Aspects of endothelial function testing in coronary vascular disease
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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2003

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Endothelial dysfunction in patients with coronary artery disease - A comparison of three frequently reported tests –


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ABSTRACT

BACKGROUND  Endothelial dysfunction is useful in predicting future cardiovascular disease. At present several tests are available to test endothelial function i.e. coronary diameter response to acetylcholine, forearm blood flow (FBF) response to acetylcholine and brachial artery flow-mediated dilative (FMD) response to post-ischemic hyperaemia. This study aimed to compare the three most frequently reported endothelial function tests.

METHODS  Twenty-eight patients (19 males and 9 females, mean age 57 years) referred for diagnostic coronary angiography were considered for endothelial function measurement in the coronary artery as well as in the forearm by FBF and FMD.

RESULTS  Acetylcholine decreased the mean coronary diameter by 7.4% (SD 6.3) and increased the mean FBF by 230% (SD 152). Hyperaemia increased the mean brachial diameter by 6.7% (SD 4.8). The effect of acetylcholine on forearm resistance vessels was significantly related to the effect of acetylcholine on the coronary conduit vessels (p = 0.039). However, FMD was not related to FBF nor to the coronary response.

CONCLUSIONS  In patients with mild coronary endothelial dysfunction, forearm vasoreactivity is related to the coronary response provided that the same stimulus is used.
INTRODUCTION

Endothelial dysfunction is a common denominator for a variety of changes the endothelium undergoes during atherogenesis (1). In recent years a number of tests has been developed in order to quantify endothelial function. The use of these tests is widespread, since coronary endothelial dysfunction is a useful marker for predicting future cardiovascular events (2;3). Endothelial function in these tests is assessed by demonstrating (lack of) nitric oxide mediated vasodilatation in an artery (4;5). Due to the invasive nature of coronary endothelial function testing, attention has shifted to forearm vasoreactivity. At present, the most feasible techniques for testing endothelial function in the forearm are: 1) local intra-brachial infusion of NO-agonists such as acetylcholine with measurement of the blood flow response by strain-gauge plethysmography (FBF; ml/100ml forearm volume/min.), 2) local NO release after forearm limb ischemia and reactive hyperaemia with measurement of the brachial flow mediated dilatation by ultrasound (FMD; μm) (6). However, the underlying mechanisms as well as the vessels that are investigated (conduit coronary vessels, or forearm micro- and macrovasculature) are quite diverse. Interestingly, several authors have shown a correlation between endothelial function in micro- and macrovasculature (7;8). And recently, evidence has accumulated that FMD in the brachial artery is useful to stratify patients at risk for future coronary revascularisation (9). However, the correlation between FMD and coronary endothelial function has been weak at best (r-square = 0.13 (7)) and the value for predicting the presence of coronary endothelial dysfunction was only 49%. The predictive value of 'semi-invasive' FBF for coronary artery disease remains to be established. This study aimed to investigate the relationship between three current endothelial function tests in a population with a high pre-test probability of coronary endothelial dysfunction. We therefore compared acetylcholine- and hyperaemia-mediated forearm endothelial responses in patients who were referred for coronary angiography with concomitant coronary endothelial function testing.

METHODS

Patient population. The Intervention Cardiology Risk Stratification (ICaRiS)-study included patients with stable angina pectoris referred for diagnostic coronary angiography study. Excluded were patients with unstable angina, recent myocardial infarction, valvular heart disease requiring surgical intervention, clinical evidence of heart failure, or a history of previous coronary intervention (PTCA or CABG). Excluded from
coronary acetylcholine infusion were patients with significant left main coronary artery narrowing (> 50%) or ischemic ECG changes and / or progressive angina pectoris during diagnostic catheterisation. In this sub study 28 patients, included between July 1998 and July 1999, were considered for endothelial function testing in both the coronary artery and the forearm (FMD and FBF).

**Data collection.** Baseline screening included history of coronary risk factors, physical examination, and fasting total serum cholesterol. A standard diagnostic catheterisation procedure with concomitant intracoronary acetylcholine and nitro-glycerine infusion was performed. The response to both stimuli was measured by automatic contour detection technique (quantitative coronary angiography). FBF responses to different stimuli and FMD of the brachial artery were determined on one day within 7 days from catheterisation (before or after). Plethysmography measured the forearm responses to intrabrachial acetylcholine and nitroprusside. The brachial artery FMD in response to hyperaemia and sublingual nitroglycerine was measured by ultrasound (wall tracking system, Pie Medical, Maastricht, The Netherlands).

**Definitions.** Angiographically significant coronary artery disease (CAD) was defined as at least one coronary vessel with > 50% luminal narrowing. Coronary endothelial function was defined as the coronary diameter in response to maximal concentration acetylcholine as percentage of the mean baseline diameter in the most-constrictive coronary artery. A negative response represents a vasoconstriction. From that most-constrictive coronary artery, the response to nitro-glycerine was also expressed as percentage of the mean baseline diameter. FBF by strain-gauge plethysmography was expressed as ml per 100ml-forearm volume per minute. Validity of individual flow measurements was verified by routine check of the correlation coefficient of the curve (> 0.90). Endothelium-dependent dilative response to intrabrachial acetylcholine and nitroprusside was defined as the maximum blood flow response to that stimulus as percentage of the mean baseline blood flow. Off-line FMD was calculated by a blinded observer using wall track system software analysis package. Brachial response to hyperaemia and nitro-glycerine was defined as the maximal brachial diameter (μm) in response to both stimuli as percentage of the mean baseline diameter.

**Coronary angiography.** Before the coronary angiogram as well as before the forearm tests, vasoactive agents were discontinued for at least 3 days (24 hours if recurrent angina was expected). Considered as vasoactive agents were long-acting nitrates, calcium channel blockers,
angiotensin converting enzyme inhibitors and angiotensin II receptor blockers. The use of β-adrenergic blockers was allowed. Smokers did not smoke for at least 4 hours before examination. Using a standard percutaneous femoral approach, a 6F diagnostic catheter was advanced into the left main coronary artery. Intracoronary nitro-glycerine was not given before the diagnostic catheterisation procedure.

**Coronary vasomotor function.** After completion of the diagnostic CAG, the diagnostic 6 French Judkins catheter was left in the left main coronary artery. For accurate vasomotor response measurement, the subsequent angiographic recordings were made with 25 frames per second and care was taken to have an adequate part of the catheter visible for calibration. A baseline CAG was done, visualizing the proximal left anterior descending artery (LAD) and the circumflex artery (LCX). Then acetylcholine-chloride (concentration 0.16 µg/ml; Clinalfa AG, Läufelfingen, Switzerland) was infused through the catheter for at least 3 minutes. To achieve a coronary blood concentration of $10^{-8}$ Molar acetylcholine, infusion rate was 82 ml/hour (with the assumption that the blood flow in the left main coronary artery was 120 ml/minute). At the end of the infusion, the acetylcholine in the catheter was removed carefully and an angiographic recording of the endothelium-dependent vasodilative response to acetylcholine was made. This procedure was repeated using 1.6 µg/ml and 16 µg/ml ($10^{-7}$ M and $10^{-6}$ M blood concentration) acetylcholine concentrations, respectively. Finally, the endothelium-independent dilative response was recorded one minute after an intracoronary bolus of 0.5-mg nitro-glycerine.

**Plethysmography.** Experiments were performed in a quiet room kept at a constant temperature of 22-24 °C. During the experiments the subjects were in supine position with both forearms stabilized slightly above the level of the heart. A 22-g needle (Arrow, Pennsylvania, USA.) was inserted into the brachial artery of the non-dominant arm after local anaesthesia. FBF in both arms was measured at 15-second intervals by R-wave triggered venous occlusion plethysmography with mercury-in-silastic strain gauges (10,11). The upper arm collecting cuffs were inflated to 40 mmHg. During FBF-measurement both hands were excluded from the circulation by small wrist cuffs, which were inflated 40 mmHg above systolic pressure. Intra-arterial blood pressure was recorded continuously. The baseline measurements started at least 30 minutes after cannulation of the brachial artery, when FBF was stable. Between the various infusions the wrist cuffs were deflated and sufficient time (at least 15 minutes) was allowed for FBF to return to baseline levels before the next infusion was started. The following drugs were infused into the brachial artery: sodium nitroprusside
(E Merck, Darmstadt, Germany) and acetylcholine (Clinalfa AG, Läufelfingen, Switzerland). Nitroprusside was administered in a cumulative dose infusion of 6, 60, 180 and 600 ng/100 ml forearm/min., each dose for at least 1.5 minutes. Acetylcholine was administered in a cumulative dose infusion of 500, 1000 and 2000 ng/100 ml forearm/min), each dose infused for 3 minutes. Both drugs were administered in random order.

Wall-tracking. Patients were studied in supine position. Three ECG leads were attached. Flow mediated dilatation was determined as published previously (12;13). In short, after a 10-15 minute rest, the brachial artery in the right ante-cubital fossa was visualized using a 7.5 MHz transducer. When a satisfactory optimal image of the brachial artery lumen diameter was obtained, the transducer was fixed and a wall tracking system was used to measure the lumen diameter. By inflation of blood pressure cuff located around the forearm for 5 minutes, ischemia was applied to the forearm distal to the location of the ultrasound probe. Ultrasonography was continued for 5 minutes to allow for lumen diameter measurements at 20 seconds intervals. Wall track data were stored digitally (respectively 3 baseline measurements; 8 measurements after cuff release, starting 30 seconds after deflation until 170 seconds after deflation at 20 second intervals). Next, 0.4-mg nitro-glycerine was administered sublingually to assess endothelium-independent dilation (respectively 3, 4 and 5 minutes after NTG administration).

Quantitative coronary angiography. Quantitative Coronary Angiography (QCA) was performed by a previously described and validated automatic contour detection technique (CMS, Medis Co., Nuenen, the Netherlands) (14;15). End-diastolic frames of a non-stenotic proximal segment of the LAD were selected for QCA. User interaction was limited to the definition of the start and end points of the coronary segment to be analysed. Easily identifiable side branches were used as anatomic landmarks to allow the analysis of the same segments in successive angiograms. The length of the analysed segment was always within a range of 10% from baseline segment length. In case of overlap with other branches, the independent analyst manually edited automatic contour detection. Segment diameter was determined in millimetres.

Statistical analysis. Baseline characteristics are presented as mean ± standard deviation. Categorical data are presented per number of valid observations. For the normally distributed continuous variables, differences were evaluated by the F-test, for skewed distributed continuous variables (p-value Shapiro-Wilk test for normality < 0.05), the Kruskal-Wallis test was used. The associations between the coronary response to acetylcholine
and the brachial blood flow by plethysmography and wall tracking system respectively, were measured by the correlation coefficient. With normally distributed responses, the Pearson correlation coefficient was calculated. In case of skewed distribution the Spearman rank-correlation coefficient was used (p-value Shapiro-Wilk test for normality < 0.05). A p-value < 0.05 was considered statistically significant. SAS version 6.12 (Cary, N.C, USA) was used for all statistical analyses.

**Ethics.** Written informed consent was obtained from all patients before the study and the Institutional Review Board of the participating centres approved the study protocol. The study was performed in the University Hospital of Groningen, University Hospital Utrecht, and St. Antonius Hospital Nieuwegein; The Netherlands, in conform with the principles outlined in the Declaration of Helsinki (16). All studies were performed in line with institutional guidelines.
RESULTS

Characteristics of the study population. The characteristics of the 28 patients at entrance of the study are listed in table 1.

<table>
<thead>
<tr>
<th>N</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender</td>
<td>68 %</td>
</tr>
<tr>
<td>Age, years</td>
<td>57 ± 8</td>
</tr>
<tr>
<td>Hypercholesterolemia</td>
<td>68 %</td>
</tr>
<tr>
<td>Hypertension</td>
<td>54 %</td>
</tr>
<tr>
<td>Current smoking</td>
<td>36 %</td>
</tr>
<tr>
<td>Diabetes Mellitus</td>
<td>14 %</td>
</tr>
<tr>
<td>Fasting total cholesterol (mmol/l)</td>
<td>5.8 ± 1.2</td>
</tr>
<tr>
<td>Medication</td>
<td></td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>14 %</td>
</tr>
<tr>
<td>Statins</td>
<td>46 %</td>
</tr>
<tr>
<td>Nitrates</td>
<td>29 %</td>
</tr>
<tr>
<td>Calcium channel blockers</td>
<td>64 %</td>
</tr>
<tr>
<td>ASA</td>
<td>57 %</td>
</tr>
<tr>
<td>Coronary artery disease by angiography</td>
<td></td>
</tr>
<tr>
<td>smooth appearance</td>
<td>21 %</td>
</tr>
<tr>
<td>wall irregularities</td>
<td>11 %</td>
</tr>
<tr>
<td>1-vessel disease</td>
<td>29 %</td>
</tr>
<tr>
<td>2-vessel disease</td>
<td>29 %</td>
</tr>
<tr>
<td>3-vessel disease</td>
<td>11 %</td>
</tr>
</tbody>
</table>

Table 1: In four patients the coronary acetylcholine infusion test was not carried out because of exclusion criteria during coronary angiography, as described in "patient population". In all other patients, mild coronary endothelial dysfunction was present, according to criteria described by Suwaidi et al (2). There were seven technical failures in measurement of the FBF (causes: unsuccessful cannulation (4), R-wave trigger failure (2), impaired responsiveness of the strain gauges due to subcutaneous fat deposition (1)). All patients had at least two endothelial function tests to evaluate; in 17 patients the results of all three tests were available.

Acetylcholine mediated coronary and brachial artery response. The mean coronary diameter before acetylcholine infusion was 2.49 ± 0.6 mm. In response to acetylcholine, the mean coronary diameter decreased by 7.4 ± 6.3 %, whereas nitro-glycerine increased the mean coronary diameter by 10.0 ± 8.9 %. The mean FBF before acetylcholine infusion was 2.72 ± 0.9 ml/100ml forearm volume/min. In response to intrabrachial acetylcholine, the mean FBF increased by 230 ± 152 %, whereas nitroprusside increased the mean FBF by 306 ± 155 %. Intra-brachial infusions of acetylcholine and sodium nitroprusside did not alter mean blood pressure, heart rate or FBF in the contra lateral control arm (data not shown). To obtain normal distribution, the FBF response to acetylcholine was transformed on a log-scale. The response to acetylcholine in both the coronary and brachial artery (N=17) showed a significant relation
(correlation $r = 0.51$; $p = 0.039$ after log-transformation), as shown in figure 1. No association could be demonstrated between both responses to nitrates ($r = -0.18$; $p = 0.50$).

![Correlation between the coronary diameter response and the forearm blood flow response (log-scale) to acetylcholine (ACH), presented as change from baseline.](image)

**Figure 1**: Correlation between the coronary diameter response and the forearm blood flow response (log-scale) to acetylcholine (ACH), presented as change from baseline.

**Brachial responses to acetylcholine and hyperaemia.** As described, the mean FBF increased by 230% and 306% in response to intrabrachial acetylcholine and nitroprusside respectively. The mean brachial diameter before hyperaemia was 4466 ± 777 μm. In response to hyperaemia, the mean brachial diameter increased by 6.7 ± 4.8%, whereas sublingual nitroglycerine increased the mean brachial diameter by 15.6 ± 6.4%. In the forearm, no significant relation could be demonstrated between the endothelium dependent responses to acetylcholine and hyperaemia ($r = -0.23$; $p = 0.33$) nor between the endothelium-independent responses to nitrates ($r = 0.06$; $p = 0.79$). When taking into account only the 17 patients with valid results in all three tests, the correlations were comparable ($r = -0.27$; $p = 0.30$ and $r = 0.04$; $p = 0.89$ respectively).
Acetylcholine mediated coronary and hyperaemia mediated brachial response. Comparison of the acetylcholine-induced coronary response and induced-induced forearm FMD did not reveal a significant correlation ($r = -0.03; p = 0.89$). Also, no association for the endothelium-independent response to nitrates was present ($r = -0.18; p = 0.39$). Using only the 17 patients with valid results in all three tests, the conclusion remained the same ($r = 0.06; p = 0.81$ and $r = -0.17; p = 0.52$ respectively).

DISCUSSION

This comparative study shows a correlation between endothelial function in the micro-vasculature of the forearm and the conduit coronary vessels. In response to acetylcholine, a more constrictive response in the epicardial coronary artery is significantly associated with a lower increase in forearm blood flow.

Since an enormous amount of studies have reported microvascular endothelial dysfunction in the forearm in presence of traditional cardiovascular risk factors, the concept of endothelial dysfunction as a generalized disease state has been put forward (1;17;18). As clearly stated in the introduction, the use of peripheral endothelial function tests would allow broader implementation of the technique in larger intervention trials. The present study aimed to compare the most feasible tests.

Ludmer et al. (19) were among the first to demonstrate that acetylcholine could be safely infused selectively into the coronary circulation to assess coronary conduit vessel vasomotion. Since then, this test has served as the gold standard for endothelial function testing. However, the assessment of coronary vascular reactivity requires an invasive approach and acetylcholine infusion has its own potential risk (20). Therefore, attention has shifted towards 'peripheral' endothelial function testing. Two techniques have been implemented for peripheral endothelial function assessment, namely the 'semi-invasive' blood flow response to acetylcholine by strain-gauge plethysmography (FBF) as first described by Hokanson et al. (21) and the brachial dilative response after forearm induced by ultrasound (FMD; mm) as first described by Celermajer et al. (22).

Recently, evidence has accumulated that FMD in the brachial artery, in an identical manner as we report here, may be useful to stratify patients at risk for future coronary revascularisation (9). The reported correlation between FMD and coronary endothelial function, using the same technique as we report here, has been weak at best (see Anderson et al.: r-square = 0.13)(7), and the predictive value of 'semi-invasive' FBF for coronary artery disease remains to be established. In reference to the correlation coefficient of 0.36 as reported by Anderson (7), in our study with 17
patients with valid results of both the coronary response to acetylcholine and FMD, the power to detect a difference of -0.36 ($r_0 = 0$, $r_a = 0.36$) would have been 43%, using a directional hypothesis test with a significance level of 5%.

The present data show that the correlation between acetylcholine-mediated FBF and acetylcholine-mediated epicardial coronary diameter response is modest, however it clearly exceeds the previously reported correlation between FMD and coronary response. In addition, no association could be demonstrated between micro- and macro vascular responses in the forearm (hyperaemia-mediated FMD and acetylcholine-mediated FBF) nor between hyperaemia and acetylcholine-mediated responses in the macrovasculature (brachial FMD and conduit coronary vessels). Our study suggests that acetylcholine-mediated forearm microvascular endothelial function parallels that in coronary conduit vessels.

**Clinical implications.** The concept of endothelial dysfunction as a generalized disease state and its relation with increased risk for future cardiovascular events makes that the implementation of forearm endothelial function testing in clinical practice is an attractive diagnostic target. However, the underlying mechanisms as well as the vessels that are investigated are quite diverse (23). Our study suggests that forearm vasoreactivity is more related to coronary endothelial function when the same stimulus is used. However, its use in population based studies or repetitive measurements will be limited due to its invasive nature.

**Limitations of the study.** All patients were referred to the catheterisation laboratory for suspected coronary artery disease. As such, the patient group we report here is rather homogeneous and the majority of patients had evidence of endothelial dysfunction. However, if endothelial function testing will ever prove to be of use, it will be in a high-risk population as ours. In contrast to earlier data by Anderson et al. (7), no association could be demonstrated between hyperaemia- and acetylcholine-mediated response in the macrovasculature (brachial FMD and conduit coronary vessels). The population studied by Anderson et al. demonstrated a larger range of coronary endothelial function (ranged from 40% constriction to 20% dilation) compared to our study population (ranged from 20% to 0% constriction). Although the main advantage of its non-invasive nature, the FMD measurement is very sensitive towards physiological changes (24) and faces technical difficulties in accurately determining the "discrete" changes in brachial artery diameter, thus resulting in a "larger" variability. These factors will limit the potential to detect coronary endothelial dysfunction. The sample size reported here is rather small. Therefore, the results of this study are merely hypothesis-
generating and further studies will be needed to conform our findings.

Acknowledgments. We thank D Versteeg, FW Asselbergs and M Hijmering for their technical assistance. Erik Stroes, Paul van Haelst and René Tio are fellows of the Netherlands Heart Foundation (D97-023, D99-020 and D95-019). Hokanson plethysmography equipment was sponsored by the Sorbo Heart Foundation (98037), St. Antonius Hospital, dept. of Cardiology, Nieuwegein, The Netherlands.
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