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REVIEW

Animal models and animal-free innovations for cardiovascular research: current status and routes to be explored. Consensus document of the ESC Working Group on Myocardial Function and the ESC Working Group on Cellular Biology of the Heart

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Abstract

Cardiovascular diseases represent a major cause of morbidity and mortality, necessitating research to improve diagnostics, and to discover and test novel preventive and curative therapies, all of which warrant experimental models that recapitulate human disease. The translation of basic science results to clinical practice is a challenging task, in particular for complex conditions such as cardiovascular diseases, which often result from multiple risk factors and comorbidities. This difficulty might lead some individuals to question the value of animal research, citing the translational 'valley of death', which largely reflects the fact that studies in rodents are difficult to translate to humans. This is also influenced by the fact that new, human-derived *in vitro* models can recapitulate aspects of disease processes. However, it would be a mistake to think that animal models do not represent a vital step in the translational pathway as they do provide important pathophysiological insights into disease mechanisms particularly on an organ and systemic level. While stem cell-derived human models have the potential to become key in testing toxicity and effectiveness of new drugs, we need to be realistic, and carefully validate all new human-like disease models. In this position paper, we highlight recent advances in trying to reduce the number of animals for cardiovascular research ranging from stem cell-derived models to *in situ* modelling of heart properties, bioinformatic models based on large datasets, and state-of-the-art animal models, which show clinically relevant characteristics observed in patients with a cardiovascular disease. We aim to provide a guide to help researchers in their experimental design to translate bench findings to clinical routine taking the replacement, reduction, and refinement (3R) as a guiding concept.

Keywords iPSC • Tissue engineering • Multiomics • Network medicine • Bioinformatics • Big data • Comorbidities • Cardiovascular disease

1. Introduction

The chronic and progressive nature of cardiovascular disease represents an enormous economical and societal challenge.[1] Economic consequences are largely due to high healthcare expenses and loss of healthy years and ability to work of affected individuals. Moreover, the burden of cardiovascular disease is high not only for affected individuals but also for their relatives. This justifies research models that resemble human cardiovascular pathology and strategies to make optimal use of obtained data. In past years, many new potential drug targets turned out to be ineffective in the treatment of ischaemic heart disease and heart failure (HF). This is principally due to a lack of reproducibility and limited translation from rodent models to large animal models and subsequently to humans. Reproducibility and validation of key research findings in experimental models that represent human cardiovascular disease characteristics is essential for the implementation of new diagnostics and therapies

Table 1 Definitions of the 3Rs[2]

	Standard	Scientific approach
Replacement	Methods which avoid or replace the use of animals	Accelerating the development and use of models and tools, based on the latest science and technologies, to address important scientific questions without the use of animals
Reduction	Methods which minimize the number of animals used per experiment	Appropriately designed and analysed animal experiments that are robust and reproducible, and truly add to the knowledge base
Refinement	Methods which minimize animal suffering and improve welfare	Advancing animal welfare by exploiting the latest *in vivo* technologies and by improving understanding of the impact of welfare on scientific outcomes

in a routine clinical setting. The design of models for studies on cardiac pathophysiology is challenging, as cardiovascular disease is complex and involves multiple causes and comorbidities, resulting in a multiple-organ disease in an ageing population. In this position paper, we focus on *replacement*, *reduction*, and *refinement* of animal experiments, also known as the 3Rs. This concept had already been introduced in 1959 by Russel and Burch[2] (*Table 1*). The objective of this consensus document is to provide an overview of current state-of-the-art in animal models, studies in human and stem cell-derived models (*Figure 1A*), and highlight how tools have been developed to advance our knowledge of cardiac muscle, vascular and valve diseases (VDs) based on the 3R principles (*Figure 1B*).

2. Cardiovascular diseases and current experimental models

2.1 Epidemiology of acquired and inherited forms of cardiovascular disease

HF has a high prevalence, is often lethal and patient care is expensive. This condition is now estimated to affect ~38 million people worldwide and represents the main cause of death and disability.[3] Despite the remarkable progress in clinical management of patients and the use of devices assisting the failing myocardium,[4] the prognosis of HF remains poor, with mortality rates ranging from 6% to 7% at 1 year in patients with stable HF to ≥25% in patients hospitalized with acute HF,[5] and with an overall mortality rate estimated at 40% at 4 years from diagnosis.[6] HF is also tremendously expensive, accounting for 2–3% of national health expenditures in high-income countries,[7] and is projected to more than double in the next 20 years as a result of the ageing population.[8] The most common progressive cardiac rhythm disorder, atrial fibrillation (AF), is associated with HF, stroke and increased mortality. AF affects 2–3% of the Western population, and this percentage will increase in the ageing population.[9] Inherited cardiomyopathies caused by pathogenic variants in genes encoding regulatory and structural cardiomyocyte (CM) proteins, and channelopathies, caused primarily by pathogenic variants in genes encoding ion channels are a major cause of sudden cardiac death and morbidity in the young.[10,11] In addition to acquired and inherited forms of heart disease and rhythm disorders, pathologies such as aortic aneurysms and valvular disease affect many individuals. Abdominal aortic aneurysms (AAAs) occur in 4–7% of men and up to 2% of women over the age of 55 and are the 10th leading cause of death worldwide.[12] Heart VD is highly prevalent, with a mortality risk ratio of 1.36 in developed countries. VD is a progressive disease that increases with the ageing of the population and up to 30% of patients undergo surgical or percutaneous interventions. Valvular dysfunction can be congenital or acquired, and in each case may lead to either stenosis or regurgitation.[13] Below we describe the main pathological features of cardiovascular diseases, animal models that mimic disease features observed in humans and the availability of animal-free models.

2.2 Heart failure with reduced ejection fraction

HF is a haemodynamic concept, and failure of the pump to deliver blood (i.e. systolic failure) is often quantified as a reduced left ventricular ejection fraction (LVEF). HF with an LVEF <40% is termed heart failure with reduced ejection fraction (HFrEF). Failure of the heart to properly relax and fill (i.e. diastolic failure) may produce similar symptoms as HFrEF, although with a preserved ejection fraction of >50% (HFpEF; Section 2.3). HF with an LVEF between 40% and 50% is termed HF with mildly reduced EF. At least half of all HF patients present with reduced systolic function.[14] Loss of contractile capacity of the heart in HFrEF is due to loss of myocytes and to adverse remodelling of the surviving myocytes, reducing their contractile function (*Table 2*). The most common cause is myocardial infarction (MI), and subsequent post-MI remodelling, due to coronary artery disease and all its underlying causes (hypertension, hypercholesterolaemia, diabetes, and obesity).[15] Other common causes of HFrEF are exposure to cardiotoxic agents, including cancer chemotherapy,[16] viral myocarditis,[17] peripartum cardiomyopathy (PPCM) (Section 6.1),[18] and genetic defects (Section 2.5).[19]

Current standard of care includes first-generation drugs: angiotensin-converting enzyme inhibitors, angiotensin receptor blockers (ARBs), β-blockers, mineralocorticoid receptor antagonists, ivabradine and, more recently, combined ARB-neprilysin inhibitors (ARNIs-sacubitril/valsartan).[20] These drugs were developed decades ago to target both myocardium and vasculature to improve haemodynamics, and they may also mitigate the adverse remodelling of CMs. Hope has been raised by the unexpected discovery of the remarkable effect on HF of gliflozins (i.e. inhibitors of the sodium–glucose cotransporter 2). However, this effect is still awaiting a molecular explanation.[21] Recently, an oral soluble guanylate cyclase stimulator, vericiguat, has been shown to reduce cardiovascular deaths or hospitalization in patients with high-risk HF.[22] The fact that not a single biological drug (protein, peptide, antibody, and nucleic acid) exists for a condition that is as prevalent as HF[23] is explained by the complex multifactorial nature of this disease.

The stalling of molecular therapeutic innovation[24] is in stark contrast to the significant progress in the understanding of HFrEF pathophysiology. Cardiac injury and coincident reduced strain results in increased myocardial stress and determines a common endpoint, largely independent from the original cause of damage and diverse response and

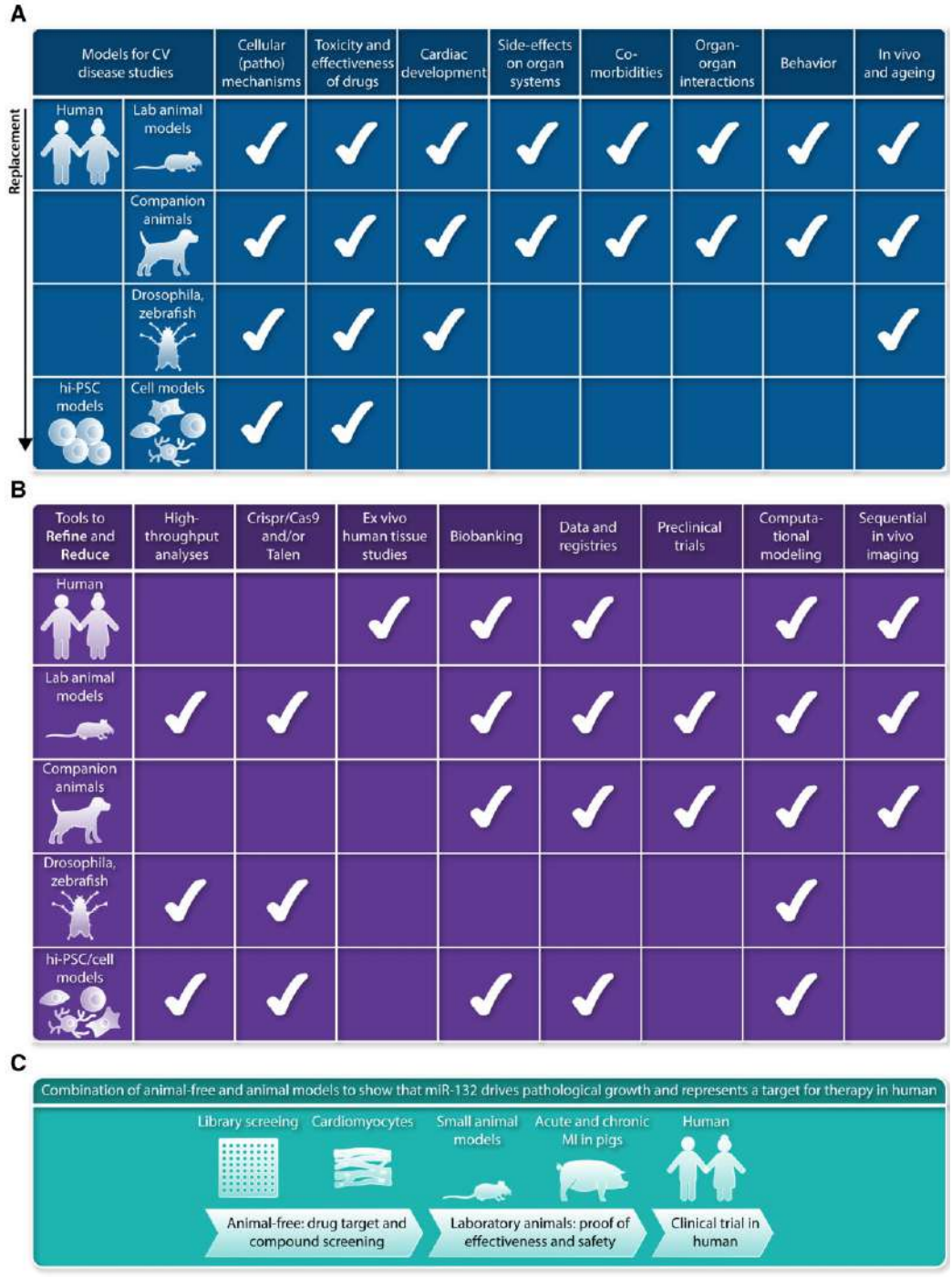

Figure 1 (*A*) Models that are available for studies on cardiovascular disease, ranging from human and laboratory animals to stem cell-derived models. Aspects that can be measured currently in the different models are indicated with the white check mark. This overview shows that several models allow to reduce the number of studies in laboratory animals, as many initial steps in identification of pathomechansisms, testing drug toxicity and drug effectiveness can be studied in cell-based models. Clearly, studies in human itself offers multiple opportunities to reduce the work in laboratory animals. (*B*) Multiple tools have been developed in past years to refine and replace studies in the models used for cardiovascular research, and range from tools and expertise to characterize human tissue samples obtained during surgery to models derived from hiPSCs (human induced pluripotent stem cells). (*C*) Example of an experimental design making use of available complementary research models based on the 3R principles.[2]

pathways triggered by the initial cardiac injury. This includes CM remodelling and alteration of metabolism, followed by progressive LV dilatation (eccentric remodelling), associated with extensive remodelling of the extracellular matrix (ECM), fibrosis and significant changes in viscoelastic properties.[25] This, in turn, reduces contraction efficiency and increases oxygen consumption, leading to the activation of the sympathetic

Table 2 Comorbidities, causes and cellular, structural and functional remodelling of the heart in HFrEF and HFpEF patients

Co-morbidities and causes	Vascular changes	Cellular changes in the heart	Structural remodelling	Cardiac dysfunction
HFrEF				
Hypertension Hypercholesteraemia Diabetes Obesity Cardiotoxic agents Viral myocarditis Peripartum cardiomyopathy Genetic defects	Coronary artery disease and ischaemia	Cell death Reduced cardiomyocyte contractility Altered metabolism Altered extracellular matrix Fibrosis Altered beta-adrenergic receptor pathway	Eccentric remodelling (dilated, thin-walled ventricle)	Reduced end-systolic pressure-volume relation Reduced response to exercise Neurohumoral activation
HFpEF				
Multiple comorbidities: hypertension, obesity, diabetes mellitus, coronary artery disease, sleep apnoea, and lung disease	*Proposed*: Systemic inflammation-mediated endothelial dysfunction	Stiff cardiomyocytes, i.e. high titin-based passive force Altered extracellular matrix Fibrosis Disturbed nitric oxide signalling	Concentric remodelling (thick-walled ventricle) Atrial dilation	Large patient heterogeneity Abnormal heart compliance and relaxation Elevated left ventricular filling pressure

nervous system and the renin–angiotensin–aldosterone system, which are initially adaptive but eventually worsen the condition.[26,27] The main features of adverse remodelling in HFrEF patients are summarized in *Table 2*. Various aspects of HFrEF pathophysiology can be mimicked in cellular or tissue models *in vitro* by applying stress factors (*Table 3*). Correlates of molecular causes of HFrEF in CMs include de-regulation of β-adrenergic receptor signalling, transition from compensatory to pathological hypertrophy, switch to a fetal type of gene expression and metabolism, changes in post-translational modification profiles, alterations in the calcium cycle and dysfunction of the sarcomere. Virtually all these cellular events can be experimentally mimicked to a significant extent in cell-based model systems where the molecular events involved can be dissected. Analogous considerations can be made for the other cell types that are involved in the myocardial response to injury, namely cardiac fibroblasts and endothelial cells.

Nevertheless, to address the wide gap in translation, and to reproduce the complex sequential events that occur in HFrEF, small and large animal models are complementary and still required.[28] Such models are essential for proof of concept of treatment strategies and for evaluation of systemic effects of cardiac insults and therapies at different stages of the disease. *Table 3* illustrates animal models showing reduced cardiac function upon acute and chronic cardiac insults, and animal-free models, including primary CMs, induced pluripotent stem cell (iPSC)-derived CMs, engineered heart tissue (EHT), and organoids.[29–48]

2.3 Heart failure with preserved ejection fraction

HFpEF prevalence is continuously increasing but many large clinical trials have failed to improve outcomes.[49] The lack of improved outcomes is due to the absence of a specific therapy because of incomplete understanding of the pathophysiology of the disease, and the recognition that the more cardio-centric view of HFrEF does not fit HFpEF. Furthermore, there is a large heterogeneity in the patient population as HFpEF is a complex syndrome with varying contribution of the pathophysiological substrate.[50,51] HFpEF is more common among the elderly and is associated with multiple comorbidities, such as hypertension, obesity, diabetes mellitus, coronary artery disease, sleep apnoea, lung disease, and remarkable sex-related differences.[52] Classic common features include abnormal LV compliance and relaxation, with resultant elevations in LV filling pressure, abnormal systemic and pulmonary vasorelaxation, and neurohumoral activation.[50,51,53] Recent principles in HFpEF management rely on the fact that the underlying mechanisms of this syndrome are not the same in all affected patients. This highlights the need to identify the specific causes that can lead to HFpEF and the different HFpEF phenotypes.[52] Recent implementation of phenomapping[54] has enabled identification of phenotypically distinct HFpEF categories to better classify pathophysiologically similar individuals who may respond in a more homogeneous and predictable way to interventions, regardless of the associated comorbidities.

An important limitation in understanding the HFpEF pathomechanisms and developing new pharmaceutical substances is the scarcity of proper animal models for this complex syndrome, leading to failure in the translation of basic research to the clinical setting. In fact, most animal models suggested to be 'HFpEF' present with elevated diastolic pressure but rarely demonstrate the development of HF, which is an essential condition to recapitulate the human situation. Excellent, in-depth reviews on this subject are available.[55–60] A true animal model of HFpEF should present with all of the following: an ejection fraction in the normal range for that animal model of at least 50%; diastolic dysfunction; exercise intolerance and pulmonary oedema (*Table 2*).[58] Concentric cardiac hypertrophy can be observed depending on the studied pathomechanism. The challenge is to reliably and reproducibly trigger these characteristic changes in small or large animal models. Several diabetes and obesity rodent models show HFpEF disease features (*Table 4*).[61–66] Unfortunately, pure gene-knockout animal models, so successful in other fields when studying a pathomechanism, are unlikely to generate the complex HFpEF phenotype, although aspects of the disease may appear.

Table 3 Examples of animal models with reduced contractile function and animal-free alternatives

Species	Experimental animal model and pathological features	Applications	Limitations animal model	Animal-free alternatives	Limitations animal-free alternatives
Mouse, rat, pig, dog	Ischaemia–reperfusion Regions of infarction, no reflow, haemorrhage, stunning, contractile function[29–31]	Assessment of cardiac contractile function, aryhthmias and long-term inflammation and LV remodelling Study sequential events	Healthy young animals are commonly used, with minimal coronary collaterals Relatively large infarcts, induced by artery ligation or balloon inflation	Mimicking ischaemia–reperfusion in primary CMs, hiPSC-CMs, EHT, cardiac organoids[32,33]	Simulated ischaemia differs from true ischaemia (altered buffer instead of blood)
Mouse, rat, pig, dog, sheep, non-human primate	Myocardial infarction by reperfused acute myocardial infarction, surgical occlusion of coronary arteries, coronary microembolization Fibrosis, systolic dysfunction[34–39]	Study systemic effects of insults (toxic compounds, inflammation) and therapies		Mimicking acute and chronic ischaemia in cell-based models[32,33]	Lack of tissue architecture, other cell types, and comorbidities CMs have an immature phenotype Only early stage effects of ischaemia can be mimicked
Mouse, pig	Spontaneous myocardial infarction in genetic mouse models, large animals on special diets. Spontaneous plaque rupture with thrombotic occlusion, MI[40,41]		High heterogeneity and unpredictability	None	
Mouse, Pig	Cancer chemotherapy cardiotoxicity. CM death, vascular injury, contractile dysfunction[42–44]		Some commonly used models do not recapitulate the dosing regime used in humans, and typically use healthy (not tumour-bearing) animals	Primary CMs[42] hiPSC-CMs, EHT Cardiac organoids[33]	Lack tissue architecture and non-myocytes Immature CMs No tumour present
Mouse	Viral myocarditis Myocardial inflammation, autoimmune reaction[45,46]		Non-physiological methods of administration (e.g. intravenous)	hiPSC-CM, EHT[47,48]	Can only assess direct effects since no inflammatory cells are present

Abbreviations: MI, myocardial infarction; CM, cardiomyocyte; LV, left ventricle; hiPSC-CMs, human induced pluripotent stem cell-derived cardiomyocytes; EHT, engineered heart tissue.

Table 4 Examples of animal models that mimic disease characteristics of HFpEF patients

Experimental model	Species	Pathological features	Strengths and limitations of model	Score as HFpEF model (− to +++)
Diabetes and obesity model	db/db (leptin deficient) and ob/ob (leptin receptor-deficient) mice[61–63]	Hypertrophy, diastolic dysfunction	Strength: mimics the HFpEF metabolic signature Limitation: confounding, adverse effects from altered leptin signalling	+
	Obese Zucker rats[64]	Hypertrophy, fibrosis, diastolic dysfunction	Strength: mimics the HFpEF metabolic signature Limitation: rarely used in HFpEF studies; possibly confounding effects due to altered leptin receptor	+/++
	ZDF (Zucker Diabetic Fatty) rats[65]	Hypertrophy, diastolic dysfunction		+/++
	Otsuka Long-Evans Tokushima Fatty rats[66]	Hypertrophy, diastolic dysfunction	Strength: mimics the HFpEF metabolic signature Limitation: mainly a type II diabetes model; rarely used in HFpEF studies	++
Hypertension models	Deocycoticosterone acetate-salt hypertensive mice[67]	Hypertension, diastolic dysfunction	Strength: mimics hypertension-mediated effects, in particular hypertrophy	-/+
	Dahl Salt-sensitive rats[68]	Hypertension, eccentric or concentric hypertrophy, and systolic and/or diastolic dysfunction depending on age-dependent timing of high-salt diet	Limitation: lacks comorbidities and the metabolic HFpEF signature; rodents can develop LV dilatation later in life, which is rarely seen in HFpEF patients	-/+
	Bilateral renal wrapping in dogs[69]	Hypertension, hypertrophy, fibrosis, diastolic dysfunction		-/+
	Deocycoticosterone acetate combined with a Western diet in pigs[70]	Hypertension, hypertrophy, impaired relaxation	Strength: combines hypertension and metabolic dysregulation Limitation: role of diabetes and obesity not addressed; no fibrosis	+
Hormones	Low dose angiotensin II in mice[71]	Diastolic dysfunction	Strength: diastolic dysfunction in the absence of hypertrophy Limitation: lacks comobidities and the metabolic HFpEF signature; no clear role of angiotensin II in HFpEF pathophysiology	-
Hypertrophy	Inbred Hypertrophic Heart in rats[72]	Hypertrophy, diastolic dysfunction	Strength: hypertrophy, diastolic dysfunction and preserved systolic function in the absence of hypertension Limitation: lacks comobidities and the metabolic HFpEF signature	-/+
Aortic constriction or banding	Mice with mild and severe transverse aortic constriction[73]	Hypertrophy, fibrosis, diastolic and systolic dysfunction	Strength: studies on myocardial remodelling, in particular cardiac hypertrophy and fibrosis	-/+
	Rats with aortic banding[74]	Hypertrophy, diastolic dysfunction	In large animal models: studies on the effect of cardiac hypertrophy on coronary perfusion Limitation: lacks co-mobidities and the metabolic HFpEF signature	
	LV pressure overload by an implantable stent or inflatable aortic cuff in pigs[75,76] or cats[77]	Hypertrophy, fibrosis, impaired relaxation, symptoms of heart failure		
	Dogs with aortic banding[78]	Hypertrophy		
Ageing models	Physiologic or accelerated ageing in mice[79,80]	Hypertrophy, fibrosis, diastolic dysfunction	Strength: captures age-related changes Limitation: lacks comobidities and the metabolic HFpEF signature	+
	Fischer F344 rats[81]			

Continued

Table 4 *Continued*

Experimental model	Species	Pathological features	Strengths and limitations of model	Score as HFpEF model (− to +++)
Cardiometabolic syndrome models	Dahl Salt-sensitive-Obese rats[65]	Diabetes, hypertension, hypertrophy, fibrosis, diastolic dysfunction	Strength: mimics human metabolic syndrome Limitation: rarely used in HFpEF studies	++
	ZSF1: ZDFxSHHF (spontaneously hypertensive heart failure)-hybrid rats[82,83]	Diabetes, hypertension, obese at older age, hypertrophy, fibrosis, diastolic dysfunction	Strength: mimics most HFpEF characteristics seen in humans, incuding exercise intolerance; well established as a HFpEF model Limitation: no identical genotype in control group (ZSF1-lean)	++/+++
	L-NAME plus high-fat diet in mice[84]	Hypertrophy, fibrosis, diastolic dysfunction	Strength: mimics most HFpEF characteristics seen in humans Limitation: no obvious LV stiffness increase found	++/+++
	Pigs with streptozotocin-induced diabetes, high-fat diet, and hypertension caused by renal artery embolization[85]	Hypertrophy, fibrosis, diastolic dysfunction	Strength: combines hypertension and metabolic dysregulation Limitation: role of obesity not addressed; no evidence of exercise intolerance	++

Abbreviations: VICs, valvular interstitial cells; CAVD, calcification aortic valve disease.

Typical examples are the db/db and ob/ob mice, two common models of type-2 diabetes mellitus that lack the leptin receptor or functional leptin, respectively and do show HFpEF characteristics. However, potentially confounding adverse effects arise from altered leptin signalling.[58,59] *Table 4* provides an overview of the different models which are used to mimic HFpEF disease characteristics based on the different comorbidities and various ways to induce cardiac remodelling.[61–85] We also indicate how well the model reflects the HFpEF phenotype observed in patients and the strengths and limitations of specific models. Questionable HFpEF models that incompletely mimic the phenotype include the classical transverse aortic constriction approach, as well as various other interventions predominantly causing hypertension and cardiac hypertrophy.[67–70,73–78] Altogether, it is unlikely that there will be a single animal model that can combine all HFpEF sub-phenotypes. This caveat notwithstanding, a good animal model of a common form of HFpEF has emerged as one that is both metabolically and mechanically stressed, similar to what is observed in patients. A recently proposed and interesting concept is that HFpEF presents as a multisystem inflammatory metabolic disease[86] driven mainly by excess adiposity linked with imbalance of nitric oxide (NO) levels.[84,87,88] An additional, commonly observed risk factor is hypertension, which is also associated with generalized imbalance in NO metabolism and bioavailability. In light of these findings, HFpEF models that recapitulate the metabolic inflammatory phenotype are warranted.

One of these rare HFpEF-mimicking models is the obese Zucker diabetic, spontaneously hypertensive Fatty (ZSF1) rat that presents with hypertension, type 2 diabetes, hyperlipidaemia, obesity, and nephropathy. This hybrid rat is a Charles River Laboratories cross between a Zucker Diabetic Fatty female rat and a Spontaneously Hypertensive Heart Failure male rat. Unlike the lean ZSF1 rat that can serve as a convenient control, the obese ZSF1 rat shows multiple HFpEF characteristics known in patients and typical cardiac hallmarks of the disease including modest fibrosis, titin modifications, and CM stiffening.[83,87] Furthermore, a large animal model of metabolic inflammatory disease has been generated, which clearly supports the concept of mechanical and metabolic hits as triggers of the disease. Manifestation of 'patient-like' HFpEF was evident in pigs with hypertension, diabetes, and hypercholesterolaemia.[85] A robust small-animal model of HFpEF was recently made by combining meta-inflammation induced by adiposity (high-fat diet) and hypertension induced by disruption of NO signalling (suppression of constitutive NO synthases) in wild-type mice.[84] Importantly, the individual insults alone did not recapitulate HFpEF pathology. A remarkable finding in this two-hit insult mouse model is the disruption of the unfolded protein response that is also linked to autophagy in various diseases.[89] Autophagy activators such as caloric restriction mimetics are pleiotropic agents that are beneficial for diastolic heart function in rodent models of ageing and hypertensive heart disease.[88]

The few available patient-mimicking animal models of HFpEF, driven by metabolic and mechanical stress, represent useful platforms for testing novel treatments in common HFpEF subtypes. The overview provided in *Table 4* highlights the progress that has been made in refinement of HFpEF animal studies. However, there remains a need to generate additional models that also represent other HFpEF phenotypes and allow for testing of specific treatments. Whether animal-free models of HFpEF can be successfully developed is questionable due to the complexity of the HFpEF pathophenotypes. iPSC-CMs may be of potential use as they can also be cultured as 3D cardiac tissues. These systems have the advantage of being derived from humans (including patients). This would be useful given the scarcity of cardiac biopsies from the HFpEF patient population. Human iPSC-CMs (hiPSC-CMs) could be used to model specific parameters of cardiac function, such as relaxation, for drug testing, and in co-culture studies to define the effect of endothelial cell dysfunction on CM performance.[90] However, with very few exceptions,[91] the application of hiPSC-CMs as well as other cell culture types has not really been explored in HFpEF research.

2.4 Atrial fibrillation

Atrial fibrillation is more than just an irregular rhythm on an ECG. It is a condition that requires a multifaceted approach and a variety of research. Known risk factors associated with AF include ageing, common cardiovascular diseases, cardiomyopathies, and channelopathies.[92,93] Furthermore, genetic studies have demonstrated an appreciable genetic component in the determination of risk for AF, and genome-wide association studies have identified ~100 risk loci.[94,95] This combination of inherited risk factors, acquired risk and DNA damage[96] makes research into AF both especially interesting and challenging. Experimental models to study AF are shown in *Table 5*. Various research groups discovered that AF perpetuates itself, 'AF begets AF', as a landmark paper put it.[97] The signalling pathways, structural, and functional alterations of this self-perpetuation have been dissected in large animal models and in patients with AF.[92] The interaction between genomic factors leading to AF and other stressors is less well understood. Small animal models like murine models, fish and *Drosophila* are useful for studying genetic and genomic modifications, and due to their shorter lifespan provide an opportunity to include research on ageing (*Figure 1A*).[96,98,99]

Animal-free innovations like human cell models, immortalized CM cell lines, and EHT will be instrumental in exploring these interactions and the underlying transcriptional and pathophysiological adaptations in detail.[100] Different forms of AF (paroxysmal, persistent, and chronic) are very difficult to mimic in animal or non-animal models. To date, there is no model for paroxysmal AF. Moreover, as AF is often a result of long-term exposure to risk factors partly on top of a genetic vulnerability it is especially difficult to copy a chronic disease like AF in cells. While experiments studying cellular adaptive processes and intracellular signalling require experiments in cells and cell-colonies allowing for genetic and pharmacological interventions, there are challenges with the use of such models for studying human chronic conditions like AF. Human iPSCs have already been differentiated into atrial CMs,[101] and atrial CMs have been generated from fetal immortalized CMs.[102] An important limitation is that such cells do not mimic all aspects of the adult CM phenotype, such as cell–cell coupling between cells (myocyte–myocyte or myocyte–fibroblast), making studies on the pathophysiology of, for example, conduction disturbances challenging. 3D formats facilitate *in vitro* maturation, and these 3D cell arrangements including EHT and bioprinting have overcome many of the previous limitations of cellular-based solutions and have been specifically adapted for AF research.[103]

As in other disease models, validation in more complex systems, occasionally large animals but ideally in patients with AF[98], will be required for successful translation of new findings into better diagnostics or therapies.[9,98,104–106] For this purpose, data collection in human cohorts should be improved and intensified by for example: analysing algorithms in smartphones and wearables, machine learning and artificial intelligence analysis, phenotyping of patients at risk of AF and with AF. This should be done not only with electrophysiological studies like high-density electrical mapping, but also imaging, biomarkers, proteomics, metabolomics, genetics, and genomics.

Table 5 Examples of animal models of atrial fibrillation and animal-free innovations

Species	Pathological features	Applications	Animal-free alternatives
Dog, pig, sheep, goat	Pacing induced tachycardia[97,104,105]	Understanding mechanisms of tachycardia-induced ion channel remodelling, therapeutic interventions to prevent electrical remodelling	Paced cell systems, immortalized myocytes
Dog, pig, sheep, goat	Electrically induced AF	Understanding the effect of stressors on electrophysiological mechanisms of AF has been extremely useful in mimicking human AF ('AF begets AF')[97] *Limitation: difficult to mimic chronic and multi-causal nature of human AF*	Cell based models are not available, but in-depth phenotyping of patients with AF may offer solutions: electrical mapping, imaging, blood/tissue biomarkers, genetics
Rodents, zebrafish, *Drosophila*	Mono-causal AF	High reproductive rates and standardized phenotyping enable high throughput studies of genetically modified animals *Limitation: difficult to mimic chronic and multi-causal nature of human AF*	Human iPSC-derived atrial cardiomyocytes[101] and engineered atrial-like heart tissue[103] *Limitation: lack of studies on chronic exposure to stressors, ageing*

2.5 Inherited cardiac diseases—cardiomyopathies, channelopathies, and ventricular arrhythmias

The clinical classification of genetic cardiomyopathies considers structural, functional, and arrhythmogenic alterations. Genetic cardiomyopathies mainly consist of dilated, hypertrophic, and arrhythmogenic phenotypes (i.e. DCM, HCM and AC).[10,107–109] Many pathogenic genetic variants in over hundred different genes encoding for sarcomeric (HCM, DCM), desmosomal (AC), nuclear (DCM), mitochondrial (DCM, HCM), and ion channel (AC, DCM) proteins have been identified. Inherited channelopathies, caused by mutations in ion channel genes and their interacting/modulating proteins, lead to a wide range of clinical phenotypes, including conduction disorders, AF and familial syndromes associated with life-threatening arrhythmias and a high risk of sudden cardiac death (e.g. long QT syndrome, Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia). The clinical variability in the expression of the phenotype, in part due to environmental factors,[110] and the genetic and phenotypic overlap among different cardiomyopathies and channelopathies,[111,112] have challenged the proper evaluation of the clinical, therapeutic, and prognostic impact of genotyping. Animal models, iPSC-CMs, and human cardiac samples (Section 4.1) are currently used to study the consequences of specific genetic variants. *Table 6* illustrates animal models and animal-free cell models that are commonly used for cardiomyopathy studies, and highlights how these models relate to the 3Rs.

Animal models of cardiomyopathies, such as mice and occasionally rats, have been obtained through genetic engineering.[113] These transgenic or knock-in models carrying human pathogenic gene variants (mutations) are the most widely used models of cardiomyopathies. Transgenic mouse models were the most often used method to show pathogenicity of mutant proteins *in vivo*. In this approach, a large number of copies of the mutant gene are introduced on top of the wild-type gene, which may lead to artificially high expression levels. Gene targeting approaches such as CRISPR/Cas9 in which a mutation is introduced in one or both alleles of the endogenous gene reflect the genetic state of cardiomyopathy patients better. Still, due to important biological and physiological differences between mice and humans, these models may not always recapitulate the human phenotypes. Recent technologies, including CRISPR/Cas9 have advanced the field helping to extend manipulation of genes to large mammals such as pigs, whose hearts are physiologically closer to humans.[114] Alternative animal models for studying genetic cardiomyopathies include *Caenorhabditis elegans*, animals with naturally occurring cardiomyopathy (Section 3.3), *Drosophila melanogaster* (Section 3.4), and zebrafish (Section 3.5). Similarities at the level of embryonic development, structure, function, and high conservation of gene function, combined with their ease of maintenance, short lifespan, and easy access to approaches for genetic manipulation, make these organisms attractive models for identifying mutations affecting proteins, signalling pathways and biological processes implicated in cardiomyopathies. They allow high-throughput screening (HTS) of gene function as well as druggable targets that can be further validated in larger animal models.

Research into inherited channelopathies traditionally employed heterologous expression systems, such as Chinese hamster ovary cells, human embryonic kidney (HEK293) cells, and *Xenopus* oocytes, for functional investigations of the consequences and putative pathogenicity of mutations. While these cell systems are inexpensive and easy to maintain and transfect, they are limited by their dissimilarities to CMs environments. Similarly, neonatal cells from rat, mouse or rabbit allow for overexpression or knock-down of genes followed by electrophysiological assessment. However, their immaturity makes them less well suited because of inherent differences in, for example, ion channel isoform expression and (t-tubule) structure. These limitations can be partly overcome by the use of transgenic animal models such as mice, rats, rabbits, and pigs. Although mice differ in certain ion current characteristics, most notably, potassium channels, heart rate, and autonomic regulation, they are easy to breed and to genetically modify by either overexpression or deletion of genes of interest, and it is easier to introduce genetic variants. More recently, rabbits have been successfully used in transgenic studies, which more closely resemble human electrophysiology. Overall, transgenic

Table 6 Examples of animal models of inherited cardiac diseases and animal-free innovations

Species	Pathological features	Applications	Animal-free alternatives
Mouse, zebrafish, *Drosophila*	Targeted deletion or transgenic overexpression of genes identified in human genetic studies, including GWAS *Refinement*	Study relevance of a specific gene Prove causality Gain novel mechanistic insight	Targeted deletion or overexpression in hiPSC-CMs ***Replacement and reduction***
Mouse, zebrafish, rabbit, pig	Transgenic animals overexpressing mutant proteins identified in patients with inherited cardiomyopathies and channelopathies *Refinement*	Study relevance of a specific gene mutation Study disease progression Prove causality Gain novel mechanistic insight Therapeutic studies	Introduce mutation in heterologous expression systems, hiPSC-CMs ***Replacement and reduction***
Mouse, zebrafish, pig	CRISPR/Cas9-induced gene mutation *Further refinement compared to transgenic models*	Mimicking heterozygous and homozygous mutations as present in cardiomyopathy patients	CRISPR/Cas9-induced gene mutations in hiPSC-CMs, and patient-derived iPSC-CMs ***Replacement and reduction***
Rat, cat, dog	Spontaneous cardiomyopathy *Refinement and reduction*	Study disease progression Gain novel mechanistic insight Therapeutic studies	Introduce mutation in heterologous expression systems, hiPSC-CMs ***Replacement and reduction***

All animal models enable *in vivo*/*ex vivo*/*in vitro* analysis of (electro)physiology, histology, and molecular biology. *Abbreviations*: GWAS, genome-wide association studies; hiPSC-CMs, human induced pluripotent stem cell-derived cardiomyocytes.

animals allow for in-depth electrophysiological studies *in vivo* (ECG, echocardiography), in the whole heart *ex vivo* (optical mapping, arrhythmia inducibility), on the CM level (patch clamp analysis, calcium fluorescence) and in combination with histological and molecular analyses as well as long-term therapeutic studies. Advances in gene editing resulted in step-wise refinement of animal models, moving from deletion or overexpression of genes of interest to transgenic overexpression of specific gene mutations, and *CRISPR/Cas9* models that mimic the heterozygous gene mutations present in most cardiomyopathy patients (*Table 6*).

Human iPSC-CMs provide an unlimited source of CMs from healthy controls and patients with inherited conditions, and thereby represent an important animal-free method for replacing animal cell studies and reducing the number of animal experiments. They maintain the patient's genotype as cells are derived from the affected patient skin biopsy or circulating cells. In addition, gene editing with CRISPR/Cas9 enables the generation of isogenic controls that allow for the characterization of the consequences of the genetic defect and rule out the confounding effect of the genetic background.[115] However, reprogramming and differentiation remains time-consuming (up to 3 months) and costly. Furthermore, hiPSC-CMs remain immature compared to human adult CMs at the metabolic, structural, and functional level (Section 4.2). For instance, hiPSC-CMs typically lack T-tubules, form only precursory intercalated disks, and their sarcomeres are relatively disorganized. Moreover, hiPSC-CMs have depolarized resting membrane potentials as a result of a lack of inward rectifier potassium current, with potential consequences for electrophysiological analyses. Human iPSC-CMs also lack the multicellular cardiac composition and neurohumoral control. Their integration into EHT with fibroblasts and/or endothelial cells has, nevertheless, been shown to increase their structural and functional maturation, as have various hormonal factors and mechanical activity.[115,116] Both hiPSC-CMs and EHTs allow molecular, functional, and electrophysiological phenotyping, facilitating research aimed at developing strategies for personalized risk stratification and therapy in inherited cardiomyopathies.[117]

Overall, there are important advantages and disadvantages of the different models. The selection of which model to use might be guided by the type of research that is being conducted. Frequently, a combination of models enabling both *in vivo* and *in vitro* studies may be required to define the molecular and functional consequences of mutations.

2.6 Valve diseases

For a long time, pathology of cardiac VD has remained elusive. Research on this subject has been limited to observational studies in small animals, such as mice, where genetic manipulation allows for a relatively rapid screening of phenotypes describing valve malformations (e.g. the development of the bicuspid aortic valve) or the evolution of valves towards a stenotic-like condition.[13] On the other hand, the lack of consistent larger animals models of valve calcification, except for sheep, has prevented an in-depth investigation of the molecular pathways underlying valve pathophysiology.

Valves contain two major cell types: valvular endothelial cells (VECs), which prevent thromboembolic events by covering the surface of the aortic and ventricular side of the aortic valve producing NO, and valvular interstitial cells (VICs), the most prevalent cell type and crucial for calcification aortic VD (CAVD) pathogenesis.[118] VICs are responsible for the homeostasis of the ECM proteins, including collagen, elastin, and glycosaminoglycans, which assure mechanical stability and elasticity of the aortic valve[119] and respond to inflammatory cues by inducing a robust calcification response.[120] Therefore, VIC functions have prompted new investigations on paracrine pathways involved in CAVD [e.g. transforming growth factor-β (TGF-β) signalling]. The human aortic valve opens and closes over three billion times over an average human lifespan and is thereby subjected to major mechanical forces. These forces include: axial stress during diastole upon valvular closure, mainly sensed by VICs, laminar shear stress on the ventricular side during systole, and oscillatory shear stress on the aortic side of the cusps during diastole both sensed by VECs.[121] Both excessive axial stress and lack of laminar shear

promote the phenotype switch of VICs towards myofibroblasts, which acting as 'mechanosensors', promote valve pathologic ECM remodelling, including fibrosis and valvular sclerosis.[120] With further progression of CAVD, increased valvular stiffness, myofibroblasts differentiate into osteoblasts.[122]

Individuals with increased mechanical strain on the aortic cusps, due to the congenital malformation of bicuspid aortic valves show increased prevalence at a younger age for the development of CAVD.[123] Moreover, calcification of the aortic valve predominantly starts at areas subjected to the highest mechanical strain and the lowest laminar shear stress, namely the non-coronary cusp.[124] It is the mechanically challenged aortic side of the valve leaflet that calcifies in contrast to the ventricular side of the leaflet. Patients with increased blood pressure, and thus valve overload, show higher risks for the development of CAVD, highlighting that therapeutic strategies should aim to reduce biomechanical forces on the valve.

Until now, no pharmacological agent was able to prevent valvular calcification or promote valve repair, as valve tissue is unable to regenerate spontaneously. Thus, heart valve replacement/repair is currently the only available treatment to prevent HF in VD. The research focuses on two approaches: animal models (mostly large animal models) and animal-free strategies. Animal models have been critical for the development of devices or innovative valve repairing/replacing techniques. Sheep is currently accepted as the gold standard model for valve replacement using defined survival surgeries that meet FDA requirements.[125] Normal cardiovascular physiological parameters of sheep approximate those of humans in blood pressure, heart rate, cardiac output, and intracardiac pressures. Also, the valve orifice diameters are similar to humans. Animal-free strategies have become exciting alternatives to promote the development of matrix-guided regenerated or bioengineered valves and studies on the cardiac impact of VD. Considering the highly controlled *in vitro* conditions, the potential of these animal-free strategies to uncover the pathophysiologic mechanisms underlying VD may even surpass the potential of animal studies. Nevertheless, animal models are still indispensable for studying specific aspects of VD. *Table 7* depicts the most commonly used animal models of VD, their potential applications and currently available animal-free alternatives.[126–135]

2.7 Vascular pathology—atherosclerosis

Atherosclerosis, the underlying process of the majority of cardiovascular diseases, is a lipid driven chronic inflammatory disease. The disease is characterized by the accumulation of lipids and immune cells in the arterial wall: the atherosclerotic plaque. Atherosclerotic plaques can cause stenosis by gradually reducing the arterial lumen or cause acute arterial occlusion by plaque erosion or rupture. These processes result in ischaemia and, depending on the arterial bed affected, result in cardiovascular events including angina pectoris, MI, stroke, or peripheral arterial disease.[136] The pathogenesis of atherosclerosis is complex and years of research in patients and experimental animal models have taught us that a combination of systemic environmental factors (e.g. flow, shear stress, oxidative stress, inflammation, endocrine factors and hyperlipidaemia) and plaque intrinsic factors [e.g. cellular lipid uptake, endothelial cell activation, vascular smooth muscle cell (SMC) migration, ECM production, immune cell recruitment and activation] and most importantly cell-cell interactions between immune cells and between immune cells and non-immune cells all drive atherogenesis.[137]

For decades, most groundbreaking insights into this complex disease have been obtained by studies in laboratory animals (*Table 8*).[40,41,138–151]

Until the 1990s, the most widely used animal models for atherosclerosis were cholesterol-fed rabbits, pigs, and non-human primates. These models, especially the pig and non-human primate, have a very similar cardiovascular physiology to humans, but need a long time (>1 year) for developing minimal disease and even longer to develop advanced atherosclerosis (see Section 3.2).[147] The design of transgenic mice that lack genes important in lipid metabolism, such as the LDL-receptor and apolipoprotein E, was a major step forward and further refined animal models for investigation of atherosclerosis. Not only do these mouse models develop widespread atherosclerotic lesions in a reproducible way within a few months, but the development, progression, and growth of lesions show features reminiscent of human atherogenesis.[148,149] A major advantage of these mouse models is that they can easily be backcrossed to other cell-type specific genetically modified mice in order to not only study the role of specific genes on plaque development, progression, and composition but also the effects of systemic alterations caused by these respective genes on atherosclerosis.[148] One of the major drawbacks of animal models of atherosclerosis is the lack of end-stage atherosclerosis with spontaneous plaque rupture.[149] Although very old $ApoE^{-/-}$ mice do develop intraplaque haemorrhages, spontaneous rupture of the fibrous cap whereby the thrombus is in continuity with the necrotic core, or spontaneous plaque erosions have only rarely been observed.[149] For studying the process of atherosclerotic plaque rupture or the post-rupture healing process, models in which acute plaque rupture is induced mechanically or by vasoconstriction have been developed. For example, in atherosclerotic mice, mechanical plaque rupture was induced by gently squeezing the plaque-bearing aortic segment of the abdominal aorta between blunt forceps.[150] Other models of plaque rupture include models in which a plastic cuff is placed around the carotid artery, followed by ligation of the artery.[151] A few genetic models, including $SRBI^{-/-}/ApoE^{-/-}$ mice[40] and $Fb1^{-/-}ApoE^{-/-}$ mice[41] show spontaneous plaque rupture with end-organ damage including stroke and MI.

Many alternative cell- and model-based efforts are currently being developed and the first results are quite promising. However, atherosclerosis is a complex, multifactorial disease which cannot be mimicked using such a 'lab on a chip' approach. As the interactions between many different immune cell types, flow, shear stress, hyperlipidaemia, and endocrine factors all affect its pathogenesis, we still need to make use of living organisms, especially mice. Noteworthy, in atherosclerosis research, we are reducing the number of laboratory animals used by carefully designing our experiments and testing aspects of the disease as much as possible in *in vitro* systems. Recent developments in single-cell technologies (transcriptomics and mass cytometry)[152–154] and the design of novel computational tools has enabled us to more carefully select our candidates and targets, thereby reducing the number of laboratory animals being used. Aspects of the disease, including endothelial cell biology, lipid uptake, leucocyte recruitment, and immune cell activation can be studied in 2D *in vitro* systems, using cell-lines or iPSCs, thereby limiting research in laboratory animals. Advanced 3D *in vitro* models are being developed. Furthermore, new and improved animal models of vascular disease (i.e. humanized mouse models) are currently under development.

2.8 Vascular pathology—aneurysms

Aortic aneurysms (AAs) are a complex cardiovascular disease, most likely to develop in the abdominal area. It is associated with risk factors such as advanced age, male gender, genetic predisposition, smoking, and other cardiovascular comorbidities. Currently, the only available treatment for AAA is surgical repair or efforts to improve general

Table 7 Examples of animal models of valve disease and animal-free alternatives

Species	Experimental animal model and pathological features	Applications	Animal-free alternatives	Refs
Calcific aortic valve disease				
Mouse	Male Notch1+/- mice fed for 10 months with a Western diet Mild phenotype: Notch1+/- mice have increased aortic valve calcification without significant valve stenosis	To study valve sclerosis early during valve disease progression	Notch-signalling can be studied in cultured aortic VICs as a model of cell-autonomous valve calcification***Replacement and reduction***	126
	Apolipoprotein E-deficient Mice (ApoE-/-) display ectopic calcification of valves showing bone-marrow-derived cells positive for osteoblast-related proteins, which might represent smooth muscle-like and osteoblast-like cells in degenerative valves; the sclerotic valves displayed frequent apoptotic cell death and chemokine expression	To study the concomitant impact of altered lipid metabolism and ageing for the development of murine aortic sclerosis To develop therapeutic strategies for aortic valve stenosis	Not available	127
Rabbit	New Zealand White rabbits subjected to one-kidney/one-clip model to induce hypertension; mild aortic valve stenosis in hypertensive rabbits, increased valve thickness and inflammation nodules, hypertrophy of valve after 4 months	To investigate the mechanisms underlying the association between hypertension and aortic stenosis and the efficacy of different medical treatments to delay, or even hinder, the disease progression	Not available	128
	High cholesterol diet for 20 and 40 weeks, atherosclerotic lesions present in aortic valves, with increased lipid deposition, inflammatory cell infiltration, osteopontin deposition, changes in collagen and elastin distribution, and mineralization; hypercholesterolemia-induced calcification in the aortic valves depends on Lrp5 receptor pathway	To study the link between atherosclerosis and aortic valve stenosis; results are similar to changes reported in human sclerotic aortic valves, suggesting the suitability of this model of atherosclerosis as a model for CAVD	*In vitro* cultured aortic valve myofibroblast model of cell proliferation. ***Replacement and reduction***	129,130
	Watanabe heritable hyperlipidaemic (WHHL) rabbits fed with a high-fat/high carbohydrate diet display a spontaneous LDLR mutation; the valve does not show significant haemodynamic stenosis but presents lipid deposition, fibrosis, calcification, and inflammatory cell infiltrations	To study early-stage of CAVD and the impact of dietary cholesterol on valve disease	Not available	130
	White rabbits fed with a standard diet supplemented with 0.5% cholesterol and 50,000 IU/day vitamin D3; non-invasive echocardiographic and invasive measurements confirmed the increase in transvalvular pressure gradient and development of valvular aortic stenosis; histology showed severe calcified and thickened aortic valve	To evaluate the haemodynamic and transvalvular gradient measurements after percutaneous balloon dilation of the valve, for translational research	Not available	131

Continued

Table 7 *Continued*

Species	Experimental animal model and pathological features	Applications	Animal-free alternatives	Refs
Pig	Yorkshire swines fed with a high-fat/high-cholesterol diet for 2 or 5 months; valves show the formation of proteoglycan-rich onlays in the fibrosa before significant lipid accumulation, inflammatory cell infiltration, or myofibroblast activation This model shows aortic valve sclerosis without calcification.	This model enables new insights into early pathogenesis, including that of proteoglycan-rich onlays; the model mimics features of early human aortic valve disease; their size makes them ideal for studies that characterize leaflet-mechanical properties and for studies requiring blood analysis	*In vitro* matrix guided regenerated valves might provide insights into the association between the valve microenvironment and pathological cell responses ***Replacement and reduction***	132
Valve insufficiency or stenosis				
Dog, pig	Severing the chorda tendinae, ischaemic injury of the posterior papillary muscle	Mitral valve regurgitation	Not available	133
Sheep	Pacing-induced heart failure with tricuspidal insufficiency	Tricuspidal valve insufficiency	Not available	134
Cat, dog, sheep, pig	Supravalvular aortic stenosis by surgical banding of the aorta	Aortic stenosis	Not available	135

Table 8 Examples of animal models that mimic human atherosclerosis

Species	Model	Main changes in the heart and vasculature	Animal-free alternatives	Refs
Pig	Familial hypercholesterolaemia	Atherosclerotic lesions of all vessels	Studies on certain aspects of atherosclerosis:	138,139
	Yucatan and Sinclair miniature pigs fed with Alloxan resulting in diabetes	Human-like atherosclerotic lesions and micro-vascular diseases		140,141
	Ossabaw pigs	Obesity and metabolic syndrome like humans	Single-cell technologies human tissue samples[152,154]	142
	PCSK9 gain-of-function mutant	Hypertension, diabetes, kidney disease, endothelial dysfunction	2D and 3D *in vitro* models	143,144
			Refinement and reduction	
Non-human primate	High-fat, high-cholesterol diet in Rhesus and cynomolgous macaques	Slow development of atherosclerosis		146
	Novel gene-modification technologies, e.g. CRISPR/Cas9	Accelerated atherosclerosis		146
Mouse	Transgenic mice with lack of genes involved in lipid metabolism (LDL-receptor, apolipoprotein E)	Accelerated atherosclerosis; spontaneous plaque rupture is rare		148,149
		Refinement: Induction of plaque rupture		150,151
	$SRBI^{-/-}/ApoE^{-/-}$, $Fb1^{-/-}ApoE^{-/-}$	Spontaneous plaque rupture with end-organ damage including stroke and MI		40,41

cardiovascular health. There are no other effective therapies or drugs because the process leading to AA is ambiguous.[155] Previous studies implicate defects in SMCs, ECM remodelling, inflammation, and oxidative stress as key factors in the pathogenesis.[156] However, treatment strategies to intervene in the oxidative stress pathway or inflammation have all failed in clinical practice. The underlying pathophysiological processes behind the long-term chronic development of AAA have to be unravelled.

Extensive studies and models have been developed to understand AAA (*Table 9*).[157–162] Research started with *in vivo* animal models. Murine models are the gold standard of experimental *in vivo* AAA research. Various different models, each with individual limitations, are capable of providing partial simulation of human pathology. One common feature of all AAA models are the required external stimuli to initiate aortic dilatation. The most common ones are angiotensin II (AngII), porcine pancreatic elastase (PPE), and $CaCl_2$ instillation.[157] Experimental AngII-induced AAAs require mice with an atherosclerosis-prone background, such as Apolipoprotein E/*ApoE* or Low-density lipoprotein receptor (*Ldlr*) deficiency. AngII-AAAs display suprarenal aortic aneurysms and are commonly associated with covered ruptures or dissections.[158] The murine PPE model presents many histo-morphological features associated with human AAA disease.[159] A promising modification of the model that utilizes external peri-adventitial elastase application in combination with β-aminopropionitrile (BAPN) to provoke acute rupture and intraluminal thrombus formation has been reported.[161] In addition to small animal models, several studies report AAA formation in large animals (mainly pigs) that have the advantage of exploiting similar anatomical and physiological dimensions to humans, allowing the application of devices and surgical techniques.[162–164] It appears evident that further advancements in small animal models as well as refinement of large animal models (e.g. using *Ldlr*-deficient mini-pigs) will enhance studies of unmet translational research questions. However, today no available model closely resembles human AAA characteristics. Recent studies are conducted on the first steps towards the development of an *in vitro* preclinical disease model for AAA (Section 4.4).

3. State-of-the-art in animal models

Animal models allow for *in vivo* and *ex vivo* functional and electrophysiological studies at various disease stages in correlation with molecular and histological findings, as well as for research into the impact of stressors such as exercise and comorbidities, ageing and chronic effects of pharmacological interventions. The latter aspects are not easily mimicked in animal-free cell and tissue models (*Figure 1A*). The following paragraphs describe limitations and opportunities of current animal models.

3.1 Rodent models

Rodent models are widely exploited as they provide biological insight at the organ and cell level, are hypothesis-generating in pathophysiological processes and provide the opportunity for body dose-response testing. The major advantages of these models are relatively easy genetic manipulation, availability of biomedical tools with rodent specificity and their relatively low cost. Below we review some of the major limitations of rodent models and provide promising perspectives to refine and improve their research use.

Rodent models are often used to study the function of a specific protein or mutation. This was initially analysed using pharmacological inhibitors and/or activators, but pharmacological treatments were increasingly criticized for their unspecific effects. Nowadays, genetically engineered mice are the standard in cardiovascular biology, because they permit the modification of a single gene or specific mutation and to examine their function in an integrated physiological system. Two genetic technologies exist, insertional transgenesis (transgenic animals in which additional copies of a gene are inserted) and gene targeting (knock-out to functionally remove a gene, or knock-in to introduce a mutation in a gene). Inducible tissue-specific gene-targeting systems based on the Cre-loxP technology are preferred, to overcome the limitations of global gene targeting which include: embryonic lethality, compensatory changes over time and effects related to gene deletion in organs not under investigation. However, numerous pitfalls have to be considered when interpreting data obtained from genetically modified animals.[165] For example,

Table 9 Examples of animal models of aneurysms and animal-free alternatives

Species	Pathological features	Applications	Animal-free alternatives	Refs
Mouse **ANGII-model**	Dilation of suprarenal aorta, dissection, covered ruptures, intraluminal thrombus formation	Therapeutic intervention studies	Not available	158
Mouse, rat **PPE-model**	Dilation of infrarenal aorta, elastic layer fragmentation, smooth muscle cell apoptosis, increased immune cell infiltration	Therapeutic intervention studies	Not available	159
Mouse **Ca_2Cl_2-model**	Dilation of infrarenal aorta, enhanced inflammation, smooth muscle cell apoptosis	Therapeutic intervention studies	Not available	160
Mouse **PPE&BAPN model**	Chronic, advanced-stage AAA with persistent growth, thrombus formation, spontaneous rupture	Therapeutic intervention studies; chronic effects of treatment strategies	Use of vasculature on a chip devices where the geometry and the flow/wall stress is modelled with computational flow dynamics (Section 4.4) ***Replacement and reduction***	161
Pigs/Mini-pigs **PPE-model**	Dilation of infrarenal aorta, elastic layer fragmentation, smooth muscle cell apoptosis, increased immune cell infiltration	Therapeutic intervention studies; device development and testing	Use of human umbilical cord-derived arteries to simulate aneurysm dilatation and stent implantation ***Replacement and reduction***	162–164

Abbreviations: ANGII, angiotensin II; PPE, porcine pancreatic elastase; BAPN, β-aminopropionitrile.

both the Cre protein and Tamoxifen, used to activate the Cre, can have cardiotoxic effects.[166,167] While overexpression of any protein might induce undesired effects, its knock-out might also affect the whole proteome.[168] Both pharmacological and genetic approaches have potential limitations and may be combined to strengthen the understanding of protein–function relationship.

Additional limitations are the difficulty in translating results generated in rodents to humans, with particular reference to novel therapeutic strategies. Firstly, rodent models are usually developed in healthy and young animals. While some models consider comorbidities, they fail to reproduce the complexity of cardiovascular disorders in humans and lack routinely used medication or other disease-influencing effectors thereby oversimplifying human disease. A second issue to consider is genetic background of mice, as phenotypes may differ significantly between different strains which may confound results. However, combining phenotypic analysis, expression data in cardiac tissue and genetics offers the unique opportunity to identify new disease-related genes and pathways.[169,170] Thirdly, rodent hearts poorly mimic the human heart, particularly in terms of heart rate and collaterals. Fourthly, while systematic reviews/meta-analyses are commonly performed to improve clinical practice,[171] they are underused in experimental research. Most rodent studies are conducted in a single research facility as a proof-of-concept study. Just like clinical trials, large multi-centre preclinical studies should be initiated to validate findings and to ensure their reproducibility (see Section 5.1), although sustainability may be challenging and require the support of large funding schemes. Societies, funding agencies, and journals should agree on common standards for experimental animal studies with regard to randomization, blinding, and information on age, sex, and comorbidities, to at least be made available as supplemental data. Standardization would allow increasing data robustness and quality, extracting new data from previous studies, reducing the number of animals, and be in compliance with the 3R policy.[172] Along the same line, an additional step forward would be establishing repositories of samples from rodent models, with biobanks maximizing tissue usage from euthanized animals. While a particular organ might be the target of a specific study, the remaining tissues could serve the goal of research groups focusing on other organs and systems, thereby reducing the number of research animals and replacing living animals with stored samples. Again, the critical aspect here is assuring that organs, tissue or cells are collected and preserved according to established protocols, to ensure high-quality samples, paired with controls and accurately linked to comprehensive databases providing relevant information. Finally, assessment of cardiovascular function in rodents should privilege methods that avoid invasive or terminal procedures, such as echocardiography, magnetic resonance imaging (MRI), and telemetry. Both echocardiography and MRI allow for complete, repeated and non-invasive assessment of systolic and diastolic function. MRI shows the advantage of providing information regarding cardiac metabolism. However, its use is limited due to its high costs. In contrast, echocardiography is widely used and standard procedures for echocardiographic assessment have been recently published aiming to increase accuracy and reproducibility of the data.[173] Telemetry systems involve surgically implanting small devices (telemeters) into the animal. These telemeters assess and emit wireless signals from conscious, non-restrained animals, to a receiver outside the cage. Progress in device miniaturization and battery duration allow for continuous recording of data and for the merging of several cardiovascular parameters in the same telemeter (ECG, blood, and intraventricular pressure) with minimal human-animal contact.[174]

3.2 Large animal models

While 'refine' and 'reduce' of the 3R principles (*Table 1*)[2] can be considered in many animal experiments, the 'replace' is difficult and is often questioned. Large animal models are mandatory for translational research before entering into clinical trials in most of the drug and class III

medical device development projects. The translational value of large animal models, including dogs, pigs, sheep, and non-human primates is high, due to their similar cardiovascular physiology and cellular biology to humans.[175–178] An additional advantage of large animal models is their size, allowing for the study of clinical imaging modalities, device implantations, and mechanical interventions. Another advantage, as compared to small rodents, is that per animal many simultaneous or serial tissue and blood samples can be taken, avoiding the need for a separate group of animals for each measurement. Despite their non-disputable advantages, large animal models are costly, require specific infrastructure and handling and lifespan and gestation times are longer. Genetic manipulation of these animals is difficult and may raise ethical questions, but if successful, genetic pig models are extremely helpful in the design of new therapies.[114] Below is a brief, non-exhaustive overview of available large animal models.

HFrEF or ischaemic–reperfusion injury without infarction mimic human ischaemic heart diseases very closely (*Table 3*).[30,35–39] In contrast to dogs, pigs (like humans) have sparse coronary collaterals. Therefore, pig or mini-pig ischaemic/reperfusion/infarction models were introduced. The porcine closed-chest reperfused MI model mimics the primary percutaneous coronary intervention in ST-segment MI, and just as in humans, cardiac function can be comprehensively investigated with cardiac MRI.[35] Such models successfully mimicked the neutral or minimal cardioprotective effect of ischaemic conditioning seen in clinical trials.[31] The size and shape of MIs in pigs are also more like those in humans as compared with infarctions in rats and mice, where infarct size often amounts >50% of LV mass, which is lethal in large animals and in humans. Therefore, results from studies on infarction in pigs are better compatible with those in humans than rodent studies. Atherosclerosis-induced vessel lesions, a major cause of HFrEF, can be simulated in large animal models with high translational power (*Table 8*).[138–146] Whereas dogs are more resistant to the development of atherosclerosis, spontaneous atherosclerosis occurs with ageing in pigs and non-human primates, as it does in humans, which can be accelerated with a Western diet.[142,146] Currently, there are four atherosclerotic pig models available: diabetic (type 1 or type 2) and/or diet-induced hypercholesterolaemic pigs; the Rapacz familial hypercholesterolaemic (LDL receptor mutant) pig; and Ossabaw pigs and PCSK9 gain of function pigs.[138,140,142–146] These porcine models produce human-like atherosclerotic plaques and importantly diagnostic and treatment studies in these models have corroborated observations in humans. Interestingly, these models also display marked coronary microvascular dysfunction and as such are excellent models for investigating microvascular disease.[140,144] Non-human primates, including rhesus and cynomolgous macaques, also recapitulate human-like hypercholesterolaemia when put on a high-fat/high-cholesterol diet, which after several years results in fibrofatty plaques.[146] This slow development of atherosclerosis, together with societal concerns, has resulted in restricted use of the non-human primate model for atherosclerosis studies. Perhaps with the advancement of genetic manipulation, accelerated atherosclerosis of primate models will be possible.[146]

Structural cardiac remodelling, such as hypertrophy or fibrosis, can be induced in pigs by implantation of stents or an inflatable aortic cuff, which results in a gradual pressure overload of the LV thereby causing hypertrophy, impairment of relaxation and HF symptoms.[75,76] The latter models may be used to model HFpEF-related structural concentric remodelling and coincident diastolic dysfunction (*Table 4*). Subcutaneous implantation of deoxycorticosteroneacetate (DOCA) pellets in combination with a Western diet resulted in chronic hypertension-induced myocardial hypertrophy with impaired relaxation and preserved LVEF in pigs,[70] while treatment with cardiotoxic cancer drugs such as doxorubicin cause remodelling of the pig heart, including fibrosis and reduced systolic function.[44] As described in Section 2.3, mimicking HFpEF in a large animal model represents a challenge, and thus far most models incompletely mimic the clinical phenotype and may show hypertrophy and diastolic dysfunction without clinical HF characteristics. The addition of relevant interventions or comorbidities is essential to trigger the microvascular dysfunction associated with systemic metabolic stress.[85,179]

An area where experiments on dogs have been indispensable for developments in understanding of disease and development of new therapy is dyssynchrony, induced by intrinsic conduction block in one of the bundle branches or by pacemaker therapy for bradycardia purposes. Dog experiments showed how abnormal conduction of the electrical impulse through the ventricles creates different contraction patterns and loading conditions in opposing ventricular wall segments, thereby lowering ventricular pump function, followed by adverse remodelling over time, with very diverse molecular abnormalities.[180] These experiments also showed how cardiac resynchronization could cure all these abnormalities.[181] Other animal species turned out to reflect the human situation less well.[182] Atrial and ventricular arrhythmias and sudden cardiac death can occur during the development of myocardial disease, or during pacing-induced rhythm disturbances in several large animal models.[97,104,105,183,184] In large animals AC, DCM and HCM are diagnosed and represent an interesting alternative model to study arrhythmias and cardiac dysfunction in genetic heart disease (described in Section 3.3). In addition, valve insufficiency and stenosis are mimicked in several large animal models[133–135] and are used to study pathomechanisms as well as to test novel therapeutic interventions. For the development and testing of heart valve prostheses large animal models became indispensable (see Section4.4). Sheep were extensively used to test prostheses based on biological materials especially as sheep had a very sensitive reaction with calcification if there were impaired graft conditions. As a result, heart valve prostheses based on decellularized allogenic valve matrices were directly introduced into clinical application after successful testing in sheep.[185,186] The pig has become a common transgenic animal model, and genetically modified porcine tissues and organs are gaining the attention of xenogeneic transplantation medicine. Furthermore, whole animals may also serve as 'humanized' recipients. Baboon, an old world monkey, lacking the prominent xenoantigen alpha-Gal is considered to be the large animal for testing immunological aspects. Therefore, genetically modified porcine tissues (e.g. decellularized heart valves, and organs) are tested in baboons.[185]

An example of the complexity and paradox of the cardiovascular system research is tissue-engineered heart valves (TEHVs), any other vascular conduits, or organic patches that can be constructed without using animals. However, to prove the safety and efficacy of the medicinal product, they must first be implanted in animals before human use. Additional comorbidities, such as diabetes and/or hypertonia-induced chronic kidney disease and related alterations in organ function would be possible to mimic in large animal models, but due to their complexity and cost, such models are rarely applied.

3.3 Companion animals

Naturally occurring large animal models have mostly been found in companion animals or livestock, as these animals ubiquitous in our society because of their emotional and economic value.[187] The most prevalent non-ischaemic cardiomyopathies in humans are commonly diagnosed in companion animals. HCM is the most common feline cardiac disease affecting around 15% of all cats.[188] Mutations have been reported in

MYH7[189] and *MYBPC3*.[190,191] DCM is more common in dogs and affects mainly large breeds, including Doberman, in which its prevalence reaches 58% and predominantly affects males.[192–197] The two main histological findings described in canine cardiomyopathies include attenuated wavy fibres, occurring in various breeds, and fibro-fatty infiltration of the myocardium, mainly observed in Boxers and Doberman Pinschers.

As in humans, canine DCM has a strong genetic basis with marked familial transmission. Human DCM-associated mutations have been reported in dogs in *PDK4, TTN, DMD*, and *PLN* gene.[194,195] Finally, AC is commonly diagnosed in Boxers and as in humans, it is characterized by fibrofatty replacement, ventricular premature complexes and ventricular tachycardia.[196,197] Being large animals, companion animals have weight, metabolism, and pharmacokinetics that are closer to humans than rodents, allowing therapeutics to be tested for efficacy and toxicity using a relevant regimen. Coupled with the fact that they are relatively outbred, share our environment, are often aged and affected by multiple comorbidities, companion animals make ideal models for testing novel therapeutic interventions (i.e. gene therapy).[198,199]

3.4 *Drosophila*

For several years, the *Drosophila* heart has been used as a tool to study various aspects of the heart, including development, mechanisms of cardiac diseases, and drug screening. The *Drosophila* heart is a linear tube, reminiscent of the primitive vertebrate embryonic heart tube. Although the final heart structure in *Drosophila* is very different compared with that in vertebrates, the basic elements for heart development, function, and ageing are conserved.[200] In addition, *Drosophila* offers the opportunity to manipulate gene expression in a highly precise spatial and temporal fashion, using the UAS/GAL4 system.[201] This system was successfully utilized to identify genes causing cardiac diseases, including AF and cardiomyopathies.[201] New techniques, such as optical coherence tomography, allow accurate phenotyping of cardiac diseases, including HF, HCM, DCM and AC as well as cardiac arrhythmias, such as AF, in flies.[96]

Because of its simplicity, ease of culturing, and genetic interventions, the *Drosophila* heart has also been successfully used for drug and genome-wide screening assays, for example, to screen for novel drugs directed at conservation of the proteostasis pathway, which underlies AF.[202] Finally, the *Drosophila* heart has been exploited to verify the outcomes of a human genome-wide association study (GWAS) on genes related to heart rate.[203] In this GWAS, 21 loci associated with the heart rate were identified. Experimental down-regulation of gene expression in *Drosophila* confirmed the relevance of 20 genes at 11 loci for heart rate regulation and highlighted a role for the involved signal transduction routes, embryonic cardiac development and the pathophysiology of DCM, congenital HF, and/or sudden cardiac death.

3.5 Zebrafish

Since their introduction into the biomedical research arena in the 1970s, zebrafish (*Danio rerio*) have become widely used to study cardiac function and disease due to their tractable genetics.[204] Sequencing the zebrafish genome in 2013 revealed that >80% of human disease-related genes have an orthologous gene in zebrafish.[205] Together with new developments in genome editing techniques, such as Talens and CRISPR/Cas9, efficient protocols were generated for gene knock-outs, knock-ins, and 'humanized' fish carrying human-specific disease alleles.[206] A promising feature is that the larvae are small, completely transparent, display similar cardiac electrophysiology to humans and readily take up chemicals from the water, so that they can be grown in a 96-well plate and used for drug screenings.[207] Several compounds that have been identified in zebrafish-based assays, are now being tested in clinical trials.

Despite clear anatomical differences, as the two-chambered zebrafish heart consists of an atrium and a ventricle, all major cardiac cell types are present, this allows for the study of their origin, regulation and function. Thus, the zebrafish has proven useful for studying numerous cardiac pathologies. Due to its regenerative capacities, cardiac regeneration remains the most frequently studied process. Upon injury, CMs are able to de-differentiate, proliferate and re-differentiate into mature CMs recapitulating embryonic development of the myocardium.[208] In addition to cardiac regeneration, inhibition or genetic deletion of pathways can be very helpful for identifying mechanisms of congenital malformations.[204]

What the zebrafish community currently lacks is a reliable method to create conditional knock-outs, allowing for the investigation of gene functions in a tissue-specific manner. Hopefully, new developments using CRISPR/Cas9 will resolve these.

4. Models and tools to reduce, refine, and replace research in laboratory animals

4.1 Human tissue samples

Research tools to study cardiovascular (patho)physiologic properties in adult myocardium and blood vessels require careful tissue sampling and storage. A pioneer in setting up a cardiac tissue bank is Prof. dos Remedios, who initiated The Sydney Heart Bank in 1989. Cardiac samples in the Sydney Heart Bank have been collected in a highly routine manner, assuring high quality of tissue samples that have been key in advancing cardiovascular science in many areas ranging from genetics to functional muscle studies.[209] RNA deep sequencing of human samples (e.g. cardiac muscle biopsies, vessels) that are obtained during cardiac catheterization or surgery from patients at different disease stages allows molecular profiling, pathway analysis and therapeutic target discovery in relation to different cardiac disease phenotypes.[210] Adult human tissue, either as membrane-permeabilized myofibrils, CMs and muscle strips, or intact CMs and SMCs, allow studying myofilament kinetics, myofilament calcium sensitivity, ATP consumption, metabolism and mitochondrial function, electrophysiology and response to different pharmacological agents.[211–217] As the preparations are derived from adult hearts, the physiological relevance and pharmacological predictivity are high. Adult CMs are relatively delicate cells, difficult to maintain in culture and have a limited lifespan and potential for expansion. Myocardial tissue slices of human samples represent a new opportunity for studying human tissue over a longer time span in culture. The methodological and technological progress associated with living myocardial slices (LMS) preparations and *in vitro* culture have increased the interest in this research platform. LMS are 200–400 μm thick sections of living myocardium where structure, function and biochemical properties of the *in situ* heart are largely preserved.[218,219] As such, LMS can be used to study the connections, networks and interplay between the different cardiac cells in a more controlled, comprehensive and realistic manner. LMS thinness allows for oxygen and nutrients diffusion which is critical during experimentation and chronic culture. A high-precision vibratome is required to produce

LMS, the slicing is very precise and automated, this is a prerequisite for higher throughput. Between 2 and 9 LMS can be prepared from mouse or rat hearts. However, this number can increase to hundreds when large portions of myocardium are available from large animals or human samples. The LMS technology may significantly reduce the number of animals needed for experimental studies. The preparation of LMS from human specimens is also crucial for translational research.[220] A large variety of assays can be applied to interrogate LMS. Functional parameters include, but is not limited to: contractility, conduction velocity, Ca^{2+} transients, action potentials and metabolism.[218,221] Structural assessment provides analysis of cellular and ECM organization, In addition, specific biomolecules can easily be labelled and visualized. Biochemical assessment can also be used to assess LMS genomic and proteomic signatures.[222,223]

Novel biomimetic technologies allow LMS to be maintained *in vitro* in a highly functional state and cultured in stable conditions for extended periods,[224,225] this allows for novel areas of cardiovascular research to be unravelled. Unique therapeutic research applications may utilize long-term efficacy prediction, RNA-based target evaluation, cell-based regeneration, and high-content analysis by RNA-seq. With standard couriers being used for tissue specimens or LMS movement, it is likely that laboratory networks will soon be formed to share human material that will reduce waste of tissue and increase data collection.

Like any other research model, LMS have limitations that should be carefully considered. Tissue damage occurs during cutting which is likely to trigger inflammatory responses and tissue remodelling. In addition, LMS are disconnected from the circulatory system and neuro-hormonal stimulation. The heterogeneity among LMS obtained from the same heart, as a result of the region that is sliced, should also be considered.[226] Furthermore, the lack of standardization across laboratories may result in variable readouts. Biomimetic approaches have enormously improved LMS *in vitro* culture, however, the preparations progressively adapt to the new *in vitro* environment that over time results in an alternative phenotype. This adaptation could potentially be controlled by culture conditions and improved biomimetic technologies. It might even level out the variability among samples from diseased individuals. Even though LMS have a bright future several challenges remain that have to be tackled. The standardization of LMS preparation and culture requiring refinement, education and validation of research readouts and applications, are a priority.

Isolated segments of human blood vessels (e.g. human mammary arteries, human coronary arteries, renal arteries, organ-specific vessels or aneurysm samples) can provide unique insights into disease pathology in patients, through western blotting, RNA studies as well as functional vasomotor studies.[227,228] Moreover, 24–48 h orgainoid culture can provide valuable pharmacological and mechanistic information. Human mammary arteries (IMAs) are most readily available as a model of systemic vascular function regulation and vascular oxidative stress. While IMA does not develop atherosclerosis, it is sensitive to local pro-atherosclerotic insults eliciting endothelial dysfunction and oxidative stress.[229] This approach may be most effectively used in combination with other methodologies described here to identify key novel mechanisms in a translational fashion.

While the demanding logistics represent a challenge, and sample availability is relatively limited, human cardiac, vascular and valvular tissue samples have proven an essential tool to uncover mechanisms of human disease and sex differences. Moreover, human tissue samples provide an excellent basis for validation of the hiPSC-derived models described below.

4.2 Human stem cell-derived cardiovascular cells and their 3D derivatives

The advent of methods to reprogramme somatic cells (e.g. from skin, adipose tissue, peripheral blood and urine) to human iPSC as well as the derivation of *bona fide* CMs and other cardiovascular cell types at principally unlimited scale, has boosted research in this area by complementing, and occasionally replacing animal experimentation. Recent advances in differentiation protocols[230] and mimicking organ-like function *in vitro* will further enhance this trend.

The human biology of hiPSC-derivatives principally increases the validity and translatability of experimental results when compared with cells from animal species, particularly rodents. Cultures of hiPSC-derivatives are generally more stable and produce more robust data than freshly isolated primary cells, tissues or organs (e.g. Langendorff-perfused hearts), which represent dying-cell-models. Human iPSC-derivatives represent a biological basis that is more physiologically relevant for mechanistic studies than the available immortalized cell lines. The genetic background of patient-derived hiPSC allows for modelling of individual disease mechanisms and susceptibility. Furthermore, direct access to pharmacological and genetic manipulation *in vitro* (e.g. by gene editing) facilitates studying direct drug/gene cause–effect relationships under controlled conditions. Moreover, cellular models can be exploited to identify both cardioprotective and pro-proliferative therapies and are particularly amenable to HTSs (Section 4.5). Co-cultures of various hiPSC-derived cell types can decipher some cell–cell interactions in a forward manner, which can be combined with tissue engineering to provide organoid-shaped and biomechanical-modelled platforms.

Human iPSC-derivatives exhibit a fetal rather than adult phenotype with only partially canonical function.[231] Human iPSC-CM, such as foetal, neonatal and immortalized cells, have poorly developed mitochondria and rely on glycolysis rather than substrate oxidation.[232] Consequently, they exhibit a high basal glucose catabolism with poor insulin responsiveness (i.e. only at supra-physiological insulin concentration).[233] Whereas differentiation protocols introduce batch-to-batch variation, reprogramming and long-term culture can induce artefacts such as karyotype abnormalities and epigenetic alterations that are difficult to control.[234] *In vitro* assays only partially capture disease-relevant whole organ functions (e.g. arrhythmias and diastolic heart function). Of the most common human pathology ischaemic damage by blood vessel occlusion, only the earliest stage of ischaemia can be modelled *in vitro* (*Table 3*). Cell–cell-based mechanisms (e.g. through the dynamic influx of inflammatory and immune cells) are difficult to explore *in vitro*. In models of iPSC-derived cardiac tissue, vascularization and ultimately perfusion are key challenges that are often underestimated in their influence on cell behaviour and in their relevance for rebuilding more physiological tissue. Moreover, the limited time lines of *in vitro* experiments impede assessment of cardiovascular disease mechanisms that often act over many years. This limitation also applies to the most common animal models, but multicellular responses could, in principle, be better assessed in animals. Major cardiovascular risk factors and comorbidities such as ageing and metabolic diseases, including hyperlipidemia and diabetes, can only partially be addressed *in vitro*. Organ–organ interactions (e.g. effects of the liver, gut or brain on heart function) cannot be captured in current *in vitro* hiPSC cultures.

Solutions to increase the applicability of hiPSC-derived cell systems for cardiovascular studies are described below:

- *Reduce experimental variation*: Employing established quality standards, such as: the obligatory use of standard operating procedures, master

and working cell banks, defined passage number, proven normal karyotype, high pluripotency marker expression, isogenic controls (e.g. by CRIPSR/Cas9 gene editing), minimum repetition of experiments in three batches from three lines, and standardizing circadian time will reduce variability.[235,236] Worldwide hiPSC banking initiatives such a hPSCreg (http://hpscreg.eu) add to this standardization. Furthermore, automation has the potential to reduce experimental variation[237] and will likely become more common in high-throughput facilities (e.g. for drug screening). The high costs for initial investment and maintenance limit a more widespread application in academia.

- *Improve maturity*: Refinement of culture media composition (e.g. energy substrates, hormones and growth factors)[238,239] as well as culturing of hiPSC-CM on matrices with tunable stiffness,[240,241] Matrigel mattresses,[242] or micropatterned surfaces[203,243] have been shown to improve the maturity. Consistently, lowering glucose and adding fatty acids have been shown to improve the metabolic maturity of hiPSC-CM, reflecting the fact that the use of glucose is inhibited by fatty acid oxidation in a fasting state and is stimulated by insulin in a fed state.[244] 3D Multicellular constructs, mechanical loading, and electrical pacing (e.g. in EHT) are some of the most effective means to improve the structural, metabolic, electrophysiological, and contractile maturity of hiPSC-CM and the spectrum of functional readouts.[245,246] Further improvements are expected from co-cultures of hiPSC-derived CMs, fibroblasts, endothelial cells, neurons, immune cells, and others.[247] So far, several differentiation protocols for the respective cell types are available,[248] but it is still not known how well these cells resemble the organ-specific cells in their respective environment (e.g. cardiac endothelial cells). More work is needed to achieve truly adult-like CMs/heart tissue from hiPSC.
- *Improve the functional readout*: Simultaneous measurements of force, calcium transients, and membrane voltage by fluorescent dyes (e.g. Fluo-4, FURA-2, Arclight, Fluovolt,[249,250] or genetically encoded calcium sensors such as GCaMP6f[116]) improve the depth of phenotypic characterization of hiPSC-CM/EHT and allow analysis, including arrhythmias, in intact preparations.[251] Sharp microelectrode action potential recordings reduce confounding influences of cell isolation and the small size of hiPSC-CM compared to patch clamp recordings.[252] However, tissue damage and localized ischaemia may occur, and patch clamp recordings in isolated hiPSC-CMs with or without dynamic clamp may be considered for certain studies.
- *Study hiPSC phenotypes under disease-provoking conditions*: Experimental setups that allow the manipulation of matrix stiffness or afterload in 3D constructs can provoke phenotypes masked under basal condition.[241,253] Influences of common comorbidities on disease phenotypes in patient-derived hiPSC-CM or the effect of simulated ischaemia may be studied by applying hyperglycaemic and hypercholesterolaemic culture conditions as shown in fetal rat myocytes.[254] *In vitro* vascularization may allow for the study of mechanisms of thrombosis and ischaemia *in vitro*.[255]
- *Study organ–organ interactions*: Organ-on-chip approaches (i.e. microfluidic culture systems in which organotypic cell types are cultured in one or multiple compartments connected by circulating medium) offer the opportunity to study organ-like function or complex interactions between organs of the human body, for example, between the drug-metabolizing liver and the heart (multi-organs-on-chips).[256] Even though perfusable tissue surrogates are available, but they are still far from replicating a vascularized organ with chambers, conduction system, and physiological function, and would therefore only enable partial replacement of animal experiments. The potential of these new approaches has to be weighed against their technical complexity. Moreover, the necessary simplification of culture conditions may interfere with the desired maturity of the respective 'mini-organs'.
- *Alternatives*: The necessary level of maturity and complexity depends on the question being asked. For some high-throughput screens, a simple and cheap cell line might be appropriate as a first choice (e.g. the rodent cardiomyoblastic cell line H9C2). These cells have primarily skeletal muscle characteristics and lack cardiac contractility. HL-1 cells, derived from a mouse atrial tumour, exhibit several cardiac-specific phenotypes but proliferate possibly involving more genetic alterations than the initial SV40 antigen expression.[257] More recently, rat atrial CMs were transduced with a doxycycline-dependent SV40 LT antigen that could be easily expanded and differentiated into excitable and contractile atrial CMs upon removal of doxycycline.[258] The rodent background of these CM-like cells has, however, a considerable limitation. More recently, a similar approach was used for generation of a human atrial immortalized cell line.[102]

As indicated above, further fine-tuning of differentiating iPSC-derived cell types and generation of multi-cellular models is ongoing. Promising developments that may be able to reduce the use of animal models, include the generation of simple 3D microtissues or organoids containing iPS-derived cardiac endothelial cells, fibroblasts and CMs,[247] most likely applying matrix-like substances,[259] containing a vascular network,[260] or using printed scaffold materials to tailor microstructural mechanical design and mimic cardiac stiffness.[261]

4.3 Animal-free strategies to mimic valve disease and vascular pathology

In recent years, animal-free strategies have been introduced to uncover the pathophysiologic mechanisms underlying VD, atherosclerosis and AAA.

For VD several studies focused on decrypting the cellular pro-calcific phenotype by evolving 3D pathology modelling involving substrates with defined chemical and mechanical characteristics using an integrated vision of 'mechano-paracrine' signalling controlling the physiological versus the pathological phenotype of VICs. The stiffness sensitivity of VICs was demonstrated, for example, in studies performed with hydrogels with tuneable mechanical characteristics,[262] as well as in the presence of paracrine signalling by TGF-β.[263] More recently, investigations have allowed for the characterization of the molecular signalling underlying the activation of VICs towards the pro-fibrotic phenotype. In particular, for describing the relevance of the mechanically activated Hippo transcriptional machinery[264] for porcine[265] and human[266] aortic VICs pro-fibrotic activation. In aortic VICs, this pathway was more active close to the calcified areas.[267] Another option relies on complex fabrication processes of valve microenvironments combining different ratios of matrix components (e.g. glycosaminoglycans, GAG) with hydrogels (e.g. Gelatin-Methacrylate) mimicking mechanical features of structural valve components such as collagen.[268] In addition to mechanical valves and valve prostheses made from fixed biological materials like porcine heart valves or bovine pericardia, prostheses made from decellularized heart valve matrices may become the gold standard as these display fundamental beneficial characteristics.[269] With these approaches, it is more feasible to investigate the complex response of valve cells to pathophysiologic stimuli in the context of valve tissue-mimicking architecture and essential biophysical characteristics (*Figure 2*).

AA for atherosclerosis, flow chambers coated with human atherosclerotic plaque lysates are being applied to study the dynamics of platelet and leucocyte plaque interactions under flow conditions. Tissue-engineered vascular grafts, composed of polymers, and implanted in bioreactors or animal models for vascular tissue regeneration, have been successfully created.[270,271] Chip-based microfluidics systems containing 3D structures with an arterial geometry build, containing iPSC-derived

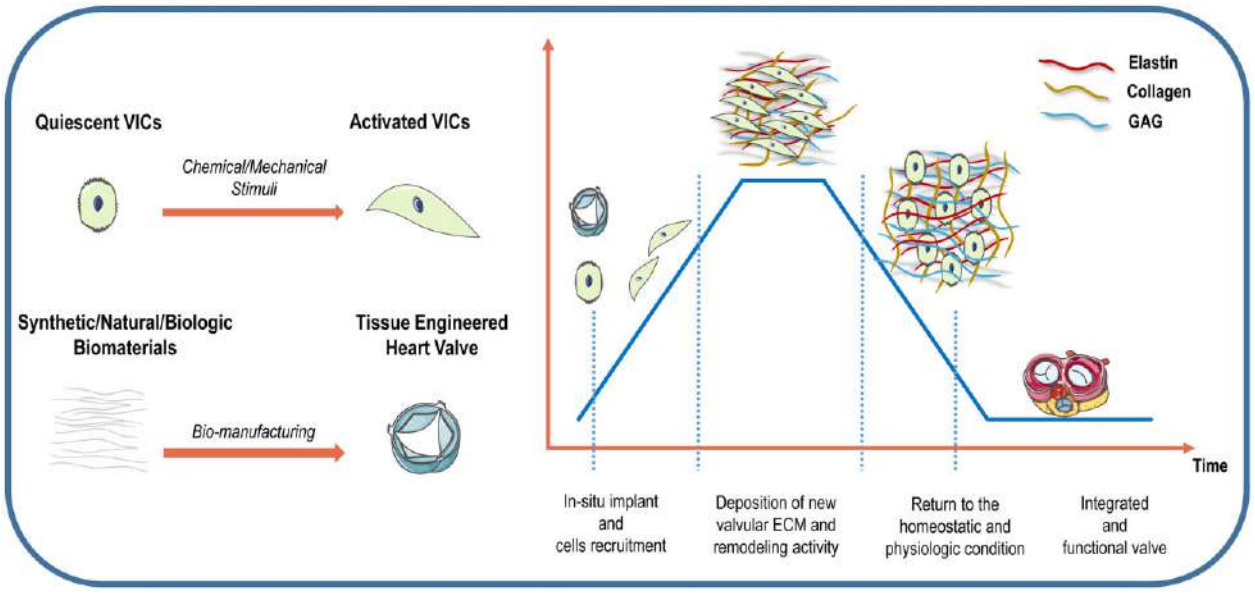

Figure 2 *In situ* valve engineering.

pericytes, vascular SMCs and endothelial cells, can be subjected to flow and shear stress. These are useful for studying the effects of flow and shear stress on endothelial cell biology, as well as arterial thrombosis.[272,273] These novel 3D tissue-engineered arteries can be considered a prelude to the 3D *in vitro* generation of atherosclerotic plaques. However, engineering an artery that contains the arterial geometry, is subjected to flow conditions, contains a plaque in which all cells are represented, immune cells are recruited, and lipids are processed, is still not possible and poses a future challenge.

AAA studies in aortic tissues or models developed with patient cells from biobanks studying the SMC contractility and AA pathophysiology (Section 4.1),[217,274] as well as novel *in vitro* 3D models to study SMC-ECM interactions are forthcoming. Advancements are made to integrate mechanical components into these models to mimic shear stress, which can activate inflammatory pathways, atherosclerosis, intima hyperplasia, and aneurysm formation.[275,276] The evolution of imaging-based models of intravascular flow dynamics has revealed that pathological programming of the vessel wall may also occur with the crucial contribution of the wall stress.[276] Recently, the concept of cell mechanosensation has come to connect the transmission of mechanical forces to cells from the ECM or vice-versa and to discrete gene regulation patterns affecting the cellular homeostasis within the cardiovascular system.[277] This has confirmed the existence of novel mechano-dependent pathologic pathways. For example, through an *in vitro* model of circumferential wall strain associated with coronary flow dynamics occurring in arterialized saphenous veins, involvement of Thrombospondin-1 (TSP-1) in pathological activation of resident myofibroblasts in the wall was revealed for the first time, with consequences for neointima accumulation and vein graft failure.[278] Since TSP-1 has a role in the formation of ascending aneurysm through a mechanism involving changes in mechanical characteristics of the vessel wall,[279] it could be a key factor connecting alterations in tissue biophysical features, modifications in cellular composition and signal transduction.

Molecular modelling with 'vasculature-on-a-chip' devices mimicking the architecture, mechanics and cell setup of arteries and veins has finally become a novel way to investigate vascular pathology programming (*Figure 3*).[280] These models have the advantage of being easily manufactured with biocompatible materials, are miniaturized and reproduce the haemodynamic patterns typical of pathologic vasculature. This is expected to allow an unprecedented multiplex analysis power with cells that can be directly derived from patient biopsies without involving animals, providing immediate translational and personalized therapeutic perspectives.

4.4 Production and testing of heart valves

Given the limited number and sizes available from human donor material, current research focuses on the development of non-immunogenic xenogeneic heart valves matrices.[281] Developed in the sheep model, orthotopically implanted acellular allogeneic pulmonary and aortic heart valve matrices get repopulated with autologous interstitial cells, whereas the lumen gets re-endothelialized by autologous endothelial cells.[269] With this, the grafts are non-thrombogenic and regain the ability to adapt to the growth of the recipient. Therefore, these animal-free based strategies are easily translated into the clinical setting as they provide the possibility to create new transplantable valves which are of utmost importance, for instance, for paediatric patients.[282]

The principle of the tissue engineered heart valve (TEHV) is based on the construction of a biodegradable heart valve-figured scaffold that develops into living valve-formed tissue by autologous cell invasion after resolving the scaffold. The basic requirements of TEHVs are: biocompatibility, non-immunogenicity, non-thrombogenicity, capacity to mimic function and structure of the heart valves, and adaptability to physiological and pathophysiological conditions.[283]

The strategies of TEHV fabrications include molded or sutured scaffolds with using: natural or synthetic polymers, decellularization, electrospinning, 3D printing, *in vivo* bioengineering, and combination of these

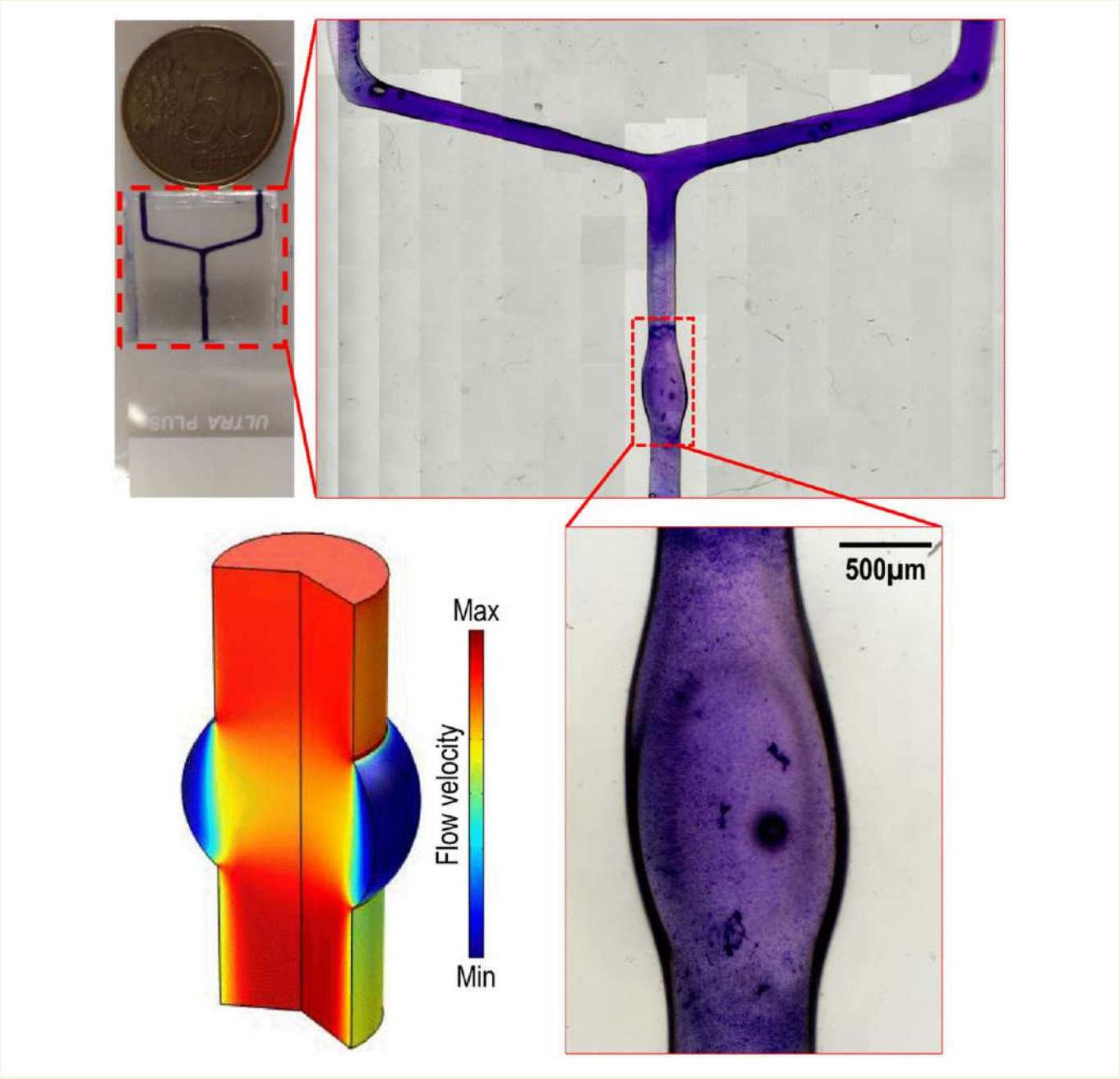

Figure 3 Aneurysm-on-a-chip manufactured with a 3D printing-based microfluidic channel patterned inside a polydimethylsiloxane (PDMS) block. The heatmap represents the distribution of the flow velocity reproducing the hemodynamic conditions occurring into aneurysms. *Figure 3* is original and contains unpublished modelling and manufacturing images.

techniques (hybrid TEHVs).[284] The majority of the TEVHs are constructed by molding of polymeric substances into a valve-like shape, or attaching to an appropriately formed stent.[285] For the engineered tissue, either natural biopolymers, such as collagen or fibrin or synthetic polymers (e.g. polyglycolic acid, polylactic acid, polyε-caprolactone, poly4-hydroxybutyrate) are used. The stent-polymeric scaffolds are then populated with different types of cells (e.g. marrow stromal or endothelial cells, or mesenchymal stem cells) in bioreactors to avoid foreign body reaction. The second most frequently used TEHV fabrication is the decellularization of animal heart valves by using detergents, immersion, or perfusion approaches.[286] Currently, two TEHVs have been approved for human use: the Cryolife's SynerGraft® in Europe and the USA, and AutoTissue GmbH's Matrix P plus N™ in Europe. Unfortunately, the safety and efficacy of these products are currently rather insufficient, showing controversial results in clinical applications.[287,288]

Electrospinning is less frequently used due to its complexity. This technique is based on creating a solid controlled fibre structure of TEHV. The construction of which fits better to the anisotropic mechanical characteristics of the natural valve, simulating the microarchitecture of the valve better than the other technologies.[289] To enable a 3D Bioprinting of a TEHV, a 3D imaging (computed tomography or magnetic

resonance) is first applied, and converted to a stereolithography computed file of the 3D printer, followed by bioprinting of the TEHVs (inkjet, extrusion or laser-assisted) by using bioinks of cell-free or cell-encapsulated biomaterial.[290] The hybrid technique to construct TEHV combines decellularization, and cell seeding technologies, as well as tubular fibrin gels, encapsulating cells followed by decellularization or the electrospinning method recombining with gelatin hydrogels, or others. The *in vivo* tissue engineering of a valve requires its implantation in an animal species chosen for the experiment (*in vivo* 'bioreactor or cell culture'), and cellularization *in vivo*, followed by orthotopic implantation.[291] Each TEHV construction technology has its advantages and disadvantageous, and a great deal more scientific and technological development is needed for human translation of the TEHVs.

4.5 High-throughput screenings

Over the last decade, there has been an explosion of studies based on HTSs of both small molecules and small nucleic acids in cultured CMs for drug and gene discovery. This was rendered possible by the development of biological assays amenable to miniaturization and automation, and by the availability of technologies for processive high content (HC) microscopy imaging, determination of mechanical forces, and electrophysiology measurements. The use of cultured cell lines of cardiac derivation, primary fibroblasts or neonatal CMs or human embryonic stem cell (hESC)/hiPSC-derived CMs has been instrumental in the possibility of identifying active compounds through large library screenings.

A number of cellular, molecular, and functional assays can be adapted to 96- or 384-well plates and thus rendered amenable to HTS analyses. To search for small molecules or nucleic acids regulating these processes at the cellular level in primary CMs or CMs derived from hESC/hiPSC lines the following has been implemented: the incorporation of thymidine analogue to measure CM proliferation,[292–294] assessment of CM cross-sectional area,[295–297] inhibition of pathologic aggregate formation,[298] protection from cardiotoxic treatments,[299–301] or regulation of Ca^{2+} handling.[302] The development of HTS assays aimed at assessing two fundamental parameters of CM function, namely electrical activity and contraction force, is definitely more demanding in terms of instrumentation and complicated by the immature nature of hESC/hiPSC-CMs. Electrophysiology assays, such as patch clamping recording, are too low throughput for HTS, although automated patch clamp technology is advancing. Nevertheless, this limitation can be overcome by using optical recording of fluorescent sensor probes of transmembrane voltage, current transients using dedicated devices or by HC microscopy.[303,304] Mechanical force exerted by CMs can be measured, in an HTS format, by culturing cells on thin films of materials that can be bent by systolic contraction,[305] or by measuring contraction and relaxation of substrates embedded with fluorescent microspheres.[306] In addition to studies in CMs, a recent HTS in primary human cardiac fibroblasts identified drug candidates to target cardiac fibrosis and diastolic dysfunction.[307]

As indicated in Section 4.2, a major limitation remains the embryonic nature of hESC/hiPSC-CMs. As some embryonic characteristics can mature *in vitro* CM maturation itself can become the read-out of specific HTS with small molecules or microRNAs. In addition to the cell studies which replace animal studies, recent advances in HTS measurements in enzymatically isolated intact single CMs from rodent hearts reduce the number of animals required for high-throughput testing of compounds and stressors.[308,309]

Finally, the possibility of growing CMs, either alone or in various combinations with cardiac fibroblasts or other cells offers the opportunity of conducting screenings in conditions of load and CM maturation closer to those of the heart *in vivo*.[310]

5. The power of data

5.1 Registration of preclinical trials: data repository for animal research

Preclinical research is pivotal to understand basic mechanisms of diseases and to provide information about the safety and efficacy of new strategies. The ultimate final goal is to make advances in medical science and to improve patient healthcare. Currently, only a relatively small number of the products from translational research finds application in the clinical setting.[311] One of the main issues with preclinical studies is publication bias. Positive and/or significant results are more likely to be published than negative study results. This leads to an overestimation of the effects of therapies and unjustified transition of interventions to clinical trials. Moreover, the lack of sharing both negative and positive results contributes to the repetition of research, and failure to comply with the 3R principles.

The development and use of an animal registry and/or preclinical network represent a possible solution for minimizing publication bias. To this end, two platforms (www.preclinicaltrials.eu[312] and www.animalstudyregistry.org[313]) were recently launched for preregistration of animal studies to increase transparency and reproducibility of bioscience research and to promote animal welfare. The registration form helps scientists plan their study thoroughly by asking detailed questions concerning study design, methods, and statistics. Although most researchers are in favour of more transparency, major disadvantages of preregistration exist, especially intellectual property (IP) issues and administrative burden. At present, these are the most likely reasons why there are only a limited number of preregistered studies. Several solutions are currently being incorporated to circumvent these obstacles. One example is when registering a study, it automatically receives a digital object identifier (DOI) that marks it as the original research idea of the investigator. In addition to this, the users can decide to restrict the visibility of their registered studies for up to 5 years. The Consortium for Preclinical Assessment of Cardioprotective Therapies (CAESAR)[314] and Mouse Phenome Database (https://phenome.jax.org/) are examples of networks in which experienced laboratories work together and share data on rodent models. The implementation of an independent and prospective animal registry and preclinical network can, therefore, support the researcher in enhancing the quality of the study, as it requires addressing blinding, randomization, sample size calculation, and power. Furthermore, they will lead to standardized protocols, and a reduction of unnecessarily repeated studies, animal use, and costs. A data repository for animal research could be exploited for advanced analysis through artificial intelligence and data mining, which could help to establish rules or formulas for predicting adverse and/or therapeutic responses.

5.2 Patient registries, biobanking, -omics studies and imaging

Further acceleration of clinical cardiovascular research will only be possible if networks are created across institutes and countries to facilitate collaborative data science. In particular, the implementation of (trans)national networks across institutes using similar data models and harmonized clinical care pathways will facilitate patient recruitment in targeted clinical trials and enable genotype–phenotype association studies with

appropriate statistical power, for example, in cardiomyopathy patient groups. Furthermore, it would provide a framework for a learning healthcare system through benchmarking, cross-validation of novel strategies and artificial intelligence algorithms in both research and routine care. Unsupervised learning allows for the clustering, structuring and compressing of the information content for a high-dimensional dataset of important features or main components. Common methods are principal component analysis, spectral clustering[315] or deep autoencoders.[316–318] A well-known extension to autoencoders are variational autoencoders that allow efficient inference and learning in directed probabilistic models.[319] Autoencoders are neural networks used to learn an efficient representation in an unsupervised manner. They contain a bottle-neck layer that then generates the latent space of compressed variables. Understanding the underlying data distribution and the effect of involved parameters with such a deep autoencoder, generates predictive models[320] and simulates the effect of different parameters, such as drug responses.[321]

Great steps in creating collaborative networks for human data exchange have been made through the creation of large biobanks, for example the, UK Biobank (https://www.ukbiobank.ac.uk/about-biobank-uk/) and Generation Scotland project (https://www.ed.ac.uk/generation-scotland). Both are resources of demographic, clinical information, biological samples and in some cases imaging data from thousands of volunteers from the South of England and Scotland, respectively. Both biobanks have established multi-disciplinary skills networks in health informatics, epidemiology, genetics, health economics, and focused data analyses from cross-sectional whole-body imaging and specific cardiac imaging. Significant ethical, legal and social issues need to be addressed to allow such complex biobanks to operate safely. The fundamental aim of such large biorepository resources is to improve the prevention, diagnosis, and treatment of a wide range of serious and life-threatening illnesses. Scotland in particular has a unique electronic health record system with data linkage dating back to its creation in 1986, the information available from the Biobankscan be data-linked with clinical outcomes and long-term follow-up, as well as genetic analysis of its participants. Whilst these Biobanks have only recently been established in the past decade, there are much older and implicitly extremely valuable long-term follow-up registries. For example, the Aberdeen Children of the 1950's, which comprises 12150 participants born between 1950 and 1956 who were subsequently deeply phenotyped every decade with state-of-the-art investigations contemporaneously available at each such time point.

An example of utilizing the maximal potential of data obtained within the different disciplines is Network Medicine. It originated from the fact that conventional scientific reductionism is inadequate for understanding complex diseases and developing precise therapies. Moreover, it views health and disease as an interplay among molecular and environmental determinants that must be fully considered in precision medicine. Network Medicine, therefore, uses big data to create an integrated set of principles and discoveries that can fully capture these inherent dependencies. Focusing on the interaction of biological components, such as proteins, mRNAs, microRNAs, or metabolites, allows us to understand molecular pathways that underlie the pathogenesis of diseases. In addition, Network Medicine has expanded to integrate molecular data with phenotypic features to clarify mechanisms driving clinical disorders.[322] The strategy used in Network Medicine to address a clinical question (i.e. absence of a priori hypotheses on the molecular mechanisms causing diseases or a priori molecular target selection) and the technologies used in network analysis are, by definition, unbiased, and do not affect how networks are defined in different data sets or network layers. Therefore, the network medicine approach can lead to a significant reduction of the number of animal experiments designed in the classical reductionist way. As a simple example, the miRNA expression fingerprint of the hypercholesterolaemic myocardium, allows to build the miRNA–mRNA target networks and predict key molecular targets in an unbiased way, thus remarkably reducing the necessary *in vivo* experiments for validation of predicted targets.[323]

The cardiovascular community should provide guidelines to establish a framework according to FAIR principles to: enhance findability using metadata catalogues of patients with clinical, genetic, imaging and -omics data; create transparency about accessibility protocols of existing data sources for external researchers and other third parties; stimulate interoperability across institutes to enable collaborative science and federated learning and promote reuse of data in spirit of open science and improve durability of financial and non-financial public investment.[324] Instead of manual curation of clinical care data, the cardiovascular community should aim to standardize clinical care pathways and harmonize phenotypes and outcomes within electronic health records to minimize the burden of data collection, and access the wealth of data available within our hospital systems including clinical notes, imaging and -omics data. To facilitate collaborative analyses a common data model should be adopted, like the one developed by the Observational Health Data Sciences and Informatics programme (https://ohdsi.org). A common data model will also enable distributed learning. Currently, collaboration across institutes is limited by privacy and security concerns of data sharing. However, with the development of federated learning, these restrictions could be resolved.[325] Instead of sharing data within a huge central data storage (data-to-code), the algorithms will be distributed across centres (code-to-data) without any actual data sharing. The created statistical models and its parameters can subsequently be validated across different clinical settings, patient characteristics (e.g. age, sex and ethnicity), and countries to ensure that those algorithms are generalizable or calibrated to the individual patient in front of us. The importance of such an infrastructure is clearly illustrated by the COVID-19 pandemic. Already existing networks such as REMAP-CAP (Randomized, Embedded, Multifactorial Adaptive Platform Trial for Community-Acquired Pneumonia, www.remapcap.org) and newly founded networks like CAPACITY-COVID (www.capacity-covid.eu) initiated by the cross-institutional Dutch CardioVascular Alliance (www.dcvalliance.nl) have accelerated clinical research to inform patients and caregivers about risk assessment and potential therapies for COVID-19 in a relatively short period. Further development and expansion of networks across countries are needed to collect real-time clinical information to perform point of care pragmatic trials across different groups of patients and healthcare systems.

Lastly, the cardiovascular scientific committee should not forget to involve the main group of interest, the patients.

> Quote from a patient: 'I have given permission to take blood and tissue for scientific research but I have never heard again about the results or outcome of the research'.

Too often, scientists forget to correspond about the results obtained with patient's data/tissues once a publication is accepted. Participation of patients and their family members is key for successful translational research, in particular in chronic cardiovascular diseases, where follow-up studies in patients and their families are central for improving our knowledge of disease pathomechanisms and effectiveness of treatments. The

fact that the questions of cardiovascular biomedical research are scientifically relevant does not necessarily mean that they are relevant from the patient's perspective. Most research questions are posed from a medical or regulatory perspective and are often based on a laboratory point of view and is focused on basic science that is often removed from the true needs of patients.[326] Patient participation in research is thus crucial for identifying patient-relevant questions and outcomes.

5.3 Computational modelling of cardiovascular function

Over the last two decades, there has been rapid development in cardiovascular research methodologies (e.g. advanced methods for quantification of cellular function, better understanding of intercellular communication, new methods for genetic targeting of selected pathways and advanced high-resolution medical imaging), which has increased the quality and quantity of available data on the complex and dynamic function of the cardiovascular system. The availability and the level of details of data have enabled the development of thoroughly validated computational models of heart and vessels.[327,328] These models capture the complex non-linear dynamics of the cardiovascular system across different scales, from genetic mutations to subcellular protein function and cellular electrophysiology, to tissue-scale myocardial and vascular mechanics, to organ-scale cardiac pump function and system-scale blood flow dynamics. Computational models provide a unique alternative research platform for integration of experimental data and for performing *in silico* experiments to better understand cardiovascular physiology and pathophysiology, support clinical decision making and improve safety and efficacy of drug and biomedical device therapies.[328]

The application of computational models for both fundamental, preclinical and clinical research in biomedicine is rapidly increasing[329] and this has led to many examples showing that *in silico* experiments can lead to refinement, reduction, and in some cases even replacement of animal experiments. For example, research has demonstrated that computational models of cellular cardiac electrophysiology can predict adverse drug effects (e.g. life-threatening arrhythmias) with higher accuracy than animal models[330] showing that human computational models can help to reduce the use of animal experiments in early stages of drug testing. This research is part of the Comprehensive *in vitro* Proarrhythmia Assay initiative (https://cipaproject.org/about-cipa/) that aims to integrate predictions by *in vitro, in silico* and hiPSC-CM models with clinical evaluation for drug safety testing and is promoted by regulatory bodies.

In fundamental cardiovascular research, *in silico* cardiovascular models have mainly been used to translate changes in cellular physiology observed *in vitro* or in animal models to cellular changes in human cells and whole-organ human clinical phenotypes. For example, in the context of cardiac myocyte Ca^{2+} handling, where *in vivo* measurements are not available, simulation studies have shown how *in silico* models can be used to extrapolate changes observed *in vitro* or in animal models into an *in vivo* human context.[331]

In a more clinical setting, multi-scale computational models of heart and vessels are being personalized using the rapidly growing wealth of patient-specific diagnostic data available in the clinic. The resulting virtual representation of the individual patient, also referred to as 'Digital Twin',[332] can be used to gain better insights into the patient's cardiovascular pathology, underlying symptoms and to predict the individual's response to therapy. Studies have demonstrated successful applications of personalized computational models, including prediction of arrhythmia risk in post-MI patients,[333] non-invasive measurement of fractional flow reserve from computed tomographic images of patients with coronary artery disease,[334] and non-invasive electrocardiographic imaging.[335]

In conclusion, computational modelling and simulation, sometimes called the third paradigm of science, already established a prominent role in the quest to refine and reduce the use of animal experiments for cardiovascular research. However, computational modelling is not likely to fully replace animal experiments in the foreseeable future. Animal models continue to provide novel insights into pathophysiological processes which have not yet been implemented in computational models. Moreover, animal experimental data are required for validation of computational models when human data are unavailable. What all aforementioned successful applications of computational models have in common is that they are the result of decades of basic research and multidisciplinary collaborations between researchers, computer scientists, and clinicians.

6. Moving from bench to clinic

Our paper highlights the evolution in the design of cardiovascular disease models that has taken place in a relatively brief time-span. Multiple animal-free models and tools to increase power of studies became available, and animal models have been refined in the past ~20 years. Translation of basic and clinical research to actual implementation in the clinic represents a major challence, and warrants a careful experimental design making use of available complimentary research models ranging from *in vitro* experiments in cells and iPSC-derived models to studies in rodents, large animals and patients. Recent examples, described below, illustrate the potential of such an approach to move from bench to clinic.

6.1 Peripartum cardiomyopathy

PPCM is a potentially life-threatening heart disease that emerges with acute or with slow progression of LV systolic dysfunction (LVEF < 45%) late in pregnancy, during delivery, or in the first postpartum months, in women with no other known causes of HF.[336] Risk factor profiles (i.e. higher risk for PPCM in women with African ancestry) for women with pregnancy-associated hypertensive complications, such as older women or women with twin pregnancies, suggests that PPCM consists of multiple pathomechanisms pointing to a syndrome and not a single defined disease.[336,337] This notion is further supported by the prevalence of cardiomyopathy-causing mutations in about 15% of patients[338,339] Experimental data confirm that different factors can induce and drive PPCM, including inflammation and immunity, pregnancy hormone impairment, catecholamine stress, defective cAMP-protein kinase A, and G-protein-coupled-receptor signalling genetic variants[336] and aberrant cardiac metabolism. Under physiological circumstances, maternal lipid metabolism is increased during the last trimester of pregnancy and normalizes after delivery. Recently, it has been shown that lipid metabolism is widely affected in hiPSC from patients with PPCM, findings that were replicated in a PPCM mouse model.[340] Evidence is accumulating that several of these mechanisms may merge into a common major pathway, which includes unbalanced oxidative stress and the cleavage of the nursing hormone prolactin (PRL) into an angiostatic, pro-apoptotic and pro-inflammatory 16 kDa-PRL fragment, resulting in subsequent vascular damage and HF.[336] Based on this common pathway, potential disease-specific biomarkers and therapies have emerged that are currently tested in a bench to bedside approach. One therapy concept has been

developed in mice where HF medication is combined with the PRL blocker bromocriptine and had already been introduced into 2018 European Society of Cardiology (ESC) Guidelines for the management of cardiovascular diseases during pregnancy.[341]

6.2 microRNAs - route to the clinic

Based on initial miRNA library screens miR-132 was identified as driver of pathological growth of CMs *in vitro* and next *in vivo* (*Figure 1C*).[342] In a number of mouse studies it was shown that oligonucleotide-based inhibiton of miR-132 halted and reverted pathological cardiac remodelling.[343] Following this, the therapeutic efficacy was tested in an acute[343] and a chronic[344] model of MI in pigs. These activities were recently translated to chronic HF patients where the miR-132 inhibitor drug showed a good safety profile and indicative therapeutic efficacy based on improvement of several parameters, such as reduction of N-terminal pro-B-type natriuretic peptide, paving the way for further clinical development of this new generation of HF medication.[345]

7. Conclusion and future challenges

Globally, there is a mounting belief that biomedical sciences can progress without animal research by replacing *in vivo* experiments with tests performed in human-derived *in vitro* models. While this is in part justified as multiple research questions can be answered without the use of animals, the use of animal pathological modelling is still necessary for several applications such as, implantation of medical devices (e.g. stents, new catheter-guided endoscopy systems, implant devices), *in vivo* drug testing, and for identifying mechanisms underlying cardiovascular disease as outlined in the current paper. Stem cell-based human pathology models have the potential to become key in testing toxicity and effectiveness of new drugs at a cellular or organ-like levels, but lack the complexity present in multiple forms of cardiovascular disease. As cardiovascular disease is a complex, multifactorial disorder, and the current knowledge is limited, we will have to continue to rely on laboratory animals, enabling thorough studies in a well-controlled *in vivo* setting.

In coming years, animal models will be further refined and made more 'human-like' on the basis of big data sets obtained in human studies. As pathomechanisms and treatment response differ between male and female cardiovascular patients, the effect of sex should be taken into account in the design of animal studies. Novel 2D and 3D *in vitro* technologies, and advanced computational analyses will certainly result in a more refined experimental design reducing the number of laboratory animals currently required to perform studies and test drugs. A major challenge in the refinement of iPSC-derived models is their validation, i.e. do models capture human pathophysiology? The iPSC-derived models may ultimately be used for precision medicine, however, currently, a gap exists between iPSC-derived heart models and the clinical phenotype of patients, as human cardiac muscle systems have not been validated (i.e. not compared to individual patient characteristics and human cardiac tissue samples). This limits their applicability for studies on pathomechanisms and use in the clinical setting. In addition, mimicking sex differences in stem cell-derived heart models is a largely unexplored area and warrants further research and development. Successful translation of cardiovascular research warrants integration of results obtained in animals, animal-free models and patients.

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Authors' contributions

All authors contributed to the design of the consensus document, and drafted and approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work, and have confidence in the integrity of the contributions of their co-authors.

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Data availability

No new data were generated or analysed in support of this consensus document.

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