Induced pluripotent stem cells (iPSCs) were first generated 10 years ago. Their ability to differentiate into any somatic cell type of the body including cardiomyocytes has already made them a valuable resource for modelling cardiac disease and drug screening. Initially human iPSCs were used mostly to model known disease phenotypes; more recently, and despite a number of recognised shortcomings, they have proven valuable in providing fundamental insights into the mechanisms of inherited heart disease with unknown genetic cause using surprisingly small cohorts. In this review, we summarise the progress made with human iPSCs as cardiac disease models with special focus on the latest mechanistic insights and related challenges. Furthermore, we suggest emerging solutions that will likely move the field forward.

**Keywords:** cardiac disease modelling; induced pluripotent stem cell-derived cardiomyocytes; molecular mechanisms

Since their discovery in 2006 [1], induced pluripotent stem cells (iPSCs) have enabled scientists to study the physiological and pathological mechanisms of both development and disease in a new way [2–4]. Inherited cardiovascular disorders and in particular channelopathies have been among the first human diseases studied using iPSCs [5–8]. Indeed, although animal models have been and continue to be essential in advancing the understanding of cardiovascular disease [9–11], interspecies differences hamper translation of many results directly to humans. Because human iPSCs (hiPSCs) can be derived from virtually any patient of interest and can usually differentiate efficiently into cardiomyocytes (hiPSC-CMs) and there are well-established techniques for their functional characterisation in vitro, they have rapidly been exploited for disease modelling and drug screening, and in the future are expected to offer new opportunities for regenerative medicine and personalised medicine.

The ambition of the Precision Medicine Initiative is to employ a combination of clinical, genetic or genomic, and molecular data to develop tailored therapies for subgroups of patients [12]. The pathogenesis of many inherited cardiac diseases remains insufficiently understood and it has been difficult to account for incomplete penetrance and variable severity. Any methodology that could help deciphering predisposition or causative molecular and cellular mechanisms therefore could be helpful. Because hiPSCs can capture the complex genetic background of a patient, expectations are that they will contribute to this goal. Whether patient-specific hiPSC-CMs can provide information that predicts disease penetrance and outcome remains to be determined. However, recent studies using hiPSC-CMs have lead to optimism with

**Abbreviations**
ALPK3, alpha kinase 3; ARVC, arrhythmogenic right ventricular cardiomyopathy; CMs, cardiomyocytes; DCM, dilated cardiomyopathy; HCM, familial hypertrophic cardiomyopathy; hiPSC, human iPSC; hPSC, human pluripotent stem cell; iPSCs, induced pluripotent stem cells; LQTS, long-QT syndrome; RBM20, RNA-binding motif protein 20.
respect to the use of this technology in providing new mechanistic insights into disease pathogenesis [13] and cardiotoxicity [14,15].

In this review we discuss the use of hiPSCs as models of inherited heart diseases, with special focus on underlying disease mechanisms that have not been evident from other approaches, current challenges, and emerging solutions for moving the field forward.

Generation of hiPSC-CMs (for cardiac disease modelling)

In using hiPSCs for disease modelling, the first step is reprogramming somatic cells collected from primary tissue samples. Most frequently, hiPSCs are derived from patients with known disease-causing mutations. An alternative, but increasingly used, approach is to introduce site-specific genetic changes (including knock-out and precise nucleotide changes) in wild-type human pluripotent stem cell (hPSC) lines by gene targeting [4]. Even in cases where no causative mutations are yet known, the rationale of using hiPSCs is that they are able to capture any genetic predisposition in patients aside from specific mutations, which are thought to play determinant roles not only in disease manifestation and progression but also in the context of external environmental factors that may precipitate the condition. These could include exercise, cardiac- and noncardiac drugs, fever, food supplements and the like [15,16].

Once hiPSCs have been obtained, several methods to induce cardiac differentiation can be used [17,18]. Although these techniques were initially inefficient and not readily transferable across cell lines, there are now a number of more robust protocols available and CMs at > 95% purity can be produced [19,20]. In addition, a number of defined media and commercial kits have become available of late which seem particularly efficient across lines, including several apparently differentiation refractory hiPSC lines. However, it is noteworthy that although the efficiency of differentiation protocols has undergone a multifold increase over recent years as a result of culture condition optimisation, this has not been paralleled by improvements in maturation of the electrophysiological properties of hiPSC-CMs: resting membrane potential is depolarised, and upstroke velocity and ion channels expression remain low in comparison with adult cardiomyocytes [21–23]. This suggests that optimisation has impacted quantitative rather than qualitative aspects of differentiation. Of note, most of these differentiation protocols result in mixed populations of ventricular-, atrial- and nodal-like subtypes, with ventricular CMs being the most represented. Some recent studies have succeeded in directing hPSC differentiation towards atrial [24,25] and pacemaker [26] subtypes, however, their application for studying molecular mechanisms related to disease is still under investigation.

Maturation of hiPSC-CMs

Improving maturity in hiPSC-CMs remains one of the major priorities of the field, since phenotypic immaturity limits their ability to successfully model critical aspects of cardiac disorders including adult-onset diseases [21]. Comparison with human fetal hearts suggests that in vitro-derived hPSC-CMs are similar to first trimester gestational stage CMs with regard to gene expression, structure and function and only in certain culture conditions do they become more similar to second trimester fetal CMs [27,28]. Channelopathies are among the cardiac diseases that suffer least from these limitations, since most (but not all) of the relevant ion channels for the generation of the cardiac action potential are expressed in hiPSC-CMs. This is the reason why the long-QT syndrome (LQTS) was one of the first cardiac arrhythmia conditions to be modelled using hiPSC-CMs [5]. Since then, approximately one-fourth of the publications on cardiac disease modelling have studied LQTS-causing mutations. Although some key features of other inherited heart diseases, such as catecholaminergic polymorphic ventricular tachycardia (CPVT) [29], arrhythmogenic right ventricular cardiomyopathy (ARVC) [30], familial hypertrophic cardiomyopathy (HCM) [31] and familial dilated cardiomyopathy (DCM) [32] have also been recapitulated, certain molecular mechanisms will only be reproduced when more mature cardiac phenotypes are achieved.

Existing hiPSC models of inherited cardiac diseases

Human iPS technology has succeeded in modelling cardiovascular and cardiometabolic diseases with different inheritance patterns: the most common autosomal dominant forms (LQTS [5–7,33], CPVT1 [29], DCM [32], HCM [31], ARVC [34]) but also the rarer autosomal recessive forms (CPVT2 [35], Jervell and Lange-Nielsen syndrome (JLNS) [36], Pompe disease [37]), the X-linked dominant forms (Danon disease [38], Fabry disease [39]), the X-linked recessive forms (Barth syndrome [40], Duchenne muscular dystrophy or DMD [41]) and also finally the nontypical Mendelian forms (hypoplastic left heart syndrome or HLHS [42]). All of these examples result from genetic
defects with cell-autonomous mechanisms of action in CMs, which means that the pathological phenotype is evident in the cardiomyocytes expressing the (mutated) gene without the need to interact with other cell types (Fig. 1). This may not always be the case and there is an increasing number of examples in which the interaction between two or more cell types is needed to reveal the disease phenotype since the cell expressing the mutation sends defective signals to its neighbours [43]. As hiPSC technology advances, the ability to establish heterotypic cultures and complex structures increases, we expect that noncell-autonomous disease mechanisms will be also recapitulated such as those leading to heart failure due to vascular diseases (thrombosis, atherosclerosis) and myocardial infarction (Fig. 1).

**Drug screening, toxicity assays and safety pharmacology**

One of the fundamental applications of hiPSC cardiac disease models is the development of treatments that ideally will eventually be translated into the clinic to cure (reverse) or relieve (delay) disease symptoms, much like that already achieved for some neurodegenerative disorders [44]. This approach is highly dependent on understanding the molecular mechanisms underlying the disease, as well as on the sensitivity of the read-out in the assay that is used for detecting the abnormal phenotype. Testing a limited number of candidate drugs based on underlying disease mechanisms is already proving the fastest way to move forward to clinical application, since it is based on repurposing previously approved compounds for a new disease [45]. In the cardiac field this has not yet led to rapid translation from the laboratory bench to patients, partly because cardiovascular diseases are often not as severe and untreatable as many neurodegenerative disorders. As an alternative to repurposing, hiPSC-CMs can be used as a platform for high throughput drug testing [46], which is most valuable to pharmaceutical companies looking for new drug and disease targets since they often have technologies for automated measurements.

In addition to drug screening and drug development, hiPSC-CMs are now also beginning to demonstrate their value in revealing cardiotoxic effects. In particular, these cells are proving a valuable tool to identify electrophysiological and transcriptional changes related to HDAC inhibitor-mediated cardiotoxicity [47]. Furthermore, Burrige and colleagues have recently shown that patient-specific hiPSC-CMs can recapitulate the predisposition of some breast cancer patients to develop late heart failure after exposure to the chemotherapeutic drug doxorubicin [15]. Although of significant interest, this study had some limitations: first, relatively few patients were included in each group (four in the *in vitro* doxorubicin cardiotoxicity assays and only three for the RNA-seq analysis of the hiPSC-CMs); second, the retrospective study design and coadministration of additional chemotherapeutic drugs in one patient group might have biased the outcome. Further validation in larger patient cohorts will be needed to determine whether different degrees of

**Fig. 1.** Cell-autonomous versus noncell-autonomous diseases. hiPSC-CMs have already proven their value in recapitulating cell-autonomous cardiovascular diseases, such as arrhythmic syndromes (LQTS, JLNS, CPVT), cardiomyopathies (DCM, HCM, ARVC, DMD), cardiometabolic disorders (Pompe disease, Fabry disease, Danon disease, Barth syndrome). More challenging to be modelled are noncell-autonomous cardiovascular disorders, such as diabetic cardiomyopathy, heart failure due to vasculature diseases, for example, thrombosis, atherosclerosis or myocardial infarction.
severity and early versus late cardiotoxic effects can be detected and whether the same approach proves valid for other patient groups such as those with tumours in other organs or paediatric patients also treated with doxorubicin [48,49]. Nevertheless, the work supports the idea that hiPSCs are able to capture complex genetic backgrounds of patients in a predictive way and therefore might contribute usefully to the realisation of the Precision Medicine Initiative [12].

Pathological phenotypes and new mechanistic insights

The successful generation of cardiac disease models with hiPSC-CMs relies on their ability to recapitulate key aspects of CM biology, including their molecular, cellular and physiological properties, and on the scientist’s tools and ability to record and capture these specific features and changes upon pathological or cardiotoxic conditions. For this, appropriate and sensitive read-out assays have been developed [13] and techniques are being continuously improved [22].

Disease-related phenotypes and read-out assays

During the first years that followed hiPSC discovery, their derivative CMs were mostly used to model known disease phenotypes to explore their potential value in recapitulating maladies and known pharmacological treatments [8]. More recently hiPSC-CMs proved helpful in providing novel mechanistic insights into inherited heart diseases with both known and unknown genetic cause using surprisingly small cohorts. The different assays used to characterise hiPSC-CMs phenotypes examine parameters such as gene and protein expression, ultrastructural organisation, electrophysiological function, calcium handling, force of contraction and metabolic profile. Here, we discuss some of the latest examples. The various kinds of diseases that have been modelled using hiPSC are illustrated in Fig. 1.

Analysis of not only gene expression in hiPSC-CMs but also the changes that take place during hiPSC cardiac differentiation has offered hints on genes and potential pathways impaired in some inherited heart conditions. In an autosomal dominant form of DCM caused by mutations in the RNA-binding motif protein 20 gene (RBM20), for example, stage-specific transcriptome profiling demonstrated early molecular perturbations during cardiogenesis in patient-specific hiPSCs [50]; these results suggested that this clinically aggressive form of DCM is a developmental disorder. In addition, using functional assays the authors demonstrated that RBM20-dependent mis-splicing of calcium-handling genes contributed to alterations in the calcium homeostasis and excitation–contraction coupling. Similarly, whole transcriptome sequencing led to the hypothesis that mitochondria were implicated in DMD cardiac pathogenesis [41]; subsequent analysis of the metabolic profile demonstrated that indeed apoptosis in DMD hiPSC-CMs is mainly induced by a mitochondrial network through the proteins DIABLO, XIAP and CASP3 rather than through cytochrome C and CASP9 cascade.

The analysis of ultrastructural CM organisation has revealed phenotypes not only in several cardiomyopathies but also in glycogen storage diseases [51]. Among these, Pompe disease was one of the first disorders characterised in depth using hiPSC-CMs, in which both specific features of the cardiomyopathy and the efficacy of recombinant enzyme therapy in patients were faithfully recapitulated [37]. However, only more recently did Raval and colleagues discover a specific deficit in the glycan synthesis in the Golgi that was initially revealed by the change in electrophoretic mobility of lysosomal-associated membrane protein LAMP1 [52]. Likewise, ultrastructural analysis by electron microscopy revealed the presence of fragmented mitochondria within autophagosomes in hiPSC-CMs carrying Danon disease, which is caused by lysosomal-associated membrane protein LAMP2 deficiency [38]; a consequent increase in oxidative stress and apoptosis was then demonstrated. This study was one of the first attempts to understand the molecular basis of some pathological features that are also characteristic of heart failure.

Interestingly, genetic polymorphisms in the cardioprotective enzyme, aldehyde dehydrogenase 2 gene (ALDH2), were studied by examining the metabolic profile of patient-specific hiPSC-CMs [53]. A new function for this enzyme was demonstrated, namely modulation of cell survival decisions through changes in the oxidative stress in hiPSC-CMs, although these finding were first observed in patient-fibroblasts and then later examined in hiPSC-CMs. Gene expression analysis highlighted overexpression of JUN and consequently the authors were able to restore ROS levels by the Jun N-terminal kinase (JNK) inhibition. Importantly, no significant differences were identified under normoxic condition, while ischaemia simulation in vitro revealed the phenotype.

Calcium influx into the cell triggers further calcium release from the sarcoplasmic reticulum to the cytosol and finally to the sarcomere resulting in cardiomyocyte contraction.
The identification of calcium handling abnormalities in hiPSC-CMs harbouring alpha kinase 3 gene (ALPK3) mutations allowed confirmation at the cellular and molecular level of strong genetic evidence that homozygous or bi-allelic truncating mutations in ALPK3 can cause paediatric cardiomyopathy [54]. However, in this *casus*, the specific role of ALPK3 remained unclear.

Channelopathies are most often characterised by examining their electrophysiological and ion channel properties. Molecular profiling coupled with measurements of action potentials and the slow component of properties. Molecular profiling coupled with measurements of action potentials and the slow component of the delayed rectifier potassium current ($I_{Ks}$) demonstrated a distinct molecular mechanism of action of two $KCNQ1$ mutations in JLNS hiPSC-CMs [36]. A recessive phenotype was associated with the amorphic mutation, while a gene dosage-dependent ion channel protein reduction at the cell membrane explained the presence of a LQTS phenotype in the heterozygously mutated hiPSC-CMs. Of note, however, the literature reports a wide range of values in the basic electrophysiological properties of hiPSC-CMs, action potentials differing an order of magnitude and beating rates anywhere between 0.5 and 1.5 Hz, but also specific ion currents (e.g. the slow $I_{Ks}$, and the rapid $I_{Kr}$ components of the delayed rectifier potassium currents, the sodium current $I_{Na}$, and the L-type calcium current $I_{CaL}$) varying widely even among wild-type control hiPSC-CMs [55,56]. Most notably, very different levels of $I_{Ks}$ have been described (ranging from ~0.3 to ~2.5 pA/pF [5,57]), variable observation leading to controversial conclusions: on the one hand, $I_{Ks}$ recapitulates physiological behaviour in playing a major role when repolarisation reserve is attenuated [58,59]; on the other, it seems to contribute to repolarisation in hiPSC-CMs even in the absence of sympathetic stimulation [5,36,60,61]. The immature phenotype of all stem cell derivatives including hiPSC-CMs is probably the reason for this variability but, independent of the cause, it is a limitation to extrapolating results obtained using hiPSC-CMs to native – healthy and diseased – adult human CMs as discussed below. The variability in protocols used for cardiac differentiation and electrophysiology further contribute to making absolute conclusions on human cardiac physiology and disease. Nevertheless, hiPSC-CMs with ion channel mutations have been able to contribute to understanding these diseases because in many cases they could recapitulate key disease features observed in patients and sometimes indicate underlying pathological molecular mechanism [56,62].

One example in which quantifiable hiPSC-CM properties were used for drug screening purposes is diabetic cardiomyopathy [63], a complex metabolic condition affecting also the heart. Here the authors built two levels of disease models with hiPSC-CMs: environmental, by modulating culture conditions to mimic the diabetes chemistry, and genetic, by deriving hiPSC-CMs from two patients with different disease severities. Interestingly, in the patient-specific cells, the diabetes phenotype appeared even in the absence of any diabetic trigger, suggesting that hiPSCs indeed capture and recapitulate genetic predisposition.

A common limitation of these studies is the small number of patients analysed; to confirm this concept, it will be necessary to validate results independently across larger cohorts.

### Choosing the right controls

The choice of controls is crucial to allow a proper definition and identification of normal versus abnormal phenotypes, including disease- and toxic-specific molecular mechanisms. Each individual harbours many genetic variants in the genome (not only single nucleotide polymorphisms, copy number variations but also heterozygous and homozygous mutations [64,65]) that may be functionally interconnected with the genetic defect underlying a disease. Gene targeting enables isogenic hPSC lines to be created that differ only at specific loci, while the rest of the genome remains identical. The advantage of isogenic lines is that any difference in the phenotype is then most likely linked to genetic change since the only difference between the disease and control line is in principle the mutation of interest. With improvement in the methodologies that can be used for precise gene targeting [66,67], genetically matched (isogenic) hiPSC lines are now becoming the first choice, although in the cardiac field only a few papers have adopted this approach [36,40,54,68–70] (Fig. 2). Hinson and colleagues demonstrated that truncating mutations in the sarcomeric protein, titin, underlie DCM sarcomeric insufficiency [69]; interestingly, when isogenic hiPSC-CMs were used, the reduction in force of contraction was still detectable in the mutated CMs but to a lesser extent than when unrelated diseased and control cells were compared. These results confirmed earlier evidence that genetic background can modify disease phenotype. Similarly, we previously generated two pairs of LQTS and control hiPSCs and hESCs harbouring the same $KCNH2$ mutation [68]; comparison of genetically matched CMs proved essential for neither under- nor overestimating the consequences of the mutation for the cardiac action potentials. Figure 2 summarises the controls used in all studies since first published in

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Human iPS cells and inherited heart disease

M. Bellin and C. L. Mummery

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Fibrillation. Protocols are becoming available to derive cells in conduction disease and atrial CMs in atrial fibrillation, for example, nodal and Purkinje other cardiac subtypes might be necessary for studying the cell type of choice for many diseases, although cell type, most often CMs. Ventricular CMs have been modelled in hiPSC-CMs and these are the major cellular players in the cardiac dysfunction in this disease [30,34,73]; however, the suspected contribution of epicardial cells to fibro-fatty substitution and the role of inflammation could not so far be studied in two-dimensional monotypic cultures. The expectation is that complex multicellular structures will be necessary to reflect fully the pathology of the condition.

We anticipate that in their second decade, iPSCs will find increasing utility when combined with cardiac tissue engineering. The necessity for better mimics of the multicellular and dynamic conditions of the cardiovascular system that can recapitulate diseases not only with both known or unknown genetic causes but also related to ageing and drug-induced cardiac damage has already encouraged engineering of three-dimensional cardiac microtissues. These are beginning to incorporate the different dynamics that reflect blood flow, mechanical stretch and strain and the electrical stimulation. Together with changes in energy substrates, it is expected that these will lead to structurally and functionally mature human myocardium into which biological and biophysical readouts can be built that allow high throughput, real-time and quantitative measurement of cardiac (patho)physiological status. The hope is that a higher degree of complexity will advance the understanding of how the human heart responds to toxic compounds and disease, improve the integrity of the disease models, and refine the predictability of drug responses.

**Predicting clinical drug response**

One of the greatest promises of hiPSC technology, but at the same time its greatest challenge, is in predicting drug responses in a patient-specific (personalised) way that disease treatment and prevention can be tailored to the individual. Because hiPSCs capture the genetic complexities of human heart disease, they are well suited for predicting the effects of drugs on patients with a particular genetic background. This is particularly important for drugs that are known to cause cardiotoxicity, such as those used in cancer treatment. By modelling the disease and testing drug responses in these models, it is possible to predict which patients are likely to respond to a particular drug, or which drugs are likely to cause severe side effects.

However, predicting drug responses is not a straightforward process. The human heart is a highly complex organ with many different cell types and functions, and it is challenging to model all of these in a single cell culture. To overcome this, researchers are developing more complex models, such as 3D cardiac microtissues, which incorporate a greater diversity of cell types and more realistic cardiac tissue structure.

Future challenges

It is now clear that hiPSC-CMs are useful for modelling inherited human cardiac diseases since there are many different examples in which these cells manifest pathogenic features of the disease. However, the predictive and instructive power of hiPSC-CMs relies on comprehensive and accurate molecular and functional characterisation [13]. Challenges that scientists are facing are the ability to model complex- and noncell-autonomous disorders, predict clinical drug response, recapitulate maturation and ageing in vitro along with difficulties in actually recognising mature CMs in culture; for these issues, emerging solutions are discussed.

**Complex and noncell-autonomous cardiovascular disorders**

Many cardiac diseases can be modelled using a single cell type, most often CMs. Ventricular CMs have been the cell type of choice for many diseases, although other cardiac subtypes might be necessary for studying different conditions, for example, nodal and Purkinje cells in conduction disease and atrial CMs in atrial fibrillation. Protocols are becoming available to derive some of these CM subtypes, [25,26] and we expect that they will soon be used in studying both pathological and cardiotoxic changes. Furthermore, some maladies might benefit from advanced culturing techniques, since certain phenotypes might become evident only under optimised conditions. For example, contractile defects were only uncovered under specific metabolic culture conditions [71] or when engineered tridimensional (3D) microtissues were used [40,69,72]. Importantly, the human heart is composed not only of CMs but also vascular, smooth muscle and epicardial cells; to better mimic its function, we predict that 3D cardiac tissue structures will be widely implemented, especially where interactions between different cell types might underlie the disease. As an example, ARVC has been modelled in hiPSC-CMs and these are the major cellular players in the cardiac dysfunction in this disease [30,34,73]; however, the suspected contribution of epicardial cells to fibro-fatty substitution and the role of inflammation could not so far be studied in two-dimensional monotypic cultures. The expectation is that complex multicellar structures will be necessary to reflect fully the pathology of the condition.

We anticipate that in their second decade, iPSCs will find increasing utility when combined with cardiac tissue engineering. The necessity for better mimics of the multicellular and dynamic conditions of the cardiovascular system that can recapitulate diseases not only with both known or unknown genetic causes but also related to ageing and drug-induced cardiac damage has already encouraged engineering of three-dimensional cardiac microtissues. These are beginning to incorporate the different dynamics that reflect blood flow, mechanical stretch and strain and the electrical stimulation. Together with changes in energy substrates, it is expected that these will lead to structurally and functionally mature human myocardium into which biological and biophysical readouts can be built that allow high throughput, real-time and quantitative measurement of cardiac (patho)physiological status. The hope is that a higher degree of complexity will advance the understanding of how the human heart responds to toxic compounds and disease, improve the integrity of the disease models, and refine the predictability of drug responses.
background of the person from whom they are derived, they are excellent candidates for recapitulating ‘in a dish’ the variability found among single patients or subgroups of patients. It is one of the few ways forward in coupling genome-wide association data, which associates disease risk with certain variants in the genome, to proof of causality in humans. This cannot be done in laboratory mice because of the genome differences. The ambition to implement hiPSC-CMs in precision medicine partially relies on their ability to predict patients’ response to administered drugs. Recent studies provide optimism in this direction [15,63,74], although additional consent and focussed investigations will be needed to determine the extent to which individual variability can be distinguished in the hiPSC-CMs, including mild or severe, acute, early or late responses. Of note, all studies so far have been based on a small number of patients per group and were conducted retrospectively. One goal in the coming years will be to demonstrate that hiPSC-CMs can be used in prospective study designs, for example, by deriving them from a large cohort of patients (> 200) that are about to undergo a specific drug treatment, applying the same drug to their hiPSC-CMs, and following-up over time the patients to find out whether the \textit{in vitro} responses matched the final clinical outcome. This approach could prove valuable especially in evaluating drug-induced cardiotoxicity.

In addition, a similar approach will ideally be applied in the evaluation of proarrhythmic risk. For example, if hiPSC-CM-based platforms for screening arrhythmic events can be combined with genetic- and FDA-collected data for the generation of reliable patient-specific arrhythmic scores, their real value will become clear in both the choice of individual patient treatment as well as in the drug development process. However, current challenges are not insignificant and suggest that expectations should be tempered in anticipation of more data. The ambition to reduce the incidence of sudden cardiac death as a result of drugs or inherent predisposition may however be a realisable goal in the coming decade.

\textbf{Maturation and ageing}

A relevant challenge in the field is to find ways to reproduce \textit{in vitro} the physiological processes of maturation and ageing that the heart naturally experiences from its formation to birth and further during the lifespan of a human being. The heart contracts many millions of times over a lifetime so that defects that are minor in CMs at birth may only be revealed with ageing. The mechanism and process of postnatal CM maturation is incompletely understood and clearly requires environmental factors including hormones, exercise and CM growth by hypertrophy. Approaches used to address this issue include prolonged culture, metabolic manipulation, tissue engineering technologies, electromechanical pacing and other biophysical approaches [21,22].

Promoting adult patterns of metabolic activity already provided a more appropriate basal condition on which to model the response to pathological stimuli, such as in ARVC [34], HCM [71] and diabetic cardiomyopathy [63].

Furthermore, anisotropic nanotopography was necessary to distinguish structural differences between control and DMD cardiomyopathy hiPSC-CMs that were otherwise masked [70].

Since both mechanical forces and molecular signalling from nonCM cell types are essential contributors to heart development, formation, ageing and disease progression [75,76], we anticipate that a combinatorial application of different strategies will likely be most successful in promoting maturation in hiPSC-CMs. Ideally 3D tissue structures will be developed, where hiPSC-CMs (subtypes) and other cells are mixed together and organised in microtissues, with or without the addition of extracellular scaffolds, and will be subjected to electrical or mechanical stimulation [77]. However, it is still unclear to what extent adult CM properties can be acquired in a culture dish. Nevertheless, some of these improvements in external parameters may contribute to the development of new and reliable methods for screening phenotypic changes also in response to drug treatments.

\textbf{Recognising a mature CM in culture}

An important question still remains: how do we recognise a mature CM in a culture dish? Our knowledge about human adult CMs relies on that of disease-free primary tissue, which is scarce and technically challenging to isolate successfully [78–80]. Nevertheless, there is consensus that adult CMs are elongated and rod shaped, the sarcomeres are highly organised, the resting membrane potential is quite negative (−80 to −90 mV), the upstroke velocity rapid (150–350 V s$^{-1}$), the sarcomeres organised in T-tubules, the excitation–contraction coupling fast and efficient, the force of contraction relatively strong (10–50 mN mm$^{-2}$), the mitochondrial content high and the metabolism mainly based on fatty acids (reviewed in [21,23]).

Ideally a combination of all of these parameters including the structural, molecular and electrophysiological characteristics associated with CM maturation...
should be assessed to determine whether hiPSC-CMs resemble myocytes of the human adult heart. However, it is common practice to test only some of these parameters, usually only those that are most important for the disease phenotype to be assessed. We propose that the most informative assays are those based on assessing all aspects of functionality of the cells, including as a minimum the electrophysiology, calcium handling properties, patterns and force of contraction.

If, for example, the expression of specific ion channel genes is examined, it is important to bear in mind that this is not always accompanied by a parallel change in the corresponding currents; there are several intermediate steps from transcription to function, including protein synthesis, post-translational regulation, protein trafficking to the membrane, anchoring of the channel to the membrane, protein turnover and channel regulation by known and unknown accessory proteins and by intracellular signalling [81]. Measuring the action potential would then seem more appropriate, since it can give some information on whether the CM population into question displays similar features to adult CMs. Drawbacks of electrophysiological measurements, especially of single-cell patch clamp, are that they are time consuming and low throughput, and the skills and technology is not readily available in all laboratories. Furthermore, the resulting data refer to the subpopulation of CMs that survived dissociation into single cells; these are usually the most immature in the population. The calcium transients can be measured using calcium-sensitive dyes and they usually reflect the action potentials, since they are closely related. In this case, complementary information is obtained from the kinetics of the calcium handling, variations of cytosolic calcium concentrations, and the extent of intracellular calcium stores. This type of analysis, much like patch clamp electrophysiology, is also low throughput although optical imaging of voltage and calcium might help increasing the measurement efficiencies. Finally, the force of contraction is another way to determine hPSC-CM maturation, although it depends on the cell shape and on substrate stiffness [82]. Techniques for measuring strain under controlled conditions have been developed [27,83–85] and we expect this will increasingly become a parameter that will be evaluated, although specialist technical implementation is required.

In summary, we believe that the intended application of hiPSC-CMs should probably determine the evaluation method to be used for assessing their maturation, but we expect that development of automated methods to analyse voltage and calcium transients and force of contraction simultaneously in both 2D and 3D settings will become a useful tool for disease modelling and drug testing, as well as for testing conditions that may eventually enhance maturation. Additional variables will need to be determined that may play essential roles in modulating CM growth, such as substrate stiffness and specific molecular cues [86] and still it is unclear whether an adult phenotype will ever be completely achieved in vitro.

**Concluding remarks**

Patient-specific models of cardiovascular diseases based on hiPSC-CMs are proving valuable in advancing our understanding of the complex and sometimes unexpected molecular mechanisms underlying pathological changes. Recent findings provide optimism on the applicability of hiPSC technology to unravel complex disorders, identify cardiotoxic drug effects and ultimately to help defining patient subtypes towards tailored drug treatments. In the future, larger cohorts of patients will be needed from which derive hiPSC-CMs and their phenotype analysis will tell until which point hiPSC in general, but in particular, their derived CMs can account for variables such as age, gender and medical treatments.

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**Conflict of interest**

CLM is cofounder of Pluriomics b.v.

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**Supporting information**

Additional Supporting Information may be found online in the supporting information tab for this article:

**Table S1.** Complete list of references used for Fig. 2.