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Anthracycline Toxicity: Light at the End of the Tunnel?

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Abstract

Anthracycline-induced cardiotoxicity (AIC) is a serious and common side effect of anthracycline therapy. Identification of genes and genetic variants associated with AIC risk has clinical potential as a cardiotoxicity predictive tool and to allow the development of personalized therapies. In this review, we provide an overview of the function of known AIC genes identified by association studies and categorize them based on their mechanistic implication in AIC. We also discuss the importance of functional validation of AIC-associated variants in human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs) to advance the implementation of genetic predictive biomarkers. Finally, we review how patient-specific hiPSC-CMs can be used to identify novel patient-relevant functional targets and for the discovery of cardioprotectant drugs to prevent AIC. Implementation of functional validation and use of hiPSC-CMs for drug discovery will identify the next generation of highly effective and personalized cardioprotectants and accelerate the inclusion of approved AIC biomarkers into clinical practice.

1. INTRODUCTION

Chemotherapy drugs from the anthracycline family are well known to cause cardiotoxicity, yet these drugs are still widely used clinically as they are considered among the most effective anticancer drugs to date (1). Anthracyclines have made a major contribution to the increase in the survival rate in cancer patients over the last 50 years and can be used to treat a variety of malignancies, including leukemias; lymphomas; sarcoma; and bladder, breast, gynecological, and other metastatic cancers in adults, adolescents, and children (1). To illustrate this, the five-year survival of childhood cancer patients currently reaches approximately 85%, and anthracyclines (doxorubicin, daunorubicin, epirubicin, and idarubicin) are utilized in nearly 60% of pediatric cancer regimens (2, 3). Development of cardiotoxicity, defined as a left ventricular ejection fraction reduction of greater than 10% to less than 53%, is the most serious and possibly fatal side effect of anthracyclines and occurs in 9% of patients (4). Anthracycline-induced cardiotoxicity (AIC) can manifest as a range of cardiac symptoms, including congestive heart failure, arrhythmias, myocardial infarction, and other forms of cardiovascular disease. The incidence of AIC could be as high as 59% if more subtle changes in cardiac function are detected, even at low doses (5). The most severe type of AIC is defined by the clinical diagnosis of heart failure, with a five-year survival rate of less than 50% and a mortality rate of approximately 100% at ten years after diagnosis (6–8) without a heart transplant.

The association between cumulative dose and AIC is well established, yet there is interpatient variability in AIC risk at any dose, and clinical variables alone yield moderate predictive power in detecting cardiotoxicity (9, 10). Identifying genetic variants associated with variable AIC risk will help to identify early those patients that will be affected by the devastating side effects of anthracyclines. In this review, we provide an overview of the function of known genes associated with AIC with a focus on cardiomyocytes and discuss the importance of genetic variant validation. We address how the utilization of human induced pluripotent stem cell–derived cardiomyocytes (hiPSC-CMs) can advance the clinical implementation of these genetic predictive biomarkers. We also review how patient-specific hiPSC-CMs can facilitate the discovery of novel cardioprotectant drugs to effectively prevent the unwanted cardiotoxic effects of anthracycline therapy.

2. CURRENT STRATEGIES FOR CARDIOPROTECTION

Clinical management of AIC mainly involves four major strategies: (*a*) limitation in the cumulative anthracycline dose for all patients, (*b*) use of cardioprotectants, (*c*) use of liposome-encapsulated versions of anthracyclines, and (*d*) treatment of cardiac symptoms in patients who develop AIC.

The most direct method of reducing AIC is anthracycline dose limitation, but this potentially reduces chemotherapy efficacy. Therefore, the maximum cumulative anthracycline dose is specifically chosen as a compromise between efficacy and cardiotoxicity. The typical dose of doxorubicin is 400–550 mg/m^2 for sarcoma patients or 240 mg/m^2 for breast cancer patients (1), and there are no plans for reduction below these limits in clinical practice.

A second method to limit AIC involves concomitant treatment with the cardioprotectant drug dexrazoxane. Dexrazoxane is thought to chelate iron, reducing the amount of superoxide radicals produced, and/or inhibit topoisomerase II beta (TOP2B), therefore reducing anthracycline-mediated DNA breaks and preventing cardiomyocyte death and mitochondrial dysfunction (11). Dexrazoxane is not routinely used in practice, and prescription to children is currently off-label in the United States. In the European Union, the previous contraindication for the use of dexrazoxane in children and adolescents was removed and its use is restricted to patients receiving $\geq$300 mg/m^2 of doxorubicin or equivalent (12). The use of dexrazoxane has been associated with risk of secondary malignancies and concerns of poor efficacy (13, 14). Even though dexrazoxane

has been proven to be effective at decreasing AIC in clinical studies, its cardioprotective activity is not complete. A systematic review and meta-analysis reported that "dexrazoxane reduced the risk of clinical heart failure (Risk ratio (RR): 0.19; 95% CI: 0.09 to 0.40; $P < 0.001$) and cardiac events (RR: 0.36; 95% CI: 0.27 to 0.49; $P < 0.001$) irrespective of previous exposure to anthracyclines" (15, p. 68). Hence the identification of more effective and specific cardioprotectants is highly desirable. The use of an encapsulated formulation of anthracyclines, in particular liposomal doxorubicin, is another method to decrease drug toxicity. Doxorubicin nanoformulations have decreased toxicity in comparison to the free drug (16). However, the number of clinical studies is small, and data on long-term follow-up are lacking. The use of angiotensin-converting enzyme inhibitors, angiotensin (II) receptor blockers, or beta-blockers is a method implemented for patients who develop AIC to prevent severe contractile dysfunction and improve cardiac function (17). However, the use of neurohormone-blocking drugs as prophylactic therapy for AIC is still controversial as the magnitude of cardiac improvement in general is small (<5%) and does not clearly improve clinical outcomes (reviewed in 18, 19). Further research is needed to strengthen recommendations for AIC treatment and to investigate new means of cardioprotection as well as to identify strategies for early detection and prevention of AIC.

3. CONTRIBUTION OF ASSOCIATION STUDIES TO THE DISCOVERY OF GENETIC VARIANTS ASSOCIATED WITH AIC

Identification of genetic variants and genes associated with AIC risk has clinical potential as a diagnostic tool and for the development of personalized therapies. To date, seven genome-wide association studies (GWASs) (20–26) and 21 candidate gene association studies (27–33) have identified more than 250 variants associated with variable risk of AIC. Many of these variants are also associated with increased risk for cardiomyopathies (e.g., variants in *MYH7*, *TTN*, and *TNNT2*) (33–35), showing that individuals predisposed to myocardial dysfunction are also at increased risk for AIC (33). Fifty-five single-nucleotide polymorphisms (SNPs) with significant statistical association with AIC ($p < 0.05$) fall within genes expressed in human cardiomyocytes (**Table 1**; **Supplemental Table 1**), suggesting a potential direct role of variability in those genes in AIC-cardiomyocyte biology. With the exception of SNPs in *ROBO2* and *POLRMT* that have been identified recently (25, 26), most of these genomic loci associated with AIC have been thoroughly reviewed (32).

Despite that the number of GWASs is growing, and many genetic variants associated with AIC risk have been identified (reviewed by 32, 36), only a few of those variants have been replicated in a fully independent cohort (37, 38). Some of the factors that contribute to the nonconcordance in variants discovered include discrepancies in study design, definition of primary end points and methods for their quantification, SNP selection approaches, limited cohort size, and cohort heterogeneity. These factors, combined with a lack of functional validation for most of the variants, hamper their implementation as biomarkers for the prediction of AIC risk in clinical practice.

4. POLYGENIC RISK SCORE FOR AIC RISK PREDICTION

Identification of genetic variants associated with AIC offers an opportunity to develop improved risk prediction models. Compared to models that only include clinical factors, incorporation of genetic risk factors can improve prediction power for the detection of AIC risk (39). Individually, genetic variants identified by association studies contribute a small proportion to AIC risk. However, the combined effect of multiple risk-increasing alleles can substantially contribute to the prediction of AIC. In this regard, AIC risk can be evaluated in the form of a polygenic risk score (PRS), a single value estimate constructed as a weighted sum of the number of risk-increasing

Table 1 Genes with SNPs significantly associated with AIC and expressed in human cardiomyocytes

Gene	Protein encoded	Functional classification
ABCB4	ATP binding cassette subfamily B member 4	Anthracycline efflux
ABCC1	ATP binding cassette subfamily C member 1	Anthracycline efflux
ABCC10	ATP binding cassette subfamily C member 10	Anthracycline efflux
ABCC2	ATP binding cassette subfamily C member 2	Anthracycline efflux
ABCC5	ATP binding cassette subfamily C member 5	Anthracycline efflux
ABCC9	ATP binding cassette subfamily C member 9	Anthracycline efflux
ATP2B1	Plasma membrane calcium-transporting ATPase 1	Ca^{+2} handling and contractility
CAT	Catalase	ROS handling
CBR3	Carbonyl reductase 3	ROS handling, Ca^{+2} handling
CELF4	CUGBP Elav-like family member 4	Ca^{+2} handling and contractility
COL1A2	Collagen type I alpha 2 chain	Contractile function
CYBA	Cytochrome b-245 alpha chain	ROS generation
CYP2J2	Cytochrome P450 2J2	Contractile function
ERCC2	TFIIH core complex helicase subunit	DNA damage
GPX3	Glutathione peroxidase 3	ROS handling
GSTP1	Glutathione *S*-transferase pi 1	ROS handling
HAS3	Hyaluronan synthase 3	ROS handling
HFE	Homeostatic iron regulator	Iron uptake
HNMT	Histamine *N*-methyltransferase	Ca^{+2} handling and contractility
MLH1	MutL homolog 1	DNA damage
MYH7	Myosin heavy chain 7	Ca^{+2} handling and contractility
NOS3	Nitric oxide synthase 3	ROS generation
*NR3C1**	Glucocorticoid receptor binding site	Ca^{+2} handling and contractility
PLCE1	Phospholipase C epsilon 1	ROS handling
*POLRMT**	DNA-directed RNA polymerase, mitochondrial	DNA binding
POR	Cytochrome P450 oxidoreductase	ROS generation
PRDM2	PR domain zinc finger protein 2	DNA damage
RAC2	Rac family small GTPase 2	ROS generation
RARG	Retinoic acid receptor gamma	DNA damage
RIN3	Ras and Rab interactor 3	Contractile function
ROBO2	Roundabout guidance receptor 2	Contractile function
SLC22A17	Solute carrier family 22 member 17	Anthracycline uptake
SLC28A1	Solute carrier family 28 member 1	Anthracycline uptake
SLC28A3	Solute carrier family 28 member 3	Anthracycline uptake
SP4	Transcription factor Sp4	ROS generation
SPG7	Paraplegin	ROS generation
WDR4	WD repeat-containing protein 4, Wuho	DNA damage
XDH	Xanthine dehydrogenase	ROS generation
ZNF521	Zinc finger protein 521	Contractile function

Abbreviations: AIC, anthracycline-induced cardiotoxicity; ROS, reactive oxygen species; SNP, single-nucleotide polymorphism.

alleles carried by an individual. Studies in long-term survivors of childhood cancer have shown the utility of PRS to predict health outcomes (40). Nevertheless, research evaluating PRSs to predict AIC risk remains limited (41).

AIC-PRSs may be calculated based on genetic variants associated with heart failure and/or AIC or on gene-environment interactions (e.g., gene-anthracycline dose). While multiple AIC-associated genetic variants have been identified to date, only a few have been successfully replicated in independent cohorts and validated in appropriate models, limiting their application for PRS development. Future studies increasing the sample size of well-phenotyped populations of all ancestries are needed to identify and validate variants robustly associated with AIC, allowing for a more accurate calculation of PRSs potentially applicable to patients of all races and ethnicities. Development of PRSs for AIC-related outcomes in the general population (e.g., cardiomyopathy, heart failure, echocardiography parameters) may also contribute to the prediction of AIC. Thus, considering multiple PRSs would be important to improve the AIC prediction.

Using PRSs to assess the risk of AIC will inform primary intervention strategies at the time of cancer treatment, including anthracycline dose reduction and/or the use of alternative treatments (e.g., cardioprotectants). AIC-PRSs will also facilitate risk stratification after completion of anticancer therapy by identifying high-risk survivors most likely to benefit from intensive preventative measures.

5. FUNCTIONAL CLASSIFICATION OF GENES ASSOCIATED WITH AIC

In this review we focus on genes directly impacting cardiomyocytes' function and response to AIC. The genes with variants associated with AIC and expressed in human cardiomyocytes can be categorized into five major groups based on their potential cardiotoxic or cardioprotectant mechanistic implication in AIC (**Table 1**; **Supplemental Table 1**). Even though many of these mechanisms are interrelated, the goal of this categorization is to further facilitate their functional validation. Of note, AIC-associated genes not expressed in cardiomyocytes could be relevant in other cell types (e.g., fibroblasts, immune cells, endothelial cells) and potentially play relevant roles in AIC.

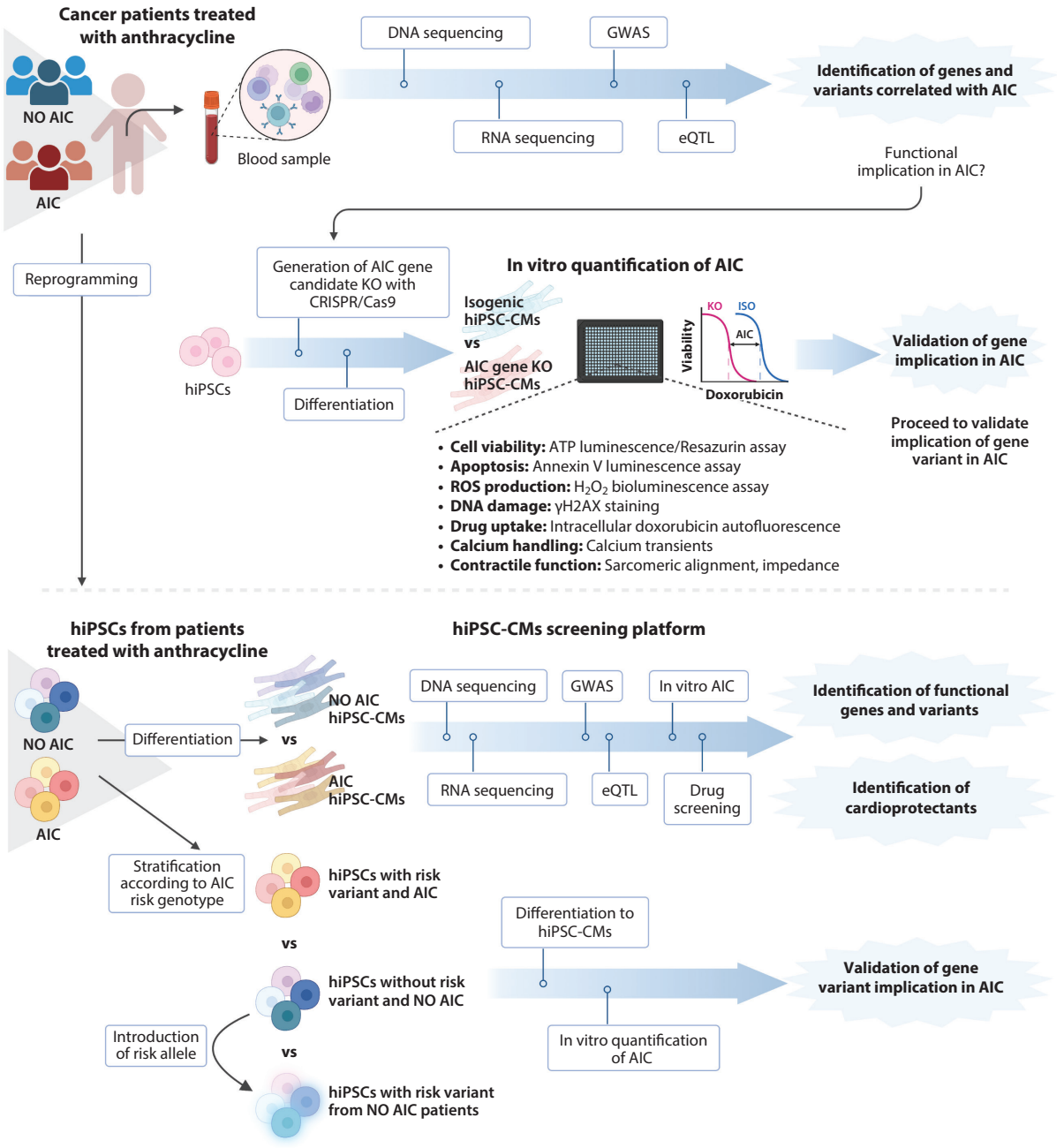
The implications of an AIC gene for hiPSC-CMs can be confirmed by generating target-based CRISPR/Cas9 knockouts followed by quantification of in vitro response to anthracycline (42). This approach can be implemented as the first step to confirm whether alteration in gene candidate expression impacts the response to anthracycline in this cell type (**Figure 1*a***). Once the implication in anthracycline response has been confirmed, the next step would consist of the validation of the role of gene variants in AIC (**Figure 1*b***). Alternative models that are useful for the validation of the role of a gene and or variants in AIC include animal models, nonhuman cell lines (e.g., murine cardiomyoblasts), human tumor-derived cell lines, and hiPSC-derived noncardiac cells.

5.1. Reactive Oxygen Species Production and Handling

Oxidative stress caused by the accumulation of reactive oxygen species (ROS) and reactive nitrogen species is one of the central mechanisms associated with anthracyclines' cardiotoxicity (43). Intracellularly, anthracyclines are reduced by several oxidoreductases and undergo redox cycling, generating ROS. Accumulation of ROS levels in excess of what the antioxidant system can handle initiates oxidative stress, resulting in cardiomyocyte damage and cardiotoxicity (44, 45).

Many of the genes associated with AIC encode enzymes with defensive mechanisms to protect the cells from ROS (**Table 1**). Catalase (CAT) converts toxic hydrogen peroxide into water and

hydrogen, and glutathione peroxidase 3 (GPX3) is a hydrogen peroxide–detoxifying enzyme. Increased expression of *CAT* and *GPX3* is associated with decreased cardiotoxicity to doxorubicin in mice (46–48) and doxorubicin-induced ROS detoxification in cardiac rat fibroblasts (47, 48). The glutathione *S*-transferase pi 1 (GSTP1) inactivates anthracycline metabolites by catalyzing



(*Caption appears on following page*)

Figure 1 (*Figure appears on preceding page*)

hiPSC-CMs for gene functional validation and as a screening platform for the discovery of novel AIC biomarkers and cardioprotectants. (*a*) The functional implication of gene candidates identified in association studies can be validated by combining genetic editing of hiPSCs and a set of assays for in vitro quantification of cardiomyocytes' response to anthracyclines. (*b*) Patient-specific hiPSC-CMs from patients with AIC or no AIC can be used as a platform for gene association and eQTL studies for the identification of functional gene candidates and screening of cardioprotectants. hiPSC-CMs can be stratified based on relevant genotypes and genetically engineered to validate the function of genetic variants in AIC. Abbreviations: AIC, anthracycline-induced cardiotoxicity; NO AIC, cells from patients without AIC; eQTL, expression quantitative trait locus; GWAS, genome-wide association study; hiPSC-CM, human induced pluripotent stem cell–derived cardiomyocyte; KO, knockout; ROS, reactive oxygen species. Figure adapted from images created with BioRender.com.

their conjugation to glutathione, protecting cells from ROS induction (49). Similarly, carbonyl reductases (CBR1 and CBR3) inactivate substrates that induce ROS cellular damage (50, 51).

Other proteins with potential protective roles in the context of oxidative stress are paraplegin, phospholipase C epsilon 1 (PLCε), and hyaluronan synthase 3. Paraplegin (SPG7) is a mitochondrial metalloprotease that degrades oxidation-damaged proteins and regulates the synthesis of respiratory chain subunits, thus regulating mitochondrial ROS (52–54). PLCε (PLCE1) is a phospholipase that protects cardiac tissue and hiPSC-CMs from oxidative stress and mitochondria-mediated cell death (55, 56). *HAS3* encodes a glycosyltransferase that is essential for hyaluronan synthesis, which is a major constituent of the extracellular matrix involved in cardiac development and regeneration. Hyaluronan protects rat cardiomyocytes from ROS-induced ischemia-reperfusion (57).

RAC2, *CYBA*, *NOS3*, *POR*, *XDH*, and *SP4* are among the genes associated with ROS generation. *RAC2* encodes a small GTPase that regulates the enzyme nicotinamide adenine dinucleotide 3-phosphate (NADPH) oxidase, and *CYBA* encodes a subunit of NADPH oxidases (54). NADPH oxidase is one of the main enzymes that produce ROS in the cardiovascular system and has an established role in AIC (58). Endothelial nitric oxide synthase 3 is a flavoprotein encoded by *NOS3* that reduces doxorubicin and synthesizes ROS in the presence of oxygen. *NOS3* overexpression correlates with increased AIC and elevated cardiac ROS in mice exposed to doxorubicin (59). *POR* encodes a flavoprotein that can reduce anthracyclines to semiquinone radical forms, generating free radicals that contribute to cellular damage (60). *XDH* encodes xanthine dehydrogenase, a key enzyme for the purine degradation that generates ROS. Xanthine dehydrogenase can be reversibly converted to xanthine oxidase (XO). Cardiac XO activity is increased in doxorubicin-treated mice, impacting hydrogen peroxide production and the upregulation of apoptotic and ferroptotic pathways (61). The protein encoded by *SP4* is a transcription factor that can bind to the GC promoter region of a variety of genes. Alteration of cardiac *SP4* expression is linked to increased levels of mitochondrial-derived ROS (62).

5.2. Iron Uptake

Anthracyclines have a high affinity for iron and can alter its intracellular metabolism through interactions with iron regulatory proteins. Interactions of anthracycline alcohol metabolites lead to the stabilization of transferrin transcripts, the inhibition of iron-sequestering protein expression, and an increase in free iron levels, which lead to ROS generation (63). Anthracyclines can also form complexes with iron, contributing to increased levels of ROS (64).

The AIC-associated gene *HFE* encodes the homeostatic iron regulator protein that impacts iron absorption by regulating the interaction of the transferrin receptor with transferrin. Hemochromatosis, a disease of iron overload in heart and other tissues, is caused by the presence of multiple polymorphisms in *HFE* (65). The resulting HFE deficiency increases susceptibility to cardiotoxicity in mice treated with doxorubicin (66).

5.3. DNA Binding and Damage

Anthracyclines bind DNA and topoisomerase II and form a ternary complex that triggers cell death. In cancer cells, the binding of anthracyclines to DNA topoisomerase II alpha (TOP2A) is one of the major mechanisms of anthracycline anticancer effects. TOP2A is not expressed in cardiomyocytes, but anthracyclines can also bind to TOP2B, which is expressed in cardiomyocytes, causing both double-stranded break–induced apoptosis and transcriptional modulation of the mitochondrial and nuclear genome that result in cardiomyocyte death and AIC.

RARG, *PRM2*, *MLH1*, *WDR4*, and *ERCC2* are among the AIC-associated genes with roles in DNA damage response. Retinoic acid receptor gamma (RARG) downregulates the expression of TOP2B and its binding to DNA, which leads to DNA double-strand break–induced apoptosis and activation of the cardioprotective extracellular regulated kinase (ERK) pathway in cardiomyocytes exposed to doxorubicin (20). *PRMD2* encodes an *S*-adenosyl-L-methionine-dependent histone methyltransferase and zinc finger transcription factor that is involved in DNA double-strand break and repair of oxidative stress. PRM2 regulates the expression of heme-oxygenase-1, which repairs doxorubicin-induced DNA double-strand breaks, impacting cardiomyocyte apoptosis (67, 68). MutL homolog 1 (MLH1) is part of a group of highly conserved proteins that repair mismatches and mispairs generated during DNA synthesis. Loss of *MLH1* is associated with resistance to anthracyclines (69). Deletion of Wuho (*WDR4*) is linked to DNA damage by a mechanism that could involve the activation of the ATM-Chk2-p53 pathway that results in cell apoptosis (70). *ERCC2* encodes for the helicase subunit of the general transcription and DNA repair factor IIH (TFIIH) core complex, which is involved in the repair of damaged DNA. TFIIH core complex helicase subunit deficiency is associated with increased levels of mitochondrial ROS and mitochondrial DNA (mtDNA) deletions, and variants in this gene lead to increased AIC risk and lower response to chemotherapy (71).

The mitochondrial RNA polymerase (POLRMT) is involved in the transcription of mtDNA and synthesizes the RNA primers required for mtDNA replication (72). A relatively recent GWAS identified that rs62134260, located in an intergenic region 4 kb upstream of *POLRMT*, is linked to AIC in breast cancer patients (26). This region shows potential binding sites for key transcription factors (homeobox protein PKNOX1, pre-B-cell leukemia transcription factor 3, CCAAT/enhancer-binding protein beta), and the variant is correlated with decreased *POLRMT* expression in heart. Given that mitochondrial dysregulation is one of the mechanisms leading to cardiotoxicity, variants impacting the expression of *POLRMT* could impact AIC risk (26), although this has not been demonstrated in human cardiomyocytes yet.

5.4. Anthracycline's Uptake and Efflux

Anthracyclines can be transported inside cells by passive membrane diffusion, and their level of lipophilicity is correlated with AIC in rat cardiomyoblasts (73). Carrier-mediated anthracycline uptake is also involved in the influx of these drugs into cardiomyocytes. Members of the solute carrier (SLC) family of transporters (encoded by *SLC* genes) mediate the influx of ions (Na^+, Ca^{2+}, Fe^{2+}) and other small molecules into the cells. SLC transporters can also mediate the uptake of anthracyclines into human cardiomyocytes. Variants that decrease the expression of *SLC28A3* and the SLC inhibitor drug desipramine decrease doxorubicin uptake and protect against AIC (74).

Conversely, ATP-binding cassette (ABC) transporters (encoded by *ABC* genes) are involved in the efflux of molecules across intra- and extracellular membranes, including chemotherapy agents, resulting in decreased intracellular drug levels (75). Variants in ABC transporters are associated with increased intracellular doxorubicin concentration and AIC (76).

5.5. Calcium Handling and Contractile Function

Calcium (Ca^{2+}) signaling is highly regulated in cardiomyocytes, and alteration of Ca^{2+} homeostasis by anthracyclines is associated with contractile dysfunction and AIC. Anthracyclines can induce cardiotoxicity by altering different Ca^{2+}-related pathways, including dysregulation of the activity of Ca^{2+} regulatory proteins, generation of Ca^{2+} release from the sarcoplasmic reticulum, and alteration of mitochondrial Ca^{2+} flux, leading to mitochondrial dysfunction (reviewed in 77).

Several of the genes with variants significantly associated with AIC have roles in cardiomyocytes' calcium handling and contractile function (**Table 1**; **Supplemental Table 1**). *ATP2B1* encodes a Ca^{2+} transporter that plays a critical role in intracellular calcium homeostasis. *ATP2B1*'s dose-dependent expression is associated with daunorubicin cytotoxicity in hiPSC-CMs (56). *CELF4* encodes a splicing factor that mediates alternative splicing of TNNT2, resulting in the expression of troponin T2 isoforms that have differential Ca^{2+} sensitivity and contractility profiles (78, 79). Expression of fetal splicing variants of TNNT2 alters the beating frequency of hiPSC-CMs exposed to doxorubicin (80). *MYH7* encodes the sarcomeric protein myosin heavy chain-β that is essential for muscle contraction. Variants in *MYH7* are associated with decreased contractility and lower maximal force-generating capacity in cardiomyocytes (81). Titin, encoded by *TTN*, is another sarcomeric protein with variants associated with increased risk of AIC in cancer survivors (33, 82). Titin plays a critical role in heart contractility, and truncation variants in this gene are associated with dilated and hypertrophic cardiomyopathies (83).

CBRs catalyze the reduction of anthracyclines to alcohol metabolites. Accumulation of these anthracycline-alcohol metabolites affects the Ca^{2+} loading of the sarcoplasmic reticulum and myocardial energy metabolism, impairing cardiomyocytes' contractile function (84). The role of histamine *N*-methyltransferase (encoded by *HNMT*) in the heart is not clear, but antihistamines with Ca^{2+} influx blocker properties are associated with decreased doxorubicin cardiotoxicity in vivo (85).

Other genes that impact contractile function in a noncalcium-dependent manner are *CYP2J2* and *RIN3*. *CYP2J2* encodes a cytochrome P450 monooxygenase that converts arachidonic acid (AA) into epoxyeicosatrienoic acids (EETs), which have cardioprotective roles and prevent arrhythmia. *CYP2J2* knockout results in AA accumulation, inhibition of potassium currents, and prolongation of the QT interval (86), while overexpression of *CYP2J2* or administration of EETs is associated with a reduction of doxorubicin-induced cardiotoxicity (87). *RIN3* encodes a Ras effector protein that interacts with and regulates RAB5A, which facilitates membrane trafficking of the voltage-gated potassium channel KCNQ1 (88, 89) that is required for the repolarization phase of the cardiac action potential.

ROBO2 and *COL1A2* encode proteins that are linked to cardiac fibrosis. Robo2 is a member of the transmembrane Robo receptors that bind to Slit glycoproteins and activate the Slit/Robo pathway. Interaction of Robo receptors with the transforming growth factor β1/suppressor of mothers against decapentaplegic (TGFβ1/Smad) pathway induces collagen production, heart dilatation, and cardiomyocyte hypertrophy (90). One of the most recent GWASs identified a SNP (rs17736312), located in the intronic region of *ROBO2*, associated with an increased risk of AIC in children (25). However, the role of ROBO2 in AIC has not been directly demonstrated in human cardiomyocytes and/or cardiac fibroblasts. Anthracyclines can upregulate genes implicated in collagen production and disposition, including *COL1A2*, resulting in impaired cardiac function and myocardial fibrosis (91).

The role of the transcription factor zinc finger protein 521 (ZNF521) in the heart and in the context of AIC is not clear. A potential mechanism involves inhibition of early B-cell factor transcription factor 1 (EBF1), which regulates the ventricular conduction system in the heart (92).

6. PATIENT-SPECIFIC hiPSC-CMs: A PLATFORM FOR FUNCTIONAL VALIDATION OF GENETIC VARIANTS AND IDENTIFICATION OF CARDIOPROTECTANTS

Association studies mostly utilize blood samples to correlate patient genetic information with clinical outcomes. This approach is relatively simple in terms of patient-specific cell acquisition and has advanced our knowledge about the genetic basis of AIC. However, the connection between these data and cardiotoxic response is limited to statistical correlations. For a successful implementation of genetic variants as clinical biomarkers, the mechanisms related to their role in AIC should be confirmed using model systems and show rescue phenotypes for the altered cardiotoxic effect.

Given the difficulty in isolating human cardiomyocytes and their nonproliferative nature, hiPSC-CMs are currently one of the best available in vitro correlates of the human heart. hiPSC-CMs have the same genetic background as donors and represent a novel technology for modeling cardiovascular diseases. hiPSC-CMs from cancer patients who have developed AIC recapitulate that increased risk in vitro when exposed to doxorubicin, with decreased cell viability; altered contraction, sarcomeric structure, and calcium handling; and increased ROS production, compared to lines from patients who did not experience AIC (42, 93, 94). hiPSC-CMs exposed to doxorubicin also show dose-dependent changes in metabolism and cell membrane permeability (95). hiPSC-CMs can resemble the phenotype of adult heart cells in diverse aspects (96), making them a unique and powerful model to study differential responses to anthracycline.

6.1. Functional Validation of AIC-Associated Gene Variants

hiPSC-CMs have recently been used as a platform to identify causal genetic variants of AIC. This is a relatively novel approach that allows the discrimination of genes and variants with high potentiality to predict clinical AIC risk.

Surprisingly, only a few genetic variants have been experimentally demonstrated to have a direct role in AIC to date. This can explain the difficulty in translating genetic variants identified in association studies for use as clinical biomarkers. The SNPs with a demonstrated causative role in AIC include coding risk variant rs2229774 in *RARG* (42, 97), cardioprotective synonymous variant rs11140490 in *SLC28A3* (linked to the GWAS-identified variant rs7853758) (74), and noncoding GWAS risk variant rs28714259 in the glucocorticoid receptor (GR) binding site (98). In addition, the implication of the SLC transporters SLC22A4 and SLC22A17 in AIC has also been confirmed in hiPSC-CMs. Their role on AIC was confirmed by a strategy that implicates the introduction of the risk allele into control patient hiPSC lines with the reference genotype. Fine mapping of the *SLC28A3* locus revealed that the coinherited SNP rs11140490 is the causal cardioprotective variant rather than the GWAS-identified rs7853758, demonstrating the importance of association study–discovered variant validation.

These studies confirmed the implication of RARG and SLC transporters in AIC by generating isogenic hiPSC lines overexpressing and/or knocking out *RARG* and *SLC* transporters followed by in vitro phenotypic characterization assays. Decreased expression of *RARG*, which downregulates TOP2B binding to DNA and DNA double-strand break induced–apoptosis, resulted in increased AIC. Conversely, knockout of the uptake SLC transporters decreased intracellular doxorubicin levels and protected cells from cardiotoxicity. These studies demonstrated that validation of the functional role of a gene in AIC is also a powerful tool to identify druggable targets for the discovery of cardioprotectant drugs. Magdy et al. (42) identified a RARG receptor agonist (CD1530) that reduces murine AIC by approximately 50%. In line with this finding, Hasbullah et al. (99) identified that the RAR agonist all-trans retinoic acid (ATRA) decreases

doxorubicin cardiotoxicity in vivo. Drug library screening of US Food and Drug Administration (FDA)-approved drugs in hiPSC-CMs identified an SLC competitive inhibitor (desipramine) with cardioprotectant activity (74).

Wu et al. (98) identified that the SNP rs28714259 risk allele locates in a GR binding site and disrupts GR binding, decreasing protective signaling, cell survival, and contractility of hiPSC-CMs pretreated with dexamethasone after doxorubicin treatment. Dexamethasone-doxorubicin combination induced cardiac hypertrophy-like signaling, a response that was attenuated by the risk allele, supporting a cardioprotective effect of dexamethasone in AIC (100).

These studies indicate that both the mechanisms underlying the effect of coding and noncoding variants and the identification of cardioprotectants targeting genes with roles in AIC can be confirmed with the hiPSC-CMs platform. This model holds great power for the identification of functional variants and provides human-tested data for clinical trials (**Figure 1**).

6.2. Strategies for the Identification of Novel Functional Targets

While association studies are helpful to identify potential loci influencing cardiotoxic risk, they do not interrogate the molecular basis of AIC. One technique commonly implemented to establish the functional impact of these genetic variants is the analysis of expression quantitative trait loci (eQTLs). eQTLs mapping allows us to identify the impact on gene expression of coding and noncoding genetic variants that are likely to be involved in gene regulation. eQTL analyses are conventionally performed in blood samples to correlate gene variant information to AIC outcomes in patients. As gene expression and regulation signatures are cell type specific, public databases are powerful tools to integrate human heart gene expression data and correlate it to genetic variants (80). However, this approach does not directly determine how genetic traits impact differential anthracycline response.

The hiPSC-CM platform does offer a model to identify molecular pathways showing differential expression when exposed to anthracyclines that can be correlated with clinical outcomes. For example, Knowles et al. (101) performed an analysis of differential gene expression after exposure to doxorubicin (24 h, concentration ranging between 0.6 and 5 μM) and identified 447 eQTLs impacting gene expression and 42 eQTLs impacting alternative splicing in a set of hiPSC-CMs from 45 healthy related individuals. Even though these donors had not been treated for cancer or developed cardiotoxicity, Knowles et al. found excellent concordance between differential expression after doxorubicin exposure and RNA expression data from hiPSC-CMs from cancer patients treated with doxorubicin, analyzed in a previous study (102). Burridge et al. (102) performed differential gene expression analysis after doxorubicin treatment and identified increased expression of programmed cell death, p53 downstream pathway genes, and transcription factors and downregulation of genes involved in muscle contraction, cholesterol biosynthesis, and anatomical structure in hiPSC-CMs from patients who developed AIC versus those that did not. In these patient-specific hiPSC-CMs, *RELA*, *NFKB1*, *RARA*, and *STAT* were the most significant differentially regulated genes that were previously reported to be implicated in doxorubicin-induced toxicity, demonstrating the potential of this platform to identify targets with roles in AIC.

Since current approaches for AIC risk biomarker discovery mostly use blood samples and this strategy has not resulted in FDA-approved markers or implementation in clinical practice, future combination of larger cohorts of patient-specific hiPSC-CMs and functional genomic studies could better guide the identification of relevant gene variants (**Figure 1*b***). Patient-specific hiPSC-CMs can be implemented as a screening platform not only for the discovery of functional variants but also to identify novel druggable pathways and additional mechanisms that could contribute to AIC risk such as, for example, epigenetics (103, 104).

7. TOWARD THE IMPLEMENTATION OF hiPSC-CM-BASED MODELS FOR DRUG DISCOVERY

Development and discovery of novel cardioprotectant drugs require understanding the mechanisms causing AIC for target identification, followed by drug testing in suitable models and clinical trials. Advances in the field of pharmacogenetics have facilitated the identification of genetic variants impacting AIC risk, and hiPSC-CMs have helped to explain molecular and cellular phenotypes observed in patients who develop cardiotoxicity. However, future progress in the identification of effective cardioprotectants in this field is conditioned by the limitations that are inherent to the models used for drug development. At the cardiomyocyte level, there is a great need to replace current models that rely on animal- or tumor-derived cell lines (105). While the use of rat cardiomyocytes has advanced the understanding of AIC, these cells differ from human cardiomyocytes in many ways, including sarcomeric structure, beat rate, electrical properties, ion channel functions, and action potential duration, making it difficult to extrapolate data to humans.

Many drug discovery programs have failed because targets validated in animal models are unreliable and nonpredictive in humans (106). In addition to this factor, laboratory animal studies are costly, labor intensive, and considered unethical. With the recent approval of the FDA Modernization Act 2.0, applicants seeking new drug market approval have the chance to opt for the use of cutting-edge alternative methods rather than animal testing to establish drug safety and effectiveness. Incorporation of preclinical platforms such as hiPSC-CM-based models will reduce production costs by refining the use of laboratory animals and identifying cardioprotectant drugs that are more relevant to humans (**Figure 2**).

Interindividual variability in drug response is a factor frequently not considered in the early stages of drug discovery, reducing the success of clinical trials. In this regard, the incorporation of patient-specific hiPSC-CMs into the drug discovery pipeline would provide a platform to identify personalized therapeutic targets and reliably stratify patients (**Figure 2**). Given this, hiPSC-CMs hold exciting potential as an essential platform for the development of the next generation of cardioprotectants to defeat AIC.

7.1. The Potential of hiPSC-CMs as a High-Throughput Platform for Cardioprotectant Drugs Discovery

In vitro functional phenotyping in high-throughput screening (HTS) format is an invaluable technique for the identification of molecular targets involved in AIC and for the discovery of cardioprotective therapeutic agents. Production of high-quantity and high-quality cells is the first step for using hiPSC-CMs as an HTS platform. Protocols for generating cardiomyocytes in a reproducible and cost-effective manner have significantly improved during the last several years (reviewed in 107). Regarding large-scale cardiomyocyte production, initial hiPSC expansion and differentiation and selection of hiPSC-CMs can currently be integrated into one process in one bioreactor to obtain a high density of functional cardiomyocytes (1.9×10^9 cells) with greater than 95% TNNT2 (108). Similarly, other protocols that use parallel differentiations can produce 2.4×10^8 hiPSC-CMs from four bioreactors (150 mL) in 22 days (109).

hiPSC-CMs can be plated in 384-well plates ($\sim$2–4 $\times 10^4$ cells per well) and easily assayed for phenotypic response to anthracyclines using fluorescence- and luminescence-based plate reader assays and high content imaging to assess cell viability, ROS, apoptosis, DNA damage, calcium handling, and sarcomeric alignment, among others (**Figure 1**). Since the molecular mechanisms by which anthracyclines induce cardiotoxicity ultimately lead to cardiomyocyte apoptosis, hiPSC-CM survival after anthracycline exposure remains the main indicator for in vitro AIC. Based on breast cancer patients who commonly receive 80 mg/m^2 of doxorubicin (cumulative dose of

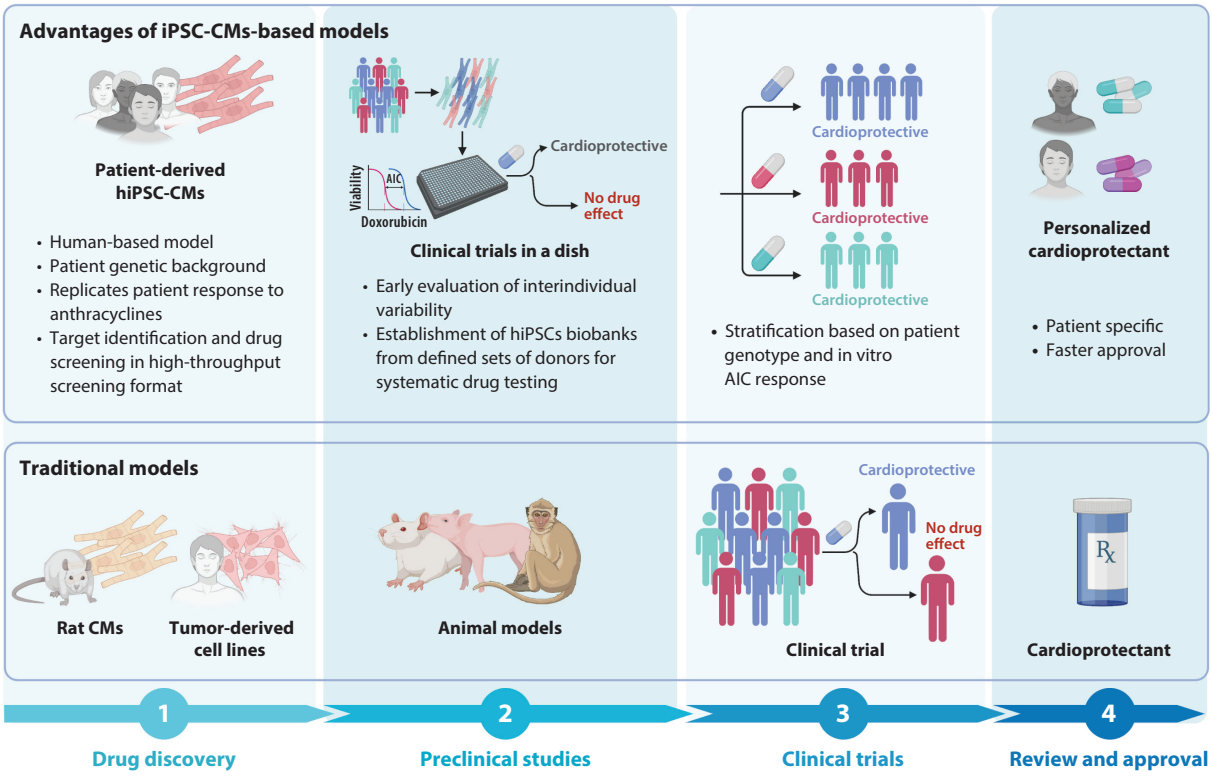


Figure 2

Advantages of the incorporation of hiPSC-CMs into the cardioprotectant drug discovery pipeline. Traditional models use rat CMs or human cancer cell lines for drug discovery followed by preclinical testing in animals. Incorporation of hiPSC-CMs into the drug discovery phase allows for the identification of targets and drug candidates more relevant to human AIC. Variability in the interindividual response to drug candidates can be evaluated earlier in the preclinical testing phase using specific cohorts of hiPSC-CMs. Clinical trials in a dish using hiPSC-CMs facilitate subsequent patient stratification in clinical trials and faster identification of effective and personalized cardioprotectant drugs. Abbreviations: AIC, anthracycline-induced cardiotoxicity; CM, cardiomyocytes; hiPSC-CM, human induced pluripotent stem cell–derived cardiomyocyte. Figure adapted from The Drug Discovery Process by BioRender.com, retrieved from **https://app.biorender.com/biorender-templates**.

240 mg/m^2), resulting in a C_{max} of 6.9 μM, we have shown that 0.1–10 μM at time points of 24–72 h is a doxorubicin physiological dose, similar to prior experiments performed by other authors in rat cardiomyocytes (110). Our team has optimized the fluorescence- and luminescence-based phenotype assays mentioned above in high-throughput format and demonstrated its usefulness in quantifying patient-specific responses to doxorubicin in vitro and in identifying novel molecular targets and cardioprotective therapies (42, 102). hiPSC-CMs are also suitable for a variety of flow cytometry–based assays, for example, quantification of intracellular levels of anthracycline (autofluorescence) and detection of membrane proteins using specific antibodies. Regarding the characterization of electrophysiological parameters in hiPSC-CMs, although patch clamp is the gold standard technique to investigate proarrhythmic effects of drugs, its implementation in drug discovery is limited due to its low throughput. Alternatively, high-throughput measurement of cardiomyocytes' extracellular field potentials, action potentials, and intracellular-like signals is possible with the multiwell microelectrode array technology, including high-density microelectrode arrays (111, 112).

Even though hiPSC-CMs are evolving to be incorporated into preclinical drug discovery, this model still has some limitations. Differentiated hiPSC-CMs typically do not demonstrate all of the facets of adult cardiomyocytes. Thus, further maturation of hiPSC-CMs is desired, with the hypothesis that this may result in these cells more accurately recapitulating the in vitro drug responses seen in patients. Recently, various strategies have been developed to promote hiPSC-CM maturation, including coculture with fibroblasts and/or endothelial cells, use of alternative matrices and substrates with micropatterns, culture with the addition of hormones (triiodothyronine and glucocorticoids), and supplementation of fatty acids (reviewed in 107). These maturation strategies allow the generation of hiPSC-CMs that further resemble adult cardiomyocytes in cell structure and sarcomeric organization, Ca^{2+} handling, electrophysiological properties, contractility, and transcription profiles. Future studies using hiPSC-CMs are also expected to increasingly incorporate advanced models such as 3D cardiac tissues. These models incorporate extracellular matrix components and additional heart cell types such as endothelial cells and fibroblasts, more accurately reflecting the functional maturation and physiological environment of the human heart.

The incorporation of hiPSC-CMs into drug discovery programs will contribute to the identification of more reliable, patient-specific, and effective cardioprotectants (**Figure 2**). Major advantages of the hiPSC-CM HTS model include its potential to be used both for target identification and compound screening in the drug discovery phase and for drug evaluation and toxicity assessment at the preclinical stage of the drug development pipeline.

7.2. hiPSC-CMs for In Vitro Clinical Trials

One of the most notable advantages of hiPSC is that they represent individual donors' genetics, which facilitates screening for drug safety and toxicity at the population level in vitro. The ability of hiPSC-CMs to recapitulate patient-specific genetic, physiologic, and pharmacological responses, including differential responses to doxorubicin even at the SNP level (42, 94, 102), confers this model with great potential as a platform for target identification and systematic drug testing in cells from defined cohorts of donors. This means that the hiPSC-CM donor cohort can be designed following the same principles of a clinical trial to predict the effectiveness of a cardioprotectant candidate in a population. Regarding the sample size needed to allow discrimination of drug effects, it is estimated that a minimum sample size of 22 hiPSC-CMs lines has a 90% probability of predicting events that occur in 10% of the population, approximately the same size as an average Phase I clinical trial (113). Additionally, hiPSC-CMs can be stratified into clinically meaningful subgroups (e.g., patients with specific genetic variants, pediatric patients, male versus female, race) to distinguish between patients who may or may not respond to drug candidates (**Figure 2**). Moreover, the establishment of biobanks can provide unlimited access to standardized hiPSC-CMs from specific cohorts for systematic drug testing and correlation with clinical observations. Currently, there are 10 major hiPSC banks worldwide, and most of those institutes (90%) are government-funded nonprofit organizations. These institutions offer hiPSC generation, characterization, and banking, and provide access to disease cell lines for both academic and industry research (114). Certainly, the creation of an hiPSC/hiPSC-CM biobank from patients treated with anthracyclines would be an invaluable tool for the systematic understanding of interindividual response to anthracyclines in vitro and correlation with clinical testing data, advancing AIC biomarker discovery and cardioprotectant development.

Considering that cardiovascular toxicity represents the most frequent adverse drug reaction resulting in drug withdrawal, this approach (also known as clinical trials in a dish) has potential applications not only for the identification of novel and more specific cardioprotectants but also for safety screening of multiple types of drugs (115). hiPSC-CMs have been shown to be useful

for assessing cardiotoxic effects of FDA-approved and marketed drugs, including doxorubicin, proving the high sensitivity and specificity of this model (94, 116).

8. THE LIGHT AT THE END OF THE TUNNEL

Patient-specific hiPSC-CM models are a powerful tool for the validation of the functional role of genetic variants. The causality of *RARG* and *SLC28A3* SNPs in AIC has been successfully validated using this technology, suggesting that these variants should be screened in clinical trials in cancer patients treated with doxorubicin. Future functional validation of remaining AIC-associated variants will allow the identification of variants relevant for clinical testing and accelerate the inclusion of approved AIC biomarkers in clinical practice. Combined AIC biomarker screening and application of polygenic risk prediction models will enable the early identification of patients at high AIC risk for the prevention of cardiotoxicity.

We anticipate that functional gene validation in hiPSC-CMs along with the implementation of patient-specific cardiomyocytes as a screening platform will identify novel druggable targets that are more relevant to AIC than those produced by traditional association studies. Integrating hiPSC-CMs and in vitro AIC data (trials in a dish) into the existing models for drug discovery will help develop the next generation of highly effective and personalized cardioprotectants.

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