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Angiotensin receptor/Neprilysin inhibitor effects in CRTd non-responders: From epigenetic to clinical beside

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ABSTRACT

Objectives: We evaluated whether Angiotensin receptor/Neprilysin inhibitors (ARNI) reduce heart failure (HF) hospitalizations and deaths in cardiac resynchronization therapy with defibrillator (CRTd) non-responders patients at 12 months of follow-up, modulating microRNAs (miRs) implied in adverse cardiac remodeling.
Background: adverse cardiac remodeling characterized by left ventricle ejection fraction (LVEF) reduction, left ventricular end-systolic volume (LVESv) increase, and the 6-minute walking test (6MWT) reduction are relevant pathological mechanisms in CRTd non-responders and could be linked to changes in miRNAs (miRs), regulating cardiac fibrosis, apoptosis, and hypertrophy.
Methods: miRs levels and clinical outcomes (LVEF, cardiac deaths, and 6MWT) were evaluated at baseline and one year of follow-up in CRTd non-responders divided into ARNI-users and Non-ARNI users.
Results: At baseline, there were no differences in levels of inflammatory markers, miR-18, miR-145, and miR-181 ($p > 0.05$) between Non-ARNI users (n 106) and ARNI-users (n 312). At one year of follow-up, ARNI-users vs. Non-ARNI users showed lowest inflammatory markers ($p < 0.01$) and miR-181 levels ($p < 0.01$) and higher values of miR-18 ($p < 0.01$)and miR-145 ($p < 0.01$). At one year of follow-up, ARNI-users had a higher increase of LVEF ($p < 0.01$) and 6MWT ($p < 0.01$) along with a more significant reduction of LVESv ($p < 0.01$) compared to Non-ARNI users. Cox regression analysis evidenced that ARNI-based therapies increase the probability of anti-remodeling effects of CRTd. Based on symptomatic improvements, echocardiographic and functional classification improvements, 37 (34.9%) patients among ARNI-users became responders, while only twenty (6.4%) patients became responders among Non-ARNi-users.

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Conclusions: ARNI might influence epigenetic mechanisms modulating miRs implicated in the adverse cardiac remodeling responses to CRTd.

1. Background

The cardiac resynchronization therapy with a defibrillator (CRTd) is recommended for selected patients with heart failure (HF), left bundle branch block, and severe reduction of left ventricle ejection fraction (LVEF) [1]. The CRTd, improving LVEF, left ventricular end-systolic volume (LVESv), and the 6-min walking test (6MWT) could reduce New York Heart Association (NYHA) class, leading to the best clinical outcomes [1]. Notably, about one-third of CRTd patients do not benefit from this treatment and are termed CRTd non-responders [2]. The CRTd non-responders evidence the worsening of LVEF, LVESv, 6MWT, and the NYHA class [3], with a higher rate of hospitalizations for HF worsening and deaths [4]. In this setting, the adverse cardiac remodeling could be seen as one of the most relevant pathological and maladaptive mechanisms in the CRTd non-responders [1–4]. Notably, adverse cardiac remodeling could be linked with miRNAs (miRs) changes. In fact, the main molecular alterations implicated in adverse cardiac remodeling as cardiac fibrosis, apoptosis, hypertrophy, and inflammation, have been influenced by various miRs pathways such as miR-18, miR-145, and miR-181 as previously evidenced [5,6]. Intriguingly, anti-HF therapies could exert anti-remodeling effects via the modulation of these adaptive cardiac processes, leading to the best clinical outcomes in HF patients [7]. In this setting, the Angiotensin receptor/Neprilysin inhibitor (ARNI) is an anti-remodeling drug for HF patients with severe reduction of LVEF [7], which works as angiotensin receptors' pathways inhibitor (blocker of angiotensin II type 1 receptor), and simultaneously activator of vasoactive peptides (inhibitor of neprilysin), [8]. Moreover, the ARNI reduces the risk of death and HF hospitalizations by improving LVEF and NYHA classes in failing heart patients [8]. Intriguingly, in a rat model, the ARNI could attenuate and revert the adverse cardiac remodeling, inhibiting cardiac fibrosis and reducing cardiac hypertrophy via epigenetic modifications as miRs change [9,10]. There are no data about the clinical effects of ARNI in the CRTd non-responders' patients in terms of NYHA class and LVEF improvement in humans. Moreover, less has been reported about the effect of ARNI on the adverse cardiac remodeling and miRs' modulation in CRTd nonresponder patients. Thus, we evaluated whether ARNI therapy influences the miRs implicated in heart remodeling as miR-18, miR-145, and miR-181, and may improve clinical outcomes (LVEF, cardiac deaths, and 6MWT) to reduce maladaptive cardiac dysfunction processes in CRTd nonresponder patients.

2. Methods

2.1. Study population

We evaluated CRTd-treated HF patients, identified according to the international guidelines on HF disease management [1–4], for a one-year follow-up. We screened a population of consecutive patients defined CRTd non-responders between Jan 1, 2016, and Jan 1, 2020 [1–4]. We defined as CRTd non-responders the HF patients who did not respect the following diagnostic criteria six months after implantation: evidence of left ventricular reverse remodeling (reduction in left ventricular end-systolic volume (LVESV) of $\geq$ 10% at cardiac echography), significant change in functional HF class (improvement of the six min-walk test improvement and Minnesota living with HF scale improvement) [1–4]. We selected the study population according to:

- **Inclusion criteria**: at least 18 years of age, with a clinical history of stable chronic HF, NYHA functional class II or III, left bundle branch block, severe left ventricle ejection fraction reduction (LVEF < 35%), stable sinus rhythm, and defined CRTd non-responders according to the diagnostic criteria [1–4].
- **Exclusion criteria:** age < 18 or > 75 years, ejection fraction $\geq$ 40%, diagnosis of CRTd responders, unstable HF, patients in IV NYHA class, hyperkalemia, systolic hypotension (systolic blood pressure < 90 mmHg); patients with an estimated glomerular filtration rate (eGFR) of at least 30 ml per minute per 1.73 m^2 of the body surface area; absence of informed patient consent, and any condition that would make survival for one year unlikely.

Thus, we enrolled a consecutive population of CRTd non-responders patients with HF. The selected patients were divided into two groups: ARNI-users, the patients treated with ARNI therapy; Non-ARNI users, the patients never treated with ARNI therapy. The identification of ARNI-user patients was achieved by administering a questionnaire investigating the treatment time of ARNI before the beginning of the study, the administration route, and the dosage. Thus, we selected the ARNI-user patients as those who had used ARNI at most for four weeks before the start of the study to avoid the effects of chronic treatment with ARNI on baseline data.

2.2. Study design

We performed a prospective observational, multicenter study conducted at the University of Campania Luigi Vanvitelli (Naples, Italy), at the Catholic University of Sacred Heart (Rome, Italy), at Gemelli Molise S.p.a. (Campobasso, Italy), at Vecchio Pellegrini Hospital (Naples, Italy), at Cardarelli Hospital (Naples, Italy) and Santa Maria Della Pietà Hospital (Naples, Italy).

The patients enrolled in the study were evaluated by clinical, instrumental assessment, and telemetric device control at baseline and quarterly during 12 months of follow-up. We reported the CRT-d effect in terms of clinical and echocardiographic parameters during quarterly visits and CRTd responder rate. The study was conducted following the Declaration of Helsinki. The Ethics Committees of all participating institutions approved the protocol. All patients were informed about the study's nature and signed informed consent to participate. All patients were submitted to clinical, hematological, and instrumental evaluations.

2.2.1. Anthropometric evaluations

We evaluated the physical examination and vital signs in the CRTd non-responders and selectively in ARNI users vs. Non-ARNI users. The authors assessed the body mass index (BMI) as the ratio between weight in kg and the height squared [11].

2.2.2. Echocardiographic evaluation

Trans-thoracic two-dimensional echocardiogram with M-mode recordings, conventional Doppler, and pulsed-wave tissue Doppler imaging (TDI) measurements was performed at baseline and quarterly during 12 months of follow-up, using Philips iE33 echocardiography (Eindhoven, The Netherlands). The echocardiographic images were acquired in the parasternal long and short-axis views. The LV end-diastolic diameter (LVEDD), end-diastolic volume (LVEDV), end-systolic diameter (LVESD), and LVESV were measured, and the LVEF was calculated by the Simpson method [12]. The amount of mitral regurgitation was calculated as the area of the color-flow Doppler regurgitant jet divided by the area of the left atrium in systole and described as low (+), moderate (++), moderate-severe (+++), and severe (++++), as previously reported [12]. Two physicians were fully trained in echocardiography and, in an independent way to the study, protocol performed

and analyzed all the echocardiographic examinations. Finally, they systematically averaged the echocardiographic measurements in five consecutive samples.

2.2.3. Laboratory analysis

After an overnight fast in all patients, we evaluated the plasma glucose, serum lipids, B-type natriuretic peptide (BNP), and N terminal pro-BNP (NT-proBNP) by enzymatic assays. We collected patient blood samples in an ice-cooled blood collection system and immediately centrifuged them at 2500 rpm for 10 min in a refrigerated centrifuge. Samples were stored at − 80 °C. GLP-1 levels (Active GLP-1 7-36, Epitope) measurements were obtained after an overnight fast and breakfast. In these patients at baseline and quarterly during follow-up, we measured inflammatory markers and miRs. Additionally, we evaluated at baseline and after 12 months of follow up, circulating serum levels of pro-inflammatory cytokines (tumor necrosis factor-α, TNF α, interleukin-6, IL6), systemic inflammatory markers (C reactive protein, CRP), and leukocytes, and neutrophils count as previously reported [13].

2.3. RNA extraction and miRs analysis

We extracted 200 μL of serum from each patient's peripheral venous blood samples, and then we used the miRNeasy Mini kit (Qiagen, 20124 Milan, Italy) to characterize the miRs expression [6]. A single reaction for RNA isolation was carried out by pooling 8 serum samples extracted from patients matched for sex, age, and clinical evaluations. The miRs were assayed from blood samples at baseline and quarterly during 12 months of follow-up in ARNI vs. Non-ARNI users. We evaluated the miRs implied in various processes of HFrEF [14] and differently expressed in CRTd responders vs. non-responders [5,6]. Thus, we spiked a 5 μL aliquot of 5 nM Syn-cel-miRNA-39 miScript miRNA-Mimic, from the total RNA, including small RNAs, before nucleic acid preparation to monitor the efficiency of miR recovery and to normalize miR expression in the subsequent real-time [6]. Furthermore, we evaluated the serum expression of the miR-18, miR-145, and miR-181. Triplicate determinations of hsa-miR-181a-5p (MIMAT000025), hsa-miR-145-5p (MIMAT0000437), hsa-miR-18a-5p (MIMAT0000072) and Ce_miR-39-3p (MIMAT0000010) were performed through CFX96 Real-Time System C1000 Touch Thermal Cycler (BioRad Laboratories, Inc), by using miScript SYBR Green PCR kit (218073, Qiagen) and specific miScript primer Assays (MS00008827, MS00009086, MS00003241, MS00003528, MS00031514 and MS00019789) [5,6,14]. qRT-PCR data were analyzed by using the $2^{-\Delta\Delta Ct}$ method, where Cycle threshold (Ct) values were determined by CFX ManagerTM Software (BioRad Laboratories, Inc) [6].

2.4. Study endpoint

The clinical outcome was the response to CRTd after one year of follow-up evaluated by the improvement of LVEF, LVESv, 6MWT, and NYHA class. Therefore, at follow-up, we re-evaluated for each patient the NYHA classification. The patients graded their overall condition as unchanged or slightly, moderately, markedly worsened, or improved by global self-assessment [1–4]. Furthermore, all the patients were instructed regularly to assess body weight, the occurrence of dyspnea, and any clinical symptoms. The clinical evaluations included physical examination, vital signs, and the review of adverse events. Fasting blood (at least 12 h from last meal) was performed for biochemical peripheral blood assay evaluation at every visit. The response to the ARNI and conventional anti-HF therapy included the assessment of cardiac dimension, volumetry, and cardiac pump for the assessment of LVEF ($\geq$ 10%) at trans thoracic echocardiography [1–4].

2.5. Statistical methods

A qualified statistician analyzed all collected data. The CRT-d patients were divided into the ARNI group of patients (ARNI-users) vs. the Non-ARNI group of patients (conventional group or controls, Non-ARNI users). We postulated that the number of patients with alterations in the study endpoints was significantly different between ARNI-users vs. Non-ARNI users patients. Safety analyses were performed on data from all enrolled patients. Continuous variables were expressed as means and standard deviations and tested by a two-tailed Student *T*-test. The categorical variables were compared by Chi-square or Fisher exact test where appropriate. Predictors of the response to CRTd were evaluated using Cox regression models in the study population adjusted for study variables: age; BMI; sex; smoking; creatine levels; diabetes; dyslipidemia; heart rate; systolic blood pressure levels; ARNI therapy. Statistical significance was considered for a p-value of less than 0.05. The statistical analysis was performed using the SPSS software package for Windows 17.0 (SPSS 23 Inc., Chicago, Illinois).

3. Results

3.1. Baseline findings

A total of 807 patient CRTd non-responders were screened. A total of 108 patients were excluded for IV NYHA class (n = 23), systolic hypotension (n = 37), unstable HF (n = 15).

chronic infection disease (n = 10), liver diseases (n = 6), kidney diseases (n = 17) (STROBE DIAGRAM, Fig. 1). Therefore, 669 patients were included in the study evaluation (Fig. 1). Among these patients, 217 were ARNI users, and 482 were Non-ARNI users. 111 ARNI-user patients and 170 Non-ARNI-user patients were excluded during the follow-up period. So, 106 ARNI users and 312 Non-ARNI users were included in the study analysis. Patients were defined as non-responders 247 ± 36 days after CRTd in the ARNI-user group and after 233 ± 47 days in the non-ARNI-user group. All patients were treated with full HF therapy as recommended by HF guidelines [14]. There were no differences in risk factors and pathogenesis of heart failure (Table 1). Baseline clinical and echocardiographic characteristics of the patients in each group are presented in Table 1. All patients had severe LV dysfunction, significant intraventricular and interventricular dyssynchrony, and severe LV dilatation. All patients were in NYHA functional class $\geq$ 3 HF and 6MWT were similar in both groups (Table 1). The ARNI-users received either 24/26 mg or 49/51 mg of ARNI twice daily (the mean time duration of the therapy was 21.4 ± 5.6 days) [8]. The Non-ARNI users were treated with ACEIs (n = 167, 53.5%) or ABRs (n 145, 46.5%) because of ARNI intolerance: dizziness n = 147, 47.1%; decreased SBP n = 72, 23.1%; general weakness n = 51, 16.3%; decreased renal function n = 11, 3.5%; other n = 31, 10%.

3.2. Effects of ARNI therapy on clinical parameters

At one year of follow-up, ARNI-users showed significantly lower values of 6MWT and a lower number of patients in the III and IV NYHA classes compared to Non-ARNI users ($p < 0.05$) (Fig. 2, Table 1). A higher number of ARNI-users were in the I and II NYHA classes compared to Non-ARNI users ($p < 0.05$) (Table 1). Conversely, at the echocardiographic exam, the ARNI-users showed the lowest diameters and volumetric dimension of the left ventricle cavity and highest values of LVEF, with a significant reduction of the degree of mitral regurgitation compared to non-ARNI users ($p < 0.05$) (Table 1). Interestingly, by analyzing the time course of echocardiographic changes during follow-up, we can see that ejection fraction and LVESv improved after six months in patients treated with ARNI while remaining unchanged in patients not treated with ARNI (Fig. 2). Finally, Non-ARNI users evidenced, at the follow-up end, a higher rate of patients under amiodarone, ivabradine, and loop diuretics drugs compared to ARNI-users

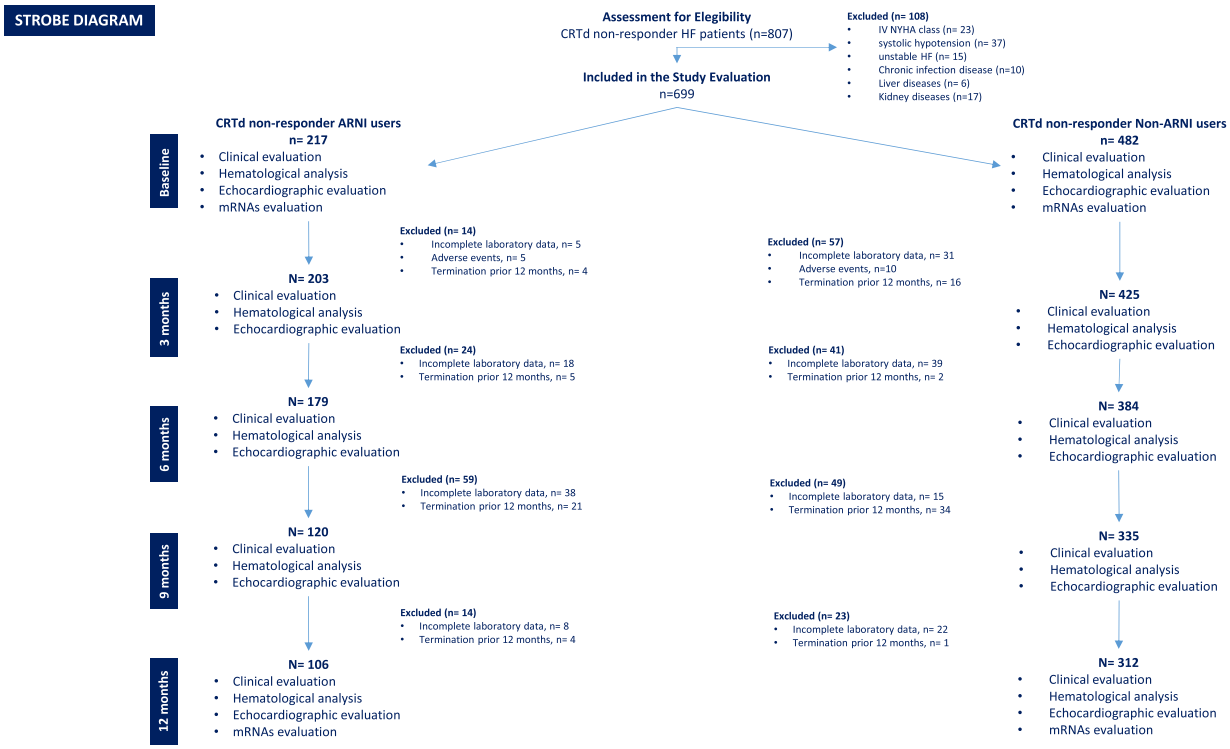

Fig. 1. STROBE DIAGRAM study population.

($p < 0.05$) (Table 1).

3.3. Effects of ARNI therapy on inflammatory markers

At baseline, there were no differences in the lymphocytes, CRP, IL6, TNFα, and the Nt-proBNP values among non-ARNI users and ARNI users (Table 1). At follow-up, the Non-ARNI users over-expressed the lymphocytes, CRP, IL6, TNFα, and the Nt-proBNP values ($p < 0.05$), while they evidenced the lowest values of BNP compared to ARNI-users ($p < 0.05$) (Table 1).

3.4. Effects of ARNI therapy on microRNA changes

As shown in Fig. 2, there were no statistically significant differences between miRNA baseline levels of Non-ARNI users and ARNI-users. After one year of ARNI therapy, the plasma levels of miR-18 and miR-145 were significantly up-regulated ($P < 0.01$), while miR-181 was reduced in the ARNI-users compared to those who were compared with Non-ARNI users. Interestingly, by analyzing the time course of miR changes during follow-up, we can see that miR-18 changed quickly after ARNI therapy began; miR-181 reversed three months and miR-145 six months after ARNI therapy remained unchanged in patients not treated with ARNI (Fig. 2). Moreover, miR changes came before functional heart improvements. Therefore, we assumed that these miRNAs might be involved in active cardiac recovery. There were significant differences in the final values of these parameters between the two groups of patients: miR-18 levels increased by 3.8-fold in ARNI-users while increasing only 0.71-fold in non-ARNI-users ($P < 0.001$); miR-145 levels increased by 1.7-fold in ARNI-users while increasing only 0.3-fold in non-ARNI-users ($P < 0.001$); and miR-181 levels were down-regulated by 1.5-fold in ARNI-users while growing 0.6-fold in non-ARNI-users ($P < 0.001$) (Fig. 3).

3.5. MicroRNA changes and clinical parameters

After one year of follow-up, continuous associations between circulating miRNA changes and EF and NT-proBNP were found: plasma miR-18 and miR-145 fold increases were directly correlated with EF improvements ($R^2 = 0.57$, $P < 0.001$; $R^2 = 0.49$, $P < 0.001$; respectively), and inversely correlated with NT-proBNP ($R^2 = -0.37$, $P < 0.05$; $R^2 = -0.41$, $P < 0.01$; respectively). Multiple linear regression analysis showed that when adjusted for confounding factors such as age, gender, functional class, and treatment (angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs), beta-blockers, spironolactone, diuretics, and digoxin), the associations between circulating miRNA changes and EF and NT-proBNP changes remained significant (EF, b-coefficient= 0.339, $P < 0.05$;.

NT-proBNP, b-coefficient= 0.4710, $P < 0.01$).

3.6. Effects of ARNI therapy on clinical outcomes

At one year of follow-up, we evidenced that, based on symptomatic improvements, echocardiographic and functional classification improvements, 37 (34.9%) patients among ARNI users became responders, while only twenty (6.4%) patients became responders Non-ARNI users. So, Cox regression analysis evidenced that patients treated with ARNI have a 8-fold probability of reverse remodeling than patients not treated with ARNI, irrespective of gender, presence of hypertension, dyslipidemia, smoking habits as well as age, BMI, plasma lipid levels, blood pressure, and heart rate (Fig. 4). Interestingly, diabetes reduces the possibility of reverse remodeling by seven-fold (Fig. 4).

4. Discussion

The most important message from this investigation on different LV functional recovery after CRTd is that the ARNI therapy improves the timing pattern of the heartbeat in CRTd non-responders patients. Interestingly, based on symptomatic recoveries, echocardiographic and

Table 1
Clinical characteristics of study population at 1 year of follow-up in ARNI users (n 106) vs. Non-ARNI users' patients (n 312).

	At Baseline			At Follow up end		
Study variables	**ARNI-users n 106**	**Non-ARNI users n 312**	**p value**	**ARNI-users n 106**	**Non-ARNI users n 312**	**p value**
Age, years	70.7 ± 4.4	71.1 ± 6.1	0.081	/	/	/
Male, n (%)	74 (69.8)	215 (68.9)	0.256	/	/	/
BMI > 30 Kg/m2, n (%)	7 (6.6)	19 (6.1)	0.720	11 (6.9)	24 (7.7)	0.591
Smokers, n (%)	54 (50.9)	155 (49.7)	0.328	83 (51.9)	174 (55.8)	0.281
Hypertension, n (%)	77 (72.6)	224 (71.8)	0.902	119 (74.4)	234 [75]	0.898
Diabetes mellitus, n (%)	71 (66.9)	209 (67.0)	0.811	114 (71.3)	222 (71.1)	0.985
Dyslipidemia, n (%)	46 (43.4)	134 [43]	0.821	69 (43.1)	144 (46.1)	0.651
COPD, n (%)	30 (28.3)	85 (27.2)	0.446	41 (25.6)	90 (28.8)	0.441
IDCM, n (%)	73 (68.9)	217 (69.5)	0.302	112 [70]	214 (68.6)	0.302
I NYHA class, n (%)	/	/	/	5 (4.7)	2 (0.6)	0.001*,‡, †
II NYHA class, n (%)	42 (39.6)	124 (39.8)	0.298	54 (50.9)	84 (26.9)	
III NYHA class, n (%)	64 (60.4)	188 (60.2)	0.195	46 (43.4)	216 (69.2)	
IV NYHA class, n (%)	/	/	/	1 (0.9)	10 (3.2)	
QRS duration, ms	142.5 ± 9.5	145.6 ± 9.2	0.309	140.8 ± 9.6	142.1 ± 8.6	0.174
6MWT	197.31 ± 25.73	194.17 ± 20.65	0.095	220.27 ± 36.54	206.18 ± 30.64	0.009*,‡
Echocardiographic parameters						
LVEF (%)	28.7 ± 5.1	28.4 ± 4.3	0.578	35.1 ± 6.7	29.6 ± 4.5	0.001*,‡
LVEDd (mm)	63.8 ± 8.1	64.7 ± 6.7	0.273	59.2 ± 7.8	62.3 ± 6.9	0.005*,‡
LVESd (mm)	43.5 ± 7.8	42.2 ± 8.6	0.241	38.9 ± 10.8	41.0 ± 8.4	0.050*,‡
LVEDv (ml)	194.3 ± 18.2	197.5 ± 22.6	0.243	175.4 ± 23.3	189.7 ± 19.6	0.001*,‡
LVESv (ml)	142.5 ± 15.2	139.4 ± 16.7	0.273	123.8 ± 18.1	134.6 ± 17.2	0.001*,‡
Mitral insufficiency						
+(%)	58 (54.7)	166 (53.2)	0.072	83 (51.9)	112 (35.9)	0.009*, †
++ (%)	43 (40.6)	132 (42.3)	0.259	72 [45]	168 (53.8)	0.143, †
+++(%)	5 (4.7)	14 (4.5)	0.133	5 (3.1)	32 (10.3)	0.043*, †
Biomarkers of inflammation						
Lymphocytes, n x 10^3	8.687 ± 1.451	8.918 ± 1.180	0.324	6.120 ± 1.352	6.302 ± 1.162	0.032*
Neutrophiles, n	5.96 ± 1.98	5.84 ± 1.86	0.214	4.97 ± 1.66	5.60 ± 1.54	0.027*
BNP, IQR (pg/ml)	295 (145–891)	253 (138–822)	0.354	385 (197–922)	307 (104–709)	0.001*
NT-proBNP, IQR (pg/ml)	1838 (375–3643)	1976 (303–3817)	0.124	1179 (303–3072)	1438 (575–3243)	0.001*,‡, †
CRP (mg/L)	10.49 ± 0.41	10.41 ± 0.82	0.520	6.18 ± 0.41	8.79 ± 0.51	0.001*,‡
IL6 (pg/ml)	6.91 ± 0.49	7.01 ± 0.91	0.254	5.79 ± 0.29	6.22 ± 0.31	0.050*,‡
TNFα (pg/ml)	6.37 ± 0.27	6.39 ± 0.35	0.302	5.49 ± 0.13	6.06 ± 0.17	0.050*,‡
Medications						
Amiodarone, n (%)	26 (24.5)	81 [26]	0.53	20 (18.9)	100 (32.1)	0.001*
ACE inhibitors, n (%)	/	167 (53.5)	/	/	158 (50.6)	/
ARB blockers, n (%)	/	145 (46.5)	/	/	121 (38.8)	/
Beta blockers:						
Carvedilol, n (%)	46 (43.4)	108 (34.6)	0.512	40 (37.7)	142 (45.5)	0.324
Bisoprolol, n (%)	35 (33.0)	124 (39.7)	0.323	37 (34.9)	108 (34.6)	0.559
Aspirin, n (%)	46 (43.4)	122 (39.1)	0.494	42 (39.6)	140 (44.9)	0.417
Tiklopidine, n (%)	2 (1.9)	7 (2.2)	0.214	4 (3.8)	8 (2.6)	0.750
Warfarin, n (%)	32 (30.2)	93 (29.8)	0.892	29 (27.4)	100 (32.1)	0.513
NOAC, n (%)	28 (26.4)	75 (24.0)	0.175	33 (31.1)	124 (39.7)	0.127
Calcium antagonist, n (%)	8 (7.5)	20 (6.4)	0.253	7 (6.6)	32 (10.3)	0.223
Ivabradine, n (%)	31 (29.2)	78 [25]	0.165	25 (23.6)	110 (35.3)	0.027*
Digoxin, n (%)	33 (31.1)	94 (30.1)	0.396	30 (28.3)	108 (34.6)	0.261
Loop diuretics, n (%)	92 (86.8)	274 (87.8)	0.414	90 [85]	294 (94.2)	0.009*
Aldosterone Blockers, n (%)	61 (57.5)	175 (56.1)	0.127	58 (54.7)	198 (63.5)	0.110
Statins, n (%)	77 (72.6)	234 [75]	0.324	82 (77.4)	258 (82.6)	0.089
Insulin, n (%)	27 (25.5)	81 [26]	0.396	28 (26.4)	90 (28.8)	0.708
Metformin, n (%)	63 (59.4)	178 (57.1)	0.442	60 (56.6)	186 (59.6)	0.649
Sulfonylureas, n (%)	21 (19.8)	59 (18.9)	0.335	21 (19.8)	78 [25]	0.278
Thiazolidinediones, n (%)	16 (15.1)	43 (13.8)	0.316	14 (13.2)	56 (17.9)	0.150
GLP-1 agonist, n (%)	15 (14.1)	41 (13.1)	0.870	15 (14.1)	52 (16.7)	0.532
DPP-4 inhibitors, n (%)	21 (19.8)	59 (18.9)	0.220	20 (18.9)	82 (26.3)	0.138

ARNI: Angiotensin receptor/Neprilysin inhibitor therapy; BMI: body mass index; COPD: chronic obstructive pulmonary disease; ICMD: ischemic dilated cardiomyopathy; NYHA: New York Heart Association; 6MWT: six minutes walking test; LVEF: left ventricle ejection fraction; LVEDd: left ventricle end diastolic diameter; LVESd: left ventricle end systolic diameter; LVEDv: left ventricle end diastolic volume; LVESv: left ventricle end systolic volume; BNP: B type natriuretic peptide; IQR: interquartile range; NT-proBNP: N terminal pro-BNP; CRP: C reactive protein; IL6: interleukin 6; TNFα: tumor necrosis factor alpha; ACE: Angiotensin converting enzyme; ARS: angiotensin receptors; NOAC: new oral anti coagulants; GLP-1: Glucagon-like peptide-1; DPP-4: dipeptidyl peptidase-4. *: $p < 0.05$ (as statistical significant) vs. Non-ARNI users; ‡: $p < 0.05$ in ARNI-users; †: $p < 0.05$ in Non-ARNI users.

functional classification improvements, 37 (34.9%) patients among ARNI users became responders, while only twenty (6.4%) patients became responders among Non ARNi users. From a clinical standpoint, at one year of follow-up, the ARNI-users showed significant improvement in the 6MWT and NYHA class compared to Non-ARNI treated patients, justified by a substantial reduction in LVESv and the LVEF improvements. As background for this association, the recovery of heart remodeling was associated with modulation of circulating miRNA patterns implicated in cardiac hypertrophy, fibrosis, apoptosis, and inflammation in CRTd patients treated with ARNI.

Previous studies evidenced that ARNI ameliorated the 6MWT and the NYHA class, leading to increased LVEF and best clinical outcomes in HF patients [7–9]. However, these studies did not investigate the clinical effects of ARNI in CRTd non-responders patients. Moreover, these

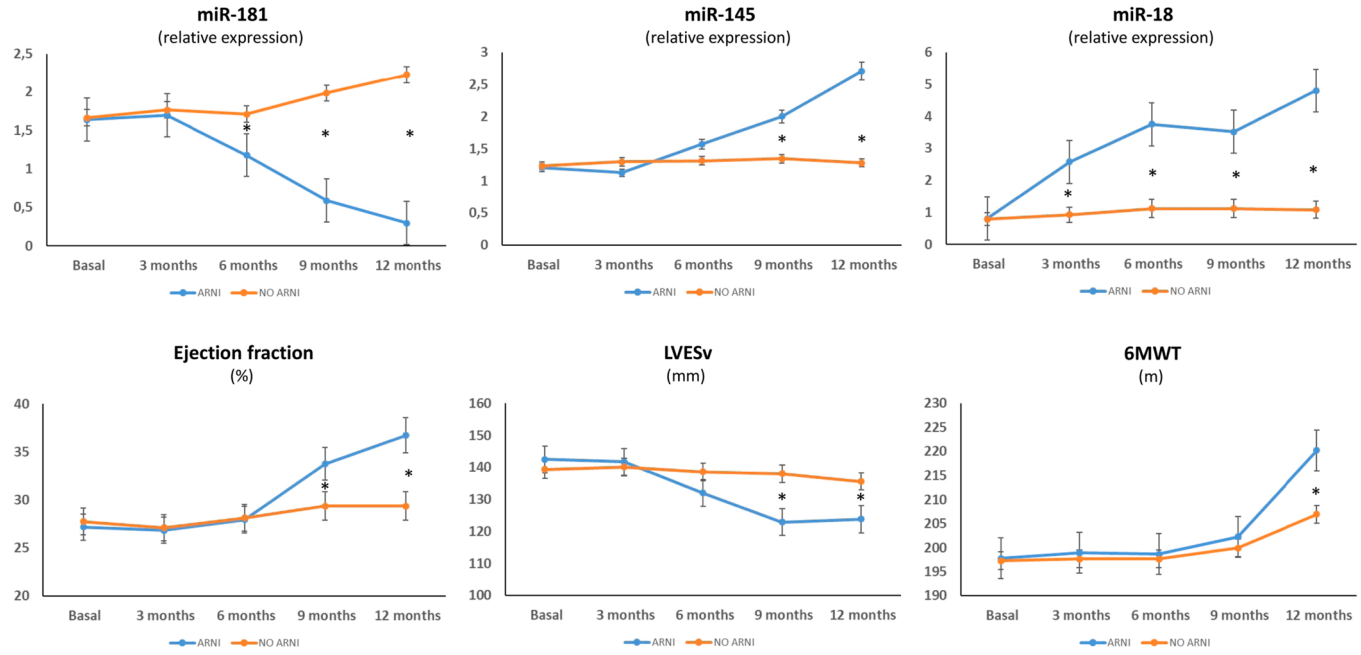

Fig. 2. miR-181, miR-145, and miR-18 serum levels in ARNI users (106 patients under Angiotensin receptor/Neprilysin inhibitor therapy) and Non-ARNI users (312 patients without Angiotensin receptor/Neprilysin inhibitor therapy), at baseline and quarterly during one-year follow-up ay 365 days of follow up. miRNA levels were calculated using Syn-Cel-miR-39 as control and expressed as $2^{-\Delta\Delta Ct} \pm$ SD. *P < 0.05 vs. ARNI treated patients.

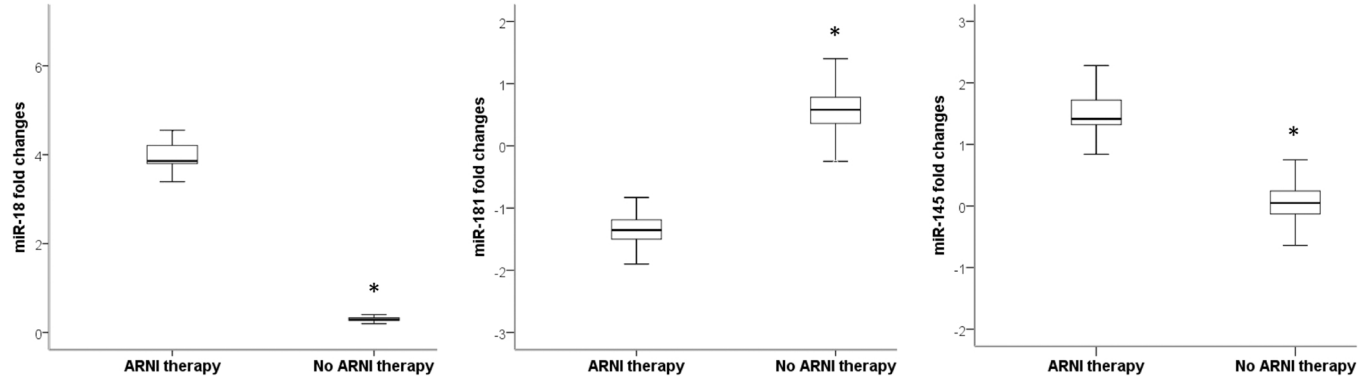

Fig. 3. miR-181, miR-145, and miR-18 fold changes between one-year follow-up and baseline levels. Boxplots show the median, 25th, and 75th percentiles, range, and extreme values. *P < 0.05 vs. ARNI treated patients.

	P	HR	95,0% CI	
			Lower	Higher
Age	,849	1,004	,961	1,049
BMI	,409	,941	,813	1,088
HR	,264	1,016	,988	1,046
Hypertension	,461	1,243	,697	2,217
SEX	,206	1,449	,816	2,573
Diabetes	,002	,206	,074	,573
Dyslipidemia	,135	,616	,327	1,162
Creatinine	,728	,745	,142	3,917
Smoking	,796	1,116	,484	2,572
Systolic blood pressure	,705	,996	,975	1,017
ARNI therapy	,000	8,088	4,410	14,836

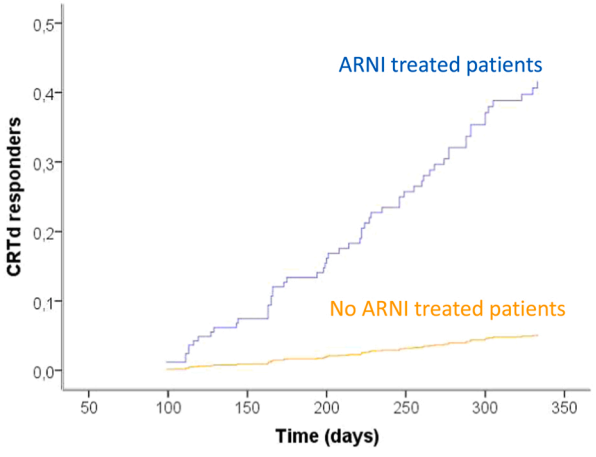

Fig. 4. Cox regression analysis in CRTd responders. Predictors of the response to CRTd were evaluated using Cox regression models in the study population adjusted for study variables: age; BMI; sex; smoking; creatine levels; diabetes; dyslipidemia; heart rate; systolic blood pressure levels; ARNI therapy.

studies did not provide any evidence about the pathway transducing ARNI therapy in epigenetic modulation in subgroups of high-risk failing patients such as that CRTd non-responders patients.

From the epigenetic standpoint, ARNI therapy was associated with the miR-18 and miR-145, increased levels, and decreased levels of miR-181 at one-year follow-up. Recent evidence suggests that these miRNAs are differentially expressed in the failing myocardium and play an important role in the progression of HF by targeting genes that govern diverse functions in the adverse cardiac remodeling process, including myocyte hypertrophy, excitation-contraction coupling, increased myocyte loss, myocardial fibrosis, and inflammation [5,6]. In particular, a recent study evidenced that the over-expression of **miR-18** caused the increase of LVEF by the significant reduction of fibrosis, hypertrophy, inflammation, and apoptosis of HF cardiomyocytes in a mice model [15]. Thus, ARNI therapy reducing miR-18 levels may confer to CRTd non-responders the protection against the adverse negative adverse cardiac remodeling effects and reduce the HF worsening with the consequent best clinical outcomes. Accordingly, we found that the highest values of miR-18 in ARNI-treated patients are associated with LVESv and LVEF improvements. Furthermore, the **miR-145** is involved in different cardiac adaptive processes of HF and selectively over-expressed in CRTd-responders [5,6,16,17]. Indeed, in CRTd-responders vs. CRTd non-responders, the circulating miR-145 increases and directly correlates with LVEF improvements and inversely with NT-proBNP values [6]. In this context, it is evidenced that mirR-145 reduced inflammation, oxidative stress, and apoptosis in the cardiomyocytes model [18]. Therefore, in line with these previous studies [6,18], we found that the highest values of miR-145 in ARNI-treated patients are associated with the reduced inflammatory burden and improvement of cardiac function and clinical outcome as NYHA class and 6MWT. Finally, the effects of ARNI on the expression of **miR-181** have been evaluated in a rodent model [19]. Indeed, the ARNI down-regulated the expression of miR-181, and the antagomir-181 showed a beneficial effect on cardiac function via the reduction of myocardial fibrosis and hypertrophy [19]. Our study found the significant downregulation of miR-181 in ARNI-users vs. Non-ARNI users' patients, and the ARNI-users showed a considerable amelioration of NYHA class, lowest NT-proBNP values. Interestingly, by analyzing the time course of miR changes during follow-up, we can see that miR-18 changed quickly after ARNI therapy began; miR-181 reversed three months and miR-145 six months after ARNI therapy remained unchanged in patients not treated with ARNI. Moreover, miR changes came before functional heart improvements [20]. Therefore, we assumed that these miRs might be involved in active cardiac recovery. Interestingly enough, diabetes reduces the possibility of reverse remodeling by seven-fold. Thus, confirming the diabetes as negative risk factor for adverse cardiac remodeling via miRs modulation [21].

To date, the differences in the miRs' serum expressions could mark the changes occurring at the myocardial tissue in HF patients [22]. In this context, in an animal model with a left bundle branch block, the authors found a close inverse relation between cardiac hypertrophy and miRs' expression [23]. Conversely, the identification of patients that will respond to CRTd represents a major challenge [24]. Moreover, identifying a predictive model of CRTd response could be relevant to predicting clinical outcomes and ameliorating clinical prognosis in HF patients. In this context, the authors investigated in circulating lymphocytes the glycation of RyR1 as a serum marker, correlating with pathologic intracellular calcium leak, and reduced CRT responsiveness [24]. However, this could confirm the over-glycation as a negative factor crossing from over-inflammation to the reduced CRTd responsiveness via altered calcium handling [24].

These data fit with recent ESC guidelines for HF, suggesting ARNI treatment for failing patients that does not evidence a cardiac functional recovery after therapy with ACEI or ARB [16]. Indeed, in a rat model, the ARNI, as compared to ARB, ameliorated cardiac and vascular function via increases in nitric oxide bioavailability [25]. Thus, ARNI showed superior cardiovascular protection in HF and improved vascular function to a greater extent than valsartan alone [25]. Notably, this favorable effect on cardiac and vascular functions [25] was linked to anti-remodeling properties and the best prognosis in HF patients under ARNI therapy [26]. Therefore, the best prognosis compared with ACEI/ARB use in HFrEF could promote the early initiation of ARNI in the HF disease process [26,27].

In this context, our data suggest that ARNI therapy may be a first-line therapy to reduce CRTd failure in HF patients. Thus, these observations support the postulate that ARNI therapy in CRTd patients could lead to sustained functional and clinical improvement by modulating the epigenetics of adverse molecular remodeling. Interestingly, we noticed similar miRs' plasma levels in both the ARNI-users and Non-ARNI-users at baseline, indicating the beneficial effects of ARNI therapy on dysfunction, dyssynchrony, or clinical HF class after CRTd could be due to a different change in plasma levels of miRs. This observation seems to indicate that non-responders treated with ARNI were more prone to a change in miRs' profiles and thus more likely to improve after CRTd. So, we speculate that the identification of miRs' ARNI-induced changes could improve the responses to current therapeutic strategies involving cardiac resynchronization therapy. Further studies using large-scale microarray profiling are needed to address this hypothesis and identify novel molecular markers of reversed remodeling after ARNI therapy in CRTd patients. However, similar to previous clinical reports, our study does not address the causative or mechanistic relationship between LV remodeling after CRTd and ARNI. Nevertheless, the relationship between BNP and EF and the positive association and miRNA changes indirectly support the favorable epigenetic effects of ARNi in high-risk failing patients as CRTd non-responders. Moreover, in addition to the regulatory roles of ARNI in signaling molecules, we must also consider that the epigenetic perspective is beginning to shed new light on how ARNI therapy may influence gene expression and sustained inflammation in HF. A fundamental question in the field of epigenetics is to understand the biochemical mechanisms underlying inflammation regulation to failure of CRTd therapy. The current study evidenced a few limitations. Regarding human research, the small sample size and the duration of follow-up cannot drive definitive conclusions about the effects of ARNI in CRTd non-responders in terms of miRs' expression and clinical outcomes. Again, the loss of an animal model of chronic HFrEF and an ex-vivo model of cultured cardiomyocytes cells could help us, soon, to test the effects of ARNI on the inflammatory cellular and molecular pathways and the miRs' expression. This model could help if added to the specific treatment with mimic-miR and/or antagomir to test the inflammation and the different remodeling cardiac processes with and without ARNI. Indeed, this advancement could then be in clinical use and may further improve outcomes in CRTd non-responders' patients. However, further studies will be designed to address this hypothesis and identify novel molecular markers of reversed remodeling after CRTd in non-responders.

5. Conclusions

As the novelty of our research, the effects ARNI induced might influence the epigenetic mechanisms modulating miRs levels implicated in the main pathways of heart dysfunction as miR-18, miR-145, and miR-181 operating also in adverse cardiac remodeling responses to CRTd.

Authors' contributions

C. S: study design, data interpretation, and writing of the study; L. S, MC. T, N. DO, and C. R performed the experiments; M. S, M. V, V. D, A. R, C.M: implantation and follow-up of CRTd; G. S: statistical analysis; L. M and F. C: data collection; M. DA, and ml. B: revised the experiments and collected the data; Ma. M, A. C, G. P, and R. M: revised and edited the article.

Data statement

The data of the current research could be shared on request.

References

[1] M. Glikson, J.C. Nielsen, M.B. Kronborg, et al., ESC Scientific Document Group, 2021 ESC Guidelines on cardiac pacing and cardiac resynchronization therapy, Eur. Heart J. 42 (35) (2021) 3427–3520.

[2] F. Leyva, S. Nisam, A. Auricchio, 20 years of cardiac resynchronization therapy, J. Am. Coll. Cardiol. 64 (10) (2014 9) 1047–1058.

[3] C. Sardu, P. Paolisso, V. Ducceschi, M. Santamaria, C. Sacra, M. Massetti, A. Ruocco, R. Marfella, Cardiac resynchronization therapy and its effects in patients with type 2 DIAbetes mellitus OPTimized in automatic vs. echo guided approach. Data from the DIA-OPTA investigators, Cardiovasc Diabetol. 19 (1) (2020) 202.

[4] C. Sardu, P. Paolisso, C. Sacra, M. Santamaria, C. de Lucia, A. Ruocco, C. Mauro, G. Paolisso, M.R. Rizzo, M. Barbieri, R. Marfella, Cardiac resynchronization therapy with a defibrillator (CRTd) in failing heart patients with type 2 diabetes mellitus and treated by glucagon-like peptide 1 receptor agonists (GLP-1 RA) therapy vs. conventional hypoglycemic drugs: arrhythmic burden, hospitalizations for heart failure, and CRTd responders rate, Cardiovasc. Diabetol. 17 (1) (2018) 137.

[5] C. Sardu, M. Barbieri, M.R. Rizzo, P. Paolisso, G. Paolisso, R. Marfella, Cardiac resynchronization therapy outcomes in type 2 diabetic patients: role of microRNA changes, J. Diabetes Res. 2016 (2016), 7292564.

[6] R. Marfella, C. Di Filippo, N. Potenza, et al., Circulating microRNA changes in heart failure patients treated with cardiac resynchronization therapy: responders vs. non responders, Eur. J. Heart Fail. 15 (11) (2013) 1277–1288.

[7] Y. Wang, R. Zhou, C. Lu, Q. Chen, T. Xu, D. Li, Effects of the angiotensin-receptor neprilysin inhibitor on cardiac reverse remodeling: meta-analysis, J. Am. Heart Assoc. 8 (13) (2019), e012272.

[8] J.J. McMurray, M. Packer, A.S. Desai, J. Gong, M.P. Lefkowitz, A.R. Rizkala, J. L. Rouleau, V.C. Shi, S.D. Solomon, K. Swedberg, M.R. Zile, PARADIGM-HF Investigators and Committees, Angiotensin-neprilysin inhibition versus enalapril in heart failure, N. Engl. J. Med. 371 (11) (2014) 993–1004.

[9] T.G. von Lueder, B.H. Wang, A.R. Kompa, L. Huang, R. Webb, P. Jordaan, D. Atar, H. Krum, Angiotensin receptor neprilysin inhibitor LCZ696 attenuates adverse cardiac remodeling and dysfunction after myocardial infarction by reducing cardiac fibrosis and hypertrophy, Circ. Heart Fail. 8 (1) (2015) 71–78.

[10] E. Vaskova, G. Ikeda, Y. Tada, C. Wahlquist, M. Mercola, P.C. Yang, Sacubitril/valsartan improves cardiac function and decreases myocardial fibrosis via downregulation of exosomal mir-181a in a rodent chronic myocardial infarction model, J. Am. Heart Assoc. 9 (13) (2020), e015640.

[11] F.Q. Nuttall, Body mass index: obesity, BMI, and health: a critical review, Nutr. Today 50 (3) (2015) 117–128.

[12] C. Sardu, P. Paolisso, C. Sacra, M. Santamaria, C. de Lucia, A. Ruocco, C. Mauro, G. Paolisso, M.R. Rizzo, M. Barbieri, R. Marfella, Cardiac resynchronization therapy with a defibrillator (CRTd) in failing heart patients with type 2 diabetes mellitus and treated by glucagon-like peptide 1 receptor agonists (GLP-1 RA) therapy vs. conventional hypoglycemic drugs: arrhythmic burden, hospitalizations for heart failure, and CRTd responders rate, Cardiovasc. Diabetol. 17 (1) (2018) 137.

[13] C. Sardu, G. Pieretti, N. D'Onofrio, F. Ciccarelli, P. Paolisso, M.B. Passavanti, R. Marfella, M. Cioffi, P. Mone, A.M. Dalise, F. Ferraraccio, I. Panarese, A. Gambardella, N. Passariello, M.R. Rizzo, M.L. Balestrieri, G. Nicoletti, M. Barbieri, Inflammatory cytokines and SIRT1 levels in subcutaneous abdominal fat: relationship with cardiac performance in overweight pre-diabetics patients, Front. Physiol. 9 (2018) 1030.

[14] E. Vaskova, G. Ikeda, Y. Tada, C. Wahlquist, M. Mercola, P.C. Yang, Sacubitril/valsartan improves cardiac function and decreases myocardial fibrosis via downregulation of exosomal miR-181a in a rodent chronic myocardial infarction model, J. Am. Heart Assoc. 9 (13) (2020), e015640.

[15] J. Na, H. Jin, X. Wang, K. Huang, S. Sun, Q. Li, W. Zhang, The crosstalk of HDAC3, microRNA-18a and ADRB3 in the progression of heart failure, Cell Biosci. 11 (1) (2021) 31.

[16] McDonagh T.A., Metra M., Adamo M., et al.; Authors/Task Force Members: ESC Scientific Document Group. With the special contribution of the Heart Failure Association (HFA) of the ESC. 2021 ESC Guidelines for the diagnosis and treatment of acute and chronic heart failure: Developed by the Task Force for the diagnosis and treatment of acute and chronic heart failure of the European Society of Cardiology (ESC). Eur J Heart Fail. 2022 Jan;24(1):4–131.

[17] C. Sardu, R. Marfella, G. Santulli, G. Paolisso, Functional role of miRNA in cardiac resynchronization therapy, Pharmacogenomics 15 (8) (2014) 1159–1168.

[18] C. Cheng, D. Xu, X. Liu, S. Bi, J. Zhang, MicroRNA-145-5p inhibits hypoxia/reoxygenation-induced apoptosis in H9c2 cardiomyocytes by targeting ROCK1, Exp. Ther. Med. 22 (2021) 796.

[19] E. Vaskova, G. Ikeda, Y. Tada, C. Wahlquist, M. Mercola, P.C. Yang, Sacubitril/valsartan improves cardiac function and decreases myocardial fibrosis via downregulation of exosomal miR-181a in a rodent chronic myocardial infarction model, J. Am. Heart Assoc. 9 (13) (2020), e015640.

[20] A. Rotini, E. Martínez-Sarrà, E. Pozzo, M. Sampaolesi, Interactions between microRNAs and long non-coding RNAs in cardiac development and repair, Pharmacol. Res. 127 (2018) 58–66.

[21] Z.Q. Jin, MicroRNA targets and biomarker validation for diabetes-associated cardiac fibrosis, Pharmacol. Res. 174 (2021), 105941.

[22] I. Ben-Zvi, N. Volinsky, L. Grosman-Rimon, I. Haviv, G. Rozen, N. Andria, N. Asulin, N. Margalit, I. Marai, O. Amir, Cardiac-peripheral transvenous gradients of microRNA expression in systolic heart failure patients, ESC Heart Fail. 7 (3) (2020) 835–843.

[23] L.B. van Middendorp, M. Kuiper, C. Munts, P. Wouters, J.G. Maessen, F.A. van Nieuwenhoven, F.W. Prinzen, Local microRNA-133a downregulation is associated with hypertrophy in the dyssynchronous heart, ESC Heart Fail. 4 (3) (2017) 241–251.

[24] J. Gambardella, S.S. Jankauskas, S.L. D'Ascia, C. Sardu, A. Matarese, F. Minicucci, P. Mone, G. Santulli, Glycation of ryanodine receptor in circulating lymphocytes predicts the response to cardiac resynchronization therapy, J. Heart Lung Transpl. 41 (4) (2022) 438–441.

[25] R.K. Trivedi, D.J. Polhemus, Z. Li, D. Yoo, H. Koiwaya, A. Scarborough, T. T. Goodchild, D.J. Lefer, Combined angiotensin receptor-neprilysin inhibitors improve cardiac and vascular function via increased NO bioavailability in heart failure, J. Am. Heart Assoc. 7 (5) (2018), e008268.

[26] Y. Sun, S. Song, Y. Zhang, W. Mo, X. Zhang, N. Wang, Y. Xia, G. Tse, Y. Liu, Effect of angiotensin receptor neprilysin inhibitors on left atrial remodeling and prognosis in heart failure, ESC Heart Fail. 9 (1) (2022) 667–675.

[27] J. She, B. Lou, H. Liu, B. Zhou, G.T. Jiang, Y. Luo, H. Wu, C. Wang, Z. Yuan, ARNI versus ACEI/ARB in reducing cardiovascular outcomes after myocardial infarction, ESC Heart Fail. 8 (6) (2021) 4607–4616.