CHAPTER 10

CEREBRAL HEREDITARY ANGIOPATHIES RVCL AND CADASIL DISPLAY DISTINCT IMPAIRED VASCULAR FUNCTIONALITY

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SUMMARY

Retinal Vasculopathy with Cerebral Leukodystrophy (RVCL) and Cerebral Autosomal Dominant Arteriopathy with Subcortical infarcts and Leukoencephalopathy (CADASIL) are cerebral small vessel diseases, which serve as monogenic models for common complex neurovascular disorders, such as stroke, dementia and migraine. Impaired vascular reactivity is likely to play a role in the pathophysiology of both monogenic disorders, but it remains unclear whether this is due to impaired endothelium-dependent or -independent (i.e., mediated by direct relaxation of smooth muscle cells) mechanisms. In this study, vascular function tests were performed at different levels of the vascular bed to investigate functional changes in (arterial) stiffness, vasodilatation of resistance vessels, and endothelial function of conduit arteries. Eighteen RVCL and 23 CADASIL patients with *TREX1* and *NOTCH3* mutations, respectively, were compared with 26 matched control subjects. Data shown are uncorrected means ± SD and corrected *p*-values.

Vascular stiffness was assessed by pulse wave analysis and pulse wave velocity. CADASIL patients displayed an elevated Aortic Augmentation Index compared to controls (22,8 ± 13,3 vs. 16,0 ± 12,7 %, $p = 0.007$); in RVCL patients a similar trend was shown (21,0 ± 13,3 %, $p = 0.06$). Pulse wave velocity was increased in RVCL patients compared to controls (7,8 ± 1,6 vs. 7,0 ± 1,4 m/s, $p = 0.01$) and showed a trend in CADASIL patients (7,5 ± 1,5 m/s, $p = 0.07$ versus controls). Vasodilatation of dermal microcirculation was reduced in CADASIL but not in RVCL, compared to control subjects as characterized by both an attenuated capsaicin-induced dermal blood flow increase 40 minutes after capsaicin application (1,38 ± 0,88 vs. 2,22 ± 1,20 Arbitrary Units (AU), $p = 0.02$) and a lower area under the curve over 40 minutes (0,52 ± 0,43 vs. 0,93 ± 0,60 AU, $p = 0.02$). Endothelium-dependent vasodilatation as assessed by flow-mediated dilatation was decreased in RVCL versus controls (2,32 ± 3,83 vs. 5,76 ± 3,07 %, $p = 0.02$ versus controls), but not in CADASIL. After correction for shear rate and blood viscosity, flow-mediated dilatation remained reduced in RVCL (3,31 ± 7,24 vs. 10,07 ± 5,73 %, $p = 0.03$ versus controls).

We identified endothelial dysfunction in *TREX1*-mutated RVCL patients and confirmed impaired smooth muscle cell relaxation in resistance vessels of *NOTCH3*-mutated CADASIL patients. Increased vascular stiffness illustrates reduced vascular functionality in both syndromes. Our findings not only improve insight in RVCL and CADASIL pathophysiology, but may also be important to understand common disorders that are part of the disease spectrum of these neurovascular disorders, such as stroke, dementia, and migraine.

INTRODUCTION

Neurovascular diseases such as migraine, (vascular) dementia and ischemic stroke are clinically and genetically heterogeneous disorders with a high population prevalence and disease burden.[1] Comorbidity of these diseases suggests, at least to some extent, shared underlying pathophysiological mechanisms. For example, stroke and dementia commonly co-occur in cerebral small vessel disease.[2] Moreover, migraineurs with aura have a doubled risk of ischemic stroke[3] and an increased risk of posterior circulation territory infarcts[4,5] and deep white matter hyperintensities.[4,6] However, as these common disorders are multifactorial and genetically complex it is difficult to identify the causal genes and disease pathways. We envisage that RVCL (Retinal Vasculopathy with Cerebral Leukodystrophy) and CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy), which are monogenic forms of cerebral small vessel disease with a high prevalence of stroke, dementia, and migraine, may serve as useful disease models for the common disorders.[7,8,9,10]

RVCL is caused by heterozygous C-terminal frameshift mutations in the *TREX1* gene, which encodes the major 3'-5' DNA exonuclease that is thought to be involved in clearing cytosolic nucleic acids.[9,11,12] In RVCL, truncated proteins seem to retain their exonuclease activity but lose normal perinuclear localization, which likely interferes with normal functioning of TREX1.[9] RVCL is a systemic vascular syndrome that primarily involves the smaller vessels in the retina and the brain.[8,13,14,15] Neurological manifestations may include cognitive disturbances, focal neurological symptoms, depression, and in 59% of *TREX1* mutation carriers migraine. In later disease stages, cerebral MRI scans frequently show characteristic contrast-enhancing intracerebral mass lesions. Several systemic symptoms can be present as well, including renal and liver dysfunction, anemia, and Raynaud's phenomenon.

CADASIL is caused by heterozygous mutations in the *NOTCH3* gene,[7] which encodes a cell surface receptor that is solely expressed on adult vascular smooth muscle cells. NOTCH3 is involved in a signal transduction pathway critical for development, homeostasis and differentiation of vascular smooth muscle cells (VSMCs).[16] Clinically, the disease is characterized by recurrent transient ischaemic attacks (TIAs) and strokes leading to cognitive decline,[17] psychiatric symptoms and dementia. Migraine with aura occurs in about one-third of patients, often as the first presenting symptom, years before the other symptoms.[18]

Both RVCL and CADASIL are small vessel vasculopathies but with different radiological features.[10] RVCL is characterized by contrast-enhancing cerebral mass lesions that develop in the end stage of the disease, typically in combination with (or preceded by the presence of) calcifications and non-specific white matter hyperintensities. The most prominent radiological features in CADASIL are white matter hyperintensities and lacunar infarcts. White matter hyperintensities are symmetrically distributed and located in the deep and periventricular white matter. Typical for CADASIL is the bilateral involvement of the anterior temporal lobes and external capsule. Other neuroradiological features in CADASIL are cortical morphologic changes,[19] subcortical lacunar lesions, lacunar infarcts and microbleeds.[18,20]

Thickening of the vessel wall leading to lumen stenosis, is a communal feature of the cerebral pathology of these arteriopathies. In addition, both in the cerebral and the systemic circulation of CADASIL patients, there is a remarkable degeneration of vascular smooth muscle cells and deposition of granular osmiophilic material (GOM) in the media/adventitia,[21] with a morphologically largely normal endothelium.[18] In contrast, in RVCL, muscle cell degeneration is minimal and electron microscopy shows irregular thickening and splitting of basement membranes in the vessel wall, which is primarily produced by endothelial cells.[22,23] These changes on cerebral vessels may affect their capability to dilate, which, in addition to luminal narrowing, may lead to hypoxia.

In CADASIL, previous studies showed reduced baseline cerebral blood flow and impaired hemodynamic reserve (i.e., vasodilation response to acetazolamide) related to the severity of white matter hyperintensities.[24-26] However, it is not yet clear whether this impaired vasoreactivity is caused by an endothelium-independent or -dependent mechanism.[27-30]

In RVCL, functional vascular properties have not been studied before. Besides radiological and other pathological alterations, the presence of Raynaud's phenomenon and migraine seems to point to altered vascular reactivity. In Raynaud's phenomenon impaired endothelium-dependent vasodilatation and a mismatch between endothelium derived vasoconstrictors and vasodilators has been observed.[31] Migraine has been linked to endothelial dysfunction,[32-36] vascular smooth muscle cell dysfunction,[37] increased peripheral arterial stiffness[34] and increased intima-media thickness.[36] However, controversy over the vascular theory in migraine pathogenesis [38-42] and migraine treatment[43,44] remains. Nevertheless, migraine (with aura) has repeatedly been linked with cardiovascular diseases[45] including ischemic stroke[3,46-48] and coronary events.[49,50]

By using different techniques at distinct levels of the vascular bed, we investigated vascular functional consequences of both RVCL and CADASIL *in vivo*, and compared results with those of a matched group healthy volunteers.

METHODS

Subjects

The study was approved by the Ethics Committee of Leiden University Medical Centre and conducted in accordance with the Declaration of Helsinki. All participants were at least eighteen years of age and gave written informed consent. RVCL and CADASIL patients with a confirmed mutation in the *TREX1* (i.e., V235fs) and the *NOTCH3* gene, respectively, were recruited from databases provided by the treating neurologist (GMT) and clinical geneticist. Subjects were invited to participate by a letter explaining the tests and study objectives. The control group consisted of spouses or friends of mutation carriers and volunteers recruited via public advertisements. Medical history, genetic and clinical information was obtained prior to the measurements and by a different investigator than the one performing the vascular measurements. During the measurements, participants were instructed not to reveal their mutation status or medical history to the investigator who performed the measurements to prevent unblinding.

Clinical assessment

Participants were asked in advance to fill out a questionnaire that included various variables on medical history such as neurological symptoms including (transient) ischemic attacks, other stepwise neurological deterioration, cognitive complaints including apathy, migraine (with or without aura), depression, visual symptoms, Raynaud's phenomenon, diabetes, thyroid function, cardiovascular disease, airway disease, cancer, multiple sclerosis, epilepsy, intestinal and stomach problems, liver disease, renal and urinary tract disease and dermatological diseases. The questionnaire also had questions on current medical condition, current medication, smoking, daily intake of alcohol, caffeine and drugs, and socio-demographic parameters such as educational level and ethnic origin. During the interview preceding the experiments, diagnosis of migraine and Raynaud's phenomenon was verified. Migraine diagnosis was made according to the International Classification of Headache Disorders-second edition (ICDH-2) and Raynaud's phenomenon was assessed according to the criteria of Miller.[51] Medication was also verified and subdivided in the following categories: anti-hypertensive, cholesterol lowering, acetylsalicylic acid (anti-platelet), anti-coagulant, acute and prophylactic migraine medication, analgesics (e.g., acetaminophen, paracetamol, NSAIDs), oral contraceptive. If possible, and in agreement with their treating physician, included subjects were asked to abstain from medication with vascular (side-)effects for one week prior to the measurements. Patients were instructed to abstain from alcohol and caffeine for 12 hours, from smoking and food for 6 hours and, if their health status allowed this, from medication for 7 days prior to the measurements. This was verified during the interview. Subsequently, weight and height were measured and BMI was calculated.

Biochemical measurements

Plasma concentrations of triglyceride (TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), total cholesterol (TC), blood fasting glucose levels, creatinine, ureum, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), lactate dehydrogenase (LDH), hemoglobin (Hb) and hematocrit (Ht) were determined according to standard procedures.

Experimental setup

All measurements were performed by the same investigator (SV) who was blinded for the diagnosis and mutation status of the subjects. Before starting the measurements, subjects had to lay down on a comfortable bed in supine position for acclimatization of at least 15 min in a room with stable ambient temperature (22 ± 1 °C).

Resting blood pressure and pulse rate were recorded at the right upper arm using a validated semi-automated oscillometric device (OMRON 705IT, OMRON Healthcare, Hoofddorp, The Netherlands). Median blood pressure and heart rate of 3 measurements was used for analyses. Subsequently, the following assessments were consecutively performed in a standardized order: i) pulse wave analysis; ii) pulse wave velocity; iii) capsaicin-induced changes in dermal blood flow; and iv) flow-mediated dilatation of the brachial artery. All these assessments have previously been described in detail, including reproducibility.[52-57]

Pulse Wave Analysis (peripheral arterial stiffness)

Augmentation index (AIx), which is a measure for arterial wave reflections and peripheral arterial stiffness, was determined via Pulse Wave Analysis (PWA) using SphygmoCor software (version 8.2; Atcor Medical, Australia). PWA was performed by applanation tonometry over the radial artery to obtain resting measurements of the peripheral AIx (Augmented Pressure / Pulse Pressure), expressing the reflected pressure wave as percentage of the forward wave (Supplementary Fig. 1). This non-invasive method has previously been shown a reproducible technique to evaluate peripheral arterial stiffness.[55] Based on 10-second PWA recordings performed at the radial artery, aortic (central) pressure waveforms can be reliably estimated by the software using a validated transfer function.[54] Subsequently, central pressure wave forms can be used for calculating the central AIx. Since AIx is influenced by heart rate,[53] an index normalized for heart rate at 75 beats/minutes (min) (AIx@HR75) was used. Mean values of at least three AIx@HR75 measurements per subject were used for statistical analysis.

Pulse wave velocity (aortic stiffness)

Aortic Pulse Wave Velocity (PWV) was measured with the same SphygmoCor device as PWA, by sequentially recording ECG-gated carotid and femoral artery pressure waves. The intersecting tangent algorithm[52] was used by the software to determine the characteristic points of the pressure wave and the R wave of the ECG for calculating the time delay. Based on 10-second recordings, the carotid-femoral transit time (T) was calculated. The carotid-femoral distance (L) used for calculating the PWV was obtained by subtracting the carotid-sternal notch distance from the sternal notch-femoral distance. PWV was calculated as L (cm) divided by T (m/s). If available, mean values of at least 3 PWV measurements per subject were used for statistical analysis.

Capsaicin-induced dermal blood flow (microcirculation in the skin)

Local application of capsaicin onto the skin results in binding to the Transient Receptor Potential Vanilloid type I receptor (TRPV1) at primary sensory neurons (Aδ- and C-fiber nociceptors). TRPV1 receptor binding of capsaicin induces a neurogenic inflammatory response due to predominant release of CalcitoninGene-Related Peptide (CGRP).[57] CGRP is a very potent vasodilator causing a local increase in dermal blood flow, which can be quantified by Laser Doppler Perfusion Imaging (LDPI)(PeriScan PIM II®; Perimed, Järfälla, Sweden). Reproducibility of this non-invasive test to evaluate dermal vascular reactivity to capsaicin was confirmed earlier.[56] After at least 20 min of supine rest the baseline dermal blood flow (DBF) was measured using LDPI. Subsequently, subjects received single topical doses of 1000 µg per 20 µL capsaicin solution (in ethanol/polysorbate 20/water) in two 10-mm rubber 'O'-rings on the volar surface of one forearm. In two rings on the opposite arm, placebo (i.e., vehicle) was applied. DBF was measured at 10, 20, 30 and 40 min after capsaicin/placebo administration. Results are presented as absolute and percentage change in DBF after capsaicin application compared to placebo.

Flow-mediated dilatation (endothelial function of conduit artery)

Flow-Mediated Dilatation (FMD) of the brachial artery was assessed following existing guidelines[58] using an echo-tracking system (Wall Track System, Pie Medical, Maastricht, The

Netherlands). The system consists of an ultrasound device (Esaote AU5, Esaote Biomedical, Genoa, Italy) equipped with a 7.5-10 MHz linear-array transducer connected to a data acquisition and processing unit. Measurements were performed as described earlier.[34] In brief, right brachial artery diameter was measured 5-10 cm proximal to the antecubital crease in a longitudinal plane, before and after an increase in shear stress induced by reactive hyperaemia. At baseline, diameter and velocity profiles were recorded 3 times and the mean was used for data analysis. After baseline measurements, a cuff (TMC7, D.E. Hokanson, Bellevue, USA), placed around the forearm, was rapidly inflated to 220 mm Hg. The cuff was released after 5 min, followed by recording of the peak velocity profile within the first 15 seconds (s). Diameter was measured at 0.5, 0.75, 1, 1.25, 1.5, 2, 3, 4 and 5 min after cuff release. Each diameter recording lasted 4 s.

After 15 min of recovery, following measuring baseline brachial artery diameter again, 400 μg sublingual nitroglycerin (NTG) was administered. Subsequently, the diameter was measured every minute for 6 min to assess NTG-induced endothelium-independent brachial artery dilatation. The endothelium-dependent FMD after 5 min of reactive hyperemia was expressed as the maximal absolute and percentage increase in diameter from baseline. FMD was corrected for the hyperemic stimulus using shear rate (= velocity/diameter) and additionally corrected for blood viscosity using shear stress (= shear rate*hematocrit) to calculate normalized FMD. Endothelium-independent vasodilatation capacity after administration of the exogenous NO donor (NTG) was expressed as the maximal absolute and percentage increase in diameter from baseline.

Statistics

P-values were calculated by independent sample t-test for normally distributed variables, Mann-Whitney U test for non-normally distributed variables and χ^2-test for categorical variables. Analysis of variance (ANOVA) was used to detect differences between controls and patient groups, followed by post-hoc Fisher LSD-test. Univariate associations were established using Spearman rank order correlations. Multiple linear regression analysis was used to adjust for confounders found with the univariate Spearman test. For all analyses p-values <0.05 were considered statistical significant. All statistical analyses were performed using SPSS 17.0 software (SPSS inc, IBM, Chicago, IL, USA).

RESULTS

Subjects

Eighteen RVCL and 23 CADASIL patients were included in the study and compared with 26 age-, BMI- and gender-matched healthy control subjects. Demographic details, clinical symptoms and laboratory values of all subjects are summarized in Table 1. All tests were well-tolerated. No subjects reported a migraine attack due to NTG when asked in a survey by phone in the first week after participation in the study. Success rates for abstaining from alcohol, caffeine and food for the predefined period prior to the measurements was 100%, except for smoking in the CADASIL group (72%; five patients smoked 1-4 cigarettes in the 6 hours prior to the measurements), abstention from alcohol in the CADASIL group (94%; one patient used 2 units 10 hours prior to the measurements), caffeine in the RVCL group (96%; one patient

Table 1. Subject demographics

Variable	Controls (n=26)	CADASIL (n=23)	RVCL (n=18)	*p-value*
Age (years)	49.0 ± 9.1	49.5 ± 12.7	50.1 ± 9.6	*0.939*
BMI (kg/m²)	25.2 ± 3.6	26.7 ± 4.3	25.2 ± 3.7	*0.362*
Female, n (%)	14 (54)	11 (48)	10 (56)	*0.867*
Smoking (Pack years)	3.0 ± 5.0	13.4 ± 16.0	3.7 ± 5.8	*0.002*
Retinopathy, n (%)	0 (0)	0 (0)	9 (56)†	*N.A.*
TIA, n (%)	0 (0)	8/21 (38)††	0 (0)	*N.A.*
Brain infarction, n (%)	0 (0)	2(9)	0 (0)	*N.A.*
Cognitive complains, n (%)	0 (0)	12 (52)	8 (44)	*N.A.*
Concentration problems, n (%)	0 (0)	8 (35)	5 (18)	*N.A.*
Depressive symptoms, n (%)	0	6 (26)	4 (22)	*N.A.*
Character change, n (%)	0 (0)	5 (28)	2 (9)	*N.A.*
Migraine, n (%)	0 (0)	6/22 (27)§	6 (33)	*N.A.*
Chronic medication use, n (%)	4 (15)	16 (70)	12 (67)	*N.A.*
Oral contraceptive use (% of women)	2 (14)	1 (9)	0 (0)	
Anti-hypertensives	0 (0)	7 (30) **(1)**	6 (33) **(2)**	
Cholesterol lowering medication	0 (0)	9 (39) **(2)**	6 (33) **(2)**	
Acetylsalicylic acid	0 (0)	13 (57) **(3)**	8 (44) **(4)**	
Prophylactic migraine medication	0 (0)	1 (4)	0 (0)	
Analgesics (acetominophen + NSAIDs)°	0 (0)	2 (9)	5 (28)	
Other°° (%)	2 (7.7)	9 (39)	8 (44)	
Laboratory parameters				
Glucose (mmol/L)	4.95 ± 0.37	5.05 ± 0.37	5.13 ± 0.38	*0.267*
TC (mmol/L)	5.25 ± 1.18	4.94 ± 1.06	5.27 ± 1.14	*0.548*
HDL-C (mmol/L)	1.51 ± 0.42	1.50 ± 0.47	1.70 ± 0.64	*0.373*
TG (mmol/L)	1.13 ± 0.76	1.00 ± 0.46	0.95 ± 0.59	*0.628*
Hb (g/dL)	8.98 ± 0.71	9.15 ± 0.73	8.10 ± 0.65	***0.000***
Ht (%)	0.43 ± 0.03	0.44 ± 0.04	0.40 ± 0.04	***0.001***
AST	23,2±6.45	24.3±6.15	28.2±8.50	*0.064*
ALT	23.3±10.6	23.8±12.1	26.8±12.9	*0.603*
GGT	21.7±12.1	24.9±6.7	99.6±117.1	***0.000***
ALP	68.4±13.4	69.7±22.7	95.73±65.0	***0.038***
LDH	163.5±31.6	158.8±19.6	147.2±22	*0.120*
Creatinine	70.0±12.3	71.4±12.9	87.4±44.8	*0.064*
Ureum	4.7±1.03	4.90±4.09	6.61±3.90	*0.014*

Values are means ± SD or number (percentage). *P*-values were calculated using a one-way ANOVA. Number of subjects that abstained from medication 7 days prior to the measurements are depicted in bold. N.A.: not applicable. † Retinopathy status for two patients unknown. †† Two patients could no be categorized as TIA or definitely no TIA. § One patient could not be classified as migraine or no migraine. ° All subjects used these painkiller for acute treatment of migraine, none of the subjects used acetaminophen or NSAIDs 24 hours prior to the measurements. °° Other medication included: glucose lowering drugs, proton pump inhibitors, gabapentin, carbamazepine, pyridoxine, folic acid, valproic acid, alfacalcidol, baclofen, cinnarezine, ursodeoxycholic acid, atovaquon.

used 1 unit 6 hours prior to the measurements). Success rates of abstaining from medication are depicted in Table 1.

Pulse Wave Analysis (PWA) and Pulse Wave Velocity (PWV)

Peripheral (brachial) and central (aortic) systolic, diastolic and pulse pressures are presented in Table 2. No differences in blood pressure values were observed between the control group and both patient groups. PWA software was unable to calculate the augmentation index and aortic pressure parameters in one subject (CADASIL patient) as the result of an atypical waveform. PWV measurements could not be performed reliably in two CADASIL patients due to obesity (BMI of 29.7 and 35.4).

Univariate analysis showed a trend towards increased AIx@HR75 in CADASIL patients versus control subjects (22,8 ± 13,3 vs. 16,0 ± 12,7%, $p = 0.08$) and a trend towards increased pulse wave velocity in RVCL patients versus control subjects (7,8 ± 1,6 vs. 7,0 ± 1,4 m/s, $p = 0.10$; Table 2). Univariate associations between clinical characteristics and PWA/PWV were assessed using Spearman correlation coefficients. (Supplementary Table 1)

Multivariate analysis resulted in a regression model for PWA (Multiple R = 0.77; Adjusted $R^2 = 0.57$) with age ($p < 0.001$; observed power > 0.99), gender ($p < 0.001$; observed power > 0.99), peripheral diastolic pressure ($p = 0.001$, observed power = 0.91) and subject group ($p = 0.02$; observed power = 0.71) as covariates. This regression model for PWA with covariates age, gender and diastolic blood pressure, revealed a significant difference in AIx@HR75 between control subjects and CADASIL patients ($p = 0.007$ and a trend in RVCL patients ($p = 0.06$) (Fig. 1).

Table 2. Blood pressure and univariate pulse wave analysis and pulse wave velocity data

Variable	Controls (n=26)	CADASIL (n=22)	*p-value*†	RVCL (n=18)	*p-value*‡
Peripheral systolic pressure (mm Hg)	129.2 ± 20.5	126.2 ± 11.1	*0.94*	132.8 ± 21.7	*0.71*
Peripheral diastolic pressure (mm Hg)	77.8 ± 10.1	75.4 ± 6.7	*0.40*	75.3 ± 8.8	*0.26*
Peripheral pulse pressure (mm Hg)	51.3 ± 12.3	50.8 ± 9.6	*0.98*	57.5 ± 15.8	*0.21*
Aortic systolic pressure (mm Hg)	119.6 ± 21.2	116.8 ± 12.3	*0.93*	122.8 ± 22.4	*0.61*
Aortic diastolic pressure (mm Hg)	79.5 ± 10.6	79.8 ± 8.2	*0.90*	76.6 ± 9.0	*0.22*
Aortic pulse pressure (mm Hg)	39.5 ± 11.7	39.2 ± 8.4	*0.77*	46.2 ± 16.9	*0.16*
Aortic Augmentation index @ HR75 (%)°	16.0 ± 12.7	22.8 ± 13.3	*0.08*	21.0 ± 13.3	*0.21*
PWV (m/s)°°	7.0 ± 1.4	7.5 ± 1.5	*0.29*	7.8 ± 1.6	*0.10*

Data presented as mean ± SD. † Difference between control subjects and CADASIL patients. ‡ Difference between control subjects and RVCL patients. ° PWA: pulse wave analysis; PWA software was unable to calculate the augmentation index and aortic pressure parameters in 1 subject (CADASIL patient) as the result of a atypical waveform. °° PWV: pulse wave velocity; PWV measurements could not be performed reliable in 2 subjects due to obesity.

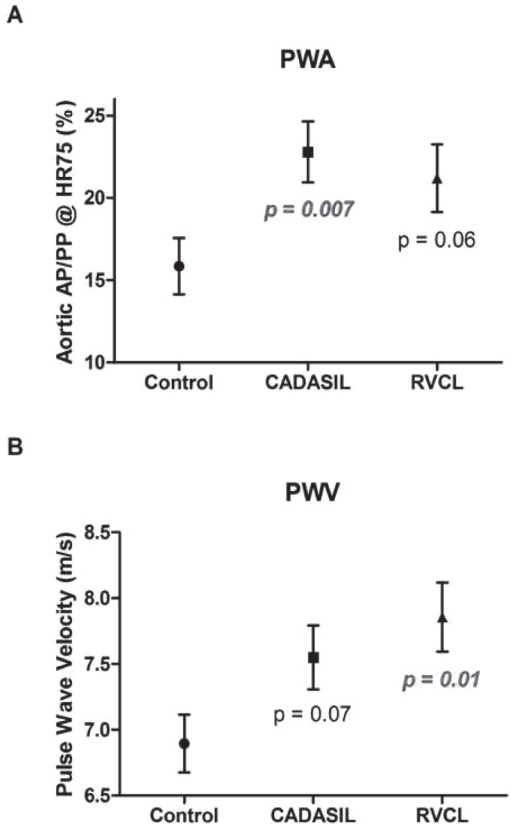

Figure 1. Increased vascular stiffness observed in CADASIL and RVCL patients measured with Pulse Wave Analysis (PWA) and Pulse Wave Velocity (PWV) respectively. (**A**) Aortic augmentation pressure corrected for heart rate and measured at the radial artery was increased in CADASIL patients using multivariate regression with age, gender and diastolic blood pressure as covariates (p=0.007). (**B**) Carotid-femoral aortic pulse wave velocity was increased in RVCL patients using multivariate regression with age and diastolic blood pressure as covariates (p=0.01).

Multivariate analysis resulted in a regression model for PWV (Multiple R = 0.70; Adjusted R^2 = 0.46; p < 0.001) with age (p = 0.003; observed power = 0.87), diastolic blood pressure (< 0.001; observed power >0.99) and subject group (p = 0.02; observed power = 0.72) as covariates. This regression model for PWV with covariates age and diastolic blood pressure, revealed a significant difference between control subjects and RVCL patients (p = 0.01) and a trend in CADASIL patients (p = 0.07) (Fig. 1).

Capsaicin-induced Dermal Blood Flow (DBF)

Results on capsaicin-induced DBF are summarized in Fig. 2. One subject did not undergo capsaicin testing due to a skin allergy to alcohol containing solutions. Another subject was excluded from analysis because of prophylactic anti-migraine medication (pizotifen) use. One subject was excluded because of coloured skin. Because measurements for RVCL and CADASIL

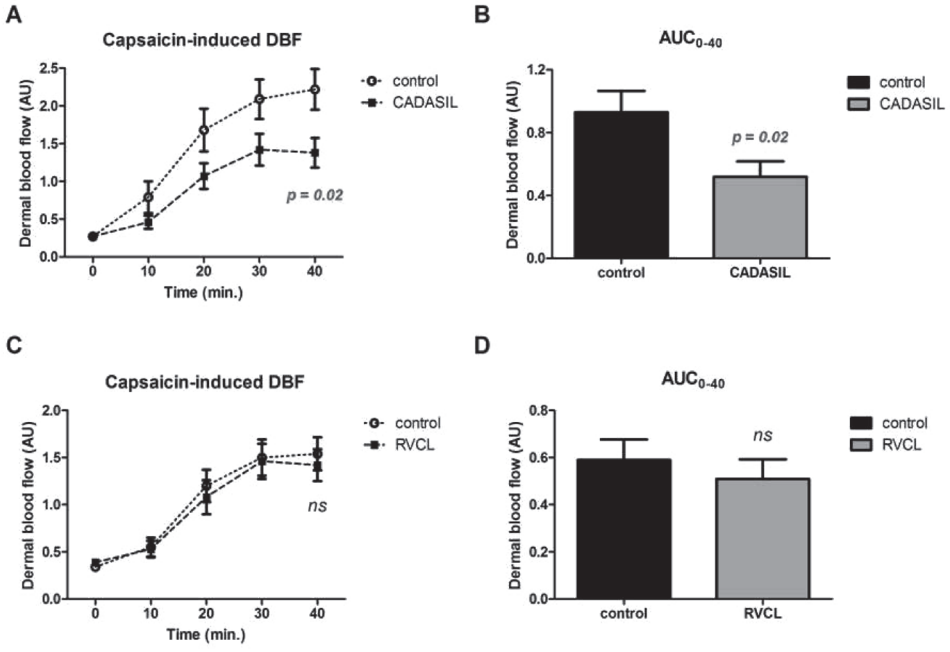

Figure 2. Microvascular reactivity of the skin after topical capsaicin application is reduced in CADASIL subjects. Dermal blood flow was assessed using laser Doppler perfusion imaging. (**A**) Maximal dermal blood flow increase 40 minutes after capsaicin application was lower in CADASIL patients than a matched control group (p=0.02). (**B**) Total dermal blood flow response over a period of 40 minutes after capsaicin application, displayed as area-under-the-curve, was reduced in CADASIL patients (p=0.02). (**C**) and (**D**) No changes in capsaicin-induced dermal blood flow were observed in RVCL patients, when compared to a matched control group.

were performed at different time points with different LDI scanners, separate matched control groups were used. No differences were found between RVCL patients and control subjects. CADASIL patients compared to control subjects displayed a lower increase in DBF 40 min after capsaicin application (1,38 ± 0,88 vs. 2,22 ± 1,20 Arbitrary Units (AU), p = 0.02) and a lower area under the curve over the total observation period of 40 min (0,52 ± 0,43 vs. 0,93 ± 0,60 AU, p = 0.02).

Flow-Mediated Dilatation (FMD)

FMD assessments were performed in 18 RVCL, 14 CADASIL patients and in 18 control subjects. Because of technical difficulties and movement artefacts, a valid set of measurements could only be obtained in 14 control subjects and 10 subjects of the two patient groups. Results are summarized in Fig. 3. In RVCL patients a decreased FMD was observed compared to control subjects (2,32 ± 3,83 vs. 5,75 ± 3,07 %, p = 0.02). In contrast, no significant differences were found between CADASIL patients and control subjects (3,77 ± 3,15 vs. 5,75 ± 3,07, p = 0.14) for any of the parameters. The decreased FMD in RVCL patients (Fig. 3) persisted after correction for shear rate (p = 0.02) and shear stress (2,32 ± 3,83 vs. 5,75 ± 3,07 %, p = 0.03) (Supplementary Table 3).

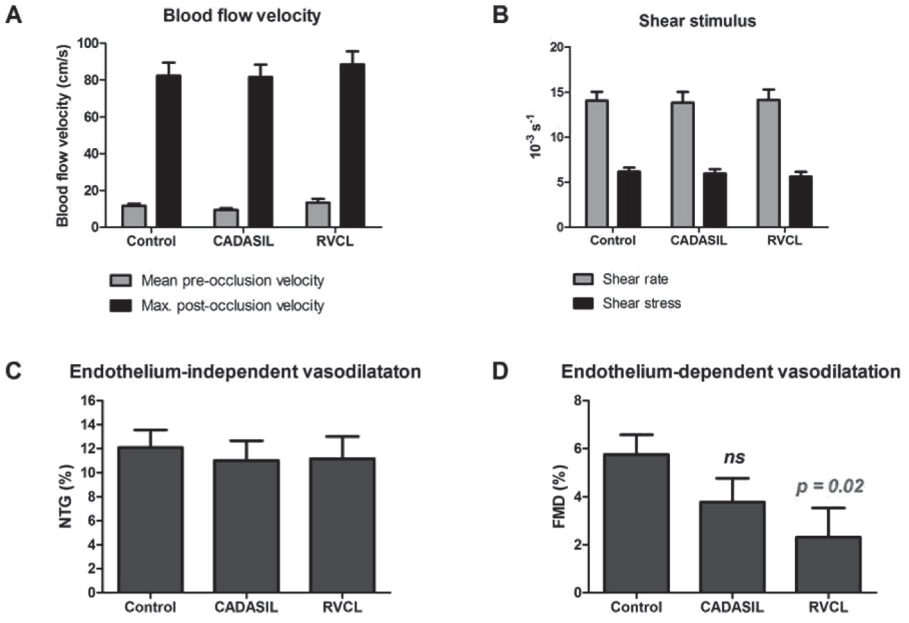

Figure 3. Endothelial dysfunction was found in RVCL patients using Flow Mediated Dilatation (FMD) at the brachial artery. (**A**) Blood flow velocity, measured pre- and post-occlusion hyperaemia, was comparable between all groups. (**B**) Shear rate, calculated as the blood flow velocity divided by artery diameter, and shear stress, calculated as shear rate multiplied by hematocrit, did not differ between all groups. (**C**) The endothelium-independent percentage increase in brachial artery diameter measured 6 minutes after sublingual nitroglycerin administration did not differ between groups. (**D**) The endothelium-dependent maximal increase in brachial artery diameter after 5 minutes of hyperaemia was decreased in RVCL patients (p=0.02).

DISCUSSION

This study provides a detailed assessment of distinct vascular properties of two monogenic cerebral small vessel diseases: RVCL and CADASIL. We are the first to find evidence for endothelial dysfunction in RVCL. In addition, we confirm impaired endothelial-independent microvascular reactivity in CADASIL. The observed increased arterial stiffness in both patient groups further illustrates RVCL and CADASIL as vasculopathies.

Endothelial dysfunction in RVCL

In RVCL patients, a decreased endothelium-dependent flow-mediated dilatation was observed in the brachial artery, supporting the hypothesis that *TREX1* mutations in RVCL cause endothelial dysfunction, as was previously hypothesized.[10,59]

In RVCL patients, capillary occlusion is suggested by histological examination of the retina and cerebral white matter, revealing thickening of the vessel wall with lumen obliteration. Additionally, nodular regenerative hyperplasia of the liver, arteriosclerosis, and glomerulosclerosis in the kidney, together with anemia and gastrointestinal bleeding, are

probably all manifestations of small vessel disease.[10,60] Thus, RVCL is a systemic vasculopathy with luminal narrowing, but in contrast to CADASIL, only minimal degeneration of vascular smooth muscle cells. Instead, fibrinoid necrosis of small and medium vessel wall, hyalinized vessels and reactive gliosis is found, which is may be a consequence of endothelial cell dysfunction. Remarkably, electron microscopy also shows irregular thickening and splitting of the basement membranes in the vessel wall[14], which may be produced by endothelial cells.[10,14]

Impaired microvascular reactivity and increased vascular resistance in CADASIL

In CADASIL patients, a decreased dermal blood flow response to capsaicin was observed. Capsaicin activates pre-synaptic TRPV1 receptors on Aδ- and C-fiber nociceptors and leads to relaxation of the VSMCs of the skin microvasculature upon CGRP release. A decreased dermal blood flow could result from defects at different levels in this pathway: i) less sensitivity of TRPV1 receptors to capsaicin; ii) impairment of CGRP release from nociceptors; iii) a decreased expression of functional TRPV1 and CGRP-receptors, or iv) a decreased relaxation of VSMCs in response to an endothelium-independent stimulus. Given the morphological alterations seen in the microvasculature of CADASIL patients, i.e., degeneration of vascular smooth muscle cells with a largely normal endothelium,[18] the last explanation seems most plausible. Thus, our findings suggest impaired endothelium independent VSMC dilatation in CADASIL.

Findings from other studies that measured microvascular vasoreactivity using different techniques (without involvement of CGRP) are in line with our results. For example, endothelium-independent skin vasoreactivity, as measured by cutaneous blood flow post-occlusive reactive hyperemia was altered in CADASIL patients.[27] In addition, lower dermal blood flow was observed after iontophoretic application of sodium nitroprusside, an exogenous Nitric Oxide (NO)-donor relaxing VSMCs. Impaired endothelium-independent vasoreactivity to glyceryltrinitrate, another exogenous NO-donor, was also found using plethysmographicendo-peripheral arterial tone (Endo-PAT) measurement at the fingertips of CADASIL patients.[30]

After measuring FMD at the brachial artery, we did not find evidence for endothelial dysfunction in CADASIL, nor did we find differences in endothelium-independent relaxation of the brachial artery after NTG administration. Thus, the vascular function of *conduit* vessels, in this case the brachial artery, remains unaltered in CADASIL. These results confirm findings of others[27,28], who also reported that FMD of the brachial artery in CADASIL patients was unaffected. One study[29] suggested endothelial dysfunction based on L-arginine induced vasoreactivity of the middle cerebral artery. However, these results might be due to baseline differences between CADASIL and control subjects. Stenborg et al.[28] reported impaired endothelium-dependent vasodilatation in forearm *resistance* arteries (i.e., the microcirculation) using venous occlusion plethysmography with intra-arterial acetylcholine infusion. Thus, although we find no evidence for endothelial dysfunction in conduit arteries of CADASIL patients, there is evidence for endothelial dysfunction of the microcirculation, which is in line with the labeling of CADASIL as a small vessel disease.

Increased vascular stiffness in both RVCL and CADASIL

We found an increased PWV and AIx in RVCL and CADASIL, which supports increased vascular stiffness in both diseases. Pulse wave velocity is a marker of aortic stiffness while the augmentation index is related to arterial stiffness in general as well as to the intensity and location of reflected waves, which is largely determined by the diameter and compliance of small arteries. In CADASIL, significance was only reached for the AIx. These results may suggest that the microcirculation is more severely affected in CADASIL than in RVCL. In RVCL significance was only reached for PWV, which may be related to endothelial dysfunction found in conduit vessels. Both PWA and PWV are validated methods which have been used for patient outcome predictions in stroke[61] and renal transplant patients,[62,63] cardiovascular disease risk in type 1 diabetes[64] as a marker for risk in cardiovascular disease[65] and brain abnormalities.[66,67] The augmentation index also predicts adverse cardiovascular events in coronary artery disease patients.[68] Consequently, a clinical implication of our results may be that for both RVCL and CADASIL, and possibly other cerebral hereditary angiopathies, intensive monitoring and treatment of cardiovascular risk factors may be important to prevent neurovascular and systemic vascular complications.[69]

Methodological considerations

Our study has important strengths. We are the first to assess four complementary vascular properties in two rare monogenic small vessel diseases (RVCL and CADASIL). All techniques were performed by one investigator (SV) who was blinded for the health status of the study subjects. A specific set of well-validated techniques was used to investigate vascular function at distinct levels of the vascular bed.[52,56,70,71] More specifically, FMD, the absolute standard for testing endothelial function, was performed according to the guidelines from the International Brachial Artery Reactivity Task Force.[58]

There are also some limitations of our study that need to be considered. Because CADASIL and RVCL are rare diseases, sample sizes are relatively small. This limits the power of our observations. Unfortunately, movement artifacts, possibly related to the fact that FMD was the last test performed during the examination (this was done because of the systemic effect of NTG), led to loss of some FMD data. Notably also is the slightly larger (non-significant) baseline brachial artery diameter in the RVCL group. Control subjects with a smaller baseline diameter who display the same absolute increase in diameter, would then automatically display higher % FMD. However, the absolute change in diameter of the RVCL group tended to be smaller than in the control group. Therefore, the validity of our results remains. Whereas the effect of hematocrit was corrected via the shear stress, the effect of slightly lower hemoglobin levels in RVCL patients may have affected our measurements as hemoglobin absorbs NO, and a lower hemoglobin results in an increased FMD.[72] However, correction for this would only strengthen our results and lead to an even lower FMD in RVCL. The slightly (not significant) reduced FMD observed in CADASIL might be the result of the mismatch in cigarette pack years, although it has to be said that mainly the two heavy smokers dramatically influenced the number of pack years. Furthermore, we did not measure endothelial function at the level of resistance arteries. Therefore, we are unable to state that the endothelial dysfunction we found

in RVCL can be extrapolated to small vessels. Therefore, iontophoresis or venous occlusion plethysmography with acetylcholine would be of interest as a follow-up study. Finally, the impact of medication use or stop of medication on our results is unknown. Although we attempted to minimize the effect by asking patients to stop their medication a week prior to the measurements, unfortunately only a few patients got permission to do so from their treating physician.

Possible implications for migraine

The increased prevalence of migraine in both CADASIL and RVCL might be related to the altered vascular properties found in the present and previous studies. In CADASIL, where patients mainly display migraine *with aura*, the increased prevalence of migraine may be due to an increased susceptibility for cortical spreading depression (CSD), which is the underlying mechanism of the migraine aura.[73,74] This hypothesis is strengthened by the fact that transgenic *NOTCH3* over-expressor mice were shown to have a decreased threshold for CSD.[75] Increased susceptibility to CSD may be due to alterations in smooth muscle cells and subsequent altered vasoreactivity of the microcirculation. These alterations may induce (subclinical) ischemia, which is a known trigger for CSD. In line with our results in the skin, indeed altered cerebral vasoreactivity in CADASIL patients has been found.[75]

A direct link with CSD is missing in the case of RVCL because migraine without aura is the more prevalent migraine subtype. A shared increased genetic susceptibility may underlie this co-morbidity, as was shown for RVCL in a family-based association study.[76] In light of the present data, an increased susceptibility for endothelial dysfunction may contribute to the development of migraine in RVCL. This seems supported by the fact that migraine itself has been linked to endothelial dysfunction.[32-36]

Conclusions

In RVCL patients endothelial dysfunction was demonstrated and, based on pulse wave velocity, increased vascular stiffness was suggested. In CADASIL patients impaired endothelium-independent vasoreactivity of the dermal microvasculature was shown. In combination with an increased augmentation index, suggestive for increased vascular resistance, these findings clearly label CADASIL as a small vessel disease. Future studies may link these vascular functional markers to disease progression and perhaps to individual symptoms of these syndromes, including, stroke, dementia, and migraine.

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SUPPLEMENTARY MATERIAL

Supplementary Table 1. Univariate associations between clinical characteristics and pulse wave analysis (PWA) and pulse wave velocity (PWV)

Spearman rank order correlations(p<0.05)	
PWA	
Gender	-0.37
Age	0.55
Systolic blood pressure	0.38
Diastolic blood pressure	0.38
Mean arterial pressure	0.24
Packyears	0.31
HDL cholesterol	0.24
Hematocrite	-0.20
Hemoglobin	-0.20
Ureum	0.22
PWV	
Age	0.56
Systolic blood pressure	0.55
Diastolic blood pressure	0.55
Mean arterial pressure	0.37
Total cholesterol	0.26
gamma-GT	0.27
Ureum	0.32

Supplementary Table 2. Capsaicin induced dermal blood flow

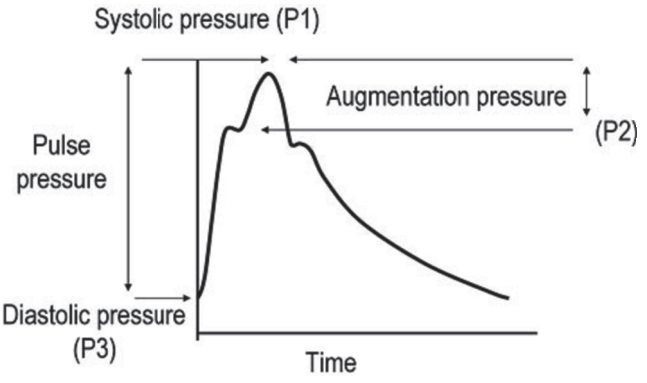
Dermal blood flow	**Control (n=20)**	**CADASIL (n=20)°**	***p-value*** †	**Control (n=18)**	**RVCL (n=18)**	***p-value*** ‡
Baseline (AU)	0.27 ± 0.15	0.27 ± 0.17	*0.98*	0.34 ± 0.08	0.39 ± 0.11	*0.12*
10 min (AU)	0.79 ± 0.94	0.46 ±0.39	*0.16*	0.55 ± 0.42	0.53 ± 0.37	*0.93*
20 min (AU)	1.68 ± 1.27	1.07 ± 0.76	*0.08*	1.20 ± 0.73	1.08 ± 0.77	*0.63*
30 min (AU)	2.09 ± 1.17	1.42 ± 0.94	*0.06*	1.50 ± 0.82	1.46 ± 0.79	*0.87*
40 min (AU)	2.22 ± 1.20	1.38 ± 0.88	*0.02*	1.54 ± 0.74	1.42 ± 0.72	*0.64*
AUC_{0-40} (AU)	0.93 ± 0.60	0.52 ± 0.43	*0.02*	0.59 ± 0.37	0.51 ± 0.35	*0.52*
Increase in DBF (%) 40min. post-capsaicin	607 ± 474	346 ± 343	*0.05*	332 ± 2.21	272 ± 2.01	*0.40*

Data presented as mean ± SD. AUC_{0-40}: area under the curve over the total observation period of 40 minutes. AU: arbitrary units. † Difference between control subjects and CADASIL patients. ‡ Difference between control subjects and RVCL patients. ° One subject did not undergo capsaicin test due to a skin allergy to alcohol containing solutions. One subject was excluded from analysis because of prophylactic anti-migraine medication (Pizotifen) use. One subject was excluded because of Indonesian coloured skin.

Supplementary Table 3. Flow mediated vasodilation (FMD) of the brachial artery

Variable	Controls (n=14)	CADASIL (n=10)	*p-value*†	RVCL (n=10)	*p-value*‡
Pre-occlusion diameter (µm)	5655 ± 1014	5934 ± 839	*0.48*	6438 ± 1103	*0.09*
Maximal post-occlusion diameter (µm)	5987 ±1109	6150 ± 828	*0.70*	6602 ± 1252	*0.22*
Absolute increase in diameter (µm)	332 ± 187	216 ± 169	*0.13*	165 ± 256	*0.08*
FMD (%)	5.76 ± 3.07	3.77 ± 3.15	*0.14*	2.32 ± 3.83	*0.02*
Pre-occlusion velocity (cm/s)	11.7 ± 4.1	9.5 ± 3.4	*0.18*	13.4 ± 6.3	*0.78*
Maximal post-occlusion velocity (cm/s)	82.4 ± 24.9	81.7 ± 20.5	*0.94*	88.5 ± 21.4	*0.56*
Shear rate (s^{-1})	0.014 ± 0.0034	0.014 ± 0.0036	*0.88*	0.014 ± 0.0035	*0.97*
Shear rate normalised FMD (s)	4.35 ± 2.31	2.99 ± 2.33	*0.20*	1.32 ± 2.91	*0.02*
Shear stress (s^{-1})	0.0062 ± 0.0016	0.0060 ± 0.0015	*0.78*	0.0056 ± 0.0015	*0.46*
Shear stress normalized FMD (s)	10.07 ± 5.73	6.96 ± 5.58	*0.23*	3.31 ± 7.24	*0.03*
Pre-NTG diameter (µm)	5710 ± 844	6198 ± 1019	*0.15*	6190 ± 1034	*0.22*
Maximal post-NTG diameter (µm)	6496 ± 899	6947 ± 891	*0.24*	6980 ± 1193	*0.27*
NTG (%)	12.08 ± 5.47	11.01 ± 5.23	*0.64*	11.16 ± 5.84	*0.70*

Data presented as mean ± SD. NTG: nitroglycerine. Peak shear rate was missing in 4 subjects, therefore no corrected FMD could be calculated in 2 healthy volunteers and in 1 of each patient groups. † Difference between control subjects and CADASIL patients. ‡ Difference between control subjects and RVCL patients



Supplementary Figure 1. Augmentation Index
Artery pressure waveform is recorded by applanation tonometry.The height of the late systolic peak (P1) above the inflection (P2) defines the augmentation pressure, and the ratio of augmentation pressure to PP defines the AIx (in percent). "In the human body, wave reflections originate in various locations, including peripheral bifurcations of conducting arteries and smaller muscular arteries. The geometry, number of arterioles, and the architecture of the microvascular network play an important role in wave reflection. Indeed, arterial and arteriolar constriction results in reflection points closer to the heart, leading to earlier aortic wave reflections. In addition, with increased arterial stiffness, as observed, for example, in older subjects or hypertensive patients, the reflected wave travels more rapidly along the arterial tree. Thus, both small and large arteries contribute to early reflected waves which arrive in early systole, superimpose on the forward wave, and boost the systolic pressure further, whereas blood pressure falls sharply in diastole with reduced diastolic fluctuations." Adapted from: Laurent S, Cockcroft J, Van Bortel L, Boutouyrie P, Giannattasio C, Hayoz D, et al. Expert consensus document on arterial stiffness: methodological issues and clinical applications. Eur Heart J 2006;27(21):2588-605.