# CAROTID ARTERY REACTIVITY PREDICTS EVENTS IN PERIPHERAL ARTERIAL DISEASE PATIENTS

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**STUDY TYPE**: Observational
**ABSTRACT WORD COUNT**: 250
**FIGURES**: 4
**TABLES**: 2

**SOURCES OF FUNDING**: ACCM - Top Institute for Food and Nutrition-grant

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**Running title:** Event prediction with carotid reactivity

**LIST OF ABBREVIATIONS**

| | |
|---|---|
| ABPI | Ankle brachial pressure index |
| AUC | Area under the curve |
| BMI | Body mass index |
| CAR | Carotid artery reactivity |
| CI | Confidence interval |
| cIMT | carotid intima-media thickness |
| CPT | Cold pressor test |
| CV | Cardiovascular |
| HR | Hazard ratio |
| OR | Odd's ratio |
| PAD | Peripheral arterial disease |
| WHR | waist-to-hip ratio |

**WORD COUNT**: 2607

**Clinical Trial Registration.** www.trialregister.nl/trialreg/index.asp, NTR-4117.

**KEYWORDS:** Endothelial function; prognosis; cardiovascular disease; atherosclerosis.

## INTRODUCTION

Peripheral arterial disease (PAD) is the result of atherosclerotic arterial stenosis and occlusions in the larger vessels supplying the lower extremities.[1] Patients with PAD have a markedly increased risk for future cardiovascular mortality and morbidity.[1-4] Endothelial dysfunction contributes to the development and progression of PAD.[5, 6] Endothelial function, often examined as the brachial artery reactivity response to ischaemia, in PAD patients is impaired[7-9] and relates to future CV events.[10, 11] The recent AHA/ACC-guideline on the management of PAD highlighted the need for an easy, simple and rapid test of endothelial function to predict future adverse events in PAD.[12] Although brachial artery reactivity shows predictive capacity for future CV events,[10, 11] concerns have been raised regarding practical limitations that prevents the clinical application of this technique.

Carotid artery reactivity (CAR) testing is a simple, non-invasive procedure to examine endothelial function. It involves measuring the carotid artery diameter responses to the sympathetic stimulation produced by the cold pressor test (CPT).[13, 14] The carotid arteries, like coronary arteries,[13, 14] dilate in response to the CPT in healthy subjects, whereas this dilation is attenuated, or reversed to vasoconstriction, in patients with cardiovascular disease.[13-16] Interestingly, the coronary arteries' response to CPT is a strong, independent predictor of cardiovascular events,[17, 18] but it is not clear if the carotid response to CPT (i.e. CAR) also predicts future cardiovascular events. This study is to our knowledge, the first to examine the prognostic value of the CAR in patients with PAD. We hypothesized that CAR-induced vasoconstriction would predict future CV events in patients with PAD, independent of subject characteristics and clinical status.

# METHODS

## Participants and study approval

We recruited 172 patients with PAD scheduled for a routine visit at the vascular laboratory (Department of Surgery, Radboud University Medical Center, Netherlands) for the study (*Figure 1*). We included PAD patients with present or prior Fontaine classification 2B-3-4, age ≥18yr, and the ability to provide informed consent. We excluded patients with Raynaud's phenomenon, chronic pain syndrome, open wounds on the upper extremities, arterial-venous shunts, scleroderma, coronary, central and/or peripheral arterial disease interventions within the prior <1 week, and unstable angina pectoris, myocardial infarction, stroke or heart failure within the prior 3 months. Patients provided written informed consent prior to participation. The study was approved by the local Ethics Committee (NL-46109.091.13) in accordance with the latest revision of the Declaration of Helsinki. This study was registered as NTR-4117 (Netherlands Trial Registration).

## Experimental design

Patients abstained from strenuous exercise for 24 hours, fasted for ≥6 hours, and abstained from caffeine and vitamin C, which are known to alter endothelial function, for ≥18 hours prior to testing in accordance with guidelines on assessing endothelial function.[20]

## Experimental measures

*General characteristics.* Age and sex were obtained from the electronic patient records. A physician obtained the history of smoking, hypercholesterolemia, hypertension, diabetes and medication use. Height (in m), weight (in kg), and waist-hip ratio (WHR) were measured by a research nurse. The same vascular surgeon assigned the patient's Fontaine clinical classification. Patients with Fontaine stage ≥2 performed walking tests to distinguish between Fontaine stages. Finally, the ankle-brachial pressure index (ABPI) was measured based on clinical requirements following recent guidelines.[21] The highest systolic pressure in the right and left posterior tibial or dorsal pedis artery and in the right brachial artery was measured twice, and the average of those two measurements was used to calculate the ABPI for each leg. The lowest ABPI of two legs was used for analysis. For the purpose of analysis, we compared those above *versus* below the median ABPI.

*Carotid artery reactivity.* Patients rested on a comfortable bed in a temperature controlled room for at least 5 minutes. Participants were in the supine position with the neck extended for assessment of the carotid artery. Left carotid artery diameter was recorded continuously for 30-seconds before and for 90-seconds during immersion of the hand up to the wrist in ice slush (4 °C). Images were obtained using a L9-3 MHz linear array probe attached to a high resolution ultrasound machine. When an optimal image was found, the probe was held stable and the ultrasound parameters were set to optimise the longitudinal, B-mode image of the lumen-arterial wall interface. Following a 30-second baseline assessment of carotid artery diameter, the hand was immersed for 90-seconds with simultaneous and continuous assessment of carotid artery diameter.

CAR% responses were assessed for diameter. Analysis of the carotid artery diameter was performed by a single blinded investigator using custom-designed edge-detection and wall-tracking software, which is largely independent of investigator bias.[22] Details of this technique can be found elsewhere.[23] Baseline diameter was calculated as the mean of data acquired across the 30 seconds preceding the CPT test. After submersion of the hand in ice slush, data were calculated as the mean value for 10-second intervals, involving 8-10 full cardiac cycles. Based on this data we calculated the peak diameter change (i.e. the 10-second bin with the highest value, CAR%). The peak diameter change can refer to a maximum constriction or dilation. The direction of this change was determined by a positive (i.e. dilation) or negative (i.e. constriction) area under the curve.

Reproducibility (coefficient of variation, CV) of diameter responses to CPT were previously assessed with a 1- and 24-hour intervals in 50 subjects. Within-day CV for baseline and peak diameters were 2.2 and 2.6%, whilst day-to-day CV were 2.3% and 2.7%. Furthermore, the CAR% (i.e. maximum change in diameter) showed a within-day reproducibility of 2.6% and between-day reproducibility of 2.8%.[14]

*Intima-media thickness*. Carotid artery intima-media thickness (cIMT), a marker for vascular structure, is related to future development of PAD.[24] To examine whether the CAR relates to future CV events, independent of the cIMT, we examined cIMT from the same section of the artery as the CAR. We obtained continuous recordings of the cIMT for 10 seconds. Analyses were performed by a blinded researcher, using observer-independent edge-detection and wall-tracking software.[25] For the purpose of analysis, we compared those above *versus* below the median cIMT.

**Follow up and assessment of adverse events**

After 12 months of follow-up, adverse events were extracted from medical records and verified by a blinded vascular surgeon (MW). The Dutch National Death Registration was used to determine mortality. Death certificates of patients who experienced a fatal event were obtained when available to categorise death into CV or non-CV related mortality. Adverse events were categorised into; *1*. Cardio- and cerebrovascular events ("CV events"; CV-related mortality, myocardial infarction, coronary revascularisation procedures, transcranial ischemic attack, cerebrovascular accident, carotid surgery, major- and minor amputations, and ischemic bowel disease), *2*. “Clinical progression” that is related to PAD (loss of patency (i.e., the presence of restenosis in a previous endovascular reconstructed vessel), endovascular reconstructive surgery using percutaneous transluminal angioplasty, and worsening in Fontaine-classification), and *3*. “All-cause Mortality”. We also grouped these 3 categories to capture all adverse events (“Adverse events”). Only the first event was included in the analyses for patients who experienced more than 1 event. All indications for PTA and revascularisation surgery were discussed prior in a multidisciplinary team of vascular surgeons and interventional radiologists, whilst preference of patient and interventional were taken into consideration. All involved members of the multidisciplinary team were blinded to the outcome of the CAR test.

**Statistical analysis**

Prior to our study, we aimed to include 200 PAD patients. This group size is in line with previous studies examining the prognostic value of measures of vascular health,[10, 19] whilst this group size also accounts for potential drop out (10-15%) and access to sufficient PAD patients (n=400/annum, 50% inclusion rate). Data are presented as mean±SD or n (%) unless stated otherwise. Statistical analysis was performed using IBM SPSS Statistics 21.0 (IBM SPSS, IBM Corp., Armonk, NY, USA). Baseline characteristics were assessed for normality with the Shapiro-Wilk test. We adopted unpaired Student’s *t*-tests (Mann-Whitney U-test for non-normally distributed parameters) to compare subject characteristics, co-morbidities, clinical status, and medication use between PAD patients with carotid constriction *vs.* dilation. We used logistic regression to assess if subject characteristics, co-morbidities, clinical status, or medication could predict presence of carotid constriction.

Analyses to examine whether presence of carotid constriction could predict future events, analyses were performed separately for “CV events”, “Clinical progression”, “All-cause mortality" and "Adverse events". Cumulative event rates of carotid constriction and dilation were estimated with Kaplan-Meier survival analyses, and were calculated with the log-rank test. Cox proportional hazard models were used to calculate hazard ratios, including correction for confounding variables (age, sex, BMI, and WHR). These variables were selected based on prior evidence of an association with measures of endothelial function.[14, 20] Analyses were repeated in subgroups in whom we examined cIMT (n=169) or ABPI (n=142). We examined if these clinical measures were related to increased risk for future events,[26] and whether they altered the analyses related to carotid measurement.

## RESULTS

Subject characteristics, co-morbidities, clinical status and adequate imaging was obtained in all 172 PAD patients. Median change in carotid artery diameter was 0.8% (95% Confidence Interval -19.3 – 11.6%). Carotid constriction occurred in n=82 patients, whereas vasodilation occurred in 90 patients. Seventy patients (41%) experienced ≥1 event, which included loss of patency (n=18), increase in Fontaine-classification (n=15), percutaneous transluminal angioplasty (n=44), transient ischemic attack (n=1), myocardial infarction (n=4), cerebrovascular accident (n=3), coronary revascularisation procedures (n=1), major (n=7) and minor (n=1) amputation, and ischemic bowel disease (n=1). Ten PAD patients died; from CV-related mortality (n=4), cancer-related mortality (n=2), and unknown cause (n=4). We found no baseline differences between PAD patients with carotid constriction *versus* dilation in subject characteristics or co-morbidities. Patients with carotid constriction reported lower antiplatelet drugs usage (*Table 1*). Logistic regression revealed that none of the subject characteristics, co-morbidities, clinical status or medication use could predict if a PAD patient would demonstrate carotid constriction (backward likelihood ratio analysis, all parameters $P>0.05$). When comparing patients with and without an adverse event, those with events showed higher prevalence of hypercholesterolemia. Other factors did not differ between groups (*Supplemental data 1*).

**Prognostic value of carotid constriction for future adverse events.**

Kaplan-Meier survival curves demonstrated that PAD patients with carotid constriction report a higher incidence of CV events (*P*=0.007), clinical progression (*P*=0.005) and adverse events (*P*=0.006) compared to carotid dilation (*Figure 3*). There were no significant differences between groups for all-cause mortality (Log-rank, *P*=0.417, *Figure 3*).

Using a multivariate Cox proportional hazard model, with the fully adjusted model correcting for potential confounders (i.e. age, sex, WHR and BMI), PAD patients with carotid constriction continued to demonstrate higher risk for CV events (HR 4.1, 95%CI 1.3-12.5), clinical progression (HR 2.0, 95%CI 1.2-3.3) and adverse events (HR 1.8, 95%CI 1.1-3.0), but not all-cause mortality (HR 1.4, 95%CI 0.4-5.1, *Table 2*).

*Added value of clinical measures*. In 30 subjects, we were unable to perform a valid ABPI because of non-compressible arteries or amputation. We were unable to perform analysis of cIMT in 3 participants because of technical problems. Analyses for cIMT (n=169) and ABPI (n=142) showed no significant effect using the Kaplan-Meier survival analysis for adverse events (*Figure 4*), CV events (Log-rank *P*=0.674 and 0.457, respectively), clinical progression (Log-rank *P*=0.484 and 0.153, respectively) or all-cause mortality (Log-rank *P*=0.198 and 0.795, respectively).

The cox proportional hazard models for the carotid constriction (including models 1 and 2) were repeated for the subgroups with data on cIMT and ABPI. Adding cIMT or ABPI to the fully adjusted model did not alter the HR of carotid constriction for future CV events, clinical progression, all-cause mortality or adverse events (*Supplemental material 2*).

## DISCUSSION

This is the first study, to our knowledge, to examine the relation between the carotid response to the cold pressor test and future CV events in PAD patients. We found that patients who demonstrated carotid constriction during the cold pressor test had a 4.1-, 2.0- and 1.8-times increased risk at 1-year of developing a CV event, clinical deterioration and other adverse events, respectively, compared to those with carotid dilation. Importantly, the ability of the carotid vasomotor response to predict CV events was independent of subject characteristics and more predictive than other common clinical measures such as ABPI and cIMT. This suggests that a measure of (generalised) vascular health is more important than the extent of the (localised) atherosclerotic lesion in PAD patients. Therefore, a simple and non-invasive measure of carotid artery endothelial function can identify PAD patients at increased risk for future adverse CV events and clinical progression.

### Dilation versus Constriction

We[14, 27] and others[13, 15, 16] have demonstrated that the CPT produces a gradual dilator *or* constrictor response. Similarly, normal coronary arteries show an endothelium-mediated dilation that is mediated by the CPT-induced catecholamine-release, which exceeds the direct constrictor effects of catecholamines on smooth muscle cells.[15, 16] However, endothelial dysfunction and/or (partial) endothelial damage impedes endothelium-mediated dilation leading to vasoconstriction.[15] Similar responses in the carotid artery probably explain the distinct dilator or constrictor responses we observed in our study. Surprisingly, other subject characteristics, co-morbidities and clinical measures did not differ between PAD patients demonstrating constriction or dilator responses. This suggests that CV risk factors do not contribute to the distinct vasomotion responses between PAD patients, and that the PAD disease state, rather than subject characteristics or CV risk factors contributes to carotid artery endothelial dysfunction.

**Relation between the carotid vascular response and subsequent events**

Current CV risk factors do not predict future CV events in patients with PAD.[12] This highlights the potential utility of the carotid artery vasomotor response in predicting future CV events. Others have demonstrated that brachial artery flow-mediated dilation predicts future CV events in PAD patients,[10, 11] but this technique is more difficult than our approach of simply measuring the relatively large carotid diameter (i.e. ~7.5 mm) in response to the CPT. Previous studies have also not measured clinical progression, whereas we found that carotid constriction had a 2-fold increased risk for loss of patency, endovascular reconstructive surgery and/or worsening in Fontaine-classification. This knowledge may allow clinicians to treat more aggressively in those patients at risk for clinical deterioration.

Others have found that the coronary artery response to CPT predicts future CV events.[17, 18] Results from the present study suggest that the carotid artery response to the CPT is a surrogate for coronary arteries. This is supported by our previous within-subject observation that a subject's carotid and coronary response to the CPT are similar.[14, 27] This observation also supports the concept that atherosclerosis is a whole body, generalised disease of the endothelium, such that an abnormal response in one vascular bed is likely also present other vascular beds. Consistent with this concept is the observation that abnormal brachial artery dilator responses to endothelial stimulation using increased flow[6] or acetylcholine[28] are associated with abnormal coronary artery responses to endothelial stimulation.

ABPI is useful in both the diagnosis of PAD[29] and predicting the need for revascularisation.[2, 30, 31] Examining cIMT has also been useful in predicting atherosclerotic risk in some, but not all, studies of the general population.[24, 32] We found no relation between either ABPI or cIMT and future CV events, clinical progression or adverse events. Furthermore, adding these clinical measures to the statistical model did not alter the relationship between carotid artery reactivity and future (CV) events. The finding that ABPI and cIMT are not related to future events contrasts with previous work performed in the general population,[2, 30, 31] but is largely in agreement with studies performed in PAD.[12, 33] Abnormal ABPI and cIMT indicate the presence of atherosclerosis, whereas coronary responses to CPT reflect endothelial function. This suggests that in individuals with known CVD, the impact of the atherosclerotic process on the endothelium is more important than the atherosclerotic lesion in predicting CV events.

A potential limitation of our study is that we did not include other biomarkers, such as high-sensitive C-reactive protein, which are demonstrated to have potential prognostic value in PAD for future CV events.[37, 38] Unfortunately, we did not assess these biomarkers to examine the potential added value of combining these markers with the CAR, which may be relevant since these biomarkers may provide additional information to our *in vivo* measure of endothelial function.

*Clinical relevance.* The carotid artery reactivity to CPT procedure is easy to perform, low-cost, non-invasive, and requires minimal time and equipment. The simplicity of the test is supported by an excellent reproducibility.[14] Moreover, in contrast to the majority of subject and/or disease characteristics, the carotid artery vasomotor response to CPT identified subjects with an increased risk for future events. The clinical relevance of the CAR may relate the identification of PAD patients who are more vulnerable to non-adherence and/or complications during surgery. For example, although symptomatic PAD should all be on drug therapy, compliance to therapy is relatively poor.[34-36] The CAR-test may help to identify individuals in whom it is of special importance to maintain compliance to drug therapy. Additionally, cardiovascular co-morbidity in PAD patients is associated with increased perioperative cardiovascular risk. Future studies are needed to assess the potential added value of the CAR to estimate perioperative risk in PAD patients.

In conclusion, our study provides the first evidence that carotid artery reactivity, independent of subject characteristics and clinical measures such as cIMT and ABPI, predicts future adverse (CV) events and clinical progression in PAD patients. The presence of carotid artery constriction during the CPT is associated with a 4-fold increased risk for future CV events and 2-fold increased risk for clinical progression. These observations suggest that the carotid artery reactivity should be further evaluated for its ability to predict future risk in patients with PAD.

## AUTHOR CONTRIBUTIONS

DHJT and MW designed the study. DHJT ensured funding of the project. ACCMM, MW, SP and JW were involved in data collection. ACCMM and DHJT performed the statistical analyses. All authors contributed to the interpretation of the data, writing of the manuscript and provided approval of the final version.

## ACKNOWLEDGEMNTS

We thank Marie-José Beelen, Ilse Rijken, Anita Theloosen-Kersten and Marijke Litjens-Frenken for their valuable contribution during the performance of the work at the vascular laboratory of the Radboudumc. We thank Ms Frederieke van Oorschot for her contribution during the study. We like to thank Dr. Maureen van der Vlugt for her advice in performing the proposed study. We acknowledge the help from Dr. Eijsvogels in the statistical part of the paper.

**SOURCES OF FUNDING**

ACCM is financially supported by a Top Institute for Food and Nutrition-grant.

**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

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**FIGURES.**

**FIGURE 1.** CONSORT diagram of the study.

**FIGURE 2.** Set-up of the practical performance of the test (A). Screen-shot of the assessment of carotid artery diameter, with the yellow box indicating the region of interest within the automated software performed analysis of the diameter (i.e. yellow lines on the artery wall) (B). Data from a representative subjects demonstrating carotid artery diameter dilatation (C) and constriction (D) during the cold pressor test. Both panels represent the carotid artery diameter (in cm) across the 30-s baseline (up to the vertical dashed line; the start of the cold pressor test) and 90-s during the cold pressor test. Data were analysed in 10-second bins to identify presence of dilatation or constriction. More detailed findings of this procedure is presented in the methods section.

**FIGURE 3.** Kaplan-Meier **s**urvival curves for adverse events (A), CV events (B), clinical progression (C) and all-cause mortality (D) in PAD patients (n=172) across a 1-year follow-up. We have dichotomised PAD patients in those who demonstrate coronary constriction (CAR constriction, dotted line) or dilation during the CPT (CAR dilation, solid line). P-values relates to a Log-rank test.

**FIGURE 4.** Kaplan-Meier survival curves for cIMT (A, 169 PAD patients), and ABPI (B, 142 PAD patients) related to occurrence of adverse events across a 1-year follow-up. The solid line represents the cIMT and ABPI above the median, the dotted line refers to the cIMT and ABPI below the median. P-values relates to a Log-rank test.

**Table 1.** Baseline characteristics of patients with PAD with carotid artery constriction (CAR constriction) or dilation (CAR dilation) during the CPT. *Indicate Mann-Whitney U test, presented as median [minimum – maximum]. P-value indicates difference between vasoconstriction *versus* vasodilation.

| **Subject characteristics** | **Total group** n=172 | **CAR constriction** n=82 | **CAR dilation** n=90 | **P-value** |
|---|---|---|---|---|
| Age*, y | 68±10 | 71 [43-85] | 67 [46-90] | 0.223 |
| Sex, males (%) | 115 (67) | 58 (71) | 57 (63) | 0.303 |
| Height (m) | 1.73±0.09 | 1.73±0.10 | 1.72±0.10 | 0.464 |
| Weight (kg) | 79.8±14.6 | 80.1±14.3 | 79.6±14.9 | 0.804 |
| Body-mass index (kg/m2) | 27±4 | 27±4 | 27±4 | 0.900 |
| Waist-to-hip ratio | 1.01±0.10 | 1.02±0.10 | 1.00±0.10 | 0.210 |
| Smoking, yes n (%) | 55 (32) | 28 (34) | 27 (30) | |
| History n (%) | 97 (56) | 45 (55) | 52 (58) | 0.839 |
| **Comorbidities** | | | | |
| Hypertension, n (%) | 138 (80) | 66 (80) | 72 (80) | 0.936 |
| Hypercholesterolemia, n (%) | 133 (76) | 61 (74) | 72 (80) | 0.380 |
| Diabetes Mellitus, n (%) | 46 (27) | 22 (27) | 24 (27) | 0.873 |
| **Medication use** | | | | |
| Antiplatelet drugs, n (%) | 135 (78) | 58 (71) | 77 (86) | *0.018* |
| Acetylsalicylic acid (ASA) | 125 (73) | 55 (67) | 70 (78) | |
| Plavix (clopidogrel) | 4 (2) | 1 (1) | 3 (3) | 0.667 |
| Dual therapy (combined) | 6 (3) | 2 (2) | 4 (4) | |
| Statins, n (%) | 141 (82) | 71 (87) | 70 (78) | 0.133 |
| Beta-blockers, n (%) | 89 (52) | 40 (49) | 49 (54) | 0.458 |
| ACE inhibitors, n (%) | 59 (34) | 26 (32) | 33 (37) | 0.494 |
| Proton pump inhibitors, n (%) | 87 (51) | 40 (49) | 47 (52) | 0.652 |
| **Clinical status** | | | | |
| Mild ischaemia (Fontaine 1-2A), n (%) | 62 (36) | 25 (30) | 37 (41) | 0.143 |
| Moderate-severe ischaemia (Fontaine 2B-3-4), n (%) | 110 (64) | 57 (70) | 53 (59) | |
| ABPI (n=142) | 0.65±0.22 | 0.65±0.22 | 0.66±0.21 | 0.679 |
| ABI left rest (n=135) | 0.72±0.22 | 0.73±0.21 | 0.76±0.25 | 0.402 |
| ABI right rest (n=132) | 0.73±0.22 | 0.74±0.25 | 0.74±0.21 | 0.978 |
| Carotid IMT* (mm) | 0.79 [0.15-2.78] | 0.80 [0.15-2.78] | 0.78 [0.35-2.06] | 0.860 |
| Baseline carotid diameter* (mm) | 7.5±1.1 | 7.7 [4.4-10.5] | 7.3 [5.1-10.9] | 0.358 |
| CAR% | 0.8 [-19.3 - 11.6] | 1.3 [-9.5 – 11.6] | -1.2 [-19.3 – 9.6] | 0.068* |

Figure 1

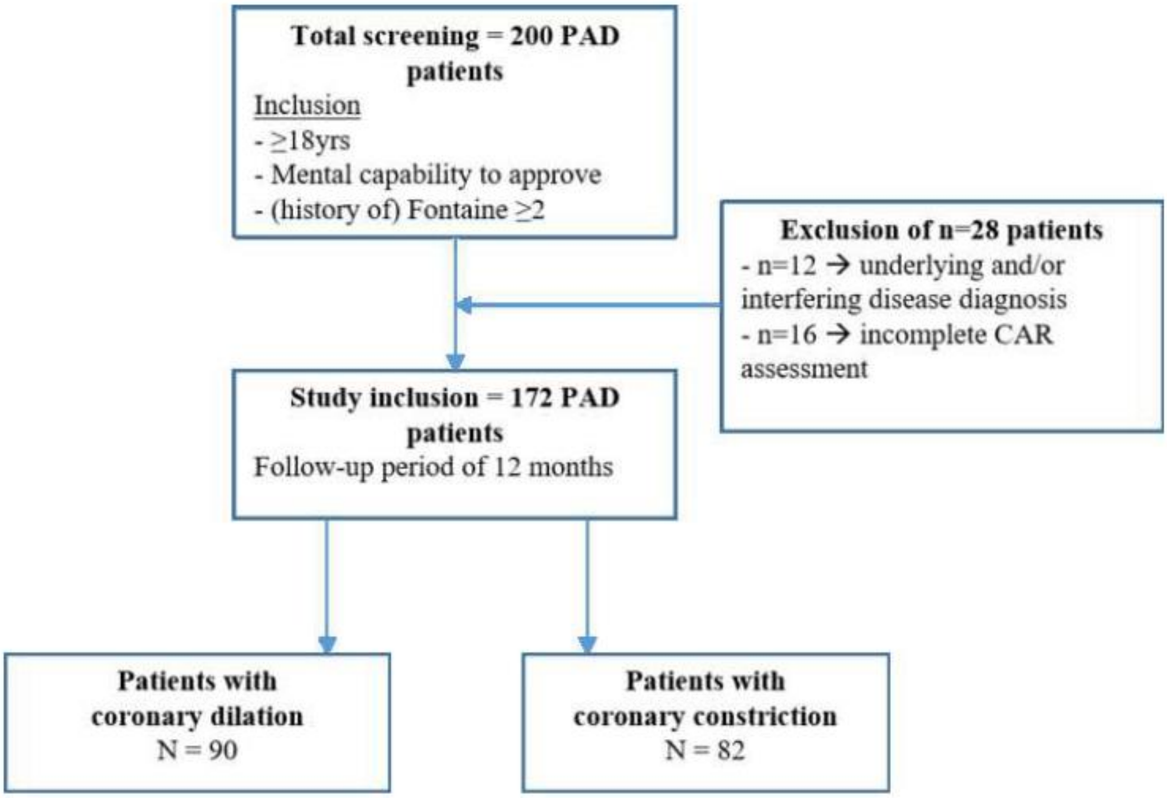

Total screening = 200 PAD patients
Inclusion
- ≥18yrs
- Mental capability to approve
- (history of) Fontaine ≥2
Exclusion of n=28 patients
- n=12 → underlying and/or interfering disease diagnosis
- n=16 → incomplete CAR assessment
Study inclusion = 172 PAD patients
Follow-up period of 12 months
Patients with coronary dilation
N = 90
Patients with coronary constriction
N = 82

Figure 2

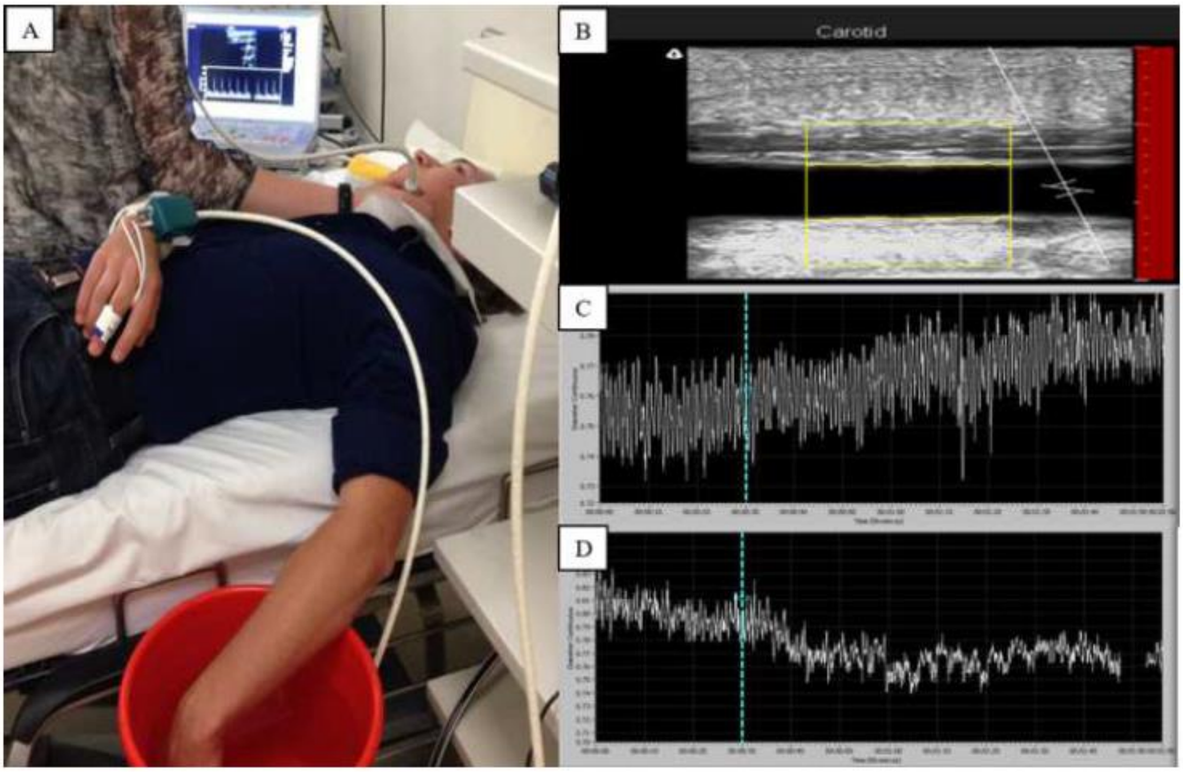


Figure 3

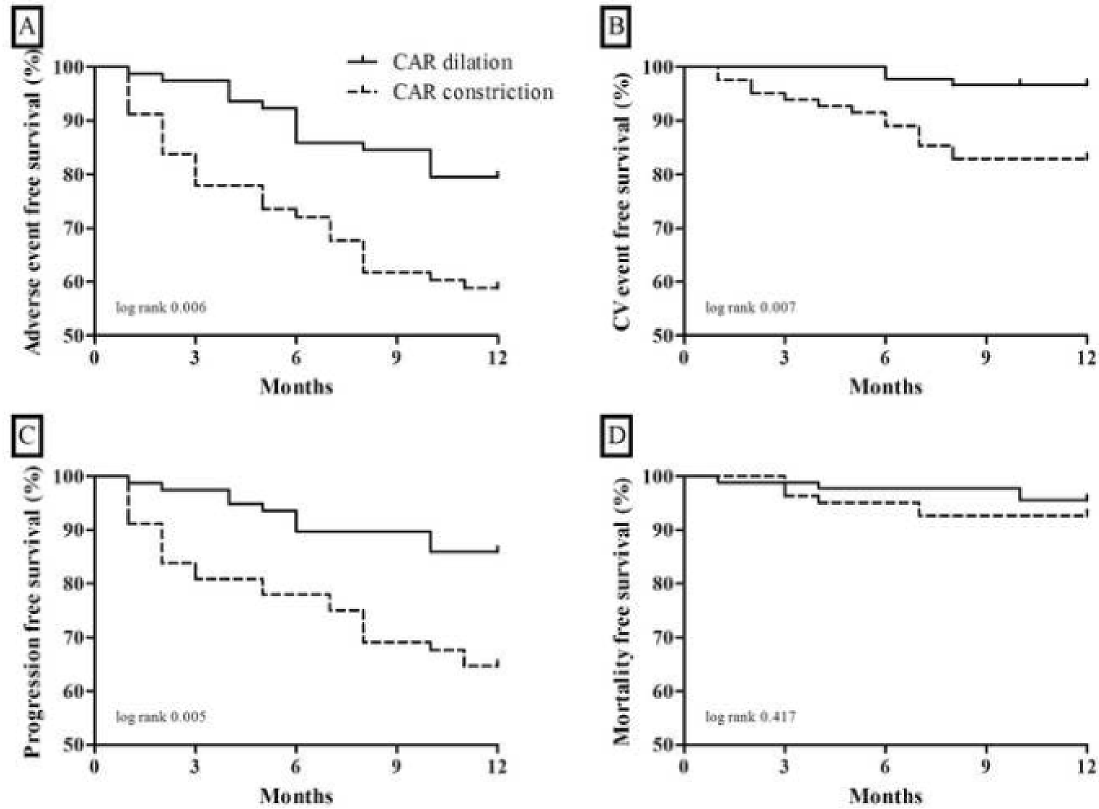
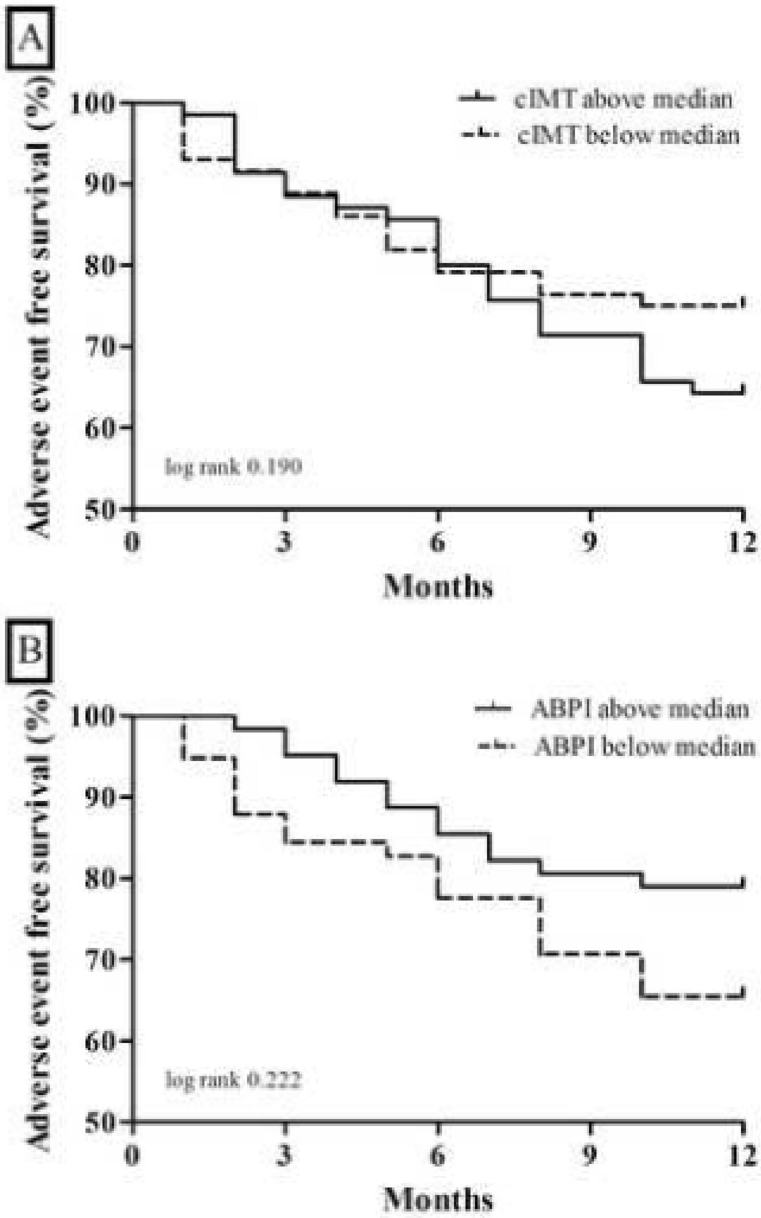

A
CAR dilation
CAR constriction
Adverse event free survival (%)
log rank 0.006
Months
B
CV event free survival (%)
log rank 0.007
Months
C
Progression free survival (%)
log rank 0.005
Months
D
Mortality free survival (%)
log rank 0.417
Months

Figure 4


A
Adverse event free survival (%)
100
90
80
70
60
50
0
3
6
9
12
Months
cIMT above median
cIMT below median
log rank 0.190
B
Adverse event free survival (%)
100
90
80
70
60
50
0
3
6
9
12
Months
ABPI above median
ABPI below median
log rank 0.222