

# NAD(P)H Oxidase and Multidrug Resistance Protein Genetic Polymorphisms Are Associated With Doxorubicin-Induced Cardiotoxicity

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**Background**—A significant number of patients treated with anthracyclines develop cardiotoxicity (anthracycline-induced cardiotoxicity [ACT]), mainly presenting as arrhythmias (acute ACT) or congestive heart failure (chronic ACT). There are no data on pharmacogenomic predictors of ACT.

**Methods and Results**—We genotyped participants of the German non-Hodgkin lymphoma study (NHL-B) who were followed up for the development of heart failure for a median of >3 years. Single-nucleotide polymorphisms (SNPs) were selected from 82 genes with conceivable relevance to ACT. Of 1697 patients, 55 developed acute and 54 developed chronic ACT (cumulative incidence of either form, 3.2%). We detected 5 significant associations with polymorphisms of the NAD(P)H oxidase and doxorubicin efflux transporters. Chronic ACT was associated with a variant of the NAD(P)H oxidase subunit NCF4 (rs1883112, -212A→G; symbols with right-pointing arrows, as edited?; odds ratio [OR], 2.5; 95% CI, 1.3 to 5.0). Acute ACT was associated with the His72Tyr polymorphism in the p22phox subunit (rs4673; OR, 2.0; 95% CI, 1.0 to 3.9) and with the variant 7508T→A (rs13058338; OR, 2.6; 95% CI, 1.3 to 5.1) of the RAC2 subunit of the same enzyme. In agreement with these results, mice deficient in NAD(P)H oxidase activity, unlike wild-type mice, were resistant to chronic doxorubicin treatment. In addition, acute ACT was associated with the Gly671Val variant of the doxorubicin efflux transporter multidrug resistance protein 1 (MRP1) (OR, 3.6; 95% CI, 1.6 to 8.4) and with the Val1188Glu-Cys1515Tyr (rs8187694-rs8187710) haplotype of the functionally similar MRP2 (OR, 2.3; 95% CI, 1.0 to 5.4). Polymorphisms in adrenergic receptors previously demonstrated to be predictive of heart failure were not associated with ACT.

**Conclusions**—Genetic variants in doxorubicin transport and free radical metabolism may modulate the individual risk to develop ACT. (*Circulation*. 2005;112:3754-3762.)

**Key Words:** drugs ■ genes ■ genetics ■ heart failure

Anthracyclines are well established as highly efficacious antineoplastic agents for various hematopoietic and solid tumors. A dose-response relationship has been demonstrated for anthracyclines and some tumors, with lower doses resulting in decreased survival and remission rates.<sup>1</sup> On the other hand, dose escalation results in a dose-dependent cardiotoxicity.<sup>2</sup> Two distinct types of anthracycline-induced cardiotoxicity (ACT) have been de-

finied. Acute ACT occurs during treatment, often immediately after the first dose, and manifests itself predominantly in the form of arrhythmias and rarely also as pericarditis-myocarditis or acute left ventricular (LV) failure. Chronic ACT presents within 1 year after anthracycline administration as congestive heart failure. In addition, recent studies postulate a distinct late-onset cardiotoxicity, which develops after years or even decades of

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asymptomatic survival. This form may well be the most frequent one, which, however, remains to be confirmed in additional studies.<sup>1</sup> The causal relationship between the individual ACT forms is unclear.

The incidence of ACT depends on the cumulative dose of the drug. For doxorubicin in bolus-injected patients, this value is 0.14% at doses <400 mg/m<sup>2</sup> and rises from 7% at 550 mg/m<sup>2</sup> to 18% at 700 mg/m<sup>2</sup>. The steep increase in cardiotoxicity at doses >550 mg/m<sup>2</sup> has led to the commonly applied upper dosing limit of 550 mg/m<sup>2</sup>. Switching from bolus to prolonged intravenous infusion<sup>3</sup> has reduced the incidence of doxorubicin-induced cardiotoxicity.<sup>4</sup>

Among many factors proposed, only cumulative anthracycline dose and previous irradiation with cardiac involvement have been confirmed as independent risk factors for ACT.<sup>5</sup> On the other hand, there is increasing evidence that the genetic makeup is a major determinant of drug response and toxicity. In regard to chronic ACT, there is ample evidence from transgenic mouse models to support this hypothesis. For example, overexpression of the multiple drug resistance gene MDR1 protects the heart from the toxic effect of doxorubicin.<sup>6</sup> In humans, the presence of a genetic component is suggested by the wide variation in the individual sensitivity to anthracyclines. Thus, doxorubicin doses >1000 mg/m<sup>2</sup> are tolerated by some patients,<sup>7</sup> whereas others develop ACT after <200 mg/m<sup>2</sup>. However, nothing is known about the identity of genes and variants underlying this variability. This knowledge may not only help in the development of individualized therapies but may also help in the understanding of drug-induced cardiotoxicity and the pathophysiology of acute and chronic congestive heart failure in general.

We investigated the role of the individual genetic makeup in doxorubicin-induced cardiotoxicity. The analysis was conducted in patients enrolled in the prospective, multicenter, randomized phase III trial called NHL-B, conducted between 1993 and 2000 by the German High-Grade Non-Hodgkin's Lymphoma Study Group (DSHNHL).<sup>8,9</sup> Genes and polymorphisms were selected with the use of a candidate approach. Among the various mechanisms suggested to mediate cardiotoxicity, increased sensitivity to anthracycline-derived reactive oxygen species (ROS) is the most plausible. Furthermore, we genotyped variants in proteins implicated in the transport of anthracyclines, in gene variants generally predisposing to heart failure, and in other genes with a conceivable role in ACT. Because the genetic identity of proteins metabolizing doxorubicin remains unknown, we genotyped only a few classic drug-metabolizing enzymes.

## Methods

### Study Design and Data Management

Study design and the demographic characteristics of the entire study population have been described in detail elsewhere.<sup>8,9</sup> The present pharmacogenomic analysis was designed as a nested case-control study including all cases and matched controls of the cohort. The study protocol was approved by the relevant institutional review boards and ethics committees, and all participants gave written informed consent. The trial included patients from 162 centers with normal lactate dehydrogenase (LDH) aged ≤60 years (NHL-B1) and patients aged 61 to 75 years (NHL-B2) with normal and elevated LDH levels. Young patients with elevated LDH levels were recruited

into a different trial addressing the role of high-dose chemotherapy with stem cell transplantation.<sup>10</sup> Because no DNA was available from patients included in this latter trial, the data from these young high-risk patients could not be included in the present analysis.

Altogether, 1697 patients aged 18 to 75 years with aggressive NHL (mainly diffuse large B cell NHL) were recruited, and eligible patients were randomized into the 4 arms of the study. Ninety-eight percent of patients were ethnic Germans. The arms were designed to compare the standard CHOP-21 chemotherapy with 3 variants including either an addition of etoposide (CHOEP-21: 100 mg/m<sup>2</sup> on days 1 to 3); shortening to 2-week intervals with the use of recombinant human granulocyte colony-stimulating factor (rhG-CSF; CHOP-14); or both (CHOEP-14). All patients received the standard CHOP scheme, defined as cyclophosphamide (750 mg/m<sup>2</sup> IV), doxorubicin (50 mg/m<sup>2</sup> IV), and vincristine (2 mg IV) all given on day 1, and prednisone (100 mg/d per os) given on days 1 to 5. All patients were intended to receive 6 cycles of chemotherapy. Acute toxicities were classified according to the World Health Organization Handbook for Reporting Results of Cancer Treatment. During treatment, ECGs were conducted in patients complaining of symptoms suggestive of impaired heart function. Follow-up examinations were conducted every 3 months in the first 2 years and every 6 months thereafter. The follow-up examinations relevant to cardiotoxicity detection were scheduled at 1, 2, and 5 years after the therapy and included ECG and echocardiography as well as a physical examination. The 2 relevant diagnoses reported to the study center were arrhythmia and heart failure. Detailed information was then obtained for the individual patients from the reporting physicians. Treatment-related cardiotoxicity cases were defined on the basis of the following criteria: cases of arrhythmia (in the absence of arrhythmia in the patient history before treatment), myocarditis-pericarditis, and acute heart failure until the end of the third cycle were defined as acute ACT. Cases recorded after the third cycles were defined as chronic heart failure cases in the absence of heart failure before chemotherapy. A reduction of the ejection fraction <50% or of the fractional shortening <25% was classified as chronic ACT.<sup>11</sup> The evaluation and classification of cases were performed by 2 independent teams of physicians, and a consensus was established during a third, joint evaluation.

### DNA Isolation and Genotyping

Peripheral blood lymphocytes were collected before therapy with patients' consent. The genotyping studies were conducted after authorization by the ethics committee of the University Göttingen, Göttingen, Germany. Genes were selected arbitrarily from functional pathways previously implicated in ACT.<sup>4</sup> Most single-nucleotide polymorphisms (SNPs) in the selected genes were chosen on the basis of frequency (>5%) and in priority order: exon variant>promoter region>intron. Genotyping was performed either by Pyrosequencing on the PSQTM HS 96A System or on the 7900 HT Sequence Detection System with the use of predeveloped assays by Applied Biosystems (SNP Assays-on-Demand) or by restriction fragment length polymorphism. The method applied is specified for each SNP in the online-only Data Supplement Table. Primer sequences and conditions for the noncommercial assays (Pyrosequencing and restriction fragment length polymorphism) can be obtained on request. Several controls were included to exclude mix-ups and other errors during genotyping. Thus, each plate contained a well with DNA-free reaction mix to detect contamination with DNA. Another well contained a dedicated DNA, which was expected to yield identical genotypes for all plates genotyped for a given genetic variant. Furthermore, 7 DNAs were genotyped twice for all variants. No genotyping errors were detected with the use of these controls.

### Statistical Analysis

The deviation of the genotype distributions from Hardy-Weinberg equilibrium (HWE) was tested with the Pearson goodness-of-fit  $\chi^2$  test. The lack of deviation of the genotype distribution among controls from HWE was necessary for the subsequent association testing. The latter was performed with the use of the procedure by Freidlin et al,<sup>12</sup> which is a modified Cochran-Armitage trend test,

**TABLE 1. Gender, Age, Weight, Doxorubicin Dose Received, and Distribution Among the Therapy Arms of Cases and Controls**

	Cases			Controls (n=363)
	Chronic (n=43)	Acute (n=44)	Chronic+Acute (n=87)	
Gender, M/F	27/16	23/21	50/37	212/151
Men, %	63 ( <i>P</i> =0.580)*	52 ( <i>P</i> =0.437)*	57 ( <i>P</i> =0.874)*	58
Age, y	62.7±7.9† ( <i>P</i> =0.303)*	61.2±13.2† ( <i>P</i> =0.959)*	62.0±10.9† ( <i>P</i> =0.627)*	61.3±11.0†
Weight, kg	74.2±13.8 ( <i>P</i> =0.784)*	74.1±15.5 ( <i>P</i> =0.463)*	74.2±14.6 ( <i>P</i> =0.489)*	74.9±13.7
Dose received, mg	515 [186.5]‡ ( <i>P</i> =0.529)*	500 [121.3]‡ ( <i>P</i> =0.961)*	504 [160.5]‡ ( <i>P</i> =0.665)*	540 [90.0]‡
Therapy				
CHOP-14	10	12	22	103
CHOEP-14	8	13	21	93
CHOP-21	11	8	19	76
CHOEP-21	14 ( <i>P</i> =0.504)*	11 ( <i>P</i> =0.942)*	25 ( <i>P</i> =0.874)*	91

\**P* values compare the controls with each case group.

†Age is given as mean±SD. Dose is given as median.

‡Square brackets indicate differences between the first and third quartiles (interquartile range).

and by Fisher exact test. Results were also analyzed by multiple logistic regression for each genetic variant individually, with adjustment for age, gender, total dose received, and dosing interval (14 versus 21 days). In addition, multiple logistic regression was used to investigate combinations of genetic variants that were individually significant. The significance level was set at 5%, as appropriate for screening purposes. The probability of clustering of associations in only some functional groups of polymorphisms given the linkage disequilibrium (LD) structure was determined by a permutation analysis, randomly allocating genes to functional groups and study participants as cases or controls. We permuted genes rather than SNPs to preserve the LD structure within each gene. The empirical quantile *q* of permutations exceeding the observed number of significant markers was regarded as an estimate, and the upper 95% confidence limit of *q*, assuming a binomial distribution, was used as a conservative overall probability value. LD was calculated as *r*<sup>2</sup> values for SNPs located on the same chromosome with the use of HAPLOVIEW software.<sup>13</sup> The population stratification was investigated with the use of genomic controls, as described by Reich and Goldstein.<sup>14</sup>

### Mouse Echocardiography

Mice with the targeted disruption of the NAD(P)H oxidase subunit gp91<sup>15</sup> were obtained from the Jackson Laboratory (Bar Harbor, Me) and maintained on a C57Bl/6 background. Female wild-type and homozygous mutant mice were given intraperitoneal injections of doxorubicin (3 mg/kg per injection) on days 1, 8, 15, and 29. In vivo echocardiographic measurements of cardiac dimensions and function were obtained at 30 MHz from short- and long-axis views at the level of the largest LV diameter with the use of a VS-VEVO 660/230 High Resolution Imaging System (VisualSonics) as described.<sup>16</sup> Mice were lightly anesthetized with 2.5% tribromoethanol (0.01 mL/g IP) and allowed to breathe spontaneously. The chest was shaved, acoustic coupling gel was applied, and a warming pad was used to maintain normothermia. Mice were imaged in a shallow left lateral decubitus position. Left ventricular end-diastolic (LVEDD) and end-systolic (LVESD) dimensions were measured from original tracings with the use of the leading edge convention of the American Society of Echocardiography. Left ventricular fractional shortening (LVFS) was calculated as LVFS=(LVEDD-LVESD)/LVEDD.

### Results

Of the 1697 patients enrolled, 147 were reported to the study center because of cardiac problems during or after chemotherapy treatment. Of those, on the basis of a detailed source data review, 38 were excluded from further analysis because there was evidence of preexisting cardiac disease or the cardiac dysfunction could not be substantiated. Of the remaining 109 patients, 55 developed acute and 54 developed chronic ACT (cumulative incidence of either form, 3.2%). No DNA samples were available for 22 of them, leaving 87 patients (44 with acute and 43 with chronic ACT) who were subjected to genetic analysis, as follows. A detailed review of the 44 acute ACT patient files revealed 12 cases of atrial fibrillation, 2 cases of myocarditis-pericarditis, and 1 case of myocardial infarction, and 5 patients showed clinical signs of acute heart failure. The 43 chronic cases showed a reduction of the ejection fraction <50% or FS values <25%. The median time between the therapy onset and first report of cardiotoxicity was 6 months, with an interquartile range of 4 to 15 months.

The ACT cases were then frequency-matched for age, gender, and weight, with 363 patients free of any clinical symptoms of arrhythmia, heart failure, or other cardiac symptoms possibly related to cardiotoxicity at any time point of the study. The data on cases and controls are given in Table 1. In each group, patients were distributed approximately equally among the 4 arms of the therapy. The cumulative total dose of doxorubicin was ≈7% lower in cases than in controls because it was necessary to abandon treatment in some patients as a result of arrhythmias. Six of 87 cases (7%) and 15 of 363 controls (4%) received irradiation of the mediastinal region (*P*=0.26, Fisher exact test).

A total of 206 polymorphisms in 82 genes with a conceivable role in ACT were genotyped. All but 17 variants were

**TABLE 2. Distribution of Genotypes and Probability Values for Gene Variants Showing Associations With Cardiotoxicity After Doxorubicin Treatment**

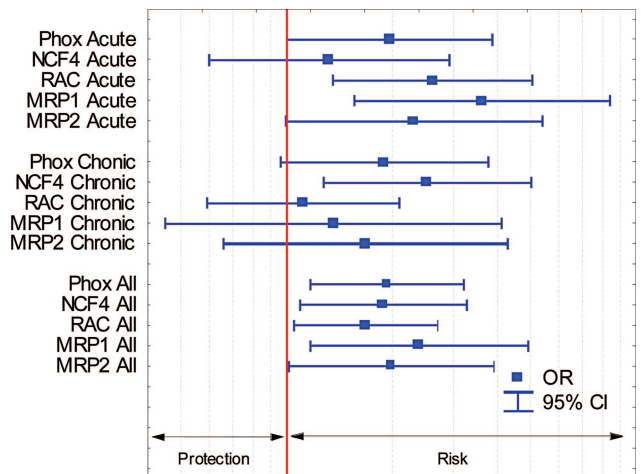
Cardiotoxicity	Gene Variant	Genotype Distribution		P vs Controls	
		Genotypes*	n	Freidlin Test	Fisher Test
Acute	CYBA His72Tyr(rs4673)	<u>CC/CT/TT</u>	13/28/3	0.048	0.054
	RAC2 7508T→A(rs13058338)	<u>TT/TA/AA</u>	15/23/5	0.002	0.005
	NCF4 -212A→G(rs1883112)	GG/GA/AA	10/22/9	0.372	0.519
	MRP1 Gly671Val	GG/ <u>GT/TT</u>	33/8/1	0.001	0.005
	MRP2 Val1188Glu(rs8187694)	<u>TT/TA/AA</u>	36/8/0	0.049	0.06
	MRP2Cys1515Tyr(rs8187710)	GG/ <u>GA/AA</u>	36/8/0	0.049	0.06
Chronic	CYBA His72Tyr(rs4673)	<u>CC/CT/TT</u>	13/27/3	0.062	0.074
	RAC2 7508T→A(rs13058338)	<u>TT/TA/AA</u>	24/15/4	0.28	0.747
	NCF4 -212A→G(rs1883112)	GG/GA/AA	14/14/15	0.007	0.013
	MRP1 Gly671Val	GG/ <u>GT/TT</u>	39/4/0	0.586	0.537
	MRP2 Val1188Glu(rs8187694)	<u>TT/TA/AA</u>	37/6/0	0.274	0.269
	MRP2Cys1515Tyr(rs8187710)	GG/ <u>GA/AA</u>	37/6/0	0.274	0.269
Chronic or acute	CYBA His72Tyr(rs4673)	<u>CC/CT/TT</u>	26/55/6	0.009	0.01
	RAC2 7508T→A(rs13058338)	<u>TT/TA/AA</u>	39/38/9	0.013	0.04
	NCF4 -212A→G(rs1883112)	GG/GA/AA	24/36/24	0.022	0.031
	MRP1 Gly671Val	GG/ <u>GT/TT</u>	72/12/1	0.012	0.029
	MRP2 Val1188Glu(rs8187694)	<u>TT/TA/AA</u>	73/14/0	0.044	0.05
	MRP2Cys1515Tyr(rs8187710)	GG/ <u>GA/AA</u>	73/14/0	0.044	0.05
None (controls)	CYBA His72Tyr(rs4673)	<u>CC/CT/TT</u>	164/154/45	1	1
	RAC2 7508T→A(rs13058338)	<u>TT/TA/AA</u>	211/132/19	1	1
	NCF4 -212A→G(rs1883112)	GG/GA/AA	106/186/62	1	1
	MRP1 Gly671Val	GG/ <u>GT/TT</u>	331/24/1	1	1
	MRP2 Val1188Glu(rs8187694)	<u>TT/TA/AA</u>	331/32/0	1	1
	MRP2Cys1515Tyr(rs8187710)	GG/ <u>GA/AA</u>	331/32/0	1	1

\*Genotype(s) predisposing to ACT is underlined.

confirmed as biallelic markers, ie, rs2032582 and rs746578 were triallelic, whereas 15 other variants were monomorphic. The genotypes of 14 additional biallelic markers were not in HWE among controls. The remaining 175 variants from 73 genes (online-only Data Supplement Table) were subsequently genotyped in cases and tested for associations. These genes play a role in the metabolism of ROS, drug transport and metabolism, DNA repair, endothelial physiology, the renin-angiotensin-aldosterone system, muscle contraction and structure, inflammation, and cell cycle. Several variants are derived from adrenergic receptors. Fifty-nine of 175 SNPs were in strong LD ( $r^2 > 0.5$ ) with at least 1 other SNP within the same gene (online-only Data Supplement Table and Figure). Using 116 independent SNPs ( $r^2 < 0.5$ ), we found no evidence for stratification of the patient population, as indicated by a variance inflation factor of 0.84.

As shown in Table 2, 6 variants showed statistically significant ( $P < 0.05$ ) associations with ACT in the Freidlin association test. Chronic ACT showed an association with the -212A→G (rs1883112) variant of the NAD(P)H oxidase subunit NCF4, which is responsible for downregulation of the enzyme. The predisposing NCF4 genotype is AA, and it conferred an odds ratio (OR) of 2.5 (95% CI, 1.3 to 5.0) (Figure 1).

Acute ACT was associated with 2 SNPs in 2 additional subunits of the same enzyme: p22phox and RAC2. The predisposing allele T at position 242 (242C→T, rs4673) in the gene CYBA, coding for p22phox, leads to the missense mutation His72Tyr. The distribution of the genotypes in cases



**Figure 1.** ORs (log scale) and CIs of developing cardiotoxicity after doxorubicin treatment conferred by the predisposing alleles identified in this study. Predisposing alleles are as in Table 2.

deviated from HWE ( $P < 0.001$ ). The homozygous carriers of T and C alleles were underrepresented (in acute ACT 3 instead of 7 expected for TT and 13 instead of 17 for CC), whereas the heterozygotes were overrepresented (28 instead of expected 21). The group of homozygous and heterozygous carriers of the T allele had an increased risk of acute ACT, characterized by an OR of 2.0 (95% CI, 1.0 to 3.9;  $P = 0.048$ ). The almost identical increase in the OR for chronic cases of 1.9 (95% CI, 1.0 to 3.8) was of borderline statistical significance ( $P = 0.062$ ). The other association with acute ACT was found for the intron 2 variant (7508T→A, rs13058338) of the regulatory subunit of the enzyme RAC2, which conferred an OR of 2.6 (95% CI, 1.3 to 5.1) for carrier of the A allele.

In addition, acute ACT was associated with 3 polymorphisms in the transmembrane efflux transporters of anthracyclines, multidrug resistance protein 1 (MRP1) and multidrug resistance protein 2 (MRP2) (Table 2). The predisposing allele A of the MRP1 variant Gly671Val conferred an OR of 3.6 (95% CI, 1.6 to 8.4). The 2 missense mutations in MRP2, Val1188Glu (rs8187694) and Cys1515Tyr (rs8187710), yielded identical frequencies and relationships with acute ACT, as characterized by an OR of 2.3 (95% CI, 1.0 to 5.4). An inspection of the individual genotypes revealed 100% LD between these variants in the samples genotyped. Probability values for all 6 associations were  $\leq 0.06$  by the Fisher exact test (Table 2).

For all 6 variants, the effects of the genotypes on the risk of cardiac disease were statistically significant when all cases were taken together (Table 2). All associations except 1 (the Val1188Glu and Cys1515Tyr haplotype) were still significant when chronic cases were defined more conservatively (ejection fraction  $< 45\%$  instead of  $< 50\%$ ). A consistent although not statistically significant trend toward increased risk was observed for all other combinations of these 6 variants and phenotypes (Figure 1). The exclusion of mediastinum-irradiated patients had no major effect on the associations, with all probability values  $\leq 0.051$  (data not shown).

Results were analyzed by multiple logistic regression analysis for each genetic variant individually, with adjustment for age, gender, cumulative dose administered until development of ATC, and dosing interval (14 versus 21 days). This analysis confirmed the results of the monovariate analysis with ORs of 1.9 ( $P = 0.010$ ) for the p22phox 242T allele (rs4673), 2.0 ( $P = 0.016$ ) for the NCF4 variant rs1883112, and 1.7 ( $P = 0.025$ ) for the RAC2 "A" allele (rs13058338). With respect to the drug transporters, an OR of 2.5 ( $P = 0.016$ ) for the MRP1 allele and an OR of 1.9 ( $P = 0.071$ ) for the 2 linked MRP2 amino acid substitutions were confirmed by logistic regression analysis. A 2-locus analysis for the various pairs of the associating CYBA (rs4673), NCF4 (rs1883112), RAC2 (rs13058338), MRP1, and MRP2 polymorphisms revealed no interactions between the genes involved (data not shown). Data on other potential confounding factors such as smoking, hypertension, and diabetes were not collected in this study.

We addressed the problem of multiple testing by calculating the probability of clustering of 5 significant associations within only 2 functional groups of genes. This was done by

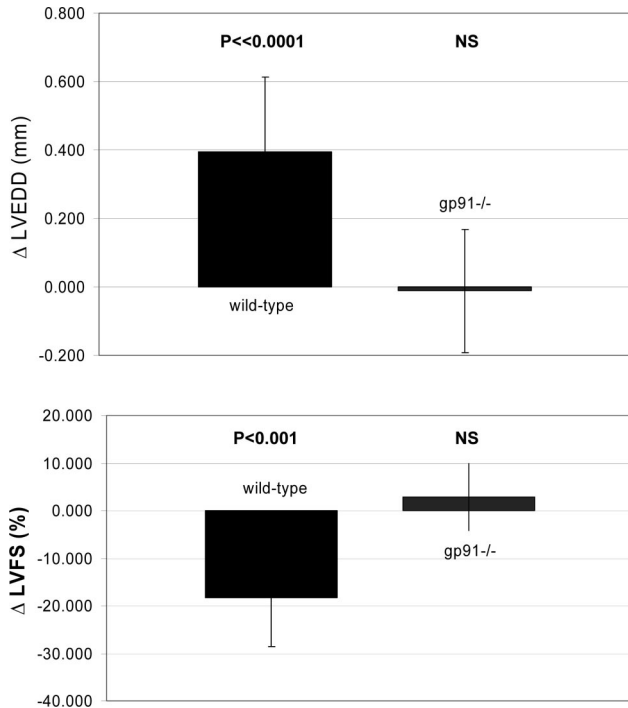
permutation analysis. Only 191 of 2500 permutations showed  $\leq 5$  genes nested within  $\leq 2$  functional groups. This translates to a robust overall probability value of 0.08, indicating that findings such as ours could be generated by chance in  $< 1$  of 12 replicas of the study.

Besides cardiotoxicity, variability in response to doxorubicin could also affect the degree of myelotoxicity and the treatment outcome. We observed no difference between cases and controls with regard to leukopenia, taken either as the highest World Health Organization leukopenia grade or as middle values of leukopenia in nadir for each patient. This was observed independently of taking acute and chronic cases separately or together. This result indicates no link between myelotoxicity and cardiotoxicity. None of the polymorphisms associated with cardiotoxicity had any effects on the treatment outcome, defined as time to treatment failure.<sup>8,9</sup> However, there was a trend of borderline significance ( $P = 0.068$ ) toward a shorter time to treatment failure in patients with acute cardiotoxicity. This was possibly caused by the reduced doxorubicin dose in these patients (Table 1).

The involvement of the NAD(P)H oxidase in ACT was verified in mice deficient in the gp91 subunit of this enzyme.<sup>15</sup> Homozygous gp91 mutants exhibit a reduced NAD(P)H oxidase activity in heart homogenates.<sup>17</sup> The kinetics of a chronic doxorubicin effect was first established in wild-type mice. Consistent with previous reports,<sup>18</sup> repeated intraperitoneal injections of doxorubicin (4×3 mg/kg per injection over 5 weeks) resulted in a robust LV dilation and severely impaired cardiac function, as indicated by a gradual increase in LVEDD and a simultaneous decrease in LVFS (data not shown). The largest changes in these parameters were observed during the last measurement, ie, 4 weeks after the last doxorubicin injection (Figure 2). In contrast to wild-type mice, homozygous gp91 knockouts on the same genetic background (C57Bl/6) showed normal LV function and geometry, indicated by no statistically significant changes in LVEDD and LVFS in comparison to untreated animals (Figure 2). We detected no statistically significant differences in body weight or in heart rate between doxorubicin-treated wild-type and homozygous gp91 knockouts (data not shown). Independent of the genotype, the animals showed no unspecific signs of poor health such as ruffled fur or hunched body form.

## Discussion

To our knowledge, this is the first analysis of the genetic predisposition to ACT in humans. The cumulative incidence of ACT in this study (6.4%) is slightly higher than recent estimates of 3% to 5%.<sup>1</sup> This was caused primarily by a much higher prevalence of acute ACT, which accounted for every second ACT case detected. The prevalence of acute ACT is usually estimated at 1%.<sup>4</sup> The much higher incidence of acute ACT in this study may have been caused by a higher detection rate, in part reflecting the increased detection of arrhythmias through improved monitoring as well as the enhanced awareness of ACT. Furthermore, we counted all cardiac events occurring during the initial 3 months as acute ACT. There is no established time cutoff between acute and chronic ACT. Classification based on symptoms rather than



**Figure 2.** Effect of chronic doxorubicin treatment on LVEDD and LVFS assessed by echocardiography in C57Bl/6 mice deficient in the NAD(P)H oxidase subunit gp91 (n=11) and in wild-type mice of the same background (n=18). Measurements were performed 4 weeks after completion of the treatment. Data are shown as mean±SD.

time points is inaccurate. Arrhythmias, usually classified as acute cardiotoxicity, may be a first and only sign of cardiac dysfunction many years after the treatment. On the other hand, congestive heart failure has been observed in patients within hours to days after a first doxorubicin administration.<sup>1</sup> In animal models, congestive heart failure develops within several weeks, ie, before completion of many typical doxorubicin treatments.<sup>18</sup> We believe that our arbitrary cutoff between acute and chronic ACT is a fair compromise between these divergent definitions and observations.

The increased frequency of acute (although not chronic) ACT may also have been increased by the comedication with cyclophosphamide, which itself may be cardiotoxic.<sup>19</sup> Mediastinal irradiation, previously reported to have a borderline effect on cardiotoxicity,<sup>5</sup> was not significantly different between cases and controls, and exclusion of irradiated patients had no effect on associations that were found. In some patients, cardiotoxicity may have been enhanced by comedications, which were, however, not among the mandatory data collected. The dosing interval applied (14 versus 21 days) had no effect on cardiotoxicity.

Heart failure has been previously associated with variants of adrenergic receptors and of the angiotensin-converting enzyme.<sup>20–23</sup> We detected no associations between any of these variants and ACT. Likewise, we found no interaction between the ADRA2C GlyAlaGlyPro322–325del and the ADRB1Gly389Arg variant,<sup>20</sup> altogether suggesting a different underlying pathophysiology. On the other hand, ACT was associated with variants of proteins implicated in 2 distinct

processes, the generation of ROS and anthracycline transmembrane transport. The NAD(P)H oxidase multienzyme complex catalyzes the 1-electron reduction of oxygen with either NADH or NAD(P)H used as the electron donor. Best investigated in endothelium and macrophages, the enzyme has been recently demonstrated in the myocardium, where it may be a major source of ROS.<sup>24</sup> The C242T (His72Tyr, rs4673) polymorphism of the CYBA gene coding for p22phox affects a heme-binding site thought to be essential for the stability of the protein. The NCF4 variant rs1883112 predisposing to ACT is located in the putative promoter of the gene. The NCF4 gene encodes the p40phox subunit of the NAD(P)H oxidase, which is responsible for the downregulation of the enzyme.<sup>25</sup> The third association pertains to the small GTPase RAC2, which is required for the NAD(P)H oxidase activity. In addition, RAC2 may induce the enzyme complex assembly,<sup>26</sup> possibly by activation of cytosolic protein kinases.<sup>27</sup> The SNP located in intron 2 of RAC2 (rs13058338) may serve as a marker for a functional, at present unknown variant. Alternatively, it could itself be functional and affect splicing or transcription of RAC2, for example.

The functional consequences of the C242T (His72Tyr, rs4673) polymorphism of the CYBA gene have been already investigated. Under ex vivo conditions, the tyrosine variant was originally reported to confer a 20% to 40% reduction in basal activity in vessel samples.<sup>28</sup> This finding has been confirmed in part by Wyche et al,<sup>29</sup> who reported a gene/dose-dependent reduction of phorbol ester-stimulated, although not of basal, NAD(P)H oxidase activity in T allele carriers. Association of reduced activity to generate ROS with ACT seems to be opposite of expectations. One may speculate that under in vivo conditions, inherited reduced activity of NAD(P)H oxidase may result in impaired ROS defense capacity and therefore in increased ROS levels under anthracycline exposure. On the other hand, Shimo-Nakanishi et al<sup>30</sup> recently reported a T allele-conferred increase in NAD(P)H oxidase activity both in human probands and in cells transfected with CYBA expression constructs. Similarly conflicting are the reported associations between the T allele and coronary artery disease, in which increased risk,<sup>31,32</sup> decreased risk,<sup>33</sup> and no change<sup>34,35</sup> in risk have been described. Altogether, the final verdict on the functional consequences of the C242T allele is still out.

The protective effect of the gp91 disruption on doxorubicin cardiotoxicity is consistent with an activating effect of the CYBA 242T allele, supporting the recent findings by Shimo-Nakanishi et al.<sup>30</sup> The cardiotoxic effect of doxorubicin at regimens similar to ours is in agreement with numerous previous investigations in mice.<sup>18</sup> The dramatic difference between wild-type and homozygous gp91 mutants provides a strong backing for the true character of the associations between variants in NAD(P)H oxidase subunits and ACT. A cardiac phenotype of the gp91 deficiency is consistent with the expression of the NAD(P)H oxidase in this organ. Although NAD(P)H oxidase is also expressed in other organs, the protective effect of gp91 deficiency was in all likelihood primarily mediated by a cardiac mechanism. In-

deed, other than a significant impairment of heart function, the overall status of the animals was inconspicuous.

The propensity to ACT is also increased in patients carrying variant alleles of the multidrug resistance proteins MRP1 and MRP2. Both genes belong to subfamily C of ATP-binding cassette (ABC) transporters and act as cellular efflux pumps for numerous endogenous and exogenous substrates. The human MRP1 confers resistance to anthracyclines and was in fact originally cloned from a doxorubicin-selected cancer cell line.<sup>36</sup> MRP1 is expressed in human<sup>37</sup> and murine<sup>38</sup> myocardium. A targeted deletion of MRP1 in the mouse increases the accumulation of etoposide in the heart,<sup>39</sup> but no data are available on the accumulation of doxorubicin. As opposed to polarized cells, in the cardiomyocytes MRP1 is found in the cytoplasm in addition to plasma membrane.<sup>37</sup> It has been postulated that such an expression may permit sequestration of doxorubicin in lysosomes, ie, away from its target the nucleus.<sup>40</sup> A low-frequency protein variant of MRP1 (Arg433Ser) has been shown to affect resistance to anthracyclines *in vitro* but has not been found in our study.<sup>41</sup> The effect on doxorubicin resistance or transport of the variant 671Val, which shows an association with acute ACT in this study, has not been investigated.<sup>42</sup>

MRP2 has a substrate spectrum similar to that of MRP1 and increases the resistance to doxorubicin when overexpressed in HEK-293 cells.<sup>43</sup> Physiologically, the MRP2 protein is expressed in the apical membrane of polarized cells in the liver and in the kidney, and there is no evidence for its expression in the heart. The observed association could be caused by a reduced biliary elimination of doxorubicin, which normally accounts for 50% of its disposition. In support of this model, an inhibition of MRP2 expression by a bacterial toxin decreased biliary clearance of doxorubicin and increased its plasma concentration.<sup>44</sup> The lower incidence of heart damage after doxorubicin infusion as opposed to bolus injection suggests a role for peak levels of the drug in doxorubicin-induced cardiotoxicity.<sup>45</sup> Significant interindividual differences in doxorubicin pharmacokinetics have been reported previously,<sup>46,47</sup> and they could result from genetic variants in transporters such as MRP2. The 2 missense variants associating with ACT, Val1188Glu (rs8187694), and Cys1515Tyr (rs8187710), were initially described in the Japanese.<sup>48</sup> No data on their functional significance have been published, and the specific variant relevant to doxorubicin treatment cannot be inferred from our results because of the 100% LD between the 2 missense mutations in our cohort.

Sufficient power and spurious associations caused by multiple testing are major problems in current molecular genetic epidemiology. Depending on the allelic frequency, the power to detect associations was between 15% and 30% for this large study. However, although low power may result in lack of detection of some associations, it has no impact on the validity of those that we describe. A separate issue is spurious associations due to the large number of markers tested. The value of this exploratory study lies primarily in the uniqueness of the cohort studied. The validation of results such as those presented here certainly requires confirmatory clinical or biochemical studies, especially because functional

data are available for only 1 of the associated polymorphisms (C242T in CYBA). In support of the role of the NAD(P)H oxidase, mice deficient in this enzyme are resistant to doxorubicin. Additional support for the associations found can be derived from the functional context of the findings and from further statistical considerations. In the case of ACT, functional support is provided by the functional similarity between the proteins. Both MRP1 and MRP2 transport doxorubicin. Therefore, an association of variants of either gene with ACT can be regarded as additional evidence for the true character of this association. Similarly supportive is our finding of the association of variants of CYBA (rs4673), NCF4 (rs1883112), and RAC2 (rs13058338), which are members of the same signaling complex. The permutation analysis demonstrated that such clustering of associations between ACT and genetic variants from functionally related genes is unlikely to have occurred by chance. Nevertheless, the validity of our results can be demonstrated only by replicating them in an independent, sufficiently powered clinical study.

The relationship between acute and chronic ACT is unclear. Acute ACT is usually attributed to damage caused by ROS arising from 1-electron reduction of anthracyclines. The chronic form has been proposed to result, at least in part, from the effects of anthracycline alcohols, which are generated by their 2-electron reduction by enzymes such as aldo-keto reductases and carbonyl reductases.<sup>49</sup> However, there is increasing evidence for a "unifying hypothesis" of ACT.<sup>50</sup> ROS have been found to induce the expression of these enzymes<sup>51</sup> and may thus facilitate the formation of the toxic alcohol metabolites and chronic ACT. Our data appear to support a common pathophysiology of acute and chronic ACT. Indeed, variants of the NAD(P)H oxidase subunits and the 2 MRP genes exhibit a consistent trend to associate with either ACT form, although not all of these associations reach the set level of significance. The generally lower number of significant associations for chronic cases considered separately could be caused by the relatively short follow-up period. Indeed, abnormal cardiac parameters have been described in 18% of patients after 4 to 10 years<sup>52</sup> and in 65% of patients after 15 years.<sup>53</sup> Therefore, it is likely that the controls used contain a significant number of patients who will develop cardiotoxicity in the future, and their genetic makeup diminishes the strength of the associations. In addition, all 6 variants show statistically significant associations when acute and chronic cases are taken together. On the other hand, the apparent relationship between acute and chronic ACT may result, at least in part, from using the same set of controls.

The ultimate goal of studies such as this is to develop a system of molecular-genetic diagnostic tests, which would help to detect persons at high risk for cardiotoxicity. Obviously, the results presented here will have to be verified in an independent clinical study before diagnostic genotyping can be considered. OR values for the significant SNPs were calculated in a univariate manner, which may have led to their overestimation. The population-based attributable risk (PAR) estimates the proportion of ACT cases caused by the carrier status of a given genetic variant in relation to all cases of

ACT. The PAR values are between 7% for MRP2 and 29% for CYBA. These values suggest that up to 29% of the possible ACT cases could be explained by the carrier status. The sensitivity varies between 15% (MRP1) and 70% (CYBA), indicating that a substantial portion of at-risk individuals could be detected. The parameter prohibiting a diagnostic application of genotyping is at present the specificity. The respective values are between 45% (CYBA) and 93% (RAC2) and indicate that many patients would erroneously be deprived of a potentially life-saving anthracycline treatment.

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### Disclosure

None.

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