Cardiovascular Research

Animal models and animal-free innovations for cardiovascular research: current status and routes to be explored

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Abstract

Cardiovascular diseases represent a major cause of morbidity and mortality, and necessitate research to improve diagnostics and discover and test novel preventive and curative therapies, all of which warrant experimental models that recapitulate human disease. 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 co-morbidities. 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, and 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 cannot provide a vital step in the translational pathway as they do provide important pathophysiological insights into disease mechanisms particularly on 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 improved current 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 3R (replacement, reduction and refinement) as a guiding concept.

7th of February 2021

Dear colleagues,

On behalf of Thomas Thum, I hereby submit a Joint Position Paper of the ESC WG on Myocardial Function together with the WG Cellular Biology of the Heart, entitled:

"Animal models and animal-free innovations for cardiovascular research: current status and routes to be explored".

Together with all members of these two working groups and experts in several specific cardiovascular research areas, we provide an overview of the current state-of-the-art in cardiovascular research, describing the complexities of different forms of cardiac disease and the available experimental models to investigate pathomechanisms and test drugs. We addressed limitations, opportunities and future perspectives.

Our objective was to describe the challenges which we need to address in the coming years, which may include new methodological developments, but also building biobanks including tissue from animal models and iPSC-derived cells. Moroever, with this manuscript we aim to communicate about the design of cardiovascular research to society and politicians to build support and understanding for the need of animal studies in the translation and implementation of results in clinical practice.

The European Society of Cardiology supports our submission. All co-authors have read the final version and agree on submission.

We hope you will find our position paper suited for publication in Cardiovascular Research.

Sincerely yours,

on behalf of Thomas Thum and all co-authors,

Jolanda van der Velden

Physiology

Amsterdam UMC

Animal models and animal-free innovations for cardiovascular research: current status and routes to be explored

Position paper by the ESC working groups Myocardial Function & Cellular Biology of the Heart

Short title: modelling cardiovascular disease

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Abbreviations

3Rs, replacement, reduction, refinement
AAA, Abdominal aortic aneurysms
AC, Arrhythmogenic cardiomyopathy
AF, Atrial fibrillation
ARBs, Angiotensin receptor blockers
CAVD, Calcification aortic valve disease
CMs, Cardiomyocytes
DCM, Dilated cardiomyopathy
ECM, Extracellular matrix
EHT, Engineered heart tissue
ES, Embryonic stem cell
ESC, European society of cardiology
HC, High content
HCM, Hypertrophic cardiomyopathy
HF, Heart failure
HFmrEF, Heart failure with mid range ejection fraction
HFpEF, Heart failure with preserved ejection fraction
HFrEF, Heart failure with reduced ejection fraction
hiPSC-CMs, Human induced pluripotent stem cell-derived cardiomyocytes
HTSs, High-throughput screenings
LQTS, Long QT syndrome
LMS, Living myocardial slices
LVEF, Left ventricular ejection fraction
MI, Myocardial infarction
MRI, Magnetic resonance imaging
NO, Nitric oxide
PPMC, Peripartum cardiomyopathy
PRL, Prolactin
SMC, Smooth muscle cell
TEHV, Tissue engineered heart valve
VD, Valve disease
2D, Two dimensional
3D, Three dimensional
VECs, Valvular endothelial cells
VICs, Valvular interstitial cells

Abstract
Cardiovascular diseases represent a major cause of morbidity and mortality, and necessitate research to improve diagnostics and discover and test novel preventive and curative therapies, all of which warrant experimental models that recapitulate human disease. 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 co-morbidities. 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, and 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 cannot provide a vital step in the translational pathway as they do provide important pathophysiological insights into disease mechanisms particularly on 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 improved current 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 3R (replacement, reduction and refinement) as a guiding concept.

Keywords: iPSC, tissue engineering, multiomics, network medicine, bioinformatics, big data, co-morbidities, cardiovascular disease, COVID-19

1. Background

The chronic and progressive nature of cardiovascular disease represents an enormous economical and societal challenge.[1] Economic consequences are largely due to high health care 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 in models which resemble human cardiovascular pathology and strategies to make optimal use of obtained data. In past years, new potential drug targets turned out to be ineffective in the treatment of ischeamic heart disease and heart failure, which appears 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 which represent human cardiovascular disease characteristics is essential to implement new diagnostics and therapies in the clinical routine setting. The design of models for studies on cardiac pathophysiology is challenging, because cardiovascular disease is complex and involves multiple causes and co-morbidities, resulting in a multi-organ disease in an ageing population. In this position paper, we focus on *replacement, reduction* and *refinement* of animal experiments, known as the 3Rs, which were already introduced in 1959 by Russel and Burch[2] (***Table 1***). The objective of this position statement is to provide an overview of current state-of-the-art in animal models, stem-cell derived models, and studies in human models, and highlight how advances have been made in cardiac muscle, vascular and valve diseases based on the 3R principles (***Figure 1***).

2. Current state-of-the-art in major cardiovascular diseases

2.1 Epidemiology of acquired and inherited forms of cardiovascular disease

Heart failure (HF) is frequent, lethal and patient care is expensive. This condition is now estimated to affect ~38 million people worldwide and to stand as 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 6-7% at one year in patients with stable HF to 25% or more 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 a projection to more than doubling 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, and affects 2-3% of the Western population.[9] Similar to the prevalence of HF, with the aging population the prevalence of AF will increase. Inherited cardiomyopathies caused by pathogenic variants in genes encoding regulatory and structural cardiomyocyte proteins, and ion 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 (AAA) 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 valve disease (VD) is highly prevalent, with a mortality risk ratio of 1.36 in developed countries. VD is a progressive disease that rises in parallel with ageing of the population, and up to 30% of patients are estimated to undergo surgical or percutaneous interventions. Dysfunctional valves can be congenital or acquired, and each may lead either to stenosis or regurgitation.[13]

2.2 Systolic heart failure

HF is a hemodynamic 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 a LVEF <40% is termed HFrEF. Failure of the heart to properly relax and fill, i.e. diastolic failure, may produce similar symptoms as HFrEF, albeit ejection fraction is preserved (>50%; HFpEF; *paragraph 2.3*). HF with a LVEF between 40-50% is termed HF with mildly reduced EF. At least half of 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, hypercholesterolemia, diabetes and obesity).[15] Other common causes of HFrEF are exposure to cardiotoxic agents, including cancer chemotherapy,[16] viral myocarditis,[17] peripartum cardiomyopathy,[18] and genetic defects (*paragraph 2.5*).[19]

Current standard of care includes first-generation drugs, developed decades ago, that target both myocardium and vasculature to improve hemodynamics, and as well may mitigate the adverse remodelling of cardiomyocytes (CMs): angiotensin converting enzyme inhibitors, angiotensin receptor blockers (ARBs), beta-blockers, mineralocorticoid receptor antagonists, ivabradin and, more recently, combined ARB-neprilysin inhibitors (ARNIs-sacubitril/valsartan).[20] Hope has been raised by the remarkable effect on HF of gliflozins, inhibitors of sodium–glucose cotransporter 2, which was however discovered unexpectedly, and 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 observation that not a single biological drug (protein, peptide, antibody, 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 pathways triggered by the initial cardiac injury. This includes CM remodelling and alteration of metabolism, followed by progressive LV dilatation (eccentric remodelling), also 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 nervous system and the renin–angiotensin–aldosterone system, which are initially adaptive but eventually worsen the condition.[26,27] 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 beta-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.

While development of HFrEF is associated with CM dysfunction, compelling evidence supports the major importance of the loss of CMs and the inability of these cells to undergo significant regeneration during adult life. CM loss can be sudden and dramatic during myocardial infarction and involve as many as 1 billion cells from the LV.[28] Chronic loss also occurs as a consequence of uncontrolled hypertension,[29] aortic stenosis,[30] or during viral myocarditis.[31] CM death accompanies virtually all forms of inherited cardiomyopathies,[32,33] including Duchenne muscular dystrophy and dilated (DCM), arrhythmogenic (AC) and hypertrophic (HCM) cardiomyopathy. Finally, CM loss is a general characteristic of physiological cardiac aging over time.[34] These observations highlight the need for developing new therapeutic strategies aimed at protecting CMs from acute or chronic loss or inducing new CM formation. This is particularly important, as the current therapy goal is to improve cardiac function, while it targets neither cardiac protection nor regeneration. Cellular models, especially based on human embryonic stem cell (ES)- or induced pluripotent stem cell (iPSC)-derived CMs, either in 2-dimensional (2D) cultures or engineered to form 3-dimensional (3D) tissues (*paragraph 4.1*),[35] can be exploited to identify both cardioprotective and pro-proliferative therapies and are particularly amenable to high-throughput screenings (*paragraph 4.3*). They can be combined with cell-free elements such as extracellular vesicles or circulating molecules.[36]

Nevertheless, to address the wide gap in translation, and reproduce the complex sequential events that occur in HFrEF, small and large animal models are still required (***Table 3***).[37] Appropriate models for genetic and acquired disease provide insight in diversity of HF mechanisms and in regional remodelling as seen in ischemic heart disease. Such models are essential for proof of concept of treatment strategies with local targeting and for evaluation of systemic effects of cardiac therapies at different stages of the disease.

2.3 Diastolic heart failure

HFpEF prevalence is continuously increasing but large clinical trials have failed to improve outcome,[38] in contrast to the progress shown in HFrEF. Reasons for this failure include 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. Another reason is the large patient heterogeneity, as HFpEF is a complex syndrome with varying contribution of the pathophysiological substrate.[39,40] HFpEF is more common among the elderly and is associated with multiple comorbidities, such as hypertension, obesity, diabetes mellitus, coronary artery disease, sleep apnoea and lung disease, with remarkable sex-related differences.[41] Classic common features include abnormal LV compliance and relaxation, with resultant elevations in LV filling pressure, abnormal systemic and pulmonary vasorelaxation, and neurohumoral activation.[39,40,42] Diagnosis is based on the presence of HF symptoms, such as preserved EF, elevation of natriuretic peptides, changes in diastolic indices, atrial enlargement, and cardiac hypertrophy. Treatment for HFpEF is still based mainly on a “one-size-fits-all” approach (e.g., reducing congestion with diuretics and controlling associated risk factors such as hypertension), which however has proven ineffective,[43] as shown e.g. by the lack of benefit of therapies effective for HFrEF.[44] 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.[41,43] Recent implementation of phenomapping[45] has enabled identification of phenotypically distinct HFpEF categories to better classify pathophysiologically similar individuals who may respond in a more homogeneous, predictable way to interventions, regardless of the associated comorbidities.

An important limitation in understanding the HFpEF pathomechanism(s) 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 arena. Indeed, most animal models suggested to be "HFpEF" models 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.[46-50] A true animal model of HFpEF should present with all of the following (***Table 3***): 1) an ejection fraction in the normal range for that animal model (at least 50%); 2) diastolic dysfunction; 3) exercise intolerance; and 4) pulmonary edema.[49] 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. 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. Typical examples are the db/db and ob/ob mice, two common models of type-2 diabetes mellitus, which lack the leptin receptor or functional leptin, respectively, and do show HFpEF characteristics; however, potentially confounding adverse effects arise from altered leptin signaling.[49,50] 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. 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. Indeed, an interesting concept proposed recently is that HFpEF presents as a multisystem inflammatory metabolic disease,[51] driven mainly by excess adiposity linked with imbalance of nitric oxide (NO) levels.[52-54] An additional, commonly observed risk factor is hypertension, also associated with generalized imbalance in NO metabolism and bioavailability. In light of these findings, HFpEF models are warranted which recapitulate the metabolic inflammatory phenotype.

One of these rare HFpEF-mimicking models is the obese Zucker diabetic, spontaneously hypertensive, Fatty (ZSF1) rat, which presents with hypertension, type 2 diabetes, hyperlipidemia, obesity, and nephropathy. This hybrid rat is a cross (Charles River Laboratories) between a Zucker Diabetic Fatty female rat and a Spontaneously Hypertensive Heart Failure male rat. Unlike the lean ZSF1 rat, which can serve as a convenient control, the obese ZSF1 rat shows multiple HFpEF characteristics known from patients and typical cardiac hallmarks of the disease, including modest fibrosis, titin modifications, and cardiomyocyte stiffening.[52,55] 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 hypercholesterolemia.[56] 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 signaling (suppression of constitutive NO synthases) in wild-type mice.[54] 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, which is also linked to autophagy in various diseases.[57] Interestingly, autophagy activators such as caloric restriction mimetics are pleiotropic agents that are beneficial, among others, for diastolic heart function in rodent models of aging and hypertensive heart disease.[53] Collectively, these findings suggest a common, convergent downstream cellular pathology in HFpEF, despite the phenotypic diversity.

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. However, there remains a need to generate additional models that also represent other HFpEF sub-phenotypes and allow testing of specific treatments. Whether animal-free models of HFpEF could be successfully developed, is questionable, due to the complexity of the HFpEF pathophenotypes. Of potential use may be iPSC-CMs, which can also be cultured as 3D cardiac tissues. These systems have the advantage that they can be derived from humans (including

patients), which is useful given the paucity 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, or for drug testing, and in co-culture studies to define the effect of endothelial cell dysfunction on cardiomyocyte performance.[58] However, with a very few exceptions,[59] 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 and therefore also AF research has many faces and methods (***Table 3***). Known risk factors associated with AF include aging, common cardiovascular diseases, cardiomyopathies, channelopathies.[60,61] Furthermore, genetic studies have demonstrated an appreciable genetic component in the determination of risk for AF and genomewide association studies have identified ~100 risk loci.[62,63] This combination of inherited risk factors and acquired risk and DNA damage[64] makes research into AF both especially interesting and challenging. Different diseases and reasons underlying AF ask for different (personalised) future treatments,[61] and these are required as current treatments are limited.

Various research groups discovered that AF perpetuates itself ('AF begets AF', as a landmark paper put it[65]), and the signalling pathways, structural, and functional alterations of this self-perpetuation have been dissected in large animal models and in patients with AF.[60] 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 to study genetic and genomic modifications with the opportunity to include aging research due to their shorter lifespan.[64,66,67]

Animal-free innovations like human cell models, immortalized cardiomyocyte cell lines and engineered heart tissue (EHT) will be instrumental in exploring these interactions and the underlying transcriptional and pathophysiological adaptations in detail.[68] Different forms of AF (paroxysmal, persistent, chronic) are very difficult to mimic in animal or non-animal models. There is thus far no model for paroxysmal AF. Morever, as AF is often a result of long-term exposure to risk factors partly on top of a genetic vulnerability, it seems 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 prone to genetic and pharmacological interventions, there are challenges with the use of such models to study human chronic disease like AF. Human iPSCs have already been differentiated into atrial CMs,[69] and using fetal immortalized CMs.[70] An important limitation is that such cells do not mimic all aspects of adult cardiomyocyte phenotype such as cell-cell coupling between cells (myocyte-myocyte or myocyte-fibroblast), making studies on the pathophysiology of e.g. conduction disturbances challenging. 3D formats facilitate *in vitro* maturation, and these 3D cell arrangements including EHT and bioprinting overcome a lot of previous limitations of cellular based solutions and have been specifically adapted for AF research.[71] Both for cellular and animal studies, long term studies under chronic conditions or ageing, and predefined protocols and analysis plans with blinded investigations more similar to clinical studies would increase the translational value of these studies.

As in other disease models, validation in more complex systems, occasionally large animals but ideally in patients with AF[66], will be required for successful translation of new findings into better diagnostics or therapies.[9,66,72] For this purpose data collection in human cohorts should be improved and intensified, e.g. by analysing algorithms in smartphones and wearables, by machine learning and artificial intelligence analysis, by phenotyping of patients at risk of AF and with AF, not only with electrophysiological studies like high density electrical mapping, but also imaging, biomarkers, proteomics, metabolomics, genetics and genomics. Biobanks including blood, plasma and tissues in clinically well characterized patients in combination with high throughput histological, genetic and molecular techniques should be used to identify the leading disease mechanisms and study their association with the clinical presentation of patients. In this way interaction between genes and environment in large prospective cohorts can be studied and specific biomarkers and individualized therapies for AF can be developed.[9]

2.5 Inherited cardiac diseases – Cardiomyopathies, Channelopathies & Ventricular arrhythmias

The clinical classification of genetic cardiomyopathies considers structural, functional and arrhythmogenic alterations. Genetic cardiomyopathies mainly consist of dilated, hypertrophic, arrhythmogenic phenotypes (i.e., DCM, HCM and AC).[10,73-75] 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 (LQTS, Brugada syndrome, catecholaminergic polymorphic ventricular tachycardia). The clinical variability in the expression of the phenotype, in part due to environmental factors,[76] and in part due to the genetic and phenotypic overlap among different cardiomyopathies and channelopathies,[77,78] have challenged the proper evaluation of the clinical, therapeutic and prognostic impact of genotyping. Animal models, iPSC-CMs, and human cardiac samples are currently used to study the consequences of specific genetic variants.

Animal models of cardiomyopathies, such as mice and occasionally rats, have been obtained through genetic engineering.[79] These knock-in models carrying human pathogenic gene variants (mutations) are the most widely used models of cardiomyopathies. 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, having hearts with a physiology more close to that of humans.[80] Alternative animal models to study genetic cardiomyopathies include *Caenorhabditis elegans*, Drosophila melanogaster and zebrafish. Similarities at the level of embryonic development, structure, function and high conservation of gene function, allied to their ease of maintenance, short lifespan and easy access to approaches for genetic manipulation make these organisms attractive models to identify mutations affecting proteins, signaling pathways and biological processes implicated in cardiomyopathies. They allow high throughput screening of gene function as well as druggable targets, to 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 investigation 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 cardiomyocytes environment. Similarly, neonatal cells from rat, mouse or rabbit cardiomyocytes allow for overexpression or knock-down of genes followed by electrophysiological assessment, but their immaturity makes them less well suited due to inherent differences in e.g., 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 genetically modify by either overexpression or deletion of genes of interest, as well as introduction of genetic variants. More recently, rabbits have been successfully used in transgenic studies, which resemble more closely human electrophysiology. Overall, transgenic animals allow for in-depth (electro)physiological studies *in vivo* (ECG, echocardiography), in the whole heart (*ex vivo*; optical mapping, arrhythmia inducibility), and on the cardiomyocyte level (patch clamp analysis, calcium fluorescence), combined with histologiocal and molecular analyses as well as long-term (therapeutic) studies.

Human iPSC-CMs provide an unlimited source of CMs from healthy controls and from patients with inherited conditions. They recapture the patient's genotype as cells are derived from the affected cardiomyopathy patient skin biopsy or circulating cells. In addition, gene editing with CRISPR/Cas9 enables to generate isogenic controls that allow to characterize the consequences of the genetic defect and rule out the confounding effect of the genetic background.[81] Still, their reprogramming and differentiation is time consuming (up to 3 months) and costly, and hiPSC-CMs remain immature compared to human adult CMs at metabolic, structural and functional level. 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 consequent to 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.[81,82] 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.[83]

Adult human cardiac tissue, either as membrane-permeabilized myofibrils, CMs and muscle strips, or intact CMs, allow studying myofilament kinetics, myofilament calcium sensitivity, ATP consumption, metabolism and mitochondrial function, electrophysiology and response to different pharmacological agents.[84-86] As the preparations are derived from adult hearts, the physiological relevance and pharmacological predictivity is high. The demanding logistics and limited sample availability are the main limitations. Adult CMs are also relatively delicate cells, difficult to maintain in culture, with a limited lifespan and potential for expansion. Myocardial tissue slices of human samples represent another new opportunity to study human tissue over a longer time span in culture, and are described in detail in *paragraph 4.2*. In addition, RNA deep sequencing of huma cardiac samples during life or end-stage along allows molecular profiling, pathway analysis and therapeutic target discovery in relation to different CM phenotypes.[87]

Overall, there are important advantages and disadvantages among the different models and 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 (e.g. mice) where genetic manipulation allows relatively rapid screening of phenotypes describing valve malformations (e.g. the development of the bicuspid aortic valve) or the evolution of valves toward 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: (a) 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 (b) valvular interstitial cells (VICs), the most prevalent cell type and crucial for calcification aortic VD (CAVD) pathogenesis.[88] 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[89] and respond to inflammatory cues by inducing a robust calcification response.[90] Thus, VIC functions have prompted new investigations on paracrine pathways involved in CAVD (e.g., TGF-β signalling). The human aortic valve opens and closes over 3 billion times over an average human lifespan and is thereby subjected to major mechanical forces. These forces include (a) axial stress during diastole upon valvular closure, mainly sensed by VICs, (b) laminar shear stress on the ventricular side during systole, and (c) oscillatory shear stress on the aortic side of the cusps during diastole, both sensed by VECs.[91] 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.[90] With further progression of CAVD, increased valvular stiffness, myofibroblasts differentiate into osteoblasts.[92]

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.[93] Moreover, calcification of the aortic valve mostly starts at areas subjected to the highest mechanical strain and the lowest laminar shear stress, namely the non-coronary cusp.[94] Solely the mechanically challenged (aortic) side of the valve leaflet 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 heart failure in VD. The research focuses on two approaches: 1) animal models (mostly large animal models) are critical for development of devices or innovative repairing/replacing valves techniques; 2) 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 might even surpass the potential of animal studies. Nevertheless, animal models are still indispensable for studying specific aspects of VD. ***Table 4*** depicts the most commonly used animal models of CAVD, their potential applications and animal-free alternatives, whenever appropriate.[95-102]

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 encroaching the arterial lumen or cause acute arterial occlusion by plaque erosion or rupture. These processes result in ischemia, and, depending on the arterial bed affected, result in cardiovascular events including angina pectoris, myocardial infarction, stroke or peripheral arterial disease.[103] 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, including flow, shear stress, oxidative stress, inflammation, endocrine factors and hyperlipidemia, as well as plaque intrinsic factors including 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, but also between immune cells and non-immune cells all drive atherogenesis.[104]

For decades, most groundbreaking insights into this complex disease have been obtained by studies in laboratory animals. 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.[105] 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. Not only do these mouse models develop widespread atherosclerotic lesions in a reproducible way within a few months, the development, progression and growth of lesions shows features reminiscent of human atherogenesis.[106,107] A major advantage of these mouse models is that they can easily be backcrossed to other (cell-type specific) genetically modified mice to not only study the role of specific genes on plaque development, progression and composition, but also the effects of systemic alterations caused by this respective gene on atherosclerosis.[106] One of the major drawbacks of animal models of atherosclerosis is the lack of end-stage atherosclerosis with spontaneous plaque rupture.[107] Although very old ApoE$^{-/-}$ mice do develop intraplaque hemorrhages, 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.[107] 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.[108] Other models of plaque rupture include models in which a plastic cuff is placed around the carotid artery, followed by ligation of the artery.[109] A few

genetic models, including SRBI$^{-/-}$/ApoE$^{-/-}$ mice[110] and Fb1$^{-/-}$ApoE$^{-/-}$ mice[111] show spontaneous plaque rupture with end-organ damage including stroke and myocardial infarction.

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, hyperlipidemia and endocrine factors all affect its pathogenesis, we still need to make use of living organisms, especially mice. Noteworthy, in atherosclerosis research, we do aim to reduce the number of laboratory animals that we use by carefully designing our experiments, by testing aspects of the disease as much as possible in *in vitro* systems. Recent developments in single cell technologies (transcriptomics and mass cytometry),[112-114] and the design of novel computational tools enable us to more carefully select our candidates and targets that are worthwhile studying, thereby reducing the number of laboratory animals being used. Aspects of the disease, including endothelial cell biology, lipid uptake, leukocyte 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. In addition, new and improved animal models of vascular disease, i.e. humanized mouse models are currently under development.

2.8 Vascular pathology – Aneurysms

Aortic aneurysms (AA) 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 cardiovascular health, but further than that no effective therapies or drugs exist. An important reason for the lack of treatments for AAA is that the exact process leading to AA is unclear.[115] Previous studies implicate defects in SMCs, ECM remodeling, inflammation and oxidative stress as key factors in the pathogenesis.[116] 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 are mostly unknown and still have to be unravelled.

Extensive studies and models have been developed to study AAA. 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 is that external stimuli are required to initiate aortic dilatation. The most common ones are angiotensin II (AngII), porcine pancreatic elastase (PPE), and $CaCl_2$ instillation.[117] Experimental AngII-induced AAAs require mice with an atherosclerosis-prone background (like 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.[118] The murine PPE model presents many histo-morphological features associated with human AAA disease.[119] A promising modification of the model that utilizes external peri-adventitial elastase application in combination with β-aminopropionitrile to provoke acute rupture and intraluminal thrombus formation has been reported.[120] In addition to small animal models, several studies report AAA formation in large animals (mainly pigs), which have the advantage of exploiting similar anatomical and physiological dimensions to humans, allowing the application of devices and surgical techniques. It appears evident that further advancements in small animal models as well as refinement of large animal models (for example using *Ldlr*-deficient mini-pigs)[121] will enhance studies of unmet translational research questions. However, no available model today closely resembles human AAA characteristics. Recent studies are conducted on the first steps towards the development of an *in vitro* pre-clinical disease model for AAA (*paragraph 4.3*).

3. Current state-of-the-art in animal models: Limitations, opportunities and future perspectives

The primary requirement of animal models is to mimic the clinical scenarios by presenting critical features that are present in patients with heart diseases. However, the animal models are frequently developed by using artificial medical processes, such as aortic banding to induce myocardial hypertrophy, or pacing-induced heart failure, and thereby may show the structural remodelling of the heart (eccentric and concentric hypertrophy), but lack relevant myocardial changes triggered by multiple co-morbidities. Furthermore, animal models often do not reflect the diversity of the patient populations with heart failure caused by the chronic burden of several co-morbidities, and other diseases that largely contribute to failure of clinical translation. Yet, animal models allow to perform *in*/*ex vivo* functional and electrophysiological studies during various disease stages, in correlation to molecular and histological findings, and study the impact of stressors such as exercise and co-morbidities, ageing and chronic effects of pharmacological interventions. The latter aspects are not easily mimicked in animal-free cell and tissue models. The following paragraphs describe limitations and opportunities of current animal models, and indicate if animal-free approaches can be applied to study cardiovascular disease mechanisms and replace studies in animals.

3.1 Rodent models

Rodent models are widely exploited as they provide biological insight at organ and cell level, are hypothesis-generating in (patho-) physiological processes and provide the opportunity for body dose-response testing. Major advantages of these models are the relatively easy genetic manipulation, the availability of biomedical tools with rodent specificity and their relatively low cost. Here 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. This was initially analyzed using pharmacological inhibitors and/or activators. Pharmacological treatments were largely criticized for their unspecific effects. Nowadays, genetically engineered mice are the standard in cardiovascular biology, because they permit the modification of a single gene and allow to examine its function in an integrated physiological system. Two genetic technologies exist, insertional transgenesis (transgenic animals) and gene targeting (knock-out (KO) animals). To overcome the limitations of global gene targeting (embryonic lethality, compensatory changes over time, effects related to gene deletion in organs not under investigation), inducible tissue-specific gene-targeting systems (based on the Cre-loxP technology) are preferred. However, numerous pitfalls have to be considered when interpreting data obtained from genetically modified animals.[122] For example, both the Cre protein and Tamoxifen (used to activate the Cre) can have cardiotoxic effects. [123,124] While overexpression of any protein might induce undesired effects, its knockout might also affect the whole proteome.[125] Thus, both pharmacological and genetic approaches have potential limitations and should be possibly combined to add solidity to protein-function relationship.

Additional limitations refer to the difficult translation of results generated in rodents to humans, with particular reference to novel therapeutic strategies. First, 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 (strain) as phenotypes may differ significantly between different strains which may confound results. However, combining phenotypic analysis, expression data in cardiac tissue and genetics also offers the opportunity to identify new disease-related genes and pathways.[126,127] Third, rodents poorly mimic the human heart, particularly in terms of heart rate, collaterals, susceptibility to atherosclerosis, etc). Fourth, while systematic reviews/meta-analyses are commonly performed to improve clinical practice,[128] 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 paragraph 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 be made available at least in supplemental data. Standardization would allow increasing data robustness and quality, extracting new data from previous studies and reducing the number of animals, in compliance with the 3R policy.[129] Along the same line, an additional step forward implies establishing repositories of samples from rodent models. Biobanks would maximize 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 by 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, entailing relevant information. Finally, assessment of cardiovascular function in rodents should privilege methods that avoid invasive or terminal procedures, such as echocardiography, MRI and telemetry. Both echocardiography and MRI allow for complete, repeated and non-invasive assessment of both systolic and diastolic function. MRI shows the advantage of providing information regarding cardiac metabolism. However, its use is limited due to its high costs. Contrarily, echocardiography is widely used and standard procedures for echocardiographic assessment have been recently published aiming to increase accuracy and reproducibility of the data.[130] 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. Progresses in device miniaturization and battery duration allow to record data continuously and to merge several cardiovascular parameters in the same telemeter (ECG, blood and intraventricular pressure) with minimal human-animal contact.[131]

3.2 Large animal models

One of the most often cited cornerstones of the animal research are the 3R principles (***Table 1***).[2] While “refine” and “reduce” can be considered in many animal experiments, the “replace” is difficult and often remained 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 cardiovascular physiology and cellular biology similar to humans.[132-135] An additional advantage of large animal models is their size, which allows 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 that for each measurement a separate group of animals is required. Beside their non-disputable advantages, the large animal models are costly, require specific infrastructure and handling, their lifespan and gestation time are longer. Genetic manipulation of mammalian animals is difficult and may raise ethical questions, but if successful, genetic pig models are extremely helpful in the design of new therapies.[80] Below we give a brief, non-exhaustive overview of available large animal models.[136-169]

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 left ventricle thereby causing hypertrophy, impairment of relaxation and HF symptoms.[136,137] The latter models may be used to model HFpEF-related structural (concentric) remodelling and coincident diastolic dysfunction. 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,[138] while treatment with cardiotoxic cancer drugs such as doxorubicin cause remodeling of the pig heart, including fibrosis and reduced systolic function.[139] As described in paragraph 2.3 mimicking HFpEF in a large animal model represents a challenge, and most models thus far incompletely mimic the clinical phenotype and may show hypertrophy and diastolic dysfunction without clinical characteristics of heart failure. Current

models may need addition of relevant interventions or co-morbidities to trigger the microvascular dysfunction associated with systemic metabolic stress.[56,140]

HFrEF or ischemic-reperfusion injury without infarction mimic human ischemic heart diseases very closely.[141-147] In contrast to dogs, pigs have sparse coronary collaterals, like humans, and therefore pig or mini-pig ischemic/reperfusion/infarction models were introduced. The porcine closed-chest reperfused myocardial infarction model mimics the primary percutaneous coronary intervention in ST-segment myocardial infarction, and, just as in humans, cardiac function can be comprehensively investigated with cardiac magnetic resonance imaging (MRI).[142] Indeed, such models successfully mimicked the neutral or minimal cardioprotective effect of ischemic conditioning seen in clinical trials.[148] The size and shape of myocardial infarctions 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 never compatible with life in large animal models 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, which are the major cause of HFrEF, can be simulated in large animal models with high translational power.[149-157] Whereas dogs are more resistant to the development of atherosclerosis, in pigs and non-human primates - as in humans - spontaneous atherosclerosis occurs with ageing, which can be accelerated with a Western diet.[153,157] Currently, there are 4 atherosclerotic pig models available: (*i*) Diabetic (type 1 or type 2) and/or diet-induced hypercholesterolemic pigs, (*ii*) the Rapacz familial hypercholesterolemic (LDL receptor mutant) pig, (*iii*) Ossabaw pigs and (*iv*) PCSK9 gain of function pigs.[149,151,153-157] 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.[151,155] Non-human primates -including rhesus and cynomolgous macaques- also recapitulate human-like hypercholesterolemia when put on a high fat/high cholesterol diet, which eventually - after several years- results in fibrofatty plaques.[157] This slow development of atherosclerosis, together with societal concerns, has resulted in selective use of the non-human primate model for atherosclerosis studies. Possibly with the advancement of genetic manipulation, accelerated atherosclerosis of primate models is possible.[157]

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 remodeling over time, with very diverse molecular abnormalities[158] and how cardiac resynchronization could cure all these abnormalities.[159] Other animal species turned out to reflect the human situation less well.[160]

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.[65,161-165] Interestingly, AC, DCM and HCM are diagnosed in large animals, and thereby represent an interesting alternative model to study arrhythmias and cardiac dysfunction in genetic heart disease (described in paragraph 3.3 Companion animals). In addition, valve insufficiency and stenosis is mimicked in several large animal models,[165-167] and are used to study pathomechanisms, but also test novel therapeutic interventions. To develop and test heart valve prostheses large animal models became indispensable (see *paragraph 6.1*). Sheep were extensively used to test protheses based on biological materials especially because sheep react very sensitive with calcification if the graft conditions were impaired. Worth mentioning, heart valve protheses based on decellularized allogenic valve matrices were directly introduced into clinical application after successful testing in sheep.[168,169] Since pig becomes more and more a transgenic animal model, genetically modified porcine tissues and organs get into the focus of xenogeneic transplantation medicine, but whole animals may also serve as “humanized” recipients. Baboon, an old world monkey, deficient for the most prominent xenoantigen alpha-Gal is considered to be the large animal for immunological

aspects to be tested. So, genetically modified porcine tissues, for example decellularized heart valves, and organs are tested in baboons.[168]

Table 5 shows which models may be (partly) replaced by animal-free experiments. In general, morphologic and functional changes of the myocardium and CMs or endothelial cells are well-modelled under *in vitro* conditions, such as 2D or 3D cell cultures or organoids or tissue-engineered cardiac structures. However, clinical symptom-derived disease entities, such as HF, certain valve diseases resulting in systemic hemodynamic changes, or heart rhythm disturbances are hard to model without large animal models. The complexity of the heart-circulatory system requires modelling of not only the isolated cardiac cell types, but also the accompanying hemodynamic changes and consequent organ failures, such as brain or kidney ischemia resulting in further cardiac damage (circulus vitiosus). An example of the complexity and paradox of the cardiovascular system research is the tissue-engineered heart valves, or any other vascular conduits, or organic patches, which can be constructed without using animals, but to prove the safety and efficacy of the medicinal product, they must be implanted in animals before first human use. Additional co-morbidities, 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, because of their emotional and economic value to our society.[170] The most prevalent non-ischaemic cardiomyopathies in humans are commonly diagnosed in companion animals. Hypertrophic cardiomyopathy is the most common feline cardiac disease affecting around 15% of all cats.[171] Mutations have been reported in *MYH7*[171] and *MYBPC3*.[173,174] DCM is more common in dogs and affects mainly large breeds, including Doberman, in which its prevalence reaches 58%, predominantly affecting male.[175-180] 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.[177,178] Finally, AC is commonly diagnosed in Boxers and, as in humans, it is characterized by fibrofatty replacement, ventricular premature complexes and ventricular tachycardia.[179,180] 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 at a relevant regimen. Coupled with the fact that they are relatively outbred, they share our environment, they are often aged and affected by multiple co-morbidities, companion animals stand as ideal models to test novel therapeutic interventions (i.e. gene therapy).[181,182]

3.4 Drosophila

Since 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.[183] In addition, *Drosophila* offers the opportunity to manipulate gene expression in a highly precise spatial and temporal fashion, using the UAS/GAL4 system.[184] This system was successfully utilized to identify genes causing cardiac diseases, including AF and cardiomyopathies.[184] New techniques, such as optical coherence tomography, allow accurate phenotyping of cardiac diseases, including heart failure, HCM, DCM and AC as well as cardiac arrhythmias, such as AF, in flies.[64]

Because of its simplicity, ease of culture, 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.[185]

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.[186] 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 heart failure, 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.[187] Sequencing the zebrafish genome in 2013 revealed that >80% of human disease-related genes have an orthologous gene in zebrafish.[188] 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.[189] 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.[190] 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, which allows studying their origin, regulation and function. Thus, the zebrafish has proven useful to study numerous cardiac pathologies. Due to its regenerative capacities, cardiac regeneration remains the most frequent. Upon injury, CMs are able to de-differentiate, proliferate and re-differentiate into mature CMs recapitulating embryonic development of the myocardium.[191] Besides cardiac regeneration, inhibition or genetic deletion of pathways can be very helpful to identify mechanisms of congenital malformations.[187]

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

4. Current state-of-the-art in animal-free models: limitations, opportunities & future perspectives

4.1 Human cardiomyocytes and alike and their 3D derivatives

The advent of methods to reprogram somatic cells (e.g. from skin, adipose tissue, peripheral blood, urine) to human iPSC and to derive bona fide CMs and other cardiovascular cell types at principally unlimited scale has boosted research in this area, complementing, and occasionally replacing animal experimentation. Recent advances in differentiation protocols [192] 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 to cells from animal species, particularly rodents. Cultures of hiPSC-derivatives are generally more stable than freshly isolated primary cells, tissues or organs (e.g. Langendorff-perfused hearts), which represent dying-cell-models, and thus produce more robust data. HiPSC-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 modeling 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. 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.

hiPSC-derivatives exhibit a fetal rather than adult phenotype with only partially canonical function.[193] Human iPSC-CM, like fetal, neonatal and immortalised cells, have poorly developed mitochondria and rely on glycolysis rather than substrate oxidation.[194] In consequence, they exhibit a

high basal glucose catabolism with poor insulin responsiveness (and only at supra-physiological insulin concentration).[195] Reprogramming and long-term culture can induce artefacts such as karyotype abnormalities and epigenetic alterations difficult to control,[196] whereas differentiation protocols introduce batch-to-batch variation. *In vitro* assays only partially capture disease-relevant whole organ functions, e.g. arrhythmias and diastolic heart function. Of the most common human pathology, ischemic damage by blood vessel occlusion, currently only the earliest stage of ischemia can be modelled *in vitro*. 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, which are often underestimated in their influence on cell behavior and in their relevance to rebuild a 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 multi-cellular responses could be principally better assessed in animals. Major cardiovascular risk factors and co-morbidities such as aging and metabolic diseases, including hyperlipidemia and diabetes, can only be partially 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 in coming years 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 3 batches from 3 lines, and standardizing circadian time will reduce variability.[197,198] Worldwide hiPSC banking initiatives such a hPSCreg (http://hpscreg.eu) add to this standardization. Automation also has the potential to reduce experimental variation[199] 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[200,201] as well as culture of hiPSC-CM on matrices with tunable stiffness,[202,203] Matrigel mattresses,[204] or micropatterned surfaces[205] have been shown to improve the maturity. Consistently, lowering glucose and adding fatty acids improved the metabolic maturity of hiPSC-CM, reflecting the fact that the use of glucose is inhibited by fatty acid oxidation in fasted state and is stimulated by insulin under fed state.[206] 3D Multicellular constructs, mechanical loading and electrical pacing (e.g. in EHT) belong to the most effective means to improve the structural, metabolic, electrophysiological and contractile maturity of hiPSC-CM and the spectrum of functional readouts.[207,208] Further improvements are expected from co-cultures of hiPSC-derived CMs, fibroblasts, endothelial cells, neurons, immune cells and others.[209] So far, several differentiation protocols for the respective cell types are available,[210] but it is still not known how well these cells resemble the organ-specific cells in their respective environment, in this case 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,[211,212] or genetically encoded calcium sensors such as GCaMP6f[213] improve the depth of phenotypic characterization of hiPSC-CM/EHT and allow analysis in intact preparations including arrhythmias.[214] Sharp microelectrode action potential recordings reduce confounding influences of cell isolation and the small size of hiPSC-CM compared to patch clamp recordings.[215] However, tissue damage and localized ischemia 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.[203,216] Influences of common comorbidities on disease phenotypes in patient-derived hiPSC-CM or the effect of (simulated) ischemia may be studied by applying hyperglycemic and hypercholesterolemic culture conditions as shown in fetal rat myocytes.[217] *In vitro* vascularization may allow studying mechanisms of thrombosis and ischemia in vitro.[218]

- 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, e.g. that between the drug-metabolizing liver and the heart (multi-organ-on-chips).[219] Thus, perfusable tissue surrogates are available, but they are still far from replicating a vascularized organ with chambers, conduction system, and physiological function enabling only partially the replacement of animal experiments. The promises of these new approaches have 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. For some high-throughput screens, a simple and cheap cell line might be appropriate as a first choice, e.g. the rodent cardiomyoblastic cell lines H9C2 and C2C12 . Of note, 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 which may involve more genetic alterations than the initial SV40 antigen expression.[220] More recently, rat atrial CMs were transduced with a doxycycline-dependent SV40 LT antigen, which could be easily expanded and differentiate into excitable and contractile atrial CMs upon removal of doxycycline.[221] The rodent background of these CM-like cells is however a considerable limitation. More recently, a similar approach was used for generation of a human atrial immortalized cell line.[70]

4.2 Myocardial tissue slices and isolated blood vessels

Ex vivo research models produced from the adult myocardium and blood vessels are fundamental tools to study cardiovascular (patho)physiology. The methodological and technological progress associated with living myocardial slices (LMS) preparation and *in vitro* culture are increasing 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.[222,223] 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 which 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 thereby significantly reduce the number of animals needed for experimental studies. The preparation of LMS from human specimens is also crucial for translational research.[224] A large variety of assays can be applied to interrogate LMS. Functional parameters include contractility, conduction velocity, Ca^{2+} transients, action potentials, metabolism and others.[222,225] Structural assessment provides analysis of cellular and ECM

organization, and specific biomolecules can easily be labelled and visualized. Biochemical assessment can also be used to assess LMS genomic and proteomic signatures.[226,227]

Novel biomimetic technologies allow LMS to be maintained *in vitro* in a highly functional state and cultured in stable conditions for extended periods,[228,229] which 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 which will reduce waste of tissue and increase data collection.

Like any other research model, LMS have limitations which should be carefully considered. Tissue damage occurs during cutting which is likely to trigger inflammatory responses and tissue remodelling, LMS are also disconnected from the circulatory system and neuro-hormonal stimulation. The heterogeneity among LMS obtained from the same heart, according to the region that is sliced should also be considered.[230] The lack of standardization across laboratories may also result in variable readouts. Biomimetic approaches have enormously improved LMS *in vitro* culture, however the preparations progressively adapt to the new *in vitro* environment which over time results in an alternative phenotype. This adaptation could potentially be controlled by culture conditions and improved biomimetic technologies, and it might level out the variability among diseased human samples. The bright future of LMS still holds several challenges that will have to be tackled. The standardization of LMS preparation and culture which implies refinement, education and validation of research readouts and applications are indeed a priority.

Isolated segments of human blood vessels (such as 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.[231,232] Moreover, 24-48 hour orgainoid culture can provide valuable pharmacological and mechanistic information. Human mammary arteries (IMA) are most readily available as a model of systemic vascular function regulation, vascular oxidative stress. While IMA does not develop atherosclerosis, it is sensitive to local pro-atherosclerotic insults elicitng endothelial dysfunction and oxidative stress.[233] This approach may be most effectively used in combination with other methodologies described here to identify key novel mechanisms in a translational fashion.

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

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

Valve disease: With an integrated vision of 'mechano-paracrine' signalling controlling the physiologic versus the pathologic phenotype of VICs, several studies focused on decrypting the cellular pro-calcific phenotype by evolving 3D pathology modelling involving substrates with defined chemical and mechanical characteristics. The stiffness sensitivity of VICs was demonstrated, for example, in studies performed with hydrogels with tuneable mechanical characteristics,[234] also in the presence of paracrine signalling by TGF-β.[235] More recently investigations allowed to characterize the molecular signalling underlying the activation of VICs toward the pro-fibrotic phenotype, and in particular, to describe the relevance of the mechanically activated Hippo transcriptional machinery[236] for porcine[237] and human[238] aortic VICs pro-fibrotic activation. Interestingly, in aortic VICs, this pathway was more active close to the calcified areas.[239] 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.[240] Beside 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 turn into the gold standard as these display fundamental beneficial characteristics.[241] With these approaches it is becoming 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***).

Atherosclerosis: Flow chambers coated with human atherosclerotic plaque lysates are being applied to study the dynamics of platelet- and leukocyte- 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.[242,243] Chip-based microfluidics systems, containing 3D structures with an arterial geometry build with iPSC-derived pericytes, vascular smooth muscle cells and endothelial cells can be subjected to flow and shear stress, and are in use to study the effects of flow and shear stress on endothelial cell biology, as well as arterial thrombosis.[244,245] 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 and 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 patients' cells from biobanks studying the SMC contractility and AA pathophysiology,[246,247] as well as novel *in vitro* 3D models to study SMC-ECM interactions are upcoming. More advanced is the integration of mechanical components into these models. As fluid shear stress, a force generated by perturbation of the laminar flow on endothelial cells due to vascular damage, could end up into activation of inflammatory pathways, atherosclerosis, intima hyperplasia and aneurysm formation.[248,249] 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.[249] Recently, the concept of cell mechanosensation has come to connect the transmission of mechanical forces to cells from the extracellular matrix (or vice-versa) and discrete gene regulation patterns affecting the cellular homeostasis within the cardiovascular system.[250] This has confirmed the existence of novel mechano-dependent pathologic pathways. For example, through an *in vitro* model of circumferential wall strain associated to coronary flow dynamics occurring in arterialized saphenous veins, the involvement of Thrombospondin-1 (TSP-1) in pathologic activation of resident myofibroblasts in the wall was revealed for the first time, with consequences for neointima accumulation and vein graft failure.[251] Since TSP-1 has a role in formation of ascending aneurysm through a mechanism involving changes in mechanical characteristics of the vessel wall,[252] it could be a key factor connecting alterations in tissue biophysical features to modifications in cellular composition and signal transduction.

Molecular modelling with 'vasculature-on-a-chip' devices mimicking the architecture, the mechanics and the cell setup of arteries and veins is, finally, a novel way to investigate vascular pathology programming (***Figure 3***).[253] These models have the advantage to be easily manufactured with biocompatible materials and are miniaturized, although they reproduce the hemodynamic 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, with immediate translational and personalized therapeutic perspectives.

4.4 High-throughput screenings

The last decade has witnessed an explosion of studies based on high-throughput screenings (HTSs) of both small molecules and small nucleic acids in cultured CMs for drug and gene discovery. This was rendered possible, on the one hand, by the development of biological assays amenable to miniaturization and automation while, on the other, 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 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. Incorporation of thymidine analogue to measure CM proliferation,[254-256] assessment of CM cross-sectional area,[257-259] inhibition of pathologic aggregate formation,[260] protection from cardiotoxic treatments,[261-263] or regulation of Ca^{2+} handling[264] have all been implemented 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 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. However, this limitation can be overcome by using optical recording of fluorescent sensor probes of transmembrane voltage or current transients using dedicated devices or by HC microscopy.[265,266] Mechanical force exerted by CMs can be measured, in an HTS format, by culturing cells on thin films of materials than can be bended by systolic contraction,[267] or by measuring contraction and relaxation of substrates embedded with fluorescent microspheres.[268] In addition to studies in CMs, a recent HTS in primary human cardiac fibroblasts identified drug candidates to target cardiac fibrosis and diastolic dysfunction.[269]

A major limitation, however, remains the embryonic nature of hESC/hiPSC-CMs. These cells are deficient in some of the ion currents present in adult CMs,[270] display an undeveloped transverse tubule (T-tubule) network and have important differences in intracellular Ca^{2+} cycling.[271] As some of these embryonic characteristics can mature *in vitro*, for example by culturing the cells on micropatterned surfaces,[272] 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.[273,274]

Finally, the possibility of growing CMs, either alone or in various combinations with cardiac fibroblasts or other cells, to form 3D structures resembling muscle tissue[275,276] or organoids[277] offer an additional layer of complexity, as described in other paragraphs of this article. However, at the same time, it also offers the opportunity of conducting screenings in conditions of load and CM maturation closer to those of the heart *in vivo*.

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, with the final goals to achieve progress in medical science and eventually improve patient health care. However, only a relatively small amount of the products from translational research finds confirmation into the clinical scenario.[278] A significant issue of preclinical studies is publication bias. Positive or significant results are more likely to be published compared to negative study results leading to an overestimation of the effects of therapies and unjustified transition of interventions towards clinical trials. Moreover, the lack of sharing both negative and positive results contributes to the repetition of research, and inadequacy to comply with the 3R principles.

The development and use of animal registry and/or preclinical network represent a possible solution to minimize the publication bias. To this end, two platforms (www.preclinicaltrials.eu[279], and www.animalstudyregistry.org[280]) 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, still major disadvantages of preregistration exist, especially intellectual property (IP) issues, and administrative burden, which most likely are the reason why at present only a limited number of studies are preregistered. Several solutions are currently being incorporated to circumvent these obstacles. For instance, with registration, the study automatically receives a digital object identifier (DOI) that marks it as the original research idea of the investigator. Also, the users can decide to restrict the visibility of their registered studies for up to 5 years.

The CAESAR (Consortium for Preclinical Assessment of Cardioprotective Therapies) Consortium[281] 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

independent and prospective animal registry and preclinical network can, therefore, support the researcher to increase the quality of the study, as it requires to address blinding, randomization, sample size calculation and power. These tools, if implemented, will lead to standardized protocols, and a reduction of unnecessarily repeated studies, animal use, and costs. Prospectively, a data repository for animal research could be exploited for advanced analysis through artificial intelligence and data mining, which can help to unveil rules or formulas able to predict adverse and/or therapeutic responses.

5.2 Patient registries, biobanking, -omics studies & imaging

Further acceleration of clinical cardiovascular research will be only possible if networks are created across institutes and countries to facilitate collaborative data science. A network of linked institutes using similar data models and harmonized clinical care pathways will facilitate patient recruitment in targeted clinical trials, enable genotype-phenotype association studies with appropriate statistical power in e.g., cardiomyopathy patient groups, and provide a framework for a learning healthcare system through benchmarking and cross-validation of novel strategies and artificial intelligence algorithms within research and routine care. Unsupervised learning allows to cluster, structure and to compress the information content of a high-dimensional dataset to important features or main components. Common methods are principal component analysis, spectral clustering[282] or so-called deep autoencoders.[283-285] A wellknown extension to Autoencoders are so-called variational autoencoders which allow efficient inference and learning in directed probabilistic models.[286] Autoencoders are neural networks used to learn an efficient representation in an unsupervised manner. They contain a bottle-neck layer which then generate the latent space of compressed variables. Understanding the underlying data distribution and the effect of involved parameters with such a deep autoencoder, allows to generate predictive models[287] and to simulate the effect of different parameters, such as drug responses.[288]

Leading steps in creating collaborative networks for human data exchange have been made through creation of large biobanks, for example the UK Biobank (https://www.ukbiobank.ac.uk/about-biobank-uk/) or 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 South of England and Scotland respectively. Both biobanks have established multi-disciplinary skills networks in health informatics, epidemiology, genetics, health economics, as well as focused data analyses from cross sectional whole-body imaging and specific cardiac imaging. Significant ethical, legal and social issue needs to be addressed to allow such complex biobanks to operate safely. Any scientist or clinician can use the resource by applying to the stakeholders of the respective repositories and upon Ethical and Caldicott (Public Benefit and Privacy Panel in Scotland) they are provided securely with the required data for their own project analyses. The fundamental aim of such large biorepository resources is improving the prevention, diagnosis and treatment of a wide range of serious and life-threatening illnesses, including cancer, heart diseases, stroke, diabetes, arthritis, osteoporosis, eye disorders, depression and forms of dementia. Particularly in Scotland which has a unique electronic health record system with data linkage dating back to its creation in 1986, the information available from the Biobanks can be data-linked with clinical outcomes and long term follow-up, as well as genetic analysis of its participans. Whilst these Biobanks have been only recently 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 12,150 participants born between 1950-1956 who were subsequently deeply phenotyped every decade with state-of-the-art investigations contemporaneously available at each such time point. A pioneer in setting up a cardiac tissue bank has been Prof. dos Remedios, who initiated the 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, which have been key in advancing cardiovascular science in many areas ranging from genetics to functional muscle studies.[289]

An example of utilizing the maximal potential of data obtained within the different disciplines is the so-called Network Medicine. Network Medicine has been born from the fact that conventional scientific reductionism is inadequate for understanding complex diseases and developing precise therapies. Moreover, Network Medicine 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. Indeed, focusing on the interaction of biological components, such as proteins, mRNAs, microRNAs, or metabolites, allow 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.[290] 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, one may realize that network medicine approach leads 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 hypercholesterolemic myocardium, allows one to build the miRNA-mRNA target networks and predict key molecular targets in an unbiased way, thereby remarkably reducing the necessary *in vivo* experiments for validation of predicted targets.[291]

The cardiovascular community should provide guidelines to establish a framework according to FAIR principles to: 1) enhance findability using metadata catalogues of patients with clinical, genetic, imaging and –omics data, 2) create transparency about accessibility protocols of existing data sources for external researchers and other third parties, 3) stimulate interoperability across institutes to enable collaborative science and federated learning, and 4) promote reuse of data in spirit of open science and improve durability of public investment, both financial and non-financial.[292] Instead of manual curation of clinical care data, the cardiovascular community should aim to standardize clinical care pathways and harmonization of 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 models should be adopted like developed by the Observational Health Data Sciences and Informatics program (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.[293] Instead of sharing data within a huge central datastorage (data-to-code), the algorithms will be distributed across centers (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 including 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) 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.

5.3 Computational modelling of cardiovascular function

Over the last two decades, rapid development of cardiovascular research methodologies employed by experimentalists, including 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, 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 such data has enabled the development of thoroughly validated computational models of heart and vessels.[294,295] 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, to support clinical decision making, and to improve safety and efficacy of drug and biomedical device therapies.[295]

The application of computational models for both fundamental and (pre-)clinical research in biomedicine is rapidly increasing,[296] which 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 demonstrated that computational models of cellular cardiac electrophysiology can predict adverse drug effects, such as life-threatening arrhythmias, with higher accuracy than animal models,[297] 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/), which aims at integrating predictions by *in vitro*, *in silico* and human iPSC-CM models with clinical evaluation for drug safety testing and which is promoted by regulatory bodies.

In fundamental cardiovascular research, *in silico* cardiovascular models have mostly 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.[298]

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',[299] can be used to gain better insights in the patient's cardiovascular pathology underlying symptoms and to predict the individual's response to therapy. Among others, studies have demonstrated successful applications of personalized computational models for prediction of arrhythmia risk in post-myocardial infarction patients,[300] for noninvasive measurement of fractional flow reserve from computed tomographic images of patients with coronary artery disease,[301] or for noninvasive electrocardiographic imaging.[302]

In conclusion, computational modelling and simulation, sometimes called the third paradigm of science, already established a prominent role in the quest to refining and reducing the use of animal experiments for cardiovascular research. Computational modelling is, however, not likely to fully replace animal experiments in the foreseeable future. The latter continue to provide novel insights into (patho)physiological processes which per se 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 experimentalists, computer scientists and clinicians.

5.4 Patient partnerships in data and tissue collection

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 (*paragraph 5.2*) are central to improve 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 they are often based on a laboratory point of view which is focused on basic science and often removed from the true needs of patients.[303] Patient participation in research is thus crucial for identifying patient-relevant questions and outcomes.

It is highly advisable for patients to participate in ethical discussions related to the studies to make sure that the information provided is clear and accessible. Moreover, there can be no autonomy as long as patients lack the information they need. Providing the population with more information about the aims of research will help to generate trust and promote participation in research. Ideally, the following aspects are taken into account in patient-professional partnerships.

The patient:

- is well-informed about his/her illness, treatment and/or research and takes ownership of this.
- must be aware of the risks, pros and cons, side-effects of treatments and/or research. Both from a medical point of view, but also by gathering and exchanging peer-to-peer experiences.
- is aware of specific patient organizations (advocacy/peer contact, exchange experiences and communities).

The professional:

- provides tailor-made patient information, which is preferably co-developed with patients and patient organizations: flyers, reliable websites with general and contextual content, including illustrations, infographics and videos.
- involves a diverse patient group for feedback. He/she does not only invite highly educated patients and benefits from the patient's professional expertise; a patient may be working in a profession (communication, IT/ finance, etc.) where the professional lacks knowledge.
- informs the patients about the current disease-specific, scientific research results and projects.
- informs the patient about the healthcare facility and research group. The researchers provide insight in their own work by e.g., showing a video of the research performed in the laboratory and clinical setting.
- lives up to expectation management; what does the study mean for the patient when treatment is started? Keep in mind the patient's condition, e.g., do not invite a HF patient on the fourth floor of a building with no elevator. What can the patient expect when he/she arrives at the ward?

In conclusion, involve patients in all phases of the study. Through patient involvement in biomedical research, the patients become actively involved in all aspects of the study, starting at the bench and moving to the clinical setting.

6. Moving from bench to clinic

While translation of basic and clinical research to actual implementation in the clinic represents a major challence, we conclude our paper with several success stories. These examples illustrate the strength of multidisciplinary research and the combination of complementary research models ranging from *in vitro* experiments in cells to studies in rodents, large animals and patients, and emphasizes the necessity of studies in animal models before implementation in the clinic.

6.1 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.[304] 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 reendothelialized by autologous endothelial cells.[241] With this, the grafts are non-thrombogenic and regain the ability to adapt to the growth of the recipient. Thus, 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 pediatric patients.[305]

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.[306] 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 techniques (hybrid TEHVs).[307] The majority of the TEVHs are constructed by molding of polymeric substances into a valve-like shape, or attaching to an appropriately formed stent.[308] For the engineered tissue, either natural biopolymers, such as collagen or fibrin, or synthetic polymers (eg. poly(glycolic acid), poly(lactic acid), poly(ε-caprolactone), or poly(4-hydroxybutyrate) are used. The stent-polymeric scaffolds are then populated with different types of cells (eg. 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, or immersion or perfusion approaches.[309] Currently, two TEHVs are approved for human use: the Cryolife's SynerGraft® in Europe and the United States 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.[310,311]

Electrospinning is less frequently used due to its complexity. This technique is grounded in creating a solid controlled fiber structure of TEHV, which construction fits better to the anisotropic mechanical characteristics of the natural valve, simulating the microarchitecture of the valve better than the other technologies.[312] 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.[313] 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 let the construct to cellularization in vivo, then explant and implant in orthotopic position.[314] Each TEHV construction technology has its beneficial and disadvantageous sites, and much more scientific and technological development is needed for human translation of the TEHVs.

6.2 Peripartum cardiomyopathy

Cardiovascular diseases account for the majority of severe complications in pregnancy worldwide. Among those, 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 heart failure.[315,316] The syndrome is associated with a high morbidity and mortality, and due to overlap with common pregnancy discomfort and the variable phenotypes of PPCM patients diagnosis is often delayed.[315] In addition, risk factor profiles, i.e. higher risk for PPCM in women with African ancestry, for women with pregnancy-associated hypertensive complications, older women or women with twin pregnancies suggests that PPCM consists of multiple pathomechanisms pointing to a syndrome and not a single defined disease.[315-318] This notion is further supported by the prevalence of cardiomyopathy-causing mutations in about 15% of patients[319,320] and a higher prevalence of cancer disease associated with mutations in the DNA damage response system.[321] Also 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 signalinggenetic variants (recently reviewed in 316) 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 could be replicated in a PPCM mouse model.[322] 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 16kDa-PRL fragment, resulting in subsequent vascular damage and HF.[316] Based on this common pathway, potential disease-specific biomarkers and therapies have emerged that are currently tested in a bench to bedside approach with one therapy concept developed in mice where HF medication is combined with the prolactin blocker bromocriptine already introduced into 2018 ESC Guidelines for the management of cardiovascular diseases during pregnancy.[323]

6.3 microRNAs – route to the clinic

Based on initial miRNA library screens the Thum group identified miR-132 to drive pathological growth of CMs *in vitro* and next *in vivo*.[324] In a number of mouse studies the group showed that oligonucleotide based inhibiton of miR-132 halted and reverted pathological cardiac remodelling.[325] Next, therapeutic efficacy was tested in an acute[325] as well as in a chronic[326] model of myocardial infarction 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 NT-proBNP paving the way for further clinical development of this new generation of heart failure medication.[327]

7. Conclusion & Outlook

There is globally a mounting belief that biomedical sciences may progress without animal research by replacing *in vivo* experiments with tests performed in human-derived *in vitro* models. While this is in part justified, due to the evident failure to translate several therapies validated in preclinical *in vivo* testing, the use of animal pathological modeling is still necessary for several applications such as, for example, implantation of medical devices (e.g. stents, new catheter-guided endoscopy systems, implant devices) and *in vivo* drug testing, as well as 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, with the current knowledge we will have to rely on laboratory animals, enabling thorough studies in a well-controlled *in vivo* setting. In coming years animal models will be made more 'human-like' on the basis of big data sets obtained in human studies. Moreover, novel 2D and 3D *in vitro* technologies, and advanced computational analyses will certainly result in a more refined experimental design, which will reduce the number of laboratory animals currently required to perform studies and test drugs. Succesful translation of cardiovascular research warrants integration of results (***Figure 1***) obtained in animals, animal-free models and patients.

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Figure Legends

Figure 1. Current state-of-the-art in animal models, stem-cell derived models, and studies in human models based on the 3R principles.

Figure 2. *In situ* valve engineering.

Figure 3. Aneurysm-on-a-chip manufactured with a 3D printing-based microfluidic channel patterned inside a PDMS block. The heatmap represents the distribution of the flow velocity reproducing the hemodynamic conditions occurring into aneurysms.

Tables

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 minimise 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 minimise 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

Table 2. Co-morbidities, causes and cellular, structural and functional remodelling of the heart in HFrEF and HFpEF patients.

Co-morbidities & causes	**Vascular changes**	**Cellular changes in the heart**	**Structural remodelling**	**Cardiac dysfunction**
Systolic heart failure, HFrEF				
Hypertension Hypercholesteremia Diabetes Obesity	Coronary artery disease & ischemia	Cell death Reduced cardiomyocyte contractility Altered metabolism Altered ECM Fibrosis Altered beta-adrenergic receptor pathway	Eccentric remodeling (dilated, thin-walled ventricle)	Reduced End-Systolic Pressure-Volume Relation Reduced response to exercise Neurohumoral activation
Cardiotoxic agents Viral myocarditis Peripartum cardiomyopathy Genetic defects				
Diastolic heart failure, HFpEF				
Multiple co-morbidities: Hypertension, obesity, diabetes mellitus, coronary artery disease, sleep apnoea, lung disease	*Proposed*: Systemic inflammation-mediated endothelial dysfunction[51]	Stiff cardiomyocytes, i.e. high titin-based passive force Altered ECM Fibrosis Disturbed NO signaling	Concentric remodeling (thick-walled ventricle) Atrial dilation	Large patient heterogeneity Abnormal heart compliance and relaxation Elevated left ventricular filling pressure

Table 3. Experimental heart failure and atrial fibrillation models.

	Cell-based models	Animal models	Patient studies
Systolic heart failure	Define molecular changes at cellular level related to ischemic and mechanic stressors. High-throughput screening: toxicity, cardioprotective, pro-proliferative effects of agents. ***Limitations:*** *Lack of disease complexity* *Lack of systemic effects*	*In vitro* and *in vivo:* Sequential events Regional remodelling Systemic effects of causes and therapies	*In vivo:* Sequential events Regional remodelling Systemic effects of causes and therapies ***Limitations:*** *Limited access to tissue; only advanced disease stages*
Diastolic heart failure	Limited number of cell models: Co-cultures to study endothelial-cardiomyocyte interaction[58] iPSC-derived cells[59] ***Limitations***: *Lack of disease complexity* *Lack of systemic effects of multiple co-morborbidities*	HFpEF model needs to show: 1) an ejection fraction in the normal range for that animal model (at least 50%) 2) diastolic dysfunction 3) exercise intolerance 4) pulmonary edema ***Limitation:*** *Many models only show structural remodelling (concentric hypertrophy) without HF features*	*In vivo:* Sequential events Systemic effects of causes and therapies ***Limitations:*** *Limited availability of tissue biopsies* *Diversity in clinical phenotypes*
Atrial Fibrillation	Human iPSC-derived atrial cardiomyocytes[69] and engineered atrial-like heart tissue[71] ***Limitation:*** *lack of studies on chronic exposure to stressors, ageing*	Small animals (rodents, zebrafish, Drosophila) to study specific mono-causal AF disease mechanisms. Goat model[65] has been extremely useful in mimicking human AF ('AF begets AF') ***Limitations:*** *difficult to mimic chronic and multi-causal nature of human AF.*	Prospective in depth phenotyping of patients with AF: electrical mapping, imaging, blood/tissue biomarkers, genetics.

Table 4. Examples of animal models of calcific aortic valve disease and animal-free alternatives.

Species	Pathological features	Applications	Animal-free	Refs

			alternatives	
Mice	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.	95
	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	96
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	97
	High cholesterol diet for 20 and 40 weeks, atherosclerotic lesions present in aortic valves, with increased lipid deposition, inflammatory cell infiltration, osteopontin (OPN) 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 AVS. 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.	98, 99
	Watanabe heritable hyperlipidemic (WHHL) rabbits fed with a high-fat/high carbohydrate diet display a spontaneous LDLR mutation. The valve does not show significant hemodynamic stenosis but presents lipid deposition, fibrosis, calcification and inflammatory	To study early-stage of CAVD and the impact of dietary cholesterol on valve disease.	Not available	99

	cell infiltrations.			
	White rabbits fed with a standard diet supplemented with 0.5%cholesterol and 50,000.0 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 hemodynamic and transvalvular gradient measurements after percutaneous balloon dilation of the valve, for translational research.	Not available	100
Swine	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 proteoglycan-rich onlays. This 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.	101
Ovine	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.	Sheep is currently accepted as the gold standard model for valve replacement using defined survival surgeries that meet FDA requirements.	Not available	102

Table 5. Large animal models of cardiovascular diseases.

Species	**Model**	**Main changes in the heart & vasculature**	**Animal-free alternatives**	**Refs**
Myocardial remodelling				
Pigs	LV pressure overload by an implantable stent or inflatable aortic cuff	Hypertrophy, fibrosis, impaired relaxation, symptoms of heart failure	*In vitro* modelling of cardiomyocyte hypertrophy and fibrosis	136,137
Pigs	Hypertension incuded by DOCA combined with a Western diet	Hypertrophy, impaired relaxation	*In vitro* modelling of cardiomyocyte hypertrophy and fibrosis	138
Pigs	Cytostatic drug-	Fibrosis, reduced LVEF	*In vitro* exposure of cells	139

	treatment		to cytostatic drugs	
HFpEF				
Pigs	Hypertension, Diabetes, hypercholesterolemia	Microvascular dysfunction, myocardial stiffening	Not applicable	56,140
Ischemic heart disease				
Pigs, dogs, sheep, non-human primates	Myocardial infarction by reperfused acute myocardial infarction, surgical occlusion of coronary arteries, coronary microembolisation	Fibrosis, systolic dysfunction	Mimicking acute and chronic ischemia in cell-based models	141-146
Pigs, dogs	Ischemia-reperfusion	Contractile dysfunction	Mimicking ischemia/reperfusion in cell cultures	147,148
Atherosclerosic vascular disease				
Pigs	Familial hypercholesterolemia	Atherosclerotic lesions of all vessels	Not applicable	149,150
	Yucatan and Sinclair miniature pigs fed with Alloxan resulting in diabetes	human-like atherosclerotic lesions and microvascular diseases		151,152
	Ossabaw pigs	Obesity and metabolic syndrome like humans		153
	PCSK9 gain-of-function mutant	hypertension, diabetes, kidney disease, endothelial dysfunction		154,155
Non-human primate	High-fat, high-cholesterol diet in Rhesus and cynomolgous macaques	Slow development of atherosclerosis		157
	Novel gene-modification technologies, e.g. CRISPR/Cas9	Accelerated atherosclerosis		157
Arrhythmias				
Dogs, pigs, sheep, goats	Pacing induced tachycardia	Arrhythmia	Paced cell systems	65, 161-163
Dogs, pigs, sheep	Infarction, arrhythmia, AV Block	Sudden cardiac death	Not applicable	164
Valve disease				
Dogs, pigs	Severing the chorda tendinae, ischemic injury of the posterior papillary	Mitral valve regurgitation		165

	muscle			
Sheep		Tricuspidal valve insufficiency		166
Cats, dogs, sheep, pigs	Supravalvular aortic senosis by surgical banding of the aorta	Aortic stenosis		167

Figure 1

Figure 1. Modelling cardiovascular disease with experimental designs based on the 3R principles: Replacement, Reduction & Refinement

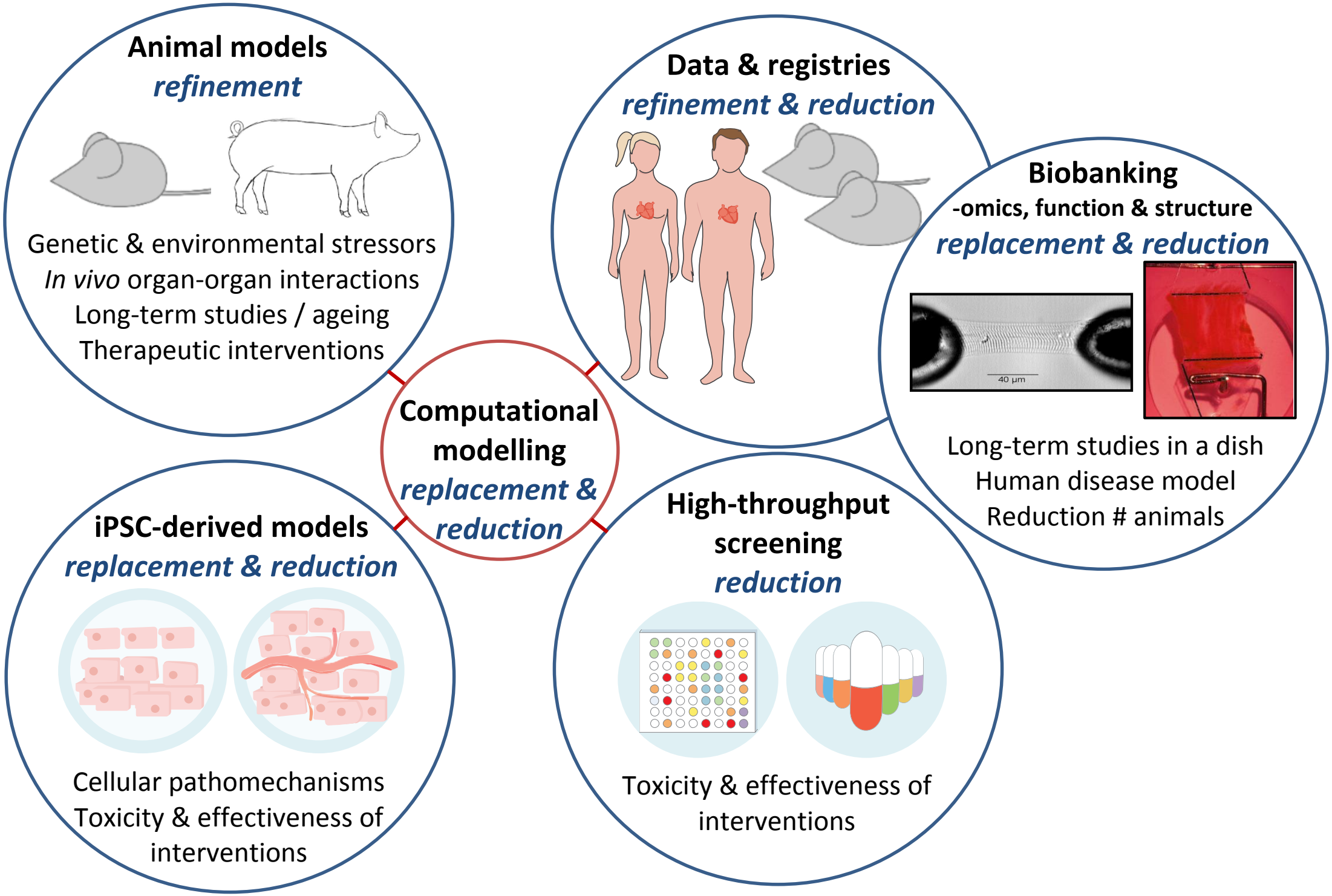

Figure 2.

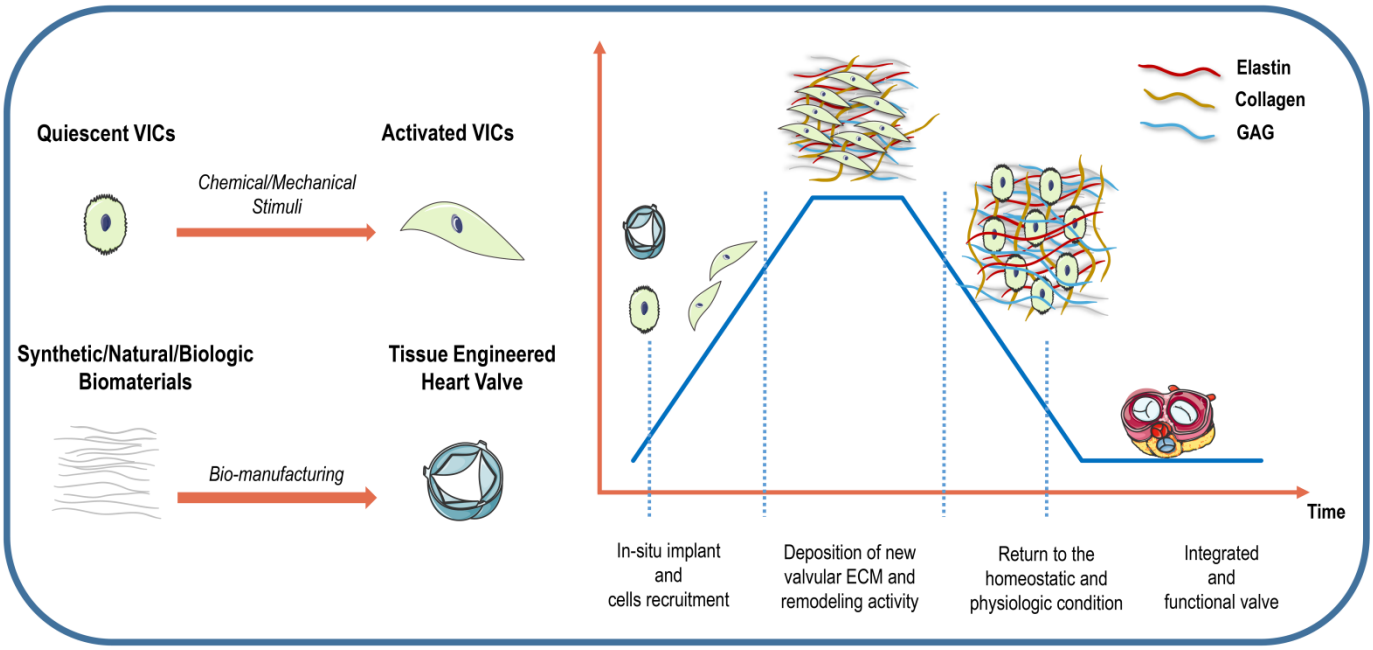

Figure 3.