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Ultrasound assessment of endothelial-dependent flow-mediated dilatation of the brachial artery: ready for the clinical arena?

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PhD thesis

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List of publications

Full papers:

I) **Fábián E,** Varga A. A simvastatin kezelés hatása hypercholesterinaemiás, coronaria X szindrómás betegek endothelfunkciójára. Orvosi Hetilap 2002; 36:2063-67.

II) Nagy L, Bajko S, **Fábián E**, Farkas K, Fazekas Á, Forster T, Járai Z, Kolozsvári E, Kovács I, Pálinkás A, Pécsváradi Zs, Rónaszéki A, Varga A, Vereckey G. Magyar konszenzus az artéria brachialis áramlásfüggő „flow mediated" vasodilatációjának vizsgálatához. Érbetegségek 2003;2:47-50.

III) Farkas K, **Fabian E,** Kolossvary E, Jarai Z, Farsang Cs. Noninvasive assessment of endothelial dysfunction in essential hypertension; comparison of the flow mediated dilatation of the brachial artery with forearm microvascular reactivity. Int J Angiology 2003;12:224-28.

IV) **Fabian E,** Varga A, Picano E, Vajo Z, Ronaszeki A, Csanady M. Effect of Simvastatin on Endothelial Function in Cardiac Syndrome -X Patients. Am J Cardiol 2004;94:652-55, **(IF: 3,059)**

V) Nagy L, **Fábián E**, Kovács I. Carvedilol és metoprolol vérnyomáscsökkentő és endothel funkcióra kifejtett hatásának összehasonlítása hypertoniás, 2-es típusú diabetes mellitusban szenvedő betegeken. Cardiologia Hungarica 2004;34:178-83.

VI) **Fábián E,** Csanády M. Simvastatin hatása coronaria-X szindrómás betegek terhelésre jelentkező ST-depressziójára és endothel funkciójára. Cardiologia Hungarica 2004, in press

VII) Fabian E, Nagy L, Kovacs I, Csanady M. Comparison of the Effects of Carvedilol and Metoprolol on Blood Pressure and Endothelial Function in Patients with Hypertension and type-2 Diabetes Mellitus. Int J Cardiol 2004, submitted

Abstracts:

I. Fabian E, Pacetti E, Nemes B, Varga A, Morelos M, Rossi PC, Stock I, Picano E. Exercise perfusion scintigraphy positivity is associated with peripheral endothelial dysfunction in patients with normal coronary arteries. Proceedings of the International Stress Echo Meeting, Florence, Italy, 1999;45.

II. Pacetti E, Talarico L, Varga A, Morelos M, **Fabian E,** Nemes B, Stock I, Rossi PC. Angina pectoris e disfunzione vascolare periferica in pazienti con scintigrafia miocardica da sforzo positiva ed albero coronarico angiograficamente normale. 4 Congresso Nazionale FADOI. 5-8 Maggio 1999,Genova. Book of proceedings, 27.

III. Fábián E, Pacetti E, Nemes B, Varga A, Morelos M, Rossi PC, Stock I. Picano E. Perifériás endothel dysfunctio pozitív perfúziós szívizomscintigraphiával és negatív coronarographiával rendelkező betegekben. Cardiologia Hungarica. Supplementum 1999;2:71.

IV. Varga A, Pratali L, **Fabian E,** Pacetti E, Nemes B, Morelos M, Stock I, Rossi PC, Picano E. Exercise perfusion scintigraphy positivity is associated with peripheral vascular endothelial dysfunction in patients with normal coronary arteries. Eur Heart J 1999;20 (S):1100 A. **(IF:6,131)**

V. R. Amyot, A.Varga, M. Morelos, **E. Fabian,** O. Rodriguez, L .Pratali, E. Picano. Stress induced ST segment depression: anatomic lie or physiologic truth. Proceedings of the International Stress Echo Meeting, Pisa, Italy, 2000;45.

VI. Fabian E, Varga A, Pacetti E, Pratali L, Morelos M, Rossi PC, Stock I, Picano E. Exercise perfusion scintigraphy positivity is associated with peripheral vascular endothelial dysfunction in patients with normal coronary arteries. J Am Coll Cardiol, 2000; 35:482 A **(IF:6,278)**

VII. Fabian E, Varga A, Plonska E, Tomcsányi J, Bedros RJ. Effect of statin therapy on the coronary and endothelial function in cardiac syndrome-X patients. Proceedings of the 8th Alpe-Adria Cardiology Meeting, Portoroz, Slovenia, 2000;51.

VIII. Varga A, Morelos M, **Fabian E**, Rodriguez O, Pratali L, Picano E. The effect of systemic endothelial dysfunction and coronary artery disease on electrocardiographic and functional signs of ischemia during stress. J Am Coll Cardiol 2000;35 :422 A **(IF:6,278)**

IX. Fábián E, Varga A, Morelos M, Pratali L, Stock I, Picano E. A szisztémás endothelium diszfunkció és a koszorúsérbetegség kapcsolata a stressz indukálta iszkémia funkcionális és EKG paramétereivel. Cardiologia Hungarica. Supplementum 2000;2:17.

X. Fabian E, Varga A, Tomcsányi J, Stock I, Picano E. The Beneficial Effect of Statin Therapy on Endothelial Function and Exercise-Induced Ischemia in Hypercholesterolemic Patients with Cardiac Syndrome X. Circulation 2000; 102: 2403 A. **(IF:10,255)**

XI. Fabian E, Varga A, Tomcsanyi J, Stock I, Picano E. The Beneficial Effect of Statin Therapy on Endothelial Function and Exercise-Induced Ischaemia in Hypercholesterolemic Patients with Cardiac Syndrome X. Proceedings of the 4 th annual meeting of the Working Group on Echocardiography of the ESC, Lisbon, Portugal, 2000;21:22.

XII. Fábián E, Varga A. statin kezelés jótékony hatása a szisztémás endothelium funkcióra coronária X syndromás betegekben. Cardiologia Hungarica. Supplementum 2001;2:95.

XIII. Fábián E, Farkas K, Kolossvary E, Járai Z, Farsang Cs. Esszenciális hypertóniás betegek endothel diszfunkciójának non-invazív vizsgálati módszerei: az a. brachiális flow-mediált dilatációjának és az alkar mikrovaszkuláris reaktivitásának összehasonlítása. Cardiologia Hungarica. Supplementum 2002; 2:79.

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XV. Fabian E, Varga A, Picano E, Soos P, Csanady M. The Chronic Statin Therapy Exerts Beneficial Effect on Systemic Endothelial Function and Exercise-Induced Ischemia in

Hypercholesterolemic Patients with Cardiac Syndrome X. J Am Coll Cardiol 2002;39:219B. **(IF:6,278)**

XVI. Farkas K, **Fabian E**, Kolossvary E, Jarai Z, Farsang Cs. Noninvasive assessment of endothelial dysfunction in essential hypertension; comparison of the forearm microvascular reactivity with flow mediated dilatation of the brachial artery. Journal of Hypertens 2002; 20: 290 A. **(IF:3,534)**

XVII. Nagy L, **Fábián E**, Kovács I, Rónaszéki A. Carvedilol és metoprolol vérnyomáscsökkentő és endothel funkcióra kifejtett hatásának összehasonlítása hypertoniás, 2-es típusú diabetes mellitusban szenvedő betegeken. Cardiologia Hungarica, Supplementum 2004;2:26.

List of abbreviations

Ach	acethylcholine
ECG	electrocardiogram
FMD	flow-mediated dilatation
HDL	high-density lipoprotein
HbA1	glycosylated hemoglobin
LDL	low-density lipoprotein
NMD	nitrate mediated dilatation
NO	nitric oxide
PAI-1	plasminogen activator inhibitor-1
SNP	sodium nitroprusside
t-PA	tissue plasminogen activator
VCAM-1	vascular cell adhesion molecule -1

I.Introduction

I.1. Physiology of endothelium

The endothelium acts to maintain vascular homeostasis through multiple complex interactions with cells in the vessel wall and lumen. The vascular endothelium as a large paracrine organ senses and responds to a myriad of internal and external stimuli through complex cell membrane receptors and signal transduction mechanisms, leading to the synthesis and release of various vasoactive factors. Specifically, the endothelium regulates vascular tone by balancing production of vasodilators, including nitric oxide (NO), and vasoconstrictors. Furthermore, the endothelium controls blood fluidity and coagulation through the production of factors that regulate platelet activity, the clotting cascade, and the fibrinolytic system. Finally, the endothelium has the capacity to produce cytokines and adhesion molecules that regulate and direct the inflammatory process (1-6).

I.2. Pathophysiology of endothelial dysfunction

The endothelium maintains normal vascular tone and blood fluidity, and there is little to no expression of pro-inflammatory factors. However, in the setting of traditional and recently discovered cardiovascular disease risk factors, such as smoking, aging, hypertension, hypercholesterolemia, diabetes and obesity, homocysteinemia, elevated C-reactive protein, chronic systemic infections, the endothelium loses its normal regulatory functions. Cardiovascular risk factors initiate a chronic inflammatory process that is accompanied by a loss of vasodilator and antithrombotic factors and an increase in vasoconstrictor and pro-thrombotic products (7-12). Clinical syndromes such as stable and unstable angina, acute myocardial infarction, claudication, and stroke also relate, in part, to a loss of endothelial control of vascular tone, thrombosis, and the composition of the vascular wall, thus, measurement of endothelial function in patients has emerged as a useful tool for vascular research (13-15). The combination of typical chest pain, non-invasive stress tests indicative of

myocardial ischaemia, angiographically normal epicardial coronary arteries with no inducible coronary artery spasm, and no known associated cardiovascular disease is referred to as cardiac syndrome-X (16). According to the recently published studies the endothelial dysfunction is able to contribute to the myocardial ischemia (17-19) during stress testing (17-22) in patients with syndrome-X. However, mechanism of functional impairment in the coronary microvasculature causing an inadequate coronary flow reserve (16,18,22-25) is not well understood. The precise extent and order in which the normal control mechanisms are affected have not yet to be fully elucidated. It has been hypothesized that the abnormal vasodilator response due to impaired endothelial function is mechanism of clinical features in this syndrome (Figure 1.)

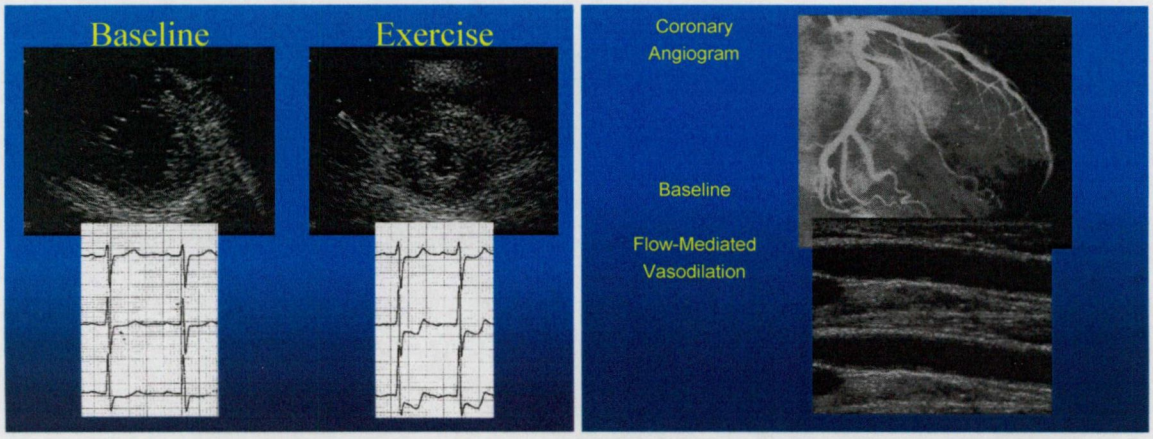

Figure 1. Cardiac syndrome-X: absense of stress-induced wall motion abnormalities, but significant ST segment depression, angiographically normal epicardial coronary arteries and reduced flow-mediated dilation.

I.3. Assessment of endothelial function in humans

Given this possible causal pathway from endothelial dysfunction to various vascular disease such as atherosclerosis, numerous methods have been employed to measure endothelial dysfunction in humans, each with its own advantages and disadvantages (26,27). The earliest studies of endothelial control of vasomotion used quantitative coronary angiography to examine the vasomotor responses of the epicardial coronary artery during infusion of acetylcholine or increased blood flow. In healthy individuals, the endothelium responds to

these stimuli by releasing vasodilator factors, particularly NO. Early studies demonstrated that patients with angiographically proven coronary artery disease (CAD) display impaired flow-mediated dilation (FMD) and a vasoconstrictor response to acetylcholine rather than the normal vasodilator response, likely reflecting loss of NO and unopposed constrictor effects of acetylcholine on vascular smooth muscle. Endothelium-independent coronary dilatation is assessed following infusion of a vasodilator such as adenosine, nitroglycerine, or papaverine (6,26-31). If resistance vessels are studied, the index of endothelial function is change in blood flow as assessed by blood flow velocity (and cross-sectional area) with Doppler wires or catheters (32). The technique requires an arterial catheter and, thus, has limited applicability for large-scale studies or future development as a clinical tool.

Impedance plethysmography reflects resistance vessel function in the forearm (33), while dorsal hand vein compliance can be assessed with the linear variable differential transducer technique (34).

Invasive measures are not suitable for evaluating large groups of patients, changes early in the development of disease in asymptomatic patients, or for repeated studies of progression of disease or reversibility after treatment.

Quantitative non-invasive assessment of myocardial blood flow and metabolic activity can be made by positron emission tomography scanning (35-37). This technique is non-invasive and has the advantage of the potential for multiple tests per patient; however, it is very expensive and limited to a small number of laboratories.

I.3.a. Laser Doppler flowmetry

Laser Doppler flowmetry is a suitable method for the noninvasive study of skin microcirculation (38,39). Iontophoresis coupled with laser Doppler flowmetry makes it possible to assess the real-time changes of the skin blood flow after the administration of different vasoactive substances without systemic effects. Acethylcholine given into the skin

by iontophoresis causes endothelium dependent vasodilation, which can be compared with the endothelium independent effect of sodium nitroprusside, a nitrogen oxide donor. The reproducibility of laser Doppler flowmetry has been studied in stable emulsion with an intra-assay coefficient of variation of about 6%. In humans, the technique shows a coefficient of variation of 20-21 % (39). The postocclusive reactive hyperaemia (PORH), mediated mainly by endothelium dependent factors, measured by laser Doppler flowmetry can be as suitable for the assessment of endothelium dependent vasodilation as the flow mediated vasodilation of the brachial artery, but on the level of capillaries and arterioles (38).

Finally, there has been considerable interest in noninvasive examination of endothelium-dependent FMD of the conduit brachial artery using vascular ultrasound.

I.3.b. Flow-mediated dilatation of the brachial artery (FMD)

This response has been shown to depend in large part on NO synthesis, but also reflects release of other endothelium-derived vasodilators. The flow mediated dilation of the brachial artery is induced by the shear stress during flow increase and the principal mediator is endothelium-derived nitrogen oxide (40-43) (Figure 2.)

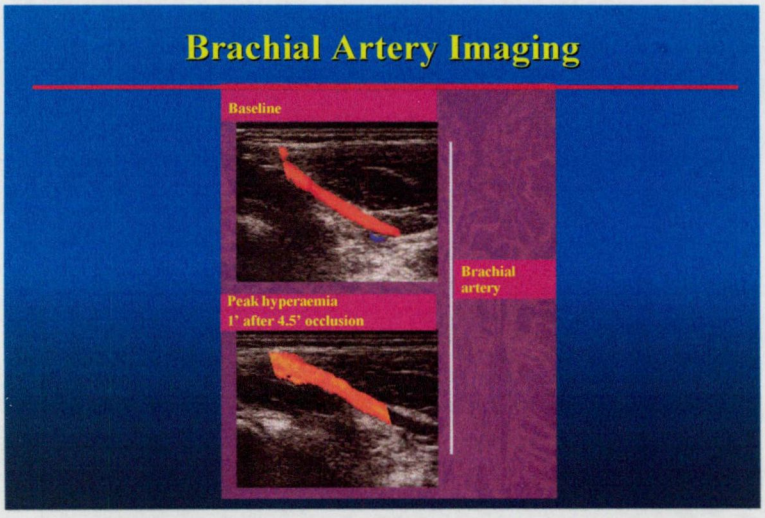

Figure 2. The upper panel: the brachial artery at baseline measurement in rest. The bottom panel: brachial artery during endothelium-dependent flow-mediated vasodilation in healthy subject.

Flow-mediated increase in diameter in the brachial artery occuring during reactive hyperaemia after release of a 4,5-minute occlusion can be within the range of 7-10% in

healthy subjects (40,44-47), whereas this flow-mediated vasodilator response is impaired in patients with risk factors for coronary artery disease or atherosclerosis and shows values of 0-5% (40,46,48-51). The endpoint of the measurements may be the absolute mean change in brachial artery diameter or the percent change in vessel diameter; blood flow is measured as the mean Doppler flow velocity multiplied by the cross-sectional area (40,52).

With intervention trials, an important parameter to report, is the time-dependent reproducibility of FMD. In the study done by Sorensen et al the mean range of interobserver difference for the measurement of percent flow-mediated dilatation with two observers was 1.7 %. From the nested analysis of variance, the estimated coefficient of variation was 1.4 % (47). An acceptable reproducibility is a mean difference of 2% to 3% in FMD over time (on a baseline vasodilation of about 10%) (53). The mean range of between-occasion within-patient variability of observed percent flow-mediated dilatation was 2.8%. From the nested analysis of variance, the estimated coefficient of variation between visits was 2.3% (47).

Several authors have shown a relationship between FMD in the brachial artery and the coronary circulation (54-56). Although there was no correlation detected between impairment of FMD and severity of coronary artery disease, the positive predictive value of FMD is similar to other non-invasive tests for detection of ischaemic heart disease (sensitivity 71%, specificity 81%) (44).

Measuring FMD is attractive because it is noninvasive and allows repeated measurements, is particularly well suited to study the earliest stages of atherosclerosis in children and young adults, thus providing maximal opportunity for prevention. A great interest exists in determining the clinical utility of brachial artery FMD. Investigators have hypothesized that endothelial function may serve as an integrating index of risk factor burden and genetic susceptibility, and that endothelial dysfunction will prove to be a preclinical marker of cardiovascular disease. Several studies suggest that the presence of endothelial dysfunction in

the coronary circulation is an independent predictor of cardiovascular events (57,58). Numerous studies have demonstrated that brachial artery reactivity improves with risk factor modification and treatment with drugs. Therefore in the future, practitioners may use brachial artery FMD to assess response to drug therapy, as well.

Despite the many parallel findings, one modest-sized study suggested that, within individual subjects, brachial artery FMD does not correlate with resistance vessel (microvascular) function as measured by infusion studies (59). Indeed, it is likely that there is differential regulation of vascular tone in conduit and resistance vessels, and that the different measures of vascular function may have relevance to different aspects of CVD.

1.3.c. Laboratory examination of endothelial markers

Endothelial function can be assessed by measuring of serum concentrations of vasoactive substances, produced by endothelium, such as endothelium derived nitric oxide, endothelium derived hyperpolarizing factor, vascular cell adhesion molecule, plasma vascular endothelial growth factor and endothelin-1, tissue plasminogen activator, plasminogen activator inhibitor, von Willebrand factor (60-67).

I.4. Pharmacologic interventions to improve endothelial dysfunction

In prospective studies, endothelial dysfunction is associated with increased incidence of cardiovascular events. Endothelial dysfunction can be reversed with appropriate interventions targeted on the most likely underlying etiological factor. (26). HMG-CoA reductase inhibitors (statins) have been demonstrated have vascular effects, moreover independent of their cholesterol lowering effect (25,35,62,63). Anderson and coworkers have found that the combination of LDL lowering and antioxidant therapy, simvastatin plus probucol resulted in near normalization in coronary endothelial function (68). Another report suggested that flow-mediated brachial artery vasoactivity responds rapidly to changes in cholesterol levels and that endothelial function improves by lowering cholesterol levels below recommendations of

current guideline (69). Endothelial dysfunction in primary hypercholesterolemia was improved by treatment with atorvastatin or simvastatin plus cholestyramine and this effect might result in the prevention of future coronary event (70). However, several studies have shown the beneficial effect of cholesterol lowering by statins on endothelial function, the role of statin is not yet elucidated in patients with syndrome-X.

It has been widely demonstrated that blood pressure reduction is not sufficient to improve or restore endothelial function. Antihypertensive drugs show contrasting effects in terms of improvement or restoration of endothelial function (71). Calcium channel antagonists, particularly the dihydropyridines, can reverse impaired endothelium-dependent vasodilation (72,73) through a mechanism probably related to an antioxidant effect. ACE inhibitors, on the other hand, seem to improve endothelial function (74,75). They can also selectively improve endothelium-dependent vasodilation to bradykinin, an effect not mediated by restoring NO availability but probably related to hyperpolarisation. Recent evidence suggests angiotensin II AT (1)-receptor antagonists can restore endothelium-dependent vasodilation (76). Little evidence is available with beta-blockers. Whereas treatment with atenolol has a negative effect in peripheral subcutaneous and muscle microcirculation, insufficient evidence is available to establish whether new compounds such as nebivolol, which activates the L-Arginine--NO pathway, and carvedilol, which has strong antioxidant activity, can improve endothelial function in patients with hypertension (77,78). Despite the considerable evidence that impaired endothelium-dependent vasodilation can be restored by appropriate antihypertensive treatment, head to head comparisons are required to demonstrate differences among drugs, such as ß-blockers.

II. Aims of the work

We hypothesed that the vascular ultrasound assessment of endothelium-dependent flow-mediated dilatation of the brachial artery is a useful tool for evaluation of effects of pharmacologic interventions in clinical setting.

Therefore we aimed:

II.1. To compare methodologically the flow mediated dilatation of the brachial artery with forearm microvascular reactivity in the assessment of endothelial dysfunction in essential hypertension

We investigated whether endothelium-dependent vasodilatation in the forearm microcirculation is related to endothelium-dependent flow mediated dilation of the brachial artery in essential hypertensive patients and normotensive subjects.

II.2. To assess the effect of chronic statin therapy on exercise-induced ST segment depression and on systemic endothelial function in cardiac syndrome-X patients with mild hypercholesterolemia.

Systemic endothelial dysfunction is associated with electrocardiographic positivity during stress testing even in the presence of normal coronary arteries (17-22). Statins can improve endothelial functions due to its non-lipid lowering effects (25,35,62,63). Starting out from these points, we aimed in this part of the study, to determine whether statin therapy have any beneficial effect on systemic endothelial function and exercise induced ischaemia in cardiac syndrome-X patients with mild hypercholesterolemia.

II.3. To compare the effects of carvedilol and metoprolol on blood pressure, endothelial function and metabolic parameters in patients with hypertension and type-2 diabetes mellitus.

The aim of this part of the study was to compare the effects of twelve weeks of antihypertensive therapy with carvedilol, a non-selective β-adrenoreceptor blocker with α1-

blocking properties, with the selective β1-adrenergic receptor blocker metoprolol on the blood pressure, endothelial function and metabolic parameters of patients with hypertension and type 2 diabetes.

III. Patients and methods

III.1. For noninvasive assessment of endothelial dysfunction in essential hypertension; comparison of the flow mediated dilatation of the brachial artery with forearm microvascular reactivity the patient population and methods used were as follows:

III.1.1 Patient population

The study population included 22 patients with essential hypertension (mean age 50.5 ± 6.9 years, males, systolic blood pressure 141.2 ± 12.25 mmHg, diastolic blood pressure 85.8 ± 7.02 mmHg) and 11 normotensive control subjects (mean age 41± 9 years, males, systolic blood pressure 116.6 ± 10 mmHg, diastolic blood pressure 68.3 ± 5.59 mmHg). Patients older than 60 years, smokers or those with diabetes mellitus or with elevated (>6.5 mmol/l) total cholesterol plasma levels were excluded. No patient was on nitrate treatment, and the antihypertensive therapy had been unchanged for at least 6 months prior to inclusion. Essential hypertensive patients were screened for FMD of the brachial artery and only patients with FMD < 5 %, confirmed impaired endothelial function (29,35), were included. The laser Doppler flowmetry, thereafter the vascular ultrasound examination were performed on the same day. Informed consent was obtained from all patients and the study was approved by the local Ethics Committee. Baseline medications were held constant through the entire study.

III.1.2 Laser Doppler flowmetry.

Measurements were carried out in a temperature-controlled room (24±1°C) with the subjects lying in the supine position, after a 20-minute acclimatization period. 15 minutes before

starting, the flexor aspect of the left forearm was gently cleaned using alcohol. We determined the blood pressure and pulse rate of the subjects.

A laser Doppler instrument (Periflux 5001, wawelength 780 mm) and a micropharmacology system (PeriIont) were used for noninvasive and continuous measurement of perfusion changes during vascular provocations in the skin (Perimed AB, Järfälla, Sweden). A drug delivery electrode was incorporated in the head of the laser probe. The probe temperature could be varied and was standardized to 32°C during drug tests. The current strength chosen for our study were well tolerated by the subjects. We have previously evaluated the effect of iontophoresis current itself on skin blood flow: practically no changes were induced by the same duration and strength of the electric current we used in the present study.

The drug delivery electrode was filled with 140 µl Acetylcholine 1% (Clinalfa AG, Swiss) and was attached with the laser probe to the volar surface of the left forearm. The position of the probe was chosen in order to avoid hair, freckles and broken skin. The dispersive electrode was attached to the volar aspect of the wrist to complete the circuit. We placed a control standard probe 4 cm laterally from the drug delivery electrode. After registration of the baseline flow (60 sec) two doses of acetylcholine was delivered using an anodal current (0.1 mA for 30 s and 0.16 mA for 30 s) with a 120 sec interval. With a new delivery electrode two doses of sodium nitroprusside 1% (Nitropress, ABBOTT, USA) were delivered using a cathodal current (0.1 mA for 20 s and 0.1 mA for 30 s) with a 120 sec interval.

During the postocclusive reactive hyperemia test after the registration of the baseline flow (60 sec) arterial occlusion was performed with suprasystolic pressure by the help of a pneumatic cuff of a sphygmomanometer for 3 minutes (biological zero), after the release of the pressure we measured the skin hyperaemia on the volar surface of the left forearm 10 cms below the elbow with a standard laser Doppler probe. Another standard probe was put on the skin of the left forearm as a control.

The laser Doppler signal is proportional to the number and velocity of moving blood cells in illuminated superficial skin microvessels. The laser beam penetrates the skin and it is partially backscattered by moving blood cells. According to the Doppler principle, a frequency shift occurs, generating a signal, that is linearly related to red cell flow, as predicted by theoretical and experimental models. The laser Doppler output is semiquantitative and expressed in PU of output voltage (1 PU = 10 mV) in accordance with general consensus (European Laser Doppler Users Groups, London 1992). The laser Doppler outputs were recorded continuously by an interfaced computer with acquisition software (Perisoft). Calibration was performed by a device composed of colloidal latex particles, the Brownian motion of which provides the standard value. Because the output cannot easily be translated into absolute values of blood flow, the magnitude of the changes in skin perfusion was calculated as the ratio between peak and mean baseline perfusions.

III.1.3. Evaluation of endothelial function by flow-mediated brachial artery dilatation

The evaluation of endothelial function was performed according to the method described by Celermayer et al (40) and as stated in the report of the International Brachial Artery Reactivity Task Force (53). All patients were studied minimum 10 hours after the last meal (53,79). The patient lay supine in a quiet, temperature-controlled room (20-25°C) for 10 minutes before the study with the right arm gently immobilized in extension. The diameter of the brachial artery was measured from 2D ultrasound images using a commercially available system (Hewlett-Packard SONOS 2000, 7.5-MHz probe). Images were recorded on an S-VHS tape. In each study, scans were taken at rest, during reactive hyperemia, at rest again and after sublingual nitrates. The brachial artery on the dominant arm was scanned in longitudinal section, 2 to 15 cm above the antecubital fossa. The settings were adjusted to optimize the lumen/arterial wall interface and held constant during testing. Measurements were taken from the anterior to the posterior "m" line at end diastole, incident with the R-wave on the electrocardiogram, at a fix

distance from an anatomic marker, such as a bifurcation. Three cardiac cycles were analyzed for each scan and measurements were averaged. Following the baseline measurements a forearm cuff occlusion was obtained for 4.5 minutes below the elbow (80-82) by inflation of pneumatic tourniquet to a pressure of 270 mmHg. The artery diameters were performed within one minute after cuff deflation during reactive hyperemia (Figure 3).

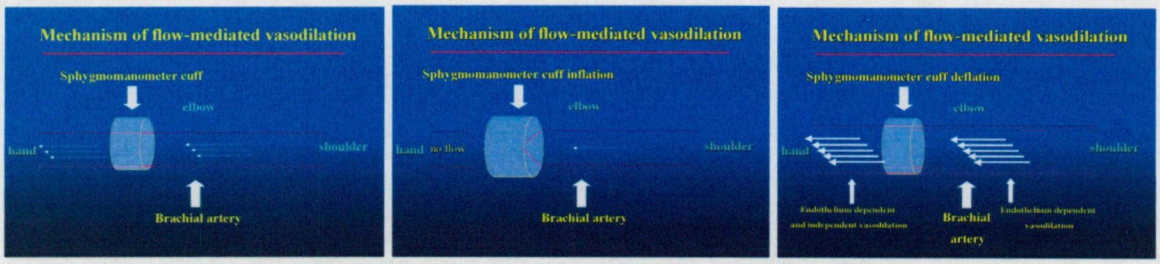

Figure 3. Mechanism of flow-mediated vasodilation. Left panel:baseline measurement; middle panel: forearm occlusion; right panel: reactive hyperemia after cuff deflation.

Following 10 minutes of recovery phase a resting scan was repeated. Sublingual nitrates (400 μg of glyceryl trinitrate) were then administered. The last set of scans was performed 3 minutes after nitrate intake, during nitrate-mediated dilatation (NMD) (Figure 4.).

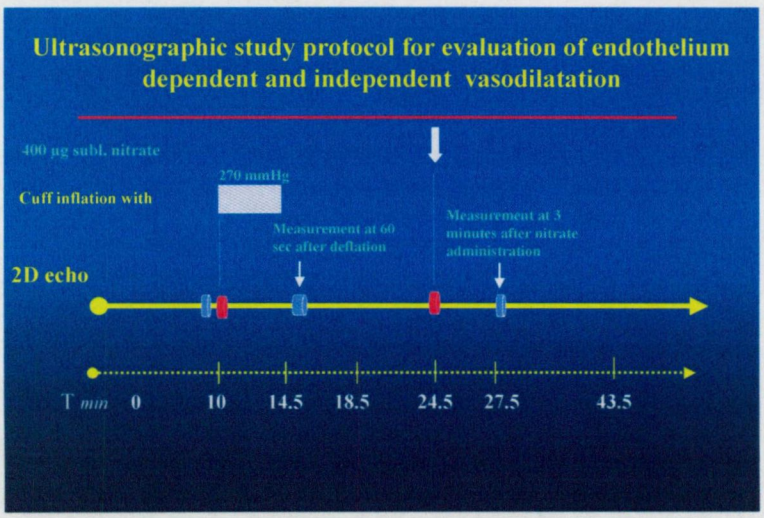

Figure 4. Ultrasonographic study protocol for evaluation of endothelium-dependent and independent vasodilatation.

The maximum FMD and NMD diameter measurements were calculated as the average of the three consecutive maximum diameter measurements after hyperaemia and sublingual nitrate. Then the FMD and NMD were calculated as the percent change in diameter compared with

baseline resting diameters. The images were read independently by 2 separate observers blinded to the patient identity and the study phase.

III.1.4. Statistical analysis

All results are expressed as the median and interquartile range. According to the sample size nonparametric procedures were performed. Results were compared in hypertensive and control subjects by Mann-Whitney U test. Effect of iontophoresis and PORH tests were analized by Friedman ANOVA. Differences were considered as statistically significant when $p<0,05$. Statistical analysis was performed by Statistica for Windows software.

III.2. For the assessment of the effect of chronic statin therapy on exercise-induced ST segment depression and on systemic endothelial function in cardiac syndrome-X patients with mild hypercholesterolemia the patient population, the study design and methods used were the following:

III.2.1. Patient population

Patient population consisted of 40 prospectively-enrolled cardiac syndrome-X patients with mild hypercholesterolemia (mean age 55.7 ± 1.05 years, 25 males). The diagnosis of cardiac syndrome-X was based on the presence of typical angina pectoris, transient > 1 mm ST segment depression during exercise stress test, transient perfusion defect during myocardial perfusion scintigraphy, angiographically normal coronary arteries in the absence of coronary artery spasm (excluded by hyperventilation), left ventricular hypertrophy and systemic hypertension. The patients underwent thallium scintigraphy and coronary angiography with hyperventilation test for exclusion of arteriospasm in 1-year period before enrolling to the study. Myocardial perfusion scintigrams with transient perfusion defects were considered as positive. Angiograms with visually no wall irregularities were accepted as normal. Patients were eligible if they met the following inclusion criteria: a normal coronary angiogram; positive exercise ECG test; positive myocardial perfusion scintigram; normal regional and

global resting left ventricular function; mildly elevated total serum-cholesterol level (>5.2 mmol/L). The exclusion criteria were previous myocardial infarction, valvular heart disease including mitral valve prolapse, congestive heart failure, cardiomyopathy, sinus node dysfunction or conducting disturbances (including left bundle brunch block), diabetes mellitus, impaired renal or liver functions, smoking. Informed consent was obtained from all patients and the study was approved by the local Ethics Committee. The investigation confirmed to the principles outlined in the Declaration of Helsinki. All patients received and were taught American Heart Association steps 2 diet and received antianginal treatment consisted of beta-blockers and/or calcium antagonists. None of them were on long-acting nitrate, NO-donor, trimetazidine and ACE-inhibitor therapy. Only sublingual nitrates were allowed for the relief of chest pain during the study. Medications were held constant through the entire study. Postmenopausal women were not on hormone replacement therapy.

III.2.2. Study design

At baseline and at the end of the study laboratory measurements, exercise ECG, and FMD studies were done. After baseline measurements the patients were randomized to placebo (n=20) or simvastatin 20 mg daily at bedtime (n=20) for a duration of 12 weeks. The data were read independently by 2 separate observers blinded to patient identity, study phase (baseline or study end), and the results of the other tests.

III.2.3. Laboratory measurements

Blood samples were analyzed for total cholesterol, high-density lipoprotein cholesterol and triglycerides. Low-density lipoprotein cholesterol levels were determined by using the formula of Friedewald. The measurements were expressed in mmol/L.

III.2.4.Evaluation of endothelial function by flow- mediated brachial artery dilatation

The evaluation of endothelial function was performed according to the method as described above under III.1.3

III.2.5. Exercise stress test

All patients performed a multistage treadmill test according to modified Bruce protocol. Patients were not allowed to take beta-blockers and sublingual nitrate 24 h before the exercise stress tests. Blood pressure, heart rate and 12-lead ECGs were recorded at rest, at 1-minute intervals during exercise, at peak exercise and for at least 5 min in the recovery phase. The ECG and the changes of ST segment were continuously displayed and measured automatically by a computer assisted system (Cardiovit AT-104 treadmill, Schiller) in all 12 leads. The reaching of age-specific target heart rate or the development of symptoms necessitating termination of the test were taken as end-points of exercise-stress test. The appearance of downsloping or horizontal ST segment depression >1 mm or 0.1 mV 0.08 sec after the J point was taken as evidence of a positive exercise electrocardiography test .The time to > 1 mm ST segment depression was defined.

III.2.6. Statistical analysis

The results are expressed as mean ± SD. Comparisons were made using the paired samples Student's t-test when assessing the differences at randomization and at study end. To assess correlation between data Pearson correlation was used. Differences were considered statistically significant at the level of $p<0.05$ (two-sided).

III.3. For comparing of the effects of carvedilol and metoprolol on blood pressure and endothelial function in patients with hypertension and type-2 diabetes mellitus the patient population, the study design and methods used were the following:

III.3.1. Patient population

The patient population was recruited from two participating outpatient care centers. 36 patients with type 2 diabetes mellitus and mild or moderate essential hypertension (systolic blood pressure > 130 and < 180 mmHg, diastolic blood pressure >85 and < 110 mmHg on repeated measurements) were recruited to the study. Patients were eligible if they met the following inclusion criteria: adequate glycemic control (glycosylated hemoglobin < 7%) with diet or oral hypoglycemic agents, unchanged and constant antihypertensive treatment for 6 months before enrollment (consisted of enalapril, ca-antagonists, diuretics), total serum-cholesterol level <6.5 mmol/L, low-density lipoprotein cholesterol level <3.4 mmol/L, triglyceride level <2.3 mmol/L and the flow mediated dilatation of the brachial artery, measured by vascular ultrasound < 5%, confirmed impaired endothelial function. The exclusion criteria were previous myocardial infarction and unstable angina, stroke within the preceding 6 months, stable angina, angiographically assessed coronary artery disease, secondary hypertension, known peripheral artery disease, impaired renal or liver function tests, microalbuminuria, nitrate, lipid lowering, hormone replacement therapy, known intolerance to nitrate, contraindications for receiving ß-blockers, smoking, childbearing age in women not using reliable contraception. Informed consent was obtained from all patients and the study was approved by the local Ethics Committee. The research protocol was carried out in accordance with the Declaration of Helsinki.

III.3.2. Study design

Our study had a prospective, randomized, open design for parallel study groups. Laboratory tests were done and endothelial function was evaluated by FMD during the initial screening

and after the treatment period. The patients were randomized either to carvedilol 2x12.5 mg starting dose daily (n=19) or metoprolol succinate 1x50 mg starting dose daily (n=17). The dose of the study drug was doubled after 4 weeks if the patient had not reached the blood pressure goal of 130/80 mmHg. The list of randomization numbers was used to label the drug boxes, which were given to the participants sequentially. Side effects, concomitant diseases, blood pressure and ECG were assessed by interview and physical examination every fourth week during the study. Baseline medications were held constant through the entire study period.

III.3.3. Blood pressure measurement

Blood pressure and heart rate were recorded in the sitting position, after at least 10 min of rest. Systolic blood pressure was recorded as the pressure noted when Korotkoff sound I appeared and diastolic blood pressure when Korotkoff sound V disappeared. Blood pressure was measured in triplicates with a sphygmomanometer on the same arm and by the same investigator, and the mean of these values was calculated.

III.3.4. Laboratory measurement

Blood samples were analyzed for laboratory measurements by standard clinical laboratory methods (glycosylated hemoglobin, plasma glucose, total cholesterol, low density lipoprotein cholesterol, high density lipoprotein cholesterol, triglyceride, liver enzyme levels, creatinin concentrations). The tissue plasminogen activator, plasminogen activator inhibitor-1 and vascular cell adhesion molecule-1 levels were measured by enzyme-linked immunosorbent assay (Asserachrom tPA and PAI-1 kits, and h-sVCAM-1 ELISA kit, Roche Inc, France).

III.3.5. Evaluation of endothelial function by flow- mediated dilatation

The evaluation of endothelial function was performed according to the method as described above under III.1.3.

III.3.6. Statistical analysis

The results are expressed as mean ± SD. Comparisons were made using the paired samples Student's t-test when assessing the differences at randomization and at study end. To assess correlation between data Pearson correlation was used. Differences were considered statistically significant at the level of $p<0.05$ (two-sided).

IV. Results

IV.1. Noninvasive assessment of endothelial dysfunction in essential hypertension; comparison of the flow mediated dilatation of the brachial artery with forearm microvascular reactivity

Endothelial dysfunction was detectable both with laser doppler flowmetry and flow- mediated dilatation of brachial artery in essential hypertension.

IV.1.1. Laser Doppler flowmetry.

Basal forearm skin perfusion. Basal forearm skin perfusion was not significantly different in the patients compared with the control subjects.

Response to acetylcholine. Iontophoresis of acetylcholine produced a significant dose-dependent increase in cutaneous blood flow both in essential hypertensive patients and control subjects, but the vasodilation to the two doses of acetylcholine was significantly lower in the essential hypertensive patients group than in the normotensive group ($p<0.01$; $p<0.05$) (Table 1).

Response to sodium nitroprusside. Table 2 shows the responses of forearm skin perfusion to the iontophoresis of the two doses of sodium nitroprusside. The cutaneous blood flow increased significantly in both groups. The vasodilation to sodium nitroprusside was lower but not significantly different in the essential hypertensive patients group compared with the normotensive subjects group.

Table 1. Changes in skin blood flow in response to iontophoresis of acetylcholine

	EHT	NT
Baseline (PU)	8.25 ± 4.48	7.25 ± 4.5
PFACh1 (PU)	21.62 ± 13.27	37.26 ± 34.57
PFACh1% (%)	177 ± 202	470 ± 509**
PFACh2 (PU)	41.94 ± 29.71	61.00 ± 74.86
PFACh2% (%)	463 ± 241	805 ± 618*

Data are median ± interquartile range.
Significant difference between EHT and NT group * $p<0.05$, ** $p<0.01$
EHT: essential hypertensive patients; NT: normotensive subjects. PFACh1 and PFACh2: maximal skin blood flow after the first and second dose of acetylcholine in perfusion units. PFACh1% and PFACh2%: maximal skin blood flow % increase after the first and second dose of acetylcholine. PU:perfusion unit.

Table 2. Changes in skin blood flow in response to iontophoresis of sodium nitroprusside

	EHT	NT
Baseline (PU)	7.60 ± 4.65	9.29 ± 8.09
PFSNP1 (PU)	33.19 ± 32.38	48.30 ± 61.34
PFSNP1% (%)	368 ± 431	440 ± 725
PFSNP2 (PU)	52.01 ± 42.43	76.21 ± 85.75
PFSNP2% (%)	641 ± 459	810 ± 877

Data are median ± interquartile range.
EHT: essential hypertensive patients; NT: normotensive subjects
PFSNP1 and PFSNP2: maximal skin blood flow after the first and second dose of sodium nitroprusside in perfusion units. PFSNP1% and PFSNP2%: maximal skin blood flow % increase after the first and second dose of sodium nitroprusside. No significant differences were found between EHT and NT. PU: perfusion unit.

Postocclusive reactive hyperemia. The three minute occlusion of the brachial artery by a pneumatic cuff produced a significant increase in the cutaneous blood flow after cuff release. The vasodilation was significantly lower in the essential hypertensive patients than in

normotensive subjects ($p<0.01$) (Table 3). Mean biological zero was not significantly different between the two groups.

Table 3. Changes in skin blood flow in response to 3 minutes arterial occlusion (Postocclusive reactive hyperemia)

	EHT	NT
Baseline (PU)	12.62 ± 9.47	8.88 ± 3.8
Peakflow (PU)	39.08 ± 29.98	44.33 ± 20.12
Peakflow% (%)	272 ± 221	409 ± 194**
Time to peakflow (sec)	9.23 ± 4.38	12.32 ± 4.65*

Data are median ± interquartile range.
Significant difference between EHT and NT group * $p<0.05$, ** $p<0.01$
EHT: essential hypertensive patients; NT: normotensive subjects.
Peakflow: maximal skin blood flow after the release of arterial occlusion. Peakflow%: maximal skin blood flow % increase after the release off arterial occlusion. Time to peakflow: the time from cuff release to maximal vasodilation. PU: perfusion unit.

IV.1.2. Flow-mediated dilation and nitrate-mediated dilation

The FMD of the brachial artery was significantly lower in essential hypertensive patients than in normotensive subjects (3.98±2.4% vs. 9.3 ± 4.9%, $p<0.001$). The NMD was lower but not significantly different in essential hypertensive patients compared with normotensive subjects (14.46 ± 6.59% vs. 18.21 ± 9.7%, p=ns) (Table 4).

There was no significant relationship either between maximal response to acethylcholine and FMD ($r = 0.28$), or between postocclusive reactive hyperemia and FMD ($r=-0.01$). No relation was found between response to sodium nitroprusside and glyceryl trinitrate ($r = 0.31$).

Table 4. Flow-mediated dilatation and nitrate-mediated dilatation

	EHT	NT
FMD (%)	3.98 ± 2.4	9.3 ± 4.9***
NMD (%)	14.46 ± 6.59	18.21 ± 9.7

Data are mean ± SD
Significant difference between EHT and NT group *** p<0.001
EHT: essential hypertensive patients; NT: normotensive subjects, FMD: flow mediated dilation; NMD: nitrate mediated dilation.

IV.2. Effects of chronic statin therapy on exercise-induced ST segment depression and endothelial function in cardiac syndrome-X patients.

The chronic statin therapy resulted in prolonged time to > 1 mm ST segment depression during exercise stress test, acted beneficially not only on lipid parameters, but also on endothelial function in cardiac syndrome-X patients with mild hypercholesterolemia.

IV.2.1. Lipid levels

Cholesterol levels were not statistically changed in the placebo group. Total serum cholesterol levels decreased significantly by 26 % (5.86 ± 0.13 mmol/L vs. 4.63 ± 0.37 mmol/L, P< 0.0001) following 12 weeks of simvastatin treatment and 38 % reduction of low-density lipoprotein levels occurred (4.47 ± 0.12 mmol/L vs. 3.22 ± 0.37 mmol/L, P<0.0001). High-density lipoprotein levels increased significantly by 7 % (0.99±0.05 mmol/L vs.1.05 ± 0.05 mmol/L, P<0.0001) in the simvastatin group.

Triglyceride levels were not significantly modified throughout the course of the study in both groups (Figure 5.).

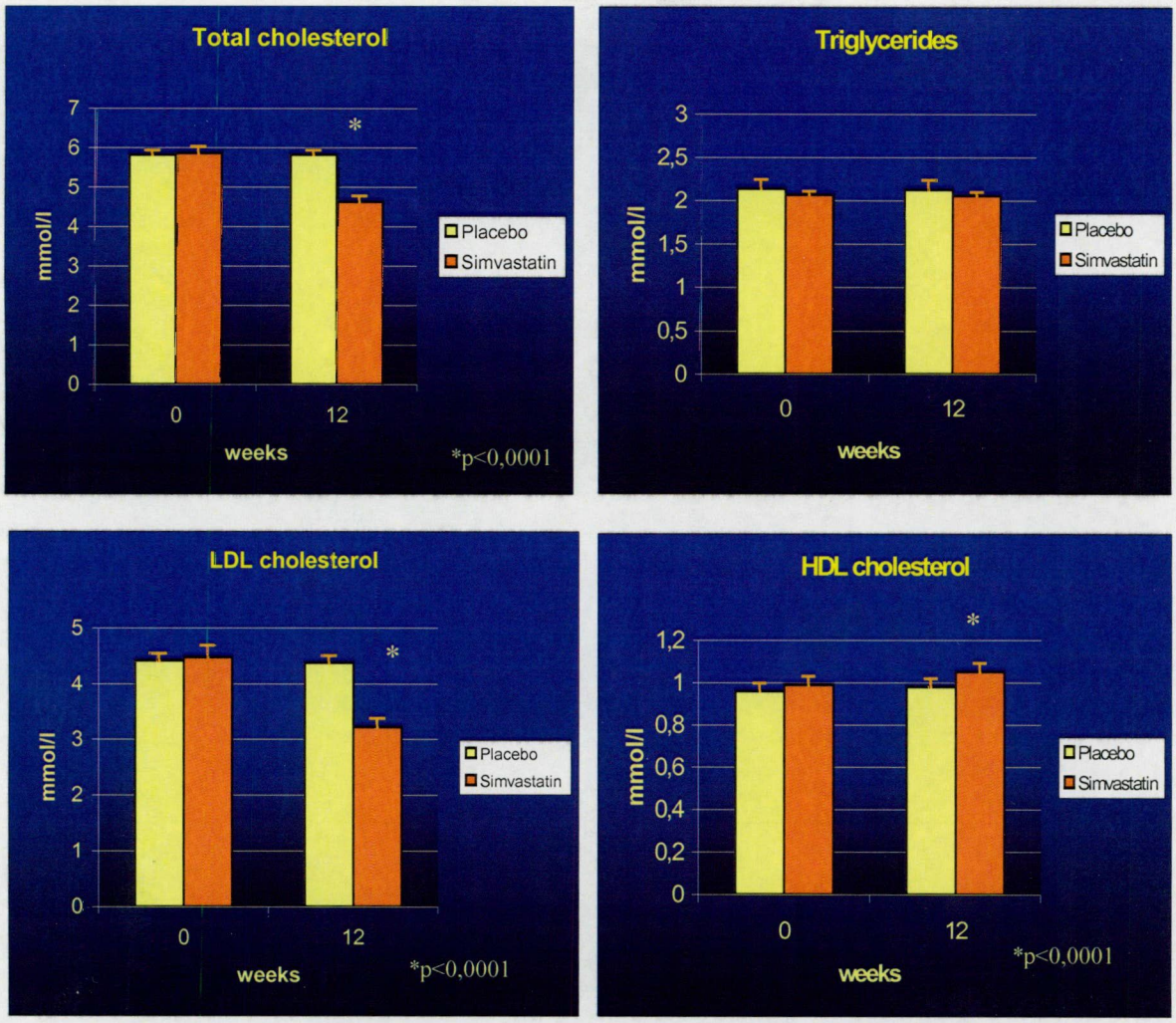

Figure 5. Changes in levels of lipid fractions at randomization to placebo (n=20) or simvastatin (n=20) at the baseline and at the study end. Total serum cholesterol (■) and LDL (■) levels decreased (P<0.0001), HDL levels (■) increased significantly (P<0.0001) following the simvastatin therapy. Cholesterol levels were not statistically changed in the placebo group (□). Triglyceride levels were not significantly modified throughout the course of the study in both groups.

IV.2.2. Flow-mediated dilatation and nitrate-mediated dilatation

Brachial artery flow mediated vasodilation (FMD) did not change significantly in the placebo group (4.07 ± 0.12 % vs. 4.06 ± 0.15, P=ns), but increased significantly (52%) in the simvastatin group (4.01 ± 0.91 % vs. 6.12 ± 0.79 %, P<0.0001) (Figure 6.). Responses to glyceryl trinitrate nitrate were similar during the time course of the study in both groups (Figure 6.).

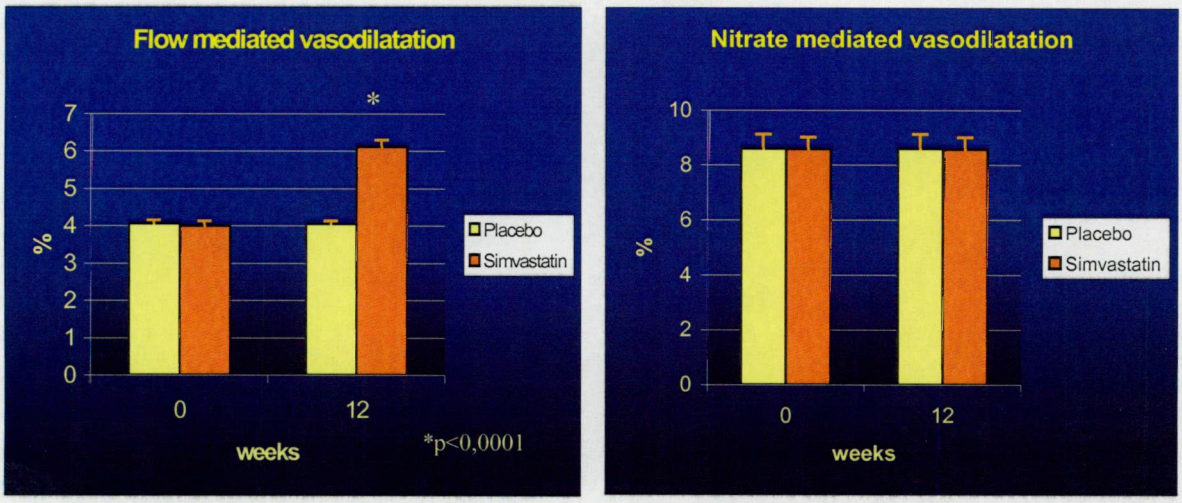

Figure 6. Endothelium-dependent flow-mediated dilatation was significantly better after simvastatin therapy (■)($P<0.0001$). In placebo group (□) there were no improvement in endothelial function at the study end. However, there were no significant differences in nitrate-induced endothelium-independent vasodilatation in both groups.

IV.2.3. Exercise stress test

No significant differences were present in the time to >1 mm ST segment depression during stress test after 12 weeks in the placebo group. It was significantly longer by the end of the study in the simvastatin group (4.45 ± 0.39 min vs. 5.33 ± 0.27 min, $P<0.0001$). In 4 patients no inducible ischaemia could be detected during the stress test in the simvastatin group by the end of the study and the exercise was finished due to reaching of age-specific target heart rate (Table 5, Figure 7.).

Table 5. Exercise stress test results during the study phase

	Placebo (n=20)		Simvastatin (n=20)	
	Baseline	**Study end**	**Baseline**	**Study end**
Significant ST depression	20 positive	20 positive	20 positive	16 positive
Time to >1 mm ST- depression (min)	4.68±0.43	4.86±0.42	4.45±0.39	5.33±0.27 *

Data are presented as mean ± SD.* P<0.0001

All patients who had significantly longer time to>1 mm ST segment depression during exercise stress test demonstrated an improvement in flow-mediated dilatation. There was no significant correlation detected between the improvement in percent FMD and the electrocardiogram parameters and fall in total- and low density lipoprotein cholesterol levels.

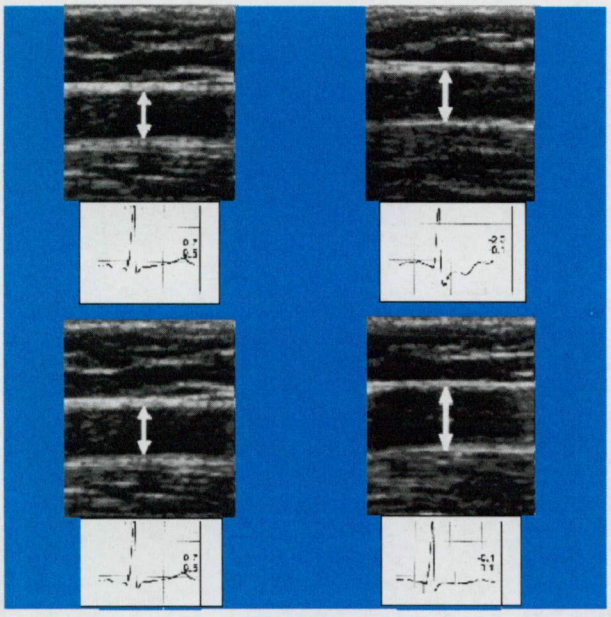

Figure 7. Changes in brachial artery diameters and exercise-induced ischemia before and after 12 weeks of simvastatin therapy. Two upper panels: the flow-mediated dilatation and the exercise ECG before treatment. Significant ST depression on the V5 lead and impaired response of the brachial artery to the forearm ischemia. The bottom panels: the same patient following the statin treatment. No inducible ischaemia on the exercise ECG but a clear-cut improvement of the flow-mediated dilatation.

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IV. 3. Comparison of the effects of carvedilol and metoprolol on blood pressure and endothelial function in patients with hypertension and type-2 diabetes mellitus.

Carvedilol treatment significantly improved the endothelial vasomotor function in patients with hypertension and type 2 diabetes, but the effect of metoprolol was not significant.

Patient characteristics at randomization are summarized in Table 6.

Table 6. Patient characteristics.

	Carvedilol (n=19)	Metoprolol (n=17)
Females/males	9/10	7/10
Age (years)	56.3±6.4	56.6±7.9
Duration of hypertension (years)	8.6±7.9	6.2±6.7
Duration of diabetes (years)	5.0±4.1	3.8± 4.9
Systolic Blood pressure (mmHg)	153.7± 9.0	151.3± 6.8
Diastolic Blood pressure (mmHg)	96.8±5.7	94.7±5.9
Heart rate (1/min)	85.1±11.4	91.7±9.9
FMD (%)	2.72±1.11	3.42± 0.89

Data are presented as mean ± SD
FMD:flow-mediated vasodilatation

There were no significant differences between the two groups in any of the characteristics.

Blood pressure and heart rate values before and after treatment are presented in Table 7.

Table 7. Blood pressure and heart rate before and after treatment.

	Carvedilol (n=19)			Metoprolol (n=17)		
	Randomi-zation (W_0)	**12 weeks (W_{12})**	**P**	**Randomi-zation (W_0)**	**12 weeks (W_{12})**	**P**
Systolic (mmHg)	153.7±9.0	133.6±7. 6	<0.0001	151.3±6.8	133.2±4. 9	<0.0001
Diastolic (mmHg)	96.8±5.7	81.6±5.3	<0.0001	94.7±5.9	82.1±2.9	<0.0001
Heart rate (1/min)	85.1±11.4	70.8±7.3	<0.0001	91.7±9.9	73.5±9.3	<0.0001

Data are presented as mean ± SD

Both treatment groups had significant decreases in systolic and diastolic blood pressure and heart rate. There were no significant differences between the two treatment groups in this respect. The doses of the study drugs were doubled after 4 weeks if the patient had not reached the blood pressure goal of 130/80 mmHg. The mean dose of carvedilol was 35.5±12.7 mg, the mean dose of metoprolol succinate was 67.6±24.6 mg during the study period.

There were no significant differences between the flow-mediated dilatation values measured one week prior to randomization, at screening (W-1), and at randomization (W0), in both groups (W-1 2.71±1.12 % vs. W0 2.72±1.11% in carvedilol group, W-1 3.41±0.97% vs. W0 3.42±0.89% in metoprolol group), which supports the reproducibility and repeatibilitiy of the method used. A twelve week treatment with carvedilol significantly improved FMD (W0 2.72±1.11% vs. W12 5.50±2.28%, p<0,0001), while treatment with metoprolol did not change it significantly (W0 3.42±0.89% vs. W12 3.99±1.18%, p=ns) (Figure 8.). Nitrate mediated dilatation, which is endothelium independent, was not influenced by either treatment (W0 13.35±4.41 % vs. W12 13.31±3.14%, p=ns in carvedilol group, W0 12.96±3.51% vs. W12 12.15±2.61 %, p=ns in metoprolol group (Figure 8.).

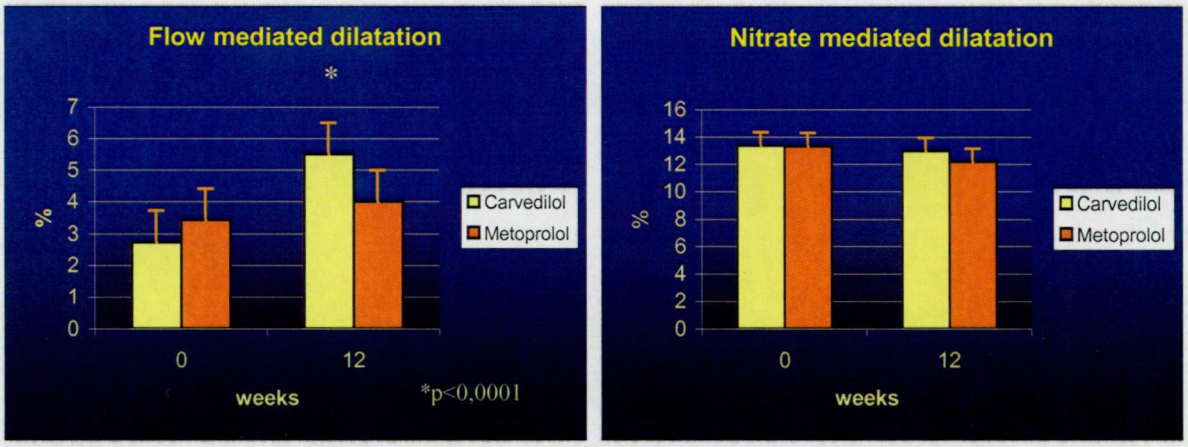

Figure 8. Endothelium-dependent flow-mediated dilatation was significantly better after carvedilol (P<0.0001) (■). In metoprolol group (□) there were no significant improvement in endothelial function at the study end. However, there were no significant differences in nitroglicerin-induced endothelium-independent vasodilatation in both groups.

Carbohydrate or lipid parameters did not change significantly throughout the study period in both groups (table 8). The plasma levels of tissue plasminogen activator (normal range:1-12 ng/mL) and plasminogen activator inhibitor-1 (normal range: 4-43 ng/mL) were elevated at the randomization, but did not change after treatment in both groups. The vascular cell adhesion molecule-1 levels (normal range: 363-484 ng/mL) were in normal range and also unchanged for the study end in both groups.

Table 8. Effects of carvedilol and metoprolol on carbohydrate and lipid parameters and tissue plasminogen activator, plasminogen activator inhibitor-1 and vascular cell adhesion molecule-1

	Carvedilol (n=19)			Metoprolol (n=17)		
Parameters	**W_0**	**W_{12}**	**P**	**W_0**	**W_{12}**	**p**
HbA_{1C} (%)	6.41±0.69	6.73±1.12	0.5421	6.44±0.55	6.81±1.35	0.4326
Fasting glucose (mmol/L)	7.15±1.93	7.35±2.57	0.7172	6.78±1.34	6.75±1.44	0.9057
Total cholsterol (mmol/L)	5.24±0.89	5.25±0.89	0.5227	5.34±0.73	5.18±1.11	0.4326
LDL–cholesterol (mmol/L)	3.02±084	3.11±0.66	0.9622	3.14±0.68	2.99±0.83	0.5509
HDL–cholesterol (mmol/L)	1.33±0.36	1.26±0.26	0.3626	1.24±0.35	1.31±0.44	0.9443
Triglycerides (mmol/L)	1.69±0.45	1.72±0.68	0.8871	1.76±0.52	1.71±0.69	0.9382
t–PA (ng/mL)	55.27±24.24	53.51±18.13	0.5694	71.81±17.89	59.75±21.82	0.0712
PAI–l (ng/mL)	248.44±149.69	220.44±129.91	0.3011	290.02±164.07	271.51±148.31	0.5303
sVCAM–1 (ng/mL)	384.36±138.47	383.63±116.81	0.8767	357.56±94.33	371.22±80.82	0.5302

Data are presented as mean ± SD. (W0-randomization, W12- 12 weeks visit).
HbA_{1C} : glycosylated hemoglobin, LDL-cholesterol: low-densitiy lipoprotein cholesterol, HDL-cholesterol: high-density lipoprotein cholesterol, t-PA: tissue plasminogen activator, PAI-1: plasminogen activator inhibitor-1, VCAM-1: vascular cell adhesion molecule-1

V. Discussion

Endothelial function in the brachial circulation is impaired in the setting of traditional and novel risk factors, such as hypertension, hypercholesterolemia, diabetes, which are frequently seen together and strongly predispose to cardiovascular disease (7-11). While atherosclerosis is associated with a broad alteration in endothelial phenotype, the assessment of endothelium-dependent vasodilation of brachial artery has emerged as an accessible indicator of endothelial function, moreover endothelial function serves as a "barometer" for cardiovascular health that can be used for patient care and evaluation of new therapeutic strategies (83).

V.1. Noninvasive assessment of endothelial dysfunction in essential hypertension; comparison of the flow mediated dilatation of the brachial artery with forearm microvascular reactivity.

Our comparative study results demonstrated that in patients with impaired FMD of the brachial artery essential hypertension is associated with endothelial dysfunction also in the skin microcirculation of the forearm. The lack of correlation between the two methods can be explained by the differences in vascular beds and mechanism involved in the hyperemic response.

The flow-mediated dilation of the brachial artery is induced by the shear stress during flow increase and the principal mediator is endothelium-derived nitrogen oxide. The endothelial cell membrane contains specialized ion channels, that open in response to shear stress (41). The effect of potassium channel opening is to hyperpolarize the endothelial cell, increasing the driving force for calcium entry (there are no voltage-gated calcium channels in endothelial cells). Calcium activates the endothelial nitric oxide synthase (eNOS) and the subsequent generation of NO appaears to account for FMD (42,43). Several mechanisms may underlie the increase in NO in response to changes in shear stress. Very acute changes may be mediated by the increase in intracellular calcium that occurs when ion channels open. Over slightly longer

time periods (minutes), shear-stress-induced phosphorylation of eNOS via a serine/theorine protein kinase, increases eNOS activity, even at low calcium concentrations, and this may be important to allow continued output of NO. Over longer time periods (many minutes or hours) eNOS gene transcription is activated, and this can result in continued increases in NO generation if shear stress is maintained at high levels.

In a previous study Farkas et al. found that microvascular vasodilatory responses to iontophoretically applied acethylcholine and to the release of transient arterial occlusion are impaired in patients with essential hypertension (84). Our group and others have used iontophoretic administration of acethylcholine combined with laser Doppler flowmetry to measure microvascular endothelial function in a variety of major cardiovascular diseases, such as atherosclerosis, coronary heart disease, essential hypertension and diabetes (85). Acethylcholine elicits vasodilation through a complex sequence of events: when applied to blood vessels acethylcholine binds to muscarinic receptors on the surface of endothelial cells, which activates specific G proteins, resulting in the production of nitrogen oxide from L-arginine (catalysed by nitrogen oxide synthase). Nitrogen oxide then diffuses across the intercellular gap, through the interstitial basement membrane and binds to the cytosolic guanylate cyclase of the smooth muscle cells. This induces a rise in cyclic guanosine monophosphate and consequently relaxation (86,87). In addition to eliciting nitrogen oxide production acethylcholine also stimulates the release of the vasodilatators prostacyclin and the endothelium-derived hyperpolarising factor. The relative contribution of these factors to cutaneous vasodilation is unclear. Morris and Shore, Berghoff et al. demonstrated that prostanoid-dependent mechanism do not contribute significantly to endothelium-dependent vasodilation in response to acethylcholine (39,85), whereas Khan et al. and Noon et al. suggested that forearm cutaneous vasodilation is mediated mainly by prostanoid-dependent mechanism (88,89). Another problem is the effect of current alone or the effect of drug

vehicles. Transcutaneous electrical nerv stimulation has been utilized to stimulate axon reflex vasodilation in the skin, but this method, in contrast to most iontophoretic studies uses painful stimuli. Abou-Elenin et al. demonstrated that NaCl solution as iontophoresis vehicle solution had a negligible vasodilatory effect (90) and we found the same at our laboratory.

The postocclusive reactive hyperemia is induced mainly by the shear stress, but vasodilator metabolite produced during the hypoxia can also contribute. Wilkin demonstrated that in cutaneous reactive hyperemia the peak hyperemic flow decreases with increasing durations of arterial occlusion, which is not consistent with changes in concentration of a hypothetized vasodilator metabolite produced during occlusion (90).

Both methods are noninvasive and allow repeated measurements, but need standardized circumstances and trained investigators. Findings in the skin microcirculation assessing endothelial function may correlate with those from other parts of the circulation. The amplitude of flow-mediated vasodilation of the brachial artery was found related to the change in coronary vascular tone induced by the intracoronary infusion of acethylcholine (56), but we have no data concerning the relation of skin microcirculation and coronary vascular tone. Importance of differences in the endothelial dysfunction of different vascular beds need further investigation, supposed, that the different measures of vascular function may have relevance to different aspects of cardiovascular disease.

In conclusion, our results show that endothelium-dependent vasodilation in forearm microcirculation is not related to FMD of the brachial artery. This can be explained by the differences in vascular beds and mechanism involved in the hyperemic response.

As we had not found correlation between laser Doppler flowmetry and FMD of the brachial artery in evaluation of endothelial function, but there is evidence of close relation between brachial artery flow-mediated dilatation and human coronary circulation (54-56), in the

following we use the flow-mediated dilatation of brachial artery for assessment of endothelial function.

V.2. Effect of chronic statin therapy on exercise-induced ST segment depression and on systemic endothelial function in cardiac syndrome-X patients with mild hypercholesterolemia.

Despite the good prognosis of cardiac syndrome-X, the chronic, frequent nature of the persistent angina and the reduced exercise tolerance can significantly impair quality of life (92). The pathophysiology of angina is not clear (24). A functional impairment in the coronary microvasculature causing an abnormal coronary flow reserve is the generally suspected mechanism of angina in this syndrome (16,22-25). In patients with microvascular angina, an impaired forearm vasodilator response to ischaemia has been observed, suggesting that the vascular involvement is not confined to the coronary circulation but is part of a generalized vasomotor disturbance also involving the peripheral conduit arteries (20-24). The fact, that endothelial dysfunction can be improved with the use of statin, due to its pleiotropic effect, is beyond doubt (25,35,62,63). We aimed to assess whether the chronic statin therapy exerts beneficial effects on systemic endothelial function mirrored by changes of ST segment during exercise stress test in cardiac syndrome-X patients with mild hypercholesterolemia. Our study is the first randomized, placebo-controlled study to investigate the effects of simvastatin in cardiac syndrome-X patients with mild hypercholesterolemia.

Hypercholesterolemia induces a number of changes on vascular homeostasis, including a decrease in NO bioactivity, an increase in superoxide production, an increase in endothelin immunreactivity, an increase in adhesion molecules and attenuation of endothelium dependent vasodilation (8,93-102). The cholesterol induced endothelial dysfunction is related to the degree of LDL oxidation and not LDL concentration itself. Oxidized LDL plays an important role in abnormal endothelial vasorelaxation. The detrimental effects of oxidized LDL on

endothelial function are likely modulated through lysophosphatidylcholine, protein kinase C and G proteins (94-96). In addition, oxygen-free radicals impair endothelial function through direct inactivation of NO (97). Statins were able to prevent the inhibitory action exerted by oxidized LDL on eNOS mRNA and protein levels. Hence, these drugs might influence vascular tone by modulating the expression of endothelial vasoactive factors (101,102). Moreover, it has been also demonstrated that 6 months of simvastatin therapy (40 mg/day) exerted a beneficial effect on lowering electronegative LDL proportion and LDL susceptibility to in vitro induced oxidation in 21 patients with heterozygous familiar hypercholesterolemia. (103). The decrease in these cytotoxic particles might be a relevant mechanism by which simvastatin protects against cardiovascular disease (104). Mechanisms which can contribute to the clinical benefit of HMG-CoA reductase inhibitors in coronary artery disease are the following: upregulation of ecNOS expression predominantly by posttranscriptional mechanisms (102), reduction of CD11b expression and inhibition of CD11b-dependent monocyte adhesion to endothelium, inhibition of the PDGF and bFGF-induced DNA synthesis in synchronized smooth muscle cells (105). Moreover, some of these effects are unrelated to the cholesterol-lowering action of this agent and appear to be mediated by enhanced endothelial release of NO and may crucially contribute to the clinical benefit of HMG-CoA reductase inhibitors (25,62,63). Our data are in confirmity with these results, showing that in cardiac syndrome X patients 12 weeks of simvastatin treatment resulted in beneficial effect on lipid parameters and significant improvement in systemic endothelial function.

In the stress imaging era, ECG positivity is regarded as a frequent source of false-positive responses (17). However, it is known that normal coronary arteries frequently coexist with abnormal endothelial function in patients with chest pain. In the study done by Palinkas et al. (19), aimed to evaluate the anatomical coronary epicardial and functional systemic endothelial

determinants of wall motion and electrocardiographic responses during stress testing, significant coronary artery disease was predicted on multivariate analysis by stress-induced wall motion abnormalities, but not by either ST segment depression or reduced flow-mediated dilation. Abnormal flow-mediated dilation was predicted by stress-induced ST segment depression, but not by either stress echo positivity or angiographically assessed coronary artery disease. Our findings show that exercise-induced ST segment depression with normal coronary arteries is associated with an impaired systemic endothelial function. In order to establish a cause-effect relationship between these 2 different factors, it was important to document the possibility to reverse ST segment depression by improving endothelial function with appropriate intervention therapy. This second approach has already been utilized in hypertensive patients with coronary microvascular disease by Virdis et al (106). Hypertensives with angiographically normal coronary arteries and dipyridamole-induced ST segment depression had higher minimal forearm vascular resistances, as assessed by venous plethysmography and using intrabrachial acetylcholine as an endothelium-dependent vasodilator. In addition, after 12 month administration of an angiotensin converting enzyme inhibitor, forearm minimal vascular resistances were significantly reduced only in patients with ECG changes at study entry, who showed disappearance of stress-induced ST segment depression (106). Our study is conceptually germane to that of Virdis et al (106), in spite of the many methodological differences in study design. They studied essential hypertensives, whereas we enrolled hypercholesterolemic patients with normal coronary arteries. They used dipyridamole as an ischemic stress, and we employed exercise (106). Their intervention therapy was obviously different, consisted of an ACE-inhibitor for 12 weeks (106), whereas we used a statin for 12 weeks. The endothelial function was also assessed with different methods, being venous plethysmography in the study by Virdis et al. (106) and high frequency brachial ultrasound in the present study. However, in spite of the many

methodological differences, the results of both studies are convergent in pointing out that exercise-induced ST segment depression is not an innocent finding, since it is associated to a true systemic endothelial dysfunction, and that it can be reversed with appropriate interventions targeted on the most likely underlying etiological factor.

Study limitations

Patients were on antianginal therapy consisted of beta-blockers and/or calcium antagonists, which drugs also could lead to an improvement of endothelial function. However, the patients receiving these medications had endothelial dysfunction before enrolling to the study and the ongoing baseline medications were kept unchanged throughout the study, and thereby were unlikely to have changed the results.

The sample size was limited to 20 patients in both groups. However, we adopted quite strict selection criteria, and the population of cardiac syndrome-X patients was narrowly defined in order to have a homogeneous patient cohort, with normal resting ventricular function, completely normal coronary arteries, transient perfusion defects on thallium scintigrams and exercise induced ischaemia by electrocardiographic criteria. In this study design, each patient acted as his/her own control, averaging out possible confounding variables affecting stress test and/or endothelial function results.

In conclusion, 12 weeks of simvastatin therapy exerts beneficial effects not only on lipid parameters, but also on endothelial function in cardiac syndrome-X patients with mild hypercholesterolemia. The prolonged time to > 1 mm ST segment depression during exercise stress test may reflect the improvement of endothelial function.

V.3.Comparison of the effects of carvedilol and metoprolol on blood pressure and endothelial function in patients with hypertension and type-2 diabetes mellitus.

In the present study, we used FMD to characterize the effects of the endothelium on vasomotor function. The effects of endothelium on fibrinolysis we investigated by measuring the levels of tissue plasminogen activator and plasminogen activator inhibitor-1, on adhesion by vascular cell adhesion molecule-1 concentration. To our knowledge, this was the first human clinical study to perform a head to head comparison of the effects of the selective β1-adrenergic receptor blocker metoprolol with the non-selective β-receptor blocker and α1 adrenergic receptor blocker carvedilol on endothelial function of patients with hypertension and type 2 diabetes mellitus.

The two drugs have been compared in animal studies before. Integnan et al. found that carvedilol significantly improved hypertension-induced endothelial dysfunction in hypertensive rats, while metoprolol had no effect on it (107). Matsuda et al. reported that carvedilol improved FMD in the brachial arteries of coronary atery disease patients (108). On the other hand, Panza et al found no effect of ß receptor blockade on the endothelial function of microvessels (109). On the basis of our results, treatment with carvedilol improved FMD significantly compared with metoprolol succinate. The mechanism of this effect is not completely understood. The effects of the two drugs on blood pressure and heart rate were similar. This suggests that the improved endothelial function seen in the carvedilol group was independent of the blood pressure lowering effect of the drug, and that blood pressure reduction alone does not correct endothelial dysfunction in hypertensive patients with type 2 diabetes. The effects of carvedilol on carbohydrate metabolism has been studied before. One study found that carvedilol had a beneficial effect on insulin sensitivity assessed by isoglycemic hyperinsulinemic glucose clamp in non-diabetic hypertensive patients (110), while others found no effect of carvedilol on glucose tolerance or carbohydrate metabolism in

patients with diabetes (111). In our study of diabetic patients we found no effects of carvedilol or metoprolol on fasting plasma glucose and glycosylated hemoglobin levels. We also found that neither treatment had a significant effect on serum lipid levels after 12 weeks, which agrees with the findings by Goto et al. in hypertensive patients treated with carvedilol for 12 weeks (112), and with other studies, which also found neutral effects of carvedilol on total-, high density-, or low density lipoprotein cholesterol levels (113,114). Thus, our findings also suggest that the improvement in endothelial function seen after carvedilol treatment was not associated with its effects on carbohydrate or lipid metabolism. Since the selective β1-adrenergic receptor blocker metoprolol showed no effect on endothelial function, we theorize that the improvement in FMD seen in our study was mediated by the α1 adrenergic receptor blocking and/or anti-oxidant properties of carvedilol.

In a recent study, Courtney et. al found that the α1-adrenergic blocker doxazosin improved endothelial function assessed by forearm plethysmography in hypertensive patients (115). Similarly, Komai et al. showed by plethysmography that doxazosin improves endothelial function in patients with essential hypertension (116).

Carvedilol has been shown to act as a scavenger of free oxidative radicals (117,118). In type 2 diabetes and hypertension the vasodilator nitric oxide is diminished, likely attributable to excessive production of reactive oxygen species (119). This could result in impaired endothelial function. As nitric oxide synthase activity, and thus, nitric oxide availability, is enhanced when reactive oxygen species are reduced, the radical scavenging property of antihypertensive agents like carvedilol could improve endothelial function via this mechanism (120).

The assessment of endothelial function is possible through examining of the endothelial plasma markers. The plasma levels of tissue plasminogen activator and plasminogen activator inhibitor-1 were elevated at the randomization, which data support the findings of majority of

clinical studies investigating these markers of fibrinolitic system (121-123). After 12 weeks of treatment neither carvedilol nor metoprolol had a significant effect on tissue plasminogen activator or plasminogen activator inhibitor-1 values. Our findings agree with those of Maqueda, who also found no effects of carvedilol on endothelial fibrinolytic activity of patients with moderate hypertension (124). The effect of endothelium on adhesion was examined in our study by measuring vascular cell adhesion molecule-1, and we found that neither carvedilol, nor metoprolol had a significant influence on it. To our knowledge, the effect of carvedilol on VCAM-1 levels has not been studied before in this aspect.

Study limitations

A part of patients were on calcium antagonist therapy, which also could lead to an improvement of endothelial function. However, the patients receiving these medications had endothelial dysfunction before enrolling to the study and the ongoing baseline medications were kept unchanged throughout the study, and thereby were unlikely to have changed the results.

In conclusion, the 12 weeks of treatment with carvedilol improves endothelial (vasomotor) function assessed by FMD, but had not significant effects on endothelial markers in patients with type 2 diabetes and hypertension, compared to metoprolol succinate. At least in part, this favorable effect of carvedilol could be due to its other, independent of the blood pressure lowering, effects.

VI. Summary and conclusions

As the flow-mediated vasodilation of brachial artery has emerged as an accessible indicator of endothelial function we aimed to investigate its use for non-invasive evaluation of endothelial dysfunction and assessment of effects of new pharmacologic interventions in clinical settings. Initially, we designed a comparative study to correlate the ability of two different non-invasive methods, such as flow mediated dilatation of the brachial artery with the laser Doppler flowmetry in the detection of endothelial vasomotor function in patients with hypertension and normotensive subjects. Following, we use the flow-mediated brachial artery vasodilation for evaluation of effects of chronic simvastatin therapy on systemic endothelial function and exercise-induced ST segment depression in cardiac syndrome-X patients with mild hypercholesterolemia. Finally, we compare the effects of carvedilol and metoprolol on blood pressure, endothelial function and metabolic parameters in patients with hypertension and type-2 diabetes mellitus.

On the basis of our results:

1. Endothelial dysfunction was detectable both with laser Doppler flowmetry and flow-mediated vasodilation of brachial artery in essential hypertension, but the endothelium-dependent vasodilation in forearm microcirculation was not related to FMD of the brachial artery.

2. Twelve weeks of simvastatin therapy exerts beneficial effects not only on lipid parameters, but also on endothelial function, detected by FMD, in cardiac syndrome-X patients with mild hypercholesterolemia. The prolonged time to > 1 mm ST segment depression during exercise stress test reflected the improvement of endothelial function.

3. Twelve weeks of treatment with carvedilol significantly improved endothelial (vasomotor) function, assessed by FMD, but had not significant effects on endothelial markers in patients with type 2 diabetes and hypertension compared to metoprolol succinate.

In conclusion, the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery is utilisable for the clinical evaluation of endothelial function and effects of appropriate pharmacologic interventions, targeted on the underlying etiological factors, in clinical settings.

VII. References

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