

# Coincidence of moderately elevated N-terminal pro-B-type natriuretic peptide, endothelial progenitor cells deficiency and propensity to exercise-induced myocardial ischemia in stable angina

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**Abstract.** *Aim:* To assess endothelial progenitor cells (EPC) counts, a novel prognostic marker, in relation to classical adverse outcome predictors – N-terminal pro-B-type natriuretic peptide (NT-proBNP), impaired left ventricular (LV) relaxation and exercise-induced ischemia – in stable coronary artery disease (CAD) with preserved LV systolic function.

*Methods:* We studied 30 non-diabetic men with one-vessel CAD, LV ejection fraction  $\geq 60\%$  and normal LV diastolic function ( $n = 16$ ) or impaired LV relaxation (by ultrasound including tissue Doppler) ( $n = 14$ ), and 14 non-CAD controls matched for risk profile and medication. CD34+/kinase-insert domain receptor (KDR)+ cells (CD34+/KDR+ cells), a leukocytes subpopulation enriched for EPC, were enumerated by flow cytometry.

*Results:* CAD patients with abnormal LV relaxation exhibited significantly elevated NT-proBNP and decreased CD34+/KDR+ cells vs. CAD with regular diastolic function and non-CAD controls. An inverse NT-proBNP–CD34+/KDR+ cells relationship was precipitated by the clustering of high resting NT-proBNP and low CD34+/KDR+ cells in the subjects with a lower Duke treadmill score.

*Conclusions:* Propensity to symptomatic exertional ischemia may underlie the coincidence of moderately elevated NT-proBNP and EPC deficiency in stable angina. Additionally, chronic subclinical ischemia can also be involved in these associations. These might result from BNP overexpression in the ischemic myocardium and a hypothetical exhaustion of the bone marrow capacity to mobilize EPC at multiple ischemic episodes, thus contributing to NT-proBNP prognostic effect irrespective of hemodynamic factors.

**Keywords:** B-type natriuretic peptide, endothelial progenitor cells, coronary artery disease, left ventricular diastolic dysfunction, myocardial ischemia

## 1. Introduction

Elevated circulating levels of B-type natriuretic peptide (BNP) or N-terminal fragment of its prohormone (NT-proBNP) are adverse outcome predictors not only in heart failure and acute coronary syndromes but also

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in stable coronary artery disease (CAD) [1–6]. The prognostic effect of BNP/NT-proBNP in stable CAD was maintained after multivariate adjustment including ejection fraction (EF), angiographic CAD extent, renal function, left ventricular (LV) end-diastolic pressure (LVEDP) and echocardiographic categories of diastolic dysfunction [1–6], which suggests that the effect extends also into low-risk subjects with discrete or absent LV function abnormalities. An excessive cardiovascular mortality risk was reported already at NT-proBNP levels over 67–120 pg/ml by an electrochemiluminescence immunoassay [1,2,5,6], i.e. close to upper limits of the normal range [7] and similar to those proposed in the diagnostics of isolated diastolic dysfunction or heart failure [8,9]. In patients with preserved EF moderate elevations of B-type natriuretic peptides were described in parallel to the degree of LV diastolic dysfunction with about 6-fold and 3-fold increases in BNP and NT-proBNP, respectively, in the earliest phase of diastolic abnormalities, equivalent to impaired LV relaxation [8, 10]. Furthermore, BNP gene is overexpressed in the hypoxic myocardium [11].

In the presence of a flow-limiting coronary stenosis impaired relaxation results from abnormal calcium handling in cardiomyocytes that predominantly affects the subendocardium [12]. Pritchett et al. [13] observed an independent rise in all-cause mortality in the presence of abnormal LV relaxation (i.e. mild diastolic dysfunction) by classical indices of transmitral and pulmonary flow in a population-based study of 2,042 residents  $\geq 45$  years of age over a 6-year follow-up. With regard to cardiac death, Wang et al. [14] have shown incremental prognostic value of an index of LV relaxation derived from tissue Doppler imaging (TDI) compared to clinical data and variables based on mitral inflow velocities in 518 subjects (including 353 patients with various cardiac diseases) over a 2-year follow-up. This index of long axis LV function, peak early lengthening velocity at the junction of the LV wall with the mitral annulus ( $E'$ ), represents a relatively preload-independent measure of relaxation and is governed by longitudinally oriented subendocardial fibers [15,16]. Lee et al. [17] reported that TDI indices of diastolic function (but not parameters derived from transmitral flow) were related to biochemical measures of endothelial dysfunction and platelet activation independently of LV systolic function in stable angina patients free of heart failure.

Endothelial dysfunction, another predictor of adverse outcome in CAD [18], coincides with deficiency of bone marrow-derived blood endothelial progenitor

cells (EPC) in subjects with atherosclerotic risk factors [19] and in CAD [20]. EPC are supposed to participate in endothelial renewal [21,22] and neovascularization of ischemic tissues [23]. Furthermore, depletion of circulating CD34/kinase-insert domain receptor (KDR)-double positive cells (a leukocytes subpopulation enriched for EPC) (CD34+/KDR+ cells) is considered, like BNP, an adverse outcome predictor in CAD [24,25].

We hypothesized that a lower CD34+/KDR+ cells count might appear already in patients with stable angina and impaired LV relaxation thus contributing to the prognostic effect of moderately elevated concentrations of BNP/NT-proBNP. On the other hand, as a single episode of exercise-dependent myocardial ischemia induced a persistent (up to 48 h) mobilization of EPC [26], patients with repetitive ischemic episodes could exhibit rather a sustained rise in CD34+/KDR+ cells numbers in addition to an elevated NT-proBNP level.

Therefore our aim was to investigate blood CD34+/KDR+ cells counts in relation to classical adverse outcome predictors: plasma NT-proBNP levels, impaired LV relaxation pattern and exercise-induced ischemia in a relatively homogenous group of non-diabetic men with stable angina, one-vessel CAD and preserved LV function.

## 2. Materials and methods

### 2.1. Subjects

We studied 2 groups of patients undergoing planned diagnostic coronary angiography in our center. The CAD group was formed by 30 men who fulfilled all four of the following inclusion criteria: stable angina class III according to the Canadian Cardiovascular Society (CCS) classification, angiographically significant one-vessel CAD, classical ECG signs of subendocardial myocardial ischemia during an exercise test [27], and an unchanged therapy with a low-dose aspirin, statin and angiotensin-converting enzyme inhibitor (ACEI) for 3 preceding months. Angiographically significant CAD was defined as the presence of at least 1 diameter stenosis of  $\geq 70\%$  within major epicardial segments of one of coronary arteries according to the American College of Cardiology/American Heart Association (ACC/AHA) guidelines on the management of patients with chronic stable angina [28].

The control non-CAD group consisted of 14 men without coronary narrowings exceeding 20%, matched

for age, BMI, GFR and other risk factors and receiving a similar medication. In the controls coronary angiography was performed due to atypical angina and an inconclusive exercise testing result.

Out of potentially eligible 115 pre-screened patients, 71 had been eliminated by a wide set of the exclusion criteria that included age below 40 or over 70 years, a history of heart failure, acute coronary syndromes, percutaneous or operative coronary revascularization, diabetes mellitus, chronic kidney disease (estimated glomerular filtration rate (GFR) below 60 ml/min/1.73 m<sup>2</sup> as calculated according to the simplified Modification of Diet in Renal Disease study equation), atrial fibrillation or flutter, arterial hypertension uncontrolled adequately by drugs, hemoglobin < 11 g/dl, thrombocytopenia (< 10<sup>5</sup>/μl), chronic obstructive pulmonary disease, pulmonary embolism, abnormalities of thyroid or liver function, malignant or inflammatory diseases, any surgery within past 6 months, any infections within past 2 months, chronic non-cardiovascular medication.

Additionally, we had excluded subjects with pericardial disease, significant valvular heart disease, congenital heart disease, abnormal LV diameters, segmental LV wall-motion abnormalities, EF below 60% (according to a modified biplane Simpson's method from apical 2- and 4-chamber views), LV hypertrophy (defined as LV mass index (LVMI) over 125 g/m<sup>2</sup> calculated according to Devereux formula) [29], and echocardiographic patterns of LV diastolic dysfunction other than its mild form, i.e. impaired LV relaxation.

## 2.2. Echocardiographic assessment of LV diastolic function

During a routine cardiac ultrasound (Vivid 7®, GE Healthcare Medical Diagnostics, Little Chalfont, UK) the impaired relaxation pattern was identified using a two-step approach proposed by Tschöpe et al. [30]. A preliminary condition was the presence of at least two abnormal indices of transmitral flow: isovolumic LV relaxation time (IVRT) > 100 ms, deceleration time (DT) of mitral E wave > 220 ms, or E/A ratio < 1.0 for subjects below 50 years of age. Respective cut-off values for those aged ≥ 50 years were IVRT > 105 ms, DT > 280 ms and E/A < 0.5 [9,31]. Then impaired LV relaxation was confirmed by TDI indices measured at the junction of the LV lateral wall with the mitral annulus: peak early myocardial lengthening velocity (E') < 8 cm/s and the ratio of early-to-late peak diastolic myocardial lengthening velocities (E'/A') below 1.0 [8,

32]. Normal LV diastolic function was defined as E/A = 1.0–2.0. IVRT = 60–100 ms for age < 50 years (60–105 ms for age ≥ 50 years) ms, DT = 150–210 (240) ms, E' > 8 cm/s and E'/A' > 1.0 irrespective of age [8,32].

In order to exclude more advanced stages of diastolic dysfunction (i.e. moderate or severe dysfunction associated with elevated LV diastolic pressures) or confirm normal LV diastolic function, E/E' ratio was calculated and flow pattern in the right upper paraseptal pulmonary vein was studied. All the patients – irrespective of the presence of the impaired relaxation pattern – had to exhibit an E/E' < 15, systolic-dominant pulmonary venous flow, peak velocity of the pulmonary venous atrial reversal wave < 35 cm/s, the difference between pulmonary venous atrial reversal duration and mitral A wave duration below 30 ms [8,9,16,32,33] and left atrial volume index (LAVI) at ventricular end-systole calculated from parasternal long-axis view and the apical 4-chamber view [8,13,34] below 29 ml/m<sup>2</sup> [13].

## 2.3. ECG exercise testing

A treadmill symptom-limited ECG exercise test according to the Bruce protocol was performed in all CAD subjects in agreement with the ACC/AHA standards as a part of the routine diagnostic procedure [27]. Patients with resting ECG abnormalities that would make ECG changes on exercise non-interpretable had been *a priori* excluded. Reasons for exercise termination was severe dyspnea, fatigue, limiting angina, a decrease in systolic blood pressure of > 10 mmHg or a ST segment depression of ≥ 0.3 mV. In no case was the test terminated due to a ST segment elevation of > 0.1 mV, serious arrhythmias, bundle-branch block, an excessive hypertensive response or central nervous system symptoms. The following parameters were computed: exercise duration, maximal workload achieved (metabolic equivalents, METs), maximal horizontal or downsloping ST segment depression in any lead during exercise or 6 min of recovery (measured in 3 consecutive beats with a stable baseline at 60–80 ms after the J point relative to the PQ junction and minus resting changes) [27], time to onset of a 0.1-mV horizontal or downsloping ST segment depression, and Duke treadmill score. The Duke treadmill score integrates exercise duration, maximal ST segment depression in any lead and presence and type of angina during the test [35].

#### 2.4. Blood collection

The Bioethical Committee of our university has approved the protocol and the patients gave informed consent. Fasting venous blood samples for assay of CD34+/KDR+ cells were drawn after an overnight fast on the occasion of routine blood sampling 0–2 days prior to a planned coronary angiography and 1–2 days after echocardiography. Simultaneously, plasma was separated and samples frozen at  $-70^{\circ}\text{C}$  until further measurements of NT-proBNP, vascular endothelial growth factor (VEGF) and high-sensitivity C-reactive protein levels.

#### 2.5. Flow cytometric analysis

The procedure was performed  $< 60$  min from the venopuncture as previously described [24,25,36,37]. In brief, 100  $\mu\text{l}$  of blood was incubated in the dark with mouse monoclonal antibodies against human VEGF receptor type 2 (KDR) (Sigma, St. Louis, MO, USA) followed by rabbit fluorescein isothiocyanate (FITC)-labeled secondary antibodies (Dako, Glostrup, Denmark) and phycoerythrin (PE)-conjugated mouse monoclonal antibodies against human CD34 (Becton Dickinson, Franklin Lakes, NJ, USA). Control blood samples were incubated with mouse isotype-matched antibodies (IgG<sub>1</sub>-FITC and IgG<sub>2a</sub>-PE,  $\gamma$ -Simultest, Becton Dickinson). After lysis of erythrocytes, flow cytometric analysis was performed including 100,000 cytometric events (FACScan, Becton Dickinson GmbH, Heidelberg, Germany). EPC were defined as CD34/KDR-double positive cells and their number was expressed as a percentage of peripheral blood mononuclear cells (PBMC) within the pre-specified lymphocyte gate [20, 24,25,36,37].

#### 2.6. Assay for NT-proBNP, VEGF and high-sensitivity C-reactive protein

NT-proBNP and VEGF were measured with commercially available immunoassays (Elecsys proBNP, Roche Diagnostics GmbH, Mannheim, Germany, and R&D Systems, Minneapolis, MN, USA, respectively). According to the manufacturers' instructions, the lower detection limit for both analytes was 5.0 pg/ml and intra-assay and inter-assay coefficients of variation  $< 4\%$  (NT-proBNP) and  $< 9\%$  (VEGF). High-sensitivity C-reactive protein was estimated by a chemiluminescent immunoassay system (Immulite 1000, DPC, Flinders, NJ, USA).

#### 2.7. Statistical analysis

Data are presented as means  $\pm$  standard deviations (SD) for variables with normal distribution, medians and interquartile ranges (25th and 75th percentiles) for not normally distributed continuous parameters (NT-proBNP levels, CD34+/KDR+ cells counts and high-sensitivity C-reactive protein concentrations) and numbers (percentages) for categorical variables. Comparisons of the not normally distributed variables between 3 groups – CAD with abnormal LV relaxation, CAD with regular LV diastolic function and non-CAD controls – were performed by the Kruskal-Wallis analysis of variance (ANOVA) on ranks followed by the Mann-Whitney  $U$  test for pairwise analyses. Intergroup comparisons of the remaining continuous variables were done by one-way ANOVA and the Tukey's honest significant difference post-hoc tests, whereas categorical variables were assessed by the Fisher's 2-sided exact test. Exercise testing results and angiographic data between 2 subgroups of CAD subjects were compared by the unpaired Student's 2-sided  $t$ -test or the Fisher's test for proportions.

In order to identify correlates of NT-proBNP levels and CD34+/KDR+ cells numbers, Spearman's rank order correlation coefficients ( $\rho$ ) were calculated separately for CAD patients and non-CAD controls. Pearson's correlation coefficients ( $r$ ) were used after prior logarithmic data transformation to obtain a normal distribution. Independent determinants of logarithmic derivatives of NT-proBNP levels and CD34+/KDR+ cells counts in CAD subjects were selected by means of backward stepwise multiple regression with a ridge regression approach to control for mutual correlations between parameters obtained from ECG exercise testing and echocardiography. Only correlates of log (NT-proBNP) or log (CD34+/KDR+ cells count) with a  $p$ -value  $\leq 0.15$  at univariate analysis were included in preliminary sets of potential covariates in the multiple regression Adjusted coefficients of multiple determination ( $R^2$ ) and standard mean regression coefficients ( $\beta \pm \text{SEM}$ ) for individual variables entering the final regression equations were shown. A  $p$ -value  $\leq 0.05$  was considered statistically significant.

### 3. Results

#### 3.1. Patient characteristics – clinical, biochemical, angiographic, echocardiographic parameters and exercise testing results

There were no significant differences between 3 groups in clinical, biochemical and angiographic char-

Table 1  
Patient characteristics – clinical and biochemical data

	Non-CAD controls (n = 14)	CAD normal diastolic LV function (n = 16)	CAD impaired LV relaxation (n = 14)	p
Age (years)	57 ± 10	56 ± 8	56 ± 9	NS
BMI (kg/m <sup>2</sup> )	27.0 ± 3.5	26.6 ± 3.4	27.0 ± 3.1	NS
GFR (ml/min/1.73 m <sup>2</sup> )	90 ± 16	92 ± 14	94 ± 15	NS
Smoking habit (%)	6 (43)	7 (44)	7 (50)	NS
Hypertension (%)	5 (36)	5 (31)	5 (36)	NS
Blood pressure (mmHg)	93 ± 9	92 ± 10	94 ± 11	NS
Heart rate (beats/min)	67 ± 7	64 ± 8	65 ± 7	NS
LDL cholesterol (mmol/l)	2.8 ± 0.8	2.9 ± 0.8	2.7 ± 0.9	NS
Fasting glucose (mmol/l)	4.8 ± 0.7	4.7 ± 0.8	4.7 ± 0.9	NS
C-reactive protein (mg/l)	1.7 (0.9–2.9)	1.9 (0.8–3.4)	1.8 (0.9–3.5)	NS
VEGF (pg/ml)	16 ± 10	19 ± 11	17 ± 12	NS
Drugs besides AA+ACEI+statins				
Long-acting nitrates (%)	12 (86)	15 (94)	13 (93)	NS
Beta-blockers (%)	12 (86)	13 (81)	13 (93)	NS
Calcium channel blockers (%)	5 (36)	5 (31)	6 (43)	NS

Data are shown as means ± SD, n (%) and medians (interquartile range). p-values by ANOVA or Kruskal-Wallis ANOVA for continuous variables and Fisher's 2-tailed test for categorical variables. AA: acetylsalicylic acid, ACEI: angiotensin-converting enzyme inhibitors, BMI: body mass index, CAD, coronary artery disease, GFR, glomerular filtration rate, LDL, low-density lipoprotein, LV, left ventricular, VEGF: vascular endothelial growth factor.

Table 2  
Patient characteristics – angiographic data

	CAD normal diastolic LV function (n = 16)	CAD impaired LV relaxation (n = 14)	p
Type of vessel involved			
LAD/LCX/RCA	8/2/6	7/2/5	
%	50/12.5/37.5	50/14/36	NS
Maximal coronary stenosis (%)	88 ± 11	87 ± 10	NS
At least one additional stenosis of ≥50% in the same coronary artery territory %	4 (25)	5 (36)	NS

Data are shown as means ± SD or n (%). p-values by unpaired Student's 2-sided t-test for continuous variables or Fisher's 2-tailed test for proportions. LAD: left anterior descending coronary artery, LCX: left circumflex coronary artery, RCA: right coronary artery.

acteristics (Tables 1 and 2).

CAD subjects with impaired LV relaxation exhibited a significantly longer IVRT and mitral E wave DT, a decreased E/A ratio as well as lower indices of relaxation by TDI, E' and E'/A' ratio, with the reference to the 2 remaining groups (Table 3; Fig. 1). EF, LVMI and parameters reflecting LV filling pressures, LAVI and E/E' ratio, were similar across the 3 groups (Table 3; Fig. 1). As compared to CAD subjects with normal LV diastolic function, CAD patients with impaired LV relaxation exhibited significant decreases in exercise duration, maximal workload achieved, time to onset a 0.1-mV ST segment depression and Duke treadmill score as well as higher maximal ST segment displacements

(Table 4).

### 3.2. Intergroup comparisons of NT-proBNP levels and CD34+/KDR+ cells counts

With the reference to the non-CAD controls (46 [28–120] pg/ml), NT-proBNP levels were significantly elevated in CAD subjects (135 [79–366] pg/ml), which was more pronounced in those with impaired LV relaxation (274 [137–423] pg/ml) relative to a subgroup with regular diastolic function (99 [59–178] pg/ml) (Fig. 2).

Additionally, in the presence of abnormal LV relaxation CD34+/KDR+ cells counts were significantly decreased (0.025 [0.02–0.03] %) as compared to the

Table 3  
Patient characteristics – echocardiographic data

	Non-CAD controls ( <i>n</i> = 14)	CAD normal diastolic LV function ( <i>n</i> = 16)	CAD impaired LV relaxation ( <i>n</i> = 14)	<i>p</i> ANOVA
EF (%)	71 ± 6	73 ± 5	71 ± 8	NS
LVMI (g/m <sup>2</sup> )	98 ± 21	97 ± 18	100 ± 22	NS
LAVI (ml/m <sup>2</sup> )	22.5 ± 4.3	22.8 ± 3.8	23.5 ± 3.3	NS
IVRT (ms)	87 ± 15	86 ± 17	121 ± 22**	< 0.001
Mitral E wave DT (ms)	192 ± 43	201 ± 39	254 ± 40*	< 0.001
E/A ratio	1.4 ± 0.3	1.4 ± 0.4	0.7 ± 0.3**	< 0.001
E' (cm/s)	11.0 ± 2.3	10.7 ± 2.0	5.3 ± 2.1**	< 0.001
E'/A' ratio	1.4 ± 0.3	1.5 ± 0.4	0.7 ± 0.2**	< 0.001
E/E' ratio	7.7 ± 2.0	8.0 ± 1.8	8.2 ± 2.3	NS
Sm (cm/s)	8.9 ± 1.6	9.2 ± 1.7	8.7 ± 1.5	NS

Data are shown as means ± SD. \**p* < 0.01, \*\**p* < 0.001 vs. CAD subjects with normal diastolic function and non-CAD controls by the Tukey's post-hoc test. CAD: coronary artery disease, DT: deceleration time, E: peak early diastolic mitral inflow velocity, EF: ejection fraction, E': peak early diastolic myocardial lengthening velocity at the junction of the LV lateral wall with the mitral annulus, E'/A': the ratio of early-to-late peak diastolic myocardial lengthening velocities, IVRT: isovolumic LV relaxation time, LAVI: left atrial volume index, LV: left ventricular, LVMI: left ventricular mass index, Sm: peak systolic myocardial lengthening velocity at the junction of the LV lateral wall with the mitral annulus.

Table 4  
CAD patients – exercise testing results

	CAD normal diastolic LV function ( <i>n</i> = 16)	CAD impaired LV relaxation ( <i>n</i> = 14)	<i>p</i>
Exercise duration (min)	5.3 ± 1.3	4.4 ± 1.1	0.05
Maximal workload achieved (METs)	6.2 ± 1.4	5.1 ± 1.3	0.03
Time to 0.1-mV ST depression (min)	3.8 ± 1.0	3.0 ± 0.8	0.02
Maximal ST depression in any lead (mV)	0.19 ± 0.03	0.22 ± 0.04	0.04
Exertional chest pain (%)	6 (37.5)	8 (57)	NS
Duke treadmill score	-5.2 ± 2.7	-7.8 ± 2.3	0.008

Data are shown as means ± SD or *n* (%). Intergroup *p*-values by the unpaired Student's 2-sided *t*-test. CAD: coronary artery disease, LV: left ventricular, METs: metabolic equivalents.

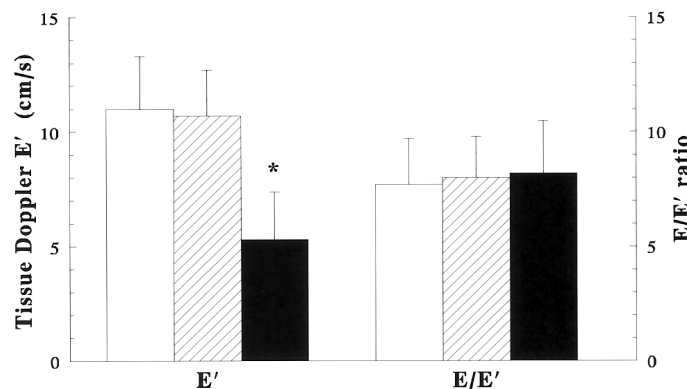


Fig. 1. Intergroup comparisons of tissue Doppler indices of LV diastolic function (means ± SD) in CAD subjects with (solid bars) or without (hatched bars) impaired LV relaxation and non-CAD controls (empty bars): E' (*p* < 0.001 by ANOVA) and E/E' ratio (*p* > 0.5 by ANOVA). \**p* < 0.001 vs. controls and CAD subjects with normal diastolic function by the Tukey's post-hoc test. Abbreviations as in Table 2.

Table 5  
CAD subjects – correlates of NT-proBNP level and CD34+/KDR+ cells count

	NT-proBNP level		CD34+/KDR+ cells count	
	<i>rho</i>	<i>p</i>	<i>rho</i>	<i>p</i>
Age	0.20	NS	0.09	NS
GFR	-0.29	0.12	0.30	0.11
LDL cholesterol	-0.03	NS	-0.07	NS
Fasting glucose	0.06	NS	-0.15	NS
High-sensitivity C-reactive protein	0.04	NS	0.01	NS
Left ventricular EF	-0.10	NS	0.05	NS
Left ventricular mass index	0.01	NS	0.04	NS
Left atrial volume index	0.22	NS	-0.05	NS
Heart rate	0.12	NS	-0.02	NS
E'	-0.46	0.01	0.40	0.03
E'/A' ratio	-0.49	0.006	0.43	0.02
E/E' ratio	0.29	0.12	-0.11	NS
Exercise duration	-0.31	0.10	0.27	0.15
Maximal workload achieved	-0.33	0.07	0.24	NS
Time to 0.1-mV ST depression	-0.40	0.03	0.25	NS
Max. ST depression in any lead	0.24	NS	-0.38	0.04
Duke treadmill score	-0.58	< 0.001	0.52	0.003
Maximal coronary stenosis	0.05	NS	0.07	NS
Additional $\geq 1$ stenoses of $\geq 50\%$	0.14	NS	-0.10	NS
Calcium channel blockers	-0.04	NS	0.08	NS

*rho*: Spearman's rank order correlation coefficient; other abbreviations as in Tables 1 and 3.

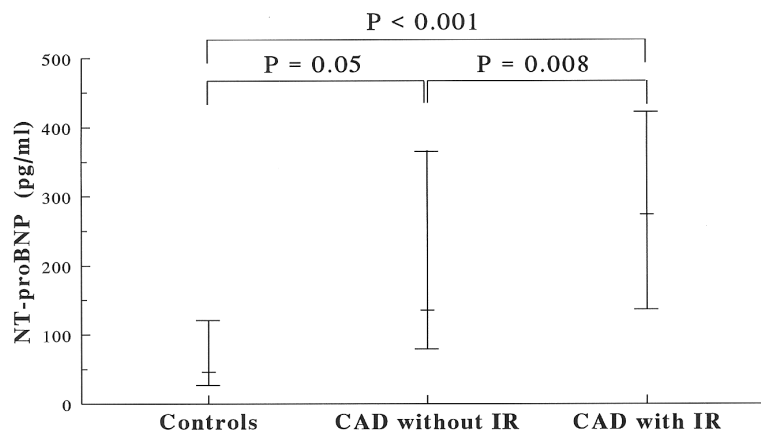


Fig. 2. NT-proBNP levels (medians and interquartile ranges) in CAD subjects with or without impaired LV relaxation (IR) and non-CAD controls. Pairwise *p*-values refer to the Mann-Whitney *U* test (*p* = 0.005 by the Kruskal-Wallis ANOVA).

non-CAD control group (0.05 [0.04–0.08] %) and CAD subjects with regular diastolic function (0.045 [0.035–0.05] %) (Fig. 3).

### 3.3. Correlates of NT-proBNP levels and CD34+/KDR+ cells counts

In CAD patients NT-proBNP concentrations correlated modestly to E' and E'/A', whereas weakly to GFR and E/E' ratio (Table 5). CD34+/KDR+ cells counts were modestly correlated with E' and E'/A' ratio, and weakly with GFR. Weak correlations were found be-

tween NT-proBNP and CD34+/KDR+ cells numbers versus parameters derived from the ECG exercise test, except for the Duke treadmill score that strongly correlated with both these variables (Table 5). The Duke treadmill score was the only variable which entered final regression equations describing log-transformed NT-proBNP levels ( $\beta = -0.54 \pm 0.15$ ,  $p = 0.001$ , adjusted  $R^2 = 0.29$ ) or CD34+/KDR+ cells counts ( $\beta = 0.46 \pm 0.16$ ,  $p = 0.007$ , adjusted  $R^2 = 0.20$ ) in CAD patients.

NT-proBNP levels and CD34+/KDR+ cells numbers were inversely correlated in CAD subjects:  $\rho = -0.48$ ,  $p = 0.007$  for untransformed data (Fig. 4A);  $r =$

Table 6  
Coincidence of elevated NT-proBNP levels and depressed CD34+/KDR+ cells counts in the CAD patients with a Duke treadmill score below the median (< -6.7)

	NT-proBNP (pg/ml)		CD34+/KDR+ cells (%)	
	> 135	< 135	> 0.03	≤ 0.03
Duke treadmill score < -6.7 (n = 15)	12 (80%)	3 (20%)	4 (27%)	11 (73%)
Duke treadmill score > -6.7 (n = 15)	3 (20%)	12 (80%)	10 (67%)	5 (33%)
<i>p</i> -value by the Fisher's test	0.003		0.035	

Numbers of patients and respective percentages (in parentheses) have been shown.

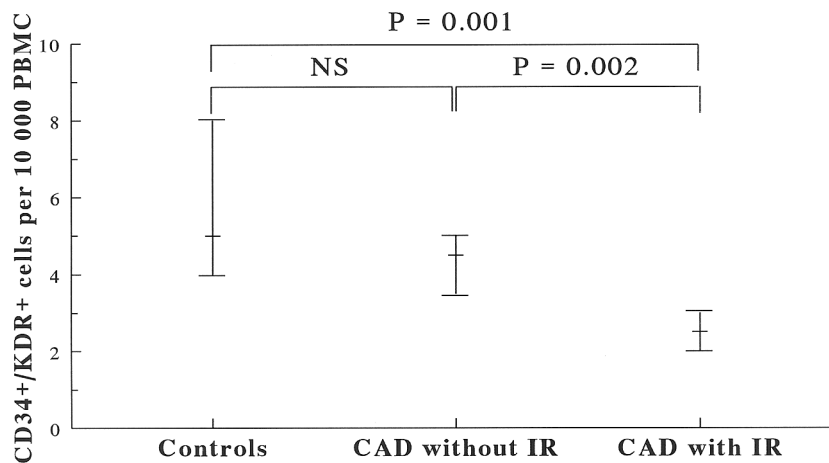


Fig. 3. CD34+/KDR+ cells counts (medians and interquartile ranges) in CAD patients with or without impaired LV relaxation (IR) and non-CAD controls. Pairwise *p*-values refer to the Mann-Whitney *U* test (*p* = 0.003 by the Kruskal-Wallis ANOVA). PBMC: peripheral blood mononuclear cells.

-0.42, *p* = 0.02 for log-transformed values. This was mainly due to the clustering of increased NT-proBNP and decreased CD34+/KDR+ cells at a low Duke treadmill score (Fig. 4A). Out of 15 patients with the treadmill score below the median (< -6.7), 12 subjects (80%) exhibited NT-proBNP levels over the median (> 135 pg/ml) and in 11 subjects (73%) CD34+/KDR+ cells counts did not exceed the respective median (≤ 0.03 %) (Table 6). The importance of the Duke treadmill score for the coincidence of elevated NT-proBNP and decreased CD34+/KDR+ cells counts was confirmed by multiple regression. When both Duke treadmill score and log (NT-proBNP) were forced into the multiple regression equation with a log-derivative of CD34+/KDR+ cells numbers as a dependent variable (adjusted  $R^2 = 0.32$ , *p* < 0.002), the effect of the treadmill score was maintained ( $\beta = 0.43 \pm 0.16$ , *p* = 0.01), whereas that of NT-proBNP lost its significance ( $\beta = -0.23 \pm 0.16$ , *p* = 0.2).

In the non-CAD controls no association between NT-proBNP concentrations and CD34+/KDR+ cells numbers was found: *rho* = 0.10, *p* > 0.7 for untransformed data (Fig. 4B); *r* = 0.21, *p* > 0.4 for log-transformed values. NT-proBNP tended to correlate weakly with

age (*r* = 0.42, *p* = 0.13) and GFR (*r* = -0.40, *p* = 0.15).

#### 4. Discussion

In our small observational study stable angina patients with preserved EF and one-vessel obstructive CAD exhibited higher plasma NT-proBNP levels, especially at impaired LV relaxation but also at normal LV diastolic function by conventional indices and TDI. Additionally, a significantly lower CD34+/KDR+ cells blood count was observed in the presence of impaired relaxation associated with a more pronounced ischemic burden.

As early as in 1980 Hirota [12] described a prolonged time constant of LV pressure decay during isovolumic relaxation in stable angina with normal LV systolic function. The importance of a decreased coronary flow reserve for LV relaxation was also confirmed by Bonow et al. [38] who demonstrated an improvement in LV early filling at rest after successful coronary angioplasty in one-vessel CAD. Importantly, our CAD patients with



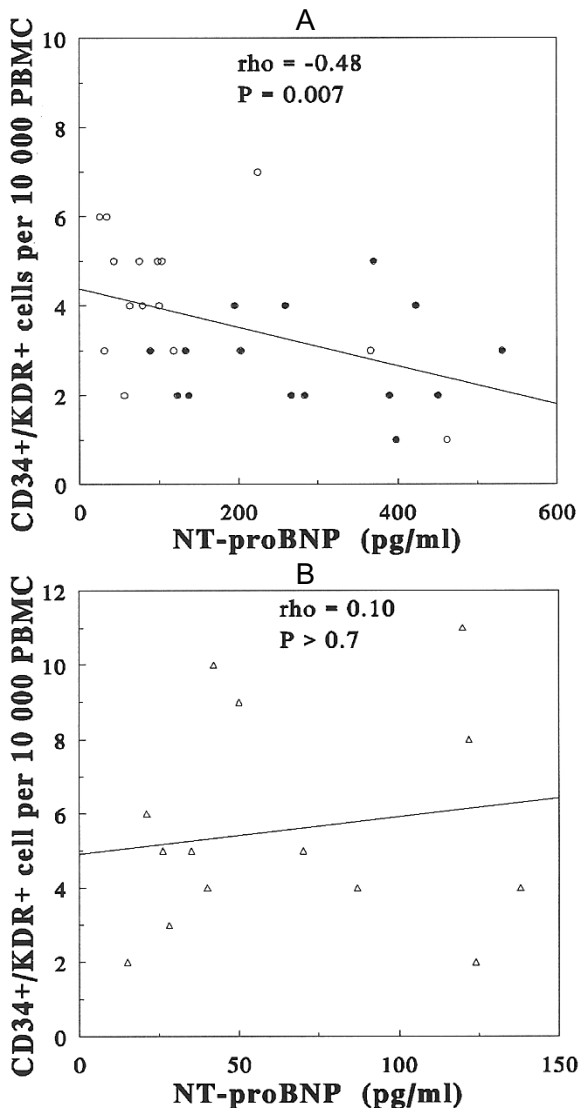


Fig. 4. (A, upper panel): A negative correlation between CD34<sup>+</sup>/KDR<sup>+</sup> cells counts and NT-proBNP levels in 30 CAD patients with Duke treadmill score below the median (< -6.7) (closed circles) or over the median (> -6.7) (open circles). (B, lower panel): The lack of association between CD34<sup>+</sup>/KDR<sup>+</sup> cells counts and NT-proBNP levels in 14 non-CAD controls (open triangles). rho: Spearman's rank order correlation coefficient, PBMC: peripheral blood mononuclear cells.

abnormal LV relaxation exhibited a more pronounced ischemic response to exercise. Finally, Duke treadmill score, a prognostic parameter reflecting ischemic burden at stress testing [35], was a stronger correlate of both augmented NT-proBNP levels and depressed CD34<sup>+</sup>/KDR<sup>+</sup> cells numbers than indices of diastolic function which were eliminated by stepwise multiple regression. Additionally, adjustment for the treadmill

score abolished the NT-proBNP-CD34<sup>+</sup>/KDR<sup>+</sup> cells relationship. Accordingly, an as yet unidentified factor, possibly related to different clinical characteristics or ischemia by itself, might have mediated the coincidence of a high NT-proBNP and CD34<sup>+</sup>/KDR<sup>+</sup> cells deficiency in symptomatic stable CAD with impaired LV relaxation.

#### 4.1. Covariates of potential influence on NT-proBNP and CD34<sup>+</sup>/KDR<sup>+</sup> cells numbers

Admittedly, we are not able to fully exclude potential alternative mechanisms that might have been responsible for the observed relationships. However, a wide set of exclusion criteria has allowed to eliminate at least some of factors which might affect CD34<sup>+</sup>/KDR<sup>+</sup> cells counts and/or NT-proBNP levels [24,25,38-41]. These included heart failure, depressed EF, chronic kidney disease and pulmonary disease. Additionally, there were no significant intergroup differences in age, prevalence of hypertension and smoking habit, GFR, BMI and EF. Second, all subjects in the 3 groups were treated with statins (known to stimulate EPC mobilization [42]) and ACEI, reported to lower BNP levels [39] and EPC counts [43]. Third, both CAD subgroups exhibited one-vessel CAD and no significant differences in the type of vessel involved, whereas angiographic CAD parameters had also been reported to affect B-type natriuretic peptide levels [1,3,6] and EPC numbers [25].

Moreover, not only EF, but also parameters reflecting LV filling pressure (E/E' ratio and LAVI) were comparable in the 3 groups, which indicates that factors other than intergroup differences in LVEDP, a parameter related to NT-proBNP [44], were responsible for a higher NT-proBNP in CAD. We cannot however exclude the possibility that at elevated heart rates and consequent shortening of the diastole, i.e. during daily physical activity, subjects with the impaired LV relaxation pattern could develop elevated filling pressures and subsequent elevations of NT-proBNP, whose half-life in plasma is about 2 h, which might have affected its values measured at rest. Nevertheless, this appears an unlikely cause of increased NT-proBNP as blood sampling was performed after overnight fast and LAVI, a more stable index of LV filling pressures, was comparable in the 3 groups. Additionally, in the presence of abnormal LV relaxation 3-6 fold elevations of baseline B-type natriuretic peptides levels were demonstrated [8, 10], including BNP [10], whose half-life is only about 18 min [39].

#### 4.2. Exercise-induced ischemia versus B-type natriuretic peptides in stable angina

Although in the studies relating BNP/NT-proBNP to isolated diastolic dysfunction CAD was present in 41–50% of subjects [8,10], quantitative parameters of ischemia during stress testing were not reported. Furthermore, among the studies that have revealed that baseline elevations of B-type natriuretic peptides are more pronounced in CAD patients with inducible ischemia on cardiac stress imaging compared to those with negative test results [45–51], indices of LV diastolic function were provided exclusively by the Heart and Soul Study Investigators [49,50]. The relationship between BNP/NT-proBNP and inducible ischemia has been demonstrated irrespective of imaging technique for nuclear perfusion imaging [45–48], exercise echocardiography [49,50] and dobutamine echocardiography [51]. Moreover, the role of the ischemic stimulus for NT-proBNP elevation in stable angina is supported by falls in the peptide level after elective angioplasty [52].

In the present study NT-proBNP correlated to both TDI indices of impaired LV relaxation at rest and parameters related to exercise-induced ischemia, nevertheless, Duke treadmill score was the sole multivariate determinant of NT-proBNP. Additionally, in our hands, NT-proBNP levels were elevated also in stable angina subjects with a normal LV relaxation pattern. Therefore it appears probable that a higher NT-proBNP concentration in our patients was attributable to repetitive ischemic episodes in their daily life. This concept had been put forward by Bibbins-Domingo et al. [49] on the basis of data of 355 Heart and Soul Study patients to explain elevated BNP at rest in stable CAD subjects with inducible myocardial ischemia, which was only slightly attenuated after adjustment for EF and echocardiographic categories of diastolic dysfunction. Moreover, in all 901 participants of the Heart and Soul Study [50], the relationship between NT-proBNP and inducible ischemia was limited to the patients with an EF > 50% and either normal diastolic function or impaired LV relaxation pattern, i.e. similar to our CAD subjects. This indicates that the NT-proBNP – ischemia relationship can be more easily detectable in the absence of co-existent abnormalities obscuring the association, such as systolic LV dysfunction or advanced diastolic dysfunction with consecutive markedly increased NT-proBNP levels.

As suggested by Tateishi et al. [53], BNP release in hypoxic conditions might be partially independent of

LV diastolic dysfunction as hemodynamic parameters were not related to transient rises in BNP in response to ischemia during balloon inflation at coronary angioplasty. Mechanistically, in stable angina patients without LV systolic dysfunction undergoing surgical revascularization, Goetze et al. [11] have shown augmented expression of BNP gene in hypoxic ventricular myocardium that correlated with several-fold elevations of plasma BNP (5-fold) and NT-pro-BNP (8–10-fold) levels. Hopkins and colleagues [54] demonstrated that chronic hypoxia was responsible for 12-fold elevations of NT-proBNP concentrations in cyanotic congenital heart disease despite low right atrial pressures. Finally, a hypoxia-inducible factor-1-responsive element has been identified in the BNP promoter [55].

#### 4.3. Exercise-induced ischemia versus CD34+/KDR+ cells counts in stable angina

Hypoxia-inducible factor-1 activates the expression of VEGF [56], a potent mediator stimulating mobilization of EPC from the bone marrow into the blood. This was a likely explanation of an over 3-fold increase in circulating EPC number in response to a single episode of myocardial ischemia, which reached its maximum 24 h after exercise and persisted until 48 h, being correlated exclusively to the preceding augmented release of VEGF (2–6 h after exercise) [26]. Moreover, this effect was similar with cells quantification by means of both flow cytometric analysis (CD34+/KDR+ cells) and the EPC *in vitro* culture assay [26]. Therefore, a selective depression of the CD34+/KDR+ cells count in our angina CCS class III patients with impaired LV relaxation was rather a surprising phenomenon, because they exhibited a shorter time to ischemic ST segment depression than those with normal relaxation pattern, which could precipitate repetitive ischemic episodes at a normal activity.

In search for a possible explanation, we hypothesize that an eventual exhaustion of the bone marrow capacity to mobilize EPC might have occurred in CAD patients exposed to multiple ischemic episodes during daily life. This hypothesis is analogous to that put forward by Fadini et al. [57] who observed a lower CD34+/KDR+ cells count in advanced lung disease and long-lasting hypoxia and suggested that the continuous hypoxic stimulation might had led to an exhaustion of the bone marrow precursor pool of EPC.

As an alternative mechanism, keeping in mind the previously reported independent relationship between coronary endothelial dysfunction and CD34+/KDR+

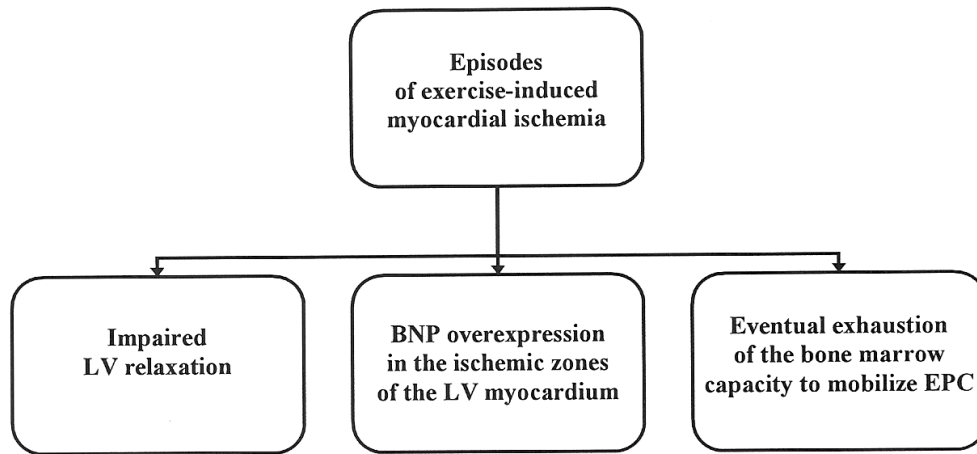


Fig. 5. The magnitude of ischemic burden as a hypothetical common mechanism underlying both elevated plasma NT-proBNP levels and circulating CD34+/KDR+ cells deficiency in stable angina CAD patients with impaired LV relaxation.

cells deficiency in stable CAD [20], it could be also proposed that lower CD34+/KDR+ cells counts might predispose to exertional myocardial ischemia via local endothelial dysfunction with consequent impairment of coronary flow reserve. In fact, Zeiher et al. [58] had demonstrated that coronary microvascular endothelial dysfunction translated into exercise-induced myocardial ischemia by nuclear perfusion imaging. These two hypothetical concepts are not mutually exclusive because endothelial dysfunction could exacerbate myocardial ischemia [58], which, in turn, may exacerbate CD34+/KDR+ cells deficiency through the former mechanism.

#### 4.4. B-type natriuretic peptides versus hematopoietic/endothelial progenitor cells and endothelial function

Irrespective of the mechanism involved, CD34+/KDR+ cells deficiency in patients with stable angina and impaired LV relaxation adds into a broad set of abnormalities associated with excessive secretion of BNP/NT-proBNP within a so-called “grey zone” of their levels. Associations between B-type natriuretic peptides and progenitor cells numbers in blood were reported in different clinical settings. First, in acute ST-segment elevation myocardial infarction NT-proBNP levels and CD34+ hematopoietic progenitor cells, although both elevated, correlated inversely with each other [59]. Second, in systolic heart failure patients the numbers of CD34+ and CD34+/CD133+/KDR+ cells were lower in NYHA classes III–IV associated with higher BNP levels [40]. Additionally, the amelioration

of decompensated heart failure symptoms was accompanied by interrelated decreases in BNP and increases in CD34+ cells counts [60]. Third, NT-proBNP and CD34+/CD133+/KDR+ cells counts were inversely correlated 72 h after cardiac surgery [61].

In a large community-based study of 1,991 subjects free of heart and renal failure, BNP and NT-proBNP concentrations predicted risk of death over a median follow-up of 5.6 years in part independently of clinical covariates and coexistent echocardiographic abnormalities including lower EF, elevated LV mass, left atrial enlargement and, importantly, diastolic dysfunction categorized by conventional indices and TDI [62]. It has therefore been proposed that elevated levels of B-type natriuretic peptides may represent a final common pathway of a variety of cardiovascular disorders beyond hemodynamic factors directly related to heart failure [62]. It cannot be excluded that endothelial dysfunction might also belong to this set of abnormalities. In line with this concept, Chong et al. [63] observed an inverse correlation between BNP and flow-mediated dilation of the brachial artery in stable heart failure patients, although neither of these parameters was related to EF or NYHA functional class.

Nevertheless, it is clear that the presence of a correlation does not imply a cause-and-effect relationship. On the basis of the present study we rather suggest that a common factor, probably repetitive ischemic burden, can be accountable for the coincidence of an elevated NT-proBNP concentration and a depressed CD34+/KDR+ cells count in stable angina patients exhibiting features of impaired LV relaxation (Fig. 5).

#### 4.5. Limitations of the study

Admittedly, we have no direct proof in support of the above proposed hypothetical mechanism of the coincidence of high NT-proBNP and low CD34+/KDR+ cells counts. It cannot be excluded that early events in the ischemic cascade (e.g. subclinical chronic ischemia owing to joint effects of epicardial stenosis and pre-existing microvascular dysfunction) might be more relevant for NT-proBNP release than the magnitude of symptomatic exercise-induced ischemia that was used for the calculation of Duke treadmill score.

Additionally, it is highly unlikely that BNP could directly negatively affect EPC mobilization controlled by a local L-arginine-NO pathway in the bone marrow [64]. Moreover, there is evidence that BNP *per se* may produce not detrimental but beneficial effects on endothelial NO generation [65] and EPC mobilization [66].

An alternative common mechanism of both high NT-proBNP and low CD34+/KDR+ cells counts might be inflammatory activation as suggested recently by Cesari et al. [61], who ascribed the observed inverse correlation between NT-proBNP levels and CD34+/CD133+/KDR+ cells numbers after cardiac surgery to an altered balance between pro- and anti-inflammatory cytokines. However we have found no differences in C-reactive protein, an approximate index of inflammation, between the 3 groups of our patients.

#### 4.6. Conclusions

Propensity to symptomatic exertional ischemia may underlie the coincidence between moderately elevated NT-proBNP and EPC deficiency in patients with stable CAD. Additionally, chronic subclinical ischemia can also be involved in these associations. These might result from BNP overexpression in the ischemic myocardium and a hypothetical exhaustion of the bone marrow capacity to mobilize EPC in response to multiple ischemic episodes, thus contributing to the prognostic effect of B-type natriuretic peptides irrespective of hemodynamic factors.

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