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New Drug Development and Clinical Pharmacology

Association of Anthracycline-Related Cardiac Histological Lesions With NADPH Oxidase Functional Polymorphisms

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Disclosures of potential conflicts of interest may be found at the end of this article.

Key Words. Anthracyclines • Secondary myocardial diseases • Genotype • Myocardial fibrosis • NADPH

Abstract

Objective. Treatment with anthracyclines may cause cardiac dysfunction, but the sequence of anthracycline-induced heart lesions has been incompletely characterized. NADPH oxidase, a key mediator of oxidative cardiac damage and remodeling, modulates anthracycline clinical cardiotoxicity. Our aim was to determine which cardiac histological lesions are specifically induced by anthracycline treatment and to investigate the role of NADPH functional genetic polymorphisms in their development.

Patients and Methods. Using a retrospective case–control design, we evaluated cardiac histological lesions and NADPH genotype (polymorphisms rs1883112, rs4673, and rs13058338) in 97 consecutive decedents with a cancer diagnosis (48 treated with anthracyclines).

Results. Myocytolysis (60%), patched myocardial necrosis (19%), and myocardial fibrosis (diffuse and patched; 62% and 23%, respectively) were associated with anthracycline treatment. In patients receiving anthracyclines, NADPH oxidase polymorphism rs4673 protected against focal myocardial necrosis (odds ratio [OR], 0.11; 95% confidence interval [CI], 0.20–0.63) whereas rs1883112 was strongly associated with cardiac fibrosis (OR, 5.11; 95% CI, 1.59–16.43), which was present in all homozygotes.

Conclusion. Anthracyclines induce a cardiac remodeling pattern characterized by interstitial or patched fibrosis. The contribution of the functionally relevant NADPH polymorphisms rs1883112 and rs4673 to anthracycline-related heart lesions provides a plausible explanation for their modulation of cardiotoxicity. If confirmed, these findings may lead to better individualized strategies for early detection and prevention of anthracycline cardiotoxicity. ***The Oncologist*** 2013;18:446–453

Implications for Practice: Although anthracycline-related cardiotoxicity is still a major problem for cancer treatment, the pathological basis of late cardiac events and clinical variability of cardiac damage is largely unknown. Our work shows that heart fibrosis, previously considered an unspecific finding, is a main component of the remodeling pattern induced by anthracyclines even after low cumulative doses. These data suggest that clinical detection of fibrosis by noninvasive image might be evaluated as a potential method to detect individuals with a higher risk of late cardiac dysfunction. The analysis of NADPH oxidase polymorphisms in our series also establishes a link between the genetic variability of NADPH oxidase-mediated oxidative stress and the development of anthracycline-induced cardiac remodeling. If confirmed, these findings could be clinically relevant for the identification of patients with different levels of risk for cardiotoxicity, thereby providing an individualized approach for decisions on dose safety thresholds and pharmacological prevention strategies.

Introduction

Anthracyclines are a fundamental component of first-line regimens for the treatment of patients with breast cancer, leukemia, lymphoma, and sarcomas. However, despite their impact on survival outcomes, anthracycline-induced cardiotoxicity is a major clinical problem [1]. An acute form of cardiotoxicity has been described, but chronic manifestations, consisting of congestive heart failure (CHF), are the most frequent in cancer patients. Demonstration of an exponential relationship between the cumulative dose of anthracyclines and the risk for heart failure [2] led to the definition of safety thresholds for the total dose of doxorubicin [3] and other drugs [4]. However, several studies have found asymptomatic ventricular dysfunction and CHF in long-term survivors of cancer even after low anthracycline doses [5–7]. Thus, the current classifications of chronic anthracycline cardiotoxicity distinguish early-onset cardiotoxicity, appearing in the first year after treatment, from late-onset cardiotoxicity, which usually occurs many years after anthracycline administration.

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Although clinical manifestations of early- and late-onset chronic cardiotoxicity are well-known [5], the pathological basis of anthracycline-related chronic cardiac dysfunction, based on a limited number of autopsy studies and endomyocardial biopsy series, has not been completely defined [8–13]. Although myocyte damage was the major lesion found in these studies, an imperfect correlation between morphological findings and clinical manifestations was observed in most cases [8]. Another key issue with many of these studies is the absence of a control group of cancer patients treated without anthracyclines. Finally, the strict definition of cardiotoxicity as myocyte damage may have overlooked other lesions, such as fibrosis, usually considered a nonspecific [10] or late [11] finding. Indeed, the effects of anthracyclines on nonmyocyte cardiac cells, such as fibroblasts, are still poorly understood [1].

It has been hypothesized that subclinical histological lesions may explain the later development of late-onset anthracycline cardiotoxicity. In fact, most asymptomatic patients treated with moderate doses of anthracyclines exhibited histological signs of cardiac myocyte damage in autopsy [9] and endomyocardial biopsy [10] series. However, the pathological basis of late-onset anthracycline cardiomyopathy has only been addressed in two small studies, both showing interstitial or replacement fibrosis in patients with late CHF [12, 13]. Because a control group was not included in any of these studies, no clear conclusions can be drawn from them regarding the specificity of fibrosis or the extent of pathological heart lesions in asymptomatic long-term cancer survivors.

Wide interindividual variability in cardiac dysfunction exists among patients receiving the same cumulative dose of anthracyclines [14]. The limited value of early detection strategies [15] and classic predictive factors such as age [16] and cardiac disease [3, 17] has turned the search for genetic predictive factors for cardiotoxicity into a priority [1, 18]. Several studies have explored the impact of genetic polymorphisms on anthracycline cardiotoxicity [19–21], and two of them demonstrated the contribution of NADPH oxidase genetic variations to the development of CHF in adults [19, 20]. These data are supported by involvement of NADPH oxidases in oxidative stress, a key mechanism for anthracycline myocardial damage, heart remodeling [22], and contractile dysfunction [23], through their modulation of myocardial damage and fibrosis [24]. However, no histological data are available regarding the contribution of these or other genetic polymorphisms to anthracycline-induced myocardial lesions.

The objective of this work was to better characterize anthracycline cardiotoxicity by comparing the histological heart lesions observed in a group of adult patients treated with anthracyclines with those found in a control group of cancer patients. To that aim, we performed an unmatched comparison between those treated with and without anthracyclines in a series of 97 consecutive necropsies. Once anthracycline-related cardiac lesions had been identified, the contribution of NADPH functional polymorphisms to their development was evaluated.

Materials and Methods

Study Design and Clinical Data

We studied the whole series of autopsies (97 consecutive autopsies) from patients with solid or hematological neoplasms in our center. Clinical variables were obtained from each patient's medical record. Autopsy data were collected from the original autopsy record. The total cumulative dose of anthracyclines was expressed as the cumulative equicardiotoxic dose of doxorubicin (mg/m^2), with the following doses considered equicardiotoxic: 450 mg/m^2 doxorubicin, 900 mg/m^2 epirubicin, 600 mg/m^2 daunorubicin, 150 mg/m^2 idarubicin, 160 mg/m^2 mitoxantrone [25]. Treatment-related cardiotoxicity cases were defined as those having CHF episodes occurring in the first month (acute) or more than 1 month (chronic) after the beginning of treatment in the absence of CHF before chemotherapy. The study protocol was approved by the hospital investigational review board and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients' families.

Pathologic Analysis of Heart Samples

Hematoxylin-eosin and Masson trichrome stainings of formalin-fixed, paraffin-embedded (FFPE) random samples from the ventricular wall of all cases were examined by a pathologist (F.P.Q.) blinded to clinical and genetic data. Cardiac lesions were systematically evaluated and classified into the following categories: myocytolysis (myocyte vacuolar degeneration), myocyte nuclear hypertrophy, myocardial or subendocardial band contraction necrosis (intense basophilic transverse bands in damaged myocytes), myocardial or subendocardial fibrosis, interstitial inflammatory infiltrate, interstitial hemorrhage, and tumor infiltration. Myocardial fibrosis and necrosis were subcategorized as diffuse or patched according to their distribution.

DNA Isolation and Genotyping

DNA was extracted from FFPE sections with the Gentra Puregene Kit (Qiagen Iberia, S.L., Madrid, Spain). DNA was quantified with a UV spectrophotometer and its integrity was evaluated using a multiple gene fragment amplification system (Specimen Control Master Mix; InVivoScribe Technologies, La Ciotat, France). Three single nucleotide polymorphisms (SNPs) of NADPH oxidase were genotyped (212A>G in the NCF4 or p40phox subunit, rs1883112; 242C>T in the p22phox subunit, rs4673; 7508T>A in the RAC2 subunit, rs13058338) using the validated Taqman SNP Genotyping Assays C_11521119_1, C_2038_20, and C_11744093_1, respectively (Applied Biosystems, Foster City, CA, http://www.appliedbiosystems.com) [20]. DNA amplifications were performed on a LightCycler 480 instrument (Roche Applied Science, Barcelona, Spain).

Statistical Analysis

The study was designed as an unmatched case–control study comparing a group of patients previously treated with anthracyclines with a control group of cancer patients without treatment or treated with nonanthracycline-based chemotherapy regimens. χ^2 and Fisher's tests were used for comparison of categorical variables. Univariate and multivariate logistic regression analyses were performed to determine the contribu-

Table 1. Patient characteristics

Characteristic	Anthracyclines ($n = 48$)	Control group ($n = 49$)	p value	Whole group ($n = 97$)
Median age (±SD), yrs	44 ± 18	60 ± 12	.001	52 (53.6%)
Sex				
Female	20 (41.7%)	12 (24.5%)	.07	32 (33.0%)
Male	28 (58.3%)	37 (75.5%)		65 (67.0%)
Cardiovascular risk factors				
Diabetes mellitus	0 (0.0%)	11 (22.4%)	.001	11 (11.3%)
Smoking	10 (20.8%)	24 (49.0%)	.004	34 (35.0%)
Hypertension	4 (8.3%)	14 (28.6%)	.01	18 (18.6%)
Dyslipidemia	1 (2.1%)	4 (8.1%)	.001	5 (5.1%)
Pretreatment cardiac disease				
Heart failure	1 (2.1%)	1 (2.0%)	.04	2 (2.1%)
Ischemic	2 (4.2%)	4 (8.1%)		6 (6.2%)
Other	2 (4.2%)	9 (18.4%)		11 (11.3%)
Diagnosis				
Acute leukemia	20 (41.7%)	3 (6.1%)	.002	23 (23.7%)
Lymphoma	15 (31.2%)	7 (14.3%)	.05	22 (22.7%)
Myeloma	7 (14.6%)	4 (8.1%)	.56	11 (11.3%)
Lung cancer	0 (0.0%)	11 (22.4%)	.005	11 (11.3%)
Other (solid tumor)	3 (6.2%)	17 (34.7%)		20 (20.6%)
Other (hematological)	3 (6.2%)	7 (14.3%)		10 (10.3%)
Treatment				
Thoracic radiotherapy	2 (4.2%)	2 (4.0%)	1.0	4 (4.2%)
Total body irradiation	5 (10.4%)	0 (0.0%)	.03	5 (5.1%)
Chemotherapy	48 (100.0%)	24 (49%)	.001	72 (74.2%)
HSC transplantation	27 (56.2%)	2 (4.0%)	.001	29 (29.9%)
Anthracyclines				
Doxorubicin	21 (43.7%)			
Idarubicin	17 (35.4%)			
Daunorubicin	14 (29.2%)			
Epirubicin	3 (6.2%)			
Mitoxantrone	20 (41.7%)			

Data are shown as number with percentages, unless stated otherwise.
p values are for χ^2 comparison between the anthracycline and nonanthracycline groups (Fisher's test when appropriate).
Abbreviations: HSC, hematopoietic stem cell; SD, standard deviation.

tions of cardiovascular diseases, risk factors, and type of treatment to the appearance of each histological lesion. p-value level for significance was ≤.05 and all statistical tests were two-sided. The Statistical Package for the Social Sciences, version 15.0 (Chicago, IL), was used for the statistical analyses.

Allele and genotype frequencies, deviations from Hardy–Weinberg expectations, haplotype analysis, and clinical–genotype associations were analyzed with SNPStats software [26] using an additive model. For the clinical–genetic association analysis, both histological cardiac lesions and treatment with anthracyclines were considered categorical binary variables. Logistic regression analysis was used to study the contribution of NADPH oxidase genetic polymorphisms to the development of cardiac lesions. Correction of statistical significance for multiple comparisons was done using the Bonferroni method [27].

Results

Clinical Characteristics and Cardiac Events

Ninety-seven consecutive white adult patients were studied. The main features of these patients are shown in Table 1. The group treated with anthracyclines included 48 patients with a median age of 44 years, whereas the control group (49 cases) was significantly older (median, 60 years; $p < .001$). The median equivalent cumulative dose of doxorubicin in the anthracycline group was 284 mg/m^2 (range, 26–639 mg/m^2), and only eight patients received a dose >450 mg/m^2. The frequency of cardiac disease ($p = .04$) and cardiovascular risk factors was higher in the control group, probably reflecting the differential age distribution and a bias toward selection of younger and healthier patients for anthracycline-based chemotherapy. Lymphoma and leukemia were the most frequent diagnoses in patients

Table 2. Association of cardiac lesions with anthracyclines

Cardiac lesions	Anthracycline group, *n* (%)	Control group, *n* (%)	*p* value
Myocyte nuclear hypertrophy	44 (92%)	40 (82%)	.23
Subendocardial fibrosis	34 (71%)	32 (65%)	.66
Myocardial interstitial (diffuse) fibrosis	**30 (62%)**	**19 (39%)**	**.019**
Myocardial patched fibrosis	**11 (23%)**	**4 (8%)**	**.045**
Subendocardial band contraction necrosis	22 (46%)	24 (49%)	.84
Myocardial diffuse necrosis	13 (27%)	11 (22%)	.64
Myocardial patched necrosis	**9 (19%)**	**0 (0%)**	**.001**
Myocytolysis	**29 (60%)**	**10 (20%)**	**<.001**
Cardiac interstitial hemorrhage	5 (10%)	3 (6%)	.48
Inflammatory infiltrates	2 (4%)	2 (4%)	1.0
Tumor infiltration	2 (4%)	2 (4%)	1.0

p value is for χ^2 comparison between anthracycline and nonanthracycline groups (Fisher's test when appropriate).
Values in bold indicate statistically significant differences.

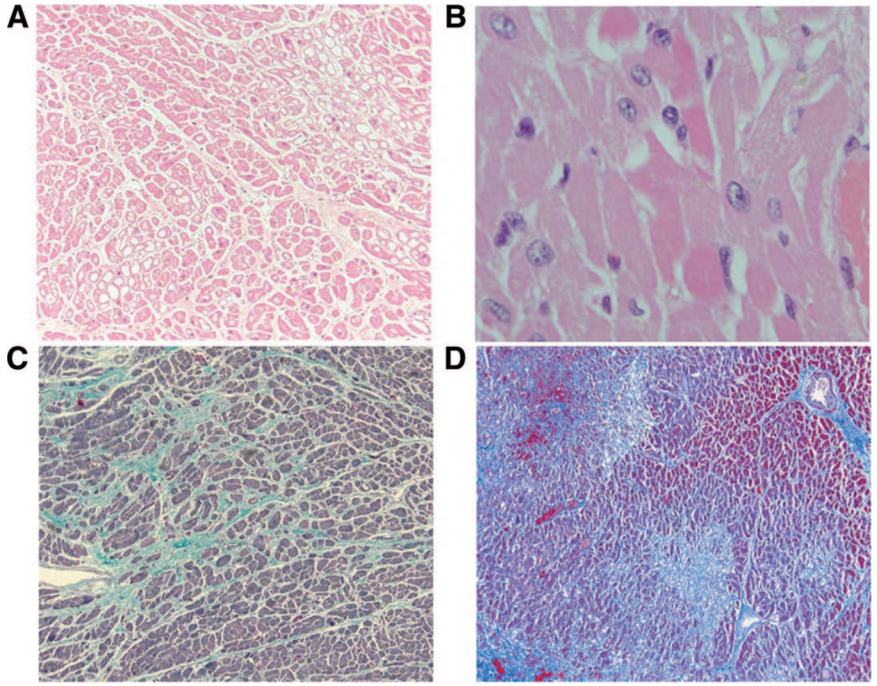

Figure 1. Pathological lesions (ventricle wall) associated with treatment with anthracyclines. **(A):** Myocytolysis or vacuolization of cardiac myocytes (hematoxylin and eosin [H&E] staining, ×125). **(B):** Patched myocardial necrosis into areas of preserved myocardium (H&E staining, ×500). **(C):** Interstitial fibrosis (Masson trichrome staining, ×62.5). **(D):** Patched myocardial fibrosis, with a multifocal distribution that disrupts the myocardial structure (Masson trichrome staining, ×12.5).

receiving anthracyclines and consequently, more patients in the anthracycline group had undergone hematopoietic stem cell transplantation and total body irradiation. No patient received trastuzumab and no differences between groups were found for the use of concomitant taxanes or other potential cardiotoxic drugs. The median overall survival duration from the time of first treatment did not differ significantly between the treatment and control groups (26 months vs. 23 months; $p = .64$).

After cancer treatment, nine patients developed heart failure (median time, 5 months), with most cases (seven vs. two) in the group treated with anthracyclines, which bordered on statistical significance ($p = .07$). Within the anthracycline group, three cases were classified as acute and four were classified as chronic CHF (all of them in the first year after treatment) (supplemental online Table 1).

Identification of Cardiac Lesions Associated With Anthracycline Treatment

The frequencies of the different cardiac lesions in the anthracycline and control groups are shown in Table 2. Direct toxic damage by anthracyclines to cardiac myocytes was reflected by the more frequent observation of myocytolysis after anthracycline treatment (60% vs. 20%; $p < .001$). A more specific pattern was found for patched myocardial necrosis, which was exclusively observed in the anthracycline group (19% vs. 0%; $p = .001$). Heart fibrosis, both in its interstitial (diffuse) (62% vs. 39%; $p = .019$) and in its patched (23% vs. 8%; $p =$

Table 3. Association of NADPH genetic polymorphisms to anthracyclines-related cardiac lesions

	NADPH polymorphism											
	rs1883112				rs4673				rs13058338			
n = 48	G/G	G/A	A/A	OR (95% CI) *p* value	C/C	C/T	T/T	OR (95% CI) *p* value	T/T	T/A	A/A	OR (95% CI) *p* value
Myocytolysis												
No	4	13	2	–	6	9	4	–	10	6	3	–
Yes	10	14	5	NS	12	10	7	NS	18	10	1	NS
Patched necrosis												
No	12	22	5	–	11	18	10	0.112 (0.20–0.63)	22	13	4	–
Yes	2	5	2	NS	7	1	1	$p = .039$	6	3	0	NS
Interstitial fibrosis												
No	9	9	0	5.11 (1.59–16.43)	7	7	4	–	10	5	3	–
Yes	5	18	7	$p = .018$	11	12	7	NS	18	11	1	NS
Patched fibrosis												
No	12	21	4	–	13	16	8	–	21	12	4	–
Yes	2	6	3	NS	5	3	3	NS	7	4	0	NS

Data are shown as absolute number.
Abbreviations: CI, confidence interval; NS, not significant; OR, odds ratio.

.045) form, was also more frequent in anthracycline-treated cases. Statistically significant differences were maintained when only patients treated with nonanthracycline chemotherapy (24 individuals) were used as the control group (data not shown). The presence of these four specific lesions (Fig. 1) was independent of the cumulative dose of doxorubicin, the time from treatment to autopsy (supplemental online Table 2), and the specific drug (data not shown).

Although our study did not allow a sequential analysis, we analyzed patterns of association among anthracycline-related lesions. Myocytolysis was associated with patched necrosis in 28% of cases, whereas only 5% of cases without myocytolysis showed patched necrosis ($p = .07$). Furthermore, we found a strong association between the patched forms of myocyte damage and stromal repair, with 78% of cases of patched necrosis also showing patched fibrosis ($p < .0001$). Myocytolysis was also associated with interstitial diffuse fibrosis in 72% of cases ($p = .08$).

Relationship of Clinical Factors and Cardiac Events With Anthracycline-Induced Lesions

No association with age, sex, cardiovascular risk factors (arterial hypertension, dyslipidemia, diabetes, and smoking), preexistent cardiac disease, transplantation, treatment with cyclophosphamide, or radiotherapy (including total body irradiation) was found for any of the anthracycline-induced heart lesions (supplemental online Table 3).

Only patched myocardial fibrosis showed a trend toward association with CHF ($p = .08$), whereas no association was found for myocytolysis, patched necrosis, or interstitial fibrosis. The lack of clinicopathological correlation was maintained when we differentiated chronic from acute CHF, with the only exception being a significant absence of interstitial fibrosis in cases of acute CHF ($p = .047$).

NADPH Functional Genetic Polymorphisms and Cardiac Histological Lesions

We studied three SNPs of NADPH oxidase: rs1883112 (NCF4 or p40phox subunit, 212A>G), rs4673 (p22phox subunit, 242C>T), and rs13058338 (RAC2 subunit, 7508T>A). Genotype and allelic frequencies for these polymorphisms are shown in supplemental online Table 4. All were in Hardy–Weinberg equilibrium. No significant differences in genotypic frequencies for the three polymorphisms were found between cases and controls (supplemental online Table 5).

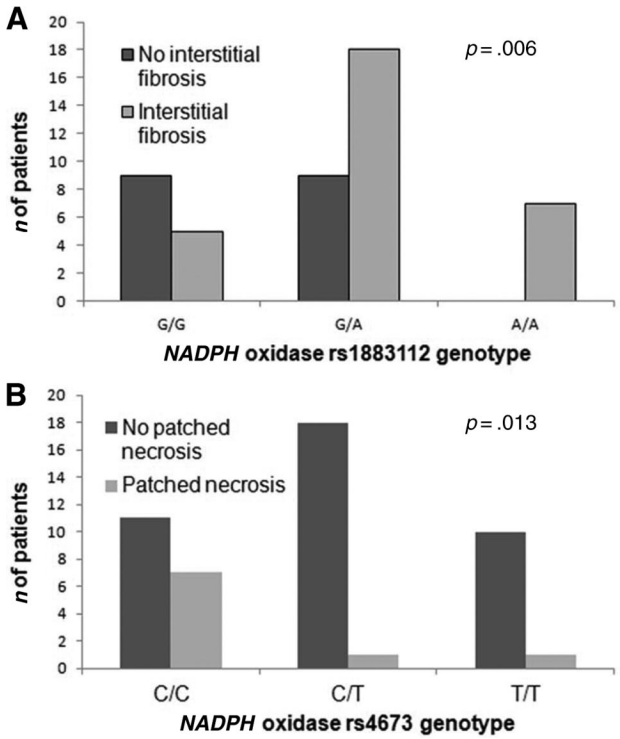

Figure 2. Bar graphs showing the effects of NADPH polymorphisms on anthracycline-related myocardial lesions. **(A):** Association of rs1883112 A/A and G/A genotypes with interstitial fibrosis. **(B):** Protective effects of T/T and C/T genotypes on patched myocardial necrosis.

Analysis of the association between NADPH polymorphisms and cardiac lesions in the anthracycline group demonstrated that cardiac interstitial fibrosis was strongly associated with rs1883112 (Table 3), which conferred an odds

ratio (OR) of 5.11 for its appearance (95% confidence interval [CI], 1.59–16.43; $p = .018$). Interestingly, this lesion was observed in all A/A homozygotes (seven of seven) and in two-thirds of heterozygotes (18 of 27), whereas only one-third of patients with the G/G genotype showed interstitial fibrosis, which strongly suggests an additive effect of allele A (Fig. 2A). Patched myocardial necrosis was associated with polymorphism rs4673 (OR, 0.11; 95% CI, 0.20–0.63; $p = .039$), with the higher frequency corresponding to the C/C genotype (7 of 18), thus showing a protective effect of allele T (Fig. 2B). No association with any of the anthracycline-related lesions was found for the NADPH variant rs13058338. In the control group, we did not find any relationship between cardiac lesions and NADPH polymorphisms (data not shown). Finally, no direct association between any of the three polymorphisms and clinical cardiac events was observed.

Polymorphic profile analysis in the anthracycline-treated group of patients showed that the profile rs4673-C/rs1883112-A significantly aggravated the risk for cardiac interstitial fibrosis (OR, 12.12; 95% CI, 1.64–89.66; $p = .033$). No profile was associated with other lesions (supplemental online Table 6) or CHF ($p = .87$).

Discussion

Anthracycline-related heart damage is a treatment-limiting toxicity for cancer patients. Here, we systematically analyzed the myocardial lesions associated with anthracyclines using a case–control design in a series of 97 consecutive necropsies, which is the largest comparative series so far reported. Because NADPH is involved in cardiac oxidative damage and remodeling, we also analyzed the role of three SNPs of NADPH previously associated with clinical cardiotoxicity. Our data suggest that the rs1883112 and rs4673 polymorphisms modify the risk for developing anthracycline-related heart lesions, thereby providing a mechanistic explanation for their impact on myocardial remodeling and cardiac dysfunction.

We found that myocytolysis, patched myocardial necrosis, patched myocardial fibrosis, and diffuse interstitial fibrosis were significantly associated with treatment with anthracyclines. Myocytolysis and interstitial fibrosis were very frequent in patients treated with anthracyclines, probably reflecting diffuse and moderate toxic heart damage. In contrast, patched myocardial necrosis and fibrosis might be more specific markers of anthracycline-related heart damage [28]. Focal or diffuse cardiac myocyte damage is the major lesion explaining anthracycline cardiotoxicity in clinical series, but the specificity of fibrosis was questioned previously [9, 10], as shown by its absence from current endomyocardial biopsy scoring systems [10, 29]. The characteristics of our study, based on a clinical necropsy series, might involve biases for selection of more fragile or poorer prognosis patients, and confirmation in larger cohorts of patients should be performed. However, the case–control design of our study allowed us to exclude the potential effect derived from other cardiotoxic treatments [30]. Additionally, the lack of association between these lesions and cardiovascular risk factors further supports their direct relationship with anthracycline-based chemotherapy. In fact, heart lesions were more frequent in the anthracycline group, even with a higher median age and a worse cardiovascular risk profile in the control group. Our findings highlight the importance not only of cardiac myocyte injury, but also of stromal participation in anthracycline-related cardiac remodeling processes even after low cumulative doses. These results are also consistent with previous experimental models of acute [31] and chronic [24, 28] cardiotoxicity in which fibrosis is a major component of the cardiac remodeling phenotype that underlies contractile dysfunction. Accordingly, the clinical appearance of late cardiotoxicity might be a result not only of direct heart damage, but also of a lack of ability of the cardiac tissue to compensate for the toxic cell loss.

The pattern of association among the four lesions, and specifically the appearance of patched necrosis almost exclusively in those cases showing myocytolysis, could suggest a common pathogenic pathway for anthracycline-induced cardiac damage and remodeling, which is a cumulative process [2]. Early after receiving anthracyclines, a substantial proportion of patients might develop diffuse myocytolysis, leading to diffuse interstitial fibrosis even after small or moderate doses. A smaller subgroup of patients with diffuse cardiac changes could also develop focal necrosis, subsequently evolving to patched fibrosis. The association of patched myocardial fibrosis with CHF ($p = .08$) suggests that patients with an established focal fibrotic remodeling pattern are specifically predisposed to early or late myocardial dysfunction and subsequent clinical events. Finally, the significantly lower frequency of interstitial fibrosis patients with acute CHF suggests a greater contribution of myocyte injury to acute cardiac events and of heart fibrosis to chronic contractile dysfunction, which is consistent with previous reports [12, 13]. Taken together, our findings point to a complex process of remodeling after anthracycline-based treatment, in which both direct cardiomyocyte death [32] and subsequent fibrosis contribute to impaired cardiac function. This pattern of development of histological changes, and in particular of fibrosis, might be useful as a surrogate marker of anthracycline cardiac damage and an increased risk for late cardiac dysfunction, allowing noninvasive image detection [33] of patients at risk and early instauration of preventive pharmacologic strategies [34, 35].

Oxidative stress is a major mechanism of heart damage by anthracyclines [36]. NADPH oxidases are key enzymes in the generation of reactive oxygen species and their expression and activity have been found to be greater in the setting of heart failure and cardiac remodeling, leading to cardiac interstitial fibrosis [22, 37]. Both the main isoforms of NADPH oxidase in the heart, Nox2 and Nox4, seem to have a role in cardiac remodeling [38]. Deficiency in Nox2 NADPH oxidase attenuates myocardial damage and cardiac interstitial fibrosis after doxorubicin chemotherapy, limiting contractile dysfunction [20, 24]. Nox4 is also involved in the regulation of oxidative stress and myocardial apoptosis [39]. Thus, individuals with less active genetic variants of NADPH might be protected from the heart damage and fibrosis induced by anthracyclines. This hypothesis is supported by the results of a case–control clinical study of doxorubicin-related cardiac toxicity showing that NADPH genetic variations can modulate the risk for acute (polymorphisms rs4673 and rs13058338) and chronic (rs1883112) cardiac events [20]. Another study confirmed the predictive value of the three NADPH oxidase polymorphisms, although only rs1883112 was significant in the

multivariate analysis [19]. Our data, showing a role for these genetic variants in the generation of cardiac lesions, provides a likely mechanistic link between NADPH functional SNPs and cardiac dysfunction. In our patients, the polymorphism 212A>G of the NADPH oxidase subunit NCF4 or p40phox (rs1883112) was strongly associated with myocardial interstitial fibrosis, which was more frequent in cases carrying allele A. The striking finding that all patients with genotype A/A showed interstitial fibrosis further supports this association and is consistent with previous clinical data demonstrating a predisposing effect of the NCF4 rs1883112 A allele for cardiotoxicity [19, 20], and in particular for its chronic form. No experimental data are available regarding its functional meaning, but rs1883112 is located in the promoter of NCF4 (p40phox), which is involved in downregulation of NADPH oxidase [40]. Our study also revealed that the polymorphism 242C>T of the p22phox subunit (rs4673), which leads to a His72Tyr substitution, protects against focal myocardial necrosis. A higher frequency of this lesion was observed in cases with genotype C/C, whereas a very low frequency was observed in carriers of allele T. The two studies cited before showed conflicting results regarding the association of the different rs4673 alleles with anthracycline cardiotoxicity [19, 20]. However, our results are consistent with functional data linking the rs4673 allele T to lower NADPH activity [41, 42]. Both our results and previous clinical data suggest that rs4673 might be mainly involved in myocyte injury whereas rs1883112 might participate in the modulation of the balance between remodeling and fibrosis, leading to late heart damage. The differential effects of the two polymorphisms could also be related to the divergent roles of Nox2 and Nox4 in cardiac remodeling [38, 39]. Polymorphism rs1883112 is located in the subunit p40phox, which is present only in the Nox2 isoform, whereas rs4673 corresponds to subunit p22phox, which is found in both the Nox2 and Nox4 isoforms. Although the impact of rs4673 on Nox2 and Nox4 functional activity remains unclear, a lower activity or expression level of both of these in patients carrying the allele 242T might explain a lesser degree of oxidative stress, apoptosis, and myocardial damage after anthracycline treatment. In contrast, a higher level of the regulatory subunit p40 in Nox2 could explain the higher degree of interstitial fibrosis associated with genotype A/A of rs1883112. Pharmacogenetic applications such as risk-adapted follow-up or the use of less cardiotoxic alternative drugs should be explored for individuals carrying the A/A genotype for the rs1883112 polymorphism, particularly in breast cancer patients receiving adjuvant anthracyclines.

Conclusion

Our work establishes not only cardiac myocyte damage, but also myocardial fibrosis as pathologic lesions specifically related to anthracyclines. These lesions might explain the later development of cardiac dysfunction through remodeling processes, which supports the current paradigm of additive sequential heart damage from the beginning of treatment. The NADPH functional polymorphisms rs4673 and rs1883112 modulate the appearance of some of these lesions, thereby providing a mechanistic link for their reported association with clinical cardiotoxicity. Although these data should be confirmed in larger cohorts of patients in whom sequential histological samples are available, they might be useful for explaining differences in cardiac tolerance to anthracyclines.

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Disclosures

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