baseline diameter was comparable (CV 2.6% vs. 3.5%). However, FMD at 60 s after cuff deflation using A-WT was lower (4.3 ± 3.6% vs. 7.5 ± 3.8% at Visit 1, and 4.6 ± 3.1% vs. 7.2 ± 4.0% at Visit 2, both $p < 0.01$) and more variable (CV 17.8% vs. 8.6%) than B-ED (Fig. 3).

Discussion

We report the opportunity, afforded by current ultrasound methodology, to evaluate endothelial function in a reproducible manner and the potential application to detect abnormality in disease states. Power curves have been constructed demonstrating that FMD measurement is practical in both crossover and parallel studies.

In 1992, we described the noninvasive method for FMD, which has been shown to be NO dependent and also to reflect coronary endothelial function (1,8,9). The technique has been widely adopted in clinical research, but, manual measurements are time consuming and subjective. Newer semiautomatic measurement software is faster, less subjective, and potentially more reproducible. This, combined with greater image stability afforded by fixing the ultrasound probe in an adjustable stereotactic clamp and adherence to a rigorous protocol, improves the accuracy and reproducibility. In contrast to early analyses, B-ED measures the whole dilation profile. We show that, of all the potential measures, FMDmax is both the most reproducible and the best discriminator of endothelial function abnormalities between health and disease. The 'area under the response curve' adds little value compared with the simpler FMDmax. The time to FMDmax and the recovery time were similar in health and disease suggesting that the magnitude rather than time course of vasodilation best reflects differences in endothelial function.

A-mode wall tracking is more variable than B-ED, despite excellent spatial resolution. This may reflect difficulty in maintaining positional stability of a single M-line without 2-dimensional image guidance. Measurements must be performed at set time points (60 s), limiting reproducibility and also producing lower FMD, missing the maximal response in most cases. Maximal dilation occurred between 24 s and 117 s (mean 59 s)

Table 1 Reproducibility of FMD Responses

	4 to 6 h (n = 32)	Mean Diff	SD Diff	CV (%)	1 Week (n = 34)	Mean Diff	SD Diff	CV (%)	1 Month (n = 37)	Mean Diff	SD Diff	CV (%)	3 Months (n = 35)	Mean Diff	SD Diff	CV (%)
FMDmax (%)	8.1 ± 3.7				7.5 ± 3.4				8.1 ± 3.7				7.8 ± 3.1			
	8.1 ± 3.8	−0.29	1.12	9.8	7.5 ± 3.4	−0.07	1.12	10.6	8.1 ± 4.0	−0.03	0.75	6.6	8.2 ± 3.7	0.38	1.04	9.2
FMD60s (%)	7.3 ± 3.7				6.7 ± 3.5				7.5 ± 3.8				7.1 ± 3.0			
	7.4 ± 3.9	−0.25	1.53	14.7	6.6 ± 2.9	−0.09	1.34	14.2	7.2 ± 4.0	−0.33	0.89	8.6	7.5 ± 3.7	0.13	1.23	11.9
FMDabs (mm)	0.3 ± 0.1				0.3 ± 0.1				0.3 ± 0.1				0.3 ± 0.1			
	0.3 ± 0.1	−0.008	0.05	8.1	0.3 ± 0.1	−0.004	0.05	10.3	0.3 ± 0.1	−0.007	0.04	6.6	0.3 ± 0.1	0.01	0.03	6.2
Time to FMDmax (s)	60.4 ± 20				59.4 ± 20				58.9 ± 14				58.6 ± 17			
	58.1 ± 15	−4.02	15.7	18.7	59.8 ± 19	0.45	18.2	21.6	63.9 ± 18	3.27	14.8	17.1	59.8 ± 21	1.14	11.22	13.4
½ rec. time (min)	1.39 ± 0.6				1.1 ± 0.6				1.4 ± 0.9				1.2 ± 0.7			
	1.4 ± 0.7	0.03	0.29	14.7	1.2 ± 0.8	0.15	0.52	32.2	1.2 ± 0.7	−0.16	0.41	22.0	1.2 ± 0.6	0.001	0.47	28.0
AUC 0 to 1 min	7.5 ± 5				5.5 ± 4				6.8 ± 5				6.5 ± 5			
	6.9 ± 5	−0.54	2.52	24.8	6.1 ± 5	0.5	3.82	46.6	5.3 ± 5	−1.95	3.46	40.5	8.1 ± 4	1.6	3.75	36.3
AUC 1 to 3 min	18.6 ± 10				15.4 ± 11				19.2 ± 10				17.9 ± 11			
	18.2 ± 11	−0.41	3.96	15.2	15.8 ± 11	−0.13	3.82	17.4	19.2 ± 11	−1.69	5.86	21.6	17.6 ± 12	−0.34	6.19	24.6
AUC 0 to 3 min	26.1 ± 1				20.8 ± 11				26.0 ± 12				24.4 ± 11			
	25.1 ± 13	−0.95	5.67	15.7	21.9 ± 14	0.37	5.82	19.2	24.5 ± 13	−3.64	7.84	22.0	25.7 ± 12	1.26	8.04	22.7
AUC 0 to 5 min	32.0 ± 15				24.2 ± 16				33.2 ± 17				31.3 ± 18			
	31.2 ± 18	−0.88	9.25	20.7	26.8 ± 20	1.8	8.68	24.1	31.4 ± 20	−4.59	11.4	25.0	30.9 ± 17	−0.43	12.18	27.7
Baseline	3.7 ± 0.8				3.7 ± 0.8				3.8 ± 0.8				3.8 ± 0.8			
Diameter (mm)	3.7 ± 0.8	0.04	0.11	2.0	3.7 ± 0.8	−0.001	0.13	2.6	3.7 ± 0.8	−0.05	0.19	3.5	3.8 ± 0.8	0.01	0.2	3.8

Characteristics of the flow-mediated dilation (FMD) response profile repeated at intervals of 4 h, 1 week, 1 month, and 3 months in Study 1 (healthy subjects). Data provided as mean ± SD for Visits 1 and 2.

AUC = area under the curve; CV = coefficient of variation of paired differences; FMD = flow-mediated dilation; FMDabs = maximum absolute change in flow-mediated dilation; FMDmax = maximum percent change in flow-mediated dilation; FMD60s = percent change in flow-mediated dilation at 60 s after cuff deflation; Mean diff = mean of paired differences; SD diff = standard deviation of paired differences; Time to FMDmax = time to reach maximal flow-mediated dilation after cuff release; ½ rec. time = time to return to one-half absolute vessel size change after cuff deflation.

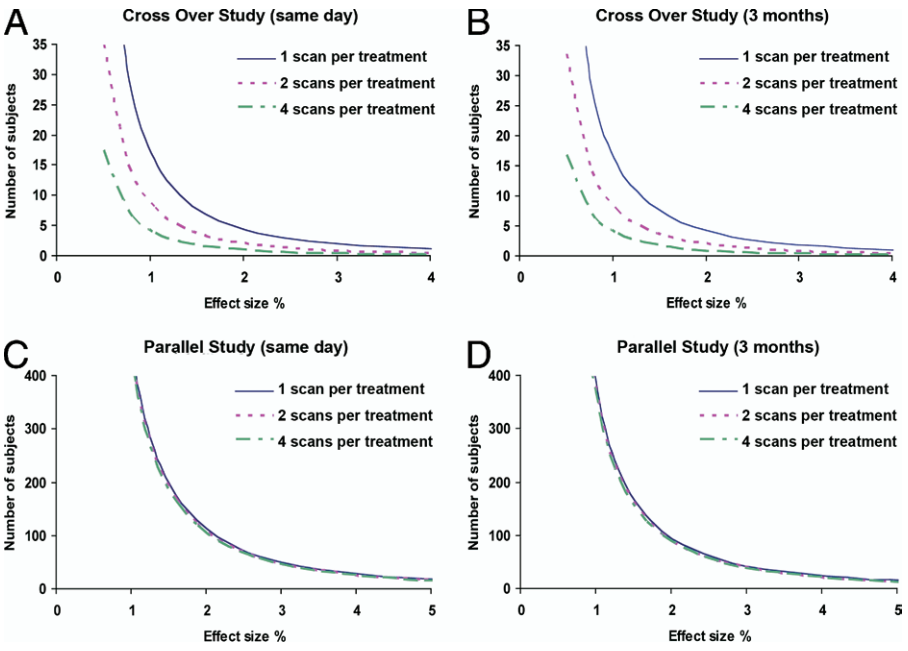

Figure 2 **Number of Subjects Required in Crossover and Parallel Designs for Given FMD Effect**

Relation between effect on maximum percent change in flow-mediated dilation (FMD) (%) and number of subjects required in crossover and parallel study designs at 80% power and 5% significance, 4 to 6 h and 3 months apart with 3 monitoring strategies: 1, 2, or 4 measures pre- and post-treatment.

after cuff release so that readings taken at 60 s are maximal for only 30% of subjects (10).

The low CV with B-ED was accomplished by meticulous standardization of the technique and operator training. Physiological variability can be reduced by controlling factors known to influence endothelial function, such as temperature, recent food and drink intake, exercise, and infection (2,11). Previous studies have reported variability during the menstrual cycle (12). Although our study was not designed to test this issue, reproducibility was similar in women and men.

We investigated subjects age 20 to 71 years, with a wide range of vessel diameters and FMD responses, using a protocol that reflects predominantly NO bioavailability (9). Dilation induced by stimuli such as hand warming, prolonged ischemia, or cuff inflation above the site of measurement are less NO dependent, less extensively studied, and require further evaluation of their biological significance (9,13).

A-mode wall tracking provided consistently lower FMD than B-ED, likely due to a more distal cuff position used to minimize movement artifact. Standardization of cuff posi-

Table 2 **FMD Response in Subjects With Diabetes or Hypercholesterolemia Compared With That in Control Subjects**

	T2DM (n = 25)	Control Subjects (n = 25)	p Value	Hypercholesterolemia (n = 25)	Control Subjects (n = 25)	p Value
FMDmax (%)	4.0 ± 2.4	6.5 ± 2.4	0.0001	4.3 ± 2.3	7.2 ± 2.3	0.0001
FMDabs (mm)	0.16 ± 0.09	0.26 ± 0.09	0.0004	0.17 ± 0.08	0.29 ± 0.08	0.000008
FMD60s (%)	3.2 ± 2.5	5.5 ± 2.4	0.0007	3.7 ± 2.3	6.4 ± 2.4	0.0004
AUC 0 to 1 min	2.6 ± 4.3	7.0 ± 4.1	0.001	2.7 ± 3.0	7.0 ± 3.4	0.0001
AUC 0 to 3 min	13.7 ± 13.6	24.5 ± 13.0	0.009	12.3 ± 10.0	26.6 ± 13.0	0.00007
AUC 1 to 3 min	11.0 ± 10.5	17.5 ± 11.8	0.05	9.7 ± 8.7	19.6 ± 11.4	0.0005
½ rec. time (s)	80.0 ± 48.0	74.5 ± 33.5	0.7	60.1 ± 28.5	79.9 ± 49.2	0.09
Time to FMDmax (s)	65.4 ± 18.0	65.2 ± 22.6	0.9	55.0 ± 13.2	58.3 ± 17.4	0.5
Baseline diameter (mm)	4.3 ± 0.7	4.21 ± 0.6	0.2	4.0 ± 0.7	4.1 ± 0.6	0.09
Baseline flow (ml/min)	32 ± 17	25 ± 12	0.1	23 ± 21	18 ± 12	0.6
Reactive hyperemia (%)	457 ± 198	518 ± 275	0.6	617 ± 231	642 ± 350	0.6

Data provided as mean ± SD and shown in descending order of significance of difference between type 2 diabetes mellitus (T2DM) cases and matched control subjects. Abbreviations as in Table 1.

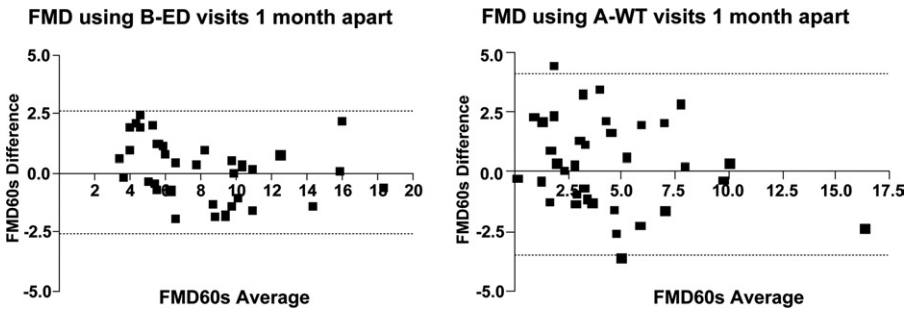

Figure 3 **Bland Altman Plots for FMD60s in Study 3 Over 1 Month Showing B-ED and A-WT**

The **dotted lines** represent the 95% confidence intervals. The x-axis shows the average flow-mediated dilation (FMD) and the y-axis the difference between pairs. A-WT = A-mode wall tracking; B-ED = B-mode edge detection; FMD60s = change in flow-mediated dilation at 60 s after cuff deflation.

tion is crucial in multicenter and interventional studies and may explain, in part, previously reported variation in FMD (14).

Flow-mediated dilation measured by B-ED is reproducible and practical for studying endothelial function in clinical research from a young age. As the major component of variability in FMD is between subjects, crossover studies can be performed with sufficient statistical power using substantially fewer subjects than for parallel trial designs. The additional complexity resulting from multiple assessments of FMD adds very little to the power of the study. This information on vascular disease biology complements studies of arterial structure and clinical outcome, which require larger and more expensive protocols.

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