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Themed Section: New Insights into Cardiotoxicity Caused by Chemotherapeutic Agents

# REVIEW ARTICLE

# Integrating cardiomyocytes from human pluripotent stem cells in safety pharmacology: has the time come?

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Cardiotoxicity is a severe side effect of drugs that induce structural or electrophysiological changes in heart muscle cells. As a result, the heart undergoes failure and potentially lethal arrhythmias. It is still a major reason for drug failure in preclinical and clinical phases of drug discovery. Current methods for predicting cardiotoxicity are based on guidelines that combine electrophysiological analysis of cell lines expressing ion channels ectopically *in vitro* with animal models and clinical trials. Although no new cases of drugs linked to lethal arrhythmias have been reported since the introduction of these guidelines in 2005, their limited predictive power likely means that potentially valuable drugs may not reach clinical practice. Human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs) are now emerging as potentially more predictive alternatives, particularly for the early phases of preclinical research. However, these cells are phenotypically immature and culture and assay methods not standardized, which could be a hurdle to the development of predictive computational models and their implementation into the drug discovery pipeline, in contrast to the ambitions of the comprehensive pro-arrhythmia *in vitro* assay (CiPA) initiative. Here, we review present and future preclinical cardiotoxicity screening and suggest possible hPSC-CM-based strategies that may help to move the field forward. Coordinated efforts by basic scientists, companies and hPSC banks to standardize experimental conditions for generating reliable and reproducible safety indices will be helpful not only for cardiotoxicity prediction but also for precision medicine.

## LINKED ARTICLES

This article is part of a themed section on New Insights into Cardiotoxicity Caused by Chemotherapeutic Agents. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v174.21/issuetoc

## Abbreviations

2D, two dimensional; 3D, three dimensional; AP, action potential; APD, AP duration; $APD_{90}$, APD at 90% of the repolarisation phase; BVR, beat-to-beat variability of the repolarisation duration; CiPA, comprehensive *in vitro* proarrhythmia assay; CMs, cardiomyocytes; ECG, Electrocardiogram; EMA, European Medicines Agency; FDA, US Food and Drug Administration; hERG, human *ether-à-go-go*-related gene; hESC-CMs, human embryonic stem-cell derived CMs; hESC, human embryonic stem cells; hiPSC-CMs, human induced pluripotent stem-cell derived CMs; hiPSC, human induced pluripotent stem cells; hPSC-CMs, human pluripotent stem-cell derived CMs; hPSC, human pluripotent stem cells; $I_{CaL}$, L-type $Ca^{2+}$ current; ICH, International Council on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use; $I_{K,ATP}$, ATP-sensitive $K^+$ current; $I_{K,slow1}$, Component 1 of the ultra-rapid delayed rectifyer $K^+$ current (4-Aminopyridine sensitive); $I_{K,slow2}$, Component 2 of the ultra-rapid delayed rectifyer $K^+$ current; $I_{K1}$, Inward-rectifier $K^+$ current; $I_{Kr}$, Rapid component of the delayed-rectifier $K^+$ current; $I_{Ks}$, Slow component of the delayed-rectifier $K^+$ current; $I_{SS}$, Steady-state $K^+$ current; $I_{to}$, Transient-outward $K^+$ current; MEA, multi electrode array; PMDA, Japanese Pharmaceuticals and Medical Devices Agency; TdP, Torsades de pointes; $V_m$, membrane potential



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## Tables of Links

| TARGETS | |
|---|---|
| **Voltage-gated ion channels**[a] | **Transporters**[b] |
| $Ca_v1.2$ | NCX |
| $K_{ir}2.1$-2.3 | |
| $K_{ir}6.2$ | |
| $K_v4.3$ | |
| $K_v7.1$ | |
| $K_v11.1$ (hERG) | |

| LIGANDS |
|---|
| Astemizole |

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b]Alexander *et al.*, 2015a,b).

## Introduction

Electrophysiological or structural damage of the myocardium as an unintentional side effect of pharmacological treatment is referred to as cardiotoxicity. The resulting contractile and electrical dysfunction often leads to abnormal heartbeat and life-threatening arrhythmias (Ewer and Ewer, 2015; Gintant *et al.*, 2016). Arrhythmia is currently an immense burden on public health, with millions of people at risk of heart malfunction because of the cardiotoxic effects of drugs (Rubinstein and Camm, 2002; Vejpongsa and Yeh, 2014; Viskin *et al.*, 2015). Individuals with an underlying but undiagnosed genetic risk may be especially vulnerable because their need for safer alternatives already on the market may be unrecognized (Svanström *et al.*, 2014; Schwartz and Woosley, 2016).

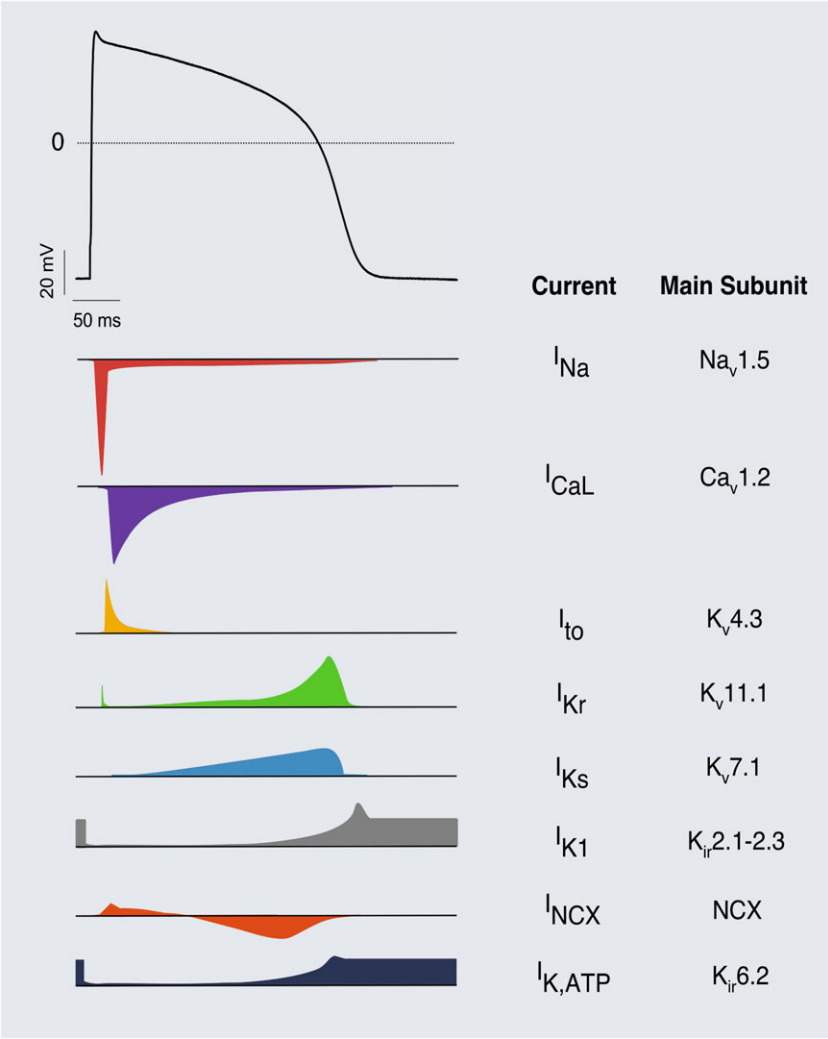
Cardiotoxicity is an important hurdle to drug discovery, with many compounds discarded during drug development because of potential toxic effects on the heart (Laverty *et al.*, 2011; Hay *et al.*, 2014) and difficulties in predicting how the human heart will respond. Cardiotoxicity is still a predominant cause of preclinical and clinical drug failure (McNaughton *et al.*, 2014; Onakpoya *et al.*, 2016; Siramshetty *et al.*, 2016). In general, it is most conveniently categorized as (i) physical damage to cardiomyocytes (CMs) or (ii) electrical disruption, depending on the molecular target or function impaired by the drug.

## Cardiotoxicity caused by physical damage to cardiomyocytes

Structural properties of CMs can be profoundly disrupted by pharmacological treatments. Physical damage is primarily associated with drugs specifically designed to be cytotoxic, such as chemotherapeutic agents in cancer treatment (Cross *et al.*, 2015; Ewer and Ewer, 2015). These drugs induce mitochondrial damage (Varga *et al.*, 2015), increase oxidative stress (Cross *et al.*, 2015), activate DNA damage response pathways (Mercurio *et al.*, 2016) and increase apoptosis (Arola *et al.*, 2000). The net outcome for the heart may be many fewer CMs, with a residual proportion being vital but unhealthy. As a result, the contractile force of cardiac muscle is reduced over time (Ewer and Ewer, 2015). In turn, this reduces the ejection fraction from the left ventricle, and heart failure ensues (Cardinale *et al.*, 2015). Indirect consequences of this are severe secondary electrical abnormalities emerging after medium- to long-term drug exposure (Albini *et al.*, 2010); the heart becomes arrhythmic and unable to cope with the physiological demand for oxygen and nutrients. Identifying these drug risks in humans has been challenging because of the paucity of predictive experimental models, but new approaches based on human pluripotent stem cell-derived CMs (hPSC-CMs) are revealing the ability to replicate cardiotoxicity as evident in the clinical context (Klimas *et al.*, 2016), most recently in precision or personalized approaches to chemotherapeutic agents (Maillet *et al.*, 2016), like the anthracyclines used for breast cancer treatment (Bellin and Mummery, 2016; Burridge *et al.*, 2016).

## Cardiotoxicity caused by electrical disruption

A second form of cardiotoxicity, commonly evident in the early phases of drug discovery, is the acute and direct, detrimental effect of drugs on the electrophysiological properties of CMs (Cook *et al.*, 2014). Electrical homeostasis is crucial for coordinating synchronous and functional contraction of the heart, and its disruption significantly reduces cardiac output. In ventricular CMs, the cardiac action potential (AP), a transient oscillation of the electrical membrane potential ($V_m$), is initiated by an electrical stimulus sufficient to overcome the threshold of voltage-gated $Na^+$ channels, which open and rapidly depolarize $V_m$ (Figure 1). Subsequently, tuned balance of the repolarizing currents [i.e. the transient outward current ($I_{to}$), rapid ($I_{Kr}$) and slow ($I_{Ks}$) components of the delayed-rectifier $K^+$ current, the inward-rectifier $K^+$ current ($I_{K1}$) and the ATP-sensitive $K^+$ current ($I_{K,ATP}$)] slowly counteract the L-type $Ca^{2+}$ current ($I_{CaL}$) until they prevail, terminating the AP. Anything that modifies the frequency or the contour of the AP dictates arrhythmogenic risk. These properties are also precisely targeted by antiarrhythmic agents.




## Figure 1

Typical ventricular action potential. Scheme of the relative contribution of ion currents to the cardiac AP. Currents and their respective α-subunits are indicated on the right.

Some of these channels, however, have been described as being particularly prone to block by some compounds, and this can initiate arrhythmias. It is a recurring condition for the $K_v11.1$ channel [encoded by the human ether-à-go-go-related gene (hERG) also called *KCNH2*], which, together with its β-subunit MinK-related peptide 1, is responsible for $I_{Kr}$. Key aromatic residues in the S6 helix ($Tyr^{652}$, $Phe^{656}$) and the pore helix ($Thr^{623}$, $Ser^{624}$, $Val^{625}$) of the $K_v11.1$ channel have been identified as the detrimental target of a broad spectrum of compounds (Perry, 2005) that impair $K^+$ ion flux through the pore and therefore reduce $I_{Kr}$. This generates a prolongation of the QT interval of the electrocardiogram (ECG), with the potential occurrence of a polymorphic form of ventricular tachycardia known as torsades de pointes (TdP), which can cause sudden death (Sanguinetti and Tristani-Firouzi, 2006).

Life-threatening arrhythmic events may arise when any drug (or its metabolites) is (i) itself pro-arrhythmic in all individuals in any population; (ii) generally safe in most of the population but cardiotoxic on specific genetic backgrounds or in the context of pre-existing conditions; and (iii) generally safe but cardiotoxic in combination with other drugs, perhaps on specific genetic backgrounds. A major goal in drug discovery is then to design drugs with low chances of interactions with ion channels other than the ones targeted and, most importantly, to be able to predict in which of the three categories above that each potential new drug belongs. This would allow adverse drug effects to be identified in early preclinical phases of development, before entering (expensive) phase III clinical trials and introducing the drug to market when it may be prescribed unknowingly to patients at risk. The lack of unequivocal predictive scores has resulted in a series of tests that can only provide an indication of clinical safety when combined together (ICH, 2005a). This is illustrated by the sharp reduction in drug withdrawal over the last decade because of arrhythmogenic events, largely attributable to the widespread introduction of compulsory hERG channel assays (Stockbridge *et al.*, 2012). What sometimes goes almost unnoticed though is the concomitant fall in the approval rate of new drugs despite the unprecedented increase in R&D investment in the last 10 years (Kola and

Landis, 2004; Bunnage, 2011; Hay *et al.*, 2014; Waring *et al.*, 2015). This indicates that current methods are still not meeting needs, perhaps providing negative indications for drugs that could be of clinical benefit. Novel and more comprehensive approaches specifically addressing risk in humans should be pursued, both in the early preclinical phases as well as later as drugs move towards human studies (Woosley and Romero, 2013), most particularly for drugs that cannot, or must not, be tested on healthy volunteers because of narrow therapeutic windows.

In this review, we describe how hPSC-CMs have been used for cardiotoxicity detection, how they compare with current alternative technologies, and whether they might eventually be introduced as effective *in vitro* predictors of cardiotoxicity *in vivo*. We also discuss how they might contribute to solving the current stagnation in drug approval.

## Cardiotoxicity screening methodologies

During the 1990s, the main drug regulatory agencies, the European Medicines Agency (EMA), the US Food and Drug Administration (FDA) and the Japanese Pharmaceuticals and Medical Devices Agency (PMDA) started investigating the interactions between approved drugs and $K_v11.1$ (hERG) channels following several cases of lethal arrhythmias associated with commonly prescribed drugs, which was later correlated with an unusually high affinity for $K_v11.1$ (Friedrichs *et al.*, 2005; Shah, 2005). Since 2005, it has become mandatory for all new drugs seeking regulatory approval to demonstrate that the $IC_{50}$ of the candidate drug for $K_v11.1$ is as distant as possible from the target of interest [ICH-S7B, (ICH, 2005b)]. A 30-fold separation margin was found to be highly predictive for hERG block in preclinical tests (Redfern *et al.*, 2003); furthermore, clinical studies on healthy volunteers [ICH-E14, Thorough QT/QTc Study (ICH, 2005a; Fermini *et al.*, 2015)] must follow *in vitro* hERG tests for new drugs, and also be carried out for any other drugs that have undergone substantial alterations in formulation, administration route or target population (Shah, 2005).

The most convenient way to assess $K_v11.1$ interaction *in vitro* is the hERG blockade assay (Hancox *et al.*, 2008). Immortalized mammalian cell lines such as HEK or CHO are genetically engineered to express $K_v11.1$ channels ectopically [or, better, combinations of selected ion channels as recently proposed in the MICE approach (Kramer *et al.*, 2013)]. These assays are extensively used in the preclinical characterization of drugs because of low cost and flexibility. Traditional low-throughput techniques for measuring effects on these channels such as the manual patch clamp, although very sophisticated and accurate, have been complemented in recent years by reliable and more affordable high-throughput technologies, which allow simultaneous automated screening of many compounds and ion channels. A recent alternative, although not yet formally approved as a replacement for the standard hERG blockade assay, is the use of [$^3$H]-dofetilide displacement from $K_v11.1$ channels assessed as radioactive decay over time when the [$^3$H]-dofetilide is displaced by higher affinity, non-radioactive compounds competing for its same binding site (Diaz *et al.*, 2004; Yu *et al.*, 2015). Although this increases the throughput over classical assays, it falls short in capturing the functional and biophysical aspects of hERG blocks, in particular for those compounds that do not compete for the dofetilide binding site on $K_v11.1$ channels.

However, although $K_v11.1$ block is a discrete indicator of arrhythmogenicity, the former does not imply the latter, as demonstrated for verapamil (Liang *et al.*, 2013) and ranolazine (Hancox *et al.*, 2008), with a significant chance of generating false-positive or false-negative outcomes. Moreover, no information is obtained on the contractile behaviour or temporal dispersion of repolarization; the latter, with a crucial role in the genesis of lethal arrhythmia (Townsend and Brown, 2013), cannot be derived from simplified *in vitro* models, which further limits the predictivity of these approaches.

The spectrum of ion channels and contractile proteins required for reliable pro-arrhythmic prediction scores should reflect that of adult CMs. Animal models recapitulate human cardiac physiology to a certain approximation so that they are widely used for arrhythmia predictivity. Small animal species like mice can also be genetically engineered to generate tailored models of either monogenic or complex diseases. Although some differences between drug effects *in vivo* and *ex vivo* have been reported (Bentzen *et al.*, 2011), isolated hearts connected to Langendorff perfusion devices have been valuable for studying drug effects in relatively controlled, but metabolically isolated conditions. This has often been carried out in combination with $Ca^{2+}$- and voltage-sensitive dyes, to analyse electrical dispersion and propagation (Akar and Akar, 2006), generate conduction maps (Yu *et al.*, 2016) and $Ca^{2+}$-handling profiles (Herron *et al.*, 2012).

Isolated CMs from medium/large animals, most particularly rabbits/dogs, are presently regarded as the gold standard for the investigation of pharmacological targets and for the prediction of the pro-arrhythmic potential of novel compounds. However, there are substantial and not negligible inter-species differences, in particular in the genetics of transgenic models because the human genome differs from that of widely used animal species; correctly mimicking the effects of genetic mutations is only performed routinely in mice, which differ substantially from humans in electrophysiology and $Ca^{2+}$-handling. A more rapid heartbeat (500 versus 60 beats $min^{-1}$ in humans), the predominant role of potassium currents [transient-outward ($I_{to}$), ultra-rapid components 1 ($I_{K,slow1}$) and 2 ($I_{K,slow2}$) of the delayed-rectifier $K^+$ current, and steady state ($I_{SS}$) $K^+$ currents], the absence of the plateau phase generated by the L-type $Ca^{2+}$ current and a smaller contribution of the $Na^+$-$Ca^{2+}$ exchanger in the removal of $Ca^{2+}$ from the cytosol, shape the AP in CMs from small rodents, limiting their arrhythmogenic predictivity, with pharmacological responses and drug sensitivities often far from those of human CMs (Nerbonne, 2004; Martignoni *et al.*, 2006; Ahrens-Nicklas and Christini, 2009; O'Hara and Rudy, 2012; Himmel, 2013). Large animal models are then required as endpoints in preclinical phases of research (Koeppel *et al.*, 2012), but they come at high cost, cannot easily be genetically modified and they still show the inter-species differences from humans. Although extremely useful and not replaceable in the short term, these models are nevertheless low throughput and do not support large-scale drug–drug interaction screening (Milan and MacRae, 2005).

Finally, primary human cardiac tissue can be obtained from biopsies collected during heart surgery. However, this is limited in quantity and is often only available from certain patient groups, for example, after sudden death from non-cardiac causes, so that they cannot be considered as representative of the general population. In addition, because samples cannot be collected repeatedly from the same source, their use in drug screening is limited. Furthermore, adult CMs de-differentiate in prolonged culture, which is also a major drawback (Mitcheson *et al.*, 1998).

### *Human pluripotent stem cell-derived cardiomyocytes*

An alternative source of functional human CMs without the limitations of collecting primary heart tissue is to derive them from human embryonic- (hESC) or induced pluripotent (hiPSC) stem cells. Protocols are available to direct cell differentiation towards many somatic cell lineages, including CMs (Mummery *et al.*, 2012; Schwach and Passier, 2016). Chemically defined media supplemented with developmentally relevant growth factors [or small molecules that activate similar pathways, (Burridge *et al.*, 2014; Chen *et al.*, 2014; van den Berg *et al.*, 2016)] are now commercially available or sold as complete kits so that deriving CMs from pluripotent stem cells (as hESC-CM or hiPSC-CM) is now feasible for most laboratories. Because legal and ethical issues restrict the use of hESC in some countries, hiPSC are preferred by most regulatory authorities seeking to establish reliable cardiotoxicity assays. Healthy and diseased hiPSC lines are increasingly available through dedicated cell banks [e.g. NIH (USA), StemBancc (EU), Human Induced Pluripotent Stem Cells Initiative (UK), Wellcome Trust (UK), New York Stem Cell Foundation (USA), and California Institute of Regenerative Medicine (USA)], and hiPSC-CMs are also commercially available (e.g. from Cellular Dynamics International iCell® CMs, Axiogenesis Cor.4U®, Pluriomics Pluricyte®.).

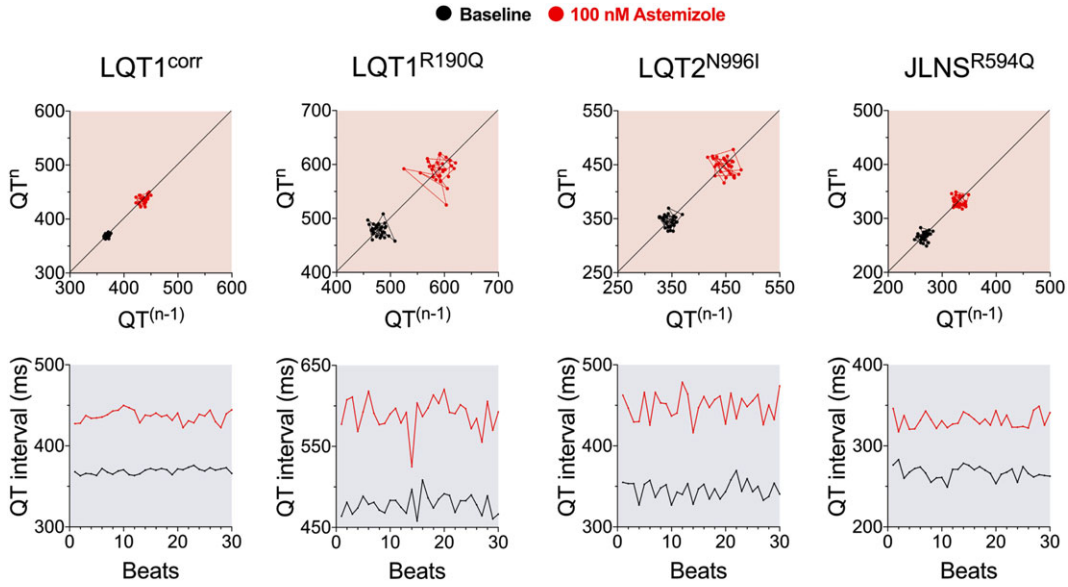
Research over the last few years has increasingly documented the potential of hiPSC in replicating (genetic) cardiovascular disease phenotypes, monogenic cardiac channelopathies being among the most common class of diseases modelled (Bellin *et al.*, 2012; Šarić *et al.*, 2014). In addition, hiPSC-CMs are also being used as model systems to test the effect of both known and novel drugs on cardiac channelopathies (Matsa *et al.*, 2011; Zhang *et al.*, 2012; Bellin *et al.*, 2013; Di Pasquale *et al.*, 2013; Zhang *et al.*, 2014), with suppression of pathological pro-arrhythmic potential and restoration of altered AP duration (APD) being two main endpoints. Clinical evidence has shown that pharmacological treatments can be of more benefit when properly calibrated in a disease-specific or even mutation-specific manner (Nebert and Vesell, 2006; Cavallari, 2012; Amin and Wilde, 2016; Itoh *et al.*, 2016; Martiniano *et al.*, 2016). This has been particularly evident for monogenic diseases targeting the cardiac electrical system (Priori, 1998; Tan *et al.*, 2006), because genetic modifiers critically determine the clinical phenotype (Duchatelet *et al.*, 2013; de Villiers *et al.*, 2014). For these reasons, a renewable source of healthy human- and patient-derived CMs represented by hiPSC have potential as valuable tools for measuring pathological phenotypes, mechanisms underlying disease and pharmacological responses.

More recently, translational crosstalk between patients and hiPSC-CMs has shown how closely they can mimic clinical drug responses. Examples include long-QT syndrome (Terrenoire *et al.*, 2013; Malan *et al.*, 2016), catecholaminergic polymorphic ventricular tachycardia (Penttinen *et al.*, 2015) and heart failure with preserved ejection fraction (Raphael *et al.*, 2016). The cardiotoxic effects of anthracyclines in cardio-oncology (Burridge *et al.*, 2016) have also been recently validated (Avior *et al.*, 2016).

## Assays and readouts

At appropriate seeding densities, hPSC-CMs can form a functional cardiac syncytium. Electrical signals resembling the QT interval of the ECG and defined field potentials can be recorded through extracellular- (Harris *et al.*, 2013; Nozaki *et al.*, 2014; Clements, 2016) or intracellular- (Fendyur and Spira, 2012; Spira and Hai, 2013) electrodes using multi electrode arrays (MEAs). Some medium-throughput MEA platforms also allow contractions (displacement) to be measured through impedance (Li *et al.*, 2016; Obergrussberger *et al.*, 2016), generating highly reproducible results (Lu *et al.*, 2015). In some cases, it can be valuable to electrically 'pace' CMs so that their beating rate (defined RR-like interval because it reflects the analogue ECG parameter) is standardized and constant rather than irregular, as often occurs if beating is spontaneous. However, in contrast to isolated cells or neuronal networks, clusters of hPSC-CMs seeded on MEA cannot efficiently be paced in all available devices, because the hardware is not suitable (Natarajan *et al.*, 2011). Although methods for electrical stimulation have been implemented in some custom-made MEAs (Kaneko *et al.*, 2012) and impedance-based systems (Xi *et al.*, 2011), these are not yet widely used. The QT interval duration in humans and in hPSC-CMs directly depends on the RR interval, such that heart rate correction methods based on Bazett's or Fridericia's formulae (Johnson and Ackerman, 2009; Kaneko *et al.*, 2012) have been developed to minimize this dependency and allow the comparison of QT intervals from samples/patients with different beat rates. Although these corrections are valid for patients (i.e. when frequencies fall in the clinical range), the absence of uniform QT-RR relationships in hPSC-CMs prevents calibration of such methodologies *in vitro* on a large scale and could cause biased interpretation of corrected QT intervals (Sala *et al.*, 2016). A standardized stimulation protocol with crescent frequencies carried out on multiple cell lines would be useful for this purpose and might address QT correction issues that have been a matter of dispute, eventually leading to more reliable cell line-specific QT correction methods for hPSC-CM disease modelling and pharmacology (Woosley and Romero, 2013).

Furthermore, because QT-like and RR-like intervals on MEAs share certain similarities to ECG signals, it could be feasible to apply some of the clinical pro-arrhythmic evaluation strategies to MEA; QT variability index (Berger, 2003) or beat-to-beat variability of the repolarisation duration [BVR (Pueyo *et al.*, 2016; Stams *et al.*, 2016)] can be informative, especially considering the pivotal role of the $K_v11.1$ channel in shaping the repolarisation stability (Altomare *et al.*, 2015). Data analysis algorithms could allow automatic quantification of both short- and long-term components of the repolarisation



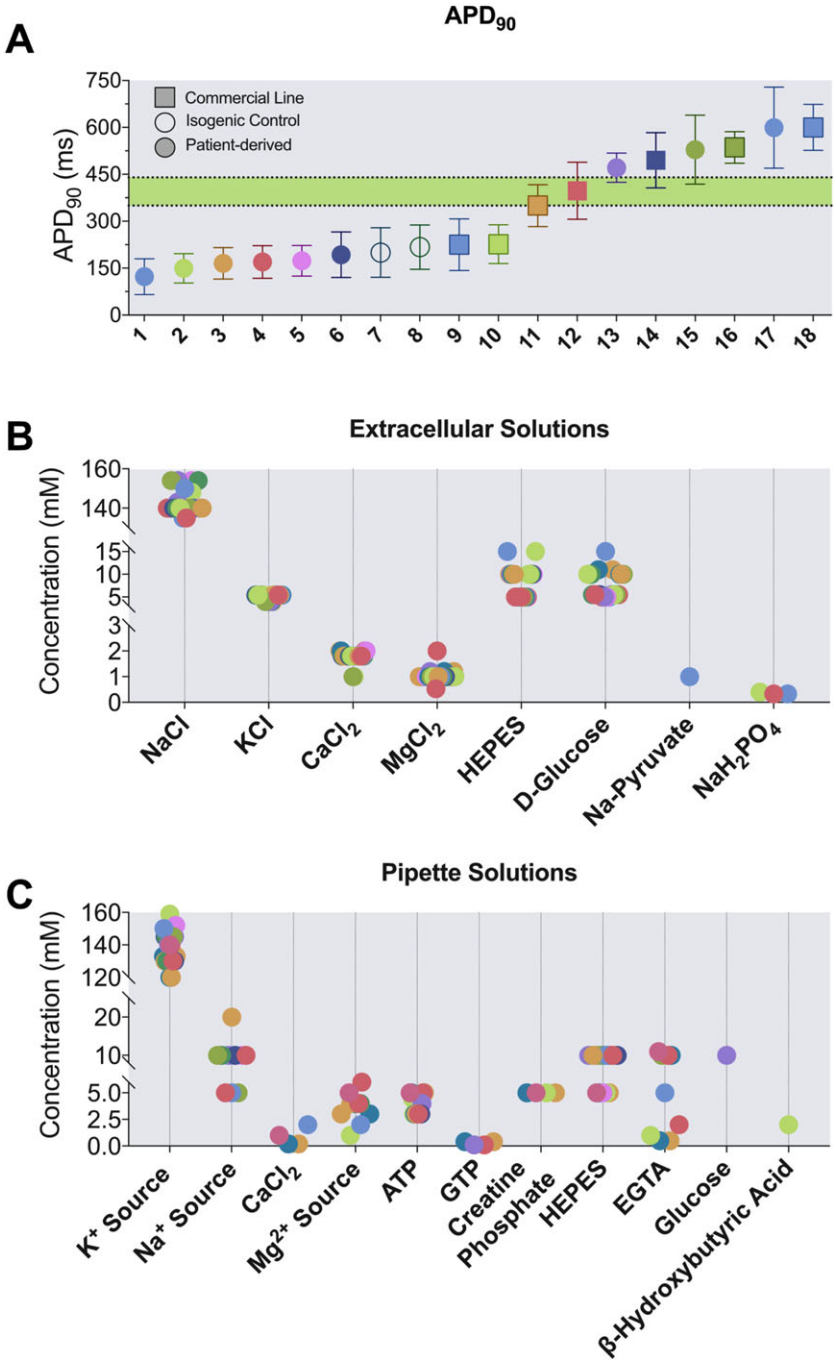

## Figure 2

BVR analysis. Representative Poincaré plots (top) and QT interval sequences for 30 consecutive beats (bottom) for individual experiments at MEA, under control conditions (black) and after $I_{Kr}$ block with 100 nM astemizole (red) in hiPSC-CMs lines with long-QT syndrome ($LQT1^{R190Q}$, $LQT2^{N996I}$, $JLNS^{R594Q}$) and one hiPSC-CMs control isogenic to the $LQT1^{R190Q}$ ($LQT1^{corr}$).

variability in a selected series of beats (Sala *et al.*, 2016). Its high dependence on the beating frequency could also allow the identification of key regulators of the RR interval variability in hPSC-CMs. Representative examples of the application of BVR analysis to hPSC-CMs are shown in Figure 2. Particular attention should be given to generating standard recording protocols for drug testing, because it has been demonstrated (for irreversible agonists/antagonists) that the volume of liquid in which a drug is dissolved may influence the outcome of the test (Cavero *et al.*, 2016).

Although straightforward to produce and maintain in culture, two-dimensional (2D) layers of hPSC-CMs on plastic or glass substrates do not entirely replicate the three-dimensional (3D) micro-environment of myocardium nor its physiological properties such as cyclic stretch, CM-non-CM or CM–CM interaction and communication, contractile force and paracrine signalling (Beauchamp *et al.*, 2015). In addition, 2D monotypic cultures do not resemble the complex cellular composition of the adult heart, where CMs represent only one third of the total number of cells: they are normally incorporated within networks of smooth muscle cells/pericytes, endothelial cells and fibroblasts (Tirziu *et al.*, 2010) and covered by epicardium on the outside and endocardium on the inside layer. The generation of cardiac microtissues (Beauchamp *et al.*, 2015), engineered heart tissues (Zimmermann and Schneiderbanger, 2002; Turnbull *et al.*, 2014) or 3D vascularized cardiac layers (Mosadegh *et al.*, 2014) have been reported. These multidimensional and heterogeneous environments promote CM organization and maturity and will thus probably contribute, in the future, to enhancing the predictivity and reliability of these platforms for drug screening when compared with 2D cultures (Eder *et al.*, 2016). Using edge-detection contractility, Ravenscroft *et al.* observed that *in vitro* measurements were more likely to replicate *in vivo* data when CMs were cultured with endothelial cells and cardiac fibroblasts, suggesting a contribution of non-CMs to drug responses (Ravenscroft *et al.*, 2016).

The immature phenotype, low levels of extracellular matrix proteins and the membrane structure of hPSC-CMs make them considerably more tolerant of enzymatic dissociation for FACS analyses or automated cell manipulation than adult CMs (Bhattacharya *et al.*, 2014; Scheel *et al.*, 2014). Ion currents and AP can be measured with a sufficient level of precision with these systems that the chances of generating large-scale, standardized methods for measuring cardiotoxicity are increased (Scheel *et al.*, 2014). However, the success of these approaches will, nevertheless, be determined by progress in improving the maturation status of hPSC-CMs, that is, the more hPSC-CMs resemble adult CMs, the less they will tolerate invasive techniques. For this reason, less invasive alternatives are being already being developed in parallel, with the interest in voltage-sensitive dyes (Burridge *et al.*, 2011) and optogenetics (Park *et al.*, 2014; Chang Liao *et al.*, 2015; Song *et al.*, 2015) significantly rising (Dempsey *et al.*, 2016). At present, their widespread implementation in drug screening is limited by the slow kinetics and the relatively low signal/noise ratio of voltage sensors, with data still requiring proper validation by low-throughput patch clamp analyses. Incorporation of these new methods in ICH guidelines would be premature and unlikely short term. For chemical and genetically encoded $Ca^{2+}$-sensitive dyes, the situation is easier, mainly due to the slower kinetics of $Ca^{2+}$ fluxes. These probes have already been widely used for high-throughput screening in both disease modelling and drug development with encouraging results (Kaestner *et al.*, 2014; Huebsch *et al.*, 2015; Lu *et al.*, 2015; Rast *et al.*, 2015; Dempsey *et al.*, 2016; Klimas *et al.*, 2016). $Ca^{2+}$-handling also affects cell contractility, which can also be measured, as recent examples with




## Figure 3

Comparison of wild-type hiPSC-CMs in the literature. (A) $APD_{90}$ values measured with patch clamp in paced (1 Hz) hiPSC-CMs at physiological temperature (~37°C); numbers 1–18 indicate wild-type lines as specified below. The green area defines the normal QT interval range for humans. Data are shown as mean ± SD: 1, (Malan *et al.*, 2016); 2, (Rocchetti/Sala *et al.*, unpublished); 3, (Zhang *et al.*, 2014); 4, (Zhang *et al.*, 2014); 5, (Davis *et al.*, 2012); 6, (Rocchetti/Sala *et al*, unpublished); 7, (Bellin *et al.*, 2013); 8, (Sala *et al.*, 2016); 9, (Bizy *et al.*, 2013); 10, (Ma *et al.*, 2013); 11, (Ma *et al.*, 2015); 12, (Ma *et al.*, 2015); 13, (Gibson *et al.*, 2014a); 14, (Itzhaki *et al.*, 2011); 15, (Gibson *et al.*, 2014c); 16, (Lu *et al.*, 2014); 17, (Gibson *et al.*, 2014b); 18, (Mehta *et al.*, 2014). (B) and (C) Composition of extracellular buffers (B) and pipette solutions (C) for current clamp experiments. $K^+$ source indicates the sum of KCl and/or K-aspartate and/or K-glutamate. $Na^+$ source indicates the sum of NaCl and/or the conjugated Na-salts to ATP, GTP or creatine phosphate. $Mg^{2+}$ source indicates the sum of $MgCl_2$ and/or the conjugated Na-salts of ATP, GTP or creatine phosphate.

sophisticated optical mapping systems show (Herron *et al.*, 2012; Hayakawa *et al.*, 2014; Maddah *et al.*, 2015). The combination of engineering and biology has allowed the precise quantification of contractile force in hPSC-CMs (Ribeiro *et al.*, 2015a), although it has been demonstrated that substrate stiffness plays a major role in the quantification of absolute force values (Feinberg *et al.*, 2012; Ribeiro *et al.*, 2015b). More recently, integrated technologies capable of simultaneously quantifying multiple parameters are being implemented with the view to providing reliable, high-throughput tools for early preclinical stages of drug development (Hochbaum *et al.*, 2014; Kijlstra *et al.*, 2015; Rast *et al.*, 2015; Song *et al.*, 2015; Dempsey *et al.*, 2016; Klimas *et al.*, 2016).

### *In silico approaches*

*In silico* approaches are also proving valuable in predicting the pro-arrhythmic potential of drugs. Computational models for ion currents are being generated based on existing electrophysiological data measured in adult animal/human CMs as well as hPSC-CMs and then integrated into complex single- or multi-cellular models (O'Hara *et al.*, 2011; Paci *et al.*, 2013). It is expected that, as computational power increases, the accuracy and complexity of such models will rise. However, the heterogeneity of hiPSC-CM phenotypes may limit the predictive power of current models (Paci *et al.*, 2013, 2015). For example, different differentiation protocols lead to different ventricular phenotypes (Figure 3), integrating reprogramming methods may disrupt genes of interest for the phenotype under study, and the kinetics of hiPSC-CM differentiation may differ between individual lines. This particularly impacts $I_{Ks}$ and $I_{K1}$, whose contribution is essential for a correct prediction of drug effects. Only few attempts have been made to promote phenotypic uniformity in hiPSC-CMs (Hwang *et al.*, 2015a; Zhu *et al.*, 2016), and we believe that the reproducibility of outcomes would benefit from standardized conditions to provide better input for computational models. On the other hand, as highlighted by Rodriguez *et al.*, the cross-institutional limitations of accessing *in vitro* propriety data profoundly slows the development of comprehensive computational models (Rodríguez *et al.*, 2015).

The result is that current mathematical AP models of hPSC-CMs (Paci *et al.*, 2012, 2013, 2015) still represent a fraction of the heterogeneity that has been experimentally observed in literature, although their usefulness and predictivity are beyond doubt (Figure 3). In this context, the coordination and promotion of standard methodologies for phenotypic characterization of differentiated cells may come from cell banks.

## Future perspectives

The comprehensive *in vitro* pro-arrhythmia assay (CiPA) initiative proposes integration of different approaches to quantify the proarrhythmic potential of drugs at three levels: (i) heterologous system, with the expression of a representative spectrum of cardiac ion channels; (ii) *in silico* mathematical models of CMs; and (iii) confirmation of the data from integrated human cellular studies in advanced systems like hiPSC-CMs.

In the coming years, the FDA plans to update the current guidelines, ICH-S7B (ICH, 2005b) and ICH-E14 (ICH, 2005a) for preclinical and clinical evaluation of drug arrhythmogenicity, and it is expected that ICH-S7B will integrate hiPSC-CMs as a platform for 'personalized' drug testing (van der Heyden and Jonsson, 2012; Chen *et al.*, 2016). A very detailed overview on the current status of the CiPA initiative has recently been provided by Cavero *et al.*, who listed in detail many of the technical and regulatory limitations that must be addressed to provide a robust set of protocols that would be needed before implementation of these assays in a safety pharmacology pipeline (Cavero *et al.*, 2016). Although significant progress has been made in this context, in particular with the standardization of patch clamp protocols to evoke $I_{Kr}$ (although only in the manual configuration) and the proposal of an *in vitro* $TdP_{risk}$ score (Crumb *et al.*, 2016), a complete list of unresolved issues and areas needing more discussion, including agreement among researchers, agencies and companies on how precisely to move forward, is at present lacking. This to some extent undermines full implementation in the short term.

## Limitations in applicability of hiPSC-CM to large-scale drug testing

### *Phenotypic immaturity*

As mentioned earlier, one major limitation of hPSC-CMs is their phenotypic immaturity compared with adult CMs. Typical fetal features that they display include automaticity of beating, depolarized diastolic $V_m$, low ion channel expression, delayed excitation-contraction coupling and low contractile force (Veerman *et al.*, 2015). Defined culture conditions (Burridge *et al.*, 2014; Ribeiro *et al.*, 2015a,b) can be used to improve the maturation of hPSC-CMs to reveal hidden disease phenotypes (Birket *et al.*, 2015) or to drive differentiation to chamber-specific cell populations (Devalla *et al.*, 2015), and drug testing might benefit from their standardization. Exogenous stimuli, such as adjusted pacing frequency (Chan *et al.*, 2013), 3D-microenvironments (Zhao *et al.*, 1999; Nunes *et al.*, 2013; Hirt *et al.*, 2014; Eder *et al.*, 2016) and heterotypic cell co-culture (Robertson *et al.*, 2013) may all contribute to cell maturation, increasing the expression of ion channels and improving functional properties. Recent advances in electrophysiology (Meijer van Putten *et al.*, 2015) and cell biology (Vaidyanathan *et al.*, 2016) allow the introduction of exogenous $I_{K1}$ conductance in hiPSC-CMs, although neither single nor combined approaches were successful in mimicking adult maturation state entirely, in particular of $Ca^{2+}$-handling, signalling and compartmentalization, mainly due to their lack of T-tubules (Kane and Terracciano, 2015). Thus, caution is needed when attempting to translate results using hiPSC-CMs to more complex systems. Furthermore, the integration of hiPSC-CMs into drug screening platforms would preferably be based on defined (*xeno*-free) reagents to implement current Good Manufacturing Practice guidelines and would extend to new coatings for substrates that are resistant to drug adsorption

and not yet broadly implemented (Rodin *et al.*, 2014; Leha *et al.*, 2016).

## *Phenotypic variability*

High line-to-line variability (although only modest clonal variability in cells from one line) is still a widespread problem that requires joint efforts to solve, although recent, robust, methodologies have contributed to mitigating the issues (Burridge *et al.*, 2011; Hartjes *et al.*, 2014; Denning *et al.*, 2016). The reproducibility of the results in terms of the absolute values of parameters measured may indeed be due to limited standardization of experimental conditions: few studies carry out head-to-head comparisons of cells from different hiPSC lines derived under identical conditions. Fully automated, high-throughput procedures to reprogramme hiPSC from somatic cells have been developed (Paull *et al.*, 2015) with the aim of limiting variability. Pharmacogenomic analysis of these cell populations may unveil the key sources of variability. A partial solution could be obtained by pooling data from large cohorts of patients to reveal unusual patterns of pharmacological responses associated with specific genotypes, contributing to insights into precision medicine (Collins and Varmus, 2015), although this is still far from the goals of personalized medicine (Food and Drug Administration, 2013), (Cuchiara *et al.*, 2015). It would then be feasible to capture key aspects of variability *in vitro* during preclinical phases of research, before the last – extremely expensive – phases of drug testing. This may reduce the failure rate of current pro-arrhythmic assays by unmasking unexpected drug sensitivities and cardiotoxic effects in the presence of specific triggers.

Individual genetic backgrounds, or DNA epigenetic status (Burridge *et al.*, 2015), appear to influence the differentiation potential (Miyamoto *et al.*, 2015) of hPSC and the cell phenotype after differentiation. A high phenotypic variability has been observed among different lines described as 'healthy controls' (sometimes age and gender matched) and used as a reference in multiple studies (Figure 3). In a study on four isogenic lines from healthy donors (Kyttälä *et al.*, 2016), up to 158 genes were found to be differently expressed among the lines; some were imprinted, evidence for a role of epigenetic memory in determining the phenotype and differentiation potential of hPSC, possibly by affecting their response to growth factors (Pekkanen-Mattila *et al.*, 2009). A

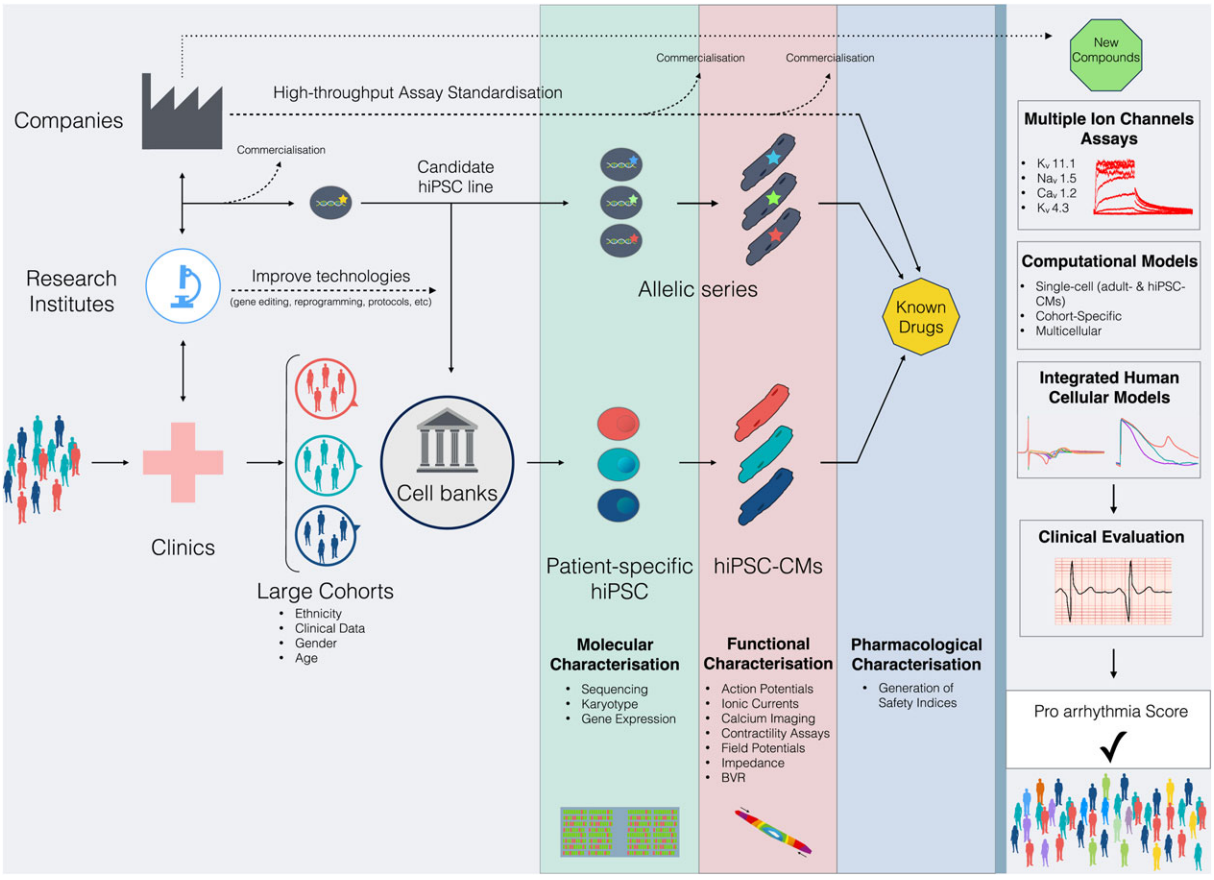
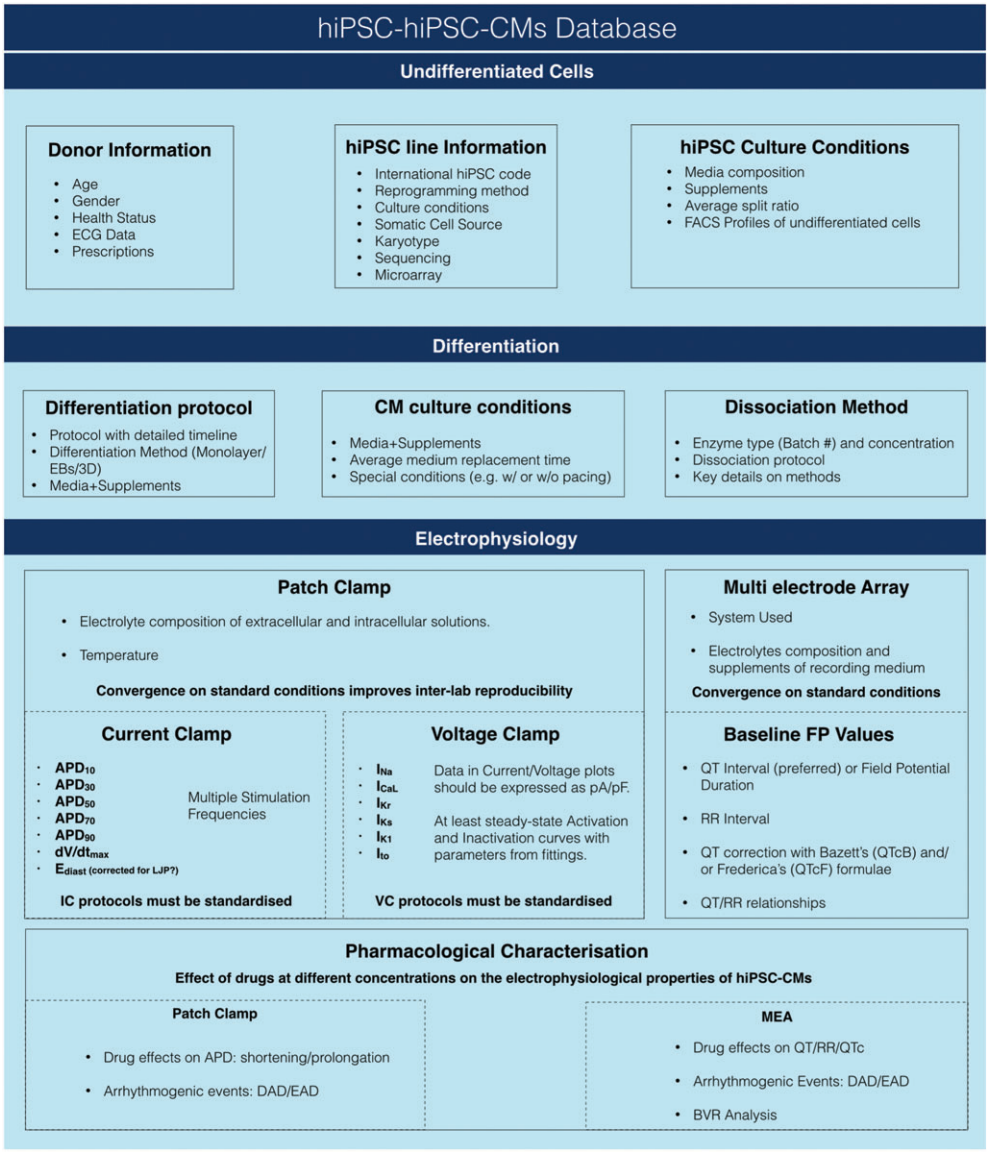



**Figure 4**

Anticipated integration of hPSC-CMs in cardiotoxicity. Samples are collected from large cohorts of patients in clinics along with patient-relevant clinical parameters. Cell banks will reprogramme somatic cells to patient-specific hiPSC using standardized methodologies. The same samples can be used to produce control and/or mutated hiPSC candidate lines in which mutations are rescued or introduced with gene editing technologies. Molecular characterisation of undifferentiated hiPSC and functional characterisations of hiPSC-CMs will outline the pharmacological response to known drugs to generate reliable *safety indices*. Novel drugs will then be tested based on the CiPA guidelines with integrated human cellular models as a predominant preclinical experimental component. The aim is to generate pro-arrhythmic scores applicable to the general population.




## Figure 5

Useful parameters for hiPSC and hiPSC-CMs banking. Data information on hiPSC lines stored in dedicated banks (top). Details of differentiation protocols and culture conditions (centre). Parameters for a comprehensive electrophysiological characterisation of hiPSC-CMs with patch clamp and MEA. EB, embryoid bodies; MEA, multi electrode array; LJP, liquid junction potential; IC, current clamp; VC, voltage clamp; FP, field potential; QTc, corrected QT interval; DAD, delayed after depolarizations; EAD, early after depolarizations.

consequence may then include differential responses to drugs. Jones *et al.* proposed $Ca^{2+}$ synchronization in aggregates of hiPSC as a reliable parameter to identify sources of variability related to culture conditions and maturation status of hiPSC-CMs (Jones *et al.*, 2015).

Even though $Ca^{2+}$-handling features appear conserved in independent hiPSC-CM cultures from different research groups (Hwang *et al.*, 2015a,b; Kane and Terracciano, 2015), heterogeneity has been widely observed in other electrophysiological properties such as the APD (Figure 3), upstroke velocity and current densities (Hoekstra *et al.*, 2012). Inconsistent results on the mechanism of hiPSC-CM automaticity have also been noted: Kim *et al.* reported a $Ca^{2+}$ clock mechanism, consistent with ivabradine having no effect on beating frequency (Kim *et al.*, 2015). In contrast, Bedut *et al.* demonstrated reduced beating rates in hiPSC-CMs at lower doses of ivabradine (1-2 μM vs 3-9 μM) (Bedut *et al.*, 2016). Furthermore, when drug concentrations were compared among different hPSC-CM lines, techniques or studies, the differences appear to be significant and functionally relevant (Kuusela *et al.*, 2016). Broader studies are still required to pinpoint the contribution of donor genetic background and subsequent epigenetic modifications on both cardiac phenotype and drug responses. This could be provided by recent advances in genome-editing techniques, with CRISPR/Cas9 being the current leading method (Sander and Joung, 2014). This approach allows rapid and precise modification of the hPSC genome so that generating isogenically matched

controls for (genetically) diseased hiPSC lines is feasible for many labs, reducing one source of variability. This type of gene repair service is also available commercially. Creating hiPSC biobanks that include not only diseased hiPSC lines as mentioned earlier but also genetically matched controls, with multiple clones of each, is an ambitious goal but could be of major benefit to the field particularly if accompanied by whole genome sequence data. A more modest goal could be the generation of smaller panels (10–50 lines) of *bona fide* wild-type hiPSC lines, representative of the gender and ethnic diversity in specific populations, which might be extremely useful to broaden comparisons from an individual perspective with major genotype classes. These small panels should be extensively characterized, by multiple laboratories and univocally validated with standard procedures under agreed conditions. Updates could be provided by the community of researchers using the panel, so that over time 'golden standards' could be created among hiPSC lines, much as already available for hESC (Figure 4).

Alternatively, very small subsets could be created with whole genome sequence data and validated high differentiation efficiency to all three germ layers. These should be made widely (commercially) available to allow the generation of allelic series for different mutations on the same genetic background (Musunuru, 2015). Commercializing disease-specific hiPSC lines with appropriate informed consents from donors for drug screening process is likely to have great value for pharmaceutical companies, because mutations and polymorphisms have been implicated as major contributors to drug sensitivity (Roden and George, 2002; Tomalik-Scharte *et al.*, 2007).

A robust, highly standardized example was recently described by Kitaguchi *et al.* (2016). With just minor discrepancies, they demonstrated, in a mixed private–public multicentre study, that commercial lines can offer reliable and consistent results, suggesting that one way to pursue a prompt integration of hiPSC-CMs in safety pharmacology could be through biotech companies; although information on protocols and reagents used (e.g. electrolyte molarity and protein supplements) may not be provided presenting difficulties in comparing and publishing results (Figure 3).

## Conclusions

The growing appreciation of the value of hiPSC as a resource in drug discovery and safety pharmacology, not only for the heart but also for the brain, kidney and liver, has also driven the establishment of novel repositories for raw data from genomics, proteomics and metabolomic studies. Some cell banks, with the Human Induced Pluripotent Stem Cells Initiative (HipSci) at the Wellcome Trust/Sanger Institute (UK) leading this approach, make genomics and FACS data publicly available for many hiPSC lines. However, at present, the data available are only phenotypic/molecular characterisations of undifferentiated cells. To the best of our knowledge, these lines have not been compared directly for their differentiation capabilities, for example, towards the cardiac lineage or the electrophysiological properties of derivative hiPSC-CMs in standard conditions. This would be extremely useful before their use in safety pharmacology (Figure 4).

Phenotypic data derived from large cohorts of patient-specific hiPSC-CMs can be categorized by variations such as ethnicity, gender, age, genotype, pre-existing (clinical) conditions and much more, as we suggest in Figure 5. Furthermore, FDA/EMA/PMDA safety profile data for active concentrations of prescription drugs can also serve as a reference for comparing hiPSC-CM data. A remarkable example has been recently presented by Burridge *et al.* (2016), in which *in vitro* doxorubicin-induced cardiotoxicity investigated in hiPSC-CMs replicated the clinical condition observed in a very small cohort of patients (Bellin and Mummery, 2016). It will be interesting to extend this type of approach to larger cohorts to reproduce different severities in drug responses or even temporal onset of the disease (early/late toxicity). Sufficiently large cohorts may make it possible to develop broadly applicable *safety indices*. This should also be encouraged in the context of cardiotoxicity evaluation.

### *Drug repurposing and 'forgotten drugs'*

Drug repurposing strategies will benefit from the introduction of more predictive and sensitive *in vitro* models, in particular those simulating specific traits of diseases of patients' cohorts (McNeish *et al.*, 2015). More refined technologies will also pave the way to rediscovering 'forgotten drugs', drugs that are no longer in production because they are no longer covered by patents or were not a commercial success for the purposes for which they were designed or were considered insufficiently safe. Having them rehabilitated in part through hiPSC technology would require (i) the predictive value of hiPSC-CMs to be confirmed; (ii) evidence that the costs of drug development justify the potential revenue and (iii) that sufficient numbers of patients are eligible for treatment in the later clinical phases of development.

We anticipate that the integration of hiPSC-CMs in early, preclinical stages of drug development is imminent and will be of benefit in: (i) the early detection of cardiotoxic drugs in safety pharmacology assays, which will help reduce the withdrawal of drugs already introduced to the market; (ii) the refinement of drug safety assays, as they are based on human cell models at early stages, which may improve the yield of drug discovery activities by selectively discarding potentially harmful compounds in '*quick win, fail fast*' approaches (Paul *et al.*, 2010); and (iii) reducing the number of animal used for drug testing.

The prerequisite, however, is process standardization within the scientific communities of academia and pharma. Joint efforts from research institutes, cell banks, biotech companies and public institutions comparing different lines in identical experimental conditions will help make hiPSC-CMs an intrinsic part of the drug testing process.

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## Conflict of interest

C.L.M. is co-founder of Pluriomics bv.

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