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REVIEW

Human In Vitro Models for Assessing the Genomic Basis of Chemotherapy-Induced Cardiovascular Toxicity

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Abstract

Chemotherapy-induced cardiovascular toxicity (CICT) is a well-established risk for cancer survivors and causes diseases such as heart failure, arrhythmia, vascular dysfunction, and atherosclerosis. As our knowledge of the precise cardiovascular risks of each chemotherapy agent has improved, it has become clear that genomics is one of the most influential predictors of which patients will experience cardiovascular toxicity. Most recently, GWAS-led, top-down approaches have identified novel genetic variants and their related genes that are statistically related to CICT. Importantly, the advent of human-induced pluripotent stem cell (hiPSC) models provides a system to experimentally test the effect of these genomic findings *in vitro*, query the underlying mechanisms, and develop novel strategies to mitigate the cardiovascular toxicity liabilities due to these mechanisms. Here we review the cardiovascular toxicities of chemotherapy drugs, discuss how these can be modeled *in vitro*, and suggest how these models can be used to validate genetic variants that predispose patients to these effects.

Keywords Human induced pluripotent stem cell · Cardio-oncology · Precision medicine · Cancer · Cardiotoxicity · Vascular toxicity

Introduction

It is well established that many cancer treatments are toxic to the cardiovascular system. Cardiovascular toxicity is seen with traditional chemotherapy agents such as anthracyclines, as well as newer targeted therapies such as trastuzumab, tyrosine kinase inhibitors, and immunotherapies. These toxicities lead to significant morbidity and mortality among cancer survivors. These effects can occur immediately after the first dose of the drug or take many years to manifest, potentially due to a reduction in baseline cardiovascular function that is then exacerbated with age [1, 2]. As cancer treatments have become more effective and overall lifespans increase, the number of cancer survivors has also increased. There are currently nearly 17 million cancer survivors in the United States alone, and this number is projected to increase significantly in the coming years [3]. As cancer survivorship increases, addressing the impact of these cardiovascular toxicities on long-term health and quality of life becomes an increasingly pressing healthcare concern.

Though many risk factors for chemotherapy-induced cardiovascular toxicity (CICT) have been identified, such as dose, patient age, BMI, and preexisting cardiovascular health, differences in interindividual incidence of toxicity exist even when risk factors are taken into account [4]. This unexplained interindividual variability suggests a genomic basis to this susceptibility [5]. Understanding the genomic predisposition to CICT will provide clinical tools that can be used to suggest alternative therapies for patients in addition to elucidating the CICT mechanisms in order to improve drug development for adjuvant therapy and novel chemotherapeutics.

To date, significant resources have been invested in identifying relevant genomic variants through large cohort-based candidate gene association studies (CGAS) and genome-wide association studies (GWAS). These studies are limited, however, as they do not establish causality, often fail to be

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replicated, and frequently have insufficient sample size to draw statistically significant conclusions [6]. Furthermore, the majority of CGAS and GWAS have focused on anthracycline-induced cardiotoxicity, which represents only a fraction of all CICTs.

Human-induced pluripotent stem cells (hiPSCs) offer a high-throughput, patient-specific platform for the study of CICTs. hiPSCs have been shown to faithfully recapitulate clinical phenotypes, provide a platform for genomic variant discovery and validation, and allow for mechanistic investigations [7, 8]. That hiPSCs recapitulate clinical phenotypes confirms the role of genomics in patient-specific predisposition to CICTs and further supports their use as a model system for CICT research.

Types of Cardiovascular Toxicity Caused by Chemotherapy

CICT manifests in a variety of ways including heart failure, arrhythmia, vascular dysfunction, and atherosclerosis. The anthracycline doxorubicin is the most well-studied [9, 10] and causes dose-dependent cardiotoxicity [11–13]. Cardiotoxic side effects experienced with doxorubicin range from asymptomatic increases in left ventricular wall stress, to reductions in left ventricular ejection fraction (LVEF), to arrhythmias and highly symptomatic congestive heart failure, often severe enough to warrant heart transplant [14–16]. Doxorubicin cardiotoxicity occurs via a number of mechanisms, including reactive oxygen species (ROS) production, DNA damage, and mitochondrial dysfunction, all of which ultimately lead to programmed cell death [7].

Trastuzumab is another chemotherapeutic with a black box warning for ventricular dysfunction and congestive heart failure. Trastuzumab is a monoclonal antibody that targets the HER2 receptor and is used in the treatment of breast cancer and some gastric cancers [17]. Trastuzumab cardiotoxicity has historically been viewed as less severe and more reversible than anthracycline-mediated cardiotoxicity, though this assertion has come into question more recently [17]. The trastruzumab target HER2 (ERBB2) has an integral role in cardiac development, and its inhibition leads to impaired autophagy and accumulation of ROS, which have been implicated in the mechanism by which trastuzumab leads to heart failure [18].

Chemotherapeutics are also frequently associated with conduction abnormalities including QT prolongation, torsades de pointes, arrhythmias, and sudden death. Both nilotinib and vandetanib have FDA-issued black box warnings for QT prolongation and sudden death. Nilotinib is a tyrosine kinase inhibitor (TKI) designed to inhibit the BCR-ABL1 fusion protein in chronic myeloid leukemia [19]. Vandetanib is a multikinase TKI used in treatment of multiple cancers [20]. Nilotinib is believed to prolong the QT interval through inhibition of a specific potassium channel, while vandetanib inhibits multiple currents in the cardiac action potential in *in vitro* studies [19, 20]. In addition to chemotherapeutics with black box warnings, many additional chemotherapeutics, including TKIs, taxanes, anthracyclines, and cyclophosphamide, have been associated with arrhythmias and QT prolongation.

CICTs are not limited to the heart and can have significant implications for vascular health. Both the angiogenesis inhibitor bevacizumab and the multikinase inhibitor cabozantinib carry black box warnings for risk of severe hemorrhage. Bevacizumab inhibits the vascular endothelial growth factor A (VEGFA) protein, while cabozantinib inhibits multiple kinases including the VEGF receptor subtypes VEGFR1, VEGFR2, and VEGFR3 [21]. The exact mechanism by which inhibition of the VEGF pathway, which is critical in angiogenesis, increases hemorrhage risk is unclear but has been hypothesized to result from endothelial cell and platelet damage or dysfunction [22]. Chemotherapeutics have also been associated with arterial thrombosis, which includes fatal myocardial infarction and stroke. Ponatinib, a TKI used for chronic myeloid leukemia, has a black box warning for this CICT. The mechanism by which ponatinib causes thrombosis remains under study, but kinase inhibition studies have shown that ponatinib inhibits multiple off-target kinases, including those with critical roles in maintenance of vascular health [23].

In addition to black box warnings for CICTs, FDA drug labels denote numerous additional cardiovascular toxicities associated with a number of chemotherapeutics. These CICTs include heart failure, arrhythmias, bradycardia, atrial fibrillation, angina, edema, hemorrhage, hypotension, hypertension, thrombophlebitis, arterial and venous thrombosis, ischemic cerebrovascular events, myocarditis, pleural and pericardial effusion, hypereosinophilic cardiotoxicity, and more. CICTs associated with specific chemotherapeutics are summarized in Table 1.

Beyond the adverse effects of individual agents, CICTs are exacerbated in the context of multiple chemotherapeutics. For example, more CICTs are observed in the context of taxane treatment coadministered with HER2 inhibitors and/or anthracyclines compared to treatment with taxanes alone [24, 25]. The breadth of CICTs caused by numerous chemotherapeutics and the long-term health consequences of these CICTs for cancer survivors has led to the emergence of the field of cardio-oncology. In order to move this field forward, it is crucial that we develop tools to understand who is susceptible to CICTs, determine the mechanism of action of CICTs, and identify novel chemotherapeutics and adjuvant therapies in order to prevent cardiovascular damage.

Pharmacogenomics of CICT

Because CICTs affect only a subset of patients and do not uniformly segregate with cardiovascular risk factors,

Table 1 Description of *in vitro* assays that could be used for validation of SNPs associated with chemotherapy-induced cardiotoxicity. CICTs that may be modeled *in vitro* with similar cell types and assays are grouped by color. Data from drugs.fda.gov

Black Box Warnings	Drug	Proposed cell type	Proposed Primary Assay
Chronic heart failure, LV dysfunction	Doxorubicin, liposomal doxorubicin, epirubicin, trastuzumab, ado-trastuzumab	Cardiomyocyte	Viability and Electrophysiology
QT Prolongation, torsades de pointes, sudden death	Nilotonib, vandetanib	Cardiomyocyte	Electrophysiology
Severe hemorrhage	Cabozantinib, bevacizumab	Endothelial, smooth muscle	Vein-on-a-chip
Arterial thrombosis (fatal MI, stroke)	Ponatinib	Endothelial, smooth muscle, megakaryocyte	Vein-on-a-chip
Warnings and Precautions			
Chronic heart failure	Cyclophosphamide, imatinib, ponatinib, sunitinib, vandetanib, bortezomib, carfilzomib	Cardiomyocyte	Viability
Cardiac dysfunction	Trastuzumab, pertuzumab, imatinib, pazopanib	Cardiomyocyte	Viability and electrophysiology
QT prolongation, torsades de pointes, sudden death	Dasatinib, crizotinib, sorafenib, sunitinib, pazopanib	Cardiomyocyte	Electrophysiology
Arrhythmias	Cyclophosphamide, doxorubicin, epirubicin, trastuzumab, ponatinib	Cardiomyocyte	Electrophysiology
Bradycardia	Paxcilitaxel, crizotinib	Cardiomyocyte	Electrophysiology
Conduction abnomalities	Paxcilitaxel	Cardiomyocyte	Electrophysiology
Ventricular repolarization abnormalities	Nilotonib	Cardiomyocyte	Electrophysiology
Atrial fibrillation	Ibrutinib	Cardiomyocyte	Electrophysiology
Myocardial ischemia, angina	5-flurouracil, sorafenib, sunitinib, regorafenib, carfilzomib	Endothelial, smooth muscle	Viability, proliferation
Cardiac and arterial vascular occlusive events	Nilotonib	Endothelial, smooth muscle	Viability, proliferation
Hypotension	Paxcilitaxel, bortezomib	Endothelial, Smooth muscle	Nitric Oxide, Contractile Phenotyping
Hypertension (HTN)	Paxcilitaxel, trastuzumab, ponatinib, cabozantinib, bevacizumab, sorefenib, sunitinib, pazopanib, vandetanib, regorafenib, carfilzomib	Endothelial, Smooth muscle	Nitric Oxide, Contractile Phenotyping
Pulmonary hypertension	Dasatinib, bortezomib	Endothelial, Smooth muscle	Nitric Oxide, Contractile Phenotyping
Thrombophlebitis	Epirubicin	Endothelial, smooth muscle, megakaryocyte	Vein-on-a-chip
Arterial thrombosis (fatal MI, stroke)	Cabozantinib, pazopanib, axitinib, bevacizumab	Endothelial, smooth muscle, megakaryocyte	Vein-on-a-chip
Venous thrombosis	Pazopanib, axitinib, carfilzomib, bevacizumab	Endothelial, smooth muscle, megakaryocyte	Vein-on-a-chip
Ischemic cerebrovascular events	Vandetanib	Endothelial, smooth muscle, megakaryocyte	Vein-on-a-chip
Hemorrhage	Vandetanib, axitinib, regorafenib	Endothelial, smooth muscle, megakaryocyte	Vein-on-a-chip
Myocarditis, pericarditis, pericardial effusion	Cyclophosphamide, ipilimuab, nivolumab, pembrolizumab	Cardiomyocyte, lymphocyte, macrophage, NK cell, eosinophil	Immune cells & cardiomyocyte
Fluid retention including pleural and pericardial effusion	Nilotonib, ponatinib	Cardiomyocyte, lymphocyte, macrophage, NK cell, eosinophil	Immune cells & cardiomyocyte
Hypereosinophilic cardiac toxicity	Imatinib	Cardiomyocyte, lymphocyte, macrophage, NK cell, eosinophil	Immune cells & cardiomyocyte
Edema	Imatinib	Cardiomyocyte, lymphocyte, macrophage, NK cell, eosinophil	Immune cells & cardiomyocyte

numerous CGAS and GWAS studies have been conducted to identify potential genetic contributors to susceptibility. To date, the majority of this research has focused on anthracycline-induced cardiotoxicity. The approximately 40 CGAS and 4 GWAS that have been performed have identified a number of SNPs that are statistically correlated with DIC [26] (see Table 2). Many of these studies have included a small numbers of SNPs (i.e., < 5). In these studies, there is variation in the definition of cardiotoxicity, recruitment methodologies, and composition of the patient populations that likely is responsible for much of the variation of the results. Many of these studies were limited by small sample size, some including as few as seven patients with toxicity [27] with even the most thorough including only 169 toxicity patients [28]. Because of these limitations, studies rarely replicate one another's findings [29–40].

The largest CGAS and GWAS were both multi-center programs [41, 42]. The first study was candidate gene-based with a 344-patient cohort and the second was genome-wide with a cohort of 562. These studies have demonstrated an association between two SNPs and a dramatically altered risk of anthracycline cardiotoxicity in both original and replication cohorts: one, rs7853758 (L461L), in *SLC28A3*, a concentrative transporter, and one, rs2229774 (S427L), in *RARG*, which encodes retinoic acid receptor-γ.

A small number of studies have examined the genomics of CICTs for non-anthracycline chemotherapeutics. Multiple CGAS, one exome association study, and one GWAS have been conducted for trastuzumab-induced cardiotoxicity. CGAS studies have consistently identified one of two variants in the trastuzumab target *ERBB2*, but variants in this gene were not identified in either the exome association or GWAS studies [43–50].

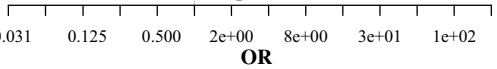
CGAS have also been conducted for vascular CICTs. Two CGAS have evaluated variants associated with venous thromboembolism with immunomodulator therapy and one CGAS assessed the association of a specific variation in the *VEGFA* untranslated region with bevacizumab-induced thrombo-hemorrhagic events [51–53].

These association studies provide clues regarding potential predictors and mechanisms of CICTs. However, they are subject to a number of limitations. Large cohorts are often required to achieve statistically significant results, and many studies are limited by small sample size [6, 54]. Additionally, as illustrated by genomic studies of trastuzumab-induced cardiotoxicity, studies may fail to replicate one another, leading to uncertainty regarding which variants to prioritize. Even when GWAS studies identify significant variants that remain significant in replication cohorts, the implications of the identified variant may still be unclear. For example, the rs7853758 variant in *SLC28A3* was identified as significantly associated with anthracycline-induced cardiotoxicity in both discovery and replication cohorts [41, 55]. However, this variant encodes a synonymous mutation, so it is likely that this variant is in linkage disequilibrium with the true causal variant, which is yet to be identified. When considering anthracycline-induced CICTs alone, almost 50 studies have been conducted, comprising thousands of patients, and we are still without a single validated causal variant. Furthermore, anthracyclines are only one class of chemotherapeutics associated with CICTs. There have been no genomic studies on the majority of CICTs.

Genomic association studies conducted to date have provided important evidence to suggest a genomic contributor to CICT susceptibility. For a subset of chemotherapeutics, these studies have identified potential variants to focus future mechanistic and variant validation research. However, the limitations of association studies and the breadth of chemotherapeutics that are currently

Table 2 Forest plot showing the top 100 significant SNPs associated with CICT

Gene	Variant	Study Size	P.Value	Reference
RARG	rs2229774	456	5.9e−08	Aminkeng et al. 2015
RARG	rs2229774	376	7.8e−08	Aminkeng et al. 2015
WDR4	rs15736	280	2.6e−06	Aminkeng et al. 2015
ZNF521	rs4381672	280	2.9e−06	Aminkeng et al. 2015
SP4	rs2282889	280	4.4e−06	Aminkeng et al. 2015
RARG	rs2229774	280	5e−06	Aminkeng et al. 2015
RIN3	rs9323880	280	6.8e−06	Aminkeng et al. 2015
SLC28A3	rs7853758	521	1.6e−05	Visscher et al. 2012 & 2013
CYP3A5	rs776746	106	1e−04	Rossi et al. 2009
SLC28A3	rs7853758	344	1e−04	Visscher et al. 2012
UGT1A6	rs17863783	521	0.00024	Visscher et al. 2012 & 2013
FMO2	rs2020870	344	0.00042	Visscher et al. 2012
UGT1A6	rs17863783	341	0.001	Leger et al. 2016
CELF4	rs1786814	331	0.001	Wang et al 2016
ABCC1	rs4148350	344	0.0012	Visscher et al. 2012
SULT2B1	rs10426377	521	0.0015	Visscher et al. 2012 & 2013
SLC28A1	rs2305364	521	0.002	Visscher et al. 2012 & 2013
SPG7	rs2019604	344	0.0021	Visscher et al. 2012
HNMT	rs17583889	257	0.0022	Visscher et al. 2013
SLC10A2	rs7319981	344	0.0029	Visscher et al. 2012
HAS3	rs223228	287	0.003	Wang et al. 2014
SLC10A2	rs9514091	344	0.0033	Visscher et al. 2012
SLC28A1	rs2305364	344	0.0033	Visscher et al. 2012
SLC22A7	rs4149178	335	0.0034	Visscher et al. 2015
SLC28A1	rs2290271	344	0.0035	Visscher et al. 2012
ABCC1	rs4148350	521	0.004	Visscher et al. 2012 & 2013
RARG	rs2229774	96	0.0043	Aminkeng et al. 2015
UGT1A6	rs4261716	344	0.0043	Visscher et al. 2012
SLC22A2	rs316019	344	0.0049	Visscher et al. 2012
HNMT	rs17645700	344	0.0053	Visscher et al. 2012
ABCB4	rs1149222	344	0.0054	Visscher et al. 2012
HNMT	rs17645700	521	0.0054	Visscher et al. 2012 & 2013
CYP4F11	rs2108623	344	0.0055	Visscher et al. 2012
HNMT	rs17583889	344	0.0057	Visscher et al. 2012
UGT1A6	rs17863783	344	0.0059	Visscher et al. 2012
FMO3	rs1736557	344	0.006	Visscher et al. 2012
UGT1A6	rs17863783	177	0.0062	Visscher et al. 2013
SLC10A2	rs9514091	521	0.0063	Visscher et al. 2012 & 2013
SLC28A3	rs7853758	156	0.0071	Visscher et al. 2012
SULT2B1	rs10426377	344	0.0071	Visscher et al. 2012
SLC22A17	rs4982753	185	0.0071	Visscher et al. 2015
SLC28A3	rs7853758	188	0.0072	Visscher et al. 2012
ADH7	rs729147	344	0.0072	Visscher et al. 2012
ABCB4	rs4148808	521	0.0072	Visscher et al. 2012 & 2013
HNMT	rs17583889	521	0.0073	Visscher et al. 2012 & 2013
SLC22A17	rs4982753	335	0.0078	Visscher et al. 2015
HNMT	rs17583889	280	0.008	Aminkeng et al. 2015
GSTP1	rs1695	58	0.008	Windsor et al. 2012
ABCB1	rs2235047	344	0.0087	Visscher et al. 2012
SLC28A3	rs4877847	344	0.0092	Visscher et al. 2012
ABCB4	rs4148808	344	0.0093	Visscher et al. 2012
SLC28A1	rs2290271	521	0.0098	Visscher et al. 2012 & 2013
RAC2	rs13058338	255	0.01	Armenian et al. 2013
ABCC2	rs8187710	255	0.01	Armenian et al. 2013
CELF4	rs1786814	341	0.01	Leger et al. 2016
CYBA	rs4673	106	0.01	Rossi et al. 2009
ABCB1	rs2229109	106	0.01	Rossi et al. 2009
HAS3	rs223228	363	0.01	Wang et al. 2014
CYBA	rs4673	450	0.01	Wojnowski et al. 2005
FMO3	rs1736557	521	0.011	Visscher et al. 2012 & 2013
CBR3	rs1056892	166	0.012	Hertz et al. 2016
SPG7	rs2019604	521	0.012	Visscher et al. 2012 & 2013
ABCB4	rs1149222	248	0.012	Visscher et al. 2013
POR	rs13240755	91	0.01376	Lubieniecka et al. 2013
HFE	rs1800562	179	0.015	Lipshultz et al. 2013
GSTP1	rs1695	106	0.015	Rossi et al. 2009
POR	rs2868177	280	0.016	Aminkeng et al. 2015
SLC10A2	rs7319981	521	0.016	Visscher et al. 2012 & 2013
SLC22A2	rs316019	257	0.016	Visscher et al. 2013
NCF4	rs1883112	450	0.016	Wojnowski et al. 2005
ABCC1	rs45511401	450	0.016	Wojnowski et al. 2005
NCF4	rs1883112	48	0.018	Cascales et al. 2013
Intergenic	rs28714259	322	0.018	Schneider et al 2017
UGT1A6	rs4261716	521	0.018	Visscher et al. 2012 & 2013
RAC2	rs13058338	106	0.019	Rossi et al. 2009
CBR3	rs1056892	487	0.02	Blanco et al. 2012
CAT	rs10836235	76	0.02	Rajic et al. 2009
ABCC1	rs246221	877	0.02	Vulsteke et al. 2015
ABCC2	rs8187710	280	0.021	Aminkeng et al. 2015
NCF4	rs1883112	106	0.023	Rossi et al. 2009
RAC2	rs13058338	450	0.025	Wojnowski et al. 2005
RAC2	rs13058338	150	0.028	Reichwagen et al. 2015
ABCB4	rs4148808	248	0.028	Visscher et al. 2013
POR	rs13240755	280	0.033	Aminkeng et al. 2015
SULT2B1	rs10426377	273	0.033	Visscher et al. 2013
ABCB1	rs2235047	521	0.036	Visscher et al. 2012 & 2013
SLC28A3	rs4877847	521	0.037	Visscher et al. 2012 & 2013
CYBA	rs4673	48	0.039	Cascales et al. 2013
FCGR3A	rs10127939	106	0.039	Rossi et al. 2009
HAS3	rs223228	341	0.04	Leger et al. 2016
HAS3	rs223228	76	0.04	Wang et al. 2014
Intergenic	rs28714259	930	0.041	Schneider et al 2017
ADH7	rs729147	521	0.041	Visscher et al. 2012 & 2013
CELF4	rs1786814	54	0.046	Wang et al. 2016
SLC22A7	rs4149178	185	0.047	Visscher et al. 2015
ABCB1	rs1045642	166	0.049	Hertz et al. 2016
HFE	rs1799945	255	0.05	Armenian et al. 2013
CBR3	rs1056892	145	0.056	Blanco et al. 2008
SLC28A3	rs7853758	177	0.058	Visscher et al. 2013
ABCC2	rs8187710	450	0.071	Wojnowski et al. 2005



unstudied point to the need for additional research methods to contribute to the identification of variants, validate a causal role for identified variants, and conduct mechanistic studies.

Model Systems for CICT

Zebrafish have been used as a model system in multiple avenues of cardiovascular research, including CICTs. Zebrafish offer a high-throughput, low cost system to study cardiovascular effects of medications, and have been well-characterized as a model system [56]. In a study of anthracycline-induced cardiotoxicity, zebrafish displayed incomplete heart development, pericardial edema, and bradycardia in response to doxorubicin [57]. While zebrafish in this study displayed an adverse, dose-dependent cardiovascular phenotype, it is important to note that the manifestation of this dysfunction in the zebrafish model differs significantly from the LV dysfunction and heart failure observed in patients, and thus underscores a limitation of this model. Additionally, zebrafish are not suited to patient-specific studies. Differences between the zebrafish and human genomes are significantly larger than the interindividual variability that determines drug response [56].

Animal models have also been employed for CICT research and have successfully identified mechanistic contributors to CICTs. For example, Miranda et al. [58] validated the role of iron in doxorubicin cardiotoxicity by showing that *HFE* knockout mice exhibited greater sensitivity to doxorubicin in cardiac assays. Zhang et al. [59] validated TOP2β inhibition as a key mechanistic contributor to doxorubicin-induced cardiotoxicity in a mouse model with cardiac-specific deletion of *TOP2B*, where knockout mice exhibited minimal cardiotoxicity in response to doxorubicin treatment. While studies like these demonstrate the utility of animal models, particularly with respect to mechanistic studies, animal models are expensive, low-throughput, and inherently limited by interspecies variability.

hiPSCs address the limitations of previous models and offer a high-throughput, patient-specific platform to study CICT. hiPSCs provide a non-invasive, renewable source of patient-specific cells that might otherwise be difficult to obtain or limited by low passage number in culture [60]. hiPSCs retain a patient's genome and are thus suited to pharmacogenomic studies of interindividual variability in drug response [60]. Additionally, hiPSCs are amenable to straightforward, specific genomic manipulation through technologies such as CRISPR/Cas9 [61, 62]. Genome editing facilitates mechanistic investigations, variant validation, and generation of isogenic controls in hiPSC systems.

While hiPSCs offer many advantages, important limitations exist which include maturity status of the differentiated cells, requisite time and labor, subtype specification, and limited integration of multiple tissue types. Current differentiation protocols for hiPSC-derived cardiomyocytes (hiPSC-CMs) result in cells that are more phenotypically similar to fetal rather than adult CMs [63]. This presents a potential concern for accurately modeling phenotypes of the adult heart, and efforts to improve maturation status of hiPSC-CMs are ongoing. hiPSC reprogramming, culture, and differentiation require an investment of time and resources. While this does not represent a significant barrier for established hiPSC laboratories, large-scale hiPSC research may not be feasible for some groups. Subtype specification is also an ongoing effort in the field of hiPSCs; for hiPSC-CMs, this involves specification to atrial versus ventricular cells, and in vascular hiPSC models, this includes specification between arterial and venous endothelial cells [64, 65]. For certain CICTs, subtype specification may be crucial, as demonstrated by a study from Shafaattalab et al. [66] where ibrutinib, a drug associated with atrial fibrillation, induced a phenotype in atrial but not ventricular hiPSC-CMs. hiPSC models often include a single cell type and consequently may not capture information about interactions among tissue types, though this is addressed to some extent by co-culture models. Even with co-culture, however, hiPSC models may not adequately capture phenotypes that result from structural features of tissues rather than phenotypes of individual cells. Efforts to address these limitations include the development of hiPSC-derived organoids and engineered heart tissue [67, 68].

Despite these limitations, hiPSC models have been shown to recapitulate patient-specific disease. Inherited arrhythmias including Brugada syndrome (BrS), catecholaminergic polymorphic ventricular tachycardia (CPVT), arrhythmia-associated calmodulinopathies, and long QT syndrome (LQTS) have been recapitulated at the cellular level in patient-specific hiPSC-CMs [69–72]. Furthermore, genome editing of the variant of interest in these studies rescued the phenotype and provided validation of mechanistic contributors and causal variants for these conditions [70–72].

hiPSC Confirm that Chemotherapy-Induced Cardiovascular Toxicity Is a Genomic Disease

Multifactorial traits such as drug response are invariably more complex to model than the monogenic conditions described above. Despite this increased complexity, multiple studies have demonstrated the ability of hiPSCs to recapitulate patient-specific drug response phenotypes. Recent work using hiPSC-CMs has shown that these cells accurately recapitulate an individual patient's predilection to doxorubicin-induced cardiotoxicity [7]. Briefly, hiPSC-CMs from breast cancer patients who developed doxorubicin-induced cardiotoxicity recapitulate that increased risk *in vitro*, with decreased cell viability, metabolic function, contraction, sarcomeric structure, calcium handling, and increased ROS production when exposed to doxorubicin, compared to hiPSC-CMs from patients who were treated with doxorubicin but did not experience cardiotoxicity [7].

Patient-specific hiPSC-CMs have also been shown to recapitulate susceptibility to trastuzumab-induced cardiac dysfunction. Trastuzumab-exposed hiPSC-CMs demonstrated impaired contractility and calcium handling, and hiPSC-CMs from patients with severe cardiac dysfunction after trastuzumab treatment were more sensitive to the drug effects *in vitro* compared to hiPSC-CMs from trastuzumab-treated patients without cardiac dysfunction [73]. The authors identified metabolic dysfunction in response to trastuzumab and suggested that therapies directed at normalizing metabolism may be promising adjuvant treatments [73]. Importantly, hiPSC-CMs did not display differences in cell death or sarcomeric disorganization, which distinguishes this *in vitro* cardiotoxicity phenotype from that seen with doxorubicin [7, 73]. This difference mirrors the differences observed clinically in the persistence of doxorubicin- versus trastuzumab-induced heart failure after treatment cessation [74].

These patient-specific hiPSC studies demonstrate that the toxicity observed *in vitro* correlates with clinically relevant *in vivo* toxicity. To date, *a priori* screening in hiPSCs to predict who will develop toxicity has not been attempted, though the above studies suggest that results would correlate. Important limitations exist to the implementation of patient-specific hiPSCs for clinical screening. The reprogramming process takes several months and requires a substantial investment of labor. In addition to the potential expense of this process, patients with cancer may not reasonably be able to delay treatment for this amount of time. Thus, patient-specific hiPSC models for screening may be most high-yield in patients with rare diseases or for severe potential side effects in patients who have the ability to delay treatment. Where patient-specific hiPSC models have the potential to impact cardio-oncology more broadly is through their ability to confirm genomic underpinnings of CICTs. Patient-specific *in vitro* phenotyping coupled with genome sequencing and genome editing techniques can be used to identify and validate key pharmacogenomic variants that could be incorporated into a clinical screen to reduce CICTs on a larger scale and target future drug discovery efforts to bypass adverse events.

In addition to patient-specific studies, many studies have been conducted with control line hiPSCs to characterize the effects and probe the mechanisms of various CICTs. For example, Maillet et al. [8] characterized the response of control line hiPSC-CMs to doxorubicin and then used CRISPR/Cas9 to generate a *TOP2B* knockout line. The authors were able to demonstrate that knockout significantly reduced hiPSC-CM sensitivity to doxorubicin, corroborating previous animal studies [8]. In another study, Kurokawa et al. [75] used control hiPSC-CMs to determine that trastuzumab is only cardiotoxic when ERBB2/4 signaling is activated in response to cellular stress, thus further elucidating the mechanism of this CICT. Sharma et al. [76] utilized non-patient-specific hiPSC-CMs and hiPSC-derived endothelial cells to characterize the cardiovascular toxicity of 21 TKIs. From these in vitro studies, the authors were able to develop a "cardiac safety index" that corresponded with clinical phenotypes and could be used in future drug screening. These studies demonstrate the utility of hiPSCs for both patient-specific recapitulation (and consequently pharmacogenomics) as well as mechanistic research.

In Vitro Modeling of Toxicity Caused by Chemotherapy Drugs

The initial step in creation of any hiPSC CICT model is identification of the appropriate cell type(s) with which to conduct *in vitro* studies. *In vivo* phenotypes are complex and involve multiple cell types. For example, while *in vitro* studies with doxorubicin have focused on cardiomyocytes, cardiac progenitor cells, fibroblasts, and vascular cells may all play a role in the development of cardiotoxicity [77]. However, hiPSC-CMs in the absence of other cell types have still proven sufficient for identifying patient-specific differences in drug susceptibility and elucidating mechanistic contributors.

With the chosen cell type, the next key consideration for an hiPSC model is identification of an assay that is an appropriate readout for the given phenotype. Multiple assays are often used in conjunction with one another, as drugs may affect multiple facets of cellular health and function, and identification of a phenotype across multiple assays increases confidence in the results. In the case of doxorubicin, effects can be seen across cell morphology, viability, apoptosis, ROS production, metabolic function, and calcium handling [7, 8]. While multiple assays can detect a phenotype with doxorubicin, the viability assay alone is sufficient to detect pharmacogenomic differences in CICT susceptibility and to identify key mechanistic contributors. Identification of a primary assay, such as viability, is necessary in order to evaluate multiple genomic or mechanistic contributors in a high-throughput manner, as all conditions can then be compared with a single readout. Identification of a primary assay also allows studies to focus on a specific output parameter, from which hypothesized effect size and consequently necessary sample size can be determined. Additional phenotypic assays can then be used to augment the primary assay and corroborate that assay. For example, an apoptosis assay can corroborate a viability assay, or provide additional information about the phenotype, or an ROS assay may provide clues about the pathways activated by a drug that ultimately causes cell death.

Identification of an assay that effectively distinguishes between affected and unaffected patients and shows a dose-response relationship with the drug of interest is a necessary early step in any hiPSC CICT investigation. Even in cases where CICT phenotypes in patients may appear similar, such as doxorubicin- and trastuzumab-induced cardiotoxicity, the primary assay to distinguish the phenotype *in vitro* may differ.

For example, a patient-specific difference in CICT susceptibility to doxorubicin could be detected in hiPSC-CMs with a viability assay [7]. Trastuzumab, on the other hand, induced no patient-specific differences in viability but instead the *in vitro* phenotype could be distinguished by calcium handling [73].

The majority of CICTs have yet to be studied in an hiPSC model. In the following section we outline potential cell types and assays of interest that may further research in these areas. These proposed model systems are outlined in Table 1 and summarized in Fig. 1.

Cardiomyocyte Toxicity

Multiple chemotherapeutics carry either black box or general warnings of a risk of heart failure or cardiac dysfunction. In these cases, one of the most straightforward assays to assess for potential use as a primary assay is viability. There are numerous ways to quantify viability but two of the most common are ATP detection luminescence assays or resazurin-based fluorescence assays [7, 76, 78, 79]. These assays use cellular metabolic function as a proxy for the number of viable cells. While there is generally a strong correlation between the level of ATP or oxidation/reduction and the number of live cells, this linear relationship is contingent on unchanged levels of metabolic activity per cell. Drugs that alter metabolic activity at the cellular level have the potential to confound interpretation of these assays [80]. Fluorescence-based assays must also account for any inherent fluorescence in the drug of interest. Other assays for viability include water-soluble tetrazolium salt or cell counts that exclude nonviable cells [76, 81].

Apoptosis assays are complementary to and may augment viability assays. Apoptosis assays include luminescence-based caspase assays, propidium iodide and annexin V staining, and lactate dehydrogenase or troponin release assays [7, 78, 79, 81]. These assays have specific strengths and shortcomings, and ultimately, the choice of assay is contingent on the objective of the given study. For example, caspase assays assume a direct relationship between caspase activation and apoptosis, which is not always the case [82]. Assays contingent on release of intracellular contents capture late stages of apoptosis and may fail to detect earlier indicators of dysfunction.

Electrophysiological Abnormalities

A significant number of chemotherapy drugs are associated with conduction abnormalities and various arrhythmias. Given the large number of arrhythmic/QT prolongation events that have been identified in approved drugs only in the post-marketing phase, drug screening in hiPSC-CMs is now required in the Comprehensive In Vitro Proarrhythmia Assay (CiPA) guidelines for cardiac drug development [83]. The ability of hiPSC-CMs to recapitulate chemotherapy-specific arrhythmias was recently demonstrated in a study of ibrutinib-induced atrial fibrillation. Shafaattalab et al. [66] showed that atrial-specific hiPSC-CMs have abnormalities in voltage and calcium transients in response to ibrutinib, and

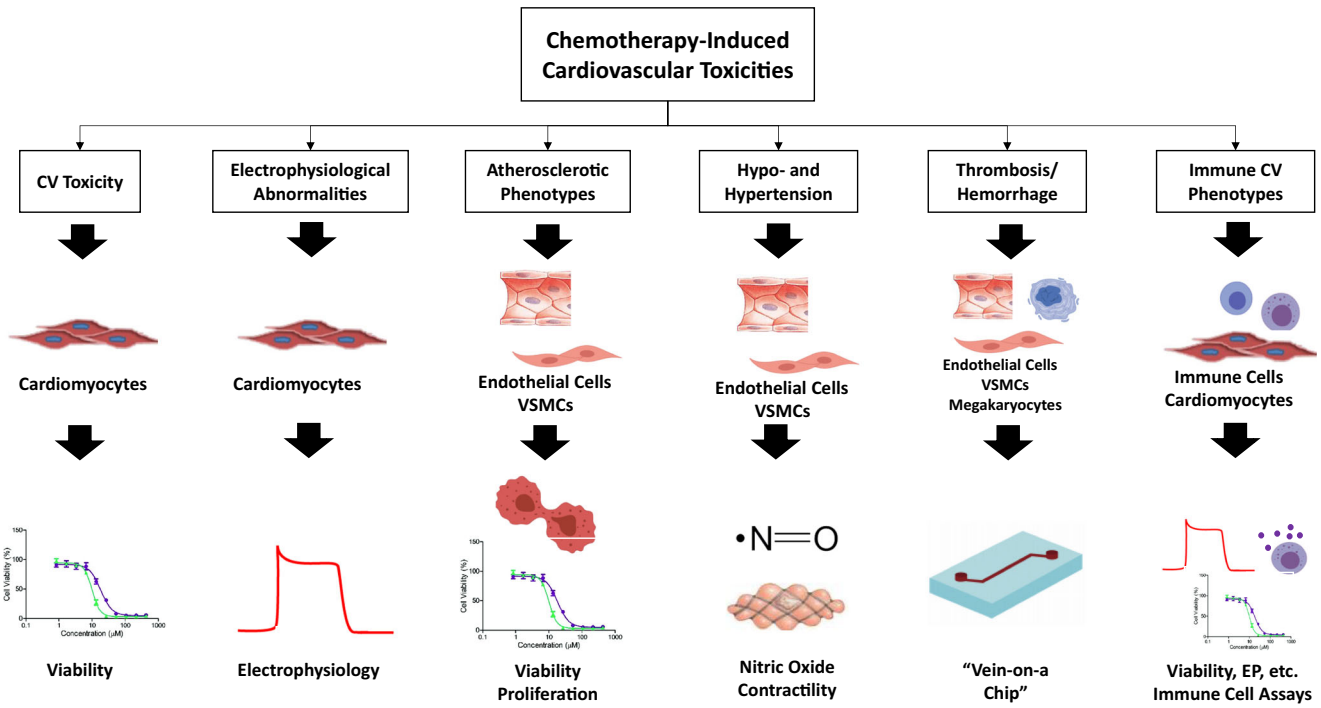


Fig. 1 A graphical depiction of the proposed workflow for hiPSC CICT modeling organized by CICT of interest

this response is not seen with other drugs in the same family that are not associated with atrial fibrillation. This study demonstrates the potential of hiPSC models to capture electrophysiological phenotypes *in vitro*.

Multiple methodologies can be applied to assess the electrophysiological properties of hiPSC-CMs in response to drug exposure. Patch clamping is the gold standard in electrophysiology and has successfully been used to assess drug response in hiPSC-CMs [76]. Higher throughput techniques include multielectrode arrays as well as voltage and calcium handling assays [84, 85]. These higher throughput techniques are not as informative with respect to certain parameters, such as upstroke velocity and resting membrane potential, but allow for longer-term electrophysiological assessment and substantially higher throughput than patch clamp techniques [84]. Calcium and voltage sensors can be introduced into hiPSC systems either as exogenous dyes or genetically encoded reporters [7, 86]. In the case of genetically encoded reporters, the reporter itself may interfere with calcium channel gating and signaling, though newer sensors are being developed to address this issue [87].

Atherosclerotic Phenotypes

The majority of CICT research to date has focused on cardiomyocyte-specific phenotypes; however, a significant number of chemotherapeutics are associated with vascular adverse events such as vascular occlusion and myocardial ischemia. These vascular phenotypes are in the atherosclerosis family and may involve two key cell types: endothelial cells (ECs) and vascular smooth muscle cells (VSMCs). EC damage is a nidus for lipid accumulation in atherosclerosis [88]. *In vitro* EC models have demonstrated drug-specific EC toxicity and impaired angiogenesis with atherosclerosis-associated chemotherapeutics such as nilotinib [89]. Atherosclerosis also involves a contractile to synthetic phenotypic switch in VSMCs that leads to extracellular matrix production and contributes significantly to vascular occlusion [90, 91]. Patient-specific hiPSC-VSMCs from patients with a genetic predisposition to coronary artery disease, a subtype of atherosclerosis, demonstrate altered contractility, proliferation, and migration *in vitro,* consistent with the more pathologic synthetic phenotype [90].

In these cell types, viability assays can be used to assess for toxicity and are similar to those listed above for cardiomyocytes. Proliferation assays in ECs would be expected to correlate directly with EC health as proliferation is a key component in angiogenesis and vascular homeostasis [89]. In VSMCs, increased proliferation would be a pathologic marker associated with the contractile to synthetic phenotypic shift observed in atherosclerosis [91]. In dividing cells such as ECs and VSMCs, many viability assays can also serve as proliferation assays. In addition to the assays described above for cardiomyocyte viability, proliferation assays such as 5-bromo-2′-deoxyuridine (BrdU), 5-ethynyl-2′-deoxyuridine (EdU), or 3H-thymidine nucleoside incorporation assays, which may employ radioactive, fluorescent, or copper-catalyzed reactions for signal detection [91, 92], can be used.

Migration assays can similarly be used to assess EC and VSMC phenotypes, where decreased migration is considered pathologic in ECs and increased migration is pathologic in VSMCs, consistent with the phenotypic switch [89, 91]. Migration can be measured through scratch assays as well as cell exclusion assays, where cells are plated in a chamber with a removable barrier. In ECs, tube formation assays are often used, as aspects of both migration and proliferation are captured in this assay of angiogenic function [89, 93].

Hypo- and Hypertensive Phenotypes

Dasatinib and bortezomib are associated with development of pulmonary arterial hypertension. Pulmonary arterial hypertension involves breakdown of lung vasculature, and consequently, hiPSC-ECs have been incorporated into studies of this condition [94]. hiPSC-ECs from patients with both heritable and idiopathic pulmonary hypertension displayed reduced adhesion, migration, tube formation, and survival compared to control subjects [94].

Numerous other chemotherapeutic drugs impact vascular tone more generally, resulting in either hypo- or hypertension. Vascular tone is regulated by multiple factors including EC nitric oxide (NO) production, VSMC contractility, and overall vascular stiffness [95–97]. As in vitro models for generalized hypertension and hypotension are sparse, the appropriate assay to assess this phenotype remains to be determined. From the physiology of vascular tone regulation, we propose some potential assays below.

NO is produced and released by ECs and acts on VSMCs to increase cyclic guanosine monophosphate (cGMP) signaling, causing smooth muscle relaxation and a reduction in blood pressure [98]. Intracellular NO in ECs can be quantified with the fluorescent dye 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM) [99]. When NO is released into cell culture media, it is quickly converted to nitrate and nitrite [100]. Nitrite can be detected with the Griess reagent. To assess total NO production, nitrate can be converted to nitrite with nitrate reductase and subsequently quantified by the Griess assay [100].

VSMC contractility can be assessed with time-lapse and traction force microscopy to evaluate the contractile response to agents such as carbachol, angiotensin II, and endothelin I [92, 101–103]. VSMC contractility, in addition to vascular stiffness, can be affected by the contractile to synthetic phenotypic shift of VSMCs, which can be assessed through

proliferation, migration, and extracellular matrix production assays as described above [91].

Thrombosis and Hemorrhage

Thrombosis is associated with a subset of chemotherapeutic drugs and can manifest as thrombophlebitis, arterial or venous thrombosis, or ischemic cerebrovascular events. The parallel plate flow chamber is a commonly used *in vitro* thrombosis system and contains a hollow rectangular space with millimeter or centimeter scale dimensions, through which blood or its components can be transported to induce physiological wall shear stresses over purified proteins (such as, von Willebrand factor or tissue factor), extracellular matrix (collagen, laminin, or fibronectin), or EC monolayers [104, 105]. Another *in vitro* approach to thrombosis involves microfluidic devices, which more accurately replicate blood vessel anatomy. A major advantage of microfluidic devices is that different anatomical structures (e.g., atherosclerotic plaques, bifurcations etc.) and complex flow patterns (pulsatile motion, rapid accelerations, and decelerations etc.) observed *in vivo* can be included in these models. These systems have successfully been used to model the process of thrombosis in various diseases [106–109]. A recent study demonstrated the potential to create microfluidic devices that exclusively utilize patient-derived blood outgrowth endothelial cells. These tissue-engineered blood vessels exhibit normal physiological function with cells from healthy individuals. However, those produced with cells obtained from diabetic pigs show the diabetic phenotype *in vitro*, demonstrating the ability of these systems to model patient-specific features [110]. Incorporation of hiPSCs into these models would allow for inclusion of multiple patient-specific cell types (some of which would otherwise be difficult to obtain) in order to investigate factors that contribute to patient-specific thrombosis in the context of chemotherapeutics.

While many chemotherapeutics are associated with thrombosis, drugs, such as vandetanib, axitinib, and regorafenib, are associated with hemorrhage. In the context of bleeding disorders, microfluidic systems successfully recapitulate the clotting dysfunction and propensity towards bleeding, suggesting that these systems are useful for the evaluation of both thrombosis and hemorrhage [109].

Immune-Related CICTs

Immune-related CICTs occur with a variety of chemotherapeutics and manifest in myocarditis, pericarditis, pericardial and/or pleural effusion, edema, and hypereosinophilic cardiac toxicity. To date, the majority of work in these conditions has been conducted in animal models [111]. Human models of immune-related CICTs would be informative and bypass interspecies differences observed in animal models. However, the use of *in vitro* cell models for these conditions has been limited. Sharma et al. [112] developed an hiPSC-CM model of myocarditis caused by coxsackievirus. Infected hiPSC-CMs displayed erratic beating with eventual cessation of beating. Responses to known antiviral compounds in this *in vitro* model were consistent with the known effects of these drugs in vivo [112]. Drug-induced immune CICTs often result from activation of autoimmunity [113]. hiPSCs have successfully been incorporated into investigations of multiple autoimmune diseases and likely hold promise for drug-induced immune phenotypes as well [114, 115]. We are not aware of any current hiPSC models of drug-induced immune phenotypes, but future models would likely include the relevant tissue type (e.g., hiPSC-CMs for myocarditis) as well as relevant hiPSC-derived immune cells. Assays would include those related to the tissue type (e.g., viability, electrophysiology, etc. for hiPSC-CMs) as well as immune-cell-specific assays such as cytokine release, migration, and proliferation [116, 117].

hiPSCs to Advance CICT Pharmacogenomics

Establishment of the appropriate hiPSC model system and assay(s) creates a platform with a defined readout for more and less susceptible CICT phenotypes. With this system established, whole genes and individual variants can be manipulated to determine how they affect this defined readout.

hiPSCs have confirmed a genomic contribution to CICT susceptibility. A major obstacle in CICT pharmacogenomics to date is that genome association studies are hindered by multiple shortcomings related to sample size, replication, and distinction of causality from correlation. hiPSCs are well-positioned to address this gap. hiPSCs can be easily modified through genome editing approaches to introduce or remove both whole genes and specific variants. Through genomic editing, hiPSCs allow for validation of significant variants identified in CGAS and GWAS studies. This approach has been used successfully to validate GWAS variants in the context of metabolic disease [118]. Additionally, hiPSC models can contribute to novel variant discovery [119, 120].

Beyond traditional CGAS and GWAS studies, more recent studies have examined the association between the genome and mRNA levels, themselves quantitative traits, in expression quantitative trait locus (eQTL) mapping [121–123]. Traditional eQTL analysis has been performed on primary patient samples (~ 60), comparing gene expression with genotypes. hiPSC models permit comparison of gene expression both with and without the chemotherapeutic of interest to establish differential eQTL (deQTL) related to the variation between patients in the gene expression response to drug. Using primary patient samples, the drug and disease status of an individual could confound the association of gene expression and their genotype. hiPSCs are not influenced by a

patient's disease status and would thus allow for a true recapitulation of the effect of their genetic variation. Though deQTL mapping has only been demonstrated a small number of times [124, 125], these studies have confirmed that deQTL mapping is uniquely powerful and that a majority of genome-wide findings would not have been uncovered via analysis of untreated (i.e., non-differential) samples.

Conclusions and Future Directions

Chemotherapeutics are increasingly effective at allowing patients to outlive their cancer. As the number of cancer survivors increases, we are developing a greater realization of the long-term adverse cardiovascular effects of some of these treatments. Understanding who is most susceptible to these adverse effects and the mechanisms by which they occur will allow providers to select alternative medications (where possible) and will promote drug discovery with respect to both adjuvant protective therapies and novel cancer therapies with fewer cardiovascular effects.

Researchers have invested substantial resources into chemotherapy side effect pharmacogenomics, but we are still without consensus regarding which variants are critical in determining patient susceptibility, and the vast majority of chemotherapeutics have yet to be studied. Additionally, the mechanisms of many side effects remain unexplained, which impairs our ability to identify potential protective strategies.

hiPSC models represent a path forward for CICT research. By providing a high-throughput, patient-specific system that is amenable to genome editing, hiPSCs will allow the field to model numerous CICTs, enabling us to better understand these side effects and identify susceptible patients in order to avert long-term cardiovascular health effects of cancer treatment. As the field of cardio-oncology continues to grow, hiPSCs will be an indispensable tool to study drug effects and develop targeted solutions that ultimately translate into more effective patient care.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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