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Relationship between cerebral arterial pulsatility and carotid intima media thickness in diabetic and non-diabetic patients with non-alcoholic fatty liver disease

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ABSTRACT. Non-alcoholic fatty liver disease (NAFLD) is considered a risk factor for atherosclerosis. The aim of the present study was to investigate the association of the pulsatility index (PI) of basilar artery (BA) and carotid intima media thickness (IMT) in diabetic and non-diabetic NAFLD patients. We compared a group of 80 stroke-free, diabetic and non-diabetic NAFLD patients and a control group of 26 healthy subjects without NAFLD. We then evaluated the PI of the BA by transcranial Doppler ultrasonography, and carotid IMT. The PI was significantly higher in diabetic NAFLD patients than in controls ($p<0.003$). Carotid IMT and asymmetrical dimethylarginine (ADMA) levels were higher in NAFLD patients than controls respectively ($p<0.003$, $p<0.04$). The PI of the BA was significantly correlated with age (R=0.369, $p<0.001$), male gender (R=0.207, $p=0.035$), diabetes (R=0.332, $p=0.001$), carotid IMT (R=0.296, $p=0.002$) and ADMA (R=0.349, $p=0.015$). A multiple regression analysis was performed with PI as the dependent variable with known clinical risk factors. Age ($\beta=3.54$, $p<0.001$), diabetes ($\beta=2.32$, $p=0.022$), gender ($\beta=2.20$, $p<0.03$), ADMA ($\beta=2.25$, $p<0.031$), and carotid IMT ($\beta=2.41$, $p<0.017$), were independent predictive factors of BA PI. Adjustment for age and gender did not alter these relative risks, exhibiting a significant independent contribution to PI. The increased PI observed in this study represents enhanced cerebrovascular resistance, and we observed that the age, male gender, diabetes, ADMA levels, and carotid IMT were independent predictive factors of BA PI.
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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) might represent the hepatic consequence of insulin resistance or metabolic syndrome. NAFLD is considered a risk factor for atherosclerosis and associated with increased carotid artery intima-media thickness (IMT) which is a marker of early generalized atherosclerosis (1, 2).

Asymmetrical dimethylarginine (ADMA), being a physiologically occurring competitive antagonist of endothelial nitric oxide (NO) synthase, is an endogenous inhibitor of the arginine-NO pathway. Low NO levels are one of the pathogenic factors starting atherosclerosis and cardiovascular diseases (CVD) (3). It is also indicated that ADMA is a strong marker of endothelial dysfunction (4). Compared with healthy people, patients with DM show more extensive atherosclerosis of extracranial and intracranial vessels, a higher prevalence of carotid artery stenosis, and increased carotid artery intima-media wall thickness (5, 6). Arterial wall IMT has been established by many studies to be an early marker and predictor of atherosclerotic disease (7). Ischemic stroke is a major complication of DM, but the optimal screening method for cerebrovascular complication has yet to be established. Previously, clinical attempts to detect subclinical cerebrovascular blood flow changes related to DM had been performed by use of single-photon emission computed tomography (8), ^{133}Xe-computed tomography (9), and positron emission tomography (10), but these methods failed to provide consistent results. In addition, the cost of magnetic resonance angiography (MRA) is high; therefore, MRA is not suitable as a screening test. In contrast, transcranial Doppler ultrasonography (TCD), as it is non-invasive and easily applicable, appears to be more suitable as a screening tool than previous methods. Nevertheless, studies involving the application of TCD to diabetic patients have rarely been reported.

Recently, some investigators did not evaluate (11, 12) hemodynamic changes in the extracranial internal carotid artery and basilar artery (BA), but diabetes-related atherosclerotic changes were more frequently noted in these vessels than the middle cerebral artery (13). Thus, we performed TCD measurements of the BA in stroke-free, normotensive diabetic and non-diabetic NAFLD patients. There is strong evidence that NAFLD is an independent risk factor for development of macrovascular complications. Meanwhile, there is no previously published data investigating the effect of NAFLD on cerebral blood flow. The present study was designed 1) to determine whether NAFLD in diabetic and non-diabetic patients affects the PI of cerebral arteries 2) to explore the relationship between carotid IMT and PI of cerebral arteries in NAFLD patients.

Key-words: Asymmetrical dimethylarginine, carotid intima media thickness, non-alcoholic fatty liver disease, Type 2 diabetes mellitus, transcranial Doppler.

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MATERIALS AND METHODS

This cross-sectional study was carried out at Fatih University School of Medicine, Departments of Endocrinology and Neurology, in Ankara, Turkey. Eighty patients affected by NAFLD and 26 control subjects without NAFLD were enrolled. There were 40 non-specified etiology (non-diabetic) NAFLD patients (30 female and 10 male), and 40 NAFLD patients (28 female, 12 male) with diabetes mellitus (DM). All patients were fully informed about the objectives of the study and agreed to participate. The diagnosis of diabetes was made by the patient history or based on the criteria of the American Diabetes Association. The control group was made up of volunteers, randomly selected from the subjects visiting Fatih University Hospital for a health screening check-up program having no history of systemic diseases or NAFLD. They also had no risk factors for cerebrovascular disease, including smoking, hypertension, dyslipidemia, obesity, and diabetes.

Patients with previous or current malignant disease, any known pancreas disease, adrenal or pituitary disease, acute or chronic liver diseases, cerebrovascular event and patients with gastrointestinal surgery, patients who had history of alcohol use were excluded. All patients were negative for hepatitis B, and C viruses. All diabetic patients underwent history screening, physical examination, and laboratory analysis, including a complete blood count, the levels of plasma electrolyte, glucose, insulin, glycosylated hemoglobin (HbA_{1c}), blood urea nitrogen, creatinine, transaminases and urinary protein levels, and lipid profile. Moreover, the patients were assessed for the presence of diabetic complications, i.e. retinopathy, neuropathy, nephropathy, a history of myocardial infarction, and the presence of *angina pectoris* and *atherosclerosis obliterans*. Complicated and insulin-treated diabetic patients were excluded from the study. All diabetic patients were taking one or two oral antidiabetics. Durations of diabetes were less than 5 yr (range 1-7 yr) and those patients were in fairly compensated conditions under diet and oral hypoglycemic agents (mean HbA_{1c}, 7.5±0.7%; range 6.0-8.1).

All subjects underwent an upper abdominal ultrasonography. The NAFLD group consisted of 80 patients with an ultrasonic view of steatosis. Although these patients were not biopsied for ethical reasons, clinical diagnosis of NAFLD was confirmed by careful exclusion of other disorders that may be associated with NAFLD. Hepatic ultrasonography scanning was performed in all participants by a single experienced radiologist, who was blinded to subjects' details. The diagnosis of hepatic steatosis was made on the basis of characteristic sonographic features (14, 15). It is known that ultrasonography has a sensitivity of 90% and a specificity of 95% in detection of moderate and severe hepatic steatosis. Although ultrasonography is not totally sensitive, particularly when hepatic fat infiltration on liver biopsy is <30%, semiquantitative sonographic scoring for the degree of hepatic steatosis was not available (16).

Transcranial doppler studies were performed with (Nicolet, EME) a 2-MHz hand-held probe below the occiput at a depth of 80 to 90 mm from the BA (17, 18). For BA, the mean, systolic, and diastolic velocities were measured and the Gosling pulsatility index (PI) was calculated automatically as (systolic velocity-diastolic velocity)/mean velocity (19, 20). At least 3 measurements were performed at a similar depth for BA; the median value was selected and used in this study. In many patients, especially in woman, we could not measure the middle cerebral artery (MCA) due to the poor temporal window and since this may affect the statistical data we excluded measurements of the MCA from the study. The systolic and diastolic blood pressures, hematocrit, and the height and weight of the subjects were checked on the same day that the TCD was performed.

Carotid ultrasound examination was performed by Siemens Sonoline Antares (Germany) equipped with a VF13-5MHz multifrequency transducer. An experienced investigator (A.K.) who was unaware of the individuals' disease status examined the right/left common carotid artery on a patient lying supine, the head directed away from the side of interest. A region 1-cm proximal to the carotid bifurcation was identified on longutidunal image, and the IMT of the far wall was evaluated as the distance between the lumen-intima interface and the media-adventitia interface. The IMT was measured on frozen frame from three contiguous sites at 1-mm intervals at a site free from any discrete plaque, and their average was used.

Serum samples for the determination of ADMA levels were taken after an overnight fast without tourniqueting the arms of patients. Serum samples were centrifuged at 4000 rpm for 10 min. Samples were stored at –80 C until analysis. ADMA measurement was carried out by a commercial ELISA kit (DLD Diagnostica GMBH, Germany). Results were expressed as μmol/l. Intra- and interassay coefficients of variation were 4.5-7.5% and 10.3-8.3%, respectively. Twenty of the serum samples collected from diabetic and non-diabetic patients were damaged during the conduction of ADMA measurements. From the samples taken simultaneously with ADMA samples, blood glucose, total cholesterol, HDL-cholesterol (HDL-C), and triglycerides were measured by standard laboratory methods on biochemistry autoanalyzer (HITACHI 912; Roche) with company's original kits. LDL cholesterol (LDL-C) was calculated with the Friedewald equation. Plasma glucose was measured with the glucose oxidase method on an automated autoanalyzer (Yellow Springs Equipment). Plasma insulin levels were measured using the dextran-charcoal radioimmunoassay method. Blood pressure was measured using a mercury sphygmomanometer. We used the mean of 2 measurements of systolic and diastolic blood pressure taken while subjects were sitting after a 5-min rest. Body mass index (BMI) was calculated as weight in (kg) divided by the square of height (m) (BMI=kg/m^2). Waist circumference, taken midway between the lowest rib and the iliac crest, and hip circumference at the level of the greater trochanters was measured to the nearest millimetre using a flexible tape. Insulin resistance was determined using homeostasis model assessment (HOMA-IR) by the following formula (21):

HOMA-IR=fasting insulin (mU/l) × fasting glucose (mmol/l)/22.5.

The study was approved by the Fatih University School of Medicine Ethics Committee and was conducted in accordance with the ethical principles described by the Declaration of Helsinki. A written informed consent was obtained from all participants.

Statistical analysis

All statistical analyses were performed using the SPSS for Windows, version 13.0 (Chicago, IL, USA). Unless otherwise stated, results were expressed as means±SD. The Mann-Whitney U test or independent-sample t test were used between 2 subject groups and analysis of variance was used to assess the significance of differences among 3 subject groups as appropriate. The same tests were used to compare the means of the examined variables in patients with different characteristics. To evaluate correlations among the BA PI and other variables in groups, Pearson correlation test or Spearman correlation test were used as appropriate. Multiple regression analysis was used to exclude the possible confounding effect of other variables on the result of each correlation analysis. $p<0.05$ was considered statistically significant.

RESULTS

The clinical and biochemical characteristics of the study groups are shown in Table 1. Twenty out of forty diabetic patients were using statins and antihypertensive drugs in addition to metformin and sulphonylurea treatments, in various dosages. Among the subject groups, there was no significant difference in gender distribution, age, systolic and diastolic blood pressures, serum cholesterol, LDL-C, HDL-C, hematocrit, C-reactive protein, and BA systolic and mean velocity. BMI, waist circumference, triglyceride, ADMA, BA resistance index (RI) and PI were higher than controls. Insulin, fasting glucose, HOMA-IR, and carotid IMT were higher in the NAFLD group than the control (Table 1). The PI of the BA was lowest in the control group and highest in the diabetic NAFLD group (Fig. 1). The diabetic and non-diabetic NAFLD group had carotid IMT values higher than the control group (Fig. 2). To assess the correlation with BA PI, a Pearson correlation analysis was performed on each variable. The variables significantly correlating with the BA PI were age, diabetes, gender, BA RI, ADMA and carotid IMT (Table 2). BA PI had a positive correlation between carotid IMT ($r=0.296$; $p=0.002$). The multiple regression analysis of BA PI and other risk factors was performed. Age, diabetes, male gender, ADMA, and carotid IMT were independent predictive factors of BA PI. Adjustment for age and gender did not alter these relative risks (Table 3).

Table 1 - *Clinical and biochemical characteristics of subjects.*

	Diabetic NAFLD	Non-diabetic NAFLD	Controls
No. of subjects	40	40	26
Age (yr)	57±8	53±8	53±7
Gender (male/female)	10/30	12/28	11/15
Hct (%)	40±5	40±5	41±4
Waist circumference (cm)	101±11*	99± 8	91±9
BMI (kg/m^2)	29±4*	28±4	26±3
FBG (mg/dl)	140±30*†	89±12	95±5
Insulin	9.3±6.0*†	6.5±2.4*	2.9±1.4
HOMA-IR	3.4±2.6*†	1.7±1.2*	1.5±0.9
Total cholesterol (mg/dl)	197±47	199±35	198±38
TG (mg/dl)	195±169*	141±75	113±57
LDL-C (mg/dl)	114±47	115±35	115±28
HDL-C (mg/dl)	50±17	56±16	61±19
SBP (mmHg)	124±7	120±9	124±6
DBP (mmHg)	79±4	79±6	79±5
ADMA (µmol/l)§	0.60±0.13*	0.55±0.11	0.49±0.09
CRP	5±4	6±5	4±3
BA S	55±16	56±15	60±14
BA M	36±11	37±11	41±8
BA RI	0.60±0.0*	0.56±0.1	0.53±0.1
BA PI (cm/sec)	0.89±0.2*	0.79±0.1	0.74±0.1
Carotid IMT (mm)	0.76±0.1*†	0.68±0.1	0.65±0.1

§: asymmetrical dimethylarginine; diabetic non-alcoholic fatty liver disease (NAFLD): no.=30; non-diabetic NAFLD: no.=30. Values are mean±SD; Hct: hematocrite; BMI: body mass index; FBG: fasting blood glucose; TG: triglyceride; HDL-C: HDL cholesterol; LDL-C: LDL cholesterol; SBP: systolic blood pressure; DBP: diastolic blood pressure; BA: basilar artery; PI: pulsatility index; RI: resistance index; S: systolic velocity; M: mean velocity; CRP: Creactive protein; IMT: intima media thickness; ADMA: asymmetrical dimethylarginine; HOMA-IR: homeostasis model assessment of insulin resistance. *$p<0.05$ *vs* control. †$p<0.05$ vs non-diabetic.

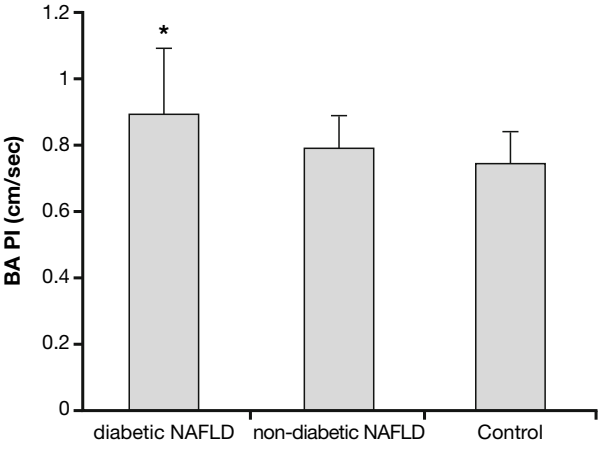

Fig. 1 - *Relationship between the pulsatility index (PI) of basilar artery (BA) and non-alcoholic fatty liver disease (NAFLD). The PI of BA was significantly increased in the diabetic NAFLD group while it was lowest in the control group and at an intermediate level in the non-diabetic NAFLD group. *p<0.05 compared with controls. Error bars represent 95% confidence interval.*

DISCUSSION

In the present study, we have shown for the first time that diabetic NAFLD is associated with an increased PI of the BA, whereas PI of the BA in non-diabetic NAFLD patients was comparable to that in control subjects. Based on these reports, it can be inferred that age, male gender, DM, ADMA levels, and carotid IMT are major factors affecting the PI.

Presence of NAFLD with diabetic and non-diabetic patients may also be linked to increased CVD risk independently of components of the metabolic syndrome. In

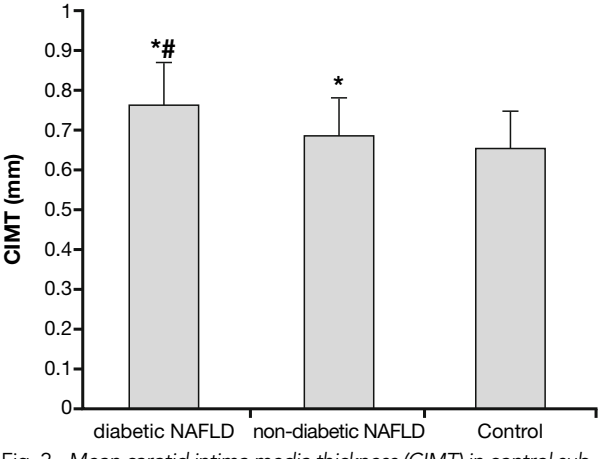

Fig. 2 - *Mean carotid intima media thickness (CIMT) in control subjects and non-alcoholic fatty liver disease (NAFLD) patients with and without diabetic patients. Error bars represent 95% confidence interval. *p<0.05 vs control. #p<0.05 vs non-diabetic NAFLD.*

some cross-sectional studies it has been shown that NAFLD is associated with increased carotid IMT, a marker of early generalized atherosclerosis (1, 2).

The Gosling PI, originally designed to measure vascular resistance, has been established in the brachial artery of normal humans (19, 20). Since PI that has been calculated from TCD values reflects the vascular resistance in the peripheral area of the cerebral vessels, it can be helpful in predicting cerebral vaso-occlusive diseases (22-24). Hemodynamic rates were measured using TCD, and the PI and RI were obtained through subsequent calculations. In a few studies, increased PI of cerebral arteries has been shown in diabetic patients. In agreement with previous trials the PI of the BA was increased in diabetic NAFLD patients in our study (22, 25, 26). Nevertheless, Lippera et al. reported that the PI is only increased in complicated diabetic patients in contrast to our study. They demonstrated a significantly increased PI of the MCA in diabetic patients with retinopathy compared with those without retinopathy (27). Our study was performed in normotensive patients, in order to avoid the possible interference of hypertension with the interpretation of data relating to diabetic cerebral microangiopathy. It has been suggested that duration of diabetes is an important factor in determining cerebrovascular reserve capacity, as demonstrated in diabetic patients with over 10-yr disease duration (28-30). In our study, although diabetic NAFLD patients were not complicated and had a relatively short duration of diabetes (mean 5 yr), PI of the BA was significantly higher than controls. On the other hand, non-diabetic NAFLD patients also had insulin resistance, elevated ADMA, and increased carotid IMT; conversely PI of the BA was not more significant than controls. Our results showed that diabetes was an independent risk factor for increased cerebral PI in NAFLD. DM is considered a very strong risk factor for acute stroke; it is assumed that the risk of stroke is increased by 1.5-3-fold for patients with diabetes. Furthermore, DM doubles the risk of stroke recurrence, and stroke outcomes and prognosis are very poor in the long term in these patients (31). In the Framingham Study the incidence of cerebrovascular disease in diabetic men was reported to be twice that of non-diabetic persons and the incidence in diabetic women almost three times greater. Similarly, we found that diabetes had 2.3 times higher risk for increased cerebral PI in NAFLD. Furthermore, men with NAFLD had 2.2 times greater risk of increased cerebral PI than females with NAFLD. Kidwell et al. reported that age, male gender, and hypertension were closely related to the increased PI of MCA in patients with lesions in the cerebral white matter (including lacunar infarction), and that the PI was the independent prognostic factor for the lesions in the white matter of the brain (23). Age was the independent risk factor for increased PI of the BA in this study. Other studies have also reported that age was correlated with the PI (25, 34, 35). Aging has been reported to reduce the flow velocity and increase the pulsatility of cerebral vessels (34). Our patients with diabetic NAFLD were slightly older than non-diabetic NAFLD, as well as controls. However, this difference in mean age does not appear to affect the present results. In our study NAFLD patients with diabetes had lower total-cholesterol and LDL-C levels than both non-diabetic NAFLD and control group. Almost half of the diabetic NAFLD patients were using statins. CRP levels were similar in NAFLD patients and the control group. This might be the result of statin therapy in diabetic NAFLD patients that has been shown to lower CRP levels (35). It is not known from our data whether statins or antihypertensives had an effect on cerebral PI in diabetic NAFLD patients.

In this study, carotid IMT was noticeably higher in NAFLD patients than the control group. Furthermore, by logistic regression analysis with adjustment for various confounders, the presence of NAFLD was related with an increased carotid IMT. Increased cerebral PI, as a clue of cerebral microangiopathy and carotid IMT, suggests

Table 2 - *Correlation coefficients determined by simple correlation between the mean basilar artery (BA) pulsatility index (PI) and other clinical factors possibly affecting the PI in non-alcoholic fatty liver disease subjects.*

Variables	Correlation coefficient	*p*
Age	0.369	0.001
Gender	0.207	0.035
DM	0.332	0.001
Waist circumference	–0.137	0.370
BMI	–0.108	0.283
Hct	0.163	0.130
Insulin	–0.23	0.820
HOMA IR	–0.063	0.532
FBG	0.140	0.062
Total cholesterol	0.055	0.498
TG	–0.009	0.929
LDL-C	0.222	0.060
HDL-C	–0.091	0.441
CRP	0.018	0.860
SBP	0.022	0.821
BA RI	0.499	0.001
DBP	0.007	0.947
BA M	–0.020	0.841
ADMA	0.349	0.015
Carotid IMT	0.296	0.002

BMI: body mass index; Hct: hematocrite; DM; diabetes mellitus; FBG: fasting blood glucose; TG: triglyceride; HDL-C: HDL cholesterol; LDL-C: LDL cholesterol; SBP: systolic blood pressure; DBP: diastolic blood pressure; BA: basilar artery; PI: pulsatility index; RI: resistance index; S: systolic velocity, M: mean velocity; CRP: C reactive protein; IMT: intima media thickness; ADMA: asymmetrical dimethylarginine; HOMA-IR: homeostasis model assessment of insulin resistance.

Table 3 - *Multiple regression analysis of clinical factors possibly affecting the mean basilar artery (BA) pulsatility index (PI) in non-alcoholic fatty liver disease subjects adjusted for age and gender.*

Variables	β	*p*
Age	3.543	0.001
DM	2.328	0.022
Gender	2.205	0.030
ADMA	2.256	0.031
Carotid IMT	2.418	0.017

R^2=0.410; BA: basilar artery; PI: pulsatility index; DM: diabetes mellitus; ADMA: asymmetrical dimethylarginine; carotid IMT: intima media thickness.

atherosclerosis occurs both in extra- and intracranial vessels in NAFLD. A correlation was found between PI of the BA and carotid IMT. Similarly, Fukuhara et al. reported that PI significantly correlated with IMT and also seems to be an indicator of cerebral atherosclerosis (37). Carotid IMT is known to be associated with the risk of coronary artery disease and stroke (8). In ischemic stroke, increased carotid IMT is more related to non-lacunar infarction than to lacunar infarction (38). Therefore, carotid IMT may be a useful marker for diabetic cerebral macroangiopathy. In contrast to macroangiopathy, diabetic cerebral microangiopathy, which may contribute to the development of lacunar infarction involving the small perforating artery, has no specific diagnostic tool.

Several studies have suggested a link between NAFLD and endothelial dysfunction, carotid IMT and carotid plaques (39, 40). An underlying mechanism linking NAFLD and CVD might be represented by increased hepatic oxidative stress and chronic inflammation. Decreased concentrations of adiponectin, a protein with anti-inflammatory and anti-atherogenic properties, might be another common, underlying, mechanism linking NAFLD and CVD. We found that ADMA levels were significantly higher in NAFLD patients than controls. Our results also showed a positive correlation between ADMA levels and PI of the BA. Elevated ADMA levels conferred an increased risk of non-fatal stroke and myocardial infarction in patients with diabetic and non-diabetic NAFLD, who are known to be at high risk of macrovascular morbidity and mortality. Nonetheless, we considered human evidence of plasma ADMA as a predictor of cardiovascular events and experimental animal data of the causal relationship between ADMA and atherosclerosis (41-43).

The adrenergic innervation of cerebral vessels is responsible for the regulation of vascular tone due to various stimulations. In diabetic patients, most probably due to β-adrenergic or sympathetic neuronal dysfunction, cerebral vasodilatory responses are impaired (44, 45). It has been shown that, in diabetic rats, the number of β-adrenergic receptors is reduced in cerebral microvessels that can cause the impaired β-adrenergic receptor-mediated vasodilatory response. One of the most important results of this reduction is the enhanced pulsatility. Vessels in the posterior cerebral circulation have fewer adrenergic neurons than the anterior cerebral circulation, which may result in limited vasodilatory response and increase the propensity of cerebral vessels to DM-related neuronal dysregulation. Because of this mechanism, earlier pulsatility changes occur in the BA, rather than the MCA and internal carotid artery. This may suggest that DM causes spread vascular changes in the whole intracranial arterial system. In many studies, a strong correlation has been shown between MCA and BA (46, 47). Moreover, PI of the BA can be used as a marker of cerebral microangiopathy when obtaining MCA flow is not possible by TCD examination. Especially in Asians and Africans in a considerable portion of subjects, TCD assessment is reported to be unsuccessful due to the poor temporal window, consistent with our findings (48-50). In many patients, especially in woman, we could not measure the MCA due to the poor temporal window. Since these may affect the statistical analysis we excluded measurements of the MCA from the study.

The biological mechanisms by which NAFLD could contribute to accelerated atherosclerosis are still poorly known. Our data suggest that in people with Type 2 diabetes, the relationship between NAFLD and increased stroke risk most likely reflects the overall atherogenic impact of the metabolic syndrome phenotype, principally diabetes, as supported by our multiple regression analyses. This study has some limitations, including a relatively small number of subjects on whom we only performed TCD measurements of the BA representing posterior cerebral circulation. MCA flow representing anterior cerebral circulation was not available by TCD examination in our study.

In summary, the increased PI observed in this study represents enhanced cerebrovascular resistance, and we observed that the age, male gender, diabetes, ADMA levels and carotid IMT were correlated significantly with the increased PI of the BA. These findings might have important clinical and public health implications. Our results support the implication that Type 2 diabetic individuals with NAFLD should be considered at high risk of stroke. Thus, the casual detection of NAFLD on an ultrasound examination in these patients should alert to the coexistence of multiple underlying stroke risk factors warranting evaluation and treatment as much as the risk for advancing liver disease. Screening with TCD as early as the disease is diagnosed and controlling diabetes and NAFLD, exercise or medical therapies may alter and modify the risk factors for future cerebrovascular diseases.

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