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# Dependency of flow-mediated vasodilatation from basal nitric oxide activity

Dennis Kannenkeril[1] | Agnes Bosch[1] | Julie Kolwelter[1,2] | Susanne Jung[2] | Kristina Striepe[1] | Christian Ott[1,3] | Christian Delles[4] | Roland E. Schmieder[1]

[1]Department of Nephrology and Hypertension, University Hospital Erlangen, Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Erlangen, Germany

[2]Department of Cardiology, University Hospital Erlangen, Friedrich-Alexander-University Erlangen-Nürnberg (FAU), Erlangen, Germany

[3]Department of Nephrology and Hypertension, Paracelsus Medical University, Nürnberg, Germany

[4]Institute of Cardiovascular and Medical Sciences, University of Glasgow, Glasgow, UK

**Correspondence**
Roland E. Schmieder, Department of Nephrology and Hypertension, University Hospital Erlangen, Ulmenweg 18, 91054 Erlangen, Germany.
Emails: roland.schmieder@uk-erlangen.de; roland.schmieder@fau.de

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## Abstract

**Background:** Flow-mediated vasodilatation (FMD) has become one of the most widely assessed parameters to analyse endothelial and vascular function in cardiovascular medicine. The degree of contribution of nitric oxide (NO) to FMD is inconclusive and varies widely depending on the device used. In this study, we used a semi-automatic ultrasound device to analyse to what extent basal NO activity contributes to FMD of the brachial artery.

**Methods:** FMD was assessed with the UNEX EF device in a cross-over single blinded randomized study at baseline and then during infusion of either a NO-synthase-inhibitor (NG-monomethyl-L-arginine (L-NMMA)) or saline. The analysis was repeated after 1 week with the alternative infusion of L-NMMA or saline. All measurements were analysed both automatically and by a technician manually.

**Results:** In total, 25 healthy men subjects completed the study. Diastolic blood pressure and heart rate significantly changed during infusion of L-NMMA. Infusion of L-NMMA reduced FMD significantly (−37%, $p = 0.002$). Saline solution had no effect on FMD (+14%, $p = 0.392$). Change in FMD was significantly different between the groups ($\Delta FMD_{L\text{-}NMMA}$ vs. $\Delta FMD_{saline}$, $p = 0.032$). There was a statistically significant correlation between automatically analysed results and those obtained by an experienced technician ($FMD_{saline}$: $r = 0.822$, $p < 0.001$; $FMD_{L\text{-}NMMA}$: $r = 0.645$, $p = 0.007$).

**Conclusion:** The influence of NO on FMD is approximately 40% if assessed using the UNEX EF. Prior to use FMD as a marker of endothelial dysfunction, we should explore different methods including various duration of forearm ischaemia to increase NO dependency of FMD.

KEYWORDS

basal nitric oxide activity, flow-mediated vasodilatation, UNEX EF

## 1 | INTRODUCTION

Endothelial dysfunction is a key pathophysiological condition leading to arteriosclerosis (Panza *et al*., 1990; Makimattila *et al*., 1996). Flow-mediated vasodilatation (FMD) has become one of the most widely assessed parameter to analyse endothelial function. Celermajer *et al*. (1992) were the first to measure the FMD-response in vivo by ultrasound technique. This non-invasive technique measures the ability of the endothelium to respond to shear stress evoked by increased blood flow as a result of reactive hyperaemia. The release of nitric oxide (NO) is considered as one of the pivotal mechanisms to induce FMD (Joannides *et al*., 1995; Green *et al*., 2014).

Peripheral endothelial function as assessed by FMD correlates with coronary artery endothelial function (Anderson *et al*., 1995). Impaired FMD has been shown to be a good predictor for cardiovascular events beyond traditional cardiovascular risk factors in patients with coronary artery disease (Yeboah *et al*., 2007). It is also predictive of the extent and severity of coronary atherosclerosis (Neunteufl *et al*., 1997). Moreover, some studies describe FMD as an independent predictor of cardiovascular disease in healthy subjects (Yeboah *et al*., 2007; 2009).

Impaired FMD can be the result of reduced bioavailability of vasodilators due to oxidative stress. Of these vasodilators, NO seems to play a major role, but the amount of its contribution to FMD is inconclusive (Joannides *et al*., 1995; Green *et al*., 2014). Studies to clarify the role of NO on FMD have been conducted by use of various methods which require extensive training and standardization. Operator's experience, study conditions, image acquisition, accurate use of edge-detection software, ultrasound probe position and cuff occlusion time are all factors which influence the FMD measurement results. Accordingly, the NO dependency of FMD varies widely, ranging from 0% to 98%, across studies (Green *et al*., 2014). Thus, each new device measuring FMD needs to be tested separately in order to determine the NO dependency precisely and thereby providing data whether and to what extent the new technology is able to assess NO activity of the vascular system.

In this study, our goal was to analyse to what extent NO contribute to FMD by measuring FMD with a recently developed semi-automatic system (UNEX EF, UNEX Corp., Japan) before and after blocking the release of NO (Corretti *et al*., 2002). This semi-automatic ultrasound system using an H-type ultrasound probe represents a new development that overcomes the limitations of traditional systems (in particular the investigator-dependency of conventional, 'hand-held' FMD measurements). No data on NO dependency of FMD measurements using the UNEX EF device are available and we therefore attempted to close this gap.

## 2 | METHODS

### 2.1 | Study design

Twenty-five healthy subjects participated in this clinical trial from November 2018 to May 2019. The study was conducted at the Clinical Research Center, Department of Nephrology and Hypertension, University Hospital Erlangen, Germany. The clinical study was registered at http://www.clinicaltrials.gov (NCT03723278). The study was approved by the Ethics Committee of the University Erlangen (Application No.: 332_18B) and performed in accordance with the Declaration of Helsinki and the principles of Good Clinical Practice guidelines. Subjects were recruited from previously conducted studies, and through the use of advertisements. All patients provided written informed consent prior to inclusion in the study. Subjects with any significant disease were excluded from the study.

After obtaining anthropometrics and medical history, physical examination was performed. Systolic and diastolic office blood pressure (BP) levels were recorded in a standardized fashion according to guideline recommendations at the first visit of the study. Blood sample was taken in order to measure glucose, lipid levels and other biochemical parameters (e.g. creatinine).

A meta-analysis described that the NO dependency of the FMD response was the most in studies with FMD measurements at brachial artery with distal placement of the cuff and 5-min occlusion (Green *et al*., 2014). We therefore applied this methodology in our study. FMD was assessed with the UNEX-device (UNEX EF 18G, Unex Co., Nagoya, Japan) in this cross-over single blinded randomized study. First, we measured FMD at baseline. At least 30 minutes after measurement of baseline FMD, the subjects were randomized to receive either NG-monomethyl-l-arginine (L-NMMA) or saline solution. L-NMMA was administered intravenously as a bolus infusion (3 mg/kg over 5 min) followed by constant infusion (2 mg/kg over 30 min). The total dose of L-NMMA was 4.25 mg/kg (Ritt *et al*., 2011). Saline solution was administered constantly at the rate of 2ml/hour without a bolus. All subjects were blinded to the solution infused. Under constant infusion of L-NMMA or saline solution, measurement of FMD was repeated. In subjects who received L-NMMA, L-arginine (100mg/kg over 30 min) was administered after FMD measurement to counteract the L-NMMA induced vasoconstriction. In control subjects, saline solution was given instead of L-arginine. This analysis was repeated after 1 week with the alternative infusion of L-NMMA or saline. Figure 1 outlines the study design. Each subject had a total of four FMD measurements: two baseline measurements (base 1 and base 2; one week apart) and one during infusion of L-NMMA and saline, respectively. We did not achieve the exact same position on the brachial artery for the two baseline measurements, thereby having a slight discrepancy in the rest diameter between those measurements.

### 2.2 | FMD protocol

Participants were instructed to fast and abstain from alcohol, caffeine and antioxidant vitamin supplements on the day of the FMD examination. All FMD measurements were conducted in the morning, with subjects being fasted overnight. Participants were asked

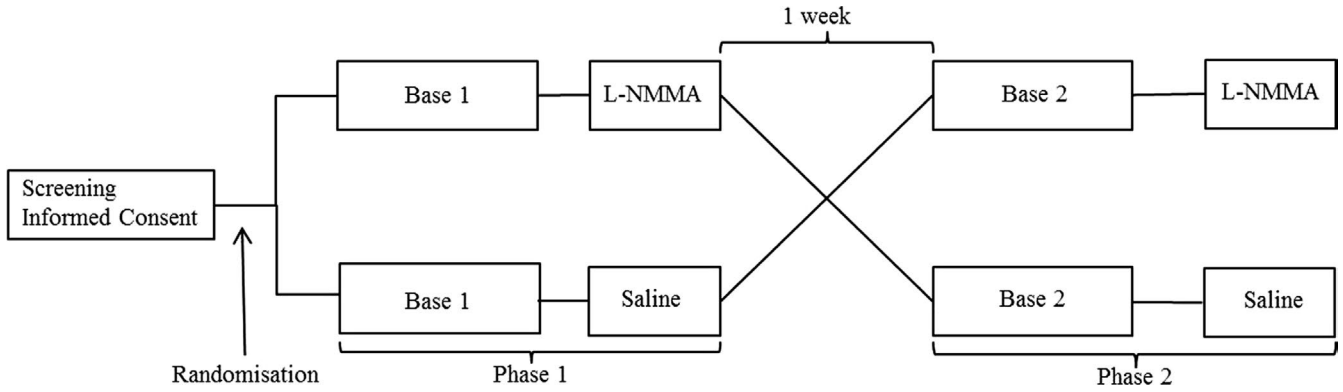


**FIGURE 1** Study design. Study consisted of two different phases (phase 1 and phase 2) lying one week apart. Each phase consist of 2 FMD measurements, one baseline (base 1/ base 2) and one measurement during infusion of either L-NMMA or saline (L-NMMA/Saline). Each box represents one FMD measurement. Base 1, first baseline FMD measurement before randomization to receive L-NMMA or saline; L-NMMA/Saline, FMD measurement during L-NMMA or saline infusion; base 2, second baseline FMD measurement before administrating alternative infusion of L-NMMA or saline. $\text{FMD}(\%) = \frac{\text{Maximum diameter (mm)} - \text{Rest diameter (mm)}}{\text{Rest diameter (mm)}} \times 100$

to rest in a quiet, dimly lighted, air-conditioned room for at least 15 min in supine position. Then, blood pressure (BP) measurement by the oscillometric method was carried out, followed by the baseline FMD measurement.

FMD was measured using the UNEX EF device connected to an online computer-assisted analysis software. This semi-automatic assessment of FMD using the UNEX EF has been described previously (Iguchi *et al*., 2013; Königstein *et al*., 2021) and has been found to be reliable across various institutions (Tomiyama *et al*., 2015). Briefly, in this system, long and short axis images of the brachial artery were recorded using a 10-MHz high-resolution H-shaped ultrasound linear array transducer. The diastolic diameter of the brachial artery was determined semi-automatically using an instrument equipped with a software for monitoring the brachial artery diameter and tracked automatically in real time by synchronization with electrocardiographic R-wave. B-mode images and A-mode waves of the brachial artery were simultaneously and continuously recorded with a probe attached to a stereotactic probe-holding device. An occlusion cuff was wrapped around the forearm with the proximal edge of the cuff at the elbow (1–2 cm above the elbow). After obtaining the rest diameter, the occlusion cuff was inflated to 50 mmHg above the systolic BP and kept inflated for 5 min. Again, images of the brachial artery were recorded continuously for 2 min following cuff deflation. Finally, FMD was estimated as the percent increase of the diameter of the brachial artery over the baseline value at maximal dilatation during reactive hyperaemia.

Automated output was generated for the rest diameter, maximum diameter and FMD of the brachial artery by the UNEX EF. All FMD images were also analysed manually by a technician of the UNEX Co. This technician was blinded to the clinical characteristics of subjects and solution infused. Of all FMD measurements ($n$ = 100), $n$ = 10 measurements have been excluded because of poor imaging quality.

**TABLE 1** Clinical characteristics of subjects

| Parameter | Healthy subjects ($N$=25) Mean ± $SD$ |
|---|---|
| Age, years | 30.3 ± 7.9 |
| Sex, % male | 100 |
| Body weight, kg | 74.8 ± 8.6 |
| BMI, kg/m$^2$ | 23.2 ± 2.4 |
| Office SBP, mmHg | 123.8 ± 9.8 |
| Office DBP, mmHg | 72.3.8 ± 8.3 |
| Office HR, bpm | 65.8 ± 8.6 |
| Serum sodium, mmol/L | 141.1 ± 5.8 |
| Serum potassium, mmol/L | 4.3 ± 0.4 |
| Serum uric acid, mg/dl | 6.0 ± 0.9 |
| Serum creatinine, mg/dl | 0.88 ± 0.10 |
| eGFR, ml/min per 1.73m$^2$ | 113.0 ± 12.9 |
| RPG, mg/dl | 85.6 ± 7.2 |
| HbA1c, % | 5.2 ± 0.19 |
| Triglyceride, mg/dl | 86.2 ± 39.0 |
| Total cholesterol, mg/dl | 188.4 ± 31.6 |
| LDL, mg/dl | 119.6 ± 24.4 |
| HDL, mg/dl | 53.0 ± 8.5 |

*NOTE:* Data are given as mean ± SD.

Abbreviations: BMI, body mass index; BPM, beats per minute; DBP, diastolic blood pressure; GFR, estimated glomerular filtration rate calculated using CKD-epi formula; HDL, high density lipids; HR, heart rate; LDL, low density lipids; RPG, Random plasma glucose; SBP, systolic blood pressure.

## 2.3 | Statistics

Data are presented as means and percentages with standard deviation (SD). Statistical significance of differences was determined

using paired *t*-test. Bivariate correlation analyses were performed using Pearson's test. Two-tailed *p*-values of <0.05 were considered statistically significant. FMD measurement reproducibility was interrogated via the coefficient of variation. Statistical analysis was performed using IBM SPSS Statistics 21.0.0.2 (SPSS Inc, Chicago, IL/USA).

## 3 | RESULTS

### 3.1 | Study cohort

Twenty-five healthy subjects participated in the study. The clinical characteristics of the study cohort are provided in Table 1. All subjects were male, average age was 30 years and none had any evidence of significant vascular disease. Subjects were not obese and did not have impaired glucose tolerance based on HbA1c or any lipid disorder. Before study participation, all subjects declared to be non-smokers and denied alcohol or drug abuse. None of the participants were on any medication.

### 3.2 | Basal NO activity by FMD

During infusion of L-NMMA with the consequence of lowering NO availability and therefore causing vasoconstriction diastolic BP (67 ± 10 mmHg vs. 74 ± 10 mmHg; $p < 0.001$) increased and heart rate (57 ± 8 bpm vs. 48 ± 6 bpm; $p < 0.001$) decreased. Infusion of L-NMMA reduced FMD significantly (Figure 2b, Table 2, $FMD_{L\text{-}NMMA(base)}$ vs. $FMD_{L\text{-}NMMA}$: −37%; $p = 0.002$). In contrast, infusion of saline solution had no effect on FMD (Figure 2a, Table 2, $FMD_{saline(base)}$ vs. $FMD_{saline}$: +14%; $p = 0.392$).Change in FMD was different between the saline and L-NMMA groups ($\Delta FMD_{LNMMA}$ vs. $\Delta FMD_{saline}$; $p = 0.032$). The time to peak FMD ($t_{L\text{-}NMMA(base)}$ 49.2 ± 11.4 s vs. $t_{L\text{-}NMMA}$ 45.9 ± 10.3 s; $p = 0.328$) and maximum blood flow ($MF_{L\text{-}NMMA(base)}$ 37.7 ± 16.6 cm/s vs. $MF_{L\text{-}NMMA}$ 45.9 ± 16.7 cm/s; $p = 0.385$) of the brachial artery during reactive hyperaemia were not different between baseline and during L-NMMA infusion.

### 3.3 | Correlation between automated and manual analysis

A significant correlation has been found between automatically analysed results and those results obtained by an experienced technician (Table 3, Figure 3). This correlation was found for rest diameter, maximum diameter and FMD in all the 4 measurements.

## 4 | DISCUSSION

The main finding of this study is that the dependency of FMD on basal NO activity is approximately 40% in healthy subjects and thus less than half. Many studies have been conducted to estimate the contribution of NO to FMD compared with other mediators of vasodilatation such as, various endothelium-derived hyperpolarizing factors (EDHF) (Bellien *et al*., 2006) like, prostacyclin (Osanai *et al*., 2000) or modulation of FMD by sympathetic nervous system (Dyson *et al*., 2006). In our study, we analysed the first time the contribution of NO to FMD assessed by a novel semi-automatic device, which has been demonstrated to be reliable (Tomiyama *et al*., 2015).

In fact, the influence of basal NO activity on FMD verified in this study is low. One possible explanation for this is the duration of ischaemia applied during FMD measurement. Previously, we found that the magnitude of NO dependency on vasodilatory response during reactive hyperaemia is dependent on the duration of ischaemia (Raff

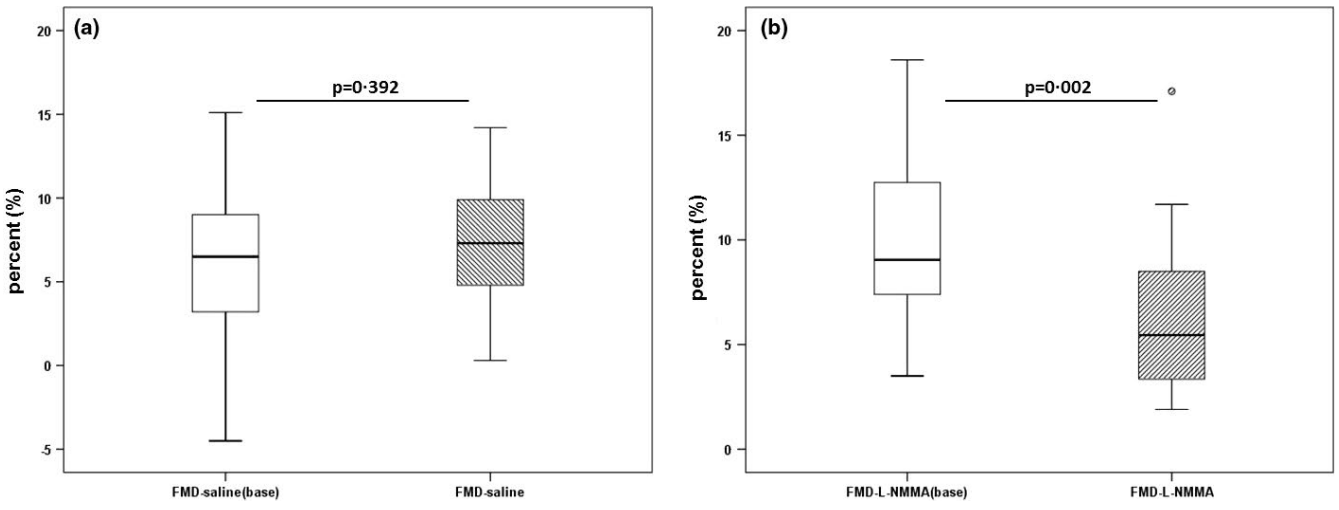


**FIGURE 2** Dependency of flow-mediated vasodilatation from basal NO activity. (a) change in FMD during saline infusion; FMD-saline (base), baseline FMD measurement before saline infusion; FMD-saline, FMD measurement during saline infusion. (b) change in FMD during L-NMMA infusion; FMD-L-NMMA(base), baseline FMD measurement before L-NMMA infusion; FMD-L-NMMA, FMD measurement during L-NMMA infusion

**TABLE 2** Dependency of flow-mediated vasodilatation from basal NO activity

| | Rest diameter (mm) | Maximum diameter (mm) | FMD (%) | *p*-value (FMD) |
|---|---|---|---|---|
| Saline(base) | 3.934 ± 0.385 | 4.221 ± 0.420 | 7.4 ± 6.3 | 0.392 |
| Saline | 3.926 ± 0.360 | 4.252 ± 0.402 | 8.4 ± 6.3 | |
| L-NMMA(base) | 3.810 ± 0.349 | 4.192 ± 0.351 | 10.2 ± 4.2 | 0.002 |
| L-NMMA | 3.951 ± 0.399 | 4.196 ± 0.367 | 6.4 ± 3.8 | |

Abbreviations: L-NMMA(base), baseline FMD measurement before L-NMMA infusion; L-NMMA, FMD measurement during L-NMMA infusion; saline(base), baseline FMD measurement before saline infusion; saline, FMD measurement during saline infusion.

**TABLE 3** Correlation between automatically analysed and technician's results

| | Rest diameter (mm) | Maximum diameter (mm) | FMD (%) |
|---|---|---|---|
| Saline(base) | $r = 0.946$, $p < 0.001$ | $r = 0.930$, $p < 0.001$ | $r = 0.497$, $p = 0.026$ |
| Saline | $r = 0.986$, $p < 0.001$ | $r = 0.965$, $p < 0.001$ | $r = 0.822$, $p < 0.001$ |
| L-NMMA(base) | $r = 0.878$, $p < 0.001$ | $r = 0.960$, $p < 0.001$ | $r = 0.441$, $p = 0.040$ |
| L-NMMA | $r = 0.956$, $p < 0.001$ | $r = 0.969$, $p < 0.001$ | $r = 0.645$, $p = 0.007$ |

Abbreviations: L-NMMA(base), baseline FMD measurement before L-NMMA infusion; L-NMMA, FMD measurement during L-NMMA infusion; saline(base), baseline FMD measurement before saline infusion; saline, FMD measurement during saline infusion.

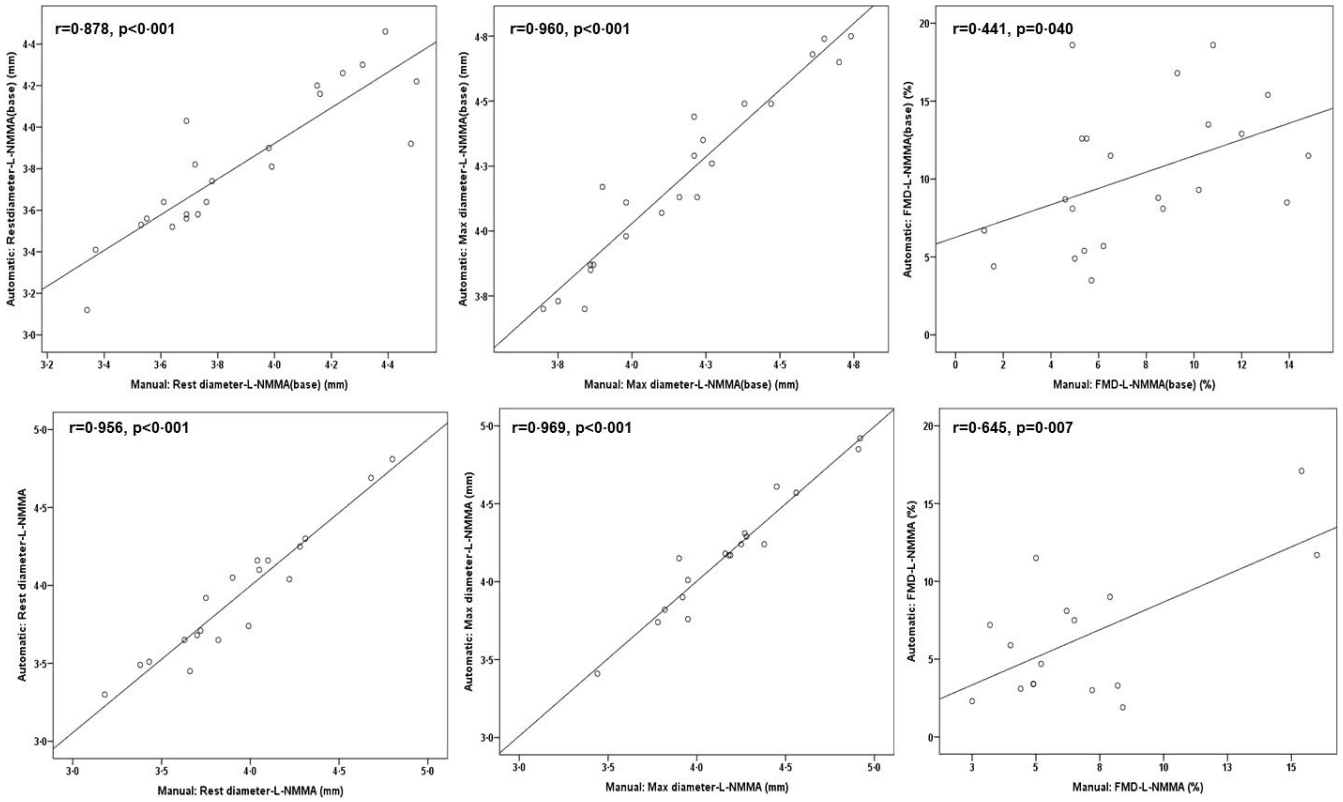


**FIGURE 3** Illustration of the relationship between automatically analysed and technician's results. Upper raw demonstrates the FMD parameters (rest diameter, max diameter and FMD) of baseline measurement before L-NMMA infusion and lower raw demonstrates these parameters during L-NMMA infusion

*et al*., 2010). It has been demonstrated that compared to ischaemia applied for 5 min, ischaemia of 1 and 2 min induced a significantly reduced vasodilatory response indicative of an increased NO activity at baseline that was blocked by L-NMMA. Of note, the contribution of NO was at maximum after 1 min of ischaemia. In accordance to that, Mullen *et al*. (2001) confirmed that the duration of cuff occlusion

plays a role in FMD response after L-NMMA infusion. They observed virtually no effect of L-NMMA infusion on maximal vasodilatation of the artery after 15-min cuff occlusion. In our study, we applied the most commonly adopted approach, which is duration of ischaemia of 5 min. The reduced contribution of NO with longer ischaemia may be explained by the formation of metabolic acid compounds, such as lactate, known to cause vasodilatation. At this point, the exact stimulus profile for maximum NO-dependent FMD response is unknown.

The location of the applied ischaemia also seems to play a relevant role. Doshi *et al*. (2001) revealed that FMD following wrist occlusion is mediated fully by NO (98%), while FMD following upper arm occlusion comprises a substantial component not mediated by NO. This might be due to the tissue ischaemia around the brachial artery induced by upper arm occlusion. At the present study, we applied ischaemia at the forearm with the edge of the cuff placed at the level of elbow and found that around 40 percent of the FMD is mediated by NO. Similar results have been found by Agewall *et al*. (2002) also applying forearm occlusion during FMD measurement. To the best of our knowledge, there are no studies comparing wrist and forearm occlusion to analyse dependency of FMD on basal NO activity.

Thirdly, it should be noted that contribution of NO activity on FMD response has been analysed using different methodological approaches, which have important impact on the response magnitude, and limit the comparability of outcomes between studies (Green *et al*., 2014). In the present study, the FMD measurements were done using a novel semi-automatic device called UNEX EF and the FMD protocol was according to guideline recommendations (Corretti *et al*., 2002; Thijssen *et al*., 2019). The UNEX EF acquire images of the brachial artery automatically using the ECG signal as a trigger and changes of the arterial diameter are tracked automatically in real time using computer-based edge-detection techniques. The reliability of FMD assessment using this device has been confirmed at different institutions in Japan (Tomiyama *et al*., 2015). We believe that using UNEX EF device with the current FMD protocol we applied optimal requirements to analyse NO-dependent FMD response to reactive ischaemia. The measurements of our present study were analysed a second time manually by experienced technicians of the UNEX Co. With regard to the rest and maximum diameter, we found excellent correlation between automated and manually analysed outputs. The significant, but poorer correlation between automated and manually analysed FMD results from very small differences in diameter and from the fact that FMD is a percentage ratio of these diameters thereby exaggerating even small differences of the raw values.

Finally, it should be discussed that in light of the result that NO activity accounts for only less than half of the FMD response, other vasodilator stimuli and interplay between these are likely to provoke the total FMD response (Engelke *et al*., 1996; Bellien *et al*., 2008). In our study, we inhibited the production of endothelial NO using L-NMMA. Inhibition of endothelial NO synthase is known to lead to compensatory increased production of prostacyclin, which is known as a potent endothelium-derived vasodilator (Osanai *et al*., 2000). The importance of other vasodilatory factors in endothelium-dependent FMD is known. An inhibition of the production of NO and other EDHF together, reduced the FMD response to a greater extent compared to inhibition of the production of NO alone (Bellien *et al*., 2008).

Another important finding of our study is that time to FMD after cuff deflation is independent of NO. Similar results have been described by intra-arterial infusion of L-NMMA in healthy subjects, which resulted in reduced FMD response, but not time to FMD. Moreover, time to FMD is not altered between healthy and diseased subjects, so it's not considered as a measure of endothelial health and cardiovascular status (Liuni *et al*., 2010).

Our study has strengths and limitations. Since it was a single blinded study, the operator conducting the FMD measurements was not blinded to the infused substances (L-NMMA/saline). However, we postulate that the bias due to unblinded operator is less as the FMD was measured semi-automatically. Furthermore, other than the study subjects the technician who manually analysed the FMD measurements was also blinded to the infusions. Our study states a diagnostic scan failure rate of 10%, which is less than reported by most studies (approximately 30%) (Charakida *et al*., 2013). This reduced diagnostic scan failure rate might be due to the semi-automatic nature of the UNEX device along with its other advantages including a stereotactic ultrasound probe holder. Apart from that, our operators of the FMD measurements were extensively trained by technicians of the UNEX Co. In this study, we analysed dependency of FMD from basal NO activity of the brachial artery and this cannot be extrapolated to other arteries, since smaller arteries dilate more than larger arteries to comparable stimuli (Celermajer *et al*., 1992).

## 5 | CONCLUSION

With this clinical trial we could demonstrate that the influence of NO on FMD is approximately 40% if assessed using the UNEX EF. Prior to use FMD as a marker of endothelial dysfunction, we should explore different methods including various duration of forearm ischaemia to increase NO dependency of FMD.

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### CONFLICTS OF INTEREST

No conflict of interests.

### AUTHOR CONTRIBUTIONS

DK designed the study, analysed all data and wrote manuscript. AB contributed to discussion and reviewed data and manuscript. JK, SJ and KS: contributed to discussion and reviewed manuscript. CO and CD: contributed to discussion, reviewed/edited manuscript. RES designed the study, contributed to discussion and reviewed data/manuscript.

## DATA AVAILABILITY STATEMENT

The data underlying this manuscript cannot be shared publicly due to the privacy of individuals that participated in the study. The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ORCID

*Dennis Kannenkeril* https://orcid.org/0000-0002-2345-238X

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