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Serum From Advanced Heart Failure Patients Promotes Angiogenic Sprouting and Affects the Notch Pathway in Human Endothelial Cells

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Abstract

It is unknown whether components present in heart failure (HF) patients' serum provide an angiogenic stimulus. We sought to determine whether serum from HF patients affects angiogenesis and its major modulator, the Notch pathway, in human umbilical vein endothelial cells (HUVECs).

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In cells treated with serum from healthy subjects or from patients at different HF stage we determined: (1) Sprouting angiogenesis, by measuring cells network (closed tubes) in collagen gel. (2) Protein levels of Notch receptors 1, 2, 4, and ligands Jagged1, Delta-like4. We found a higher number of closed tubes in HUVECs treated with advanced HF patients serum in comparison with cells treated with serum from mild HF patients or controls. Furthermore, as indicated by the reduction of the active form of Notch4 (N4IC) and of Jagged1, advanced HF patients serum inhibited Notch signalling in HUVECs in comparison with mild HF patients' serum and controls. The circulating levels of NT-proBNP (N-terminal of the pro-hormone brain natriuretic peptide), a marker for the detection and evalutation of HF, were positively correlated with the number of closed tubes ($r = 0.485$) and negatively with Notch4IC and Jagged1 levels in sera-treated cells ($r = -0.526$ and $r = -0.604$, respectively). In conclusion, we found that sera from advanced HF patients promote sprouting angiogenesis and dysregulate Notch signaling in HUVECs. Our study provides in vitro evidence of an angiogenic stimulus arising during HF progression and suggests a role for the Notch pathway in it.

The serum of HF patients contains elevated levels of inflammatory cytokines and pro-angiogenic markers (Torre-Amione et al., 1996; Vila et al., 2008). Levels of inflammatory cytokines are highly predictive of cardiovascular events in HF patients (Harutyunyan et al., 2013; Osmancik et al., 2013) and a large body of evidence suggests that this may be related to the induction of endothelial dysfunctions (Yoshizumi et al., 1993; Agnoletti et al., 1999a; Crook et al., 2002). It is unknown whether or not the high levels of circulating pro-angiogenic factors vascular endothelium growth factor A (VEGFA) and interleukin 6 (IL6) in HF promote new vessels formation. It has been suggested that they may reflect disturbances of endothelial integrity or play a role in the maintenance and repair of the damaged luminal endothelium (Chong et al., 2004; Lip and Chung, 2005; Vila et al., 2008). Several studies have reported that the stimulation of angiogenesis positively affects clinical outcome in HF (Pearlman et al., 1995; Givvimani et al., 2014; Mozid et al., 2014) while impaired angiogenesis is associated with poor prognosis (van der Meer et al., 2005; Gouya et al., 2013; Rengo et al., 2013). However, there are scant data showing that angiogenesis actually takes place in HF (Mozid et al., 2014).

Angiogenesis requires a coordinated cross-talk between soluble pro-angiogenic cytokines and Notch signaling. Notch signaling is a major determinant of cell fate decision during the development and in self-renewing tissues in post-natal life (Espinoza and Miele, 2013). Mammals express four Notch receptors (Notch1-4) and five ligands (Jagged1-2, Delta like1, 3, and 4) on the surface of cells. Notch-ligand binding leads to two proteolytic cuts producing the active Notch intracellular domain (NICD) (Espinoza and Miele, 2013) which binds to the transcriptional regulator CSL (CBF1/Suppressor of Hairless/LAG-1) and induces the transcription of target genes such as basic helix-loop-helix family (Hes1 and Hes5) and hairy and enhancer of split related (HESR) family (Hey1 and Hey2) (Espinoza and Miele, 2013). During the development of the vascular system Notch determines endothelial artery-vein cell fate specification (Swift and Weinstein, 2009) whereas, in post-natal life the Notch pathway protects endothelial cells from apoptosis induced by tumor necrosis factor α (TNFα) (Quillard et al., 2010) and lipopolysaccharides (LPS) (MacKenzie et al., 2004b) and it regulates angiogenesis (Gerhardt et al., 2003; Sainson et al., 2008).

During angiogenesis, Jagged1/Delta-like ligand4/Notch1-mediated signals control the formation of new blood vessel branches from pre-existing ones by modulating the number of sprouts induced by VEGFA in endothelial cells (sprouting angiogenesis) (Gerhardt et al., 2003; Sainson et al., 2008). Specifically, VEGFA induces the expression of Dll4, which, in turn, activates Notch in surrounding cells. thus promoting “branching” of neovascular sprouts. Jagged1 and Dll4 signals are qualitatively different, and balance one another to determine the number of branches. Inhibition of Dll4 results in uncontrolled proliferation of endothelial cells without branching, and formation of non-functional capillaries (Gu et al., 2009).

The aim of this study was to establish whether the serum of HF patients affects angiogenic sprouting and Notch signaling of endothelial cells. For this purpose, we treated HUVECs with HF patients' serum in: (1) Three dimensional cultures (collagen gel) to evaluate the effect on cell networks formation as a measure of sprouting angiogenesis. (2) Standard two-dimensional cultures to characterize the effects of treatment on the Notch signaling.

Methods

Patients

Twenty-nine patients with different degree of clinical HF according to the New York Heart Association (NYHA) classification (n = 12 NYHA class I/II and n = 17 NYHA class III/IV) were enrolled in the study. Blood was withdrawn in the morning and collected into Vacutainer tubes with clot activator. Tubes were inverted five to six times to mix clot activator and blood and incubated in upright position at room temperature for 30–40 min to allow clotting. Tubes were spun at 2,000*g* for 15 min without brake. Serum was then removed, aliquoted and stored at −80°C until use. Diagnosis of HF was based on history of HF of at least 6 months duration, reduced exercise tolerance, objective LV (left ventricle) functional impairment and levels of the N-terminal of the prohormone brain natriuretic peptide (NT-proBNP) above the normal range of 125 pg/ml (McMurray et al., 2012). Control sera (6) and the control pool of sera (from 46 subjects) were obtained from age-matched healthy volunteers. Approval from the Ethics Committee of Azienda Ospedaliero-Universitaria Sant'Anna-Ferrara and written informed consent from patients and healthy subjects control were obtained in compliance with the guidelines of the World Medical Association Declaration of Helsinki.

Cell culture

Four different batches (each batch made of cells pooled from 22 donors) of HUVECs purchased from Invitrogen (Carlsbad, CA) were utilized for the experiments. Cell culture conditions are described in Supplementary methods.

HUVECs network formation in collagen

Collagen gels were prepared as described (Yang et al., 1999; Montanez et al., 2002). Each well of a 24-well plate was covered with 320 μl of collagen solution and incubated for 30 min at 37°C (in absence of CO_2) to allow solidification. 1.6×10^5 cells were then seeded on the surface of collagen and let to adhere for 1 h (37°C, 5% CO_2). Three hundred and twenty

microliter of collagen solution were then added to the cells to form a “sandwich” and allowed to solidify for 30 min at 37°C (in the absence of CO_2). One milliliter of medium containing 20% of patients or control serum was then added to each well. Cells network formation was assessed 48 h later by counting the number of closed tubes in eight fields per well, as previously described by Paschoalin et al. (2007) and Caliceti et al. (2013). Images were acquired by phase-contrast microscopy (20 × magnification) using Nikon Eclipse TE300 Stereo Inverted Microscope System (Nikon Instruments Inc., Melville, NY).

Cytokines analysis

Serum samples were thawed only once before performing the MILLIPLEX MAP Human Cytokine/Chemokine Panel (Merck Millipore, Billerica, MA) as previously described by Voltan et al. (2014). Only cytokines detectable in more than 50% of patients (20) were used for our analysis.

Western blotting

Western blot analyses to assess the expression levels of Notch1, Notch2, Notch4, Jagged1, Dll4, and β – actin were performed as previously described by Caliceti et al. (2013). Densitometric analyses were performed to determine the intensity of each band relative to the β-actin band (Image J analysis software - http://imagej.nih.gov/ij/). The obtained values were normalized to the same parameters obtained from cells treated with control sera pool.

RNA analyses

Total RNA was extracted after 48 h of treatment using RNeasy Mini Kit (Qiagen, Carlsbad, CA). mRNA retrotranscription, Real time PCR and determination of changes in gene expression were performed as previously described by Caliceti et al. (2013).

Notch4 and VE-cadherin detection by immunofluorescence

Immunofluorescence was performed as previously described by Caliceti et al. (2013). Primary antibodies used were VE-cadherin (F-8) (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and Notch4 (H-225) (1:100). The secondary antibody used were Alexa Flour 546 Rabbit Anti-Mouse IgG (1:500; Life Technologies, Carlsbad, CA) and Alexa Flour 488 Goat Anti-Rabbit IgG (1:500; Life Technologies, Carlsbad, CA). Images were acquired on laser confocal microscope Zeiss LSM 510 (Zeiss, Jena, Germany) (40 × magnification).

Apoptosis detection

Active caspase 3 and VE-cadherin staining were performed at the end of the 12 days-culture. Active caspase 3 positive (apoptotic) cells (Cleaved caspase 3 antibody #9661) (1:100; Cell Signaling Technology, Danvers, MA) were counted in each well. Nikon Eclipse TE300 Stereo Inverted Microscope System was used to acquire images for prolonged serum-treated cultures (Nikon Instruments Inc., Melville, NY) (40 × magnification). Apoptosis was also assessed with the Annexin V-FITC binding assay. HUVECs were grown for 48 h in the presence of 20% serum from patients then cells were collected and stained with Annexin V-FITC (Life Technologies, Carlsbad, CA) (100 ng/ml) and propidium iodide (Sigma-Aldrich, Saint Louis, MO) (10 mg/ml) at room temperature for 15 min. Flow cytometric analysis was

performed with BD FACSCalibur (Becton-Dickinson Biosciences, San Jose, CA), for each sample 20,000 cells were counted. Data analysis was performed with Kaluza Flow Analysis Software (Beckman Coulter, Brea, CA).

Statistical analysis

RT-PCR and densitometric data were analyzed using the GraphPad Prism Software (GraphPad Software Inc., CA) by two-tailed Student's *t*-test and a *P*-value < 0.05 was considered statistically significant.

Linear discriminant analysis (LDA) was performed using Statistica 6.0 software (2001; Statsoft, Tulsa, OK). LDA was applied to the standardized data matrix of NT-proBNP and of 20 cytokines from a panel of 29 inflammatory cytokines determined for these sera in a previous study (Voltan et al., 2014). LDA allowed scoring of the cases (i.e., serum of 29 HF patients) as a function of the first two roots (canonical discriminant functions) to visualise similarities among studied HF patients' sera according to their NYHA classification (n = 12 NYHA class I/II and n= 17 NYHA class III/IV). The statistical significance of each discriminant function was evaluated on the basis of Wilks' lambda factor, which ranges from 1.0 (no discriminatory power) to 0.0 (perfect discriminatory power).

Cluster analysis of cytokines expression was performed using GeneSpring GX software (Agilent Technologies, Palo Alto, CA). Manhattan correlation was used as similarity measure.

Linear correlation between protein levels was measured by calculating the Pearson's correlation coefficient (r) for each pair of variables. Associated *P*-values were computed for determining statistical significance ($P < 0.05$). Statistical computations were performed with the XLSTAT package (http://www.addinsoft.com).

Results

Patient demographics

The characteristics of the studied patients, including NYHA class, cardiovascular risk factors, functional parameters, and therapy are shown in Table 1. Patients were divided in two groups according to the severity of HF. The majority of patients (79.3%) were suffering from ischaemic HF. At the time of serum collection, patients were receiving standard pharmacological therapy, consisting of angiotensin- converting enzyme (ACE) inhibitors or angiotensin receptors, β-blockers, anti-aldosterone drugs, diuretics and digitalis, according to individual clinical needs. Some patients also received antianginal drugs such as nitrates, calcium antagonists and two received ivabradine to further reduce their heart rate.

In order to assess the variation among HF patients' sera a Linear Discriminant Analysis (LDA) was carried out using as variables the expression levels of NT-proBNP and of 20 cytokines determined in these sera in a previous study (Voltan et al., 2014) (Suppl. Table SII). Figure 1A shows the scatterplot of the 29 HF patients' sera on the space defined by the first two canonical functions. The Wilks' Λ value was 0.03426 ($P < 0.0003$) indicating a good discriminatory power of the applied model. The 29 analyzed sera were clustered into

three main groups (a–c). The first cluster (a), located in the North-Western and South-Western quadrants of the scatterplot, included the sera of 11 patients classified as NYHA III/IV. The second cluster (b) located in the South-Eastern quadrant is formed by the sera of four patients belonging to NYHA III/IV class. The cluster (c) included the sera of 12 NYHA I/II patients and of two NYHA III/IV patients. Overall, the discriminatory power of the proposed model based on the expression level of NT-proBNP and 20 cytokines in distinguishing between patients belonging to NYHA class I/II and NYHA class III/IV was equal to 93.1% (27 patients correctly classified out of a total 29 patients). The data suggested that the whole panel of selected biomarkers (NT-proBNP and 20 cytokines) can provide valuable information with respect to the NYHA classification of HF patients, but not completely satisfactory with a probability of erroneous assignment equal to 6.9%.

The first discriminant function (Wilks' $\Lambda = 0.034256$, $P = 0.000071$) which explains 85% of cytokine variation in HF patients, discriminated better between cluster a (11 sera belonging to NYHA III/IV group) and clusters b,c (12 sera belonging to NYHA I/II group and six sera belonging to NYHA III/IV group). On the first discriminant function the variables NT-proBNP, GMCSF, GCSF, IL7, IP10, VEGF, IL1b, IL12p40, and IL12P70 (first canonical discriminant function equal to − 1.79, − 1.41, − 4.32, − 1.10, − 1.19, − 1.21, − 4.47, 3.50, 3.64, respectively) appeared to be the most important in separating group a from groups b,c. The second discriminant function (Wilks' $\Lambda = 0.364087$, $P = 0.102892$), which explains 15% of the variation, was not statistically significant and consequentially cannot be considered in the discriminatory interpretation.

Cluster analysis showed that NT-proBNP and IL6 were the most differentially expressed proteins between sera from NYHA classes I/II and III/IV patients (Fig. 1B). Indeed, there was a significant difference between sera from the two groups of patients in the levels of NT-proBNP (363.3 ± 96.8 pg/ml vs. 1,688.4 ± 344.6 pg/ml, $P = 0.0039$, respectively) and of IL6 (1.26 ± 0.40 pg/ml vs. 9.30 ± 1.80 pg/ml, $P = 0.0014$, respectively) (Fig. 1C). The natural log (ln)-transformed IL6 levels correlated with the ln-transformed NT-proBNP levels ($r = 0.652$, $P < 0.001$) (data not shown). No significant difference between the two groups of patients were found when considering TNFα or VEGFA levels (Fig. 1C).

Serum from advanced HF patients promotes HUVECs network formation in collagen gel

NYHA class III/IV patients' serum, after 48 h treatment, promoted an increase in collagen gel-induced HUVECs network formation compared with control sera (1.47 ± 0.04 vs. 1.08 ± 0.12, $P = 0.0011$) and with NYHA class I/II patient's serum (1.47 ± 0.04 vs. 0.9 ± 0.06; $P < 0.0001$) (Fig. 2A,B). There were no differences between the number of closed tubes in cells treated with control and NYHA class I/II patient's sera. Inhibition of Notch activation by adding 5 μM DAPT to the control pool-treated cells induced an increase in the number of closed tubes compared with control pool-treated cells (1.62 ± 0.04 vs. 1.08 ±0.12, $P = 0.004$) (Fig. 2A,B). In cells treated with patient's sera the number of closed tubes at 48 h positively correlated with the ln-transformed values of NT-proBNP ($r = 0.485$, $P = 0.0069$, Fig. 2C) and with the ln-transformed values of IL6 ($r = 0.514$, $P = 0.0037$, Fig. 2D). Consistent with the presence of an angiogenic stimulus, VE-cadherin staining showed the presence of filopodia-like structures when HUVECs were treated for 48 h with serum which caused

increased network formation in collagen gel (Suppl. Fig. S1). Forty-eight hour treatment or prolonged exposure (12 days) of two dimensional HUVECs cultures to selected sera (n = 5 sera from each group of patients) caused apoptosis. The levels of apoptosis were higher in cells treated with serum from NYHA class III/IV compared with serum from NYHA class I/II patients (annexin V positive cells: 9.47 ± 0.9 vs. 6.98 ±0.16, respectively, $P = 0.024$; active caspase 3 positive cells: 20 ± 0.7 vs. 6.4 ± 1.7, respectively, $P < 0.0001$) (Fig. 3A, middle parts and B). Furthermore, the treatment with serum from NYHA class III/IV patients, but not with serum from NYHA class I/II patients, caused the disruption of HUVECs monolayers (Fig. 3A, top and bottom parts).

Serum from advanced HF patients dysregulates Notch signaling in HUVECs

Differences in the protein expression levels of Notch receptors (Notch1, Notch2, and Notch4) and their ligands (Jagged1 and Dll4) in HUVECs grown in the presence of serum from controls or NYHA class I/II and III/IV patients were assessed by comparison with the levels of the same receptors and ligands in HUVECs exposed to a pool of sera obtained from 46 healthy subjects. Figure 4A shows a representative Western blot for each receptor and ligand tested after treatment of cells with 20% of serum. The specific bands identified by the antibody for each receptor and ligand in HUVECs had been previously validated (Caliceti et al., 2013). The bands represent: Notch1TM (110 KDa, transmembrane form which requires activation through -γ-secretase-catalyzed cleavage), Notch2IC (100 KDa, active form), Notch4IC (55 KDa, active form), Dll4 (65 KDa, active form), Jagged1 (150 KDa) (Caliceti et al., 2013). Notch1TM levels were lower in cells treated with NYHA class I/II sera compared with controls (0.71 ± 0.05 vs. 1.07 ± 0.06; $P = 0.0006$) and higher in cells treated with NYHA class III/IV sera compared with NYHA class I/II sera (0.99 ± 0.03 vs. 0.71 ± 0.05, $P < 0.0001$, Fig. 4B). There were no differences in Notch1TM levels between cells treated with NYHA class III/IV sera and controls. On the contrary, the levels of Notch4IC were decreased in NYHA class III/IV- compared with controls- (0.65 ± 0.07 vs. 0.94 ± 0.09, $P = 0.04$) and NYHA class I/II sera-treated cells (0.65 ± 0.007 vs. 1.11 ± 0.09, $P = 0.0004$ Fig. 4B). Immunofluorescence staining of Notch4 in HUVECs confirmed changes in Notch4 expression levels matching the changes observed in Western blot (Suppl. Fig. S2). No significant differences in the expression level of Notch2IC were observed between the three groups of sera (Fig. 4B). Among Notch ligands, there were no differences between controls and NYHA class I/II sera-treated cells in Jagged1 expression whereas the expression of this ligand was decreased in NYHA class III/IV sera treated-cells compared with controls- and NYHA class I/II sera-treated cells (0.87 ± 0.04 vs. 1.09 ± 0.08, $P = 0.02$ and 0.87 ± 0.04 vs. 1.11 ± 0.07, $P = 0.0057$, respectively, Fig. 4B). Dll4 expression levels were unchanged between the studied groups of sera (Fig. 4B).

To exclude the possibility that these results were dependent on the particular cell type used, we treated mesenchymal stem cells isolated from adipose tissue of healthy subjects (adMSCs) with two sera utilized for HUVECs treatment and determined the expression levels of Notch4 and Dll4. Supplementary Figure S3 shows that serum from patient 10 (NYHA class I) and patient 20 (NYHA class IV) had the same effect on Notch4IC and Dll4 expression levels both in adMSCs (A) and HUVECs (B).

The effect of HF patient's serum treatment on transcriptional Notch activity was evaluated by measuring the expression levels of Notch target genes Hey1, 2, and Hes1. There were no differences in the mRNA levels of these genes between HUVECs treated with NYHA class I/II and NYHA class III/IV patients' serum (Suppl. Fig. S4).

The ln-transformed values of NT-proBNP in patient's sera negatively correlated with the decrease in Notch4IC and Jagged1 (r=−0.526, $P = 0.003$ and r=−0.604, $P = 0.0005$, respectively after removal of an outlier), (Fig. 5B,D). No correlations were found between the NT-proBNP values and Dll4 or Notch1TM (Fig. 5A,C). In HUVECs treated with patient's sera, the number of closed tubes at 48 h correlated with Notch1TM and Notch4IC levels (r = 0.583, $P = 0.0007$ and r=−0.529, $P = 0.0027$, respectively) (Fig. 6A,B). No correlations were found between the number of closed tubes and Dll4 or Jagged1 levels (Fig. 6C,D).

Discussion

Treatment with serum from HF or acute myocardial infarction patients has been shown to induce apoptosis of cultured endothelial cells (Agnoletti et al., 1999b; Rossig et al., 2000; Valgimigli et al., 2003). In this study, we used a similar approach to establish whether exposure of endothelial cells to HF patients' serum has an effect on sprouting angiogenesis and on the Notch pathway, a major regulator of angiogenesis. We found an increase in cells network formation (closed tubes) and a significant decrease in active Notch4 (Notch4IC) and Jagged1 levels in HUVECs treated with serum from advanced HF patients in comparison with serum from mild HF patients and control subjects. There were no differences, other than a decrease in the transmembrane form of Notch1 (Notch1TM), in the expression of Notch pathway components and in cells network formation between HUVECs treated with serum from mild HF patients and controls. Of interest, treatment with advanced HF serum restored the same levels of transcriptional inactive Notch1TM observed in control sera-treated cells. The number of closed tubes induced by patients' serum was positively correlated with the ln-transformed values of NT-proBNP and with the levels of Notch1TM and negatively correlated with the levels of Notch4IC. In conclusion, our study shows that, in HUVECs: (1) Only serum of advanced HF patients causes enhanced sprouting angiogenesis in comparison with control and mild HF patient's serum. (2) Serum of mild and advanced HF patients differentially affects Notch signaling.

Stimulation of angiogenesis positively affects clinical outcome in HF and its impairment has been associated with HF progression and poor prognosis (Hilfiker-Kleiner et al., 2004; van der Meer et al., 2005). Consistent with the attempt to stimulate angiogenesis, increased levels of pro-angiogenic markers (Vila et al., 2008) and circulating endothelial progenitor cells (EPCs) (Valgimigli et al., 2004) have been observed in HF patients. There is no evidence confirming that angiogenesis occurs in HF, and it has been suggested that the increase in pro-angiogenic markers may reflect an attempt to repair a dysfunctional endothelium rather than promoting angiogenesis per se (Chong et al., 2004; Lip and Chung, 2005). Our finding of increased network formation in HUVECs grown in the presence of serum from advanced HF patients confirms that components of the serum may have pro-angiogenic activities. We also found that the prolonged exposure of HUVECs to the serum

of patients with advanced HF caused increased apoptosis compared with serum from mild HF patients. Taken together, these findings suggest that HF patients' serum may provide a pro-angiogenic stimulus which could be counteracted by the pro-apoptotic effect of inflammatory cytokines. The balance between these two effects may determine whether productive angiogenesis occurs. In this study, we used two independent in vitro assays (3D-tube formation and 2D-apoptosis detection) to estimate pro- and anti-angiogenic stimuli present in the sera. The question of how these two stimuli interact in the modulation of angiogenesis in HF patients should be addressed by specifically designed in vivo experiments.

In cells treated with patient's sera we found a correlation between decreased levels of active Notch4 and Notch1 and increased endothelial sprouting, suggesting that serum from advanced HF patients could promote sprouting angiogenesis in part by downregulating Notch signaling. Angiogenesis involves VEGFA-mediated specification of tip cells (from which new branches of blood vessels originates in the direction of the angiogenic stimulus) and stalk cells (that constitute the trunk of the growing vessels) (Gerhardt et al., 2003). The Notch pathway is a major regulator of tip/stalk cells balance. Specifically, inhibition of Notch causes increased endothelial cells sprouting (Estrach et al., 2011; Caliceti et al., 2013). The consequences of the increased sprouting on angiogenesis depend on modality and duration of Notch inhibition. In tumour vasculature, inhibition of Dll4-mediated Notch1 signaling leads to increased sprouting but unproductive angiogenesis (Ridgway et al., 2006). In a rodent model of hind limb ischemia, temporary Notch inhibition with the γ-secretase inhibitor DAPT promoted angiogenesis and enhanced regional vascularization (Cao et al., 2009). TNFα promotes angiogenesis by inducing a tip cell phenotype and inhibiting Notch signaling (Sainson et al., 2008). Activation of Notch4 has been linked to angiogenesis inhibition (Leong et al., 2002; MacKenzie et al., 2004a) but also to endothelial cells survival under inflammatory conditions (MacKenzie et al., 2004c; Quillard et al., 2008; Quillard et al., 2009). Taken together with our data, these studies suggest that the extent of Notch4 inhibition and the cell context in which it occurs could determine whether it will promote angiogenesis or induce endothelial cells apoptosis.

Downstream Notch effectors relevant to angiogenesis are still being investigated (Lee et al., 2014). We did not detect differences in the expression of the most commonly studied Notch target genes Hey1, Hes1, and Hey2 between the two groups of patients. These results are not entirely surprising, considering that: (1) Expression of target genes depends on the levels of expression of all receptors and ligands as well as on other pathways, and it is highly cell context-dependent. (2). The number of genes regulated by Notch receptors is significantly larger than the few canonical basic helix-loop-helix (bHLH) transcription factors usually associated with Notch activity (Wang et al., 2011; Yun et al., 2013). (3) Notch4 activity in angiogenesis is also independent of its transcriptional activity (MacKenzie et al., 2004a). In this study, there were no significant differences between the two HF classes in the levels of Notch2 and Dll4, both known to be important factors in the expression of the canonical target genes we tested (Quillard et al., 2010; Caliceti et al., 2013). Further studies are required to identify the molecular targets downstream of the Notch pathway affected by HF patients' serum treatment and whether they would regulate sprouting angiogenesis.

Further investigation is needed to identify the factors involved in promoting angiogenesis and in dysregulating Notch in endothelial cells exposed to severe HF patients' serum. Although VEGFA and Dll4-activated Notch signalings play a major role in angiogenesis (Gerhardt et al., 2003), we did not find differences in VEGFA and Dll4 levels between patients with mild and advanced HF. Our results could be explained by differences in the levels of sFlt-1 (the soluble version of VEGFR1 acting as a specific antagonist of VEGFA (Kendall and Thomas, 1993)) or VEGFC, which activates VEGFR3 and VEGFR2 (Tammela and Alitalo, 2010). Upregulation of VEGFR3 following the treatment with advanced HF sera could also play a role since the kinase activity of VEGFR3, but not the binding of VEGFC, is pro-angiogenic in endothelial cells with low or no Notch signaling activity, as was the case in HUVECs treated with sera from advanced HF (Benedito et al., 2012). Similarly, same levels of Dll4 in the the two groups of patients do not necessarily exclude a role for Dll4 in the observed modulation of angiogenesis. High levels of circulating Dll4 in HF patients serum, as observed for Dll1 (Norum et al., 2015), could act as decoy and inhibit Dll4 activated-Notch signaling. Additionally, other molecules circulating in HF patients serum could affect Notch independently of Dll4 (and Jagged1). Elevated levels of the Wnt pathway antagonist secreted frizzled-related protein 3 (sFRP3) have been associated to adverse outcome in HF (Askevold et al., 2014a). In neuroblastoma, homeobox gene MSX1 induces both sFRP1 and the soluble Notch inhibitor Delta-like 1 homolog (DLK1) (Revet et al., 2008; Revet et al., 2010). In retinal neurogenesis sFRPs inhibit Notch signaling by downregulating ADAM10 (Esteve et al., 2011). Lastly, we cannot exclude a role for the carbohydrate-binding protein galectin3 in the stimulation of angiogenesis. Galectin3 is elevated in serum of HF patients and it is able to predict disease progression better than NT-proBNP (Chen et al., 2013). Of interest, galectin 3 is also a marker of angiogenesis (Markowska et al., 2010) and it has been shown to inhibit Notch signalling (Fermino et al., 2013). The analysis of the levels of sFlt-1, VEGFC, sFRP3, DLK1, or galectin 3 in the studied sera and of the expression levels in treated cells of VEGFR1, 2, and 3 could help to fully characterize the molecular mechanism underlying the angiogenic stimulus of HF serum and the role played by Notch in this context.

Of interest, we observed a difference in the IL6 levels between the two groups and a positive correlation between IL6 and the ln-transformed values of NT-proBNP and the number of HUVECs closed tubes, suggesting that IL6 may be involved in the pro-angiogenic stimulus caused by exposure to advanced HF patients' serum. IL6 is elevated in HF patients and promotes cardiomyocyte survival and hypertrophy (Askevold et al., 2014b). IL6 signaling is active in endothelial cells (Marin et al., 2001) and is a potent stimulator of angiogenesis after a stroke and in ovarian cancer (Nilsson et al., 2005; Gertz et al., 2012). Cross-talks between IL6 and Notch signaling have been described (Zhang et al., 2011; Kayakabe et al., 2012; Zeng et al., 2012). However, the lack of correlation between IL6 and the expression levels of Notch4, Jagged1, and Notch1, rules out an effect of IL6 on the observed dysregulation of this pathway.

HF is still associated with a high morbidity and mortality and early identification of high-risk patients may favorably affect outcome (Dickstein et al., 2008). NT-proBNP is used to guide the management of HF patients, although it is influenced by many factors including age, renal function, and anemia and new biomarkers of HF progression are being

investigated (Gouya et al., 2013; Lok et al., 2013). In our study, the number of closed tubes could discriminate between HF classes better than NT-proBNP values suggesting that the assessment of ex vivo sprouting angiogenesis could represent a novel biomarker of HF progression.

In conclusion, we provide ex vivo data suggesting an attempt to stimulate compensatory angiogenesis in advanced HF that may involve the Notch signaling. Given the small number of samples analyzed, our findings should be considered “hypothesis generating” rather than definitive conclusions on the modulation of angiogenesis and Notch by soluble components in HF patients' serum. Further studies are also needed to determine whether the expression levels of Notch components and/or the number of closed tubes together with NT-proBNP could help to identify HF patients at high risk of mortality. This study, together with our recent work showing Notch signaling dysregulation and functional impairment in mesenchymal cells isolated from adipose tissue of HF patients (Fortini et al., 2014), provides evidence suggesting that Notch should be further investigated as a potential therapeutic target in HF. As multiple tools for the manipulation of Notch signaling are being developed and targeted delivery of drugs by nanotechnology is rapidly progressing, the possibility to target the Notch pathway in HF warrants further investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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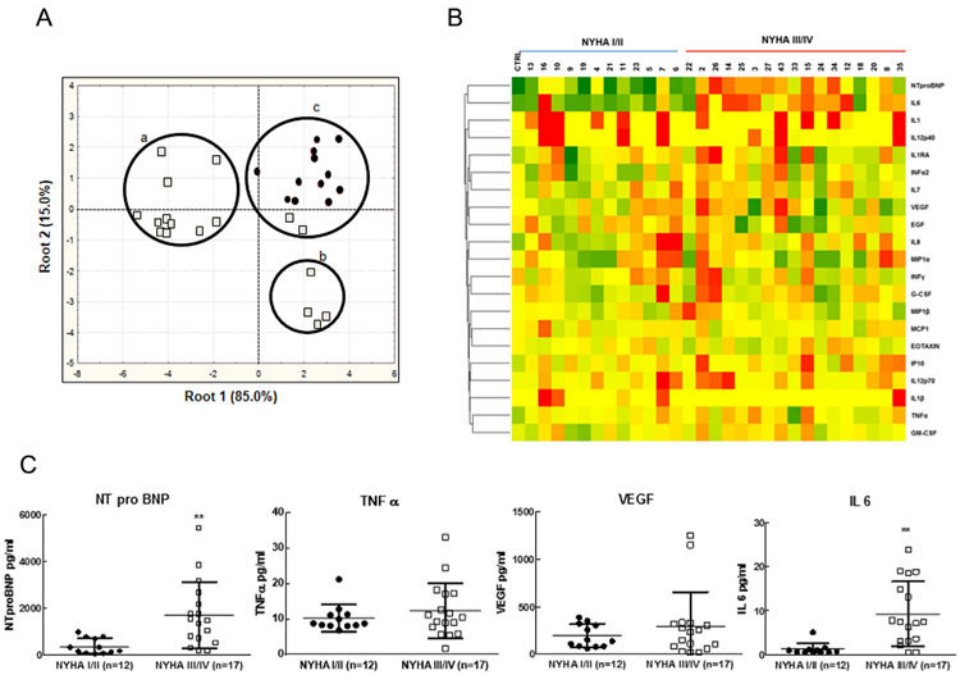

Fig. 1.

Characterization of circulating cytokines in serum from NYHA classes I/II and III/IV heart failure patients. (A) Scatterplot of the 29 HF patients' serum defined by the first two canonical functions (Root 1 and Root 2, representing 85% and 15%, respectively, of cumulated variability). Circles and squares refer to the classes NYHA I/II and NYHA III/IV, respectively. (B) Cluster analysis of cytokines in NYHA classes. Cytokines were grouped according to Manhattan correlation. Samples were grouped in the two groups corresponding to NYHA classes I/II and III/IV. (C) Differences in NT-proBNP, TNFα, VEGFA, and IL6 levels between sera from NYHA class I/II and NYHA class III/IV patients groups. Circles and squares refer to the classes NYHA I/II and NYHA III/IV, respectively. Data are expressed as mean ± SEM of each group. $^{**}P < 0.01$, two-tailed Student's *t*-test.

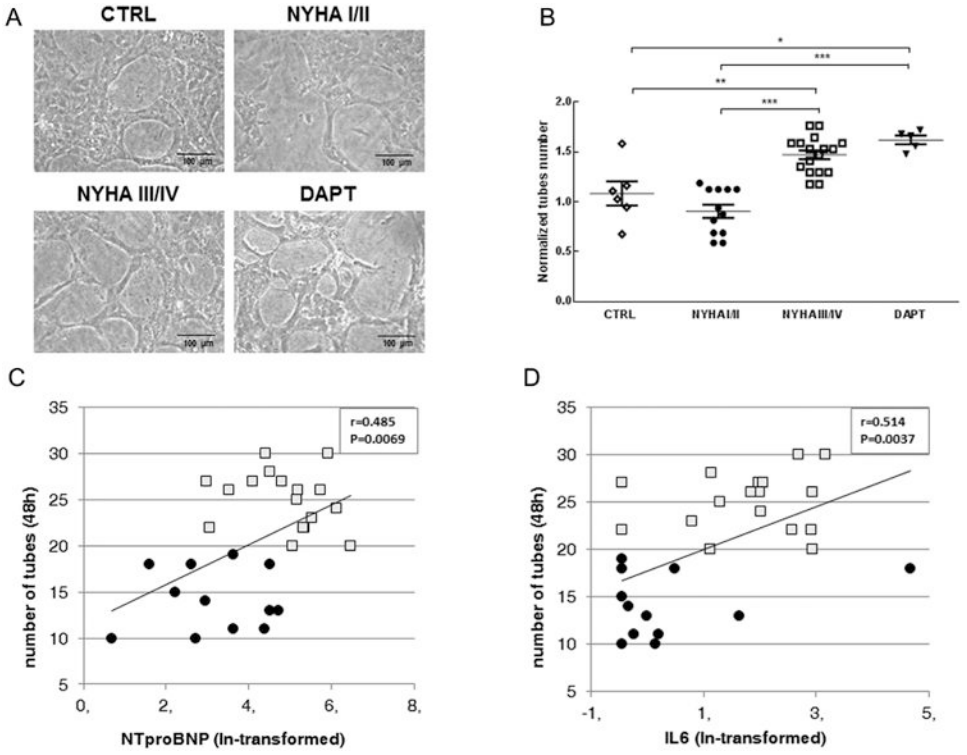

Fig. 2.

Effect of serum treatment on HUVECs network formation in collagen sandwiches. (A) Representative pictures showing network formation in HUVECs treated for 48 h with 20% serum from healthy subjects (n = 6), NYHA class I/II (n = 17) or NYHA class III/IV (n = 12) patient's sera and with 5 μM DAPT added to control serum. (B) After 48 h of network formation, the number of closed tubes was determined in eight fields (20 × magnification). The results are shown as the ratio between number of closed tubes in cells treated with the controls and patients sera and tubes in cells treated with pool of sera. Results are expressed as mean ± SEM. ***$P < 0.001$, two-tailed Student's t-test. (C) Correlation between the number of closed tubes in HUVECs treated with sera from NYHA class I/II and NYHA class III/IV patients and the corresponding ln-transformed values of NT-proBNP (NYHA class I/II sera, circles; NYHA class III/IV sera, squares). (D) Correlation between the number of closed tubes in HUVECs treated with sera from NYHA class I/II and NYHA class III/IV patients and the corresponding ln-transformed values of IL6 levels (NYHA class I/II, circles; NYHA class III/IV, squares).

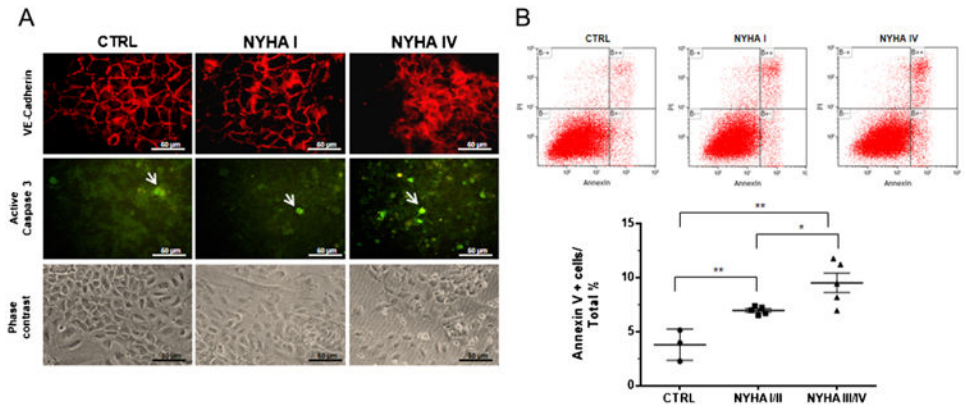

Fig. 3.

Effect of serum treatment on apoptosis in HUVECs. (A) After 12 days of serum treatment, HUVECs were immunolabelled with VE-cadherin and active caspase three antibodies, then treated with 546- and 488-conjugated secondary antibody. Representative phase-contrast microscopy images are shown (40 × magnification). White arrows point to apoptotic cells. (B) After 48 h of serum treatment HUVECs were stained with Annexin V and PI then cytometric analysis was performed. Representative PI/Annexin V plots are shown for each group (Control, NYHA I/II and NYHA III/IV). Percentage of apoptotic cells (ratio of Annexin V positive cells/Total cells) is shown. Circles, squares and triangles refer to control sera, classes NYHA I/II and NYHA III/IV, respectively. Data are expressed as mean ± SEM of each group. *$P < 0.05$, **$P < 0.01$ two-tailed Student's *t*-test.

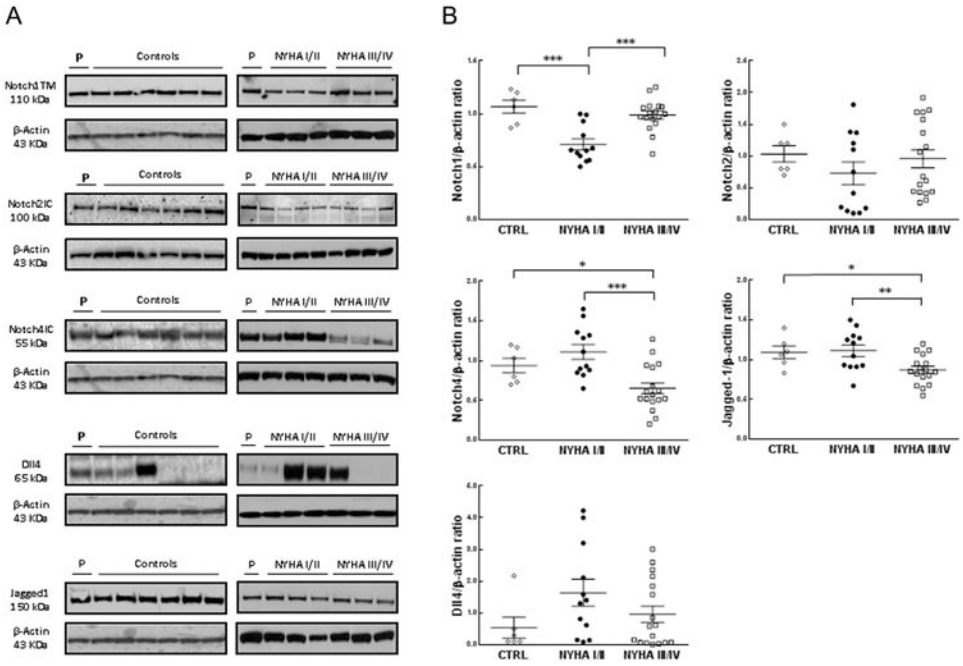

Fig. 4.

Effect of serum treatment on Notch signaling components in HUVECs. (A) After 48 h of serum treatment, cell lysates were electrophoresed and immunoblotted with Notch1, Notch2, Notch4, Dll4, Jagged 1, β-actin antibodies. Representative pictures for each blot are shown. (B) Densitometric analysis of Western blot assay to quantify Notch1TM, Notch2IC, Notch4IC, Dll4, Jagged1, and β-actin protein levels in analyzed samples. Circles and squares refer to the classes NYHA I/II (n = 12) and NYHA III/IV (n = 17), respectively. Diamonds identify cells treated with control sera (n = 6). Data are expressed as mean ± SEM of each group.** P <0.01, *** P <0.001 two-tailed Student's t-test.

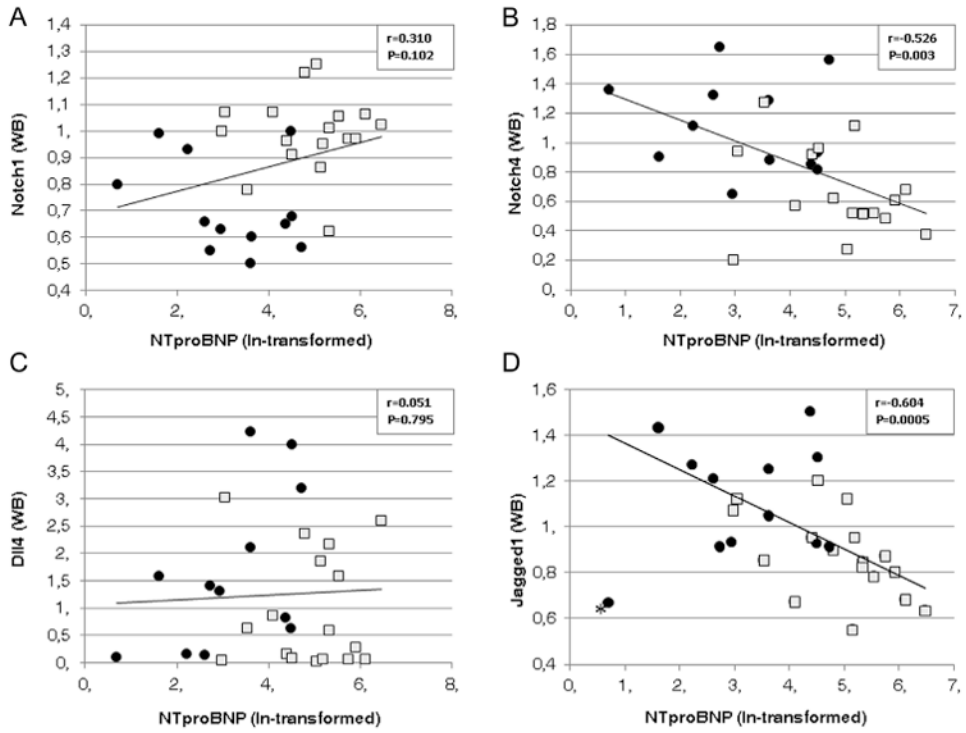

Fig. 5.

Correlation analysis in serum treated HUVECs. Correlation analysis between the ln-transformed values of NT-proBNP (NT-proBNP (ln-transformed)) and (A) Notch1TM, (Notch1(WB)), (B) Notch4IC, (Notch4(WB)), (C) Dll4, (Dll4(WB)), and (D) Jagged1, (Jagged1(WB)) expression levels. The correlation between NT-proBNP and Jagged1 was calculated considering the sample marked with an asterisk as an outlier. Circles and squares refer to the classes NYHA I/II and NYHA III/IV, respectively.

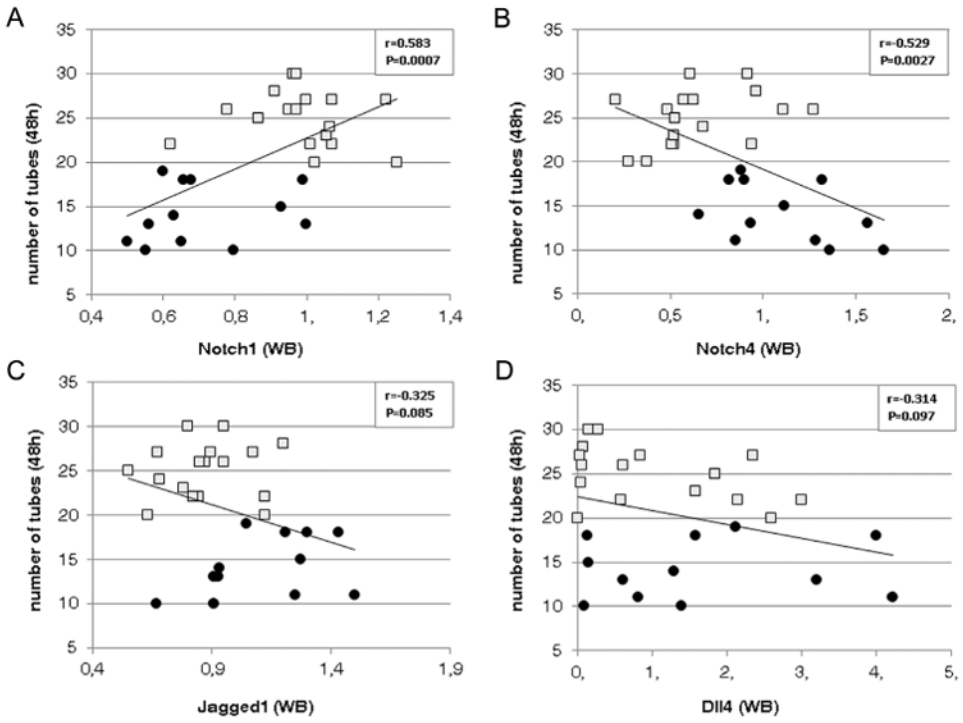

Fig. 6.

Correlation analysis in serum treated HUVECs. Correlation analysis between the number of HUVECs closed tubes at 48 h and (A) Notch1TM (Notch1(WB)), (B) Notch4IC (Notch4(WB)), (C) Dll4, (Dll4(WB)) and (D) Jagged1, (Jagged1(WB)) expression levels. Circles and squares refer to the classes NYHA I/II and NYHA III/IV, respectively.

Table 1
Baseline clinical parameters of the studied population

	All patients, n = 29	NYHA I/II, n=12	NYHA III/IV, n= 17	*P*
Age (years)	72.2 1.8	69.5 2	74.1 2.7	NS
Male (%)	24 (82.7)	12 (100)	12 (70.6)	0.039
Ischaemic etiology (%)	23 (79.3)	8 (66.7)	15 (88.2)	NS
BMI	26.7 ± 0.9	26.5 ± 0.6	26.8 ± 1.4	NS
Heart rate (bpm)	74.7 ±3.5	67.2 ± 3.9	80 ± 5	
LVEF (%)	32.2 ± 1.4	31.3 ± 1.7	32.8 ± 2.1	NS
VEGF (pg/ml)	254 ± 53.3	194.5 ± 35.1	296 ± 87.3	NS
NT-proBNP (pg/ml)	1,140.1 ± 237.7	363.3 ± 96.8	1,688.4 ± 344.6	0.0039
TNFα (pg/ml)	11.5 ± 1.2	10.3 ± 1.1	12.3 ± 1.9	NS
Diabetes (%)	8 (27.6)	2 (16.7)	6 (35.3)	NS
Hypercholesterolaemia (%)	16 (55.2)	4 (33.3)	12 (70.6)	0.047
Smoking habits (%)	19 (65.5)	10 (83.3)	9 (52.9)	NS
CAD Familiarity (%)	9 (31.1)	4 (33.3)	5 (29.4)	NS
ACE Inhibitors (%)	16 (55.2)	7 (58.3)	9 (52.9)	NS
ARB (%)	10 (34.5)	7 (58.3)	3 (17.6)	0.023
β-Blockers (%)	24 (82.8)	10 (83.3)	14 (82.4)	NS
Anti-Aldosterone (%)	12 (41.4)	4 (33.3)	8 (47.1)	NS
Diuretics (%)	26 (89.6)	9 (75)	17 (100)	0.029
Digitalis (%)	1 (3.4)	0 (0)	1 (5.9)	NS
Nitrates (%)	9 (31.1)	1 (8.3)	8 (47.1)	0.026
Calcium antagonists (%)	2 (6.9)	0 (0)	2 (11.7)	NS
Statins (%)	20 (70)	10 (83.3)	10 (58.8)	NS
PUFA (%)	9 (31.1)	4 (33.3)	5 (29.4)	NS
Ivabradine (%)	2 (6.9)	2 (16.7)	0 (0)	NS

ACE, Angiotensin-Converting Enzyme; ARB, Angiotensin II Receptor Blockers; BMI, Body Mass Index; CAD, Coronary Artery Disease; LVEF, Left Ventricular Ejection Fraction; NS, Not statistically Significant; NT-proBNP, N-Terminal of the pro-hormone Brain Natriuretic Peptide; NYHA, New York Heart Association; PUFA, PolyUnsaturated Fatty Acids; TNF, Tumor Necrosis Factor; VEGF, Vascular Endothelial Growth Factor.

P: intra NYHA classes analysis by two-tailed Student's *t*-test and χ^2 test. *P*-value <0.05 was considered to be statistically significant. Data are expressed as percentage (%) or mean ± SEM.