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REVIEW

Alternative strategies in cardiac preclinical research and new clinical trial formats

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Abstract

An efficient and safe drug development process is crucial for the establishment of new drugs on the market aiming to increase quality of life and life-span of our patients. Despite technological advances in the past decade, successful launches of drug candidates per year remain low. We here give an overview about some of these advances and suggest improvements for implementation to boost preclinical and clinical drug development with a focus on the cardiovascular field. We highlight advantages and disadvantages of animal experimentation and thoroughly review alternatives in the field of three-dimensional cell culture as well as preclinical use of spheroids and organoids. Microfluidic devices and their potential as organ-on-a-chip systems, as well as the use of living animal and human cardiac tissues are additionally introduced. In the second part, we examine recent gold standard randomized clinical trials and present possible modifications to increase lead candidate throughput: adaptive designs, master protocols, and drug repurposing. *In silico* and N-of-1 trials have the potential to redefine clinical drug candidate evaluation. Finally, we briefly discuss clinical trial designs during pandemic times.

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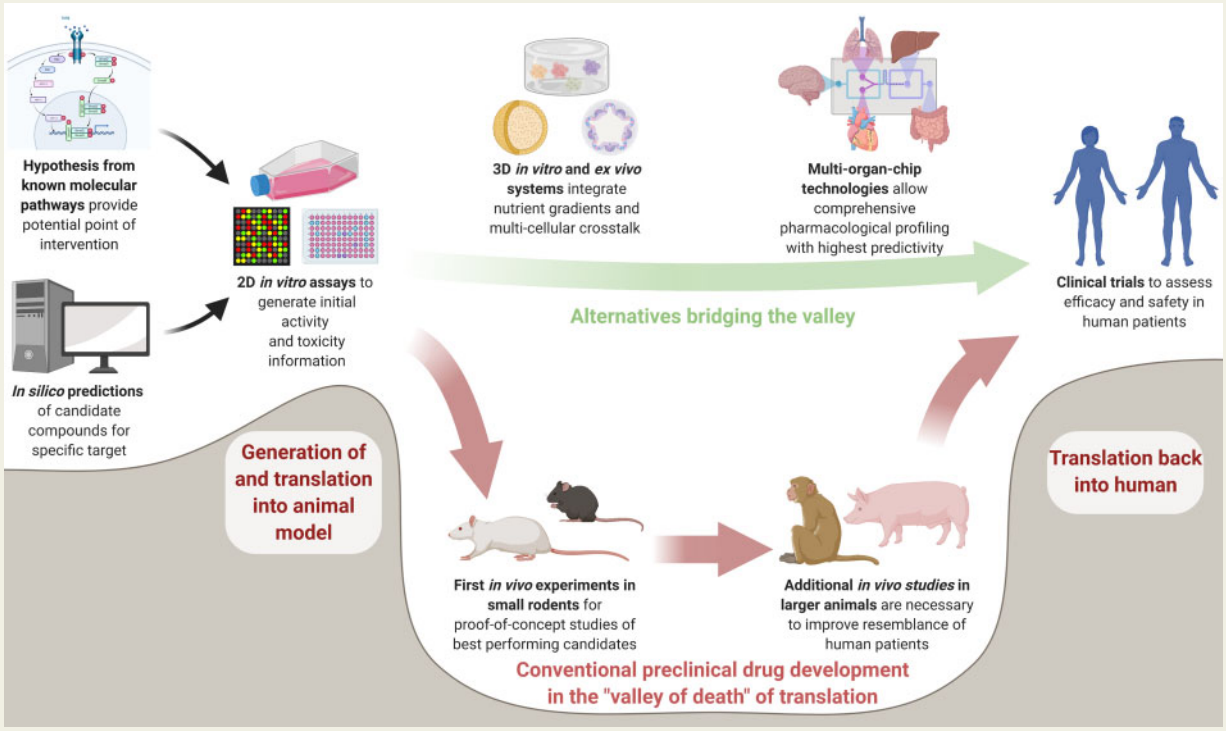
Graphical Abstract



Keywords Preclinical research • Alternatives to animal models • 3D cell culture • Organ-on-a-chip • Clinical trials • Adaptive design • Master protocols N-of-1 trials • Pandemic trials

1. Introduction

Drug discovery and development is a lengthy and complex process describing the multi-stage process from a treatment hypothesis to a drug on the market. It comprises the identification and validation of target–function and compound–target relationships in basic research, the discovery of drug parameters in preclinical development and the confirmation of efficacy and safety in clinical trials.

Notwithstanding a positive trend in the past 5 years,[1] pharmaceutic output has been low for a long-time coupled with increasing costs to successfully launch a drug since the 1950s.[2–4] In fact, merely approximately 8% of selected drug candidates successfully pass all clinical phases and can eventually be introduced to the market.[5] The predominant reason for clinical trial failure is lack of efficacy, which hints at limited predictability and transferability of preclinical research to human patients.[6,7] Especially, the cardiovascular drug development appears to be in a drought. Due to expensive research costs, relatively poor funding, and low expectancy of success, only few companies and research labs focus on the investigation of novel treatment strategies.[8] Moreover, translational success in academic research is often accomplished by reporting moderately positive results in a single animal model, often with low n-numbers, which is only a first small step towards true translational success—the regular use of a new safe drug to treat patients.

We provide the reader an overview of traditional preclinical research and clinical studies, with a focus on cardiovascular research, and highlight recent disruptive alternatives in preclinical and clinical drug development.

2. Preclinical research

2.1 General aspects and history of preclinical development

On the way to a successful drug, preclinical investigations of potential lead structures aim to assess the safety and therefore are essential to minimize the risk of harming human subjects. To file an application for clinical trials of an Investigational New Drug, clear and efficiently documented preclinical data are necessary and usually many different experiments, ranging from computational simulations to animal models, are employed.[9] Preclinical studies generally cover pharmacodynamic, pharmacokinetic, and toxicologic properties of compounds to predict adverse outcomes, define safety windows and estimate dose ranges to support and design subsequent clinical trials.[9–11]

Prior to initiating clinical trials, descriptions of chemical characteristics of the drug and the formulation have to be submitted to regulatory authorities [e.g. the European Medicines Agency (EMA, EU) or the Food and Drug Administration (FDA, US)]. Results from *in vitro* and *in vivo* experiments regarding molecular, cellular and systemic modes of action as well as absorption, distribution, metabolism, and elimination are reviewed. Moreover, a variety of toxicological models must be assessed

covering the investigation of acute and chronic toxicity, impact on reproductivity, mutagenic, and carcinogenic effects.[12] First analyses of molecular modes of action and cellular toxicity are usually performed in two-dimensional (2D) cell cultures (*Figure 1A*). However, due to their low complexity 2D cell cultures are highly artificial and often unable to adequately recapitulate systemic effects and *in vivo* conditions, thereby limiting their significance for the determination of drug safety.[13,14] Gathering comprehensive information on *in vivo* dose-responses for human risk assessment, systemic effects, interactions between tissues and organs, specific organ sensitivity, chronic effects, and the pharmacokinetic profile of the Investigational New Drug is the major preclinical focus.[15] To generate valuable results regarding these issues, the employment of animal models is prevalent throughout history.

Starting with the Ancient Greeks such as Aristotle and Hippocrates (400–300 BCE) and Galen of Pergamon in the 2nd century, the use of animal experiments increased strongly and multiple scientific milestones have been reached.[16] These landmarks ranged from the proof of Charles Darwin's evolution theory in 1859[17] to the discovery and development of alkaloid-based anaesthetics around 1900[18,19] to even the cloning of an entire organism in 1996.[20] Since the beginning of the 20th century, small rodents, especially mice and rats, have emerged as the predominant model organisms in biomedical research.[21,22] Uncomplicated handling, short lifespan and high reproduction rate as well as relatively cheap housing costs led to establishment of a myriad of experimental procedures, which makes these animals attractive for many research projects.[23] Moreover, inbred mouse strains show high genetic uniformity, indicating good reproducibility of research results.[24] As biomedical research advances and multiple therapeutic approaches have to be preclinically examined, many laboratories specialize in generating customized mouse strains leading to more than 1000 genetically defined inbred strains.[21]

2.2 Use of animal models in cardiovascular drug research

The most commonly used model organisms used for proof-of-concept experiments are mice and rats (*Figure 1B*).[25,26] As a way of example, a recent study employed mice with angiotensin II-induced hypertension and

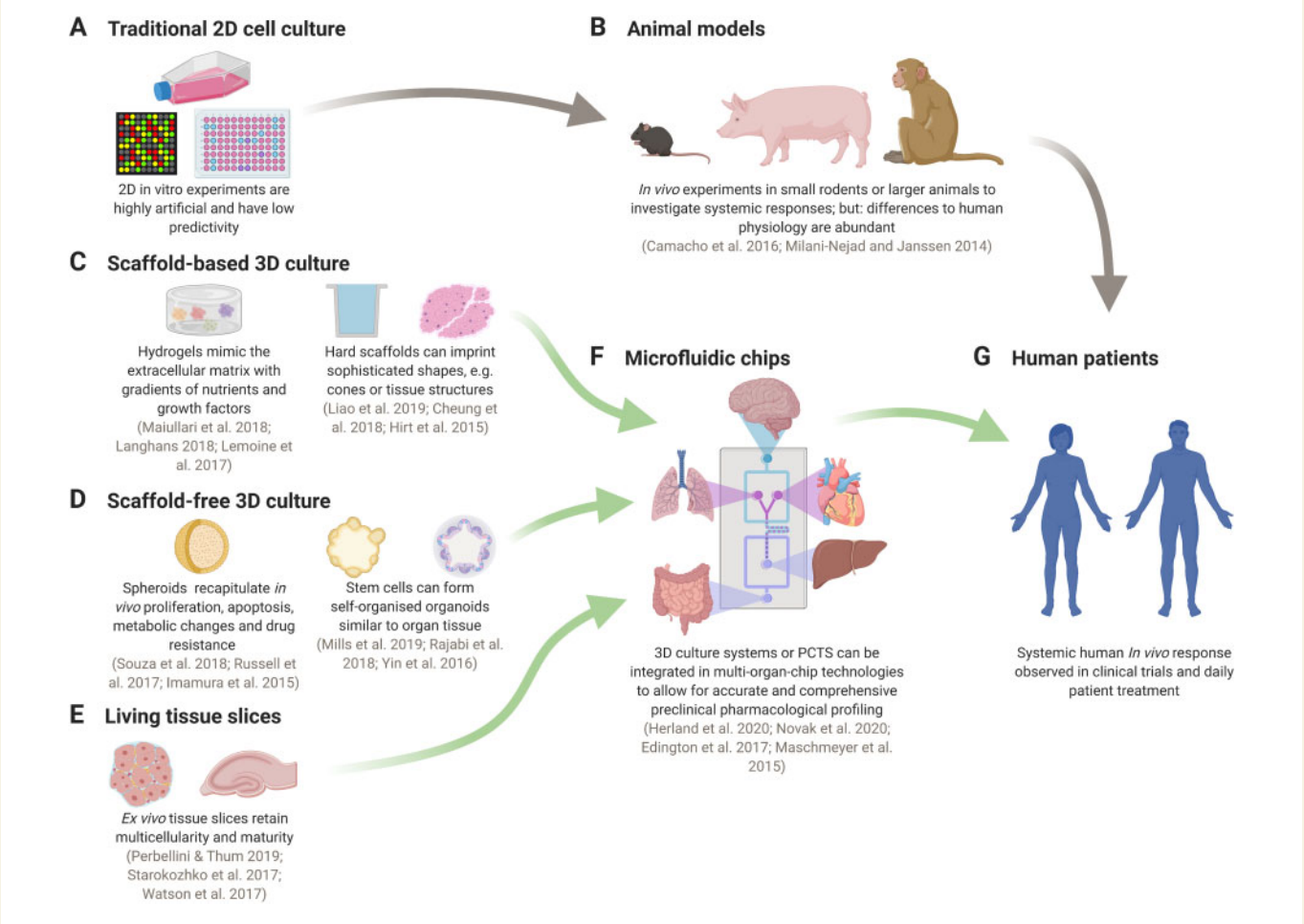


Figure 1 Alternatives to 2D cell culture and animal models. (*A*) Traditional 2D cell culture usually has low predictivity of human physiology. (*B*) To investigate systemic effects, small and large animal models used, but due to limited representation of human physiology and disease in animals resemblance is limited. (*C,D*) 3D cell culture methods improve on several parameters and have to be chosen according to the research question. (*E*) The explantation of tissue from adult animals or humans preserves multicellular tissue structure and maturity. (*F*) All previous systems can be combined with microfluidic devices to generate organ-on-a-chip systems. If Chips of multiple organ are combined, limited systemic responses can be modelled. (*G*) The response seen in human often differs from animal data, but novel methods (*C–F*) can improve predictability.

Dahl salt-sensitive hypertensive rats as model for cardiac fibrosis to study ameliorating effects of natural compounds.[27] Similarly, the preclinical testing of the anti-fibrotic small molecule pirfenidone included several small rodent cardiomyopathy and myocardial infarction models ranging from transverse aortic constriction and ischaemia–reperfusion injury over angiotensin II, doxorubicin, or diphtheria toxin treatment to streptozotocin-induced diabetes.[28] However, even if 99% of human genes have a murine orthologue, it is long known that cardiac physiologies of mice and rats exhibit profound differences to humans, thus limiting their predictivity.[23,29] One way to improve predictability of animal experiments is the use of humanized animal models.

Most commonly, the species-specific ortholog is replaced with the human version of the gene of interest, which allows to study pharmacological agents specifically targeting the human version.[30,31] Importantly, these models still base on genetic background of the animal, which may still not reflect the complex and multi-genetic response in many human diseases or lead to unknown or unintended interactions in down-stream pathways. The often insufficient observance or reporting of the limitations of novel humanized animal models recently led to publication of 'Minimal Information for Standardization of Humanized Mice'.[32] The introduction of human genes can be for the whole body, e.g., via microinjection of a plasmid or using promiscuous viral vector such as Sendai virus into embryos,[33,34] or tissue-specific, such as AAV9 for heart specific introduction of transgenes.[35] Cre recombinase systems even introduce temporal specificity through selective tamoxifen dependent transgene expression.[36] Transgenic strains for modelling different cardiomyopathies or atrial fibrillation as well as established methods to induce heart failure are abundantly available.[26] For example, calsequestrin overexpressing mice with dysfunctional beta-adrenergic receptor signalling have been used to study the inhibition of heart failure progression by the G-protein βγ blocking compound gallein.[37] In a recent study, a canine calsequestrin expressing mouse strain was cross-bred with strains featuring human renin and angiotensinogen genes resulting in triple transgenic animals to inspect the effect of a renin inhibitor on heart failure.[38] Sapra *et al.*[39] even bred a genetic mice model of dilated cardiomyopathy with a mouse carrying cardiac-specific risk factors to assess the cardioprotective effect of the nicotinic amidoxime derivate BGP-15 in the combination of heart failure and atrial fibrillation. An interesting future addition to preclinical animal research is monitoring of disease progression to identify the optimal time-point of treatment in order to maximize efficacy and minimize occurrence of adverse effects. Just recently, Hess *et al.* monitored CXCR4 levels in mice using positron emission tomography. Specifically on-peak blockage of CXCR4 in coronary artery ligated mice significantly reduced left ventricular rupture incidence and improved contractile function, whereas later blockage did not improve the outcome.[40]

Larger mammals including dog, sheep, swine, and non-human primates have a more complex immune system than small rodents and, in some aspects, are physiologically closer to humans. Consequently, data are more representative and can more easily be translated into human, justifying their use to confirm proof-of-concept studies (*Figure 1*). Recent advances in CRISPR technology, especially the possibility of multiple edits in one animal, let us predict that the remaining differences will only further dwindle in the coming years; the inactivation of all 62 loci of porcine endogenous retroviruses (PERVs) in pigs provided a recent milestone towards xenotransplantation.[41] On the other hand, representativity is still limited and experimenting with larger animals faces substantial difficulties in terms of cost and housing capacity as well as research resources such as techniques or methods and last but not least ethical considerations and regulations.[23,25]

2.3 Ethical issues with animal experimentation and alternative non-mammalian methods

The abundance of studies involving animal experimentation has rapidly elevated and continuously gained scientific relevance. In fact, the FDA recommends the inclusion of one or more animal experiments for in-depth assessment of PK/PD (pharmacokinetic/pharmacodynamic) relationships before admission to clinical trials.[42,43] Still, many ethical concerns about animal experimentation have been risen.[44] Starting with advocating rights for animals in the 18th century by the philosopher Jeremy Bentham, animal welfare societies and protection acts accumulated over the following centuries and many countries adopted Animal Welfare Acts.[45] Since 2013, the Directive 2010/63/EU demands 'the protection of animals used for scientific purposes' in all member states of the European Union.[46] It is fundamentally based on the 'Three Rs'—Replacement, Reduction, and Refinement—that were first postulated by William Russel and Rex Burch in 1959.[47] In concordance with more restrictive ethical regulations and legislation, novel systems, models, and methods for preclinical drug development as well as alternative non-mammalian organisms including *Drosophila melanogaster, Danio rerio,* and *Caenorhabditis elegans* have been established.[48] The zebrafish (*D. rerio*) in particular is frequently used in cardiovascular research and drug development,[49] and various genetic and inducible models of cardiomyopathies are broadly used to understand disease pathology and to explore novel treatment strategies.[50] Low costs and *in vivo* imaging methods among others are advantageous for high throughput screenings. Recently, transgenic zebrafish have been employed in a screening of 10 000 small molecules for activation of fibroblast growth factor (Fgf)/Ras/Mapk signalling to discover novel probes in heart development.[51] Moreover, zebrafish hearts are able to regenerate throughout their adult lifetime whereas the hearts of mammals lose their regenerative capacity postpartum. This led to invaluable insights in cardiac regeneration, e.g., the importance of Wnt signalling for cardiac regeneration and post-infarction repair.[52] Nevertheless, non-mammalian organisms are anatomically and physiologically even more distant to humans than rodents, which leads to limitations in terms of translational interpretation of experimental results.[48]

Although *in vivo* models are abundantly used, findings in model animals often cannot be translated. A multitude of solutions circumventing any animal usage whatsoever has been proposed and both the US National Toxicology Program (NTP) and the EU Reference Laboratory for alternatives to animal testing (EURL ECVAM) list over 100 distinct methods replacing animal testing.[53,54] For example, *in vitro* cell transformation assays are approved for predictions of carcinogenicity in humans due to their ability to sufficiently resemble key stages of *in vivo* carcinogenesis.[55] Furthermore, the Neutral Red Uptake cytotoxicity test can be used to determine acute oral toxicity of substances.[56] In our view, the most promising action to both solve this so-called 'valley of death' of translation[57] and the ethical complications of animal experiments is the development and refinement of advanced cell culture techniques (*Figure 1*).

2.4 Emerging 3D cultures to bridge the 'valley of death' of translation

In the past 20 years, ever increasing effort has been poured into the development of three-dimensional (3D) cell culture with the aim to reduce

or even replace *in vivo* models for drug development.[13,58] Such cell culture systems provoke intensified cell-cell contacts or the establishment and interaction with extracellular matrix, thereby providing a more *in vivo*-like environment and cellular behaviour.[13] Multiple studies showed that 3D cultures outperform 2D cultures in terms of *in vivo* representation. For instance, profound differences in diffusion and uptake of substances have been observed.[59] Especially in cancer research, the superiority of 3D cultures in studying tumour pathology and response to chemotherapy has been proven. Spheroids possess hallmark features of *in vivo* neoplasia such as hypoxia, proliferative activity, apoptosis inhibition, metabolic changes, and resistance to e.g. paclitaxel, docetaxel or 5′-FU.[60–62] Nowadays, a variety of methods for hundreds of cell lines is available.[63] Due to recent advancements in biotechnology and material sciences, 3D culture systems have emerged as an indispensable translational tool in biomedical research and preclinical drug development.[13,64] In the following, we want to highlight some non-animal models that are already developed and/or could potentially be used for pharmaceutical cardiovascular research with the aim to reduce or even replace *in vivo* models for drug development.

2.5 Engineered heart tissue and spheroids

In brief, there are two types of 3D culture techniques: scaffold-based and scaffold-free. In scaffold-based methods hydrogels or other materials are introduced to facilitate 3D growth of the cells (*Figure 1C*). Hydrogels e.g. consisting of agarose or gelatine can mimic the extra-cellular matrix (ECM) by allowing diffusion and establishing gradients of nutrients and growth factors.[14] Stiffness and composition need to be individually adjusted to suit the requirements of certain cell types for their *in vivo*-like development and behaviour. In the field of stem cell research, Matrigel is often used to guide specific differentiation.[65–67] Lemoine *et al.*[68] reported successful 3D cultivation of iPSC-derived cardiomyocytes in a mixture comprising Matrigel, fibrin, and thrombin as this scaffold closely represented adult atrial and ventricular myocardium in terms of sodium currents. Instead, collagen I has been identified as most suitable 3D scaffold for mature rat cardiomyocytes due to superior cell-binding properties and facilitation of cellular migration.[69] However, since most hydrogel materials are natural products they can contain additional by-products such as cytokines or growth factors. Moreover, contamination of such signalling molecules as well as chemical composition can vary strongly between different charges,[70] thereby limiting their use in drug development.[71] This issue could be avoided by using synthetic nanofibres. Such materials with similarity to collagen were proposed to be advantageous as they are easily modifiable allowing to adjust pH-gradients and cell attachment sites. Unfortunately, gelation procedures of hydrogels often lack homogeneity resulting in deviating cellular behaviour within one setup.[72] Novel bioprinting methods could be a promising tool to overcome this prevalent drawback of hydrogels. In 2018, a microfluidic printing device was used to assemble 3D cell cultures in an alginate and polyethylene glycol monoacrylate-fibrinogen matrix. This led to functional 3D cardiac tissue by co-cultivating iPSC-derived cardiomyocytes with human umbilical vein endothelial cells.[73] Notwithstanding, suitability of cells to bioprinting and the stress resulting from the printing process itself (e.g. mechanical stress, or temperature and solvent conditions) need to be considered.

An interesting use of scaffolds was recently reported by Cheung *et al.*: they coated silica microrods with a lipid bilayer to imitate antigen presenting cells, which strongly improved antigen-specific expansion of rare T cell subpopulations in mice and human. This may be an important evolution in the production of CAR T cells in immunotherapy.[74] Additionally, hard scaffold can be used to force the development of a specific shape, e.g., to mimic heart tissue. As such, Hirt and colleagues prepared engineered heart tissue (EHT) from rat and induced hypertrophy by afterload enhancement. Similar to the changes seen *in vivo*, EHT showed reduced contractile force and reactivation of a foetal gene program.[75] Furthermore, inhibition of the pro-fibrotic miRNA-21 could prevent progression of fibrosis just as previously described in live animals.[75,76] Successively, the same group transferred their method to human iPS cells which they differentiated into cardiomyocyte to form EHT which showed contractile functions after 2 weeks in culture.[77] For example, rat EHTs were utilized as a screening platform to assess proarrhythmic side-effects of 47 inhibitors targeting different ion channels and 28 chemical compounds not known to alter cardiac rhythm. In their experiments contractile behaviour of EHT and Ca^{2+} transients were monitored.[78] Just recently, Afshar *et al.* applied a similar approach to generate 3D skeletal tissue. Moreover, this group could progress to a 96 well format suitable for small- to medium-sized screening approaches.[79] Aside from improving scalability, an important milestone of EHT towards regular use in preclinical pipelines is standardization. In a recent blinded multi-centre study, an multinational consortium of academic groups and biotech companies used 36 compounds with known effects on cardiac physiology to assess whether EHT are able to consistently predict cardiotoxic effects across different institutions. After harmonization of SOPs, they reported up to 93% accuracy in prediction when using EHT, comparable to commonly used animal models.[80]

Still, as EHTs are artificially engineered, their functionality is highly dependent on the precise composition of cell types and culture materials and comparability to human cardiac tissue has to be closely monitored.

In contrast to scaffold-based methods, scaffold-free 3D cell culture uses gravity or magnetic levitation to gently push cells towards each other and form spheroids (*Figure 1D*). Probably, the simplest way to create spheroids without scaffolding material is to apply U-shaped microtiter plates. This method was exemplarily used to create spheroids from human iPSC-derived cardiomyocytes which were then implanted into infarcted mouse hearts.[81] Interestingly, in a successive study, the group was able to evade immune rejection of grafts by knocking out mayor histocompatibility complex proteins in the cells of the spheroid.[82] However, the assembly of 3D aggregates within U-shaped wells or hanging drops can be time-consuming for certain cell types. A more effective model at promoting 3D structure and cell–cell interaction networks may be microwells with a conical shape. Such microwells can e.g. be created with 3D printing or using simple stamp-like tools in an agarose matrix. As seeded cells sediment in these microwells, they are pushed towards another and form spheroids.[83,84] The assembly of uniform spheroids has been shown for hepatic and hepatocellular carcinoma cell lines as well as breast cancer cell lines alone and in coculture with bone marrow stromal cells.[85] Due to the cost-effectiveness and multi well-format, such methods are assumed to hold good potential for high throughput drug screenings. The magnetic levitation method provides another fast way to create spheroids.[86] For example, gadolinium can be added to culture media for magnetization of cells, which can then be assembled to 3D clusters in a magnetic field.[87,88] In a study by Timm *et al.*, ring-shaped 3D structures were formed with HEK293 cells and tracheal smooth muscle cells using magnetic levitation. In a high throughput manner, they assessed ring closure in dependence of treatment with ibuprofen and sodium dodecyl sulphate in various concentrations.[89] Importantly, the impact of the magnetic particles itself is often unknown and not investigated in detail. Moreover, as with all scaffold-free systems, the lack

of cell-ECM contacts is highly artificial and careful consideration during interpretation of data.

Considering that cardiomyocyte cultures often lack cell maturity and sufficient complexity, e.g., in terms of vascularization, innervation and immune system, comparability to adult hearts and physiological relevance in general is debatable.[90] Thus, all these different methods can further profit from improved co-culture of different cell types. In line, several groups have reported on the co-culture of cardiomyocytes, endothelial cells, and cardiac fibroblasts (the three main cell types of the heart) in 3D environments. Figtree *et al.* co-cultivated rat neonatal cardiac myocytes, endothelial cells, and fibroblasts in hanging drops. Exposure to profibrotic stimulants TGF-β and doxorubicin resulted in an increased ECM deposition and remodelling.[91] In a recent study, a similar model was investigated for effects of hypoxia, α/β-adrenergic receptor modulation, VEGF (vascular endothelial growth factor) inhibition, and TGF-β stimulation. By using endothelial cells with fluorescence reporters, treatment- and time-dependent variations in vascularization and cellular physiology could be observed.[92] In addition, Verheijen *et al.* recently studied the effects of the cardiotoxic doxorubicin (used in chemotherapy) on spheroids of human iPSC derived cardiomyocytes co-cultivated with cardiac fibroblasts. Transcriptomic analysis was able to retrieve the known mechanisms of doxorubicin cardiotoxicity, while acute and chronic effects of doxorubicin treatment could be recapitulated using different doses.[93] Similarly, 3D cardiac tissue obtained via liposome fusion (using ketone and oxyamine groups) profited from firm intercellular adhesion and shows contractility and intended response when exposed to isoprenaline and doxorubicin.[94] In another application, human embryonic stem cell (hESC)-derived cardiomyocytes and endothelial cells were co-cultivated in a scaffold-free manner with HUVECs and murine embryonic and neonatal human dermal fibroblasts to create pre-vascularized heart tissue, which was subsequently transplanted onto the hearts of nude rats. These patches showed improved integration into myocardium of rats and prolonged viability when compared to patches consisting solely of cardiomyocytes.[95] Nevertheless, the reproducibility of such co-cultures are limited, as even small alterations in cell type or medium composition will lead to pronounced differences from batch to batch.

Spheroid cultures of other organs have also been utilized for pharmaceutical assessment. As example, *in vitro* liver tissue consisting of fused spheroids bio-printed with primary hepatocytes and mouse fibroblasts has been reported to resemble key liver functions for a long time. Metabolic functionality as well as stable expression of metabolic enzymes such as CYP3A4 could make this system a promising tool to evaluate metabolization and toxicity of drug candidates.[96] Also, adipose tissue spheroid cultures have been shown to be sensitive to toxin exposure. Scaling to 384-well format is expected to make these models suitable for high throughput screenings.[97] In conclusion, all these 3D cell culture methods can improve physiological similarity in certain aspects. However, the comparability of new methods is often not studied systematically, and comparisons with human physiology, or at least established animal models, are scarce. We believe stringent comparison with human healthy and patient tissue, e.g., via transcriptomics and proteomics, is essential.

2.6 Organoids

In contrast to 3D cultures generated out of differentiated cell types, stem cells can be encouraged to differentiate into self-organized multicellular 3D structures (*Figure 1D*). If the seeded cells further develop cellular orientations and into multiple distinguishable cell types, the developing structures are called organoids.[98] As this is a highly complex process, limited reproducibility is still limited and represents the major bottleneck. Still, in recent years, organoid systems resembling liver, lung, or brain have advanced significantly and are a valuable tool for studying developmental as well as pathological aspects of these organs.[99,100] In the field of cardiac organoids, the maturity of cardiomyocytes and the unique composition of various cell types, ideally electrically coupled, represent major hurdles.[101] Due to the insufficient maturity of cardiomyocytes, todays hCOs mostly resemble foetal heart tissue and are predominantly used for research of cardiac development and regeneration.[102] Nevertheless, alterations in cardiac frequency of hCOs after exposure to the non-selective cardioactive β-adrenergic receptor agonist isoproterenol[100,103] and heart rhythm modulators quinidine and astemizole have been reported.[103] In addition, hCOs and liver organoids have been used to evaluate toxicity of the environmental pollutants lead and glyphosate by monitoring beating behaviour, ATP production, and viability, suggesting the general applicability for heart- and liver-toxicological analyses of small molecules.[104] Mills *et al.* reported the successful generation of more mature hCOs by modulating nutrient supply and other extracellular factors to modulate metabolic activity, a key difference from immature to adult cardiomyocytes.[105] In more recent work, these matured hCOs were used for a multi-step screening of small molecules, thereby identifying two candidates with pro-regenerative potential and without cardiotoxic effects.[106] In another approach, Rajabi *et al.* decellularized a rat heart and repopulated the remaining ECM scaffold with hESC-derived cardiovascular progenitor cells, which subsequently differentiated into cardiomyocytes, smooth muscle cells, and endothelial cells resulting in a beating artificial heart reconstruct.[107] While there is still a long way towards fully 3D printed miniature hearts, given the recent advances in scaffolding and 3D printing the successful generation of miniature humanized hearts appears to be only a matter of time.

2.7 Living tissue slices

The best resemblance of the processes seen in human is the direct use of tissue *ex vivo*, thereby preserving the multicellular and mature environment also finally seen in patients (*Figure 1E*). Protocols for generation, maintenance, and investigatory treatment of such precision cut tissue slices (PCTS) are available for various organs of many different animals and humans.[108,109]

For instance, the airway hyperresponsiveness response generated by murine lung slices after exposure to increasing doses of the chemical allergen methacholine was strikingly similar to the response seen in mouse lungs challenged *in vivo*.[110] Later, Hirn *et al.* utilized rat lung PCTS for assessment of silver, zinc oxide, and quartz nanoparticle toxicity by monitoring cell viability and secretion of inflammatory cytokines. However, multiphoton imaging revealed an incomplete penetration of the slice tissue by the nanoparticles, thereby avoiding interaction with some cells in deeper tissue layers. This highlighted the importance of slice thickness as potential limitation of PCTS for toxicology screenings.[111] Subsequently, rat lung PCTS were used to assess inhalation toxicity in a medium scale screening of 20 chemicals in different concentrations by measuring lactate dehydrogenase (LDH) release and mitochondrial activity.[112]

Other important organs in toxicology studies and drug development are intestine and liver due to their key roles in metabolism, often being the cause for later observed toxic side effects. As common example, HepG2 liver cells are routinely used as surrogate for liver toxicity in early phases of drug discovery. While this offers a cost-effective and straightforward approach towards excluding potentially hazardous candidates, resemblance of actual liver function and toxicity is severely limited.[113] Several models of intenstine or liver slices have been proposed as

alternative with increased similarity to human physiology. As example, human jejunum slices were subjected to treatment with the non-steroidal anti-inflammatory drug diclofenac to elucidate intestinal side effects. The resulting depletion of ATP production, morphological damage, as well as increased caspase 3 activity and LDH release indicated a cytotoxic effect of diclofenac to intestinal tissue, as seen *in vivo*.[114] To assist ongoing clinical trials, Gore and others recently used murine and human jejunal as well as liver slices for an in-depth investigation of the mode of action of the anti-fibrotic small molecule Omipalisib. In liver PCTS, the treatment resulted in a reduction of fibrosis via inhibition of the PI3K/Akt pathway, whereas an increased cell damage was observed in jejunum tissue, indicating potential toxicity as limitation of this therapy.[115] As for assessing liver toxicity, Granitzny *et al.*[116] proposed measurement of ATP and albumin production as well as histomorphology assessments as the most significant readouts based on an analysis of rat liver slices treated with different concentrations of acetaminophen. Hepatic *ex vivo* models might also be suitable for pharmacokinetic investigations, as human liver slices keep the ability to transport as well as metabolize drugs and preserve other functions such as albumin production or glycogen storage for several days.[117] Although these developments hold promise, adoption of 3D or *ex vivo* hepatic models in the pharmaceutical industry is still limited due to insufficient or substandard characterization of those novel model systems.[113]

Apart from these examples, the use of living myocardial slices also becomes more and more popular in cardiovascular research. Canine and human myocardial PCTS have been used to examine the activity of fibroblasts in the process of cardiac fibrosis.[118] In a recent study, human iPSC-derived cardiomyocyte 3D culture and human myocardial PCTS were treated with a potassium channel blocker. Importantly, only native myocardial PCTS showed early afterdepolarizations and conclusive force–frequency relationships, and remaining parameters were less pronounced when compared to iPSC-derived cultures due to insufficient maturity.[119] Some groups described long-term cultivation (up to 4 weeks) of human myocardial slices with maintained high viability and tissue functionality, thereby giving the possibility for chronic exposure experiments.[118,120] Still, the comparability between such long-term cultivation systems, those only suitable for short-term cultivation, and the human physiology as eventual target, is highly debated and more work is needed to optimize long-term cultures. Pharmacological treatment of the isolated tissue with dofetilide and rilmakalim, known to interfere with hERG (Kv11.1) and ATP dependent potassium channels,[121] respectively, reliably modulated the action potential.[122] Importantly, living myocardial slices are not subjected to the same pressure as seen inside the heart. While up-to-date protocols include different variants of 'stretchers' pulling on cardiomyocytes, optimally in conjunction with electrical stimulation paced similar to the physiological heart, to imitate cardiac load and prevent overgrowth of resident cardiac fibroblasts,[120] more progress is needed to properly address and replicate this important parameter. Explantation and *ex vivo* cultures still induce alterations of the tissue and thus are not completely mimicking the *in vivo* effects. This should be especially noted, when sensitive analyses, e.g., transcriptomics via RNA-seq, are performed and compared with 'freshly explanted' control samples. An additional drawback is the limited access to human tissue for many researchers.

That said, we want to encourage those researchers to use animal PCTS as intermediate step to at least reduce the number of animals needed: as numerous slices can be acquired from an organ, multiple experimental conditions can be assessed in a single animal in parallel. Moreover, the lack of an explicit head-to-head comparison, e.g., blinded drug testing studies, may be the biggest hurdle towards accepting such systems as standard in preclinical research. Therefore, we propose to compare the reaction to approved CV (cardiovascular) drugs in the most promising models to current gold standard cell culture and animal models, and rank by resemblance of real life patient data, similar to those multi-centre studies in human iPS-derived cardiomyocytes or EHT.[80,123] We believe that the introduction of more researchers, especially as interdisciplinary teams, will accelerate and broaden such a comparative study to allow meaningful conclusions. Nevertheless, today's PCTS can already be used as a model themselves, and may soon be able to reduce or replace animal experiments.

As a last step, one can combine such engineered 3D cultures or sliced tissues with microfluidic devices to build organ mimics.

2.8 Organ-on-a-chip

In recent years, organ-on-a-chip (OOC) technologies have been studied increasingly and emerged as promising *in vitro* tools for preclinical drug development.[124] The basic concept of OOC is to combine 2D and 3D cell culture or explanted tissue with microfluidic devices (*Figure 1F*). Due to constant perfusion, these miniaturized tissue or organ replicas can mimic *in vivo* conditions more closely than cell culture alone.[125] By now, such chip-based systems have been established for various organs including lung, liver, kidney, and intestine.[126] For example, Huh *et al.* generated a lung-on-a-chip model that they comprehensively characterized regarding exposure to bacteria and pro-inflammatory stimuli. Moreover, the processing and toxicity of nanoparticles in mechanically stressed lung tissue revealed close similarity to the reaction of the complete organ *in vivo*.[127] Above mentioned lung-on-a-chip cultures were later used to model IL-2-induced pulmonary oedema, an adverse effect observed in IL-2 treated cancer patients, and subsequently used to assess novel therapeutic approaches including angiopoietin-1 administration and the novel ion channel inhibitor GSK2193874.[128] Similarly, liver-on-a-chip technology was adopted for an in-depth analysis of metabolism of HIF prolyl hydroxylase inhibitors adaptaquin and analogues, identifying CYP3A4 and CYP2B6 as the most important enzymes.[129] Just recently, a bioprinting approach was proposed to create fully functional liver-on-a-chip models. Preliminary toxicity experiments with acetaminophen implied promising applicability for this model in preclinical drug testing.[130]

Due to the constant, high workload and low regenerative capabilities of the heart, cardiac dysfunction is a major factor of adverse drug effects, especially under high dose conditions, as for the treatment of cancer.[131] Therefore, the prediction of cardiotoxicity is a major factor of safety-related drug attrition and an integral part of drug candidate evaluation.[132,133] Still, to date these experiments are mainly performed *in vitro* using single isolated or iPS-derived cardiomyocytes, which often does not recapitulate the detrimental effects observed in animal models or clinical trials.[132,134] Consequently, robust prediction of the cardiac effect of drugs via heart-on-a-chip systems could become a true alternative to animal models.[135] Although heart tissue has been cultured for over 100 years,[136] development of heart-on-a-chip models appears to be more demanding due to the highly complex interplay of constant mechanical load, synchronized electrophysiology, and supply with nutrients and oxygen.[120,131] Electrical stimulation has been identified to guide cellular alignment, coupling, and contractile function.[137,138] Similarly, by coating thin elastomeric films (to measure contractility in muscular thin film methods) with the central extracellular matrix component fibronectin, Grosberg *et al.*[139] observed improved self-assembly and -organization of seeded rat cardiomyocytes.

In the past years, the mechanical forces came more and more into focus of optimization. Marsano *et al.* used hanging posts instead of the previously described elastic films to induce uniaxial mechanical stimulation to improve resemblance of the *in vivo* mechanical load. They observed early spontaneous beating, improved contractility, and more sensitive response to the β-adrenoreceptor agonist isoprenaline in a human iPSC-derived cardiac tissue model.[140] Zhang *et al.* presented a heart-on-a-chip model, which showed loss of contractile function and decline of cell viability after administration of the chemotherapeutic doxorubicin.[141] In another direction, more and more products containing nanoparticles reach the market, although some studies indicated potential cardiotoxic effects.[142] Upon exposure to TiO_2 and Ag nanoparticles (e.g. present in antiperspirants or sunscreen lotions) to a heart-on-a-chip model, decreased contractility due to structural tissue damage was observed.[143]

Despite the successful improvements of the past years, heart-on-a-chip models mostly feature ventricle-derived tissue, thereby limiting themselves to respond for ventricular effects. In that way, as it was recently proposed by Zhao *et al.*, we believe additional knowledge of chamber specific development and generation as well as miniaturization is required to properly mimic the four-chamber structure as well as electrophysiology of the heart, ultimately having the possibility to reliably predict cardiotoxicity in its full picture.[144] Moreover, meaningful comparisons to human physiology and today's gold standard animal models are essential and presuppose careful characterization of all novel and refined models.

2.9 Towards body-on-a-chip technologies in drug development

As shown above, drug action and safety can be evaluated in increasing detail using 3D culture, tissue slices or OOC methods. Each of these systems only represent a single organ or even cell type. To comprehensively investigate systemic effects of drugs and their metabolites, mimics of several organs can be connected to multi-organ-chips (*Figure 1F*).[135] In an early design, Sin *et al.* circulated culture medium through a three-chamber system ('lung'-'liver'-'other') using a chip of only about 2.5 square centimetres.[145] The addition of a liver fabrication to an engineered heart abolished the reduction in beating to the beta-blocker propranolol due to hepatic metabolization and thereby inactivation. Moreover, in a triple organ microfluidic model additionally featuring lung epithelium, the pro-inflammatory and -fibrotic chemotherapeutic bleomycin displayed a yet unidentified cardiotoxic side-effect via the induction of IL-1β secretion from lung tissue.[146] By now, the circulation of media through connected organs-on-a-chips can be individually adjusted to recapitulate *in vivo* blood flow and thereby distribution of administered substances.[147] Additionally, separated microfluidic circuits representing e.g. blood and excretory circulation can further discriminate effects of drugs and their metabolites.[148] In the coming years, further improvement is expected to introduce multi-organ-chips including every relevant organ. Especially if using human-only material, these body-on-a-chip systems hold great promise to allow pre-clinical investigation of drugs or drug candidates. Just a year ago, a team of Wyss Institute could show a breakthrough by combining eight vascularized, two-channel organ chips into a single automated system. Of great interest, these chips could be used to predict pharmacokinetic parameters of nicotine and cisplatin administration in human, and cisplatin PD matched patient data.[149,150]

To no surprise, OOC technology was named one of the top 10 emerging technologies of 2016.[151] However, before a broad and routine application in preclinical drug development, it is necessary to overcome the current disadvantage of low-throughput of organ- or body-on-a-chip technologies.[152] Therefore, considerable effort is invested to enhance analytical readout possibilities by integrating sensors for various physiological parameters and advancing long-term use of multi-organ-chips for investigation of chronic effects.[153] Such systems could be used to rapidly and reliably evaluate chemicals, toxins or pathogens and inhibitory strategies, e.g. under conditions of deliberate release or emerging infectious diseases. Given these promises, and due to the exceptional rich funding [e.g. by the US Defense Advanced Research Projects Agency (DARPA), FDA, and NIH], further research to generate the 'human-on-a-chip' is ongoing and will certainly reshape early drug development.

3. Clinical trials

Once preclinical safety has been confirmed, novel drug candidates advance to the clinical trial stage, which describes the actual testing procedure in humans. Standards in medical treatment changed from anecdotal medical practice to scientific evidence in the last century until binding medical guidelines and best practices were enacted.[154–156] On the way towards today's gold standard of clinical trials, the randomized double-blind placebo-controlled trial format (RDBPCT, *Figure 2A*),[157,158] several key steps have been documented: (i) concurrently treated control groups first performed by J. Lind in 1747,[159] described in detail in reference 160 (ii) sham[161] and placebo treatment by H.G. Sutton in 1863 as reviewed by reference 162 (iii) blinding between different interventions (at best subjects and experimenters: 'double-blind');[163] and (iv) randomization of subjects into intervention groups.[164] In the 20th century, applications were randomized and multicentre trials were established until the first RDBPCT was performed in 1980.[162] By now, clinical trials follow an established structure including design, funding, and protocol development before patient recruitment can actually start, and the various roles of contributors, e.g., sponsor or investigator, are distinct and clearly defined. After final treatment of the volunteers, follow-up and patient close-out are mandatory procedures prior to final study conclusion.[162]

To negate bias from unpublished negative results and to globally provide comprehensive information on the status of all potential drugs and procedures in clinical testing, the Canadian Institutes for Health Research expressed the necessity of prior registration for all upcoming clinical trials, now known as the Ottawa statement.[165] Furthermore, *a priori* registration can improve patient participation and enrolment as well as prevent redundant trials with the same aim.[166] The trials should be registered following the principles of the International Committee of Medical Journal Editors (ICMJE) ensuring an independent non-profit operator and the free charge for users and registrants. Next to smaller registers of different countries the largest global register is the US-based ClinicalTrials.gov platform.[167] In 2004, the WHO implemented the International Clinical Trials Registry Platform (ICTRP) to generate one database for all clinical trials registers and therefore to strengthen the public accessibility.[168] In principle, candidates are tested for safety in healthy volunteers (Phase I), efficacy in patients (Phase II), and a confirmatory study of safety and efficacy in a larger cohort of patients (Phase III). If successful, this is followed by post marketing studies to constantly monitor a drug's risk and benefit during its use (Phase IV). Importantly, for every phase sample size, required time and consequently cost for the sponsor substantially increase.[169]

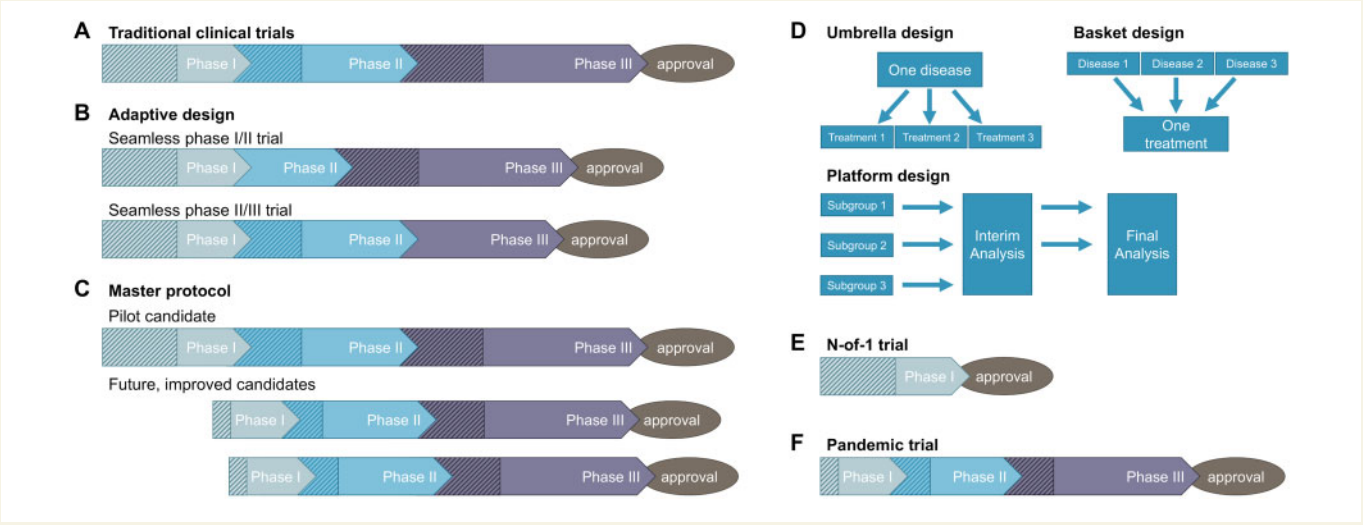


Figure 2 Advancements in clinical trial structure. (*A*) While traditional clinical designs display long approval procedures in all three phases (cross-hatchings in the figure), several designs can shorten this process and rise efficacy of clinical trials. (*B*) Adaptive design allows to combine two phases in a single application and can include interim analyses to adapt the trial on-the-go. (*C*,*D*) Master protocols can investigate several interventions (umbrella design) or diseases (basket design) or subgroups (platform design) in parallel. As special cases, (*E*) N-of-1 trials investigate only one subject and (*F*) pandemic trials follow a shortened procedure due to the emergency pandemic situation.

Unfortunately, only a small number of drug candidates passed all phases of clinical trials. According to Dowden *et al.*[170] the likelihood of successful launch to market of a candidate in Phase III is about 60%, 25% in Phase II and only 7% in Phase I. These 'success rates' in Phase I and Phase II did not significantly change over the past years.[170–172] Unfortunately, most new candidate drugs fail in Phase III due to a lack of efficacy.[6] For example, the lipoprotein-associated phospholipase A2 (Lp-PLA2)-inhibitor Darapladib (SB-480848) was investigated for treatment of coronary heart disease and atherosclerosis by targeting inflammatory processes.[173,174] Subsequently, several phase I and II clinical trials were completed and deemed successful.[175–178] Nevertheless, Darapladib could not show improvement of the primary endpoint in three independent Phase III clinical trials for treatment of acute coronary syndrome (SOLID-TIMI 52, NCT01000727[179,180]) stabilization of atherosclerotic plaques in coronary heart disease patients (STABILITY, NCT00799903[179,181]) or improvement of coronary endothelial function (NCT01067339[182]) In a more comprehensive analysis of overall clinical trial failure from the National Academies Forum on Drug Discovery, Development, and Translation, Galson *et al.*[170] identified three fields of failure in phase III clinical trials: expertise (lack of training and critical disciplines), execution (inappropriate trial design and endpoint selection as well as 'overenthusiastic interpretation of data'), and knowledge (regarding the mechanism, biomarkers or patient populations).

Despite the expanding research and development in drug discovery leading to an ever increasing amount of drug candidates in the past years, the number of approved drugs is stagnating and the cost doubling about every 9 years.[4] Hence, the progress of medical innovation becomes limited as clinical trials display less frequent success, high costs, the need of a large study population, and a long study duration. Importantly, we wish to advice the reader that the view of drug development as a determined pipeline is useful to compare different clinical trial formats (as will happen below, see *Figure 2*), but the whole drug development process may better be represented as a complex and highly interconnected network.[183,184] Nevertheless, the traditional structure of clinical trials has provided additional room for improved success rates and reduced costs.

3.1 Adaptive designs and master protocols

The traditional RDBPCT format allows for two readily recognizable optimizations: the adaptive modification of trial parameters based on newly acquired knowledge, using predefined rules, as well as reusing a clinical trial for additional interventions or indications. To this end 'adaptive designs' allow expansion or termination of study arms depending on preliminary results, e.g., through identification of previously unknown (non)-responder subgroups during the study (*Figure 2B*).[185–187] Adaptive designs containing such interim analyses, e.g., sample size re-estimation, a dose-selection rule or a change of the primary endpoint, have been increasingly accepted by national authorities.[188]

Adaptive Platform Trials have an algorithm with several subgroups of multiple treatments and re-evaluation steps which allow participants to switch or exit study arms.[187] As example, the phase III trial CHAMPION PCI (NCT00305162) and the companion trial CHAMPION PLATFORM (NCT00385138) investigated the potential of the ADP (adenosine-diphosphate)-receptor antagonist cangrelor in patients before or after percutaneous coronary intervention.[189,190] Both trials included interim power analyses after enrolling 50% and 70% of the study population to assess whether sample size should be increased, additional patient groups should be included or the trials should be stopped for futility. Indeed, the second analysis of both trials at the 70% threshold showed insufficient superiority of cangrelor over clopidogrel, and consequently enrolment was stopped immediately in both trials.[189,190] In a follow-up trial (CHAMPION PHOENIX, NCT01156571), a similar interim power analysis was used utilizing predefined zones to decide if sample size adjustment was necessary to ensure a successful outcome of the study.[191,192] In this final trial, the study was continued and completed as planned without changes to the sample size, and cangrelor significantly

decreased the rate of stent thrombosis and other ischemic events.[191] Interim analyses are also part of population-enrichment designs, where patients are enrolled in placebo-controlled subgroups depending on their biomarker status (*Figure 2D*, platform design). The effects noticed in the interim analysis decide whether all subgroups are treated and analysed as planned or if a subgroup will be terminated prematurely to improve study conclusiveness.[193]

In seamless trial formats, recruited and randomized subjects remain in the study while the study is upgraded from phase I to II, e.g., through inclusion of PK/PD monitoring (see NCT04045405), or Phases II to III (*Figure 2B*). A recent and stunning example of seamless design, although outside of the field of cardiology, is the seamless Phase I/II/III trial to evaluate the SARS-COV-2 RNA Vaccine Candidate Comirnaty (also known as BNT162b2, BioNtech/Pfizer, EudraCT 2020-002641-42), where the seamless design allowed for rapid and constant review by EMA and FDA and (conditional) marketing approval.[194]

Importantly, 'using an adaptive design implies that the statistical methods control the pre-specified type I error, that correct estimates and confidence intervals for the treatment effect are available, and that methods for the assessment of homogeneity of results from different stages are pre-planned'.[195] To follow this guideline, control of the type 1 error (the false-positive rate) throughout all analyses and pre-planned adjustments represents a major challenge, as other statistical parameters, e.g., Z statistics, have to be adapted accordingly.[193] Further disadvantages arise from the necessity to unblind data for any kind of interim analysis as well as the increased time and complexity of planning an adaptive trial format compared to a trial in traditional design.[193]

Additional novel study formats are umbrella designs which test several drug candidates for the same indication in parallel, currently mostly used in the field of oncology (*Figure 2D*, umbrella design). For example, a clinical trial of treatment with different combinations of pembrolizumab to treat non-small cell lung cancer (NCT04165798) is designed as an umbrella master protocol to enrol patients which are subsequently transferred into one of three Phase II substudies, depending on previous treatment history as well as PD-L1 expression in the neoplasm. In contrast basket designs investigate the impact of the same drug candidate for several indications or patient subgroups (*Figure 2D,* basket design). Again, basket study designs are mostly used in trials of anti-cancer drugs targeting a specific mutation that occurs in neoplasms originating from various tissues.[196] For instance, the STARTRK-2 basket trial (NCT02568267) simultaneously examines the small molecule Entrectinib as a potential treatment in solid tumours with mutated NTRK, ROS1, or ALK variants, respectively. In addition, platform trials can share one control group for several interventions, thereby reducing the number of patients not receiving a potentially life-prolonging new treatment.[197] All these designs can be further combined into master protocols for maximal flexibility[185] (*Figure 2C*).

For now, adaptive designs are mainly used in the field of orphan diseases and master protocols in the field of oncology. Implementing those novel designs in larger clinical trials, e.g., those with over 10 000 participants increasingly happening in cardiology, may gift the necessary flexibility option for successful translation into clinical use. As of writing, at least 186 trials using a master protocol design are available in the clinicaltrials.gov database.

3.2 Drug repurposing

So far, we described trials following the long-time paradigm 'one drug—one target—one disease', but a remarkable shift could be noticed in the past few years. Already established and approved drugs can be repurposed for other diseases with relative ease, or candidates proven safe but ineffective in previous clinical trials can be assessed for novel indications. One of the most important and well-known examples of drug repurposing took place in cardiovascular medicine: although already in use for centuries to millennia (probably since ancient Egypt[198]) three independent but back-to-back articles first described the antiplatelet activity of Aspirin in 1971.[199–201] Today, the antiplatelet use of low-dose ASS is the most important pharmaceutical therapy in primary and secondary prevention of cardiovascular diseases.[202,203] As another commonly known example, the phosphodiesterase-5 inhibitor sildenafil was originally developed to improve cardiac functional capacity and exercise performance but is now mainly used to treat erectile dysfunction.[204,205] To take drug repurposing another step forward, the combination with in-depth *in silico* analyses could allow routine testing of large safety approved drug libraries for novel identified targets. With many options available, the main questions is no longer how to find but how to decide on the right database.[206] As starting point, we found the Drug Repurposing Hub, currently annotating about 6800 compounds with preclinical and clinical data, to be an effective tool to guide decision-making.[207]

A few years ago, in several large-scale clinical trials investigating SGLT2-Inhibitors (gliflozines) for treatment of diabetes, a significant reduction of cardiovascular risk was observed in the treated patient cohorts.[208,209] This potential in cardiovascular therapy was then further explored in the subsequent Declare-TIMI58 trial (Phase III, 17 160 included subjects, NCT01730534), where treatment with dapagliflozin indeed significantly reduced the incidence of hospitalization for heart failure as well as the rate of cardiovascular death in patients with type 2 diabetes and a high risk for CV disease.[210] In addition to glucose-lowering, SGLT2-inhibitors alleviated ventricular loading, improved cardiac metabolism as well as reduced cardiac necrosis and fibrosis.[211] Similarly, the hydroxylamine derivate BGP-15 was initially developed to prevent insulin resistance,[212,213] but subsequent animal studies indicated improvement of cardiac function, decreased cardiac fibrosis as well as reduced arrhythmogenic episodes.[39] Currently, a multitude of Phase III studies specifically investigate the potential of SGLT2-inhibitors in patients with heart failure or other cardiac diseases, with or without accompanying diabetes.[214]

In the area of autoimmune diseases, the common effective drugs are mostly antibodies and recombined proteins (biologics) which need parenteral application. As small molecules often can be administered orally,[215] they are of high interest to autoimmune research. The janus kinase inhibitor tofacitinib, approved for rheumatoid arthritis, was successful in a Phase III trial treating inflammatory bowel disease.[216] Unfortunately, this inhibitor class has been linked to increased infection rates, especially herpes zoster, due to their modulation of several cytokine pathways.[217,218] Moreover, novel immunosuppressive small molecules seem to amplify the risk of developing cardiovascular complications. Consequently, during repurposing significant attention has to be focused on previously reported and novel adverse effects, e.g., using networks integrating known pathways,[219] off-targets and adverse effects with drug similarity algorithms.[220] Notwithstanding, drug repurposing could provide advantages when using combinations of drug biological and clinical profiles and *in silico* analysis for repurposing potential and prediction of adverse effects.[221]

3.3 Computer-based *in silico* clinical trials

In silico clinical trials describe the concept to model and treat patient cohorts in computer simulations.[222] The recent advancements in machine learning facilitate novel ways to analyse on- and off-targets as well

as side effects of drug candidates and, in some first cases, already replaced preclinical *in vivo* animal studies.[223] Next to a wider spread of candidates, machine learning algorithms and *in silico* prediction lead to accelerated drug discovery and increased safety prior to clinical trials.[224] As an example of computer simulations, the Virtual Human Physiology project started in 2005 to translate computational physiology into clinical practice.[225] For instance, the cardiac action potential was computationally modelled with algebraic and differential equations.[226,227] For assembling all these mathematic models, OpenCOR was created as a user-friendly software.[228] *In silico* clinical trials could improve success rates of real clinical trials if optimized planning and estimation of outcomes lead to a reduction of sample size and duration as well as increased safety for participants by a streamlined design of the study.[229,230]

As finding the recommended dose for a Phase II study via treating 6–9 subjects in a Phase I trial is based on dose escalation and observed toxicity, severe safety issues for the participants cannot be excluded. To increase the safety, Yan *et al.* propose a computer-based algorithm to complement first-in man studies to predict the recommended dose. Therefore, they established Phase I–II trials where first efficacy outcomes were noticed in parallel to safety results.[231]

With new machine learning approaches large unstructured datasets can be analysed, which allows research of drug use overall. Choi *et al.*[232] recently proposed a framework to allow such post marketing analysis from data of Electronic Health Records (EHRs). This could be useful because a wide and diverse population, not limited by extensive exclusion criteria as usual in clinical trials, could be analysed for pharmacodynamic and pharmacokinetic effects of a post marketing drug. Using EHRs could provide the great opportunity to analyse treatment effects and to monitor adverse effects of a whole population. However, prior to (global) application important aspects regarding ethics and data protection have to be discussed intensively.[233,234]

3.4 'N-of-1' randomized controlled clinical trials

As a large portion of the high costs for Phase III clinical trials stem from the enormous number of patients, reducing this number would be an elegant way to reduce costs and encourage clinical research. Taking this reduction to the extreme, N-of-1 clinical trials are studies of only one participating patient (*Figure 2E*). In contrast to a descriptive case report, treatment in a N-of-1 trial consists of at least two phases. In random order, the patient is treated both with the drug candidate or a corresponding placebo (cross-over), and the order is revealed neither to the clinician nor the patient (double-blind).[235] The basis of N-of-1 trials, time-series research (meaning the introduction of experimental change into the periodic measurement of an outcome variable[236]) was mainly developed in behavioural research during the 1960s and 70 s.[237] Shortly thereafter, guidelines were established for the use of such N-of-1 time-series trials in the treatment of patients not being covered by guideline treatment, e.g., because of meeting exclusion criteria.[238,239] As important limitation, quick onset of the effect of the drug or intervention is essential, and this effect cannot to remain active after treatment is withdrawn, to allow reliable correlation of the patient's status to the current treatment.[238]

Beyond patients meeting exclusion criteria, N-of-1 trials may be useful in a number cases: as a proof of concept model or to generate hypotheses to justify larger clinical trials,[240] to fulfil the promise of personalized medicine by finding the optimal drug for an individual patient, e.g., suffering from chronic diseases,[241,242] or for very rare and severe diseases where years of preclinical analysis are not profitable and even multi-centre approaches cannot recruit sufficient patient numbers for traditional RDBPCTs.[243,244] In recent years, N-of-1 trials gained new momentum with the onset of antisense oligonucleotide (ASO) treatments,[131,245] especially in the field of spinal muscular atrophy, the leading genetic cause of infant mortality.[246] Formerly without any treatment option, in late 2016 the drug *Nusinersen*, an ASO altering the splicing of SMN2, was approved by the FDA.[247] Whereas development of *Nusinersen* took about 7 years for the first-in-man study and 11 years until market approval, ASO treatments have the potential for much more rapid development. Kim *et al.* reported on the N-of-1 study of *Milasen*, an ASO drug specifically designed for treatment of one particular patient suffering from the ultra-rare disease neuronal ceroid lipofuscinosis 7. Within the first year after contact with the patient, the group identified a novel mutation causing a splice defect, established patient-derived cell lines, identified a functional ASO, performed toxicology testing in rats, and concluded a N-of-1 clinical trial with the patient.[243] Especially in the field of rare and ultra-rare diseases caused by simple mutations, we expect more examples of such rapid development using N-of-1 studies within the upcoming years.

3.5 Trials during pandemics

Due to the current pandemic spreading of the virus SARS-CoV-2 and the high need of rapidly available vaccines, novel arrangements of clinical trials are necessary.[248] Research and development have to start from the very beginning with this novel virus. As seen with the first pandemic SARS virus, clinical trials of vaccine candidates only started when the pandemic situation was already concluded.[249,250] Over time, several initiatives set out to develop antiviral drugs until Phases I or II to make sure effective and safe drugs are already available 'on the shelf' before future pandemics can spread worldwide.[251]

Under acute pandemic settings the standard clinical trial setting of treatment versus placebo and long patient follow-up may not be suitable. Instead, 'challenge trials', where healthy individuals receive different vaccine candidates and then are voluntarily infected may be an alternative strategy.[252] Whether this can withstand ethical concerns is currently under intense debate.[253,254] The regulations of the WHO and EMA allow for 'conditional' emergency approvals during a pandemic situation to allow vaccination or treatment despite limited clinical data. Additionally, the final evolution before an approval could be shortened to less than a day in such circumstances Annex 10 of [255] (*Figure 2F*). At the time of writing, at least 251 vaccine candidates, ranging from inactive or attenuated viral vectors over protein subunits or virus-like particles to DNA or RNA vaccines, as well as 323 (repurposed) treatments, including antibodies, antiviral compounds, RNA candidates, and even cell-based therapies, are under development.[256] Currently, only a minority of these candidates already advanced into clinical studies, and it will be of interest how many different platforms and variants eventually will be investigated in clinical trials and reach the market.

Similar to the ASO treatment *Milasen* described above, RNA therapeutics held the potential to actually be the first drugs or vaccines available due to their rapid development: the mRNA vaccine candidate mRNA-1273 (Moderna[257]) took only 63 days from release of the genetic sequence of the novel SARS-CoV-2 to the application of the first dose within Phase I trial (NCT04283461) and 139 days until the application of the first dose within phase II trial (NCT04405076).[258,259] Similarly, the mRNA SARS-COV-2 RNA Vaccine Candidate BNT162b2 (marketed as Comirnaty, BioNtech/Pfizer) needed only 102 days to the application of the first dose and, aided by the seamless Phase I/II/III trial, quickly

advanced through clinical trials.[194,260] Indeed, this rapid progress allowed the mRNA vaccines to be the first with (conditional) market approvals in US and EU, and in light of this rapid development we believe more RNA-based vaccines and therapeutics for other diseases will follow.

In conclusion, these novel strategies in clinical trial design underlined in this review offer attractive opportunities to improve efficacy and success of such essential studies as well as to improve and streamline the clinical trial system as a whole.

4. Outlook

In the past decades, preclinical and clinical drug development have consolidated, but output reaching the market is stagnating. In this review, we present a number of alternatives which can expand todays gold standards by maximizing knowledge gained in a single experiment or study while minimizing the valley of death of translation. Of note, recent work on the discovery of new candidates expands the available chemical space, e.g., through non-natural products[261] or non-coding RNAs,[131] and aims to improve initial hit quality with ultra-large virtual screening[262] or artificial intelligence.[263] Combined, translational researchers of all stages now have the necessary tools to reflect their strategies, optimize their projects and streamline drug development as a whole.

We here highlight recent advantages of preclinical drug discovery, including novel *ex vivo* models of human heart tissue, as well as new clinical trial designs that will facilitate improved development of more efficient and safer drugs in the cardiovascular disease market.

Authors' contributions

F.P.K., A.M., and K.S. wrote the initial draft of the manuscript and designed the initial drafts of the figures, F.P.K. revised the figures. F.P.K., A.M., and T.T. revised the manuscript. All authors critically read the manuscript and approved the final version of the figures and the manuscript.

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Conflict of interest: T.T. and J.F filed several patents about ncRNAs and natural compounds in cardiac disease. T.T. is co-founder of Cardior Pharmaceuticals. F.P.K., A.M.K.S., and A.M. declare no competing interest.

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