



Original article

Plasmatic and chamber-specific modulation of cardiac microRNAs in an acute model of DOX-induced cardiotoxicity



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ARTICLE INFO

Keywords:

microRNAs
Circulating microRNA
Doxorubicin
Cardiotoxicity
Biomarkers
Heart chambers

ABSTRACT

Background: Doxorubicin (DOX) is a chemotherapeutic drug limited in its usefulness by an adverse side effect, cardiotoxicity. The mechanisms leading to this detrimental occurrence are not completely clear, and lately many authors focused their attention on the possible role of microRNAs (miRNAs), small regulators of cardiovascular functions, in this phenomenon. Notably, these molecules recently emerged also as potential circulating biomarkers of several cardiac diseases. Thus, the aim of this study was the simultaneous investigation of circulating and cardiac tissue miRNAs expression upon DOX treatment *in vivo*.

Methods: Twenty C57BL/6 female mice were administered with 24 mg/Kg cumulative dose of DOX or saline (CTRL) for 2 weeks. Echocardiography was performed at baseline and at the end of treatment (T1). Plasma and heart samples were collected at T1, separating atria from left (LV) and right (RV) ventricles, and miRNAs expression was tested by RT-qPCR-based arrays. All putatively DOX-regulated candidates were then validated by single assays *in vivo* and then evaluated also in murine immortalized cardiomyocytes (HL-1) treated with 1 μM DOX for 24 h. In the end, bioinformatics target prediction was performed for all DOX-miRNAs.

Results: Cardiotoxicity onset was diagnosed upon impairment of six cardiac functional parameters in DOX-treated mice at T1. Samples collection, followed by screening and validation steps, identified eleven miRNAs dysregulated by the drug in plasma, while seven resulted as altered in separate heart chambers. Interestingly, miR-34a-5p and miR-451a showed a dysregulation in both plasma and tissue samples of DOX-administered animals, whereas five additional miRNAs presented chamber specific modulation. Of note, *in vitro* experiments showed a very modest overlap with *in vivo* results. Bioinformatics prediction analysis performed on miR-34a-5p and miR-451a identified several putative targets presenting no significant association with cardiotoxicity. Anyhow, the same analyses, conducted by combining all miRNAs regulated by DOX in each heart chamber, evidenced a possible dysregulation of the adherens junctions gene network, known to be involved in the onset and progression of dilated cardiomyopathy, an established detrimental side effect of the drug.

Conclusions: This is the first work investigating miRNAs regulation by DOX both in plasma and heart districts of treated animals. Our results indicate a strong association of miR-34a-5p and miR-451a to DOX-induced cardiotoxicity. In addition, the observed altered expression of diverse miRNAs in separated cardiac chambers hints at a specific response to the drug, implying the existence of different players and pathways leading to dysfunction onset.

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1. Introduction

Doxorubicin (DOX) is a potent chemotherapeutic agent belonging to the family of anthracycline drugs, discovered in the late 1960s and isolated from *Streptomyces peucetius*. It is used to treat many types of tumours such as breast, liver, colon cancer, lymphomas, and leukemia [1].

Doxorubicin treatment quickly led to an improvement in the survival rates of oncologic patients, thus, at length, showing its toxic side effects on many organs, particularly on heart. Indeed, the major limiting factor of DOX administration relies in its dose-related cardiotoxicity [2], ranging from subclinical ventricular dysfunction to severe cardiomyopathy, leading to heart failure, cardiac transplantation and, eventually, death [3]. Cardiotoxicity can occur early during treatment or after several years from administration, and individual risk assessment is very difficult. Despite the lack of a universally accepted definition, it is described as a decline of left ventricular ejection fraction (LVEF) of more than 10 percentage points to a value < 53% (normal reference value for two-dimensional echocardiography), according to a recent expert consensus paper [4]. The approaches currently used to detect and monitor cardiotoxicity are echocardiography, nuclear imaging, and, when indicated, endomyocardial biopsy [5,6], all techniques leading to a late diagnosis. In the last few years, Brain Natriuretic Peptide (BNP) and cardiac Troponins (cTns) have been adopted [7,8] as additional cardiac damage/dysfunction indicators. MicroRNAs (miRNAs) are small endogenous non-coding RNAs of 21–24 nucleotides, acting as post-transcriptional gene regulators via inhibition and/or degradation of target messenger RNAs (mRNAs). miRNAs play an important role in different biological processes including proliferation, development, differentiation and cell death [9]. In addition, several miRNAs are implicated in the regulation of heart development from embryonic to adult stage and their dysregulation leads to various heart diseases such as cardiomyopathy, heart failure, arrhythmias and cardiac hypertrophy [10]. In recent years, numerous groups investigated the potential use of circulating miRNAs as possible specific biomarkers and therapeutic targets of cardiac maladies [11]. To date, though, there are only few articles about circulating and tissue miRNAs association with DOX-induced cardiotoxicity. In addition, data derived from these studies are heterogeneous, in particular concerning cardiotoxicity evaluation methods and the selection of the samples used for the analyses [12]. On these bases, we aimed at identifying specific plasma and heart-chambers miRNAs associated with DOX-induced cardiotoxicity *in vivo* during the acute phase of treatment.

2. Materials and methods

2.1. Animal model

Twenty C57BL/6 female mice (Charles River Laboratories), aged 10 weeks old, were administered either with saline (CTRL, n = 10) or doxorubicin hydrochloride (DOX, n = 10, cumulative dose 24 mg/kg, Sigma-Aldrich) by six intraperitoneal injections (4 mg/kg) 3 times a week for 2 weeks (Fig. 1), as previously reported [13]. The study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Milan and by the Italian Ministry of Health (approval number 379/2015-PR). Female mice were chosen in order to create an animal model as close as possible to female breast cancer patients.

2.2. Cardiotoxicity assessment

Before treatment (baseline) and after the last DOX-injection (T1) cardiac function was monitored by echocardiography using a VEVO 2100 high-resolution imaging system (VisualSonics). Induction of

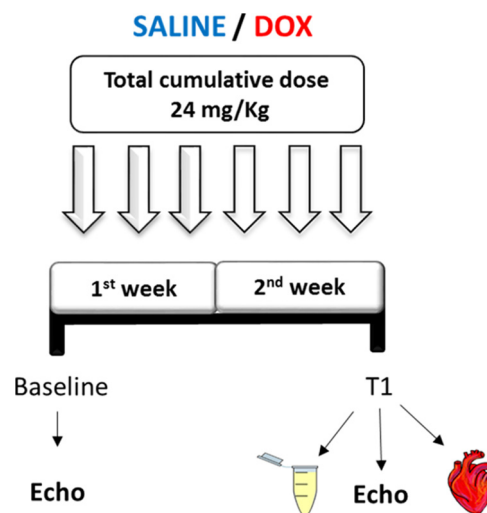


Fig. 1. Experimental Design. Treatment protocol for our animal model of DOX-induced cardiotoxicity. Arrows indicate each single 4 mg/kg intraperitoneal DOX/saline injection. Black vertical bars indicate experimental endpoints when echocardiography (Echo) was performed and samples (plasma, hearts) collection took place. Each group was composed of ten animals.

anaesthesia was performed using 2% isoflurane (Merial) mixed with 100% oxygen (2 min) in an induction chamber. Mice were then placed on a heat pad in the supine position and kept at 37 °C to minimize fluctuations of body temperature. Data acquisition was performed in mice lightly anesthetized with 0.5%–1% isoflurane, in order to maintain heart rate \geq 450 beats/min. Two-dimensional short-axis M-mode echocardiography was performed to evaluate the following parameters: left ventricular ejection fraction (LVEF), left ventricular fractional shortening (LVFS), left ventricular end-systolic volume (LVSV), left ventricular end-diastolic volume (LV DV), internal end-systolic and end-diastolic diameters (LVSD, LVDD).

2.3. Plasma cardiac troponin

Cardiac Troponin I was measured in plasma samples at T1 using the Mouse Cardiac Tn-I (High Sensitivity) kit (Life Diagnostics), following the manufacturer's instructions.

2.4. Plasma and cardiac tissue collection

Blood was collected after sacrifice at T1 using EDTA-coated tubes, and immediately centrifuged to separate plasma as previously described [14]. At the same time, cardiac chambers (atria, LV and RV) were isolated after heart excision, and immersed in Allprotect Tissue Reagent (Qiagen) following manufacturer's indications to prevent RNA degradation. Due to technical limitations, right and left atria were collected as a single chamber. All samples were stored at -80 °C until further use.

2.5. Cell culture

Immortalized murine cardiomyocytes (HL-1) were grown in Claycomb complete medium (Sigma Aldrich), according to vendor's instructions. Cells were plated in six-well plates (250.000/well); the following day, fresh medium was added with either 1 μ M (final concentration) DOX or an equivalent volume of sterile PBS. After 24 h, supernatants and cells were collected. Supernatants were centrifuged at $500 \times g$ for 10' at 4 °C to remove cells, and then at $16000 \times g$ for 10' at 4 °C to remove remaining debris.

2.6. Total RNA purification

RNA purification from plasma (200 µL/sample) was conducted using TRIzol (Life Technologies) following a modified protocol for liquid samples [15]. Total RNA was resuspended in 50 µL of RNase-free water. RNA isolation from cardiac chambers was performed by homogenizing the tissues in 1 ml/100 mg of TRIzol reagent using a Tissue-Lyser (Qiagen), according to manufacturer's instructions, similarly to total RNA from HL-1 cells. Heart and HL-1 RNA pellets were resuspended in 32 µL RNase-free water and quantified using a NanoDrop One (ThermoFisher Scientific). All samples were stored at -80°C until further use.

2.7. Circulating microRNAs screening

Circulating miRNAs expression profiles were conducted at T1 using the TaqMan Rodent MicroRNA A Array v2.0 (Applied Biosystems), following manufacturer's protocol for liquid samples. The screening was performed on four chosen samples from each group. Data were analyzed using ExpressionSuite v1.0.3 dedicated software (Life Technologies), and Global Normalization method was applied. All miRNAs presenting Ct values > 32 were considered as not expressed.

2.8. MicroRNAs screening of cardiac tissues

Tissue miRNAs expression profiles were conducted at T1 by OpenArray® Real-Time PCR System according to manufacturer's instructions (Applied Biosystems), using a custom plate with 224 candidate microRNAs (supplementary table A1). The screening was performed on four chosen samples from each group. RT was conducted using the TaqMan Advanced miRNA cDNA Synthesis Kit. After pre-amplification and dilution, a mixture composed of 2.5 µL of each sample and 2.5 µL of TaqMan OpenArray Real Time PCR master mix (Applied Biosystems) was dispensed on a OpenArray plate using the AccuFill automated sample loading system (Applied Biosystems), and run using a QuantStudio 12k Flex Real Time PCR System. Data analysis was conducted using a cloud-based Thermo Fisher Scientific proprietary software (Thermo Fisher cloud, <https://apps.thermofisher.com/apps/>). Samples presenting a Cq value > 35 , an amplification score (Amp Score) < 1.24 , and a Cq confidence (Cq conf) value < 0.8 were rejected.

2.9. Single microRNA assays

MicroRNAs reverse transcription was performed using TaqMan Advanced miRNA cDNA Synthesis Kit (Life Technologies) starting from either 2 µL (liquid samples) or 10 ng (tissues and cells) of total RNA. Expression levels of selected miRNAs were evaluated using single TaqMan Advanced miRNA assays (Life Technologies), according to manufacturer's protocol. RT-qPCR experiments on plasma were normalized using miR-27b-3p, while on tissues were normalized on miR-186-5p levels, in both cases basing on screening results as previously described [14–16]. Results from HL1 cells were normalized using miR-16.

2.10. DOX-miRNA target prediction

In order to identify putative mRNA targets for miR-34a-5p and miR-451a, we performed a target prediction analysis by the means of the Cystoscape v.3.5.0 [17] plug-in 'Cytargetlinker v.4.0.1' [18], taking advantage of the miRTarBase database v.6.1 [19], which gathers the largest amount of experimentally validated miRNA-target interactions. Then, the functional analysis of the identified miRNA target genes was performed by the Cystoscape plugin 'ClueGO v.2.5.2' [20], using the Gene Ontology Biological Process as ontology reference. Combined target prediction of district specific DOX-regulated miRNAs was

performed using DIANA-miRPath v3. 0 (<http://diana.imis.athena-innovation.gr/DianaTools/index.php>) [21].

2.11. Statistical analysis

Two-way ANOVA was used in combination with Tukey's test to compare the mean levels of the six measured echocardiographic parameters across two conditions (CTRL vs. DOX) and two time points (baseline vs. T1). The tests were performed by using R (version 3.5.0) native statistical functions. The differences in miRNAs expression level were assessed by either *t*-test or Kruskal–Wallis test, when appropriate, after evaluation of normal distribution of the data (GraphPad Prism 5 Software). All P values < 0.05 were considered statistically significant.

3. Results

3.1. Evaluation of cardiotoxicity

Since the principal purpose of the present study was to identify potential dysregulation of miRNAs upon DOX-induced cardiac dysfunction, we firstly assessed the presence of cardiotoxicity by echocardiography (supplementary figure A1). No differences were evident between the two groups at baseline. Contrarily, we found that heart impairment was evident in drug treated animals at T1. In particular, DOX mice presented a significant reduction of LVEF (-6.1% , $p = 0.01$) and LVFS (-5.4% , $p = 0.03$) when compared to the CTRL group, and a concomitant increase of LVSD ($+ 0.3\text{ mm}$, $p < 0.01$) and LVSV ($+ 4.3\text{ µL}$, $p < 0.01$) (Fig. 2 and supplementary table A2). Besides, despite not being statistically significant, both LVDD ($+ 0.1\text{ mm}$, $p = 0.38$) and LVDV ($+ 3.9\text{ µL}$, $p = 0.37$) showed an increasing trend in treated mice. Doxorubicin administered animals showed significant modifications in all six parameters during treatment. In particular, both LVEF (-8.5% , $p < 0.01$) and LVFS (-7.9% , $p < 0.01$) showed a decrease, while LVSD ($+ 0.4\text{ mm}$, $p < 0.01$), LVSV ($+ 6.2\text{ µL}$, $p < 0.01$), LVDD ($+ 0.2\text{ mm}$, $p < 0.01$) and LVDV ($+ 8.5\text{ µL}$, $p < 0.01$) presented an increase vs baseline values. Of note, we observed statistically significant variations also in CTRL animals through time in regard of two parameters, LVFS (-5.2% , $p = 0.04$) and LVSD (-0.2 mm , $p < 0.01$). In addition, plasma cardiac troponin I was measured at T1 as marker of cardiac damage. Interestingly, no differences were observed between DOX and CTRL animals (supplementary figure A2).

3.2. Plasmatic microRNAs profiling and validation

With the aim of identifying plasma miRNAs potentially regulated by DOX, we conducted an array-based screening comparing CTRL ($n = 4$) and treated ($n = 4$) samples. We observed forty miRNAs potentially regulated ($p < 0.05$) at T1 between DOX and CTRL groups (supplementary table A3), among which all miRNAs showing a fold change > 2 and < 0.6 were selected for validation by single assay on all samples ($n = 10/\text{group}$). Moreover, a few additional miRNAs were analysed, basing on previously known involvement in cardiac diseases: miR-1-3p, -133a-3p and miR-499a-5p [14].

Our results showed eleven miRNAs regulated by DOX at T1. In particular miR-32-5p, -34a-5p, -34b-3p, -34c-5p, -133a-3p, -301b-3p, -499-5p were upregulated, while miR-93-5p, -140-3p, -431-5p, and -451a were downregulated in DOX-administered mice vs. CTRL (Fig. 3 and supplementary table A4).

3.3. Cardiac tissue microRNAs profiling and validation

Concurrently, we validated the expression of miRNAs in cardiac tissues obtained from the same animals, keeping the three different cardiac districts (LV, RV and atria) separated. Our screening showed fifteen miRNAs putatively presenting a dysregulation in heart chambers from DOX animals (supplementary table A5). After validation, seven

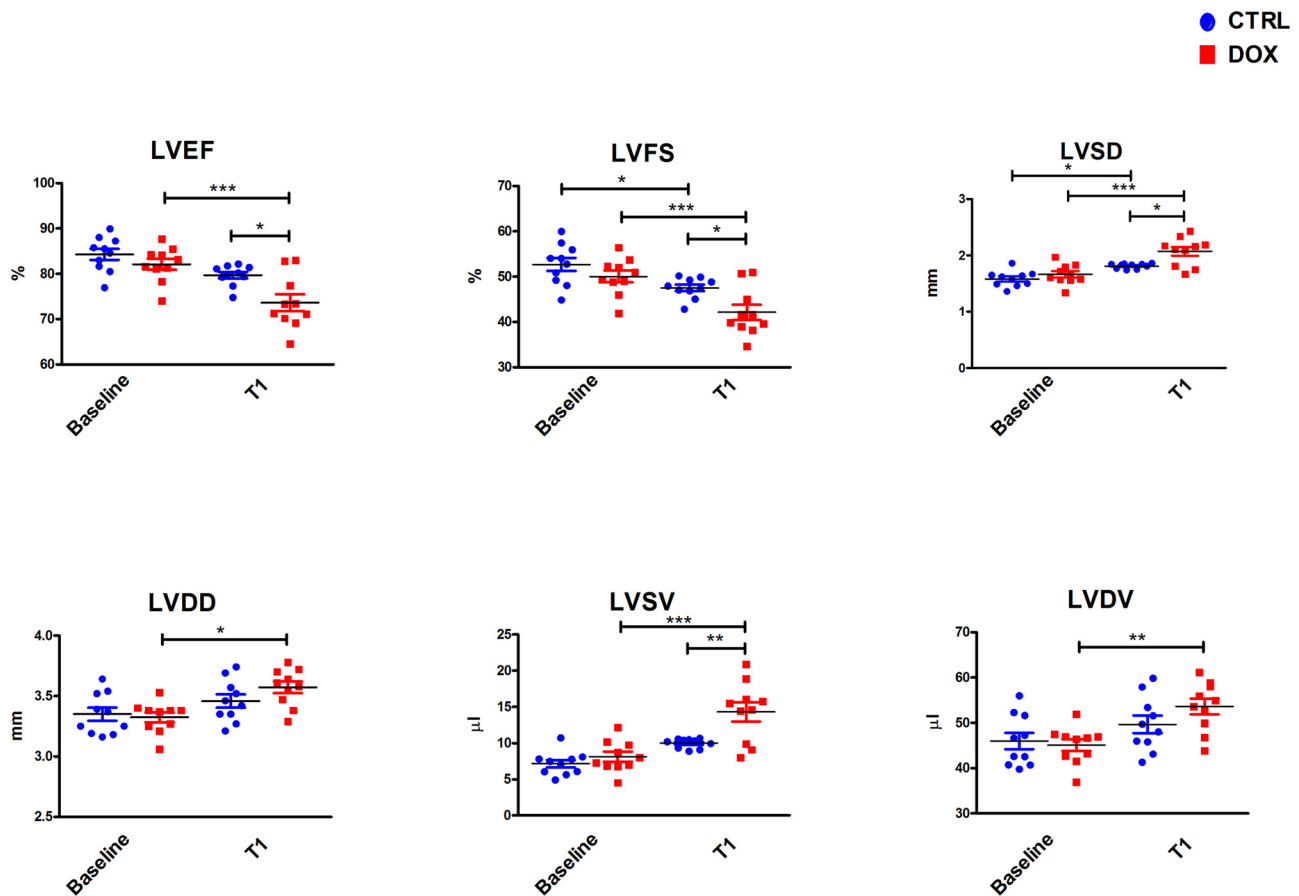


Fig. 2. Cardiac dysfunction assessment. Cardiac functional parameters were analyzed in controls (CTRL, blue) and DOX (red) mice at baseline and T1. LVEF = left ventricular ejection fraction; LVFS = left ventricular fractional shortening; LVSD = left ventricular internal end-systolic diameter; LVDD = left ventricular internal end-diastolic diameter; LVSV = left ventricular end-systolic volume, LVDV = left ventricular end-diastolic volume. Data are depicted as dot plots, lines represent mean value \pm SD. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. CTRL n = 10; DOX n = 10 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

miRNAs presented a differential expression among the cardiac districts obtained from drug-treated mice vs CTRL. In particular, two miRNAs showed a dysregulation in more than one chamber. Indeed, miR-34a-5p was upregulated in atria and downregulated in both ventricles (LV and RV) of DOX treated mice. At the same time, miR-451a showed a decreased expression in both atria and RV (Fig. 4 and supplementary table A6). Differently, three miRNAs presented a dysregulation only in the atria: two (miR-128-3p and miR-190a-5p) were increased by DOX, while miR-484a was diminished in treated animals. At the same time, both miR-146a-5p and -351 were downregulated in RV (Fig. 5 and supplementary table A6).

3.4. DOX-miRNA expression in vitro

We decided to investigate whether a murine cardiomyocyte model could produce results comparable to our *in vivo* experiments. Thus, we analysed the expression of cardiac DOX-miRNAs in both cells lysates and supernatants from HL-1 cells treated with doxorubicin (1 μ M) for 24 h. Interestingly, miR-451a showed a downregulated expression (vs. CTRLs) in supernatants from DOX-treated HL-1 cells, (supplementary figure A3). Conversely, cellular expression analysis indicated five miRNAs as upregulated by DOX in HL-1 cells (supplementary figure A4 and supplementary table A7): miR-34a-5p, -34b-3p, -34c-5p, -140-3p, and miR-451a.

3.5. Target prediction of DOX-miRNAs

We performed target prediction analysis for the two miRNAs presenting a DOX-driven dysregulation in all chambers, miR-34a-5p and miR-451a. Our results showed 16 high- and 39 low-confidence (basing on the number of the number of validation techniques) targets for miR-34a-5p, and 5 high- and 43 low-confidence targets for miR-451a (supplementary table A8). There was no evident overlapping between the two groups both in terms of target genes and of predicted targeted gene networks (supplementary figures A5 and A6). Nevertheless, two previous unrelated works involving the 2 miRNAs showed their possible involvement (upon miR-34a-5p increase and miR-451a decrease) in dilated cardiomyopathy, a known long-term side effect of DOX-induced cardiotoxicity [22,23]. This “paucity of results” prompted us to expand our analyses to all other miRNAs regulated by DOX in the different heart districts: miR-128-3p, miR-190a-5p and miR-484a in the atria, and miR-146a-5p and miR-351 in RV. The combination of these miRNAs with miR-34a-5p and miR-451a evidenced their possible involvement in several molecular networks in both atria and RV (supplementary table A9). Interestingly, among these, we observed the presence of 3 gene networks (Rap1 and Notch signaling in atria, adherens junction in RV), involved in the regulation of intercellular connections and known to be dysregulated in dilated cardiomyopathy and other cardiac diseases [24].

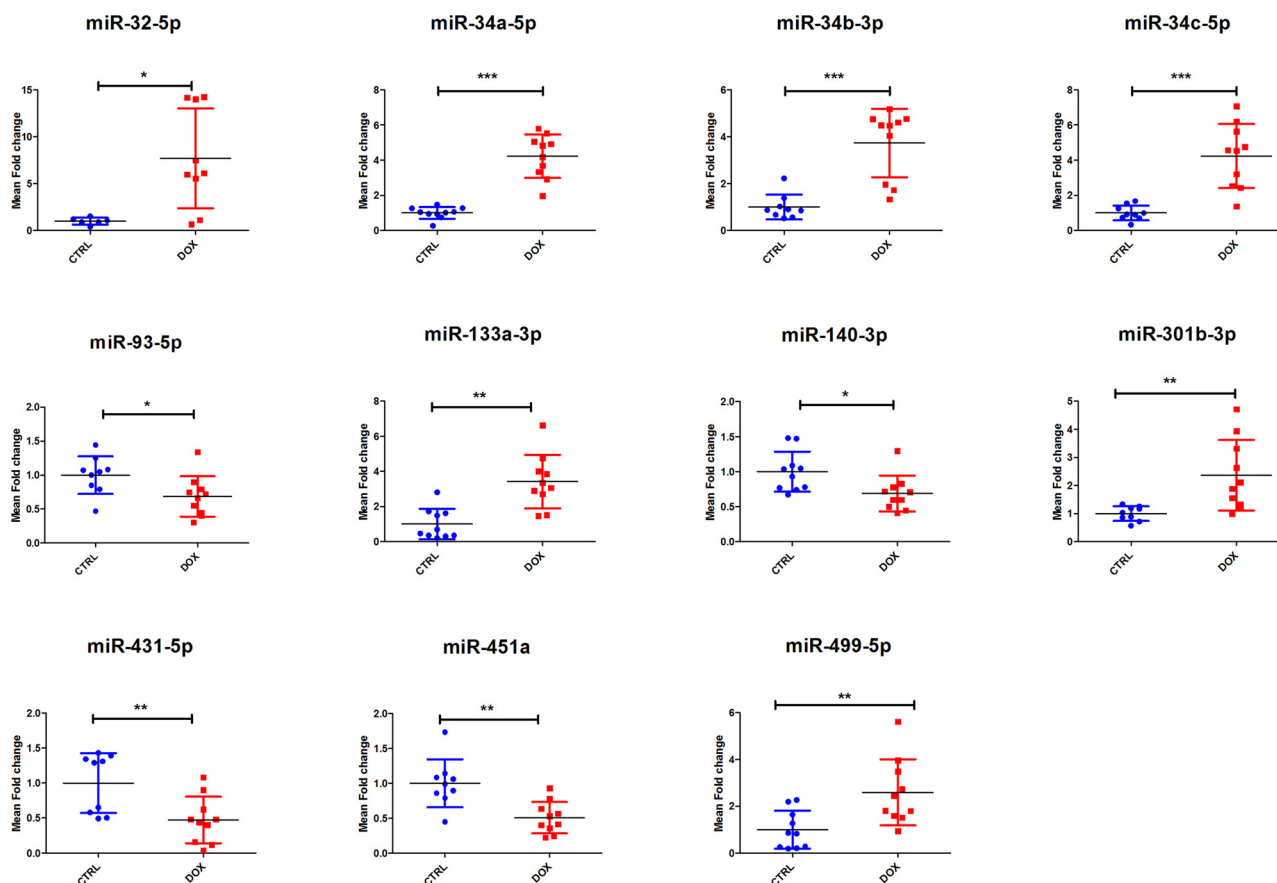


Fig. 3. Plasma miRNAs regulation upon Doxorubicin treatment. Eleven miRNAs showed dysregulated expression upon DOX-treatment (vs. controls) at T1. Data are depicted as scatter plots and expressed as mean fold change \pm SD vs. CTRL, arbitrarily set to one. Controls (CTRL): blue dots, Dox-treated (DOX): red dots. * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$. CTRL $n = 10$, DOX $n = 10$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

4. Discussion

This is the first work reporting the effects of acute DOX treatment on the levels of miRNAs evaluated in both plasma and specific heart chambers from the same animals. In particular, the differential drug-response of each specific cardiac district represents an element of novelty in this kind of studies, particularly in the miRNA field. Interestingly, echocardiography showed the expected presence of cardiac dysfunction in our murine model of DOX-induced cardiotoxicity [13]. Surprisingly, though, CTRL mice showed a quite modest but significant alteration in both LVFS and LVSD, hinting at possible detrimental cardiac effects of mock treatment combined with physiological stress due to manipulation and stabulation. Despite that, when comparing CTRL and DOX animals at T1, cardiotoxicity symptoms were evident only in the latter group, characterized by a significant decrease in both LVEF and LVFS, and by the increase in systolic LV diameters and volumes. In this setting, we decided to assess the expression of plasma miRNAs upon cardiac dysfunction onset. Our screening, and the following validation step, evidenced the dysregulation of eleven miRNAs, seven of which displayed a positive regulation by DOX, while four decreased when we compared treated vs. CTRL mice. Of note, three (miR-34a-5p, -133a-3p, -499-5p) of the regulated circulating miRNAs were, to some extent, previously associated with DOX-induced cardiotoxicity [25–27]. Increased plasma levels of these miRNAs were also shown by us and others also in relation with other types of cardiac injury (miR-133a-3p and miR-499-5p) [14,28] and with heart remodelling (miR-34a) [29], hinting at a specific role in cardiac response to damage and triggered here by DOX. Notably, two other members of the miR-34 family, miR-34b-3p and -34c-5p, were already known to be

regulated by the drug in the heart [12], the former being modulated also in circulating form [30], in line with our results. Among the other DOX-miRNAs, miR-32-5p was never associated to cardiotoxicity, and was recently described as involved in aortic calcification [31]. Interestingly, similarly to what observed in our setting, plasma levels of miR-301b-3p were shown to be increased in patients with acute myeloid leukaemia (AML) after standard chemotherapy [32]. Among the DOX-decreased miRNAs, circulating miR-93-5p is of particular interest because of its previously proposed role as exosome-vehiculated attenuator of cardiac damage [33]. Thus, its decrease upon DOX administration could contribute to the onset and progression of cardiotoxicity. Of note, increased plasma levels of miR-140-3p were associated to the onset of coronary events, but only in the very early phases [34], possibly explaining the discrepancies with our observations. As for miR-431-5p, no previous data are available about its possible role as marker of cardiac injury.

The results obtained from the separate heart districts evidenced seven DOX-regulated miRNAs. Among them, two miRNAs were dysregulated upon drug administration both at tissue and at circulating levels, miR-34a-5p and miR-451a. Of note, the former did not show a uniform response to DOX treatment in all heart chambers. Indeed, while the results from treated atria are in line with the current literature [12], both RV and LV showed an unexpected downregulation of this miRNA. Intriguingly, our results could indicate a differential DOX effect on those players regulating miR-34a expression in the three separate heart districts, although the exact mechanisms at the base of this phenomenon remain elusive. Differently from miR-34a-5p, miR-451a presented a uniform trend of regulation both in RV and in atria, where it exhibited a marked decrease in treated animals. Interestingly, miR-

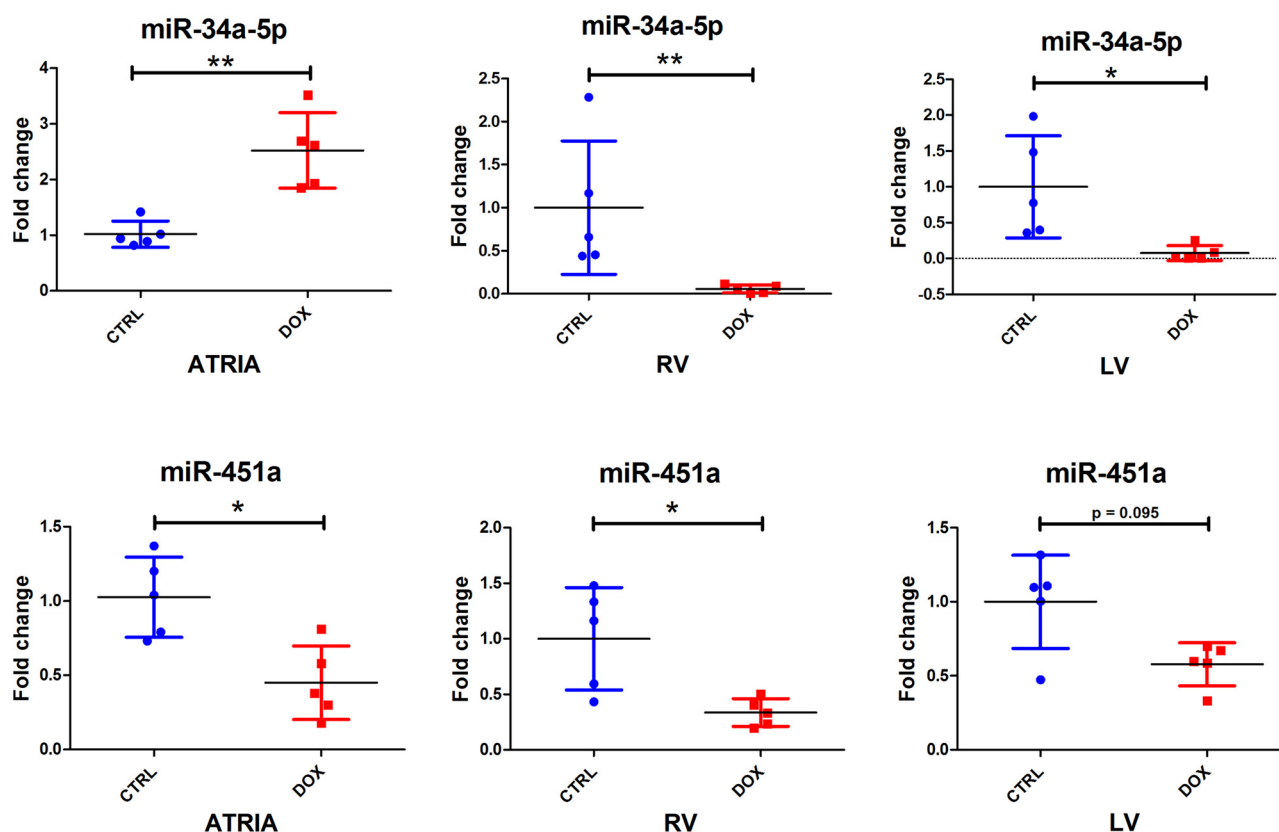


Fig. 4. MiRNAs regulated in multiple cardiac chambers upon Doxorubicin treatment. Two miRNAs showed dysregulated expression upon DOX-treatment (vs. controls) in atria, LV and RV at T1. Data are depicted as scatter plots and expressed as mean fold change \pm SD vs. Controls (CTRL), arbitrarily set to one. Controls (CTRL): blue dots, Dox-treated (DOX): red dots. * = $p < 0.05$; ** = $p < 0.01$. CTRL n = 5, DOX n = 5 (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

451a was shown to be a candidate marker of chemoresistance in triple-negative breast cancer patients administered with DOX [35], and decreased levels of this miRNA were associated to hypertrophic cardiomyopathy, one of the main diseases associated with cardiotoxicity [36]. Among the miRNAs regulated by DOX in heart tissues, only miR-146a had a previous history of “suggested” regulation by the drug in rodent hearts [30], even if its modulation in the cardiac tissue was never shown. In addition, miR-128 and miR-190 were described as implicated in the onset of heart impairment. Indeed, miR-128 deletion was correlated with a reduction of the levels of fibrosis and attenuation of cardiac dysfunction in response to MI [37], suggesting a possible role for this miRNA also in the DOX-triggered heart remodelling. Conversely, miR-190 down-regulation was associated with transverse aortic constriction-induced hypertrophy [38], seemingly in contrast with our observations, limited as they are to the acute phase of the treatment. None of the other miRNAs showing a modulation by DOX in the three heart districts presented an association with cardiac failure in the literature.

Our *in vitro* results clearly demonstrate that cellular models are not completely reliable in recapitulating *in vivo* experiments. Indeed, only two miRNAs showed DOX-induced modulation both *in vitro* and *in vivo*, miR-34a-5p and miR-451a. Of note, while DOX-induced down-regulation of the latter in HL-1 supernatants was in line with its murine plasma counterpart, it showed an opposite trend when comparing hearts and cells. Interestingly, miR-34a-5p displayed a decreased expression upon DOX treatment in HL-1 cells, a result in line with the atrial origin of these cardiomyocytes.

The natural step following the validation of DOX-regulated miRNAs in murine tissues was target identification. Our bioinformatics prediction performed for miR-34a-5p and -451a did not produce any appreciable result in terms of (possibly) modulated genes and gene networks.

Interestingly, though, the same analyses performed combining all DOX-modulated miRNAs (regardless of heart chamber specificity), clearly evidenced a possible dysregulation in the physiological expression of cardiac adherens junctions, important players in the onset and progression of cardiotoxicity-related cardiomyopathies. These results could open the way to new investigations aiming at shedding some light on the many still unveiled mechanism leading to DOX-triggered heart dysfunction.

We should acknowledge some limitations of our work. We decided to rely on echocardiography assessment of cardiac dysfunction, instead of histology, in order to use all our tissue samples to evaluate miRNA expression. In addition, we did not observe any change in circulating cardiac troponin I, a known marker of cardiac damage. In a previous paper from our group [13], we showed an increase of plasma cTnI one week after the end of DOX administration, using the same animal model. Thus, it is possible to speculate that the two-weeks-time-frame (T1) presented in this work is too short to appreciate a significant increase in TnI levels (at least in our experimental conditions). Female reproductive hormones were previously proposed to be associated with cardioprotective states [39]. However, although several mechanisms have been suggested for this phenomenon, no study elucidated the influence of fluctuating hormones on cardiac tissue remodelling. Noteworthy, estrogen was demonstrated to have no significant effects on LV diastolic function in premenopausal woman [40]. In addition, it was shown that estrous cycle does not influence cardiac ischemia-reperfusion injury in rats [41]. Since oestrous cycle was not evaluated in our animals, though, its possible influence on cardiotoxicity cannot be excluded.

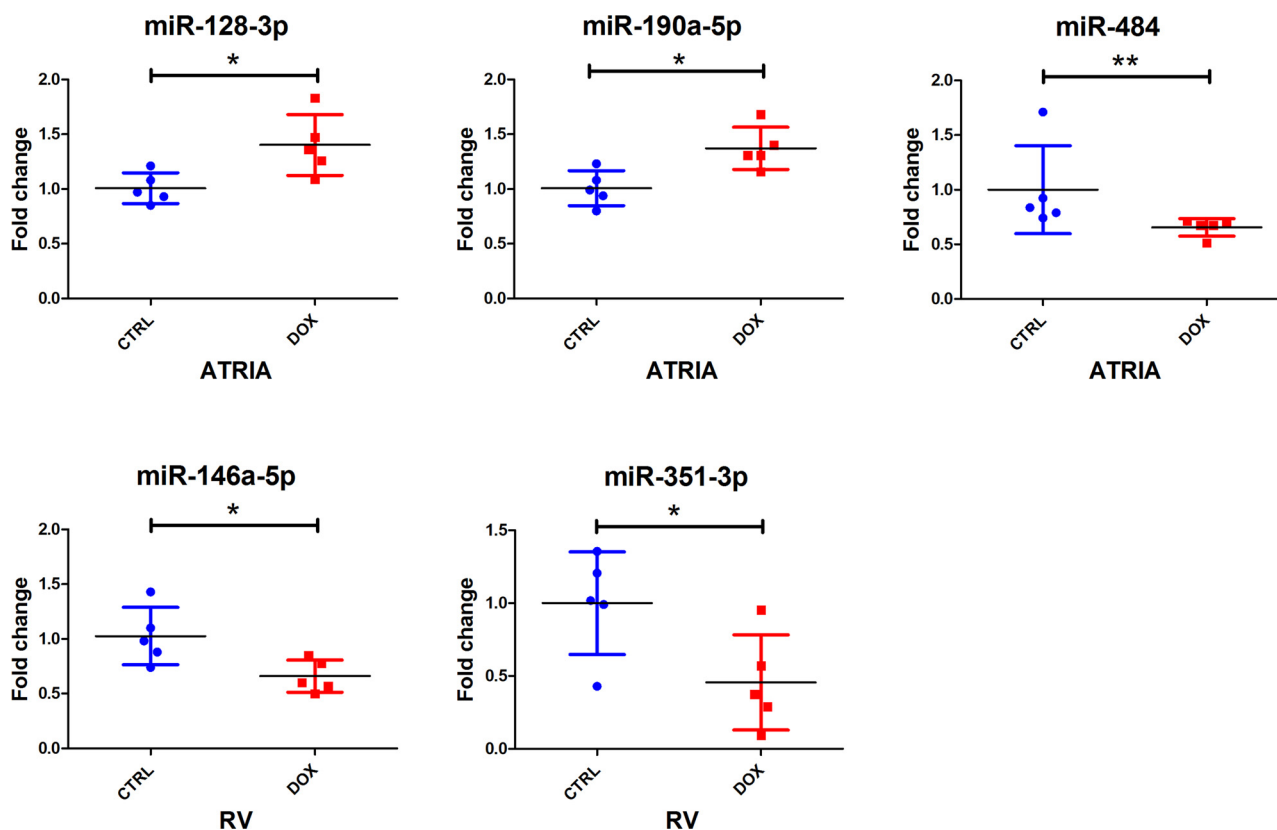


Fig. 5. MiRNAs regulated only in a specific cardiac district upon Doxorubicin treatment. Three miRNAs showed dysregulated expression upon DOX-treatment (vs. controls) in atria while two showed dysregulation in right ventricle (RV) at T1. Data are depicted as scatter plots and expressed as mean fold change \pm SD vs. CTRL, arbitrarily set to one. Controls (CTRL): blue dots, Dox-treated (DOX): red dots. * = $p < 0.05$; ** = $p < 0.01$. CTRL $n = 5$, DOX $n = 5$ (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

5. Conclusions

In conclusion, despite the limited overlap in terms of drug-response between plasma and heart districts, our results suggest an important role for specific miRNAs both as biomarkers and as mechanistic factors in the DOX-induced and chamber-specific heart dysfunction onset. In particular, both miR-34a-5p and miR-451a seem to represent the master players in this still poorly defined and complicated network of pathways triggered by the drug. Future studies focusing on identification of chamber specific triggers and validation of their targets will help unravelling their exact role in the pathology. The results obtained in the three considered cardiac chambers could lead to a better understanding of how the investigated drug affects specific functional districts of the heart. Indeed, this could pave the way to new therapeutic and diagnostic strategies to prevent and contrast DOX-induced cardiotoxicity.

Funding

This work was supported by GR-2011-02346742 grant (Ricerca Finalizzata) from Italian Ministry of Health to Y.D.

Declarations of interest

None

Acknowledgements

None.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.biopha.2018.11.042>.

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