Università Campus Bio-Medico di Roma

Corso di dottorato di ricerca in

Scienze della Plasticità d'Organo e della Rigenerazione Tissutale per il Recupero Funzionale

XXVI ciclo anno 2011

Titolo tesi

Cerebral hemodynamics and systemic endothelial function are impaired in metabolic diseases

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28 Maggio 2014

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Abstract

Metabolic diseases are associated with an increased risk of cerebrovascular and cardiovascular diseases. Several mechanisms could contribute to this increased risk of vascular events; among them the role of an impairment in vascular reactivity has been considered. Cerebral vasomotor reactivity (VMR) represents the capability of cerebral vessels to modify their caliber in response to a stimulus. Impaired VMR is associated with an increased risk of ischemic events in subjects with carotid disease. Endothelial dysfunction is considered an important pathogenic factor for atherosclerosis and can be non-invasively assessed by flow-mediated vasodilation (FMD) evaluation. We found that VMR and FMD did not correlate in subjects without a history of vascular diseases probably due to physiological differences between cerebral and peripheral vascular districts and vasodilatatory stimulus used.

We also found a slight still significant impairment in cerebral hemodynamics and systemic endothelial function in patients with type-2 diabetes with optimal metabolic control and preserved autonomic balance, but with clinical features of metabolic syndrome. This change in vasomotor function could be responsible for the increased risk of stroke and silent cerebral ischemia observed in patients with diabetes mellitus. Finally, acute hyperglycemia was found to reduce VMR both in patients with metabolic syndrome and in controls. Glycemic variability, increased in a condition of insulin resistance (i.e. metabolic syndrome), appeared to be the major predictor of this VMR reduction induced by hyperglycemia, possibly representing the earliest cause of cerebrovascular damage in diabetes.

Introduction

Metabolic diseases are associated with an increased risk of cerebrovascular and cardiovascular diseases. A meta-analysis of prospective studies found that diabetic subjects have a hazard ratio for stroke of 2.3 (95% CI 2.0 – 2.7) versus non-diabetic ones (Sarwar et al 2010). Similarly, prospective studies suggest that metabolic syndrome is associated with a 1.5- to 2-fold higher risk of stroke and a 1.4-to-4.5-fold higher cardiovascular morbidity and mortality, even after adjusting for conventional risk factors (Wild et al 2009, Grundy 2007, Boden-Albala et al 2008, Rodriguez-Colon et al 2009). In the prospective Framingham study, both diabetes and the metabolic syndrome were powerful risk factors, with a 10-year risk of ischemic stroke associated with diabetes of 14% in men and of 10% in women compared with 8% and 6% in non-diabetic men and women, respectively, with the metabolic syndrome alone (Najarian et al 2006).

Several mechanisms could contribute to this increased risk of vascular events. Among them the role of an impairment in vascular reactivity, in particular in cerebral hemodynamics, has been considered.

The brain has a high metabolic demand and is very sensitive to over- and under-perfusion; therefore, maintaining an appropriate blood flow is of pivotal importance. Cerebral autoregulation is the specific capability of brain circulation, which ensures adequate and stable cerebral blood flow (CBF) even during changes in the cerebral perfusion pressure (CPP) (mABP between 60 and 160 mmHg).

When CPP is normal, CBF stabilizes according to the metabolic rate of the tissue (Stage 0). A drop in CPP produces a reflex dilation in arterioles in order to maintain normal blood flow (Stage I). This compensatory condition is granted by myogenic (i.e. modification of arteriolar tone in response to changes in trans-mural pressure) and metabolic mechanisms (i.e. vasculature sensitivity to changes in interstitial pH and pCO2). Neurogenic control is probably also present by means of autonomic cholinergic innervations of cerebral vessels. With more severe CPP reductions, when

this compensatory vasodilation is exceeded, autoregulation fails, and CBF begins to decline. In this condition, metabolic requirement is assured through an increase in Oxygen Extraction Fraction (OEF); this stage of initial hemodynamic failure is defined misery perfusion (Stage 2) (Grubb et al 1998).

Resistance arterioles dilate via endothelium-dependent and endothelium-independent mechanisms. In cerebral vasculature, both mechanisms are involved although the relative importance of each one is still debated.

These autoregulatory mechanisms may be impaired in subjects with brain microvasculature affecting conditions, such as smoking, aging, arterial hypertension, which are proven to increase arterial stiffness, and diabetes.

In this thesis, the characteristics of non-invasively assessed cerebral and systemic vasomotor reactivity in metabolic diseases are considered, mainly focusing on those studies regarding conditions of chronic and acute hyperglycemia conducted during the PhD period.

Chapter 1

Cerebral Vasomotor Reactivity and Flow-mediated Dilation

Cerebral Hemodynamics Cerebral vasomotor reactivity (VMR) represents the capability of cerebral blood vessels to modify their caliber in response to a stimulus, i. e. hypercapnia. Under normal conditions a fall in perfusion pressure or an increase in metabolic requirements of brain tissue are counterbalanced by a proportional dilation of cerebral arterioles (Markus and Harrison 1992). This cerebrovascular reserve can be evaluated by measuring the cerebral blood flow change that occurs in response to a vasodilatatory stimulus such as carbon dioxide or infusion of acetazolamide or L-Arginie. VMR to L-Arginine specifically evaluates cerebral endothelial function (Pretnar-Oblak et al. 2007; Micieli et al. 1999). In the presence of normofunctioning endothelium, L-Arginine induces NO synthesis, determining the dilation of resistance vessels (Micieli et al. 1999).

Transcranial Doppler (TCD) assessment of hypercapnia-induced VMR is considered the simplest and most non-invasive method for evaluating the cerebrovascular reactivity. It is also thought to explore the metabolic component of vasomotor function. In fact, CO_2-induced vasodilation is only partially related to NO-dependent mechanisms (Iadecola et al. 1994), and hypercapnia is a well-known metabolic stimulus, with a shorter latency effect compared to L-Arginine (Micieli et al. 1999). So far, there is ample evidence in the literature of the clinical importance of VMR in stroke patients. In patients with cortical (ie, large artery atheromatous) as well as lacunar stroke, VMR was reduced compared with normal subjects (Stevenson et al. 2010). Endothelial function is also altered in subjects with vascular risk factors (Girouard and Iadecola 2006) and by drugs used for stroke prevention (Webb et al. 1995). VMR to hypercapnia as well as to L-Arginine is significantly reduced in patients with severe internal carotid artery stenosis and impaired collateral circulation (Vernieri et al. 2001; Micieli et al. 1999) and this difference is potentially reversible after carotid endarterectomy (Micieli et al. 1999). In addition, impaired VMR

distal to the stenosis or occlusion is associated with an increased risk of ischemic events in subjects with carotid artery disease (Silvestrini et al 2000, Vernieri et al. 2001, Palazzo et al 2010).

VMR to hypercapnia should be assessed in a quiet, temperature-controlled room, with the subject lying in a comfortable supine position without any visual or auditory stimuli. Two TCD dual 2-MHZ transducers, fitted on a headband and placed on the temporal bone windows, are commonly used to obtain bilateral continuous measurement of mean flow velocity (MFV) in the middle cerebral arteries (MCAs) insonated at a depth of 50±4 mm. During the experiment, end-tidal expiratory CO_2 should be measured by means of a capnometer (Drager Capnodig, Lübeck, Germany). Once the recorded signals stabilize, MFV and end-tidal CO_2 at rest are obtained through the continuous recording of a 120-second period of normal room air breathing. Hypercapnia can induced by the breath-holding test or by a CO_2-reactivity test by means of the inhalation of a mixture of 7% CO_2/air. This second measurement allows to evaluate VMR during a longer and more stable period of hypercapnia. During the breath-holding test, subjects are asked to hold their breath for 30 seconds after a normal inspiration. The length and effectiveness of breath-holding should be checked by means of the capnometer. End-tidal CO_2 during the first exhalation after apnea should be evaluated. Before proceeding to the definitive recording, subjects should be trained to perform the procedure correctly. Breath-holding index (BHI) is obtained by dividing the percent increase in MFV, occurring at the end of breath-holding, by the length of time (seconds) that subjects hold their breath after a normal inspiration {[(MFV at the end of breath-holding – rest MFV)/rest MFV]X100/second of breath-holding}(Markus and Harrison 1992; Vernieri et al. 2001). To ensure an effective hypercapnia period is evaluated, BHI should be calculated when the rise in end-tidal CO_2 from baseline to the first expiration after breath-holding is >8 mm Hg.

Regarding the CO_2-reactivity test, during the inhalation of the mixture of 7% CO_2/air, subjects are asked to breathe through a mask until MCA flow velocity stabilizes. Once equilibrium is reached, a further 30-second recording is made (plateau period), followed by a 90-second recovery period. The maximal vasodilatory range, or reactivity to 7% CO_2, is determined by the percentage increase in

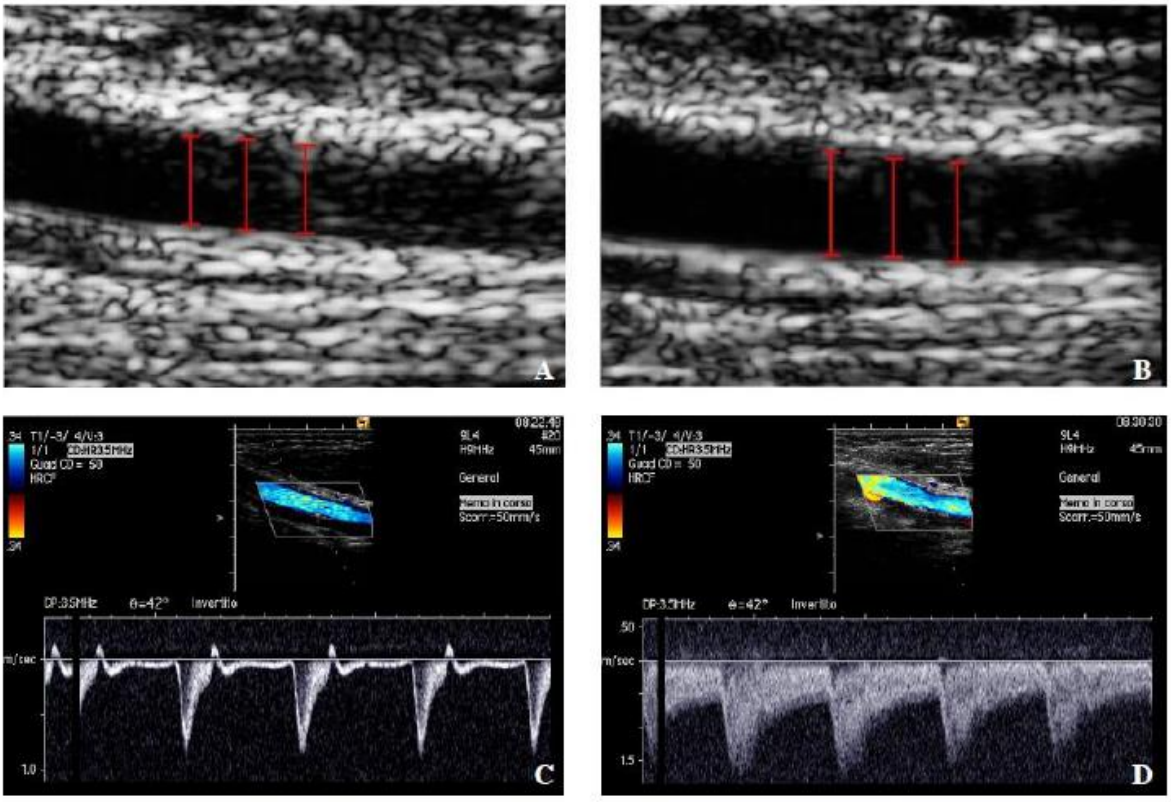
MCA flow velocity recorded during the administration of 7% CO_2 (Vernieri et al. 2004), according to the following formula:

$$CVR = \left[\frac{(MFV_{CO2} - MFV_{Baseline})}{MFV_{Baseline}} \right] \cdot 100$$

Systemic endothelial function Endothelial function is fundamental in regulating vascular tone, cell growth and platelet-leukocyte interactions. Endothelial dysfunction is considered an early pathogenic factor for atherosclerosis and its detection could allow appropriate intervention to prevent its progression (Corretti et al. 2002). Endothelium-dependent flow-mediated vasodilation (FMD), tested by high-frequency ultrasonographic probes after transient brachial artery occlusion, represents a non-invasive method for assessing endothelial function. Endothelial-dependent arterial dilation is mediated by the release of nitric oxide (NO) from the endothelium, which is the main determinant of vascular smooth muscle tone. FMD can allow non-invasive identification of systemic endothelial dysfunction, which is considered an early and fundamental step in the atherosclerotic disease process (Corretti et al. 2002; Yeboah et al. 2009). Its presence is a risk factor for vascular events (Yeboah et al. 2009), and may represent a marker of atherothrombotic burden (Correia and Haynes 2007). In fact, it has been observed in stroke patients and related to stroke physiopathology, stroke subtypes, clinical severity and outcome (Roquer et al. 2009). Endothelial dysfunction contributes to enhanced plaque vulnerability, may trigger plaque rupture, and favors thrombus formation. The assessment of endothelial reactivity is a useful marker of atherosclerotic vascular disease (Roquer et al. 2009).

Flow-mediated dilation is measured as brachial artery blood flow and diameter changes induced by transient ischemia, according to guidelines (Corretti et al. 2002). All examinations should be performed in a quiet, temperature-controlled room, by experienced vascular sonographers using an ultrasound system with a broadband 8-14 MHz transducer. With the subject in the supine

position, the brachial artery should be scanned over a longitudinal section, 3-5 cm above the elbow. Depth and gain settings should be optimized to identify the lumen-to-vessel wall interface. A cuff should be placed around the arm and inflated to 50 mm Hg above systolic blood pressure for 5 minutes. After deflation of the cuff, the FMD should be assessed by measuring the change in brachial artery diameter after 50, 60 and 70 seconds of reactive hyperemia, compared with baseline measurements. Arterial diameter is determined as the distance between intima layers from the far to near vessel wall. The mean diameter is calculated from three measurements of arterial diameter performed at end-diastole incident with the R wave on a continuously recorded ECG.



The response of the vessel diameter to reactive hyperemia (FMD) is expressed as a percent change relative to the diameter before cuff inflation. However, FMD depends on the shear stress on the blood vessels, which is directly related to the velocity and the viscosity of the blood but inversely related to the vessel diameter. Vessels with different diameters may have the same flow but substantially different levels of shear stress and thus a different degree of stimuli for FMD. To

avoid this bias, FMD raw values need to be corrected for flow velocity and diameter. A shear rate should then be estimated as velocity divided by diameter (Pyke and Tschakovsky 2005). Peak shear rate, expressed as peak flow velocity divided by baseline diameter, is calculated to quantify the FMD stimulus. FMD responses should be normalized by dividing the maximal percentage change in diameter by the peak shear rate (Pyke and Tschakovsky 2005).

Lack of correlation between cerebral vasomotor reactivity and flow-mediated dilation

Controversial results have been obtained in several experimental studies evaluating the role of endothelial NO (the main FMD mediator) on cerebrovascular reactivity (White et al. 1998; Iadecola et al 1994). While a weak correlation between FMD and retinal vessels reactivity was found in diabetic and hypertensive patients (Pemp et al. 2009), the only study which compared cerebral and peripheral endothelial function simultaneously did not demonstrate a correlation between FMD and cerebral VMR to L-Arginine (Pretnar-Oblak et al. 2007). So far, no study had investigated possible associations between VMR to hypercapnia and FMD.

In a previous paper of our research group, we tested the hypothesis that cerebral vascular function is not just a local phenomenon but may correlate with systemic endothelial function (Palazzo et al, 2013). We therefore contextually evaluated VMR and FMD in subjects without a medical history of vascular events in order to define a possible correlation between these two different modalities of vasomotor function evaluation.

Subjects and Methods We evaluated 30 Caucasian subjects (60% women, mean age 56 ± 6 years) selected from consecutive volunteers undergoing clinical and ultrasonographic evaluation after being directly called for and having accepted our study proposal. Inclusion criteria were: medical history negative for any previous ischemic event, absence of carotid artery occlusion or stenosis > 30% according to ECST criteria (European Carotid Surgery Trialists' Collaborative Group 1998), normal vertebral and proximal subclavian arteries, no differences between right and left brachial

artery blood pressures, normal brachial arteries blood flow pattern, good insonation of the temporal bone window, and normal intracranial vessels. All subjects underwent a careful neurological and cardiovascular evaluation. Clinical history with particular attention paid to the major vascular risk factors (hypertension, diabetes, smoking, and hyperlipidemia) was obtained from each subject and the European Society of Cardiology (ESC) score was calculated. A regular 12-lead ECG was assessed to exclude abnormalities compatible with previous asymptomatic myocardial infarction. None of the subjects had rhythm abnormalities.

All subjects underwent color-coded duplex sonography of the neck vessels (ACUSON C512 Sequoia, Siemens, Erlangen, Germany) and cerebral vessels examination (DWL MultiDop X4, Elektronische Systeme GmbH, Singen, Germany). A high-resolution B-mode system with linear ultrasound transducers at 9 MHz was used, and depth of focus, frame rate and gain settings were adjusted in order to obtain optimal image quality. In each subject, IMT was measured, by a single experienced ultrasonographer, at the far wall of the distal common carotid artery, 1 cm proximal to the bifurcation, with the mean value calculated on a 10-mm segment of the artery. In order to improve reliability and reproducibility of measurements, semiautomatic software was used (Touboul et al. 2007).

The study was performed between 8 and 9 AM in a quiet, temperature-controlled room (22- 24°C). All subjects were studied after a 12-hour overnight fast. They were asked not to take caffeine, theine, or alcohol. Smokers refrained from smoking in the 12 hours preceding the study. Vasoactive drugs were discontinued for the same time period. Female subjects were investigated during the first week of their menstrual cycle (Hashimoto et al. 1995). None of the subjects were taking estro-progestinic drugs.

FMD was measured as brachial artery blood flow and diameter changes induced by transient ischemia, according to guidelines (Corretti et al. 2002). All examinations were performed by a single experienced vascular sonographer, who was unaware of the subjects' clinical background, using an ultrasound system (ACUSON C512 Sequoia, Siemens, Erlangen, Germany) with a

broadband 8-14 MHz transducer. In our ultrasound laboratory the coefficient of variation for FMD repeated measurements was 15%. The FMD protocol has been previously described.

In addition to FMD assessment, each subject's VMR was also evaluated, with an at rest 30-minute interval between these two tests. The order of the tests was determined by a simulated coin toss. VMR to hypercapnia was assessed in the same quiet room, with the subject lying in a comfortable supine position without any visual or auditory stimuli, according to previously described procedure. Hypercapnia was induced by the breath-holding test. All the study subjects were able to hold their breath for the required period. Twenty subjects were also asked to take a CO_2-reactivity test by means of the inhalation of a mixture of 7% CO_2/air. This second measurement was added in order to evaluate VMR during a longer and more stable period of hypercapnia. Those subjects who underwent both tests had a 5-minute rest interval of normal room air breathing between each. The order of the tests was determined by a coin toss.

The experimental protocol was approved by our Institutional Review Board, and all subjects signed a written informed consent.

Statistical analysis For both BHI and CO_2–induced VMR, inter-hemispheric concordance was measured by Intra-Class-Correlation index (ICC) and the resulting systematic difference between the two sides was evaluated with paired t-test. This analysis allowed justifying the computation of the average of right and left-sided VMR as a reliable measure of vasomotor reactivity.

To study the correlation between VMR and FMD, Pearson's r correlation was used. Since inference on this index relies on specific assumptions (bivariate gaussianity of variables, robustness with respect to the effects of eventual outliers), we evaluated the departures from gaussianity and re-ran the procedure even after deleting the outliers. The same analysis was applied to assess the correlation between CO_2-induced VMR and BHI.

Statistical analyses were performed with the SPSS 16.0 software (SPSS Inc).

Results Demographic characteristics, vascular risk factors, and medication of the study population are shown in the table. Patients' median ESC score was 1% (min-max 0-4).

Baseline characteristics of the study population (n = 30)*

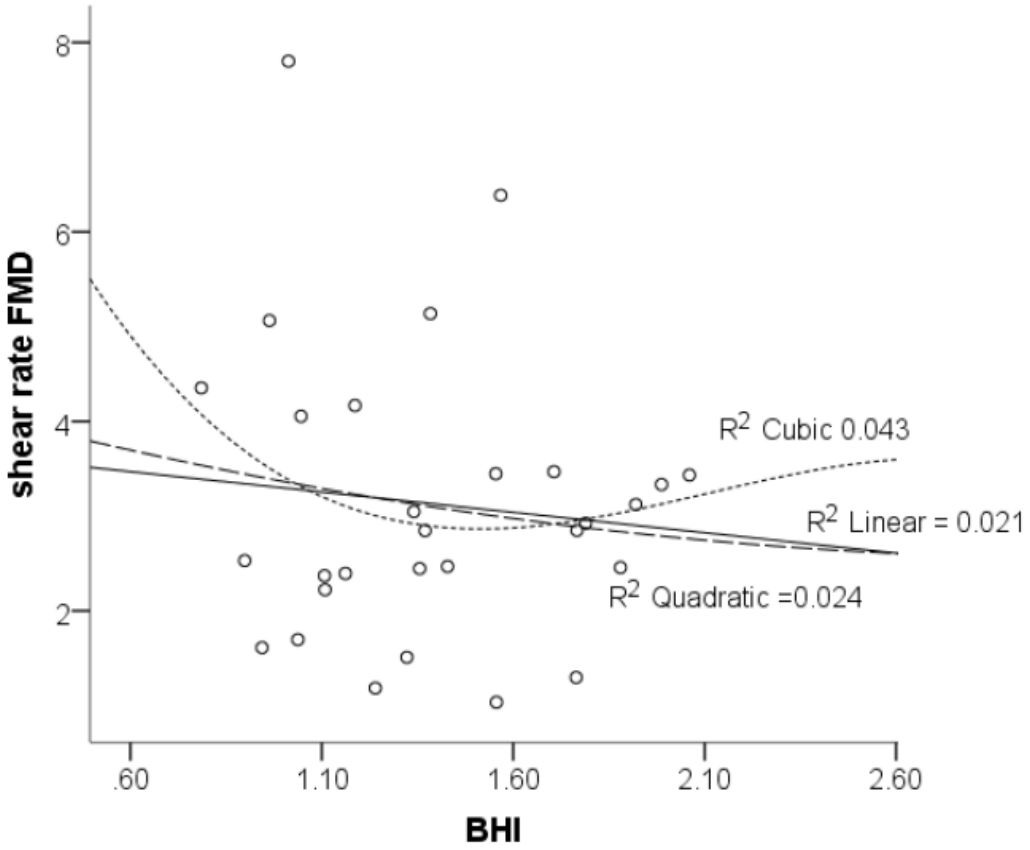
Variable	Value
Age, mean (SD)	56 (6)
Male gender	12 (40)
Hypertension	4 (13)
Diabetes mellitus	6 (20)
Smoking	10 (33)
Hypercholesterolemia	18 (60)
BMI, mean (SD)	26 (4)
Carotid artery stenosis 0% ≤30%	 17 (57) 13 (43)
Lipid-lowering medication	7 (23)
Anti-hypertensive drugs	2 (7)
Oral hypoglycemic drugs	2 (7)

* Values are n (%) unless otherwise indicated.

No systematic differences between right and left-side values of the CO_2-induced nor apnea-induced VMR were observed (t(19)=-0.69, p=.499 and t(19)=1.86, p=.078, respectively), with a high correlation between the 2 sides (ICC=0.96, 95% CI= 0.91, 0.99, p<.001 and ICC=0.91, 95% CI= 0.77, 0.97, p<.001, respectively), allowing reliable computation of the average of right and left values as unique indexes of VMR.

In our study, VMR and FMD did not appear to be correlated. In fact, the size of Pearson's linear correlation between BHI and FMD was low and non-significant (BHI and raw FMD: r = 0.05, p=0.792; BHI and shear rate FMD: r=0.15, p = 0.444). Even after the remedial procedures to

avoid biasing effects of asymmetrical residuals distribution, the correlation between BHI and FMD remained non-significant ($p>0.20$) and characterized by a small effect size (less than 7% of the explained variance). In addition, neither FMD shear stress data nor BHI values appear to correlate with IMT ($r<0.20$, $p>0.25$).



In those (n=20) subjects who performed the breath-holding and CO_2-inhalation test, a clear correlation between CO_2-induced VMR and BHI ($r = 0.77$, $p< 0.001$), without systematic differences ($t = – 0.607$, $DF= 19$, $p = 0.551$), was observed. More precisely, the mean CO_2-induced VMR was 40.1 (SD = 11.1), while the mean BH-induced value was 41.1 (SD = 10.3).

Comments In our study, evaluation of VMR and FMD in subjects without a history of cerebrovascular and cardiovascular disease did not show a correlation between these two parameters. We believe that this lack of association is due to the differences in the vascular district evaluated and to the vasodilatatory stimulus used.

Before our study, there were only a few clinical papers which compared cerebral and peripheral vasomotor reactivity. In a previous study regarding patients with lacunar infarctions, an association between systemic (FMD) and cerebral (VMR to L-Arginine) endothelial function was not observed (Pretnar-Oblak et al. 2007). On the contrary, a weak correlation was observed between FMD and retinal vessel response to flickering light, which represents local endothelial reactivity induced by augmented retinal metabolic requirements (Pemp et al 2009). To our knowledge, no study had investigated a possible correlation between FMD and VMR to hypercapnia before.

Similarly, several experimental studies evaluating the role of endothelial function mediators on cerebrovascular reactivity led to discordant results (Wang et al 1995; White et al. 1998; Iadecola et al 1994). In particular, in a number of studies, the cerebral vasodilatory response to hypercapnia has been inhibited by L-arginine analogues (Wang et al 1995; Iadecola et al 1994), suggesting the involvement of NO. On the contrary, White et al. (1998) found no effect of NO synthase inhibitor on cerebrovascular reactivity to CO2, as assessed by internal and common carotid artery volume flow and MCA flow velocity.

In addition, in our study IMT did not appear to correlate either with FMD or with VMR. Despite contrasting data regarding this issue (Juonala et al. 2004, Yan et al. 2005), it is generally accepted that FMD and IMT are independent surrogates that measure early stages but different aspects of atherosclerosis (Corretti et al. 2002; Touboul et al. 2007).

Finally, performing both CO_2- and apnea-induced VMR in a subgroup of subjects, we found that these two modalities of evaluating VMR to hypercapnia appeared to be significantly associated, and this is consistent with previously published data (Markus and Harrison 1992).

Chapter 2

Cerebral Vasomotor Reactivity and Flow-mediated Dilation in Patients with Diabetes Mellitus

Type 2 diabetes mellitus (T2DM) is associated with an increased risk of cardiovascular and cerebrovascular diseases with high mortality and disability. Among the factors associated with cerebrovascular events, endothelial dysfunction and cardiac autonomic neuropathy (CAN) seem to play a major role (Shechter et al 2009, Santos-García et al 2011). The mechanisms of glucose-mediated endothelial dysfunction (Moens ET AL 2005) include reduced NO bioavailability due to increased reactive oxygen species formation (Cosentino et al 1997), and glucose auto-oxidation (Wolff and Dean 1987, Ceriello et al 2008).

Data on vascular reactivity in diabetes are few and unclear. Impaired VMR and FMD were found in selected subgroups of T2DM patients with long-term disease (Fülesdi et al 1999) or in type 1 diabetic patients with nephropathy (Kozera et al 2009). VMR appeared to be inversely related to the duration of diabetes (Fülesdi et al 1999) and albuminuria (Vuletić ET AL 2011). However, VMR was normal in unselected patients (van Oers ET AL 2006), and no data were available in well-controlled patients with short-term disease. These controversial results are likely due to the heterogeneity of the studied populations, since it is well known that the degree of metabolic control and the presence of diabetic complications strongly contribute to determine the different phenotypes of type 2 diabetic patients. Particularly, CAN seems to play a fundamental role, since autonomic innervation is known to regulate systemic endothelial function (Engelke et al 1997, Dyson et al 2006), and a role, although still unclear, of the vegetative nervous system on cerebral vasomotor reactivity has been demonstrated (Vernieri et al 2010, LeMarbre et al 2003).

In our study (Palazzo et al 2013), we evaluate cerebral hemodynamics, systemic endothelial function and sympatho-vagal balance in a selected population of well-controlled T2DM patients with short-term disease and without overt CAN.

Subjects and Methods Twenty-six T2DM patients with short-term disease (duration of diabetes <10 years), good metabolic control (HbA1c <8%), and normo-albuminuria (albumin excretion rate, AER, <30 mg/gr urinary creatinine), treated with diet and/or metformin, were consecutively recruited from those followed at the Department of Endocrinology and Diabetes, S. Giovanni Calibita Fatebenefratelli Hospital of Rome. Eighteen age- and sex-matched controls were enrolled also.

Exclusion criteria were: a medical history of any vascular ischemic event; history of arterial hypertension or anti-hypertensive treatment; documented CAN; micro and macroalbuminuria; proliferative retinopathy; estro-progestinic therapy; vasoactive drugs; carotid artery stenosis > 40% according to ECST criteria (ECST 1998); vertebral and proximal subclavian artery abnormalities; differences between right and left brachial artery blood pressures; brachial arteries blood flow pattern alterations; poor insonation of the MCA through transtemporal bone windows; intracranial artery abnormalities.

All control subjects underwent OGTT to exclude diabetes and impaired glucose tolerance. All subjects underwent a careful clinical evaluation and the European Society of Cardiology (ESC) score was calculated. Color-coded duplex sonography of the neck vessels (ACUSON C512 Sequoia, Siemens, Germany) and TCD (DWL MultiDop X4, Elektronische Systeme GmbH, Germany) evaluation were performed on all subjects. In each subject, intima-media thickness (IMT) was measured, by a single experienced ultrasonographer, as previously described.

In order to exclude CAN, cardiac autonomic function was assessed in T2DM patients using the battery of cardiovascular tests proposed by Ewing (Ewing et al 1985) and recommended by the Toronto Consensus (Tesfaye et al 2010). Blood and urinary samples were obtained for the determination of Albumin Excretion Rate (AER), cholesterol, triglycerides and HbA1C. HbA1c was analysed by High Performance Liquid Chromatography (HPLC, VARIANT 2, BIORAD Laboratories, Munich, Germany), with intra- and inter-assay coefficients of variation of 0.46-0.77 % and 0.69-0.91 %, respectively. Total cholesterol, HDL-cholesterol and triglycerides were determined by an enzymatic colorimetric test (Cobas, Roche Diagnostic, Indianapolis, USA). AER was determined by immunoturbimetric-assay (Tina-quant, Cobas, Roche Diagnostic, Indianapolis, USA).

FMD and CO_2 VMR tests were performed according to previously described protocol (Chapter 1).

Twenty-one subjects (10 T2DM patients and 11 controls) also underwent a 5-minute ECG monitoring. Using fast Fourier transformation, fluctuations in RR interval widths were transformed into a frequency waveform that depicted periodic oscillations in sympathetic and parasympathetic functions. The frequency domain variables included total power (0.01 to 0.40 Hz), very low frequency (0.01 to 0.04 Hz), low frequency (LF 0.04 to 0.15 Hz) and high frequency (HF 0.15 to 0.40 Hz). To better define the role of the sympathetic-parasympathetic system, normalized LF (LFN) and HF (HFN) and LF/HF ratio were calculated. LFN was calculated according to the formula LFN=[LF/(Tot Power-VLF)]*100; HFN was calculated according to the formula HFN=[HF/(Tot Power-VLF)]*100.

Statistical analysis VMR inter-hemispheric concordance was measured by using the Intra-Class-Correlation index (ICC). The systematic difference between the two sides was evaluated with paired t-test, which showed no differences between right and left VMR. This permitted the use of the average of right- and left-sided VMR as a reliable measure of vasomotor reactivity.

All continuous variables were assessed for normal distribution of the values. Since their distribution was not different from gaussianity assumptions, independent samples Student's t-test was performed to compare continuous variables between groups. Chi-square test was used to compare categorical variables, Mann-Whitney test to compare ordinal data.

To study the correlation between FMD, shear rate FMD and VMR, Pearson's r correlation was used. Since inference on this index relies on specific assumptions (bivariate gaussianity of variables and robustness with respect to the effects of eventual outliers), we evaluated the departure from gaussianity and re-ran the procedure even after deleting the outliers.

Statistical analyses were performed with the SPSS 19 software (SPSS Inc).

Results Demographic characteristics and vascular risk factors are shown in the table.

Demographic characteristics and vascular risk factors of the study population

Variable	**T2DM patients (n= 26)**	**Controls (n= 18)**	**p**
Age, years	58 (9)	54 (8)	.08
Male gender	11 (42)	4 (22)	.167
BMI	27 (3)	25 (3)	.052
Waist circumference, cm	93 (10)	81 (5)	**<.05**
Smoking	11 (42)	3 (17)	.073
Systolic Blood Pressure, mmHg	128 (11)	115 (13)	**<.001**
Diastolic Blood Pressure, mmHg	80 (6)	77 (7)	.07

Total Cholesterol, mg/dl	202 (34)	218 (33)	.171
LDL Cholesterol, mg/dl	126 (27)	131 (31)	.595
HDL Cholesterol, mg/dl	51 (12)	71 (14)	**<.001**
Triglycerides, mg/dl	121 (45)	73 (28)	**<.05**
Familiar history of cerebrovascular disease	17 (65)	8 (44)	.168
ESC score	1.5 (0-8)	0 (0-3)	**.003**
Mean IMT	0.7 (0.5-1.3)	0.6 (0.5-0.9)	**.004**

BMI: body mass Index; IMT: intima media thickness. Significant interactions are evidenced in bold. [a] *For continuous variables, values were expressed as mean ± SD; for categorical variables, percent were used. ESC score and IMT were expressed as median (min-max range).*

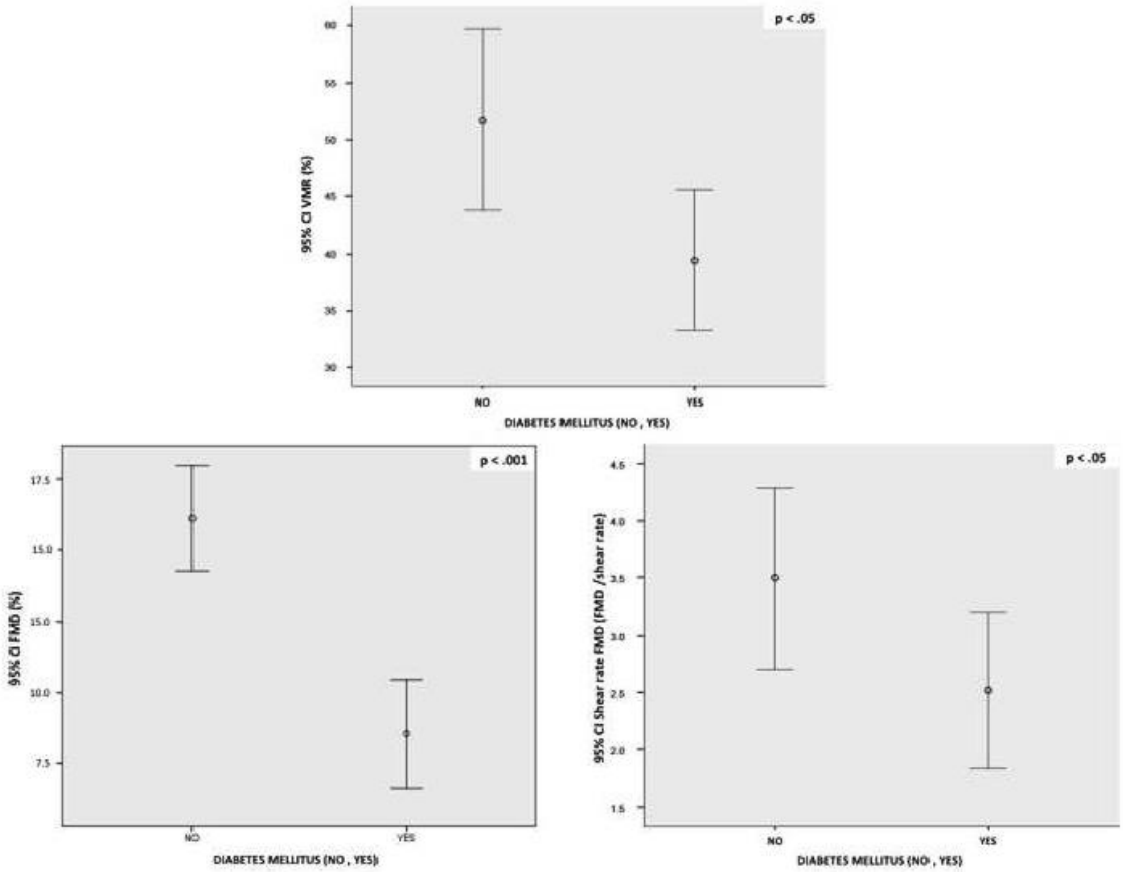
In T2DM, mean duration of diabetes was 4.40 ± 4.80 years, HbA1C 6.71 ± 1.29%, AER 7.1 ± 7.6 mg/gr of urinary creatinine.

FMD and shear rate FMD were found to be reduced in T2DM subjects (8.5% SD 3.5 and 2.5 SD 1.3, respectively) compared to controls (15.4% SD 4.1 and 3.5 SD 1.4; $p<.001$ and $p<.05$).

No systematic differences between right and left-side values of the CO_2-induced VMR were observed, with a high correlation showed between the 2 sides (in both cases $r = 0.8$, $p < 0.001$). Therefore, the average of right and left values was considered in our statistical analysis.

VMR also appeared to be significantly reduced in T2DM patients (39.4% SD 12.4) compared to controls (51.7%, SD 15.5) ($p <.05$).

After correcting for HDL cholesterol levels, smoking habits and BMI, the above mentioned differences were confirmed.



LF/HF ratio (1.2, SD 0.7 vs 1.5, SD 0.9, p = .442) and the single LFN (49.6 SD 16 vs 54.8 SD 15.2, p=.455) and HFN components (50.3 SD 16 vs 45.1 SD 15.2, p=.455) were similar in T2DM compared to controls.

No correlation between FMD and shear rate FMD respectively with VMR was found, both in T2DM patients and in controls (p>.05).

Comments Well-controlled T2DM patients with short-term disease and normoalbuminuria already displayed reduced, still not clearly impaired, VMR values when compared to age- and sex-matched

controls. In fact, VMR value was >20% in all patients, which is considered the cut off between normal and altered reactivity to 7% CO_2 (Markus and Cullinane 2001).

Even at this initial stage of the disease, without clinically relevant vascular and autonomic complications, cerebral hemodynamics is slightly still significantly altered. This early cerebral hemodynamic dysfunction could be one of the main pathophysiological mechanisms underlying the increased risk of ischemic and atrophic brain damage as well as cognitive impairment observed in patients with diabetes (Verdelho ET AL 2010, Silvestrini et al 2006).

We also found that endothelial function was reduced in our group of diabetic patients compared with controls. These data are consistent with previous studies demonstrating impaired endothelial function in type 2 diabetic patients. However, these previous data were obtained in diabetic patients with chronic complications (Yokoyama et al 2011), or poor metabolic control (Kotb et al 2012). Our patients with short-term disease were accurately selected with optimal metabolic control, in order to exclude the impact of marked hyperglycemia on endothelial function. However, clinical characteristics of metabolic syndrome (low HDL cholesterol, high triglyceride, increased waist circumference, increased BP), suggestive for insulin resistance, were significantly increased in the group of diabetic patients compared to controls. Therefore, our finding of reduced VMR and FMD in this selected population of T2DM patients strongly suggests that insulin-resistance has an impact on vascular function, even in the absence of metabolic derangement. Activation of oxidative stress pathways by increased glucose variability, as previously demonstrated (Di Flaviani et al 2011), might also have a negative impact on endothelial function.

The exclusion of CAN and the observation of a preserved sympatho-vagal balance, proven by LF/HF values within the normal range according to age (Agelink et al 2001), allowed us to rule out the role of impaired autonomic innervation of the cerebral vessels in early alterations of cerebrovascular reactivity.

The lack of correlation between FMD and VMR confirms previous data obtained in patients with lacunar infarction (Pretnar-Oblak et al 2007), and in subjects with no history of vascular disease (Palazzo et al 2013), suggesting different responses in different vascular districts.

Chapter 3

Cerebral Vasomotor Reactivity during Acute Hyperglycemia in Patients with Metabolic Syndrome

Metabolic syndrome (MS) is associated with a 1.5- to 2-fold higher risk of stroke, even after adjusting for conventional risk factors (Wild et al 2009, Grundy 2007, Boden-Albala et al 2008, Rodriguez-Colon et al 2009). An impairment in VMR, recently reported in a cohort of patients with atherosclerotic disease and MS, probably contribute to this higher rate of cerebrovascular ischemic events in these patients (Giannopoulos et al 2010).

In experimental animals, acute exposure to elevated glucose concentration, a well-recognized negative prognostic factor in acute ischemic stroke, selectively alters endothelium-dependent cerebral vasodilation via the activation of protein kinase C (Mayhan and Patel 1995). Besides, acute hyperglycemia also impairs cerebrovascular resistance and control of cerebral blood flow in dissected posterior cerebral arteries of rats, via an endothelium-mediated mechanism that involves nitric oxide and prostaglandins (Cipolla et al 1997). In addition, the effect of hyperglycemia on myogenic tone seems to be concentration-dependent. In fact, exposure to 25 mM glucose in the isolated ophthalmic artery of rats causes an increase in pressure-dependent tone, while a decrease in pressure-dependent tone was observed in the presence of 40 mM glucose (Ito et al 2006).

Similar findings were observed in pre-diabetic stages. In fact, in insulin-resistant rats endothelium-dependent dilation of isolated MCAs was found to be impaired probably because of a defect in COX-mediated pathways (Erdös et al 2002) with an increase in reactivity to constrictor stimuli (Phillips et al 2005). Such vascular impairment seems to correlate with the severity of MS, since it was demonstrated in obese insulin-resistant rats compared to lean ones (Phillips et al 2005).

In our paper (Giordani et al 2014), we evaluated the impact of acute hyperglycemia on cerebrovascular reactivity, mainly in a condition of insulin resistance (i.e. MS).

Subjects and methods Eighteen consecutive patients (8 men e 10 women) with MS, diagnosed according to the IDF criteria (Alberti et al 2006) but without DM, 26 patients with short-term (4.40 ± 4.80 years) and well-controlled (HbA1C = 6.7 ±1.3%, 50 ± 14.1 mmol/mol) type 2 DM, treated with diet and/or metformin, and 9 age- and sex-matched controls (C) were consecutively recruited. Exclusion criteria were a history of cardiovascular or cerebrovascular diseases, systemic or neoplastic diseases, carotid stenosis greater than 40% according to the European Carotid Surgery Trialists' (ECST) criteria, vertebral and proximal subclavian artery abnormalities, poor insonation of the MCA through the transtemporal bone windows, intracranial artery abnormalities. All subjects underwent an oral glucose tolerance test, for the diagnosis of DM, and a 7-day wash-out for anti-hypertensive and anti-dyslipidemic drugs, before being evaluated.

On the first day, anthropometric parameters were taken. After an overnight fast, all subjects simultaneously underwent 24-h continuous glucose monitoring with the Ipro2 System (Medtronic, Northridge, CA) and Ambulatory Blood Pressure Monitoring (ABPM; TM2430, Intermed). On the following day, VMR was basally evaluated in the three groups and, following a 2-hour hyperglycemic clamp (HC), in MS and C.

Hyperglycemic clamp (HC) A hyperglycemic clamp study was performed, as described elsewhere (Malandrucco et al 2012). All studies were carried out at 8 am after a 12-h overnight fast, while the subjects were lying in bed, and lasted 180 min. Two intravenous catheters were inserted into an antecubital vein (retrogradely) and into a wrist vein for substance infusion and sampling of arterialized blood, respectively (Marfella et al 2000). After a 60-min period to establish baseline (-60 to 0 min), a hyperglycemic glucose clamp was carried out for the following 120 min (Malandrucco et al 2012). Plasma glucose was measured with Glucocard G Meter blood glucose monitoring system (Menarini Diagnostic, Florence, Italy) at bedside every 2–5 min as needed and was clamped at +126 mg/dL (+7.0 mmol/L) from basal.

In order to avoid the possible confounding effect of endogenous hyperinsulinemia, insulin secretion was inhibited by octreotide (bolus 25 μg in 1 minute, followed by a continuous infusion, 0.5 μg/min).

All subjects underwent a careful clinical evaluation and the ESC score was calculated. Color-coded duplex sonography of the neck vessels (ACUSON C512 Sequoia, 124 Siemens, Germany) with IMT measurement and TCD (DWL MultiDop X4, Elektronische Systeme GmbH, Germany) evaluation were performed in all subjects.

Cerebral vasomotor reactivity was assessed in all the study subjects accordingly to previously described protocol.

Continuous Glucose Monitoring System The day before HC, a subcutaneous sensor (Enlite; Medtronic, Northridge, 144 CA) for Continuous Glucose Monitoring System (CGMS) was applied. To calibrate CGMS, Glucocard G Meter blood glucose monitoring system (Menarini Diagnostic, Florence, Italy) was used. Data were downloaded using USB connection to CareLink iPro™. From CGMS data, the following indexes of GV were calculated (Picconi et al 2012):

- Mean amplitude of Glucose Excursions (MAGE), defined as the average of all blood glucose excursions of more than 1 SD of the 24-h mean blood glucose value;
- "J-index": J = 0.324 x (MBG + SD)2, where MBG is the Mean Blood Glucose level measured in mmol/l and SD is the SD of glucose levels;
- Mean absolute glucose (MAG), that calculates the sum of the differences between successive glucose values divided by the total time measured in hours;
- Lability Index (LI): this formula processes three glucose values to calculate a lability value and then moves to the next three glucose values, and so on. The LI is the mean of these values (Rodbard 2009);
- Standard Deviation (SD): rate of variation or dispersion from average glycemia.

Ambulatory Blood Pressure Monitoring ABPM was monitored by the non-invasive oscillometric technique, validated by the British Hypertension Society (O'Brien et al 1991). The percentage change from day to night (Δ) for systolic and diastolic BP (SBP and DBP) was defined as [(mean value during the day-mean value during the night) ×100]/mean value during the day. A ΔBP of less than 10% indicated a non-dipper BP profile (Spallone te al 2011).

Statistical analysis VMR inter-hemispheric concordance was measured by using the Intra-Class-Correlation index (ICC). The average of right- and left-sided VMR was used as a reliable measure of vasomotor reactivity.

When frequency distributions did not show significant departures from gaussianity assumption, independent samples Student's t-test was applied to compare continuous variables between groups. Chi-square test was used to compare categorical variables, Mann-Whitney test to compare ordinal data. When DM group was also considered, Kruskal-Wallis non parametric tests were applied and comparisons among two groups were tested for statistical significance using the Mann-Withney's non parametric U test with Bonferroni correction for multiple testings. Regarding VMR, ANOVA was applied and post-hoc pairwise comparisons were adjusted according to Tukey's procedure. ANOVA for repeated measures (rm-ANOVA) was applied to evaluate the changes induced by hyperglycemic clamp (within-subjects factor: Time) and their interaction with Group (between subjects factor). Pearson linear correlation (bivariate and partial) was used to assess the association between variables. A two tailed $p<0.05$ was considered significant. All statistical analyses were performed using the SPSS 16 software (SPSS Inc).

Results Clinical and laboratory characteristics of MS, DM and C are reported in the table (Giordani et al 2014).

	Controls (n = 9)	**Metabolic Syndrome (n = 18)**	**Diabetes Mellitus (n = 26)**	$p^{2,3}$
Age (yrs)	52.6 ± 1.2	59.1 ± 2.5	59.2 ± 2.1	0.09
Sex (M: F)	4:5	8:10	11:15	0.167
Waist Circumference (cm)	82.6 ± 1.9	100.7 ± 3.2*	93.4 ± 2*	0.001
BMI (kg/m^2)	24.5 ± 1.1	28.8 ± 1*	26.9 ± 0.7	0.03
Total cholesterol [mg/dl (mmol/l)]	213 ± 12 (5.5 ± 0.3)	208 ± 6 (5.38 ± 0.15)	201 ± 8 (5.2 ± 0.2)	0.604
HDL cholesterol [mg/dl (mmol/l)]	69 ± 3 (1.78 ± 0.09)	45 ± 2 (1.16 ± 0.06)†	50 ± 2 (1.3 ± 0.06)*	<0.001
LDL cholesterol [mg/dl (mmol/l)]	130 ± 10 (3.35 ± 0.26)	137 ± 5 (3.53 ± 0.14)	126 ± 4 (3.26 ± 0.1)	0.547
Triglycerides [mg/dl (mmol/l)]	74 ± 10 (0.84 ± 0.11)	145 ± 12 (1.64 ± 0.13)*	124 ± 9 (1.4 ± 0.1)*	0.002
Glycemia [mg/dl (mmol/l)]	84.6 ± 1.8 (4.7 ± 0.1)	97.2 ± 3.6 (5.4 ± 0.2)*	127.8 ± 5.4 (7.1 ± 0.3)†‡	<0.001
Systolic Blood Pressure (mmHg)	118.8 ± 2.8	135.1 ± 2.2*	124.8 ± 2.9	0.003
Diastolic Blood Pressure (mmHg)	74.4 ± 2.4	78.1 ± 1.9	74.3 ± 1.4	0.209
Δ Systolic Blood Pressure (%)	17.6 ± 3.5	14.2 ± 1.6	11.7 ± 1.3	0.183
Δ Diastolic Blood Pressure (%)	20.4 ± 3.6	16.3 ± 1.8	12.8 ± 1.5	0.256
Baseline Cerebrovascular Reactivity (%)	59.6 ± 6.7	61.6 ± 4.2	39.4 ± 2.9*§	0.001

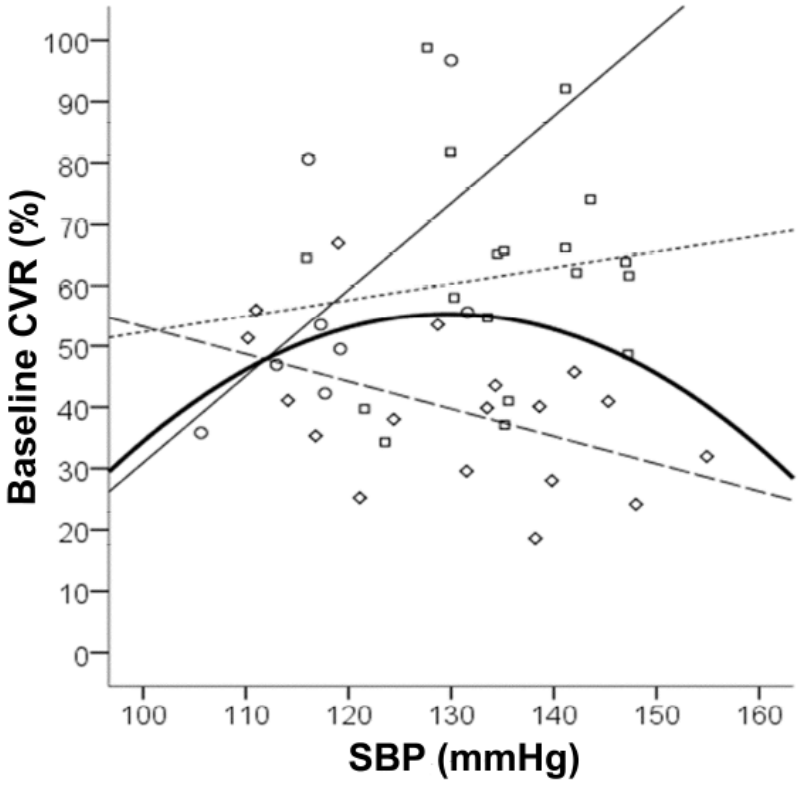
1 All values are mean ± SEMs
*2 p value by Kruskal-Wallis non parametric tests. When Kruskal-Wallis test was statistically significant (p < 0.05), all comparisons among two groups were tested for statistical significance using the Mann-Withney's non parametric U test. Comparisons that were significantly different from one another are indicated by superscripts as follows: * p <0.05 versus controls; † p <0.001 versus controls; ‡ p<0.001 versus metabolic syndrome; § p <0.05 versus metabolic syndrome.*
*3 For baseline Cerebrovascular Reactivity: p value by ANOVA. When ANOVA test was statistically significant (p < 0.05), all post-hoc pairwise comparisons among two groups were tested for statistical significance using the Tukey's. Comparisons that were significantly different from one another are indicated by superscripts as follows: * p <0.05 versus controls; † p <0.001 versus controls; ‡ p<0.001 versus metabolic syndrome; § p <0.05 versus metabolic syndrome.*

As expected, fasting glucose, HDL cholesterol, triglycerides and waist circumference were significantly lower in C than in MS and DM.

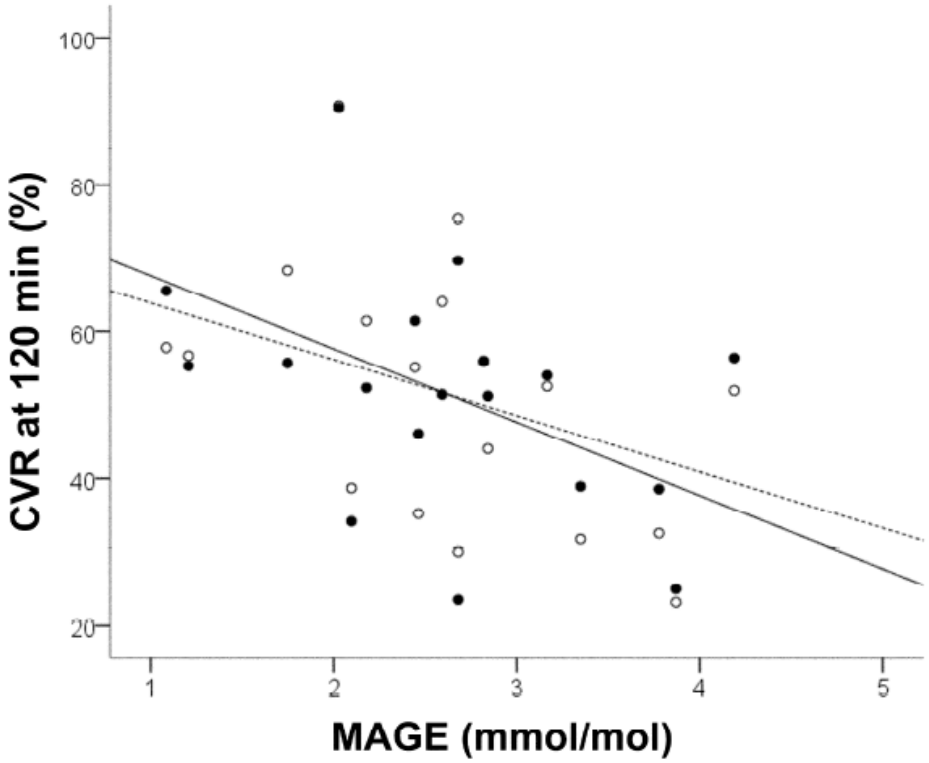
According to ANOVA, ΔSBP and ΔDBP were normal and similar in the three groups (p = 0.26 and p = 0.18, respectively). Baseline VMR was higher in MS and C than in DM (ANOVA F(2,43)=8.840, 199 p= 0.001; Tukey's post-hoc MS-DM = 22.2; 95% CI: 8.6,35.8; p= 0.001 and C-DM = 20.2; 95% CI: 3.5,36.9; p = 0.014), but similar in MS and C (MS-C=2.0; 95% CI: -14.7,18.7; p = 0.643). When VMR behavior after hyperglycemia was evaluated, according to rm-ANOVA, the main effect of Time was significant (F(2,48)=7.374; p= 0.002) indicating that, in the whole sample of MS patients and C, VMR changed after acute hyperglycemia. In particular, a significant decrease was found after 1 hour and 2 hour vs. baseline (p= 0.004 and p= 0.0007, respectively). No difference was found between 1 hour and 2 hour of hyperglycemia (p= 0.677). The interaction between Time and Group was not significant (F(2,48)=0.302, p= 0.741), indicating that VMR changes were quite similar in C and MS. After hyperglycemic clamp, in both MS and C groups, VMR reached values more comparable to DM (three-groups ANOVA, p= 0.139 at 1 hour, p= 0.081 at 2 hours). In MS, all GV indexes were increased vs. C, but lower than in DM (Table 2); MAGE was increased vs. C (p< 0.000), and similar to DM (p = 0.58).

	Controls (n = 9)	Metabolic Syndrome (n = 18)	Diabetes Mellitus (n = 26)	p^2
Mean Amplitude of Glucose Excursions [mg/dl (mmol/l)]	23.4 ± 1.8 (1.3 ± 0.1)	46.8 ± 3.6 (2.6 ± 0.2)†	57.6 ± 5.4 (3.2 ± 0.3)†	0.002
J-index $(mg/dl)^2$ or $(mmol/l)^2$	3337 ± 389 (10.3 ± 1.2)	4536 ± 227 (14 ± 0.7)*	8068 ± 680 (24.9 ± 2.1)‡†	0.026
Mean Absolute Glucose [mg/dl (mmol/l)]	14.4 ± 1.8 (0.8 ± 0.1)	23.4 ± 1.8 (1.3 ± 0.1)*	37.8 ± 3.6 (2.1 ± 0.2)‡†	< 0.000
LabilityIndex $[(mg/dl)^2/h \cdot week^{-1}]$ or $[(mmol/l)^2/h \cdot week^{-1}]$	130 ± 32 (0.4 ± 0.1)	389 ± 65 (1.2 ± 0.2)*	616 ± 97 (1.9 ± 0.3)§†	0.005
SD [mg/dl (mmol/l)]	13.5 ± 4.32 (0.75 ± 0.24)	19.8 ± 1.8 (1.1 ± 0.1)*	24.48 ± 1.26 (1.36 ± 0.07)*§	0.001

Correlation analysis: The effect of blood pressure on baseline VMR was statistically different in the three groups (F(2,38)=3.5; p= 0.040): there was a positive correlation in C (r = 0.59), a correlation close to 0 in MS (r = 0.136) and a negative correlation in DM (r = -0.481). It is noteworthy that, since BP was always lower than 130/85 mmHg in C, the positive correlation observed in this group indicates that, under physiological conditions, lower BP values are associated to lower values of VMR. In the other two groups, which included a relevant percentage of subjects with BP higher than 130/85 mmHg (for SBP 72% of MS and 58% of DM; for DBP 22% of MS and 23% of DM), the negative correlation between BP and VMR indicates a negative effect of pathologically high BP values on VMR. Regarding the three groups as a single sample, in three different chronological phases, we noted a U-shaped relationship, particularly for SBP.



In MS, but not in C, negative correlations were observed between VMR at 120 min and mean 24-h DBP ($r=-0.469$, $p<0.05$) and between VMR at 120 min and MAGE ($r=-0.48$, $p=0.007$). The correlation between VMR at 120 min and MAGE did not change after controlling for mean 24-h DBP (from $r= -0.48$ to $r = -0.42$, Fig. 2). In MS, VMR changes between baseline and 2 hours of hyperglycemia [ΔVMR: (baseline VMR – VMR at 120 min)/baseline VMR] were significantly correlated to MAGE, with larger decreases associated to larger MAGE scores ($r=0.55$, $p= 0.02$). We could not assess such correlation in C, because of the very low variability in terms of MAGE in this group (min=0, max=1.85). No significant correlations were found between VMR and or ΔVMR and age, HbA1c, fasting glucose or lipid profile.



Comments The main finding of this study was the impact of glucose variability on VMR during experimentally induced hyperglycemia in subjects with insulin resistance, but without the confounding effect of chronic hyperglycemia (i.e. DM).

In order to examine whether insulin resistance or hyperglycemia is the key predisposing factor for reduced VMR observed in subjects with MS (Giannopoulos et al 2010), we selected a cohort of patients with MS but without diabetes, cardiovascular disease or carotid stenosis greater than 40%. In these subjects, basal VMR was normal. Since VMR was reduced only in diabetic patients, we investigated the impact of a hyperglycemic peak in subjects with normal VMR, i.e. control subjects and patients with MS, demonstrating that experimentally induced hyperglycemic peak significantly reduces VMR in both groups similarly reaching values comparable to DM.

Since experimental hyperglycemic clamp procedure may resemble a single peak of glucose variability, our data suggest repeated glucose oscillations (i.e., intra-day glucose variability) could have an impact on VMR. All indexes of intra-day GV progressively increased from C to MS and to DM, respectively. Interestingly, MAGE, one the most widely used indexes of GV, did not

statistically differ between MS and DM. However, in our study, patients with DM were particularly selected for having very strict metabolic control and no insulin therapy; thus, they had a low GV. In MS patients, MAGE was significantly related to the VMR reduction after 2 hours of hyperglycemia. This correlation did not present itself in control subjects; probably due to the very low variability of glucose present in normal subjects.

Since blood pressure might have its own effect on VMR, and about 80% of the patients with MS were hypertensive, we analyzed the correlation between these two variables. A correlation between VMR at 120 min and mean 24h diastolic blood pressure was found in patients with MS. To evaluate the relative role of blood pressure and glucose variability, a regression analysis was performed. MAGE entered as first variable in the regression model; mean 24-h DBP did not account for additional variance, thus predominant impact of GV on VMR was supported.

To our knowledge, this was the first report of an impaired response of VMR to acute hyperglycemia in patients without DM and cardiovascular or cerebrovascular disease; the accurate selection of the study population allowed the exclusion of possible confounding effects of severe hypertension, chronic hyperglycemia and atherosclerotic damage on our results. Acute hyperglycemia induced a decrease in VMR also in control subjects. This observation sustains a very direct and early effect of glucose oscillations on endothelium, probably due to oxidative stress activation. Control subjects, differently from patients with MS, do not normally experience glucose oscillations; therefore, in control subjects, reduced VMR is present only in experimental conditions, as in our research study and not present in everyday life. On the contrary, this is likely to happen in subjects with MS, due to increased GV. Whether reduction in VMR in response to glucose oscillations is involved in the pathogenesis of cerebrovascular damage needs further study. In conclusion, experimentally induced acute hyperglycemia reduces cerebrovascular reactivity possibly through the activation of oxidative stress pathways. Our findings have important clinical implications on the possible negative prognostic meaning of glucose variability on cerebral vessels, even before the onset of diabetes.

References

Agelink MW, Malessa R, Baumann B, Majewski T, Akila F, Zeit T, Ziegler D. Standardized tests of heart rate variability: normal ranges obtained from 309 healthy humans, and effects of age, gender, and heart rate. Clin Auton Res 2001;11:99-108.

Alberti KG, Zimmet P, Shaw J. Metabolic syndrome – a new world-wide definition. A consensus statement from the International Diabetes Federation. Diabetic Medicine 2006;23:469–80.

Boden-Albala B, Sacco RL, Lee HS, Grahame-Clarke C, Rundek T, Elkind MV, Wright C, Giardina EG, DiTullio MR, Homma S, Paik MC. Metabolic Syndrome and Ischemic Stroke Risk. Northern Manhattan Study. Stroke 2008; 39:30-5.

Ceriello A, Esposito K, Piconi L, Ihnat MA, Thorpe JE, Testa R, Boemi M, Giugliano D. Oscillating glucose is more deleterious to endothelial function and oxidative stress than mean glucose in normal and type 2 diabetic patients. Diabetes 2008;57:1349-54.

Cipolla MJ, Porter JM, Osol G. High glucose concentrations dilate cerebral arteries and diminish myogenic tone through an endothelial mechanism. Stroke 1997;28:405-10.

Correia ML, Haynes WG. Arterial compliance and endothelial function. Curr Diab Rep 2007;7:269–275.

Corretti MC, Anderson TJ, Benjamin EJ, Celermajer D, Charbonneau F, Creager MA, Deanfield J, Drexler H, Gerhard-Herman M, Herrington D, Vallance P, Vita J, Vogel R, International Brachial Artery Reactivity Task Force. Guidelines for the ultrasound assessment of

endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. Journal of American College of Cardiology 2002;39:257-265.

Cosentino F, Hishikawa K, Katusic ZS, Lüscher TF. High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. Circulation 1997;96:25-8.

Di Flaviani A, Picconi F, Di Stefano P, Giordani I, Malandrucco I, Maggio P, Palazzo P, Sgreccia F, Peraldo C, Farina F, Frajese G, Frontoni S. Impact of glycemic and blood pressure variability on surrogate measures of cardiovascular outcomes in type 2 diabetic patients. Diabetes Care 20114;34:1605-9.

Dyson KS, Shoemaker JK, Hughson RL. Effect of acute sympathetic nervous system activation on flow-mediated dilation of brachial artery. Am J Physiol Heart Circ Physiol 2006;290:1446-53.

Engelke KA, Williams MM, Dietz NM, Joyner MJ. Does sympathetic activation blunt nitric oxide-mediated hyperemia in the human forearm? Clin Auton Res 1997;7:85-91.

Erdös B, Miller AW, Busija DW. Impaired endothelium-mediated relaxation in isolated cerebral arteries from insulin-resistant rats. Am J Physiol Heart Circ Physiol 2002;282:2060-5.

European Carotid Surgery Trialists' Collaborative Group. Randomised trial of endarterectomy for recently symptomatic carotid stenosis: final results of the MRC European Carotid Surgery Trial (ECST). Lancet 1998;351:1379-87.

Ewing DJ, Martyn CN, Young RJ, Clarke BF. The value of cardiovascular autonomic function tests: 10 years experience in diabetes. Diabetes Care 1985;8:491–498.

Fülesdi B, Limburg M, Bereczki D, Káplár M, Molnár C, Kappelmayer J, Neuwirth G, Csiba L. Cerebrovascular reactivity and reserve capacity in type II diabetes mellitus. J Diabetes Complications 1999;13:191-9.

Giannopoulos S, Boden-Albala B, Choi JH, Carrera E, Doyle M, Perez T, Marshall RS. Metabolic syndrome and cerebral vasomotor reactivity. Eur J Neurol 2010;17:1457-62.

Giordani I, Diflaviani A, Picconi F, Malandrucco I, Ylli D, Palazzo P, Altavilla R, Vernieri F, Passarelli F, Donno S, Lauro D, Pasqualetti P, Frontoni F. Acute hyperglycemia reduces cerebrovascular reactivity: the role of glycemic variability. The Journal of Clinical Endocrinology & Metabolism. *In press*.

Girouard H, Iadecola C. Neurovascular coupling in the normal brain and in hypertension, stroke, and Alzheimer disease. J Appl Physiol 2006;100:328–335.

Grubb RL Jr, Derdeyn CP, Fritsch SM, Carpenter DA, Yundt KD, Videen TO, Spitznagel EL, Powers WJ. Importance of hemodynamic factors in the prognosis of symptomatic carotid occlusion. JAMA 1998;280:1055-60.

Grundy SM. Metabolic syndrome: a multiplex cardiovascular risk factor. J Clin Endocrinol Metab 2007;92:399-404.

Hashimoto M, Akishita M, Eto M, Ishikawa M, Kozaki K, Toba K, Sagara Y, Taketani Y, Orimo H, Ouchi Y. Modulation of endothelium-dependent flow mediated dilatation of the brachial artery by sex and menstrual cycle. Circulation 1995;92:3431-3435.

Iadecola C, Pelligrino DA, Moskowitz MA, Lassen NA. Nitric oxide synthase inhibition and cerebrovascular regulation. J Cereb Blood Flow Metab 1994;14:175–92.

Ito I, Jarajapu YP, Guberski DL, Grant MB, Knot HJ. Myogenic tone and reactivity of rat ophthalmic artery in acute exposure to high glucose and in a type II diabetic model. Invest Ophthalmol Vis Sci 2006;47:683-92.

Juonala M, Viikari JS, Laitinen T, Marniemi J, Helenius H, Rönnemaa T, Raitakari OT. Interrelations between brachial endothelial function and carotid intima-media thickness in young adults: the cardiovascular risk in young Finns study. Circulation 2004;110:2918-23.

Kotb NA, Gaber R, Salah W, Elhendy A. Relations among Glycemic Control, Circulating Endothelial Cells, Nitric Oxide, and Flow Mediated Dilation in Patients with Type 2 Diabetes Mellitus. Exp Clin Endocrinol Diabetes ;120:460-5.

Kozera GM, Wolnik B, Kunicka KB, Szczyrba S, Wojczal J, Schminke U, Nyka WM, Bieniaszewski L. Cerebrovascular reactivity, intima-media thickness, and nephropathy presence in patients with type 1 diabetes. Diabetes Care 2009;32:878-82.

LeMarbre G, Stauber S, Khayat RN, Puleo DS, Skatrud JB, Morgan BJ. Baroreflex-induced sympathetic activation does not alter cerebrovascular CO2 responsiveness in humans. J Physiol 2003;551:609-16.

Malandrucco I, Pasqualetti P, Giordani I, Manfellotto D, De Marco F, Alegiani F, Sidoti AM, Picconi F, Di Flaviani A, Frajese G, Bonadonna RC, Frontoni S. Very-low-calorie diet: a quick therapeutic tool to improve β cell function in morbidly obese patients with type 2 diabetes. Am J Clin Nutr 2012;95:609-13.

Marfella R, Nappo F, De Angelis L, Paolisso G, Tagliamonte MR, Giugliano D. Hemodynamic effects of acute hyperglycemia in type 2 diabetic patients. Diabetes Care 2000;23:658-63.

Markus H, Cullinane M. Severely impaired cerebrovascular reactivity predicts stroke and TIA risk in patients with carotid artery stenosis and occlusion. Brain 2001;124:457-67.

Markus HS, Harrison MJ. Estimation of cerebrovascular reactivity using transcranial Doppler, including the use of breath-holding as the vasodilatatory stimulus. Stroke 1992;23:668–673.

Mayhan WG, Patel KP. Acute effects of glucose on reactivity of cerebral microcirculation: role of activation of protein kinase C. Am. J. Physio Z. 1995;269:1297-1302.

Micieli G, Bosone D, Zappoli F, Marcheselli S, Argenteri A, Nappi G. Vasomotor response to CO_2 and L-Arginine in patients with severe internal carotid artery stenosis; pre- and post-surgical evaluation with transcranial Doppler. J Neurol Sci 1999;163:153–158.

Moens AL, Goovaerts I, Claeys MJ, Vrints CJ. Flow-mediated vasodilation: a diagnostic instrument, or an experimental tool? Chest 2005;127:2254-63.

Najarian R, Sullivan L, Kannel W, Wilson P, D'Agostino R, Wolf P. Metabolic syndrome compared to type 2 diabetes mellitus as a risk factor for stroke. Arch Intern Med 2006;166:2106–2111.

O'Brien E, Mee F, Atkins N, O'Malley K. Accuracy of the Spacelabs 90207 determined by the British Hypertension Society protocol. J Hypertens 1991;9:573–574.

van Oers CA, Manschot SM, van Huffelen AC, Kappelle LJ, Biessels GJ. Utrecht Diabetic Encephalopathy Study Group. Cerebrovascular reserve capacity is preserved in a population-based sample of patients with type 2 diabetes mellitus. Cerebrovasc Dis 2006;22:46-50.

Palazzo P, Balucani C, Barlinn K, Tsivgoulis G, Zhang Y, Zhao L, Dewolfe J, Toaldo B, Stamboulis E, Vernieri F, Rossini PM, Alexandrov AV. Association of reversed Robin Hood syndrome with risk of stroke recurrence. Neurology 2000;75:2003-8.

Palazzo P, Maggio P, Altavilla R, Di Flaviani A, Giordani I, Malandrucco I, Picconi F, Passarelli F, Pasqualetti P, Ercolani M, Vernieri F, Frontoni S. Cerebral hemodynamics and systemic endothelial function are already impaired in well-controlled type 2 diabetic patients, with short-term disease. PLoS One 2013;8:e83287.

Palazzo P, Maggio P, Passarelli F, Altavilla R, Altamura C, Pasqualetti P, Vernieri F. Lack of correlation between cerebral vasomotor reactivity and flow-mediated dilation in subjects without vascular disease. Ultrasound Med Biol 2013;39:10-5.

Pemp B, Weigert G, Karl K, Petzl U, Wolzt M, Schmetterer L, Garhofer G. Correlation of flicker-induced and flow-mediated vasodilatation in patients with endothelial dysfunction and healthy volunteers. Diabetes Care 2009;32:1536–1541.

Phillips SA, Sylvester FA, Frisbee JC. Oxidant stress and constrictor reactivity impair cerebral artery dilation in obese Zucker rats. Am J Physiol Regul Integr Comp Physiol 2005;288:522-30.

Picconi F, Di Flaviani A, Malandrucco I, Giordani I, Frontoni S. Impact of glycemic variability on cardiovascular outcomes beyond glycated hemoglobin. Evidence and clinical perspectives. Nutr Metab Cardiovasc Dis 2012;22:691-696.

Pretnar-Oblak J, Šabovič M, Zaletel M. Associations between systemic and cerebral endothelial impairment determined by cerebrovascular reactivity to L-Arginine. Endothelium 2007;14:1–8.

Pyke KE, Tschakovsky ME. The relationship between shear stress and flow mediated dilatation: implications for the assessment of endothelial function. Journal of Physiology 2005;568:357-369.

Rodbard D. New and improved methods to characterize glycemic variability using continuous glucose monitoring. Diabetes Technol Ther 2009;11:551-65.

Rodriguez-Colon SM, Mo J, Duan Y, Liu J, Caulfield JE, Jin X, Liao D. Metabolic syndrome clusters and the risk of incident stroke: the atherosclerosis risk in communities (ARIC) study. Stroke 2009;40:200-5.

Roquer J, Segura T, Serena J, Castillo J. Endothelial dysfunction, vascular disease and stroke: The ARTICO Study. Cerebrovasc Dis 2009;27:25–37.

Santos-García D, Blanco M, Serena J, Rodríguez-Yáñez M, Leira R, Castillo J. Impaired brachial flow-mediated dilation is a predictor of a new-onset vascular event after stroke. Cerebrovasc Dis 2011;32:155-62.

Sarwar N, Gao P, Seshasai SR, Gobin R, Kaptoge S, Di Angelantonio E, Ingelsson E, Lawlor DA, Selvin E, Stampfer M, Stehouwer CD, Lewington S, Pennells L, Thompson A, Sattar N, White IR, Ray KK, Danesh J. Emerging Risk Factors Collaboration. Diabetes mellitus, fasting blood glucose concentration, and risk of vascular disease: a collaborative meta-analysis of 102 prospective studies. Lancet 2010; 375:2215–22.

Shechter MK, Issachara A, Maraia I, Koren-Moraga N, Freinark D, Shahar Y, Shechter A, Feinberg MS. Long-term association of brachial artery flow-mediated vasodilation and cardiovascular events in middle-aged subjects with no apparent heart disease. International Journal of Cardiology 2009;134:52–58.

Silvestrini M, Pasqualetti P, Baruffaldi R, Bartolini M, Handouk Y, Matteis M, Moffa F, Provinciali L, Vernieri F. Cerebrovascular reactivity and cognitive decline in patients with Alzheimer disease. Stroke 2006;37:1010-5.

Silvestrini M, Vernieri F, Pasqualetti P, Matteis M, Passarelli F, Troisi E, Caltagirone C. Impaired cerebral vasoreactivity and risk of stroke in patients with asymptomatic carotid artery stenosis. JAMA 2000;283:2122-7.

Spallone V, Ziegler D, Freeman R, Bernardi L, Frontoni S, Pop-Busui R, Stevens M, Kempler P, Hilsted J, Tesfaye S, Low P, Valensi P; on behalf of the Toronto Consensus Panel on Diabetic Neuropathy. Cardiovascular autonomic neuropathy in diabetes: clinical impact, assessment, diagnosis and treatment. Diabetes Metab Res Rev 2011;27:639-653.

Stevenson SF, Doubal FN, Shuler K, Wardlaw JM. A systematic review of dynamic cerebral and peripheral endothelial function in lacunar stroke versus controls. Stroke 2010;41:434-42.

Tesfaye S, Boulton AJ, Dyck PJ, Freeman R, Horowitz M, Kempler P, Lauria G, Malik RA, Spallone V, Vinik A, Bernardi L, Valensi P. Toronto Diabetic Neuropathy Expert Group. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care. 2010;33:2285-93.

Touboul PJ, Hennerici MG, Meairs S, Adams H, Amarenco P, Bornstein N, Csiba L, Desvarieux M, Ebrahim S, Fatar M, Hernandez Hernandez R, Jaff M, Kownator S, Prati P, Rundek T, Sitzer M, Schminke U, Tardif JC, Taylor A, Vicaut E, Woo KS, Zannad F, Zureik M. Mannheim carotid intima-media thickness consensus (2004-2006). An update on behalf of the Advisory Board of the 3rd and 4th Watching the Risk Symposium, 13th and 15th European Stroke Conferences, Mannheim, Germany, 2004, and Brussels, Belgium, 2006. Cerebrovasc Dis 2007;23:75-80.

Verdelho A, Madureira S, Moleiro C, Ferro JM, Santos CO, Erkinjuntti T, Pantoni L, Fazekas F, Visser M, Waldemar G, Wallin A, Hennerici M, Inzitari D; LADIS Study. White matter changes and diabetes predict cognitive decline in the elderly: the LADIS study. Neurology 2010;75:160-7.

Vernieri F, Assenza G, Maggio P, Tibuzzi F, Zappasodi F, Altamura C, Corbetto M, Trotta L, Palazzo P, Ercolani M, Tecchio F, Rossini PM. Cortical neuromodulation modifies cerebral vasomotor reactivity. Stroke 2010;41:2087-90.

Vernieri F, Pasqualetti P, Matteis M, Passarelli F, Troisi E, Rossini PM, Caltagirone C, Silvestrini M. Effect of collateral blood flow and cerebral vasomotor reactivity on the outcome of carotid artery occlusion. Stroke 2001;32:1552-8.

Vernieri F, Tibuzzi F, Pasqualetti P, Rosato N, Passarelli F, Rossini PM, Silvestrini M. Transcranial Doppler and near-infrared spectroscopy can evaluate the hemodynamic effect of carotid artery occlusion. Stroke 2004;35:64-70.

Vuletić V, Cengić L, Basić S, Sporis D, Rahelić D, Demarin V. Impaired cerebral vasoreactivity in type 2 diabetes mellitus. Coll Antropol 2011;35:27-31.

Wang Q, Pelligrino DA, Baughman VL, Koenig HM, Albrecht RF. The role of neuronal nitric oxide synthase in regulation of cerebral blood flow in normocapnia and hypercapnia in rats. J Cereb Blood Flow Metab 1995;15:774–778.

Webb DJ. The pharmacology of human blood vessels in vivo. J Vasc Res 1995;32:2–15.

White RP, Deane C, Vallance P, MarkusHS. Nitric oxide synthase inhibition in humans reduces cerebral blood flow but not the hyperemic response to hypercapnia. Stroke 1998;29:467–472.

Wild SH, Byrne CD, Tzoulaki I, Lee AJ, Rumley A, Lowe GD, Fowkes FG. Metabolic syndrome, haemostatic and inflammatory markers, cerebrovascular and peripheral arterial disease: the Edinburgh Artery Study. Atherosclerosis 2009; 203:604-9.

Wolff SP, Dean RT. Glucose autoxidation and protein modification. The potential role of 'autoxidative glycosylation' in diabetes. Biochem J 1987;245:243-50.

Yan RT, Anderson TJ, Charbonneau F, Title L, Verma S, Lonn E. Relationship between carotid artery intima-media thickness and brachial artery flow-mediated dilation in middle-aged healthy men. J Am Coll Cardiol 2005;45:1980-6.

Yeboah J, Folsom AR, Burke GL, Johnson C, Polak JF, Post W, Lima JA, Crouse JR, Herrington DM. Predictive value of brachial flow-mediated dilation for incident cardiovascular events in a population-based study: the multi-ethnic study of atherosclerosis. Circulation 2009;120:502-9.

Yokoyama H, Sone H, Saito K, Yamada D, Honjo J, Haneda M. Flow-mediated dilation is associated with microalbuminuria independent of cardiovascular risk factors in type 2 diabetes - interrelations with arterial thickness and stiffness. J Atheroscler Thromb 2011;18:744-52.