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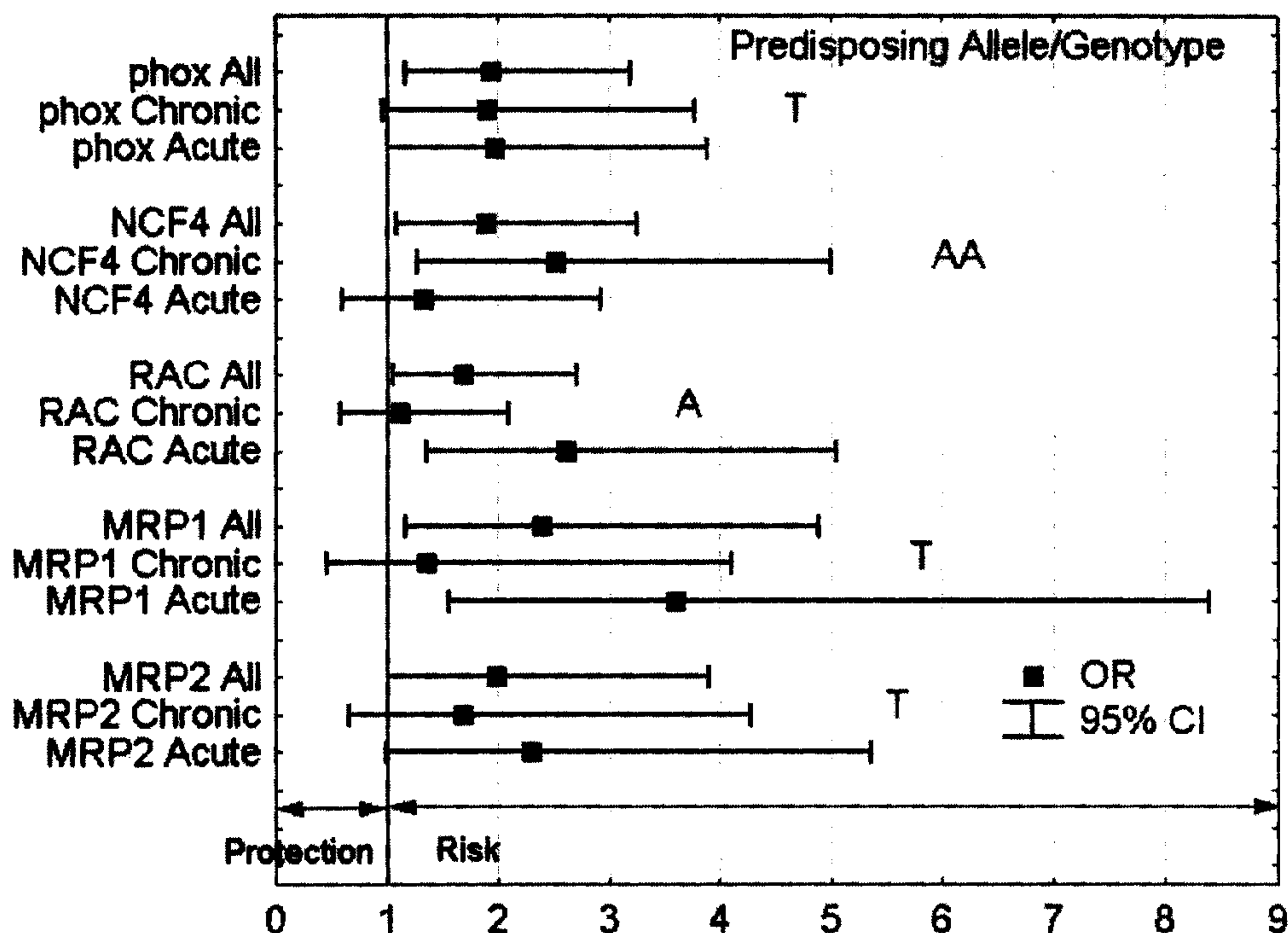
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(57) Abrégé/Abstract:

A system of molecular-genetic diagnostic tests is provided, which help to identify and treat populations of patients who have a genetic predisposition to cardiac disorders. In particular, a method of determining the genetic predisposition of a subject to be at a higher risk for drug induced cardiotoxicity is described.



Abstract

A system of molecular-genetic diagnostic tests is provided, which help to identify and treat populations of patients who have a genetic predisposition to cardiac disorders. In particular, a
5 method of determining the genetic predisposition of a subject to be at a higher risk for drug induced cardiotoxicity is described.

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**Means and methods for individualized drug therapy and
for predicting adverse drug reaction**

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Field of the invention

The present invention relates to the individualization of therapy. More specifically, the present invention relates to systems and the use of genotyping aiming at the individualization of therapy and/or individualization of drug dosing. with a therapeutic agent or a class of therapeutic agents in accordance with a novel method, which is particularly designed for the identification and medication of populations of patients who have a genetic predisposition to cardiac disorders. In particular, the present invention relates to a method of determining the genetic predisposition of a subject to be at risk for a cardiac disease or dysfunction comprising assaying a sample of a subject for the presence of a variant allele of at least one gene selected from the group consisting of genes involved in (a) the generation of reactive oxygen species (ROS) or (b) drug transmembrane transport; wherein the presence of the variant allele is considered indicative of a higher risk for cardiotoxicity compared to a control. The present invention further concerns methods for the treatment of a disease comprising administering to a subject in need of such treatment a non-cardiotoxic amount of a drug or a non-cardiotoxic analog or substitute thereof, wherein said subject carries at least one of said variant alleles.

Background of the invention

Anthracyclines are well established as highly efficacious antineoplastic agents for various haematopoietic 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 (Shan et al., Ann. Intern. Med. 125 (1996), 47-58). On the other hand, dose escalation results in a dose-dependent cardiotoxicity (Von Hoff et al., Ann. Intern. Med. 91 (1979), 710-717). Two distinct types of anthracycline-induced cardiotoxicity (ACT) have been defined. Acute ACT occurs during the treatment, often immediately after the first dose, and manifests itself predominantly in the form of arrhythmias, rarely also as pericarditismyocarditis or acute left ventricular failure. Chronic ACT presents within one 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 asymptomatic survival. This form may well be the most frequent one, which, however,

remains to be confirmed in additional studies (Shan et al., *Ann. Intern. Med.* 125 (1996), 47-58). 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-
5 injected patients it is 0.14 % at doses below 400 mg/m² and raises from 7 % at 550 mg/m² to 18 % at 700 mg/m² (Von Hoff et al., *Ann. Intern. Med.* 91 (1979), 710-717). The steep increase in cardiotoxicity at doses higher than 550 mg/m² has led to the commonly applied upper dosing limit of 550 mg/m². Switching from bolus to prolonged intravenous infusion (Legha et al., *Ann. Intern. Med.* 96 (1982), 133-139) has reduced the incidence of
10 doxorubicin-induced cardiotoxicity (Zucchi and Danesi, *Curr. Med. Chem. Anti-Canc. Agents* 3 (2003), 151-171).

Among many factors proposed, only cumulative anthracycline dose and previous irradiation with cardiac involvement have been confirmed as independent risk factors for ACT (Torti et
15 al., *Ann. Intern. Med.* 99 (1983), 745-749). On the other hand, there is increasing evidence that the genetic makeup is a major determinant of drug response and toxicity. Regarding 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 (Dell'Acqua et al., *Hum. Gene Ther.* 10 (1999),
20 1269-1279). In humans, the presence of a genetic component is suggested by the wide variation in the individual sensitivity to anthracyclines. Thus, doxorubicin doses exceeding 1000 mg/m² are tolerated by some patients (Henderson et al., *J. Clin. Oncol.* 7 (1989), 560-571), whereas others develop ACT after less than 200 mg/m². However, nothing is known about the identity of genes and variants underlying this variability. This knowledge
25 may not only help to develop individualized therapies, but may also help to understand drug induced cardiotoxicity and the pathophysiology of acute and chronic congestive heart failure in general.

Thus, there is a need for diagnostic tests which would allow the detection and pre-selection of
30 patients who are at high risk for cardiotoxicity, in particular in response to drug therapy and generally therapeutic intervention.

The solution to said technical problem is achieved by providing the embodiments characterized in the claims, and described further below.

Summary of the invention

The present invention is directed to a system aiming at the individualization of therapy and/or individualization of drug dosing method including determining the genetic predisposition of a subject to be at risk for a cardiac disease or dysfunction. In particular, the method of the present invention comprises assaying a sample of a subject for the presence of a variant allele of at least one gene selected from the group consisting of genes involved in (a) the generation of reactive oxygen species (ROS) or (b) drug transmembrane transport; wherein the presence of the variant allele is considered indicative of a higher risk for cardiotoxicity compared to a control.

Furthermore, the present invention relates to a method for the treatment of cancer comprising administering to a subject in need of such treatment a non-cardiotoxic amount of an anthracycline or a non-cardiotoxic analog or substitute thereof, wherein said subject carries at least one variant allele in a gene involved in the NAD(P)H oxidase multi-enzyme complex or encoding multidrug resistance-associated protein 1 (MRP1) or multidrug resistance-associated protein 2 (MRP2) as well as to a method for the treatment of cancer comprising administering to a subject in need of such treatment an anthracycline, wherein said subject does not carry any one of said variant alleles. Preferably, a sample of the subject to be treated has been assayed in accordance with the method of the present invention. The use of the anthracycline in accordance with the method of the present invention may be accompanied by the use of further therapeutic agents such as cardioprotective and/or other antineoplastic agents

It is thus also an object of the present invention to provide a pharmaceutical composition comprising an anthracycline and an agonist/activator of a gene or gene product involved in the NAD(P)H oxidase multi-enzyme complex or transmembrane transport of multidrug resistance-associated protein 1 (MRP1) or multidrug resistance-associated protein 2 (MRP2).

It is another object of the present invention to provide oligonucleotides, e.g. in form of a primer or probe, for use in determining the presence of variant allele in a gene involved in the NAD(P)H oxidase multi-enzyme complex or encoding multidrug resistance-associated protein 1 (MRP1) or multidrug resistance-associated protein 2 (MRP2) for diagnosing a higher risk for cardiotoxicity, especially anthracycline induced cardiotoxicity. Usually, said oligonucleotides comprise at least 10, preferably at least 15 nucleotides, more preferably at

least 20 nucleotides in length while a hybridizing polynucleotide to be used as a probe usually comprises at least 50, preferably at least 100, more preferably at least 200, or most preferably at least 500 nucleotides in length. Also comprised in accordance with the method of the invention are hybridizing oligo- and polynucleotides which are useful for analyzing DNA-
5 protein interactions via, e.g., electrophoretic mobility shift analysis (EMSA).

The present invention also concerns a kit for use in a method of the present invention comprising poly- or oligonucleotides, a chip or array, reference samples, amplification and/or sequencing means, buffer, detergents, biochemical reagents, detection means, or the like, and
10 optionally a reformulated drug with a drug dose which is tailored to a patient carrying a variant allele as defined herein.

For the purpose of the present invention the following terms are defined below.

15 The term "individualization" as it appears herein with respect to therapy is intended to mean a therapy having specificity to at least an individual's phenotype as calculated according to a predetermined formula on an individual basis.

The term "sample" is intended to mean a sample usually obtained from a biological entity and
20 includes, but is not to be limited to, any one of the following: tissue, cerebrospinal fluid, plasma, serum, saliva, blood, cells like peripheral blood lymphocytes, nasal mucosa, urine, synovial fluid, tumor sample, microcapillary, microdialysis and breath.

The term "therapeutic agent" or "drug" are used interchangeably herein and are intended to
25 mean an agent (s) and/or medicine (s) used to treat the symptoms of a disease, physical or mental condition, injury or infection.

The term "treatment" is intended to mean any administration of a pharmaceutical compound to an individual to treat, cure, alleviate, improve, diminish or inhibit a disease, physical or
30 mental condition, injury or infection in the individual.

The term "individual treated" is intended to mean any individual being subjected to the administration of (i) a pharmaceutical compound, for treating, curing, alleviating, improving,

diminishing or inhibiting a disease, physical or mental condition, injury or infection, or (ii) a probe substrate for determining multi-determinant metabolic phenotype.

5 The term "dosage" includes the size, frequency, formulation, co-medication and number of doses of at least one therapy to be given to a patient. This also includes newly prescribed therapies and/or therapies, both singly and in combination and is irrespective of the way of administration.

10 The term "patient" or "subject" includes any organism, particularly a human or other mammal, suffering from a disease, in need or desire of treatment for a disease, or in need of testing or screening for a disease. A patient includes any mammal, including farm animals or pets, and includes humans of any age or state of development.

15 The term "therapeutic agent regime" or "dosage regimen" is the course of action or use of a therapeutic agent or drug, or combination of therapeutic agents or drugs in treating a patient including, for example, at least one of dosage, schedule of administration, choice and/or combination of therapeutic agents.

20 As used herein, the terms "nucleic acid", "polynucleotide", or "nucleic acid sequence" refer to a polymer of deoxyribonucleotides or ribonucleotides, in the form of a separate fragment or as a component of a larger construct. Polynucleotide or nucleic acid sequences of the invention include DNA, RNA, including mRNA and cDNA sequences. The polynucleotides of the sample of the present invention are typically genomic DNA or RNA.

25 As used herein, the terms "polypeptide" and "protein" are used interchangeably herein and refer to a polymer of amino acid residues in the form of a separate fragment or component of a larger construct. A polypeptide may encode for a functional protein or fragments of a protein.

30 In the context of the present invention the term "variant allele" of the given target gene including, nucleotide and amino acid sequences, respectively, as used herein means that the nucleotide, and optionally amino acid sequence of said variant allele differs from the wild type nucleotide and amino acid sequences by way of nucleotide, and optionally amino acid substitution(s), addition(s) and/or deletion(s). Genomic, cDNA and marker sequences of

various target genes including those specifically described herein can be retrieved from, for example Genbank, <http://www.ncbi.nlm.nih.gov/Genbank/GenbankOverview.html> and EMBL Bioinformatics. Reference or wild type sequences for the NCF4, CYBA, RAC2, MRP1 and MRP2 polynucleotides are for example the GeneBank accession Nos. given in Table 2.

Unless stated otherwise the term "variant allele" and "allelic variant" are used interchangeably herein. Furthermore, in the following, said genes involved in (a) the generation of reactive oxygen species (ROS) or (b) drug transmembrane transport may also be designated "target genes". Likewise, any one of said genes in the generation of ROS or drug transmembrane transport may be designated "target gene". At some places the terms "target sequence" or "target protein" may be used synonymously.

The term "corresponding" as used herein means that a position is not only determined by the number of the preceding nucleotides and amino acids, respectively. The position of a given nucleotide or amino acid in accordance with the use of the present invention which may be deleted, substituted or comprise one or more additional nucleotide(s) may vary due to deletions or additional nucleotides or amino acids elsewhere in the gene or the polypeptide. Thus, under a "corresponding position" in accordance with the present invention is to be understood that nucleotides or amino acids may differ in the indicated number but may still have similar neighboring nucleotides or amino acids. Said nucleotides or amino acids which may be exchanged, deleted or comprise additional nucleotides or amino acids are also comprised by the term "corresponding position". Said nucleotides or amino acids may for instance together with their neighbors form sequences which may be involved in the regulation of gene expression, stability of the corresponding RNA or RNA editing, as well as encode functional domains or motifs of the protein of the invention.

The term "pharmaceutical composition" as used herein comprises the substances of the present invention, and optionally one or more pharmaceutically acceptable carriers.

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Brief description of the drawing

Fig. 1: Odds ratios for the development of ACT (chronic cases, acute cases, all cases) conferred by the predisposing alleles identified in this study.

Detailed description of the invention

The present invention is based on a method which involves the genotyping of the patients prior to administering a drug so that its dosage is tailored to the patient's genetic profile. The method also relates to pharmaceutical compositions and the preparation of them, which are tailored to the genetic profile of these patients. Thus, in a first aspect the present invention relates to a method of determining the genetic predisposition of a subject to be at risk for a cardiac disease or dysfunction comprising assaying a sample of a subject for the presence of a variant allele of at least one gene selected from the group consisting of genes involved in (a) the generation of reactive oxygen species (ROS) or (b) drug transmembrane transport; wherein the presence of the variant allele is considered indicative of a higher risk for cardiotoxicity compared to a control.

Genotyping is performed by analyzing the genetic sequence of a gene coding for a specific enzyme often by a polymerase chain reaction assay (PCR) or a PCR with a restriction fragment length polymorphism assay (PCR-RFLP) or a PCR and dideoxysequencing. Preferably genotyping methods are employed, which are amenable to high-throughput detection of multiple genetic polymorphisms, for example with mismatch primers in allele-specific polymerase chain reaction as described in Ishiguro et al., *Anal. Biochem.* 337 (2005), 256-261. The genes are examined for the presence of genetic mutations that can be linked to increased or decreased enzyme levels or activity, which in turn result in a specific phenotype, i.e. a higher susceptibility to cardiac disorders. The genotype is a theoretical measurement of what an individual's phenotype should be. In principle, the genetic mutation or allele may present in any region of the gene including the coding region, promoter, enhancer sequences, intron, exon, intron/exon junction and vice versa, poly signal and the like, but also including polymorphisms which are not located within the gene encoding the metabolic factor but which nevertheless display a tight linkage to the observed phenotype.

The present invention is based on observations made in experiments aiming at investigating 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) (Pfreundschuh et al., *Blood* 104 (2004), 634-641; Pfreundschuh et al., *Blood* 104 (2004), 626-633). Genes and polymorphisms were selected

using a candidate approach. The experiments performed in accordance with the present invention suggest that among the various mechanisms suggested to mediate the cardiotoxicity, increased sensitivity to anthracyclines –derived reactive oxygen species (ROS) is involved in this disease. Furthermore, genotyping of variants in proteins implicated in the transport of anthracyclines revealed that this class of proteins and genes is involved as well.

Without intending to be bound by theory it is believed that genetic variants in anthracycline, e.g., doxorubicin transport and free radical metabolism modulate the individual risk to develop cardiotoxicity (ACT), mainly presenting as arrhythmias (acute ACT) or congestive heart failure (chronic ACT). In particular, significant associations, including polymorphisms of the NAD(P)H oxidase and doxorubicin efflux transporters could be determined.

Thus, the present invention provides a method of determining the genetic predisposition of a subject to be at risk for a cardiac disease or dysfunction comprising assaying a sample of a subject for the presence of a variant allele of at least one gene selected from the group consisting of genes involved in (a) the generation of reactive oxygen species (ROS) or (b) drug transmembrane transport; wherein the presence of the variant allele is considered indicative of a higher risk for cardiotoxicity compared to a control.

The test sample may be obtained using any technique known in the art including biopsy, blood sample, sample of bodily fluid (e.g., urine, lymph, ascites, cerebral spinal fluid, pleural effusion, sputum, stool, tears, sweat, pus, etc.), surgical excisions, needle biopsy, scraping, etc.; see also the Examples.

A sample, e.g. nucleic acid sample may be obtained from the test sample using any techniques known in the art (Ausubel et al. Current Protocols in Molecular Biology (John Wiley & Sons, Inc., New York, 1999); Molecular Cloning: A Laboratory Manual, 2nd Ed., ed. by Sambrook, Fritsch, and Maniatis (Cold Spring Harbor Laboratory Press: 1989); Nucleic Acid Hybridization (B. D. Hames & S. J. Higgins eds. 1984); the treatise, Methods in Enzymology (Academic Press, Inc., N.Y.); each of which is incorporated herein by reference). The nucleic acid may be purified from whole cells using DNA or RNA purification techniques. The sample may also be amplified using PCR or in vivo techniques requiring subcloning. In a preferred embodiment, the sample is obtained by isolating mRNA from the cells of the test

sample and reverse transcribing the RNA into DNA in order to create cDNA (Khan et al., Biochem. Biophys. Acta 1423 (1999), 17-28; incorporated herein by reference).

5 Methods for assaying a sample of a subject for the presence of a variant allele of a target gene utilize the principle that specific sequence differences can be translated into reagents for allele differentiation. These reagents provide the necessary backbone for the development of diagnostic tests. Examples for such reagents include, but are not limited to oligonucleotides that deviate from the wild type target gene sequence in the alteration(s) in the nucleotide sequence or which are capable of priming the synthesis of a cDNA or cRNA through said
10 alteration(s) in the template gene or transcripts derived therefrom. Frequently, the principles of diagnostic tests for the determination of the individual target gene status include, but are not limited to differences in the hybridization efficiencies of such reagents to the various variant alleles. In addition, differences in efficacy of such reagents in, or as different substrates for, enzymatic reactions, e.g. ligases or polymerases or restriction enzymes can be
15 applied. The principles of these are well known to experts of the field. Examples are PCR and LCR techniques, Chip-hybridizations or MALDI-TOF analyses. Such techniques are described in the prior art, e.g., PCR technique: Newton, (1994) PCR, BIOS Scientific Publishers, Oxford; LCR-technique: Shimer, Ligase chain reaction. Methods Mol. Biol. 46 (1995), 269-278; Chip hybridization: Ramsay, DNA chips: State-of-the art. Nature
20 Biotechnology 16 (1998), 40-44; and MALDI-TOF analysis: Ross, High level multiplex genotyping by MALDI-TOF mass spectrometry, Nature Biotechnology 16 (1998), 1347-1351. Other test principles are based on the application of reagents that recognize specifically or that are being specifically recognized by the allelic variant as translated expressed protein. Examples are allele-specific antibodies, peptides, substrate analogs, inhibitors, or other
25 substances which bind to (and in some instances may also modify the action of) the various protein forms that are encoded by the variant alleles. In addition, conventional functional biochemical assays or cell-based in vitro assays may be used.

30 Additionally, the presence of a variant allele can be monitored by using a primer pair that specifically hybridizes to a region of the target gene and by carrying out an amplification reaction according to standard procedures. Specific hybridization of the above mentioned probes or primers preferably occurs at stringent hybridization conditions. The term "stringent hybridization conditions" is well known in the art; see, for example, Sambrook et al., "Molecular Cloning, A Laboratory Manual" second ed., CSH Press, Cold Spring Harbor,

1989; "Nucleic Acid Hybridisation, A Practical Approach", Hames and Higgins eds., IRL Press, Oxford, 1985. Furthermore, the genomic DNA obtained from the subject may be sequenced to identify mutations which may be characteristic fingerprints of mutations in the target gene. The present invention further comprises methods wherein such a fingerprint may
5 be generated by RFLPs of DNA obtained from the subject, optionally the DNA may be amplified prior to analysis, the methods of which are well known in the art. For example, the sample nucleic acid, e.g., amplified or cloned fragment, may be sequenced by dideoxy or other methods. Hybridization with the variant sequence may also be used to determine its presence, by Southern blots, dot blots, etc. The hybridization pattern of a control and variant
10 sequence to an array of oligonucleotide probes immobilized on a solid support, as described in US patent no. 5,445,934, or in international application WO95/35505, may also be used as a means of detecting the presence of variant sequences. Single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), mismatch cleavage detection, and heteroduplex analysis in gel matrices are used to detect
15 conformational changes created by DNA sequence variation as alterations in electrophoretic mobility. Alternatively, where a polymorphism creates or destroys a recognition site for a restriction endonuclease (restriction fragment length polymorphism, RFLP), the sample is digested with that endonuclease, and the products size fractionated to determine whether the fragment was digested. Fractionation is performed by gel or capillary electrophoresis,
20 particularly acrylamide or agarose gels. Corresponding methods may be applied on basis of the expressed proteins, for example using proteases which cleavage site is destroyed in the variant allele.

In a preferred embodiment of the method of the present invention the genetic predisposition is
25 determined by genotyping. It is a well known fact that genomic DNA of individuals, which harbor the individual genetic makeup of all genes, including any one of the target genes described herein, can easily be purified from individual blood samples. These individual DNA samples are then used for the analysis of the sequence composition of the alleles of the target gene that are present in the individual which provided the blood sample. The sequence
30 analysis can be carried out by PCR amplification of relevant regions of said genes, subsequent purification of the PCR products, followed by automated DNA sequencing with established methods (e.g. ABI dyeterminator cycle sequencing); see also supra and Example 1.

Depending on the nature of the allele, i.e. whether it said allele is dominant or recessive one important parameter that has to be considered in the attempt to determine the individual genotype by direct DNA-sequencing of PCR-products from human blood genomic DNA is the fact that each human harbors (usually, with very few abnormal exceptions) two gene
5 copies of each autosomal gene (diploidy). Because of that, care has to be taken in the evaluation of the sequences to be able to identify unambiguously not only homozygous alleles but also heterozygous alleles.

It is well known in the art that genes comprise structural elements which encode an amino
10 acid sequence as well as regulatory elements which are involved in the regulation of the expression of said genes. Structural elements are represented by exons which may either encode an amino acid sequence or which may encode for RNA which is not encoding an amino acid sequence but is nevertheless involved in RNA function, e.g. by regulating the stability of the RNA or the nuclear export of the RNA.

15 Regulatory elements of a gene may comprise promoter elements or enhancer elements both of which could be involved in transcriptional control of gene expression. It is very well known in the art that a promoter is to be found upstream of the structural elements of a gene. Regulatory elements such as enhancer elements, however, can be found distributed over the entire locus of a gene. Said elements could be reside, e.g., in introns, regions of genomic DNA which
20 separate the exons of a gene. Promoter or enhancer elements correspond to polynucleotide fragments which are capable of attracting or binding polypeptides involved in the regulation of the gene comprising said promoter or enhancer elements. For example, polypeptides involved in regulation of said gene comprise the so called transcription factors.

Said introns may comprise further regulatory elements which are required for proper gene
25 expression. Introns are usually transcribed together with the exons of a gene resulting in a nascent RNA transcript which contains both, exon and intron sequences. The intron encoded RNA sequences are usually removed by a process known as RNA splicing. However, said process also requires regulatory sequences present on a RNA transcript said regulatory sequences may be encoded by the introns.

30 In addition, besides their function in transcriptional control and control of proper RNA processing and/or stability, regulatory elements of a gene could be also involved in the control of genetic stability of a gene locus. Said elements control, e.g., recombination events or serve to maintain a certain structure of the DNA or the arrangement of DNA in a chromosome.

Therefore, single nucleotide polymorphisms can occur in exons of a gene which encode an amino acid sequence as discussed supra as well as in regulatory regions which are involved in the above discussed process. The analysis of the nucleotide sequence of a gene locus in its entirety including, e.g., introns is in light of the above desirable. The polymorphisms comprised by the polynucleotides of the present invention can influence the expression level of protein activity via mechanisms involving enhanced or reduced transcription of the target gene, stabilization of the gene's RNA transcripts and alteration of the processing of the primary RNA transcripts.

Usually, said variant allele results from a nucleotide deletion, addition and/or substitution compared to the corresponding wild type gene. In one embodiment of the method of the invention a nucleotide deletion, addition and/or substitution results in altered expression of the variant gene compared to the corresponding wild type gene. Preferably, said nucleotide substitution(s), addition(s) or deletion(s) referred to in accordance with the method of the present invention result(s) in one or more changes of the corresponding amino acid(s) of the proteins encoded by the target genes.

Without intending be bound by theory it is believed that said variant allele generally results in lower amount of protein activity compared to a control, for example because of reduced expression of the target gene or altered enzymatic activity. The decreased amount or level of protein activity as referred to herein includes in addition to a significantly decreased amount of transcripts encoding a functional gene product also a normal or even elevated amount of transcripts encoding a gene product which has no activity or a significantly decreased activity. The term "lower amount of protein activity" means that the level of activity of the gene products of the mentioned target genes is determined and the level is compared to a reference standard. As a reference standard, preferably a sample is obtained from cells or tissues of a subject having the wild type allele of the respective target genes in its genome. Preferably, said subject is homozygous or hemizygous with respect to the selected target gene.

As discussed supra, the variant alleles which correspond to coding regions of the target gene effect the amino acid sequences of the polypeptides encoded by said variant alleles. The variant polypeptides preferably, but not necessarily exhibit altered biological and/or immunological properties when compared to their corresponding wild type counterpart. Preferred variant polypeptides in accordance with the use of the invention are those, which exhibit an altered biological activity, i.e. altered enzymatic function resulting in reduced,

enhanced or complete loss of catalytic activity or altered transport function resulting in reduced, enhanced or complete loss of transport activity or altered binding to receptors or other drug targets resulting in altered activation of signal transduction pathways or altered inhibition of transporter or enzyme function. It might be necessary to obtain a sample comprising biological material such as isolated cells or tissue from the subject prior to perform said methods for determination of the activities of the wild type and the variant polypeptides, respectively. Whether a variant polypeptide has an altered activity or level of expression compared to its wild type corresponding counterpart can be determined by standard techniques well known in the art. Such standard techniques may comprise, e.g., ELISA based assays, RIA based assays, HPLC-based assays, mass spectroscopy-based assays, western blot analysis or assays which are known in the art; see also the references cited herein.

As described in the examples, the method of the present invention has been established inter alia on the basis of specific alleles of genes involved in the generation of ROS, i.e. encoding proteins of the NAD(P)H oxidase multi-enzyme complex, i.e. NCF4, CYBA and RAC2. In particular, chronic ACT associated with a variant of the NAD(P)H oxidase subunit NCF4 (-212A>G, OR: 2.5, 95 % CI: 1.3 - 5.0). Acute ACT was associated with the His72Tyr polymorphism in the p22phox subunit (OR: 2.0, 95 % CI: 1.0 - 3.9) and with the variant 7508T>A (OR: 2.6, 95 % CI: 1.3 - 5.1) of the RAC2 subunit of the same enzyme. Therefore, in a preferred embodiment of the present invention said variant allele to be determined is a polymorphism corresponding to the C242T (His72Tyr) polymorphism of the CYBA gene, a polymorphism corresponding to the 212A>G polymorphism in the NCF4 gene or a polymorphism corresponding to the 7508T>A polymorphism in the RAC2 gene.

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In addition, it could be shown that genes involved in drug transmembrane transport, i.e. genes encoding multi-drug resistance protein, especially multidrug resistance-associated protein 1 (MRP1) or multidrug resistance-associated protein 2 (MRP2) associate with anthracycline cardiotoxicity. In particular, acute ACT was associated with the Gly671Val variant of the doxorubicin efflux transporter MRP1 (OR: 3.6, 95 % CI: 1.6 - 8.4) and with the Val1188Glu-Cys1515Tyr haplotype of the functionally similar MRP2 (OR: 2.3, 95 % CI: 1.0 - 5.4). Therefore, in another and additional preferred embodiment of the present invention, respectively, said variant allele to be determined is a polymorphism corresponding to the Gly671Val polymorphism in the MRP1 gene or a polymorphism corresponding to the

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Val1188Glu or Cys1515Tyr polymorphism in the MRP2 gene. Further, polymorphisms in the human gene for the multidrug resistance-associated protein 1 (MRP-1) and means and methods for their diagnosis are described, for example in international application WO02/059142, the disclosure content of which is incorporated herein by reference. In the background section WO02/059142 also provides a review over the human multidrug resistance-associated protein (MRP) family, a subfamily of the ATP-binding cassette (ABC) protein superfamily, said information is likewise incorporated herein.

A subject may not suffer from cardiotoxicity at first place but only after medication with a given drug. For example, as explained above and in the examples, a significant number of patients treated with anthracyclines develop cardiotoxicity (ACT), mainly presenting as arrhythmias (acute ACT) or congestive heart failure (chronic ACT). Accordingly, the method of the present invention is preferably applied in cases, wherein said cardiac disease or dysfunction the risk of which a subject is to be diagnosed for is induced by a drug. In this case the method of the present invention is particularly useful for selecting patients who have to undergo chemotherapy and who have been diagnosed to be at high risk of developing cardiotoxicity to the intended drug in order to provide an adapted such low dose and/or combination therapy, or an alternative treatment.

Preferably, said drug is an antineoplastic agent, most preferably said drug is an anthracycline. Anthracyclines belong to a member of a family of chemotherapy drugs that are also antibiotics and that originally come from the fungus *Streptococcus peucetius*. The anthracyclines act to prevent cell division by disrupting the structure of the DNA and terminate its function. They do so in two ways: (1) they intercalate into the base pairs in the DNA minor grooves; and (2) they cause free radical damage of the ribose in the DNA. The anthracyclines are frequently used in leukemia therapy. The anthracyclines include daunorubicin (Cerubidine), doxorubicin (DOX, Adriamycin, Rubex), epirubicin (4'epi-doxorubicin, Ellence, Pharmorubicin), and idarubicin (4-demethoxy-daunorubicin, Idamycin), and as well as their isomers and analogs, for example morpholino-and cyanomorpholino-doxorubicin (morpholino-DOX and cyanomorpholino DOX). Anthracyclines, for example doxorubicin and epirubicin are widely used in cancer therapy.

In a preferred embodiment of the present invention the methods and therapeutic uses described herein are applied to cancer treatment with doxorubicin.

As summarized in the background section and in the examples there is a lower incidence of heart damage following doxorubicin infusion as opposed to bolus injection suggesting a role for the drug's peak levels in drug-induced cardiotoxicity. Accordingly, besides the initial dose also the route of administration should be taken into account. For example, in case a patient is
5 diagnosed positive for risk of cardiotoxicity in accordance with a method of the present invention it is recommended to administer the drug by intravenous infusion rather than by bolus injection.

With respect to the particular cardiac disease or dysfunction these usually comprise
10 arrhythmia and/or congestive heart failure. For example, patients treated with anthracyclines develop cardiotoxicity (ACT), mainly presenting as arrhythmias (acute ACT) or congestive heart failure (chronic ACT). Accordingly, in one embodiment said cardiac disease or dysfunction a subject may be at risk for comprises anthracycline-induced cardiotoxicity (ACT). Said ACT may be acute ACT or chronic ACT.

15

As is evident from the above, a prerequisite for selecting a suitable therapy is the knowledge of the presence or absence of a variant allele referred to in accordance with the method of the present invention. Therefore, the method of the present invention encompasses the determination of the presence or absence of said variant alleles in a sample which has been
20 obtained from said subject. The sample which is obtained by the subject comprises biological material which is suitable for the determination of the presence or absence of said variant alleles, such as isolated cells or tissue. For example, the said sample can be a plasma sample, a blood sample, a saliva sample, a tumor sample, a tissue sample or bodily fluid sample.

25 Additionally, the presence or expression of variant target gene can be monitored by using a primer pair that specifically hybridizes to either of the corresponding nucleic acid sequences and by carrying out a PCR reaction according to standard procedures. Specific hybridization of the above mentioned probes or primers preferably occurs at stringent hybridization conditions. The term "stringent hybridization conditions" is well known in the art; see, for
30 example, Sambrook et al., "Molecular Cloning, A Laboratory Manual" second ed., CSH Press, Cold Spring Harbor, 1989; "Nucleic Acid Hybridisation, A Practical Approach", Hames and Higgins eds., IRL Press, Oxford, 1985. Furthermore, the mRNA, cRNA, cDNA or genomic DNA obtained from the subject may be sequenced to identify mutations which may be characteristic fingerprints of mutations in the polynucleotide or the gene of the invention.

The present invention further comprises methods wherein such a fingerprint may be generated by RFLPs of DNA or RNA obtained from the subject, optionally the DNA or RNA may be amplified prior to analysis, the methods of which are well known in the art. RNA fingerprints may be performed by, for example, digesting an RNA sample obtained from the subject with
5 a suitable RNA-Enzyme, for example RNase T1, RNase T2 or the like or a ribozyme and, for example, electrophoretically separating and detecting the RNA fragments as described above. Further modifications of the above-mentioned embodiment of the invention can be easily devised by the person skilled in the art, without any undue experimentation from this disclosure; see, e.g., the examples. An additional embodiment of the present invention relates
10 to a method wherein said determination is effected by employing an antibody of the invention or fragment thereof. The antibody used in the method of the invention may be labeled with detectable tags such as a histidine flags or a biotin molecule.

In a preferred embodiment of the present invention, the above described methods comprise
15 PCR, ligase chain reaction, restriction digestion, direct sequencing, nucleic acid amplification techniques, hybridisation techniques, immunodiagnostic methods or biological or enzymatic activity assays. Diagnostic screening may be performed for polymorphisms through the use of microsatellite markers or single nucleotide polymorphisms (SNP). The microsatellite or SNP polymorphism itself may not phenotypically expressed, but is linked to sequences that result
20 in altered activity or expression. Two polymorphic variants may be in linkage disequilibrium, i.e. where alleles show non-random associations between genes even though individual loci are in Hardy-Weinberg equilibrium. Linkage analysis may be performed alone, or in combination with direct detection of phenotypically evident polymorphisms. The use of microsatellite markers for genotyping is well documented. For examples, see Mansfield et al.,
25 Genomics 24 (1994), 225-233; and Ziegle et al., Genomics 14 (1992), 1026-1031. The use of SNPs for genotyping is illustrated in Golevieva, Am. J. Hum. Genet. 59 (1996), 570-578; and in Underhill et al., Proc. Natl. Acad. Sci. USA 93 (1996), 196-200.

Hence, the variant alleles of the target genes of the present invention are preferably determined by genotyping. Further means and methods for genotyping and phenotyping,
30 respectively, which can be used in and/or adapted to methods of the present invention are described in international application WO01/55432.

The foregoing methods are preferably conducted at least twice on a given sample using, for example, at least one different primer pair specific for particular alleles. Following detection,

one may compare the results seen in a given patient with a statistically significant reference group of normal subjects and a patient to be treated. In this way, it is possible to correlate the number and kind of variant alleles with various clinical states or disease prognosis. The method of the present invention can include the combination of two, three or all of the alleles,
5 weighted according to F-power in a multivariate analysis, to increase the accuracy of the method. One of ordinary skill in the art recognizes that Genbank accession numbers refer to numbers used to identify nucleotide sequences available in Genbank (Benson et al., "GenBank", Nucleic Acids. Res. 30 (2002), 17-20).

10 In addition, the present invention relates to the use of an oligonucleotide for determining the presence of variant allele in accordance with the method of the present invention. Preferably, said oligo- or polynucleotide is about 15 to 50, preferably 20 to 40, more preferably 20 to 30 nucleotides in length and comprises the nucleotide sequence of any one of the primer or target sequences disclosed in Table 2, *infra*, or a complementary sequence.

15 Hence, in a still further embodiment, the present invention relates to a primer or probe consisting of an oligonucleotide as defined above. In this context, the term "consisting of" means that the nucleotide sequence described above and employed for the primer or probe of the invention does not have any further nucleotide sequences of the target gene involved in
20 the generation of ROS or drug transmembrane transport immediately adjacent at its 5' and/or 3' end. However, other moieties such as labels, e.g., biotin molecules, histidin flags, antibody fragments, colloidal gold, etc. as well as nucleotide sequences which do not correspond to the particular target gene may be present in the primer and probes of the present invention. Furthermore, it is also possible to use particular nucleotide sequences of the respective target
25 gene and to combine them with other nucleotide sequences derived from the target gene, wherein these additional nucleotide sequences are interspersed with moieties other than nucleic acids or wherein the intervening nucleic acid does not correspond to nucleotide sequences of the target gene.

30 Furthermore, it is evident to the person skilled in the art that the oligonucleotide can be modified, for example, by thio-phosphate-backbones and/or base analogs well known in the art (Flanagan, Proc. Natl. Acad. Sci. USA 96 (1999), 3513-8; Witters, Breast Cancer Res. Treat. 53 (1999), 41-50; Hawley, Antisense Nucleic Acid Drug Dev. 9 (1999), 61-9; Peng Ho, Brain Res. Mol. Brain Res. 62 (1998), 1-11; Spiller, Antisense Nucleic Acid Drug Dev. 8

(1998), 281-93; Zhang, J. Pharmacol. Exp. Ther. 278 (1996), 971-9; Shoji, Antimicrob. Agents Chemother. 40 (1996), 1670-5; Crooke, J. Pharmacol. Exp. Ther. 277 (1996), 923-37). In one embodiment of the invention, an array and chip, respectively, of oligonucleotides are provided, where discrete positions on a solid support of the the array or chip are
5 complementary to one or more of the provided polymorphic sequences, e.g. oligonucleotides of at least 12 nt., frequently 20 nt. or larger, and including the sequence flanking the polymorphic position. Such an array may comprise a series of oligonucleotides, each of which can specifically hybridize to a different polymorphism. For examples of arrays, see Hacia et al., Nature Genetics 14 (1996), 441-447; Lockhart et al., Nature Biotechnol. 14 (1996), 1675-
10 1680; and De Risi et al., Nature Genetics 14 (1996), 457-460.

The term "solid support" as used herein refers to a flexible or non-flexible support that is suitable for carrying said immobilized targets. Said solid support may be homogenous or inhomogeneous. For example, said solid support may consist of different materials having the
15 same or different properties with respect to flexibility and immobilization, for instance, or said solid support may consist of one material exhibiting a plurality of properties also comprising flexibility and immobilization properties. Said solid support may comprise glass-, polypropylene- or silicon-chips, membranes oligonucleotide-conjugated beads or bead arrays.

20 Thanks to the present invention the particular drug selection, dosage regimen and corresponding patients to be treated can be determined in accordance with the present invention. The dosing recommendations will be indicated in product labeling by allowing the prescriber to anticipate dose adjustments depending on the considered patient group, with information that avoids prescribing the wrong drug to the wrong patients at the wrong dose.

25 Furthermore, the present invention relates to a kit useful for performing the method of the present invention, said kit comprising polynucleotides, oligonucleotides, a chip or array, reference samples, amplification and/or sequencing means, buffer, detergents, biochemical reagents, detection means, or the like, and optionally a reformulated drug with a drug dose
30 which is tailored to a patient carrying a variant allele as defined hereinabove, and optionally instructions for carrying out a method of the invention.

The kit of the invention may contain further ingredients such as selection markers and components for selective media suitable for the generation of transgenic cells and animals. The kit of the invention can be used for carrying out a method of the invention and could be,

inter alia, employed in a variety of applications, e.g., in the diagnostic field or as research tool. The parts of the kit of the invention can be packaged individually in vials or other appropriate means depending on the respective ingredient or in combination in suitable containers or multicontainer units. Manufacture of the kit follows preferably standard procedures which are known to the person skilled in the art. The kit may be used for methods for detecting expression of a mutant form of the polypeptides, genes or polynucleotides in accordance with any one of the above-described methods of the invention, employing, for example, immunoassay techniques such as radioimmunoassay or enzymeimmunoassay or preferably nucleic acid hybridization and/or amplification techniques such as those described herein before and in the Examples.

In a further aspect the present invention relates to the use of a drug or prodrug for the preparation of a pharmaceutical composition for the treatment or prevention of a disorder of a subject diagnosed by the method described hereinbefore.

In one embodiment, a given drug as defined herein is used for the preparation of a pharmaceutical composition for the treatment of a disease which is amenable to a corresponding therapy with said drug, wherein said pharmaceutical composition is designed for administration to a patient who carries at least one variant allele as defined hereinbefore, wherein the drug dose is lower than compared to the use for administering to a patient without carrying said variant allele. By "wherein the drug dose is lower than compared to the use for administering to a patient without carrying said variant allele" a standard dose is meant which is routinely administered to patients in need thereof without regarding the genotype. Such a general population of patients is considered as having the normal genotype, i.e. wild type genotype.

The methods of the invention may also provide, for example, the optimization of therapy for a disease such as cancer. The invention also provides a method of designing a therapy for a patient, and a method of prescribing a therapy for a patient, including making recommendations for drugs and/or combinations of drugs not yet prescribed for that patient. Accordingly, in a further embodiment the present invention relates to a method for the preparation of an medicament individual subject or subpopulation of subjects comprising reformulation of the drug or pro-drug, wherein said individualized dosage is calculated from the dose usually applied and recommended for in-patients, without taking gene

polymorphisms into consideration, as taken from manufacturers' recommendations factored against the metabolic quotient determined in accordance with the pharmacokinetic model of the present invention. The definitions and explanations of the terms made above apply mutatis mutandis to all of the methods described herein. The term "suitable therapy" as used herein
5 means that a substance according to the invention is selected and said substance is administered in a certain dosage to a subject, wherein said substance and said dosage are selected based on the knowledge of the presence or absence of at least one, preferably at least two, more preferably at least three and most preferably at least four variant alleles referred to in accordance with the use of the invention. Said substance and said dosage of the substance
10 are selected in a way that they are most effective on one hand and on the other hand they do not cause toxic or undesirable side effects.

In one embodiment, the present invention relates to a method for the treatment of a disease comprising administering to a subject in need of such treatment a non-cardiotoxic amount of a
15 drug as defined herein or a non-cardiotoxic analog or substitute thereof, wherein said subject carries at least one variant allele as defined above. For example, a drug as defined above may be used for the preparation of a pharmaceutical composition for the treatment of a disease which is amenable to a corresponding therapy with said drug, wherein said pharmaceutical composition is designed for administration to a patient who carries at least one variant allele
20 as defined herein, wherein the drug dose is lower than compared to the use for administering to a patient without carrying said variant allele.

In addition, or alternatively suitable analogs and derivatives of drugs such as anthracyclines, which are less cardiotoxic may be used; see, e.g., Danesi et al., Toxicology 70 (1991), 243-
25 253, which describes cardiotoxicity and cytotoxicity of the anthracycline analog 4'-deoxy-4'-iodo-doxorubicin. Sugar-modified derivatives of antitumor anthracycline, daunorubicin, are described in Pawlowska et al., Oncol. Res. 14 (2004), 469-474, and characterization of anthracenediones and their photoaffinity analogs is described in Chou et al., Biochem. Pharmacol. 63 (2002), 1143-1147. Pouna et al., Cancer Chemother. Pharmacol. 35 (1995),
30 257-261, describe evaluation of anthracycline cardiotoxicity with the model of isolated, perfused rat heart exemplified by a comparison of new analogues versus doxorubicin. Furthermore, reduced toxicity by a novel disaccharide analogue is reported by Minotti et al. in Chem. Res. Toxicol. 13 (2000), 1336-1341 and Br. J. Pharmacol. 134 (2001), 1271-1278.

In addition, or alternatively the drug may substituted, for example an anthracycline-based chemotherapy may be changed to a non-anthracycline-based therapy.

In another embodiment, the present invention relates to a method for the treatment of a disease comprising administering to a subject in need of such treatment a drug as defined herein, wherein said subject does not carry any one of said variant alleles as described above. Put in other words, a drug such as an antineoplastic drug may be used for the preparation of a pharmaceutical composition for the treatment of a disease which is amenable to a corresponding therapy with said drug, wherein said pharmaceutical composition is designed for administration to a patient who does not carry any one of said variant alleles of, for example MRP1 and MRP2.

Hence, a particular object of the present invention concerns drug/pro-drug selection and formulation of pharmaceutical compositions for the treatment of diseases which are amenable to chemotherapy taking into account the polymorphism of the variant form(s) of genes involved in (a) the generation of reactive oxygen species (ROS) or (b) drug transmembrane transport, which co-segregate with the affected phenotype of the patient to be treated. This allows the safe and economic application of drugs which for example were hitherto considered not appropriate for therapy of, e.g., cancer due to either their side effects in some patients, i.e. cardiotoxicity. The means and methods described herein can be used, for example, to improve dosing recommendations and allows the prescriber to anticipate necessary dose adjustments depending on the considered patient group. Accordingly, prior to treatment a sample of the patient has preferably been assayed in accordance with the method of the present invention as described above.

25

In a particular preferred embodiment of the present invention said drug to be used is an antineoplastic agent as defined hereinbefore said disease is cancer.

Chemotherapy for cancer is varied, because there are so many different forms of this disease. Treatment may rely on a single anticancer medication - that is, single agent chemotherapy - or it may involve combination chemotherapy with a number of different anticancer drugs. Such drugs destroy cancer cells by preventing them from growing and dividing rapidly. Drugs and chemotherapy regimens which may be improved in accordance with the present invention include but are not limited to:

30

- ABVD: Doxorubicin (Adriamycin®), bleomycin, vinblastine, and dacarbazine
- ACVBP: Doxorubicin, cyclophosphamide, vindesine, bleomycin, prednisone, intrathecal methotrexate
- ASHAP-mBACOS-MINE: Doxorubicin, methylprednisolone, cytarabine, platinum,
5 methotrexate, bleomycin, cyclophosphamide, vincristine
- BEP: BCNU (carmustine), etoposide, procarbazine
- CEOP-B: Cyclophosphamide, epirubicin, vincristine, methylprednisolone, bleomycin
- CEOP-Bleo: Cyclophosphamide, epirubicin, vincristine, prednisone, bleomycin
- CEP: Cyclophosphamide, etoposide, prednisolone
- 10 CEVOP: Cyclophosphamide, epirubicin, etoposide, vincristine, prednisone
- CIOP-B: Cyclophosphamide, idarubicin, vincristine, prednisone, bleomycin
- CHOP: Cyclophosphamide, doxorubicin, vincristine, prednisone
- CHOP-B: Cyclophosphamide, doxorubicin, vincristine, methylprednisolone, bleomycin
- CHOP-R: Cyclophosphamide, doxorubicin, vincristine, prednisone, rituximab
- 15 CHOEP: Cyclophosphamide, doxorubicin, vincristine, etoposide, prednisone
- CNOP: Cyclophosphamide, mitoxantrone, vincristine, prednisone
- CTVP: Cyclophosphamide, pirarubicin, teniposide, prednisone
- CVP: Cyclophosphamide, teniposide, prednisone
- ChIVPP: Chlorambucil (Leukeran®), vinblastine sulfate (Velban®), procarbazine
20 hydrochloride (Matulane®), and prednisone;
- CVP: Cyclophosphamide, vindesine, prednisolone
- EVA: Etoposide, vinblastine, and doxorubicin
- EVAP: Etoposide (VP-16; VePesid®), vinblastine sulfate (Velban®), doxorubicin (Adriamycin®), and prednisone
- 25 ISHAP-mBICOS-MINE: Idarubicin, methylprednisolone, cytarabine, platinum, methotrexate, bleomycin, cyclophosphamide, vincristine
- LOPP: Chlorambucil (Leukeran®), vincristine sulfate (Oncovin®), procarbazine hydrochloride (Matulane®), and prednisone
- MACOP-B: Methotrexate, doxorubicin, cyclophosphamide, vincristine, bleomycin,
30 prednisone
- m-BACOD: Methotrexate, bleomycin, doxorubicin, cyclophosphamide, vincristine, prednisone
- MEP: Mitoxantrone, etoposide, procarbazine
- MiCEP: Mitoxantrone, etoposide, cyclophosphamide, prednisone

MOPP: Mechlorethamine, vincristine (Oncovin®), procarbazine, and prednisone;

NOVP: Mitoxantrone (Novantrone®), vincristine (Oncovin®), vinblastine, and prednisone

PAdriCEBO: Prednisolone, doxorubicin, cyclophosphamide, etoposide, bleomycin, vincristine

5 PMitCEBO: Prednisolone, mitoxantrone, cyclophosphamide, etoposide, bleomycin, vincristine

ProMACE-CytaBOM: Cyclophosphamide, doxorubicin, etoposide, cytarabine, bleomycin, vincristine, methotrexate, prednisone

ProMECE-CytaBOM: Cyclophosphamide, epirubicin, etoposide, cytarabine, bleomycin, 10 vincristine, methotrexate, prednisone

PVABEC: Etoposide, doxorubicin, cyclophosphamide, vincristine, bleomycin, prednisone

P-VEBEC: Epirubicin, cyclophosphamide, etoposide, vinblastine, bleomycin, prednisone

T-COP, THP-COP: Pirarubicin, cyclophosphamide, vincristine, prednisone

THP-COPE: Pirarubicin, cyclophosphamide, vincristine, prednisone, etoposide

15 VMP: Etoposide, mitoxantrone, prednimustine

VNCOP-B: Cyclophosphamide, mitoxantrone, vincristine, etoposide, bleomycin, prednisone

VEEP: Vincristine, epirubicin, etoposide, and prednisolone;

20 Preferably, method and therapeutic use of the present invention, respectively, involves any one of the aforementioned drugs or drug regimen wherein said drug or at least one of said drugs belongs to class of anthracyclines; see also supra.

The pharmaceutical compositions and formulations referred to herein are administered at least once in accordance with the use of the present invention. However, the said pharmaceutical compositions and formulations may be administered more than one time, for example once 25 weekly every other week up to a non-limited number of weeks.

Thanks to the method of the present invention, it is possible to efficiently select a suitable therapy for a subject, preferably a human, suffering, for example, from lymphoma, Hodgkin's and Non-Hodgkin's Disease, respectively, colorectal cancer, cervical cancer, gastric cancer, 30 lung cancer, malignant glioma, ovarian cancer, and pancreatic cancer. Thereby, mistreatment of patients based on wrong medications and the results thereof, such as development of resistance towards cancer therapy, and subsequent increased costs in health care, can be efficiently avoided. Furthermore, patients that are at high risk can be excluded from therapy prior to the first dose and/or dosage can be adjusted according to the individual's genetic

makeup prior to the onset of drug therapy. Also, agonists/activators for the mentioned target genes (e.g. MRP1) can be applied in genetically defined patient subpopulations. Thus, adverse effects can be avoided and the optimal drug level can be reached faster without time-consuming and expensive drug monitoring-based dose finding. This can reduce costs of medical treatment and indirect costs of disease (e.g. shorter time and less frequent hospitalization of patients).

Accordingly, the present invention also relates to combination preparations, i.e. pharmaceutical compositions comprising an antineoplastic agent and an agonist/activator of a protein involved in the generation of reactive oxygen species (ROS) or drug transmembrane transport.

Agonists and activators of NAD(P)H oxidase activity are well known in the art and include, for example, hormones such as Endothelin-1 and angiotensin II. Furthermore, thrombin, interleukin-1 (IL-1), platelet-derived growth factor (PDGF), tumor necrosis factor- α (TNF- α), and lactosylceramide also stimulate NAD(P)H oxidase activity. In addition, activation of the oxidase can be mediated by intracellular second messengers, including calcium. For review of NAD(P)H oxidase system and regulation see Griendling et al., Circulation Research 86 (2000), 494, and references cited therein. Furthermore, methods and materials involved in identifying agonists NADPH oxidase activity are described for example in international application WO03/095667.

Agonists and activators of drug transmembrane transport are also known in the art. For example, Worm et al. (Biol. Chem. 276 (2001), 39990-40000) describe up-regulation of P-glycoprotein in response to 5-aza-2'-deoxycytidine and 5-aza-2'-deoxycytidine-induced up-regulation of multidrug resistance-associated proteins. In addition, methods and compositions for regulating MRP2 are described in international application WO03/042400. Hence, preferably said protein involved in the generation of ROS is derived from the NAD(P)H oxidase multi-enzyme complex and the protein involved in transmembrane transport is MRP1 or MRP2.

As already explained for the preceding embodiments the antineoplastic agent to be used in the methods of the present invention is preferably an anthracycline, and most preferably doxorubicin; see also *infra*.

The appropriate concentration of the therapeutic agent might be dependent on the particular agent. The therapeutically effective dose has to be compared with the toxic concentrations; the clearance rate as well as the metabolic products play a role as do the solubility and the formulation. Therapeutic efficacy and toxicity of compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50 % of the population) and LD50 (the dose lethal to 50 % of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50.

10 The term "pharmaceutical composition" as used herein comprises the substances of the present invention and optionally one or more pharmaceutically acceptable carriers. The substances of the present invention may be formulated as pharmaceutically acceptable salts. Acceptable salts comprise acetate, methylester, HCl, sulfate, chloride and the like. The pharmaceutical compositions can be conveniently administered by any of the routes
15 conventionally used for drug administration, for instance, orally, topically, or by inhalation. The substances may be administered only after the genotype of the patient has been determined. Depending on a patient's genotype three different dosage forms which are suited to treat patients who have three (ultrafast metabolizers), one (intermediate metabolizers) or no wild type gene (poor metabolizers) are given the patient. These dosage forms are prepared by
20 combining the drugs with standard pharmaceutical carriers according to conventional procedures. These procedures may involve mixing, granulating and compressing or dissolving the ingredients as appropriate to the desired preparation. It will be appreciated that the form and character of the pharmaceutically acceptable character or diluents is dictated by the amount of active ingredient with which it is to be combined, the route of administration and
25 other well-known variables. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof. The pharmaceutical carrier employed may be, for example, either a solid or a liquid. Exemplary of solid carriers are lactose, terra alba, sucrose, talc, gelatin, agar, pectin, acacia, magnesium stearate, stearic acid and the like. Exemplary of liquid carriers are phosphate
30 buffered saline solution, syrup, oil such as peanut oil and olive oil, water, emulsions, various types of wetting agents, sterile solutions and the like. Similarly, the carrier or diluents may include time delay material well known to the art, such as glyceryl mono-stearate or glyceryl distearate alone or with a wax. The substance according to the present invention can be administered in various manners to achieve the desired effect. Said substance can be

administered either alone or in the formulated as pharmaceutical preparations to the subject being treated either orally, topically or by inhalation. Moreover, the substance can be administered in combination with other substances either in a common pharmaceutical composition or as separated pharmaceutical compositions.

5

The diluents are selected so as not to affect the biological activity of the combination. Examples of such diluents are distilled water, physiological saline, Ringer's solutions, dextrose solution, and Hank's solution. In addition, the pharmaceutical composition or formulation may also include other carriers, adjuvants, or nontoxic, nontherapeutic, nonimmunogenic stabilizers and the like. A therapeutically effective dose refers to that amount of the substance according to the invention, which ameliorate the symptoms or condition. Therapeutic efficacy and toxicity of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50 % of the population) and LD50 (the dose lethal to 50 % of the population). The dose ratio between therapeutic and toxic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50.

The dosage regimen will be determined by the attending physician and other clinical factors; preferably in accordance with any one of the above described methods. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's weight, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Progress can be monitored by periodic assessment.

25 The pharmaceutical compositions and formulations referred to herein are administered at least once in accordance with the use of the present invention. However, the said pharmaceutical compositions and formulations may be administered more than one time, for example from one to four times daily up to a non-limited number of days.

30 Specific formulations of the substance according to the invention are prepared in a manner well known in the pharmaceutical art and usually comprise at least one active substance referred to herein above in admixture or otherwise associated with a pharmaceutically acceptable carrier or diluent thereof. For making those formulations the active substance(s) will usually be mixed with a carrier or diluted by a diluent, or enclosed or encapsulated in a

capsule, sachet, cachet, paper or other suitable containers or vehicles. A carrier may be solid, semisolid, gel-based or liquid material which serves as a vehicle, recipient or medium for the active ingredients. Said suitable carriers comprise those mentioned above and others well known in the art, see, e.g., Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pennsylvania. The formulations can be adopted to the mode of administration comprising the forms of tablets, capsules, suppositories, solutions, suspensions or the like.

The dosing recommendations will depend on the genotype of the patient and will be indicated in the product labeling. This will allow the doctor to select the drug formulation that contains the drug concentration which is suited to the specific genotype of a patient. It is a prerequisite that the gene for the drug metabolizing enzyme is first analyzed before the specific drug formulation is prescribed. This will avoid that the wrong drug is prescribing the wrong patients at the wrong dose.

The present invention also encompasses all embodiments described in connection with pharmaceutical compositions in US patents: 4,695,578; 4,753,789; 5,578,628; 5,955,488; 6,063,802; 4,886,808; 6,294,548; 4,906,755.

The kit of the present invention may be tailored for phenotypic and/or genotypic screening. According to one embodiment, the assay system and kit preferably employ antibodies specific to a plurality of metabolites on a suitable substrate allowing for detection of the preferred metabolites in a biological sample of an individual after consumption of a corresponding probe substrate. The assay systems of the present invention may be provided in a plurality of forms including but not limited to a high-throughput assay system or a dipstick based assay.

These and other embodiments are disclosed and encompassed by the description and examples of the present invention. Further literature concerning any one of the materials, methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries and databases, using for example electronic devices. For example the public database "Medline" may be utilized, which is hosted by the National Center for Biotechnology Information and/or the National Library of Medicine at the National Institutes of Health. Further databases and web addresses, such as those of the European Bioinformatics Institute (EBI), which is part of the European Molecular Biology Laboratory (EMBL) are known to the person skilled in the art and can also be obtained using internet

search engines. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

5 The above disclosure generally describes the present invention. Several documents are cited throughout the text of this specification. Full bibliographic citations may be found at the end of the specification immediately preceding the claims. The contents of all cited references (including literature references, issued patents, published patent applications as cited throughout this application and manufacturer's specifications, instructions, etc) are hereby
10 expressly incorporated by reference; however, there is no admission that any document cited is indeed prior art as to the present invention.

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific examples which are provided
15 herein for purposes of illustration only and are not intended to limit the scope of the invention.

EXAMPLES

20 The examples which follow further illustrate the invention, but should not be construed to limit the scope of the invention in any way. Detailed descriptions of conventional methods, such as those employed herein can be found in the cited literature; see also "The Merck Manual of Diagnosis and Therapy" Seventeenth Ed. ed by Beers and Berkow (Merck & Co., Inc. 2003).

25

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art.

Methods in molecular genetics and genetic engineering are described generally in the current
30 editions of Molecular Cloning: A Laboratory Manual, (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press); DNA Cloning, Volumes I and II (Glover ed., 1985); Oligonucleotide Synthesis (Gait ed., 1984); Nucleic Acid Hybridization (Hames and Higgins eds. 1984); Transcription And Translation (Hames and Higgins eds. 1984); Culture Of Animal Cells (Freshney and Alan, Liss, Inc.,

1987); Gene Transfer Vectors for Mammalian Cells (Miller and Calos, eds.); Current Protocols in Molecular Biology and Short Protocols in Molecular Biology, 3rd Edition (Ausubel et al., eds.); and Recombinant DNA Methodology (Wu, ed., Academic Press). Gene Transfer Vectors For Mammalian Cells (Miller and Calos, eds., 1987, Cold Spring Harbor Laboratory); Methods In Enzymology, Vols. 154 and 155 (Wu et al., eds.); Immobilized Cells And Enzymes (IRL Press, 1986); Perbal, A Practical Guide To Molecular Cloning (1984); the treatise, Methods In Enzymology (Academic Press, Inc., N.Y.); Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (Weir and Blackwell, eds., 1986). Reagents, cloning vectors, and kits for genetic manipulation referred to in this disclosure are available from commercial vendors such as BioRad, Stratagene, Invitrogen, and Clontech. General techniques in cell culture and media collection are outlined in Large Scale Mammalian Cell Culture (Hu et al., Curr. Opin. Biotechnol. 8 (1997), 148); Serum-free Media (Kitano, Biotechnology 17 (1991), 73); Large Scale Mammalian Cell Culture (Curr. Opin. Biotechnol. 2 (1991), 375); and Suspension Culture of Mammalian Cells (Birch et al., Bioprocess Technol. 19 (1990), 251); Extracting information from cDNA arrays, Herzel et al., CHAOS 11, (2001), 98-107.

Example 1: Patients and methods for analysis of the genetic predisposition to a cardiac disorder

Study design and data management

Study design and the demographic characteristics of the entire study population have been described in detail elsewhere (Pfreundschuh et al., Blood 104 (2004), 634-641; Pfreundschuh et al., Blood 104 (2004), 626-633). 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 $\leq$ 60 years (NHL-B1) and patients aged 61–75 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 (Kaiser et al., J. Clin. Oncol. 20 (2002), 4413-4419). Since 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 four arms of the study. The arms were designed to compare the standard CHOP-21 chemotherapy with three variants including either an addition of etoposide (CHOEP-21: 100 mg/m² on days 1-3), the shortening to 2-week intervals using 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/m² i.v.), doxorubicin (50 mg/m² i.v.), vincristine (2 mg i.v.) all given on day 1, and prednisone (100 mg/ day per os) given on days 1–5. All patients were planned to receive 6 cycles of chemotherapy. Acute toxicities were classified according to the WHO Handbook for Reporting Results of Cancer Treatment. During treatment, electrocardiography was 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 electrocardiography and echocardiography as well as physical examination. The two 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 based on the following criteria: Cases of arrhythmia (in the absence of arrhythmia in the patient history prior to treatment), of myocarditis-pericarditis and of 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 prior to chemotherapy. A reduction of the ejection fraction (EF) below 50 % or of the fractional shortening below 25 % was classified as chronic ACT. The evaluation and classification of cases was performed by two 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 of Goettingen. Genotyping was performed either by pyrosequencingTM on the PSQTM HS 96A System, or on 7900 HT Sequence Detection System using pre-developed assays by Applied Biosystems (SNP Assays-on-DemandTM). Genetic variants genotyped by the latter, commercially available assays; see Example 2, *infra*. Primer sequences for the other assays can be obtained upon 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, seven DNAs were genotyped twice for all variants. No genotyping errors were
5 detected using these controls.

Statistical analysis

The deviation of the genotype distributions from Hardy-Weinberg equilibrium (HWE) was tested with Pearson's goodness-of-fit Chi-square test. The lack of deviation of the genotype
10 distribution among controls from HWE was necessary for the subsequent association testing. The latter was performed using the procedure by Freidlin (Freidlin et al., Hum. Hered. 53 (2002), 146-152), which is a modified Cochran-Armitage trend test. Pearson's (Von Hoff et al., Ann. Intern. Med. 91 (1979), 710-717) test was used to validate the results of Freidlin's test especially for genotypes where no or few homozygotes of the variant allele were detected.
15 Wherever necessary due to small cell counts, Fisher's exact test was used to validate results. Results were also validated using multiple logistic regression for each genetic variant individually, adjusting for age, gender, total dose received, and for 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
20 appropriate for screening purposes. Therefore, no adjustment for multiple testing was made.

Example 2: Polymorphisms in the genes involved in the generation of reactive oxygen species (ROS) and drug transmembrane transport are indicative for cardiac disorders

25 Of the 1697 patients enrolled, 147 were reported to the study center because of cardiac problems during or after chemotherapy treatment. Of those, based on a detailed source data review, 38 were excluded from further analysis since there was evidence of pre-existing cardiac disease or the cardiac dysfunction could not be substantiated. Of the remaining 109
30 patients, 55 developed an acute and 54 a chronic ACT (cumulative incidence of either form 3.2 %). No DNA samples were available for 22 of them, leaving 87 patients (44 with an acute and 43 with a chronic ACT) who were subjected to genetic analysis presented in the following. A detailed review of the 44 acute ACT patient files revealed 12 cases of atrial fibrillation, 2 cases of myocarditis-pericarditis, in 1 case myocardial infarction and 5 patients
35 showed clinical signs of acute heart failure. The 43 chronic cases showed a reduction of the ejection fraction (EF) below 50 % or fractional shortening values below 25 %. The median

time between the therapy onset and first report of cardiotoxicity was 6 months, with the interquartile range of 4 to 15 months.

The ACT cases were then 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.

				N=87		N=363	
27/16 63%		23/21 52%		50/37 57%		212/151 58%	
62.7±7.9		61.2±13.2		62.0±10.9		61.3±11.0	
74.2±13.8		74.1±15.5		74.2±14.6		74.9±13.7	
515 [186.5]		500 [121.3]		504 [160.5]		540 [90.0]	
CHOP-14	10	CHOP-14	12	CHOP-14	22	CHOP-14	103
CHOEP-14	8	CHOEP-14	13	CHOEP-14	21	CHOEP-14	93
CHOP-21	11	CHOP-21	8	CHOP-21	19	CHOP-21	76
CHOEP-21	14	CHOEP-21	11	CHOEP-21	25	CHOEP-21	91

Table 1: Gender, age, weight, received doxorubicin dose and distribution among the therapy arms of cases and controls. Age given as mean +/- SD. Dose given as median. Interquartile differences are given in brackets.

In each group, patients were distributed approximately equal among the four arms of the therapy. The cumulative total dose of doxorubicin was by about 7 % lower in cases compared to controls, due to the necessity to abandon treatment in some patients due to arrhythmias. Six out of 87 cases (7 %) and 15 out of 363 controls (4 %) received irradiation of the mediastinal region ($p=0.26$, Fisher's exact test). A total of 210 polymorphisms in 82 genes with conceivable role in ACT were analyzed in controls. The genes play a role in the metabolism of reactive oxygen species, in drug transport and metabolism, DNA repair, endothelial physiology, the renin angiotensin aldosterone-system, muscle contraction and structure, inflammation and cell cycle. Furthermore, variants of adrenergic receptors were analyzed. All

but 17 variants were confirmed as biallelic markers. The Ser893Ala variant of MDR1 and rs746578 TTN/Titin proved to be triallelic, whereas 15 other variants were monomorphic in the DNA samples screened. The genotypes of all but 14 biallelic markers were in Hardy-Weinberg equilibrium among controls.

5 **Table 2:** Primer and target sequences of the MRP1, MRP2, , CYBA, RAC2, and NCF4 genes.

Gene	Entrez Gene GeneID	mRNA sequence	Target Sequence context	rs number
MRP1	4363	<u>NM_004996</u>	TCTCCATCCCYGAAG [G/T] TGCTTTGGTGGCCGT	-
MRP2	1244	<u>NM_000392.1</u>	TGAAACACAATGAGG [T/A] GAGGATTGACACCAA	8187694
MRP2	1244	<u>NM_000392.1</u>	GGAAGATTATAGAGT [G/A] CGGCAGCCCTGAAGA	8187710
CYBA	1535	<u>NM_000101.1</u>	CCCAGGGGACAGAAG [C/T] ACATGACCGCCGTGG	4673
RAC2	5880	<u>Z82188.2</u>	ACGGAGGAAGGATGG [A/T] GCATTCAAGGAACCC	13058338
NCF4	4689	<u>AL008637.1</u>	AAGACACCCTGATG [A/G] CTGGGACCCCATCTC	1883112

Gene	Effect	Sequence Forward Primer	Sequence Reverse Primer
MRP1	Gly671Val	BIOTIN-CTGAGCCAGGTGTGTTGTG	TTGTCCATCTCAGCCAAGAG
MRP2	Val1188Glu	GCAGCGATTTCTGAAACACA	BIOTIN-CCTCCCACCGCTAATATCAA
MRP2	Cys1515Tyr	TGGTCCTAGACAACGGGAAG	BIOTIN-CTAACCCATGGGGCCTTCT
CYBA	His72Tyr	GTTTTGTGGGAGGAAAGAGG	BIOTIN -CGGCCCCGAACATAGTAATTC
RAC2	intron 2	Assay ID: C_11744093_1	
NCF4	promoter region	Assay ID: C_11521119_1	

Gene	Sequence Sequencing Primer	PCR Fragment length (bp)	Method	Assay
MRP1	CGGCCACCAAAGCA	132	Pyro.	reverse
MRP2	TTCTGAAACACAATGAGG	155	Pyro.	forward
MRP2	CAACGGGAAGATTATAGAG	147	Pyro.	forward
CYBA	CCCCAGGGGACAG	184	Pyro.	forward
RAC2		Celera RefSNP ID hCV11744093	TaqMan	
NCF4		Celera RefSNP ID hCV11521119	TaqMan	

As shown in Table 3, six variants showed statistically significant ($p < 0.05$) associations with ACT in either association test.

5 **Table 3:** Distribution of genotypes and p values for polymorphisms showing associations with ACT.

				$p=0.008$	$p=0.048$
Chron	13	27	3	$p=0.062$	$p=0.062$
				$p=0.008$	$p=0.008$
Control	164	154	45		
				$p=0.008$	$p=0.008$
Chron	39	4	0	$p=0.586$	$p=0.586$
				$p=0.012$	$p=0.015$
Control	331	24	1		
				$p=0.008$	$p=0.008$
Chron	37	6	0	$p=0.274$	$p=0.274$
				$p=0.040$	$p=0.044$
Control	331	32	0		
				$p=0.008$	$p=0.008$
Chron	37	6	0	$p=0.274$	$p=0.274$
				$p=0.044$	$p=0.044$
Control	331	32	0		
				$p=0.008$	$p=0.008$
Chron	24	15	4	$p=0.280$	$p=0.741$
				$p=0.013$	$p=0.028$
Control	211	132	19		
					25
Acute	10	22	9	$p=0.372$	$p=0.484$
				$p=0.008$	$p=0.008$
Chron	24	15	4	$p=0.022$	$p=0.022$
Control	106	186	62		

Chronic ACT showed an association with the -212A>G variant of the NAD(P)H oxidase
 30 subunit NCF4, which is responsible for down-regulation of the enzyme. The predisposing
 NCF4 genotype is AA and it conferred an OR of 2.5 (95 % CI: 1.3 - 5.0) (Fig. 1).

Acute ACT was associated with two SNPs in two further subunits of the same enzyme: p22phox and RAC2. The predisposing allele T at position 242 (242C>T) in the gene CYBA, coding for p22phox, leads to the missense mutation His72Tyr. The distribution of the genotypes in cases 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 odds ratio of 2.0 (95 % CI: 1.0 - 3.9, $p = 0.048$). The almost identical increase in the odds ratio for chronic cases of 1.9 (95 % CI: 1.0 - 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) of the regulatory subunit of the enzyme RAC2, which conferred an OR of 2.6 (95 % CI: 1.3 – 5.1) for carrier of the A allele.

In addition, acute ACT was associated with three polymorphisms in the transmembrane efflux transporters of anthracyclines, MRP1 and MRP2 (Table 3). The predisposing allele A of the MRP1 variant Gly671Val conferred an odds ratio of 3.6 (95 % CI: 1.6 – 8.4). The two missense mutations in MRP2, Val1188Glu and Cys1515Tyr, yielded identical frequencies and relationships with acute ACT, as characterized by an odds ratio of 2.3 (95 % CI: 1.0 – 5.4). An inspection of the individual genotypes revealed a 100 % linkage disequilibrium between these variants in the samples genotyped.

For all six variants, the effects of the genotypes on the risk of cardiac disease were statistically significant when all cases were taken together (Table 3). A consistent, albeit not statistically significant trend towards increased risk was observed for all other combinations of these six variants and phenotypes (Fig. 1). The exclusion of mediastinum-irradiated patients had no major effect on the associations, with all p values ≤ 0.051 .

Results were validated by using multiple logistic regression analysis for each genetic variant individually, adjusting for age, gender, cumulative dose administered until development of ATC, and for dosing interval (14 versus 21 days). This analysis confirmed the results of the monovariate analysis with odds ratios of 1.9 ($p = 0.010$) for the p22phox 242T allele, of 2.0 ($p = 0.016$) for NCF4, of 1.7 ($p = 0.025$) for the RAC2 "A" allele. With respect to the drug transporters, an odds ratio of 2.5 ($p = 0.016$) for the MRP1 allele and an odds ratio of 1.9 ($p = 0.071$) for the two linked MRP2 amino acid substitutions were confirmed by logistic

regression analysis. A two-locus analysis for the various pairs of the associating CYBA, NCF4, RAC2, MRP1, and MRP2 polymorphisms revealed no interactions between the genes involved.

5 Example 3: Implications for the findings in Example 2

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 % (Shan et al., Ann. Intern. Med. 125 (1996), 47-58). This was primarily caused 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 % (Zucchi and Danesi, Curr. Med. Chem. Anti-Canc. Agents 3 (2003), 151-171). 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, all cardiac events occurring during the initial three months as acute ACT were counted. There is no established time cut -off between acute and chronic ACT. Classification based on symptoms rather than 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 (Shan et al., Ann. Intern. Med. 125 (1996), 47-58). In animal models, congestive heart failure develops within several weeks, i.e. prior to completion of many typical doxorubicin treatments (Forrest et al., Cancer Res. 60 (2000), 5158-5164). The arbitrary cut -off between acute and chronic ACT established in accordance with the present invention is believed to be a fair compromise between these divergent definitions and observations.

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In addition, it cannot be excluded that in some patients arrhythmia might have been caused by other factors such as fluid overload and may therefore have not been related to doxorubicin. Finally, the increased frequency of acute (although not chronic) ACT may also have been increased by the co-medication with cyclophosphamide, which itself may be cardiotoxic (Gharib and Burnett, Eur. J. Heart Fail. 4 (2002), 235-242). Mediastinal irradiation, previously reported to have a borderline effect on cardiotoxicity (Torti et al., Ann. Intern. Med. 99 (1983), 745-749), was not significantly different between cases and controls and exclusion of irradiated patients had no effect on associations found. In some patients, cardiotoxicity may have been enhanced by co-medications, which were, however, not among

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the mandatory data collected. 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 (Small et al., N. Engl. J. Med. 347 (2002), 1135-1142; Borjesson et al., Eur. Heart J. 21 (2000), 1853-1858; Kaye et al., Pharmacogenetics 13 (2003), 379-382; Andersson and Sylven, J. Am. Coll. Cardiol. 28 (1996), 162-167). In the present experiments no associations between any of these variants and ACT have been detected. Likewise, no interaction between the ADRA2C GlyAlaGlyPro322-325del and the ADRB1Gly389Arg variant (Small et al., N. Engl. J. Med. 347 (2002), 1135-1142) has been found, altogether suggesting a different underlying pathophysiology. On the other side, ACT was associated with variants of proteins implicated in two distinct processes, the generation of reactive oxygen species and drug, i.e. anthracycline transmembrane transport. The NAD(P)H oxidase multi-enzyme complex catalyzes the 1-electron reduction of oxygen using either NADH or NAD(P)H 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 reactive oxygen species (Heymes et al., J. Am. Coll. Cardiol. 41 (2003), 2164-2171). The C242T (His72Tyr) polymorphism of the CYBA gene coding for p22phox affects a heme-binding site thought to be essential for the stability of the protein. Under *ex vivo* conditions, the tyrosine variant was originally reported to confer a 20-40 % reduction in basal activity in vessel samples (Guzik et al., Circulation 102 (2000), 1744-2747). This finding has been in part confirmed by Wyche et al. (Hypertension 43 (2004), 1246-1251), 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 to what would be expected. One may speculate that under *in vivo* conditions, inherited reduced activity of NAD(P)H oxidase may result in impaired ROS defence capacity and therefore in increased ROS levels under anthracycline exposure. On the other hand, Shimo-Nakanishi et al. (Shimo-Nakanishi et al., Atherosclerosis 175 (2004), 109-115) have recently reported an 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, where an increased (Cai et al., Eur. J. Clin. Invest. 29 (1999), 744-748; Cahilly et al., Circ. Res. 86 (2000), 391-395), decreased (Inoue et al., Circulation 97 (1998), 135-137), as well as no change (Li et al., Am. J. Med. Genet. 86 (1999), 57-61;

Gardemann et al., *Atherosclerosis* 145 (1999), 315-323) in risk have been described. Altogether, the final verdict on the functional consequences of the C242T allele is still out. The NCF4 gene encodes the p40phox subunit of the NAD(P)H oxidase, which is responsible for the downregulation of the enzyme (Lopes et al., *Biochemistry* 43 (2004), 3723-3730). The
5 NCF4 variant predisposing to ACT is located in the putative promoter of the gene, but its functional consequences are at present unknown.

The NAD(P)H oxidase requires for its activity also binding of the small GTPase RAC2, which may in addition induce the enzyme complex assembly (Price et al., *J. Biol. Chem.* 277
10 (2002), 19220-19228), possibly by activation of cytosolic protein kinases (Shalom-Barak and Knaus, *J. Biol. Chem.* 277 (2002), 40659-40665). A single nucleotide polymorphism located in intron 2 of RAC2 may serve as a marker for a functional, at present unknown variant. Alternatively, it could itself be functional and affect e.g. splicing or transcription of RAC2. Altogether, the association with variants encoding three proteins of the of NAD(P)H oxidase
15 complex is a strong evidence for the role of this enzyme in anthracycline-induced heart failure.

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
20 cassette (ABC) transporters and act as cellular efflux pumps for numerous endo- and exogenous substrates. The human MRP1 confers resistance to anthracyclines, and was in fact originally cloned from a doxorubicin-selected cancer cell line (Cole et al., *Science* 258 (1992), 1650-1654). MRP1 is expressed in human (Flens et al., *Am. J. Pathol.* 148 (1996), 1237-1247) and murine (Wijnholds et al., *Nat. Med.* 3 (1997), 1275-1279) myocardium. A
25 targeted deletion of MRP1 in the mouse increases the accumulation of etoposide in the heart (Wijnholds et al., *J. Clin. Invest.* 105 (2000), 279-285), 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 (Flens et al., *Am. J. Pathol.* 148 (1996), 1237-1247). It has been postulated that such an expression may permit sequestration
30 of doxorubicin in lysosomes, i.e. away from its target the nucleus (Rajagopal and Simon, *Mol. Biol. Cell* 14 (2003), 3389-3399). A low-frequency protein variant of MRP1 (Arg433Ser) has been shown to affect resistance to anthracyclines *in vitro*, but has not been determined in the present study (Conrad et al., *Pharmacogenetics* 12 (2002), 321-330). 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 (Conrad et al., *J. Hum. Genet.* 46 (2001), 656-663).

MRP2 has a substrate spectrum similar to that of MRP1 and increases the resistance to doxorubicin when overexpressed in HEK-293 cells (Cui et al., *Mol. Pharmacol.* 55 (1999), 929-937). 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 (Hidemura et al., *Antimicrob. Agents Chemother.* 47 (2003), 1636-1642). The lower incidence of heart damage following doxorubicin infusion as opposed to bolus injection suggests a role for the drug's peak levels in doxorubicin-induced cardiotoxicity (Hortobagyi et al., *Cancer* 63 (1989), 37-45). Significant inter-individual differences in doxorubicin pharmacokinetics have been reported previously (Jacquet et al., *Cancer Chemother. Pharmacol.* 27 (1990), 219-225; Piscitelli et al., *Clin. Pharmacol. Ther.* 53 (1993), 555-561) and they could result from genetic variants in transporters such as MRP2. The two missense variants associating with ACT, Val1188Glu and Cys1515Tyr, were initially described in the Japanese (Itoda et al., *Drug Metab. Dispos.* 30 (2002), 363-364). No data regarding their functional significance have been published and the specific variant relevant to doxorubicin treatment cannot be inferred from our results, due to the 100 % linkage disequilibrium between the two missense mutations in our cohort.

Additional support for the associations found can be derived from the functional context of the findings of the present invention. In the case of ACT, this 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 an additional evidence for the true character of this association. Similarly supporting is our finding of the association of variants of CYBA, NCF4 and RAC2, which are members of the same signalling complex.

The relationship between the acute and chronic ACT is unclear. Acute ACT is usually attributed to damage caused by reactive oxygen species arising from one-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 two-electron reduction by enzymes such as aldo-keto reductases and carbonyl reductases (Mordente et al., *Biochem. Pharmacol.* 66 (2003), 989-998). However, there is increasing evidence for a "unifying hypothesis" of ACT (Mordente et al., *IUBMB Life* 52 (2001), 83-88). Reactive oxygen species have been found to induce the expression of these enzymes (Spycher et al, *Faseb J.* 11 (1997), 181-188) and may thus facilitate the formation of the toxic alcohol metabolites and chronic ACT. The present data appear to support a common pathophysiology of acute and chronic ACT. Indeed, variants of the NAD(P)H oxidase subunits and the two 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 4-10 years (Steinherz et al, *Jama* 266 (1991), 1672-1677) and in 65 % of patients after 15 years (Lipshultz et al., *N. Engl. Med.* 324 (1991), 808-815). Therefore, it is likely that the controls used contain a significant number of patients which will develop cardiotoxicity in the future and their genetic makeup diminishes the strength of the associations. On the other hand, all six variants show statistically significant associations when acute and chronic cases are taken together.

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. 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.

Claims

1. A method of determining the genetic predisposition of a subject to be at risk for a cardiac disease or dysfunction comprising assaying a sample of a subject for the presence of a variant allele of at least one gene selected from the group consisting of genes involved in (a) the generation of reactive oxygen species (ROS) or (b) drug transmembrane transport; wherein the presence of the variant allele is considered indicative of a higher risk for cardiotoxicity compared to a control.
2. The method of claim 1, wherein said variant allele is in the coding region of the gene.
3. The method of claim 1 or 2, wherein said variant allele is in the promoter region or in an intron of the gene.
4. The method of any one of claims 1 to 3, wherein said variant allele results from a nucleotide deletion, addition and/or substitution compared to the corresponding wild type gene.
5. The method of any one of claims 1 to 4, wherein said variant allele results in lower amount of protein activity compared to a control
6. The method of any one of claims 1 to 5, wherein said gene involved in the generation of ROS encodes a protein of the NAD(P)H oxidase multi-enzyme complex.
7. The method of claim 6, wherein said gene is selected from the group consisting of NCF4, CYBA and RAC2.
8. The method of claim 7, wherein said variant allele is a polymorphism corresponding to the C242T (His72Tyr) polymorphism of the CYBA gene, a polymorphism corresponding to the 212A>G polymorphism in the NCF4 gene or a polymorphism corresponding to the 7508T>A polymorphism in the RAC2 gene.
9. The method of any one of claims 1 to 8, wherein said gene involved in drug transmembrane transport encodes a multi-drug resistance protein.

10. The method of claim 9, wherein said protein is multidrug resistance-associated protein 1 (MRP1) or multidrug resistance-associated protein 2 (MRP2).
11. The method of claim 10, wherein said variant allele is a polymorphism corresponding to the Gly671Val polymorphism in the MRP1 gene or a polymorphism corresponding to the Val1188Glu or Cys1515Tyr polymorphism in the MRP2 gene.
12. The method of any one of claims 1 to 11, wherein said cardiac disease or dysfunction is induced by a drug.
13. The method of claim 12, wherein said drug is an antineoplastic agent.
14. The method of claim 12 or 13, wherein said drug is an anthracycline.
15. The method of claim 14, wherein said drug is doxorubicin.
16. The method of any one of claims 12 to 15, wherein said drug is administered by infusion or bolus injection.
17. The method of any one of claims 1 to 16, wherein said cardiac disease or dysfunction comprises arrhythmia or congestive heart failure.
18. The method of any one of claims 1 to 17, wherein said cardiac disease or dysfunction comprises anthracycline-induced cardiotoxicity (ACT).
19. The method of claim 18, wherein said ACT is acute ACT or chronic ACT.
20. The method of any one of claims 1 to 19, wherein said sample is a plasma sample, a blood sample, a saliva sample, a tumor sample, a tissue sample or bodily fluid sample.
21. The method of any one of claims 1 to 20, wherein said variant allele is determined by genotyping and/or genotyping.

22. The method of any one of claims 1 to 21 comprising PCR, ligase chain reaction, restriction digestion, direct sequencing, nucleic acid amplification techniques, hybridization techniques; immunodiagnostic methods or biological or enzymatic activity assays.
- 5
23. An oligonucleotide for use in determining the presence of variant allele in accordance with the method of any one of claims 1 to 22.
24. The oligonucleotide of claim 23, wherein said oligonucleotide comprises the
10 nucleotide sequence of any one of the primer or target sequences depicted in Table 2.
25. A primer or probe consisting of an oligonucleotide as defined in claim 24.
26. A kit for use in a method of any one of claims 1 to 22 comprising polynucleotides, a
15 chip or array, reference samples, amplification and/or sequencing means, buffer, detergents, biochemical reagents, detection means, or the like, and optionally a reformulated drug with a drug dose which is tailored to a patient carrying a variant allele as defined in any one of claims 1 to 22.
- 20 27. A method for the treatment of a disease comprising administering to a subject in need of such treatment a noncardiotoxic amount of a drug as defined in any one of claims 1 to 22 or a noncardiotoxic analog or substitute thereof, wherein said subject carries at least one variant allele as defined in any one of claims 1 to 22.
- 25 28. A method for the treatment of a disease comprising administering to a subject in need of such treatment a drug as defined in any one of claims 1 to 22, wherein said subject does not carry any one of said variant alleles as defined in claims 1 to 22.
29. The method of claim 27 or 28, wherein a sample of the subject has been assayed in
30 accordance with the method of any one of claims 1 to 22.
30. The method of any one of claims 27 or 29, wherein said drug is an antineoplastic agent as defined in any one of claims 13 to 15 and said disease is cancer.

31. A pharmaceutical composition comprising a drug as defined in any one of claims 1 to 22 and an agonist/activator of a gene or gene product involved in the generation of reactive oxygen species (ROS) or drug transmembrane transport.
- 5 32. The pharmaceutical composition of claim 26, wherein said gene or gene product is involved in the NAD(P)H oxidase multi-enzyme complex or transmembrane transport of MRP1 or MRP2.
- 10 33. The method of any one of claims 27 to 30 or the pharmaceutical composition of claim 31 or 32, wherein said drug is an antineoplastic agent.
34. The method or pharmaceutical composition of claim 33, wherein said drug is an anthracycline.
- 15 35. The method or pharmaceutical composition of claim 34, wherein said anthracycline is doxorubicin.

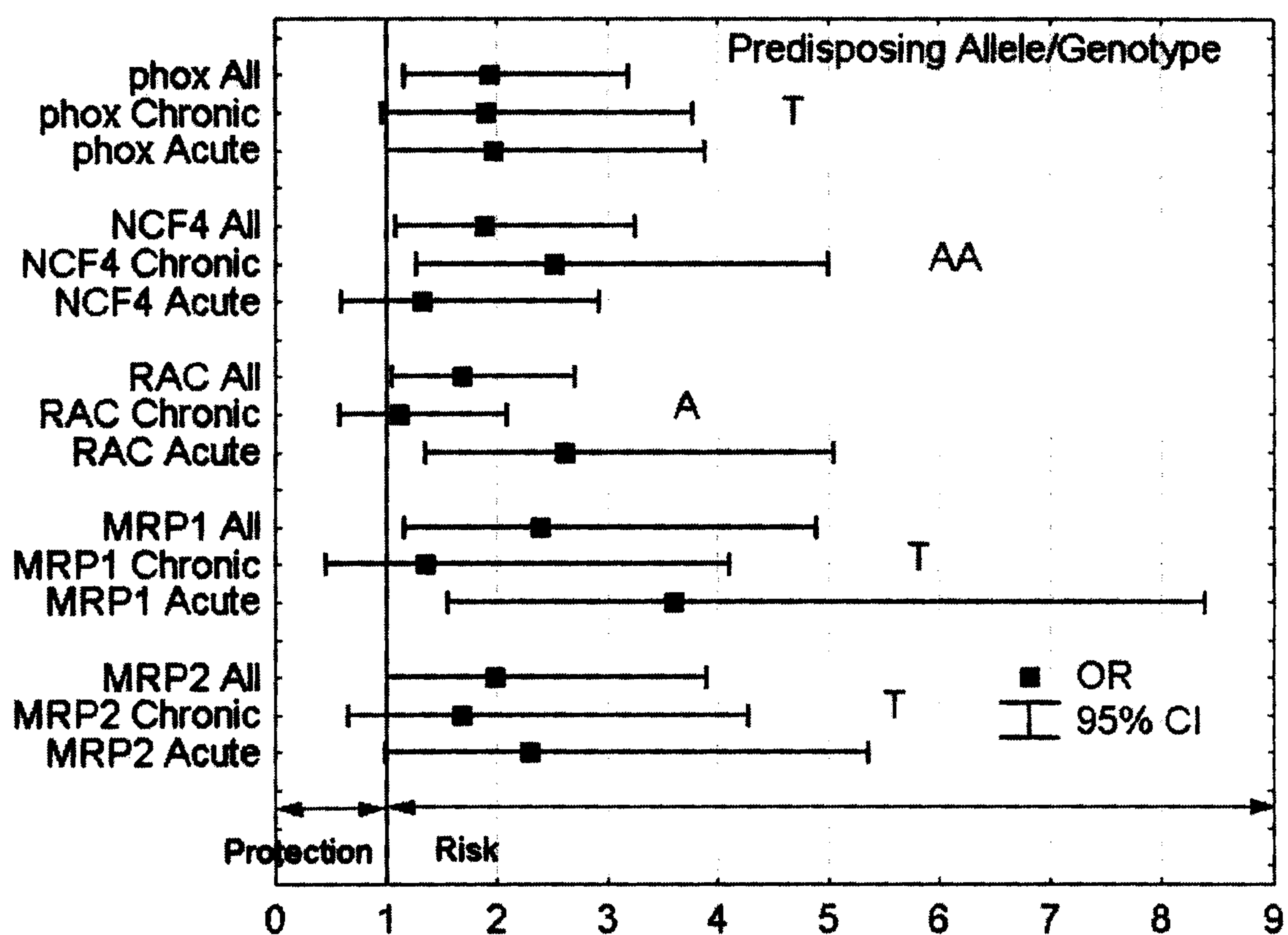


Fig. 1

