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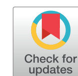
Neuromuscular and cardiac organoids and assembloids: Advanced platforms for drug testing



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ABSTRACT

The inherent technical difficulties, ethical/regulatory issues and costs of experimental studies in animal models is prompting investigators to replace as much as possible living organisms with *in vitro* physiological models named organoids and assembloids. Generated from induced pluripotent stem cells, these three-dimensional structures approximate the complexity of tissues and their interactions, enabling personalized disease modelling and drug testing. The integration of multiple components in assembloids further enhances their predictive value for multi-system interactions and toxicities. This review describes how neuromuscular organoids, incorporating functional neuromuscular junctions and contractile muscle tissue, have been used to replicate, *in vitro*, complex neuromuscular morpho-functional structures, offering very valuable platforms to study molecular mechanisms and drug effects in models of incurable diseases such as spinal muscular atrophy and amyotrophic lateral sclerosis. In the cardiological field, cardiac organoids and assembloids are proving reliable models for testing drug effects at molecular, morphological, electrophysiological and mechanical level. Recently, the integration of neuronal components into cardiac organoids has provided a potential approach to investigate autonomic function, a fundamental aspect of many neurological, neuromuscular and cardiac diseases. Challenges and limitations still remain, including the non-uniform differentiation protocols across studies, the incomplete maturation of cell phenotypes, and the lack of integrated pharmacokinetic modelling. We discussed some future developments aimed at overcoming such hurdles. Despite their current limitations, organoids and assembloids clearly hold great promises and will help advancing many fields of biomedicine.

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Abbreviations: AAV, adeno-associated virus; ACIT, acitretin; ALS, amyotrophic lateral sclerosis; ANP, atrial natriuretic peptide; ATR, atrial organoid; AVC, atrioventricular canal organoid; BMP, bone morphogenetic protein; BRD4, bromodomain-containing protein 4; CDK, Cyclin-dependent kinase; CRW, contractility-repolarization window; DM1, myotonic dystrophy type 1; ECM, extracellular matrix; EMD, electro-mechanical delay; EHT, engineered heart tissue; eIF2A, eukaryotic translation initiation factor 2 A; EGF, epidermal growth factor; FGF, fibroblast growth factor; FPD, field potential duration; GSK2, GSK2606414; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; IL, interleukin; iPSCs, induced pluripotent stem cells; LV, left ventricle; MEA, microelectrode array; NCCs, neural-crest cells; NMJs, neuromuscular junctions; PERK, protein kinase R (PKR)-like endoplasmic reticulum kinase; OFT, outflow tract; PolyGA, poly-glycine-arginine; PD03, PD 0332991; Pt, platinum; RV, right ventricle; SA-βgal, senescent-associated β galactosidase; SASP, senescent-associated secretory phenotype; SMA, spinal muscular atrophy; SMN, survival motor neuron; STAT1, signal transducer and activator of transcription 1; TGF-β, transforming growth factor beta; THALID, thalidomide; TNF, tumor necrosis factor; t-RET, trans-retinol; TXB, T-box transcription factor; vWF, von Willebrand factor..

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1. Introduction

Preclinical studies of drug development utilize cell cultures and animal models to predict safety, efficacy and mechanisms of action of pharmacological compounds or biological molecules. The ability of these models to faithfully replicate pharmacological effects in humans is crucial for minimizing safety risks, accelerating the discovery of effective drugs, and reducing late-stage failures. Each model has specific characteristics, with distinctive strengths and limitations. Cellular models commonly consist of two-dimensional cultures allowing a precise control of experimental conditions that can be fine-tuned to meet the researchers' needs. These systems are usually easy to analyse, as they are promptly accessible and relatively cheap: within the volume of a culture hood and an incubator, their ease of handling consents to a single operator to manage hundreds of replicates. However, two-dimensional models poorly replicate the complex physiology of organs and the interplay among different systems, limiting their clinical relevance. Animal models offer a far more complex environment that overcomes the limitations of traditional cell cultures. Many species are used in pharmacological studies, from small animals, mostly rodents, to large animals, mostly pigs and dogs. The biomedical community owes much of the extraordinary progress that has characterized medicine and surgery over the past six decades to the experimentation in animal models. They suffer from limitations, yet they are still indispensable. However, the inherent technical difficulties, ethical/regulatory issues and costs of animal studies is prompting investigators to replace as much as possible living organisms with *in vitro* physiological models. Together with "reduction" and "refinement", the word "replacement" defines indeed one of the "3R" that must be mandatorily pursued when investigators design studies in animal models (European Medicines Agency, 2016). Moreover, despite physiological similarities between certain species and humans, animal models often do not faithfully mimic pathological conditions, especially genetic diseases. A paradigmatic example, pertinent to the topic of the present review, is represented by murine models of type 1 myotonic dystrophy (DM1). In humans, the disease is caused by a triplet expansion in the *DMPK* gene. Since mice with expansions in the *Dmpk* gene do not exhibit pathological hallmarks of the disease, models have been developed by inducing nonsense mutations in *Dmpk*, *Six5*, *Mbnl1*, and *Dmwd*. However, each model reproduces only partially the characteristic phenotype (Lei & Finnell, 2020). Prompted by these limitations, a progressively larger number of investigators are developing *in vitro* models named organoids and assembloids. Organoids are three-dimensional, self-organizing *in vitro* culture systems that mimic certain features of *in vivo* complexity (Lei & Finnell, 2020), recapitulating select aspects of organ architecture and functions. They can be generated using induced pluripotent stem cells (iPSCs) derived from a variety of somatic cells (skin fibroblasts, blood-circulating hematopoietic cells, urinary exfoliated epithelial cells) harbouring the same genotype of the patient, thus functioning as personalized disease models (Soto-Gamez et al., 2024; Wang et al., 2022; Zhao et al., 2022) (Fig. 1). Investigators have been exploring the possibility to recreate the *in vitro* replica of almost every organ (Zhao et al., 2022); moreover, organoids of different types can be combined together or with additional cell types to form the so called "assembloids" (Paşca et al., 2022). Organoids and assembloids, which enhance complexity while maintaining the ease of handling and analysis typical of cell cultures, represent a promising platform for drug development. Due to their small size, these systems can be cultured on microplates, such as 96 or 384-well formats, enabling high-throughput screening and parallel experimentations. To overcome the limitations of animal models in DM1, Morelli and colleagues generated cortical organoid models from DM1 patient–derived iPSCs, replicating key pathogenetic mechanisms of the disease (Morelli et al., 2022). Recently, the complexity of cardiac organoids has been enhanced by incorporating neurons. Kostina and colleagues generated neural crest cells and cardiac organoids from iPSCs, demonstrating that parasympathetic neurons integrate with cardiac cells, forming functional neuro-cardiac junctions, and that the integration promoted the maturation of the cardiac conduction system (Kostina et al., 2024). By integrating the neuronal component, these models are well-suited for further studies on dysautonomia associated with neurological and neuromuscular disorders (*e.g.*, Parkinson's disease (Pfeiffer, 2020), muscular dystrophies (Smith et al., 2014)). Moreover, neuromuscular and cardiac organoids share critical developmental and functional characteristics, such as electrical excitability, contractility, and calcium signalling, rendering them particularly suited for assessing pharmacodynamics. Additionally, many

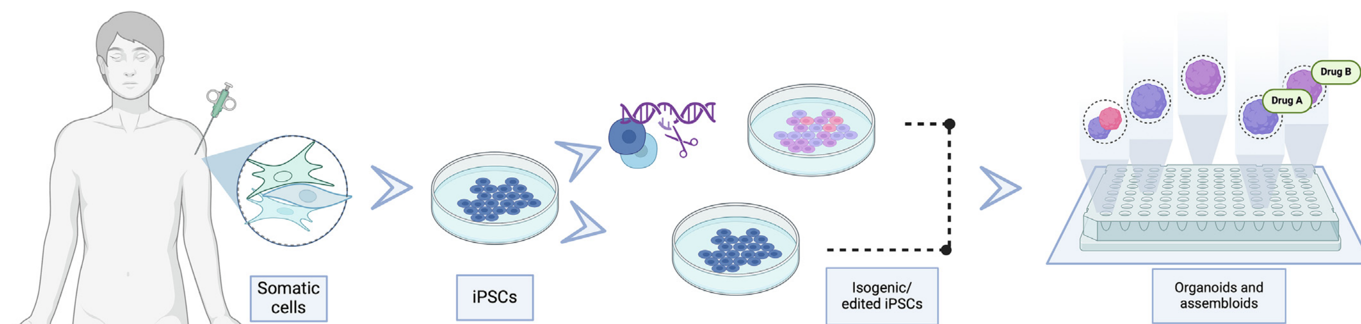

Fig. 1. Generation of organoids and assembloids. Somatic cells (*e.g.*, skin fibroblasts) from patients or healthy subjects are reprogrammed into iPSCs, which can be genetically edited to create isogenic controls, to introduce pathological mutations or investigate the role of genes. Organoids are then generated by sequential exposure to transforming factors, so replicating *in vivo* ontogenesis, and optionally using scaffolds and bioprinting techniques (not shown). Organoids of different types can be combined together to form assembloids. Considering at least three biological replicates (a standard in molecular biology), the 96-wells plate shown in figure allows testing up to 32 different conditions.
Acronyms. iPSCs: induced pluripotent stem cells.
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pharmacological and biological molecules exert their actions on different tissues, for instance cardiac and neuromuscular, underscoring the need for *in vitro* models that can predict multi-system effects and toxicities.

This review aims to describe the use of neuromuscular and cardiac organoids and assembloids as platforms for drug testing.

2. Generation and characterization of organoids and assembloids: basic principles

Unlike two-dimensional cultures, in which cells grow adhering side by side along the plate surface, cells arrange themselves in organoids forming 3D cultures. Organoids can be generated employing different methods, either based on extracellular matrix or scaffold-free approaches (Zhao et al., 2022). In scaffold-free approaches, after being suspended in medium as free cells, iPSCs are cultured in low-adhesion environments, such as ultra-low attachment plates. They subsequently form the so-called "embryoid body" which, as the name suggests, is reminiscent of the early stage of an embryo, potentially developing into any tissue type. Their generation involves the sequential, time dependent exposure to selected growth factors, in some cases analogous of those expressed during ontogenesis, in others synthesized *ad hoc*. Scaffolds provide mechanical and biochemical cues (Dutta et al., 2017) that influence stem cells differentiation (Engler et al., 2006), proliferation, and migration (Lo et al., 2000). Matrigel (Mavrommatis et al., 2023), an extracellular matrix (ECM) derived from mouse sarcoma tumours, or other scaffolds such as synthetic hydrogels (Kozlowski et al., 2021) and agarose microwell inserts (Scalise 2025) have been used to support various models, each tailored to mimic specific tissues or physiological environments. Scaffolds also allow the generation of organoids using differentiated cells and the development of larger-scale systems. Three-dimensional structures can also be formed from pre-differentiated cells (*e.g.* cardiomyocytes with endothelial cells and fibroblasts). When these cells are induced to aggregate and organize within a scaffold, the resulting models are referred to as "engineered heart tissue" (EHT); if the pre-differentiated cells are let free to organize themself on plates, the model is referred to as "cardiac microtissue" (Groen et al., 2024). Examples of engineered heart tissues are the models used by Mills and colleagues and Arhontoulis and colleagues to mimic the effects of inflammation on heart function, described in chapter 4. Over the years, organoids of increasing complexity have been generated and characterized and proved able to establish functional and biologically relevant interactions with other organoids or cells, forming what are referred to as 'assembloids'. Examples of assembloids are the complex system generated by Andersen and colleagues (Andersen et al., 2020), comprised of functional cortical, spinal, and muscle spheroids, and the heart assembloids with resident macrophages, as shown by O'Hern and colleagues (O'Hern et al., 2024). To be clinically relevant, these models must have a cellular composition, structure and function similar to their *bona fide* counterparts. Given their peculiar features, a plethora of methods can be applied to study organoids characteristics, to assess their relevance and the effects of exposure to exogenous molecules. From a morphological point of view, these systems can be analysed using conventional immunohistochemistry and immunofluorescence techniques, in slices or whole mounts (Zhao et al., 2022), as well as more advanced approaches, such as optical coherence tomography (Ming et al., 2022). These methods permit the analysis of cellular composition, tissue architecture and spatial distribution of specific markers, and even 3D imaging. The detailed composition of organoids can be further assessed by flow cytometry (Ostrop et al., 2021) and their deep molecular characterization can be achieved by single-cell RNA sequencing for high-resolution analysis of cellular diversity and gene expression profiles (Kanton et al., 2019). From a functional perspective, cell electrophysiology can be assessed in intact organoids by patch clamp, sometimes with the same type of instruments utilized for *in vivo* assessments (Landry et al., 2023), or by optical methods. These latter have been used to obtain live calcium imaging in whole organoids, either *via* transfection of an adeno-associated virus 1 (AAV1)-encoded ultrasensitive protein calcium sensor such as GCaMP6f (Samarasinghe et al., 2021) or by using exogenous Fluo4-AM solution (Invitrogen)(Lewis-Israeli, Volmert, et al., 2021). In both cases, engineered proteins or probes dynamically interacts with Ca^{2+} and emit a signal proportional to the ion concentration. Similarly, the potentiometric Di-4-ANEPPS dye has been successfully used for measuring voltage activity (Volmert et al., 2023). The force generated by cardiac organoids contraction has been measured with an optical method tracking the displacement of organoid poles (Voges et al., 2023), showing concordance with measurements obtained with classical force transducers (Mills et al., 2017). Yin and colleagues merged electrophysiological and mechanical measurements (Yin et al., 2025) to assess excitation-contraction coupling dynamics in real-time. In their system, cardiac organoids are placed between a silicone polymer diaphragm engineered on a nano-cracked platinum film and a microelectrode array. The nano-cracked platinum component acts as a resistive sensor changing its conductivity as a function of strain, while the microelectrode array registers field potentials. The authors could measure electro-mechanical window and electro-mechanical delay, two established physiological parameters (Constantino et al., 2013; Shingu et al., 2011) as well as new parameters based on contraction-repolarization window and excitation-contraction duration. The use of this system to evaluate the dose-response to an antiarrhythmic drug or the acute doxorubicin-induced cardiotoxicity will be discussed in section 4.1.2.2 of this review.

These various methodologies support the remarkable versatility and potential of organoids in preclinical research. Compared to two-dimensional cultures and animal models, organoids allow the same control of experimental conditions obtainable in classical cultures, while approximating more closely the complexity of animal models, with the additional advantage of being generated from human-derived cells. As demonstrated by various group (Giacomelli et al., 2020; Kostina et al., 2024), co-cultures of different histotypes favour cell maturation, and the interplay among different cells is fundamental for better modelling diseases. Consequently, these are currently the best-suited models to infer, *in vitro*, the effects of drugs in humans. Since organoids are relatively new in the preclinical scenario, their clinical significance still needs more ample evidence. Nonetheless, their biological complexity, relatively easy handling, and scalability point to high usefulness in preclinical studies. Table 1 compares the characteristics of two-dimensional models, organoids, assembloids, animal models and humans.

3. Neuromuscular assembloids

The generation of neuronal and muscle organoids, designed to model the pyramidal tract, allowed morphological and physiological studies that could not be previously performed *in vivo*, due to anatomical constraints. The control of movement, achieved by the coordinated contractions of various muscles, requires the integration of multiple elements. Voluntary movements are planned in the cortex and encoded as action potentials reaching effector muscles through the pyramidal tract. The first motoneurons project from cortex to the second motoneurons through the spinal cord. Second motoneurons extend from the spinal cord to the muscles and, at their ends, highly specialized structures named neuromuscular junctions (NMJs) transduce the signal and trigger muscle contraction. One of the distinctive features of the motor system, aside from its considerable complexity, is its challenging accessibility. *In vivo*, direct access to constitutive elements is hindered by their extensive anatomical distribution, spanning the length of the entire body, and by their difficult accessibility through various protective structures, including the cranial vault, the vertebral canal, and the muscle and fascial sheaths that envelop peripheral nerves. Furthermore, certain neuromuscular phenotypes are difficult to replicate in animal

Table 1
Key features of two-dimensional models, organoids and assembloids, animal models and humans.

	2D models	Organoids and assembloids	Animal models	Humans
Control of experimental condition	High	High	Medium	Low
Biological complexity	Low	Medium	High	Very high
Clinical relevance	Low	Potentially high	Variable	High

Organoids and assembloids increase biological complexity while maintaining the ease of handling and high control of experimental conditions typical of classical 2D models. The culture of different histotypes enhances cell maturation, which may improve physiological relevance (see in the text). Compared to animals, the ability to derive *in vitro* models from human cells carrying the same genotype of patients may help recapitulate specific disease mechanisms, especially for genetic diseases. While their clinical relevance is still being investigated due to their relative novelty, organoids and assembloids hold significant potential for capturing key aspects of *in vivo* physiology and disease processes.

models, as is the case of intermediate phenotypes for spinal muscle atrophy or nerve degeneration in mice with amyotrophic lateral sclerosis (Jucker, 2010). Such difficulties limit the usefulness of these animal models for drug testing. Therefore, the use of human-derived cells with the same genotype of patients, namely the iPSCs, capable of generating anatomically relevant structures, open perspectives for *in vitro* models, which can potentially propel drug discovery in neurology. To generate physiological models, iPSCs must undergo processes similar to ontogenesis. Mammalian brain develops from neuroectoderm (Götz & Huttner, 2005), a specialized region of the outermost germ layer in early embryonic development. With the onset of neurogenesis, these cells turn into radial glial cells and, eventually, into mature neurons. *In vitro*, strong inhibition of the SMAD pathway, obtained by a double inhibition of TGFβ and BMP signals with the small molecules SB431542 and dorsomorphin, is crucial to avoid cells commitment to non-neural fates, such as mesodermal and endodermal lineages. Mirroring what is observed *in utero*, the exposure of neuronal progenitors to FGF2 and EGF2 regulates precursors proliferation and neurogenesis, while sequential exposure to neurotrophins induces further differentiation and formation of functional circuits (Park & Poo, 2013). By replicating ontogenesis according to a robust protocol developed by Pașca and colleagues (Pașca et al., 2011), Andersen and colleagues (Andersen et al., 2020) generated functional cortical organoids that were later combined with spinal cord organoids. *In vivo*, the spinal cord consists of a ventral part formed by the descending motor pathways, and a dorsal part formed by the ascending sensory pathways. Several morphogens regulate the formation of such structures. In particular, BMP and Wnt signalling direct the cells towards a dorsal phenotype, while SHH determine ventral differentiation (Hor & Ng, 2020). Rostro-caudal differentiation relies on retinoic acid gradient and FGF. In the same work, using a combination of WNT activator, SHH modulator, FGF and other neurotrophic factors, Andersen and colleagues (Andersen et al., 2020) were able to generate spinal organoids that integrate with the cortical structure. It is worth noting that rhythmic patterns, such as those required for strolling, can be maintained by spinal central pattern generators (Marder & Bucher, 2001), without a continuous involvement of the cortex. Remarkably, Faustino Martins and colleagues (Faustino Martins et al., 2020), successfully generated human trunk neuromuscular organoids from neuromesodermal progenitors that contain central pattern generators-like circuits. Moreover, their model displays functional NMJs as well as contractile muscle tissue. These NMJs mimic *in vivo* responses: curare, an antagonist of nicotinic acetylcholine receptors, blocks muscle contraction in organoids as it does *in vivo*; moreover, when challenged with immunoglobulins purified from patients affected by myasthenia gravis, a disease caused by autoantibodies against NMJ-specific proteins such as the acetylcholine receptor, a reduction in the rate and amplitude of muscle contraction was observed. In addition to being generated concurrently with the neural component, muscle organoids can also be derived separately from iPSCs by the initial activation of the Wnt pathway combined with BMP inhibition, followed by exposure to FGF, HGF and IGF. The organoids can then be integrated with spinal and cortical components. This method was employed by Andersen and colleagues (Andersen et al., 2020), whose cortico-spinal-motor assembloid replicated the entire pyramidal pathway within a single well and consisted of neurons, astrocytes, oligodendrocytes that faithfully reproduced NMJs. Optogenetic stimulation of the cortical component determined muscle contraction, confirming the functional integration of these components. In the future, the integration of extrapyramidal components, such as basal ganglia (Miura et al., 2020) and cerebellum (Atamian et al., 2024), into neuromuscular assembloids will create more enriched platforms for modelling the various components that control movement. The ability to faithfully replicate human structures and functions, combined with the diversity of cell types in organoids and assembloids, holds promise for developing reliable preclinical models for drug testing.

3.1. Testing drugs in neuromuscular assembloids

3.1.1. Models of spinal muscular atrophy

Spinal muscular atrophy (SMA) is an autosomal recessive disease caused by loss-of-function mutations in the Survival Motor Neuron 1 (*SMN1*) gene. Humans are usually endowed with at least one copy of the paralog SMN2, but a cytosine-to-thymine variant on exon 7 in this second gene leads to its inefficient translation and reduced protein stability (Singh, Singh, & Androphy, 2006). Reduced expression of SMN leads to a progressive loss of lower motoneurons and muscle weakness (Mercuri et al., 2022; Soler-Botija et al., 2002). Four classical phenotypes, based on disease severity and age of onset, have been described, with SMA Type I being the most serious. While animal models have provided, and continue providing (Xie et al., 2024), relevant information on the disease and its potential therapeutical options, several key limitations exist. For example, only humans express *SMN2* and certain regulatory elements such as the antisense transcript SMN-AS1 (Signoria et al., 2023). Importantly, SMN-AS1 is considered as a therapeutic target for SMA (d'Ydewalle et al., 2017). Moreover, while patients display a spectrum of phenotypes, animal models develop either extremely severe or relatively mild forms of disease, making it difficult to study intermediate phenotypes of SMA.

Currently, three different disease-modifying drugs are available to treat SMA. While they have yielded striking results when administered in infants and children, much less evidence is available in adults (Fontanelli et al., 2024), hence reliable models to test therapeutic effects at different stages of disease progression are pressingly needed. When considering this aspect, neuromuscular assembloids are especially promising tools: despite the intrinsic difficulties to correlate *in vitro* and *in vivo* progression of the pathological processes, these models allow sequential evaluations of molecular, functional and morphological changes. This provides a dynamic platform to understand key disease mechanisms at different stages and facilitates the identification of stage-specific therapeutic options. Indeed, determining the time of the disease onset and exploring protective pathways that may restore motoneurons is crucial for the optimization and development of new therapies, either as self-sufficient agents or complementary to established treatments. Since the disease affects principally the second motoneuron, several studies have focused on models of ventral spinal cord organoids. Grass et al. (Grass et al., 2024) obtained three iPSC lines from patients with types I, II, and III SMA and generated corresponding isogenic controls by altering the variant on exon 7 in *SMN2* to increase SMN translation. They generated ventral spinal cord organoids from all the mutated iPSCs lines, isogenic controls and healthy wild type iPSCs lines. Interestingly, they found that, during development, compared to wild type, SMA ventral spinal cord organoids displayed lower expression *of DCX*, a marker of immature neurons, and of *ISL1* and *NKX6.1*, canonical markers of motoneurons. Moreover, the impaired

motoneuron development was restored in the respective isogenic ventral spinal cord organoids, suggesting impaired motoneuron development in SMA (Fig. 2a). Accordingly, therapeutic approaches aiming at restoring SMN expression during the embryonic period may represent a breakthrough in SMA treatment. To further increase the cellular diversity of ventral spinal cord organoids, approaching the complexity observed *in utero*, investigators induced neuromesodermal progenitors from iPSCs. During ontogenesis, neuromesodermal progenitors are located in the caudal extremity of the forming spine, they respond to WNT/FGF2 signalling and, within a specific time window, give rise both to neural and mesodermal precursors (Metzis et al., 2018). To investigate whether this process is disrupted in SMA, the organoid cellular composition was analysed at different time points, revealing an altered neuronal/mesodermal ratio in SMA ventral spinal cord organoids, similar to what is observed in SMNΔ7 mouse embryos, a classical animal model of disease. At molecular level, WNT/β-catenin pathway effectors were highly expressed in SMA ventral spinal cord organoids in a disease-severity manner, in line with observation in mice model (Wishart et al., 2014). Since WNT signalling directs motoneuron progenitors towards mesodermal fate (Gouti et al., 2017), this pathological mechanism may play a pivotal role during tissue maturation. Many drugs can target the WNT/β-catenin signalling (Jung & Park, 2020) and it is known that pharmacological inhibition of the β-catenin pathway with quercetin (Wishart et al., 2014) ameliorates the SMA phenotype in mice. Therefore, ventral spinal cord organoids represent a promising platform to model the disrupted WNT/β-catenin signalling

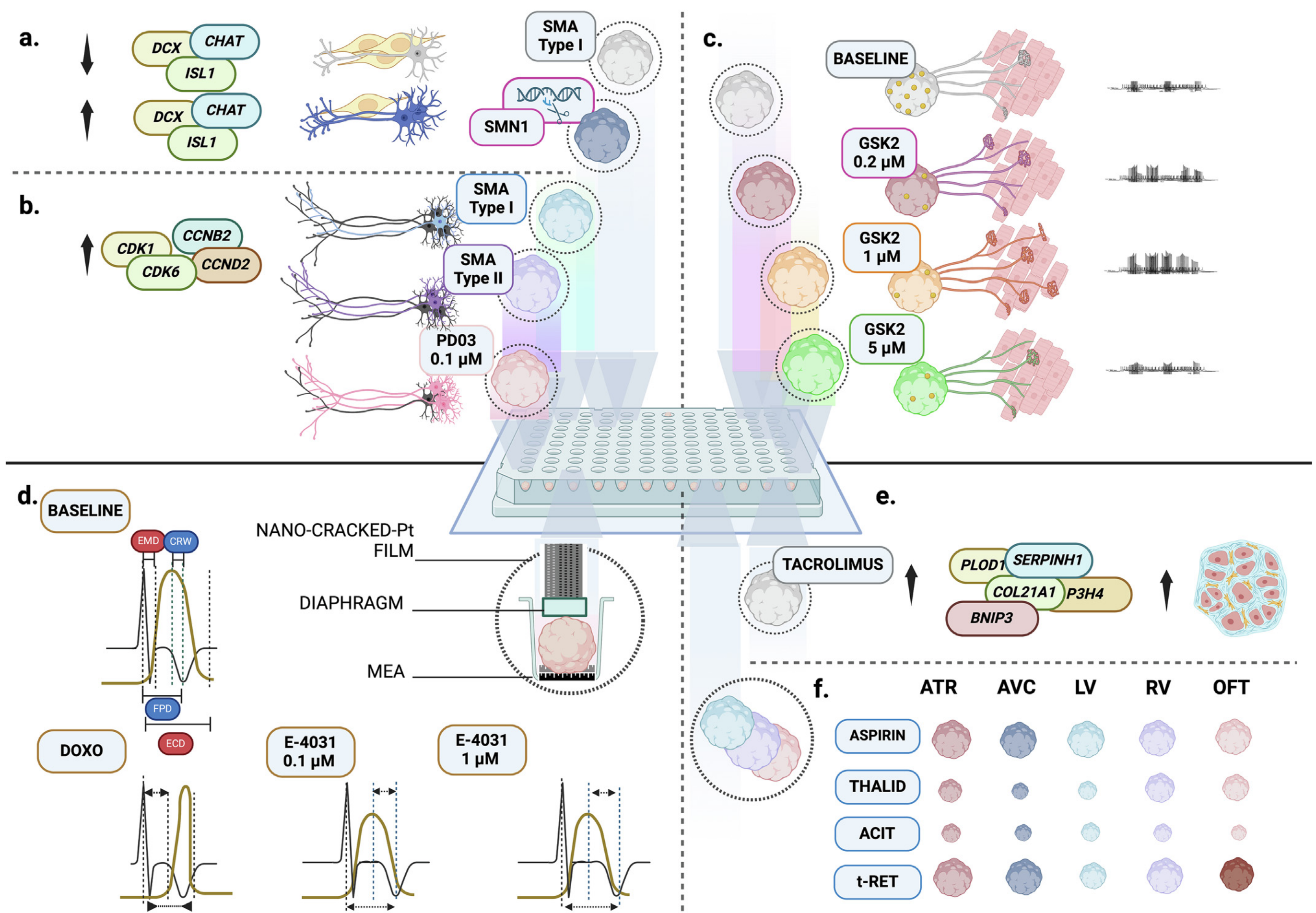

Fig. 2. Suitability of neuromuscular and cardiac organoids and assembloids for drug testing.
a. SMA patient-derived organoids were developed along with corrected isogenic models, in which a copy of *SMN2* was substituted with a version of *SMN1* gene (right), thus enhancing SMN production. Patient-derived organoids display altered neuronal differentiations and less mature motoneurons (centre) and disruption of key neuron-specific genes (left). Notably, the phenotype was ameliorated in the isogenic corrected version (Grass et al., 2024). **b.** Ventral spinal organoids from SMA type I and type II patients displayed neurodegeneration (Hor et al., 2018). PD02, a CDK4/6 inhibitor, partially reversed neurodegeneration and proved a potential candidate for further preclinical and clinical studies. **c.** Neuromuscular organoids from ALS patients (Gao et al., 2024). Patient-derived neuromuscular organoids showed characteristic polyGA inclusions (left, schematically represented as yellow dots), signs of denervation (centre) as well as less contractions (right, shown as black lines over time) compared to healthy controls. The PERK inhibitor GSK2606414 (GSK2) enhanced muscle contraction at low concentrations, whereas prolonged exposure to higher concentrations were toxic, reducing acetylcholine receptors. **d.** Silicone diaphragm engineered on a nano-cracked platinum film to transduce the force generated by beating cardiac organoids (Yin et al., 2025). A micro-electrode array recorded the electrical activity. Doxorubicin prolonged the electro-mechanical delay and shortened excitation-contraction duration, while E-4031, an antiarrhythmic drug, induced a concentration-dependent increase in field potential duration and contractility-repolarization window. **e.** Exposure of cardiac organoids to tacrolimus caused upregulation of genes associated with profibrotic responses (centre). Consistent with molecular changes, collagen production was significantly higher, suggesting a profibrotic effect of tacrolimus in heart tissue (Sallam et al., 2022). **f.** Cardiac assembloids employed to identify drug teratogenicity in different components of the heart (Schmidt et al., 2023). Thalidomide determined concentration-dependent dimension reductions, especially of the atrioventricular canal. Low concentrations of acitretin and isotretinoin (not shown) caused morphogenetic defects of all components, while trans-retinol determined morphological and gene expression alterations only in the outflow tract and upregulation of ventricular genes.
Acronyms. ACIT: acitretin; ATR: atrial organoid; AVC: atrioventricular canal organoid; CDK: cyclin-dependent kinase; CRW: contractility-repolarization window; ECD: excitation-contraction duration; EMD: electro-mechanical delay; FPD: field potential duration; GSK2: GSK2606414; LV: left ventricle organoid; MEA: microelectrode array; OFT: outflow tract organoid; Pt: platinum; RV: right ventricle; SMA: spinal muscular atrophy; SMN1: survival motoneuron 1; THALID: thalidomide; t-RET: trans-retinol; PD03: PD 0332991.
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observed in SMA, offering a relevant system to investigate disease mechanisms and evaluate potential therapeutic interventions. In another seminal study, Hor and colleagues (Hor et al., 2018) developed ventral spinal cord organoids using iPSCs from patients affected by Type I and Type II SMA and compared them to those obtained with wild type iPSCs. These organoids, while displaying a neurogenesis similar to wild type controls, underwent a progressive loss of motoneurons. Intriguingly, the degenerative phenotype was more severe in Type I SMA organoids compared to those derived from Type II patients. At molecular level, motoneurons displayed higher expressions of the cell cycle genes *CDK1*, *CDK2*, *CCNA2*, *CCNB1*, and *CCNB2*, confirming previous evidence obtained from 2D cultures (Ng et al., 2015). In an attempt to reverse the phenotype by targeting cyclin kinases, those investigators treated 4-week-old SMA organoids for seven days with one of the following: CDK1 Inhibitor, CDK2 Inhibitor, CDK4 Inhibitor, and PD 0332991, a CDK4/6 inhibitor. While CDK1 and CDK2 inhibition did not rescue SMA ventral spinal cord organoids, CDK4 and CDK4/6 inhibition led to improved motoneuron survival, as demonstrated by an increase of SMI-32 and ISL1 positive cells (Fig. 2b).

3.1.2. Models of amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease affecting both upper and lower motoneurons. The genesis of this neurodegeneration may lay anywhere along the motor pathway, as the first symptoms can appear either in upper or lower limbs as well as in the bulbar district, in the form of upper or lower motoneuron dysfunction. Wherever the disease begins, it relentlessly spreads throughout the body, leading to paralysis and muscle wasting, and, eventually, death. How this neurodegeneration, focal at least in the beginning, spreads throughout the motor nervous system, reaching also the regions involved in cognition and executive functions, is still unknown. Causative mechanisms of disease are poorly understood as well; the suspected culprits in ALS aetiology include glutamate excitotoxicity, protein misfolding and aggregation, mitochondrial dysfunction, endoplasmic reticulum stress, neuroinflammation (Thonhoff et al., 2018), and impaired autophagy. Importantly, the role of non-neuronal cell (*e.g.*, astrocytes, oligodendrocytes, vascular cells) in the genesis and spreading of the disease is not completely known yet. The most common genetic mutation, found both in sporadic and familial ALS, is the non-coding G_4C_2 repeat expansion in an intronic region of chromosome 9 open reading frame 72 *(C9ORF72)*. In affected individuals, this portion is unconventionally translated in polyglycine-arginine (PolyGA) (Zhang et al., 2016) peptides that, once precipitated in the cytoplasm, form characteristic inclusions. Transgenic mice models of the disease, albeit carrying the pathological G_4C_2 repeat expansion, do not show classical signs of neurodegeneration nor survival impairment and motor deficits (Mordes et al., 2020), thus they are unsuitable models to study the disease. To date, drugs tested in mouse genetic models have not successfully translated into effective human therapies (Morrice et al., 2018), highlighting a critical need for novel paradigms that can both identify mechanisms of damage and faithfully replicate drug effects in patients.

Pereira and colleagues (Pereira et al., 2021) generated sensorimotor organoids containing many different cell types, *i.e.*, motor and sensory neurons, astrocytes, and mesodermal derivatives such as vascular cells, microglia, and skeletal muscle cells. Similar to previous findings, motoneurons made connections with muscle tissue *via* functional NMJs, which were blocked by curare and, as occurs clinically, by botulinum toxin after a longer delay. Those investigators generated organoids using iPSCs from patients affected by sporadic ALS and two familial ALS lines harbouring mutations in *C9ORF72* and *FUS*. To reduce variability among different cell lines, they also generated organoids from three isogenic iPSCs lines edited to harbour mutations in *TARDBP*, *SOD1*, and *PFN1,* three genes associated with familial ALS. Consistent with the disease phenotype, organoids derived from ALS iPSC lines exhibited fewer contractions and structural deficits in NMJs.

Compared to monocultures, the interactions among different histotypes in organoids, by enhancing cellular crosstalk and microenvironment, may activate physiological and pathological mechanisms. To study the role of inflammation in ALS pathogenesis, Guo and colleagues (Guo et al., 2024) generated *C9O*RF*72* knockdown iPSCs and derived from them ALS spinal cord organoids as well as cultures of neurons and astrocytes. Consistent with previous findings indicating an autoinflammatory state in ALS patients (McCauley & Baloh, 2019), *IL-6*, *IL-1*β and *TNF*α and *TGF-*β expression were significantly higher in ALS spinal cord organoids compared to healthy controls. Notably, these differences were even more pronounced when using the organoid platform compared to astrocyte or motoneuron monocultures, evidencing that the complex interplay among cells plays a pivotal role in *C9ORF72* ALS neuroinflammation. Further exploring *C9ORF72* ALS pathology, Gao and colleagues (Gao et al., 2024) generated neuromuscular organoids from patients. Their system replicated classical neuronal and astrocytic polyGA inclusions found in *C9ORF72* ALS as well as evidence of NMJs denervation, a pathological hallmark observable *in vivo* in cases of second motoneuron degeneration. The ability to look closely and longitudinally into the denervation process, allowed by this model, offers major advantages when studying the core element of the pathology and the effects of therapeutic approaches. In their study, Gao and colleagues (Gao et al., 2024) tested the effect of GSK2606414, a selective inhibitor of protein kinase RNA-like endoplasmic reticulum kinase functioning as a repressor of translational inhibition caused by unfolded protein response. Since unfolded protein response is thought to be involved in ALS (Montibeller et al., 2020), GSK2606414, by reducing endoplasmic reticulum stress, may hold potential as a disease-modifying agent. The molecule was already tested on ALS brain organoids (Szebényi et al., 2021) where it reduced the ratio of total p-eIF2α/eIF2α, polyGA levels and DNA damage in neurons. The effects of different GSK2606414 concentrations (0.2, 1, and 5 μM) over time was then evaluated in neuromuscular organoids. Either short (18 h) and prolonged (5 days) treatments resulted in a significant reduction of polyGA inclusions and astrocytic autophagy, while only higher concentrations were effective in reducing neuronal autophagy. The lower concentration increased muscle contraction, while the prolonged exposure to the higher concentration reduced the acetylcholine receptors area of neuromuscular organoids, suggesting a potential dose-dependent toxic effect (Fig. 2c).

Taken together, experimental evidence has so far supported the versatility of organoid models in replicating ALS physiology, response to treatments and potential toxic effects.

4. Cardiac organoids and assembloids

Bioengineered systems developed with cardiomyocytes have proved valuable for modelling specific features of cardiac physiology and pathophysiology. Tu and colleagues (Tu et al., 2024) engineered a three-dimensional heart tissue generated from iPSCs-derived cardiomyocytes that provided important insights in the metabolic alterations characterizing tachycardia-induced cardiomyopathy, consistent with *in vivo* findings. For other studies, *in vitro* constructs of higher complexity might be necessary to approximate more closely cardiac tissues architecture. Cardiomyocytes constitute most of the cardiac mass and display distinct characteristics depending on their anatomical location, with differences between atrial and ventricular ones. The inner surface of cardiac chambers is lined by the endocardium, a thin layer of endothelial cells and connective tissue. Endothelial cells also line the internal surface of blood vessels. Additional cell types in the heart include fibroblasts, nerve cells, adipocytes, macrophages, and others belonging to the myeloid and lymphoid lineages (Litviňuková et al., 2020). Mammalian heart cell progenitors derive from two distinct regions of the mesoderm. During the first weeks of embryonic development, a subset of mesodermal cells migrates to form the cardiogenic cords, which eventually canalize, fuse together, and form the primitive heart tube. Since this region

of mesoderm was long considered as the only one giving rise to cardiac structures, it is referred to as the 'first heart field'. Further evidence, however, showed that additional cells originating from the pharyngeal mesoderm migrate and integrate into the primitive heart tube. This mesodermal region is known as the 'second heart field' and can be further divided into an anterior and a posterior component. Cells from the first heart field give rise to part of the left ventricle, the anterior second heart field gives rise to outflow tract region, while precursors of atrial and right ventricular myocardium derive from the posterior second heart field (Kelly, 2012). The primitive heart tube subsequently develops into the heart. The morphogenic process that leads to heart formation relies on a temporally coordinated molecular modulation, with a central role played by WNT and BMP signalling (Li et al., 2022; van Wijk et al., 2007). Giacomelli and colleagues, by modulating the WNT and BMP pathways to mimic the *in vivo* processes, established a protocol for simultaneous 2D differentiation of cardiomyocytes and endothelial cells from iPSCs through mesoderm induction (Giacomelli et al., 2017). In their seminal work, they further demonstrated that the presence of cardiac fibroblasts and endothelial cells enhance cardiomyocytes maturation in cardiac microtissues (Giacomelli et al., 2020). Hofbauer and colleagues (Hofbauer et al., 2021) demonstrated that adding specific molecules of extracellular matrix (*i.e.*, laminins 521/511) to two-dimensional iPSCs cultures before mesodermal induction resulted in self-assembling, hollow, 3D beating structures. These models, named "cardioids" were further enriched with epicardial cells that integrated within cardiomyocytes. When cryoinjured, they produced fibronectin and collagen, consistent with the injury response observed *in vivo* (Moore-Morris et al., 2014). More recently, Lewis-Israeli and colleagues (Lewis-Israeli et al., 2021) generated self-assembling human heart organoids from human iPSCs that replicate human foetal cardiac tissue. Their model consists of an organoid formed by cardiomyocytes with an atrioventricular phenotype, and also by fibroblasts, epicardial and endocardial cells, and vascular structures. The same group integrated autologous cardiac tissue-resident macrophages into cardiac organoids to generate human heart-macrophage assembloids, demonstrating the formation of gap junctions, hence synchronized calcium transients and action potentials between these two cell types (O'Hern et al., 2024). Prompted by the evidence that a pro-inflammatory state contributes to the onset and progression of atrial fibrillation (Ajoolabady et al., 2022), O'Hern and colleagues (O'Hern et al., 2024) used their assembloids as the first *in vitro* model to study the effects of inflammation in arrhythmogenesis (Korantzopoulos et al., 2018). They exposed human heart-macrophage assembloids to pro-inflammatory triggers like lipopolysaccharides, interferon-gamma, and IL-1β, and demonstrated that inflammation increased the rate of electrophysiological events characterizing, *in vivo*, atrial fibrillation, such as early afterdepolarizations, delayed afterdepolarizations and spontaneous calcium fluctuations. Notably, these abnormalities were more common in cardiomyocytes expressing atrial rather than the ventricular phenotype, in line with the more pronounced impact of inflammation on those cells *in vivo* (Dobrev et al., 2023). In parallel, they successfully integrated neural crest cells in cardiac organoids (Kostina et al., 2024) and demonstrated that neural crest cells migrate towards the sinoatrial region of cardiac organoids, then differentiating into parasympathetic neurons and cardiac glia. Parasympathetic neurons functionally integrate with pacemaker cells and improve cardiomyocyte maturation. Cells of the cardiac glia showed increased expression of genes linked with outflow tract formation. Indeed, neural crest cells have been shown to migrate to the outflow tract in mouse embryo, where they contribute to the development of the aorticopulmonary septum and of semilunar valvular leaflets (Chen et al., 2021). The use of this model to test teratogenic effects of drugs is discussed in section 4.1.2.4.

To further increase the complexity, hence the relevance of these models, Schmidt and collaborators (Schmidt et al., 2023) replicated the morphogenesis observed *in utero* and generated three different cardioid progenitors similar to first heart field, anterior second heart field, and posterior second heart field. When combined, these cardioids became electrochemically connected and started contracting in a coordinated manner, eventually forming cardiac assembloids with various compartments replicating atrium, left ventricle, right ventricle, outflow tract, and atrioventricular canal (Schmidt et al., 2023). The use of this multi-chambered organoid to test drug effects and teratogenicity will be discussed in the next chapter.

Scaffolding techniques have allowed developing organoids with increased dimensions, similar contractile properties of the human heart, where physiological data can be acquired with the same technology utilized *in vivo*. Voges and colleagues (Voges et al., 2023) differentiated endothelial cells and cardiomyocytes/fibroblasts separately from iPSCs, mixed them at a ratio of 20/80, similar to the cellular composition of the foetal human heart (Sim et al., 2021), and used the silicone polymer PDMS to fabricate vascularized cardiac organoids. They demonstrated that the addition of vascular cells improves organoid contractile force up to 30–60 mN/mm^2, similar to human cardiac muscle (Lunkenheimer et al., 2004). Kupfer and colleagues (Kupfer et al., 2020) firstly developed a bioink in which stem cells can proliferate and differentiate into cardiomyocytes and then, to replicate cardiac anatomy, bioprinted a millimetric structure with 2 chambers, a vessel inlet and outlet. By inserting a pressure transducer combined with a conductance catheter into one of the organoid chambers, they were able to obtain pressure-volume loops and ejection fraction during spontaneous activity or after pharmacological challenges. The ability to measure, *in vitro*, physiological parameters that, *in vivo*, would require invasive approaches based on cardiac catheterization, opens the possibility to generate high throughput data, optimizing time and costs.

4.1. Modelling the effect of drugs on cardiac assembloids

4.1.1. Effects of pharmacological agents

4.1.1.1. Models to test cardioactive therapies. Currently available organoid models are already well-suited to assess the effects of cardioactive pharmacological therapies. Kupfer and colleagues used their system to test the *in vitro* effects of isoproterenol, a nonselective β-adrenoreceptor agonist, and of the calcium blocker verapamil. As expected, isoproterenol exerted its positive chronotropic effect in a dose-dependent manner, while verapamil decreased calcium currents amplitude (Kupfer et al., 2020). Schmidt and colleagues (Schmidt et al., 2023) tested the effects of ivabradine, isoprenaline, and Bay K 8644 on Ca^{2+} signal propagation in different components of their multi chambered model as well as in the whole assembloid. Isoprenaline, a beta-adrenergic agonist, increased the speed of calcium signal propagation in whole assembloids, while ivabradine, a selective blocker of channels responsible for the pacemaker current (DiFrancesco & Camm, 2004), reduced calcium signal in atrial cardioids, thus lowering the assembloids beating rate, in line with the negative chronotropic effect of this pharmacological agent. Conversely, Bay K 8644, a L-type calcium channel agonist, stimulated both atrial and atrioventricular beating. Therefore, this setting allowed to easily test the effect of drugs on specific component of the assembloids.

Yin and colleagues (Yin et al., 2025) evaluated the effects of increasing concentrations of E−4031, a class III antiarrhythmic agent that selectively blocks delayed rectifier potassium channels, on the electromechanical properties of organoids. As expected, treatment with E-4031 determined a concentration-dependent reduction in organoid beating rates as well as an increase in field potential duration, which is the *in vitro* counterpart of QT interval prolongation. Moreover, rising E−4031 concentrations caused a decline in mechanical beating amplitude, probably due to the prolongation of action potential duration caused by the inhibition of the delayed rectifier potassium channel, and an increase in contractility-repolarization window caused by the delayed repolarization wave (Fig. 2d). The contractility-repolarization window represents the delay between maximal contraction force and

ventricular repolarization wave peak, a physiologically vulnerable period with higher arrhythmogenic susceptibility. Overall, these findings highlight the capacity, given by the possibility to simultaneously monitor electrical and mechanical parameters in a 3D model, to identify physiologically relevant drug effects in a preclinical setting, offering valuable insights into arrhythmogenic risks and cardiotoxicity.

4.1.1.2. Models to test anti-inflammatory drugs in SARS-CoV-2 infection. Engineered heart tissue (EHT) has been employed to study the impact of anti-inflammatory drugs in SARS-CoV-2 infection. These platforms effectively model some aspects of the inflammatory cardiac damage associated with the disease and have led to the identification of candidate molecules that subsequently proved effective in animal studies (Arhontoulis et al., 2022; Mills et al., 2021). When exposed to elevated cytokine levels, as occurs in severe COVID-19, the EHT developed by Mills and colleagues exhibit diastolic dysfunction characterized by prolonged contraction-relaxation times (Mills et al., 2021), mirroring the cardiac dysfunction observed in patients (Szekely et al., 2020). Additionally, treatment with IL-1β impaired EHT contractility, stimulated fibrosis and altered transcriptional profiles, including α-sarcomeric actinin downregulation, akin to findings in heart tissues from COVID-19 patients (Arhontoulis et al., 2022). At molecular level, cytokines-exposed EHT showed hyperphosphorylation of the site S727 in the signal transducer and activator of transcription 1 (STAT1), and of two sites in the bromodomain-containing protein 4 (BRD4). Notably, these molecular sites are targetable with small molecules. To exploit the effect in modulating STAT pathway, two groups of investigators screened the effects of the JAK/STAT inhibitors baricitinib and ruxolitinib and of the CDK8 inhibitors SEL120-34A and BI-134, since CDK8 is the putative STAT1-S727 kinase (Mills et al., Yin et al., 2025). They further tested the effects of flavopiridol, a broader CDK inhibitor, and of the bromodomain extraterminal inhibitors INCB054329 JQ-1 and ABBV-744. None of the agents acting on the STAT pathway rescued cytokine-induced diastolic disfunction, whereas flavopiridol caused moderate cardiotoxicity. Conversely, JQ-1 and especially INCB054329 showed positive concentration-dependent effects. To validate BRD4 as a target, Mills and colleagues (Mills et al., 2021) confirmed that BRD4 knockdown, obtained by adeno-associated virus 6 (AAV6)-mediated delivery of short interfering RNA, prevents cytokine-induced diastolic dysfunction. Given the robust *in vitro* evidence of positive effects of NCB054329, those authors tested this agent in a mouse model, demonstrating beneficial effects on cardiac function and even on animal survival. Further tests in EHT, of immunomodulatory agents utilized to treat the SarsCov2 infection were performed by Arhontoulis and colleagues (Arhontoulis et al., 2022). They screened different molecules, already used during the 2020 pandemic, such as an IL-1 receptor antagonist with mechanisms of action similar to anakinra, the IL-6 receptor inhibitor tocilizumab, and dexamethasone. The potentially repurposable drug baricitinib was tested too. They found that only dexamethasone ameliorated contractility in IL-1beta treated EHT, but it also increased the vWF/CD31 ratio, suggesting a possible prothrombotic effect, consistent with findings in clinical studies (Brotman et al., 2006).

4.1.2. Models to test cardiotoxicity and teratogenicity

Cardiotoxicity is the second leading cause of drug withdrawal from the market for safety reasons, accounting for approximately 20 % of cases (Craveiro et al., 2019). It is therefore critical to identify potential cardiotoxic effects early along the drug development pipeline. Moreover, certain drugs have exerted teratogenic effects exclusively in the human species. Therefore, reliable preclinical models capable of predicting cardiotoxicity and teratogenicity would be highly desirable to support first administrations in humans.

4.1.2.1. QT prolongation. Many drugs interfere with ventricular repolarization, leading to prolonged QT interval and, potentially, to serious arrhythmias like the *torsade de pointes*. According to current European guidelines, preclinical assessments of delayed ventricular repolarization risk rely on *in vitro* assays to the drug impact on rectifier potassium current (I_{Kr}) channels, and on animal models. However, those channels are species-specific and are variably expressed across different cardiac regions (European Medicines Agency, 2005).Models as similar as possible to the human heart are therefore needed for trustworthy evaluations. Of note, the annex of the European guidelines published in 2020 considers organoids as suitable platforms to conduct such analyses (European Medicines Agency, 2022). Schmidt and colleagues (Schmidt et al., 2023) evaluated the effect of 4-aminopiridyne (4AP) on their multichambered organoids. 4AP is a voltage-gated potassium channel (K_v) blocker which exerts its effect especially on delayed rectifier K_v and is used as a symptomatic reliever in multiple sclerosis. They observed a prolongation of ST and RT intervals, indicating elongation of field potentials consistent with what it is expected *in vivo*.

4.1.2.2. Doxorubicin cardiotoxicity. Doxorubicin is a potent chemotherapeutic agent used against a wide range of cancer types, but its cardiotoxic effects limits the administrable dose. Acute effects are seen in about 11 % of patients and include arrhythmias and other electrocardiographic alterations. While several pathways have been proposed to explain doxorubicin cardiotoxicity, the development of effective cardioprotective strategies remains a challenge, as the precise molecular mechanisms underlying its detrimental effects in cardiac tissue are not fully understood (Avagimyan et al., 2024). For these reasons, further exploring the pathogenic factors that drive doxorubicin cardiotoxicity would help designing complementary therapies to mitigate deleterious effects and potentially permit higher doses. The electro-mechanical changes identifiable with the model developed by Yin and colleagues (Yin et al., 2025) allowed obtaining mechanistic insights in acute cardiotoxicity. Acute treatment with doxorubicin increased organoids beating rate and mechanical contraction kinetics, resulting in shorter rise and decay times, as well as in a reduction of the electro-mechanical window, a prolongation of the electro-mechanical delay and a shortening of excitation-contraction duration (Fig. 2d). These findings suggested arrhythmogenic alterations induced by acute exposure to doxorubicin and documented, for the first time, a delay between repolarization and contraction onset. The generated force was not affected by doxorubicin, therefore the contractile apparatus was not damaged by acute exposure. Consequently, further studies should seek the potential reversibility of functional effects through targeted interventions restoring the electro-mechanical coupling.

4.1.2.3. Models to test cardiac effects of immunosuppressive drugs. Immunosuppressive drugs used in heart transplantation have been associated with cardiac graft remodelling and evidence that an increased left ventricular mass leads to adverse outcomes (Sallam et al., 2022), but the molecular mechanisms responsible for such alterations are poorly understood. Detailed molecular characterizations of the effects of tacrolimus and sirolimus – two immunosuppressant drugs utilized in post-transplant management – have been obtained by Sallam and colleagues (Sallam et al., 2022) who designed two parallel studies in patients and in cardiac organoids. Firstly, they longitudinally evaluated transplanted patients using serial echocardiographic assessments for two and a half years and found that the ones treated with sirolimus displayed a statistically significant reduction in left ventricular mass compared to those treated with tacrolimus. To obtain mechanistic insights at molecular level, they generated cardiac organoids from iPSCs and treated them with tacrolimus, sirolimus or vehicle. By performing single-cell RNA sequencing, the authors found that, compared with those treated with sirolimus, fibroblasts from organoids treated with tacrolimus developed a pro-fibrotic phenotype, with higher expression of genes associated with collagen and extracellular matrix production, and of genes encoding for cell adhesion proteins, such as *SERPINH1*, *P4HB*, *PLOD1*, *COL21A1*, and *P3H4*. Similarly, cardiomyocytes and fibroblasts from the tacrolimus

group displayed a significant upregulation of *BNIP3*, a gene associated with profibrotic responses. Consistent with their molecular phenotype, cardiac organoids treated with tacrolimus produced more collagen compared to the other groups, while fibroblasts in the sirolimus group proliferated at a significantly reduced rate (Fig. 2e). These findings suggest that cardiac remodelling observed especially after tacrolimus treatment may be causally related to a drug induced profibrotic environment. Therefore, those authors are paving the way to the development of strategies aimed at limiting the progression of fibrosis and its detrimental impact on cardiac function. It should also be emphasized the capacity of organoids to replicate complex phenomena in a well. Cardiac biopsies could have been used for the same purpose, but such study would have implied invasive procedures, not devoid of potential complications. Furthermore, a cardiac biopsy collects only a small portion of the cardiac wall, potentially failing to capture the full extent of pathological changes across the organ. Instead, organoids allow studying drug effects across different histotypes, providing a comprehensive overview of the treatment response.

4.1.2.4. Models to test cardiac teratogenicity. The multi-chamber assembloid platform developed by Schmidt and colleagues has been used also to test drug teratogenicity (Schmidt et al., 2023). Firstly, investigators exposed these assembloids to aspirin, a known non-teratogenic molecule, observing that the drug did not cause morphological and transcriptomics changes. The effects of thalidomide, a well-known teratogenic drug, were then tested. It is known that the teratogenic effect of thalidomide, supposedly mediated by a negative interference with the T-box transcription factor (TXB5) function, does not occur in rodents nor other animal models, further reinforcing the importance, in this case, of human-derived *in vitro* models. Exposure to thalidomide in a concentration-dependent fashion determined smaller dimensions of the various assembloid components, especially of the atrioventricular canal compartment, as well as the downregulation of the TBX5 target natriuretic peptide A and the dysregulation of *NR2F2*, *IRX1*, and *IRX4*, three major factors involved in cardiac development. Acitretin and isotretinoin, two other known teratogenic drugs, caused defects in specification, patterning, and morphogenesis of all assembloid components even at low concentrations, while exposure to trans-retinol determined a marked morphological alteration only in the outflow tract, thus supporting the reliability of this model for studies on localized and selective effects of drugs on cardiac development (Fig. 2f).

The neuro-cardiac assembloids developed by Kostina and colleagues (Kostina et al., 2024) have been used to investigate the teratogenic effect of antidepressants drugs such as paroxetine, venlafaxine, sertraline, citalopram, fluoxetine, escitalopram and fluvoxamine. These investigators exposed the neuronal crest cells to different drug concentrations mimicking maternal plasma levels and found in neural crest cells a dose-dependent downregulation of *PHOX2B*, a gene selectively expressed in neurons and involved in the differentiation of autonomic neurons. They also found a downregulation of the neuronal markers *PHOX2B*, *NEFM* and *PRPH*, the parasympathetic marker *SLC18A3* and the glial markers *S100B* and *GAP43* in neuro-cardiac assembloids. Moreover, neuro-cardiac assembloids exposed to paroxetine and sertraline showed increased beating rate and less developed neurites, suggesting a potential teratogenic effect of these drugs *in vitro*.

4.1.3. Models of cardiac aging

Scalise and colleagues (Scalise 2025) demonstrated that cardiac organoids treated with doxorubicin express hallmarks of aging, both at gene and protein expression level. They displayed collagen accumulation and upregulation of *COL3A1*, *COL1A2*, *VIM*, *ANP* and *BNP* genes, recapitulating fibrosis and cardiac remodelling observed in aging hearts. Moreover, those organoids expressed higher levels of the cyclin dependent kinase inhibitors $p16^{INK4A}$, p15, p19, p21 and of the senescence-associated beta-galactosidase, with an enhanced secretion of monocyte chemoattractant protein-1, interleukin 6 and 8, typical of the senescence-associated secretory phenotype. Treatment with the senolytic combination of dasatinib and quercetin reduced fibrosis and the expression of senescence-related cyclin dependent kinase, beta-galactosidase and secretory phenotype, while restoring atrial and brain natriuretic peptides expression. Moreover, dasatinib + quercetin treatment restored beating efficiency and frequency, exerting positive effects on morphological, molecular and functional parameters.

5. Limitations

Several limitations still pose significant challenges to the development, refinement and evolution of organoid and assembloid models. Some of them relate to the methods utilized to generate them. In fact, one of the major current issues is the lack of standardized protocols. On one hand, the flexibility in designing organoids and assembloids allows researchers to tailor them and model specific anatomical and physiological features of interest, on the other hand it can also hinder reproducibility and comparability of results across studies. Another key aspect is the possible inconsistency of differentiation processes of the various cell lines, which further complicates standardization. It is therefore essential to properly characterize the model for every cell line employed in it. Of note, achieving an adequate cell maturation remains an obstacle, as differentiated cells frequently exhibit immature, foetal-like characteristics. This immaturity can result in altered receptor expression and functional properties of organoids, causing responses to pharmacological agents that differ from those of the mature counterparts. Another important limitation derives from the very nature of the iPSCs generation process. During reprogramming, the epigenetic signature is erased. Importantly, epigenetic modifications are thought to play a pivotal role in both neuromuscular (Coppedè, 2020) and cardiac (Prasher et al., 2019) diseases. One approach to avoid epigenetic resetting consists of directly differentiating somatic cells into the desired histotypes. Through direct lineage reprogramming, fibroblasts have been successfully converted into neuron-like cells (Yang et al., 2019) and cardiomyocytes (Yang, 2011). Assembloids generated from such cells could retain the patients' epigenetic signatures, leading to more faithful and relevant phenotypes, particularly for diseases in which aging plays a pivotal role.

Other limitations arise from the complexity of pharmacological *in vivo* effects compared to the relative simplicity of these systems. In fact, pharmacokinetic properties such as absorption, distribution, metabolism and excretion are very partially replicated in these platforms. Current approaches rely on the addition of pharmacological agents to the organoid culture medium. Besides the obvious corollary that, in order to obtain meaningful results, the concentrations must be compatible with those of the active drug (*i.e.* not bound to serum proteins) achievable in tissues, it must be noted that the vehicles necessary to dissolve the tested drugs may be toxic or exert themselves other effects. It is therefore essential to test the effects of the vehicle alone in the system. The problem of drug distribution is particularly relevant for neuromuscular applications, since the central nervous system is protected by the blood-brain barrier, a highly selective screen between capillaries and neurons (Ballabh et al., 2004) primarily formed by endothelial cells and selectively permeable to certain molecules. The blood-brain barrier controls molecules directed to the central nervous system and, by selectively extruding therapeutic agents through drug efflux pumps, limits their bioavailability. Blood-brain barrier organoids have been developed and successfully replicated the selective permeability to drugs observed *in vivo* (Bergmann et al., 2018; Cho et al., 2017). In the future, integrating the blood-brain barrier into neuromuscular assembloids will further increase their physiological relevance and reliability for drug testing. Another limitation of simplified *in vitro* systems is the absence of liver-like drug metabolizing systems that prevents the study of a full range of effects exerted not only by pharmacological agents, but also by their metabolites. The interplay of functionally

Table 2
Cardiac and neuromuscular organoids and assembloids used for drug testing.

Reference	Organoid type	Progenitor	Drugs tested	Outcomes	Relevance
Neuromuscular organoids and assembloids					
Grass et al., 2024	Ventral spinal cord organoids	iPSCs from patients affected by SMA type I, II, and III, and isogenic controls	–	More immature neurons in SMA organoids; motoneuron development was restored in isogenic controls;a ltered WNT/β-catenin pathway in SMA organoids.	Insights in the WNT/beta catenin pathway during tissue maturation and neurogenesis in SMA.
Hor et al., 2018	Ventral spinal cord organoids	iPSCs from patients affected by SMA type I and II, and from normal iPSCs	CDK1 inhibitor CDK2 inhibitor CDK4 inhibitor CDK4/6 inhibitor	SMA organoids shown progressive loss of motoneurons, especially those derived from SMA Type I patient, and higher expression of the cell cycle genes *CDK1*, *CDK2*, *CCNA2*, *CCNB1* and *CCNB2;* CDK4/6 inhibition improved motoneuron survival.	Organoids replicate patients' phenotype severity. This model is suitable to study the effects of CDK inhibitors and, potentially, other drugs in SMA.
Pereira et al., 2021	Sensorimotor organoids	iPSCs from patients affected by sporadic and familial ALS and isogenic controls	–	ALS organoids displayed denervation, fewer contractions and alterations in NMJs structure, indicative of motor neuron dysfunction.	Organoids replicate findings in familial and sporadic ALS and could serve as a promising platform for high-throughput drug screening.
Guo et al., 20243	Spinal cord organoids	C9orf72-knockdown iPSCs and unaffected iPSCs	–	ALS organoids displayed higher expression of IL-6, IL-1β, TNF-α and TGF-β mRNA and higher IL-6, IL-1β, TNF-α protein levels.	Suited to study certain aspects regarding inflammation in ALS. Notably, differences in inflammatory mediators *versus* healthy controls were more pronounced using mutated organoids rather than monocultures, further reinforcing the relevance of models with integrated multiple cell types.
Gao et al., 2024	Neuromuscular organoids	iPSCs from ALS patients with *C9ORF72* mutations	GSK2606414 (selective PERK inhibitor)	ALS organoids displayed denervation and classical polyGA inclusions. Higher concentrations of the tested drug reduced neuronal autophagy, the ratio of total p-eIF2α/eIF2α, polyGA levels and DNA damage, but the prolonged exposure caused toxic effects.	Organoids replicates characteristic aspects of *in vivo* pathology. This platform is suitable to test protein unfolding responses and to study longitudinally the effects of drugs.
Cardiac organoids and assembloids					
O'Hern et al., 2024	Assembloids with cardiac macrophages	iPSCs	–	Pro inflammatory triggers increased the rate of electrophysiological disturbances linked with atrial fibrillation.	Suitable to study the effects of inflammation on cardiac electrophysiology, potentially serving as a platform for drug screening.
Schmidt et al., 2023	Cardiac assembloids replicating atrium, left ventricle, right ventricle, outflow tract, and atrioventricular canal	iPSCs	ivabradine isoprenaline Bay K 8644 (L-type calcium channel agonist) 4-aminopiridyne Aspirin thalidomide acitretin isotretinoin *trans*-Retinol	Isoprenaline increased the speed of calcium signal propagation in whole assembloids; ivabradine reduced calcium signal in atrial cardioids and had negative chronotropic effects; Bay K 8644 had a positive chronotropic effect on atrial and atrioventricular organoids; 4-aminopiridyne induced a prolongation of field potentials; no effects of aspirin in organoid generation and morphology;t halidomide determined morphological alterations in organoids, downregulation of natriuretic peptide A and dysregulation of NR2F2, IRX1, and IRX4 expression;a citretin and isotretinoin altered morphogenesis of all assembloid components; *trans*-Retinol determined a marked morphological alteration only in the outflow tract.	Faithful replication of the electrophysiological effects of drugs to assess pro-arrhythmogenic parameters such as the prolongation of field potentials. Reliable evaluation of the teratogenic effects of multiple drug concentrations on different cardiac components.
Kupfer et al., 2020	Bioprinted cardiac organoids	iPSCs	isoproterenoli vabradine	Isoproterenol exerted positive chronotropic effects;i vabradine reduced calcium currents amplitude	Organoids replicated electrophysiological effects of drugs. The increased dimensions of bioprinted organoids allow obtaining pressure-volume loops and ejection fraction using commercially available equipment, further enhancing functional analyses.

Table 2 (*continued*)

Reference	Organoid type	Progenitor	Drugs tested	Outcomes	Relevance
Sallam et al., 2022	Cardiac organoids	iPSCs	tacrolimuss irolimus	In accordance to *in vivo* findings, tacrolimus induced pro-fibrotic changes, with increased fibroblasts proliferation, collagen deposition and upregulation of *SERPINH1*, *P4HB*, *PLOD1*, *COL21A1*, *P3H4* and *BNIP3* genes.	Organoids replicate certain aspects of drug-induced cardiac graft remodelling allowing to study its molecular pathway, avoiding limitations of cardiac biopsy.
Yin et al., 2025	Cardiac organoids	iPSCs	E-4031 (class III antiarrhythmic agent)d oxorubicin	E-4031 had negative chronotropic effect and caused an increase in field potential duration; acute exposure to doxorubicin reduced electro-mechanical window, prolonged electro-mechanical delay and shortened excitation-contraction duration without affecting force generation.	This platform enables real-time assessment of excitation-contraction coupling dynamics, identifying drug-induced arrhythmogenic alterations.
Scalise 2025	Cardiac organoids	iPSCs	doxorubicind asatinib + quercetin	Doxorubicin induced premature senescence of cardiac cells; dasatinib + quercetin rescued senescence phenotype reducing fibrosis, p16^{INK4A}, p21, p15, p19, SA-βgal expression, SASP production, ANP and BNP expression and restoring beating efficiency.	Organoids can model certain aspects of cardiac aging-related pathophysiology, thus allowing morphological and functional assays, and are suitable for the assessment of senolytic treatments effects.
Kostina et al., 2024	Neuro-cardiac assembloids	iPSCs	paroxetine venlafaxine sertraline citalopram fluoxetine escitalopram fluvoxamine	Dose-dependent downregulation of PHOX2B in neural crest cells, especially after exposure to paroxetine, venlafaxine and sertraline; downregulation of neuronal markers PHOX2B, NEFM and PRPH, parasympathetic marker SLC18A3 and glial markers S100B and GAP43 in neuro-cardiac assembloids; increased beating rate of neuro-cardiac assembloids integrating NCCs exposed to paroxetine and sertraline; less developed neurites in neuro-cardiac assembloids exposed to paroxetine and sertraline.	Neuro cardiac organoids may be helpful in predicting teratogenicity; In the future neuro cardiac organoids could be used to study dysautonomia in neurological and neuromuscular disorders.

Abbreviations - ANP: atrial natriuretic peptide; ALS: amyotrophic lateral sclerosis; BNP: brain-derived natriuretic peptide; CDK: cyclin-dependent kinase; eIF2α: eukaryotic translation initiation factor 2 A; IL: interleukin; iPSCs: induced pluripotent stem cells; NCCs: neural-crest cells; PERK: protein kinase R (PKR)-like endoplasmic reticulum kinase; SA-βgal: senescent-associated β galactosidase; SASP: senescent-associated secretory phenotype; SMA: spinal muscular atrophy; TGF: transforming growth factor; TNF: tumor necrosis factor.

specialized organoid might help overcoming this limitation, as described in the following paragraph.

6. Future perspectives

The literature reviewed in the previous paragraphs, summarized in Table 2, clearly points to a highly promising future of the development of organoids and assembloids in the neurological and cardiovascular fields. Numerous studies performed over the past ten years show their ease of handling, high scalability, and suitability for multiple parallel tests, which render them excellent systems for preclinical evaluation of new therapeutic strategies, of untoward effects and of interactions among key cell types forming a given tissue. Organoids and assembloids are greatly versatile and especially suited for high-throughput screenings. They can be analysed at multiple levels based on biochemical and functional parameters already established *in vivo*. For instance, organoids could help understanding the molecular mechanisms that cause spreading and progression of currently incurable diseases such as ALS. Through longitudinal analyses of these systems, researchers can monitor changes in cellular behaviour, gene expression, and signalling pathways as the disease evolves.

And there is a lot more to come. Ideally, a highly reliable system would include a component responsible for drug absorption, such as monolayers of intestinal epithelial cells derived from intestinal organoids, as demonstrated by Takahashi and colleagues (Takahashi et al., 2023). Following absorption, drugs could be conveyed *via* microfluidic pumps to a liver organoid. Notably, the liver organoids developed so far displays key metabolic functions, such as secretion of albumin for protein binding and cytochrome P450 activities that could allow xenobiotic metabolism (Kim & Park, 2025). Subsequently, the microfluidic system would deliver the drug and its metabolites, in combination with carrier proteins, to the target assembloid. An excretion system would complete the platform, eliminating unbound compounds. Albeit a functional kidney organoid capable to faithfully model renal filtration has not been developed yet, nephron-like structures replicating the various components have been successfully generated from iPSCs (Morizane et al., 2015). Moreover, micro-physiological systems replicating the kidney proximal tubule with established polarity and organic anion transporters have proved successful (Ma et al., 2024). Future optimization and integration of these components could allow incorporating the absorption, distribution, metabolism and excretion axis in complex *in vitro* systems, enhancing their predictive value as drug the testing platforms (Fig. 3).

Another promising application of organoids and assembloids is the assessment of gene therapy, a seasoned and nonetheless still cutting-edge strategy proposed in various medical fields, including the neurological and cardiovascular. The possibility to generate models with gene mutations directly from patients' iPSCs provides *in vitro* models

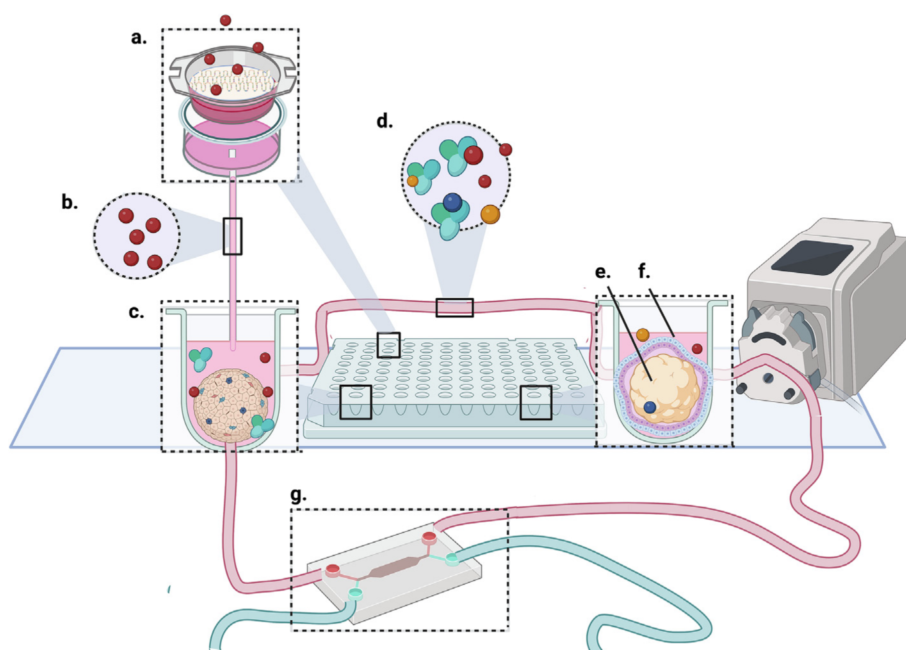

Fig. 3. Challenges in replicating pharmacokinetics with organoids. Lack of pharmacokinetic properties such as absorption, distribution, metabolism and excretion limit the possibilities of drug testing in organoids. Potential toxicity of drug solvents, poor replication of the physiological barriers that hinder drug distribution to certain tissues (*e.g.*, brain), absence of drug metabolites, and difficult mimicking of protein-drug binding complicate active concentration determination in standard organoids. Potential future systems could include: (a) an intestinal organoid capable of absorbing drugs. Once absorbed, molecules are transported *via* (b) a microfluidic system to (c) liver organoids. These organoids can secrete albumin and exhibit cytochrome activity, potentially metabolizing drugs as it would occur *in vivo* (d) The drug and its metabolites, variably bound to albumin, are then transported to (e) the target organoid or assembloids potentially protected by (f) physiological barriers such as the blood-brain barrier. Finally, an excretion system (g), similar to the one proposed by the group of Ma (Ma et al., 2024), would complete the platform.
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well-suited to test potential gene restorative strategies. This approach has been developed by Papes and colleagues (Papes et al., 2022), who used brain organoids harbouring TCF4 mutations generated from children with the Pitt-Hopkins syndrome, a disorder characterized by developmental delay. After functional and molecular characterization of the organoids, they restored the TCF4 expression *via* CRISPR-Cas9 technique or virus-mediated gene transfer, demonstrating improved dimensions and function of treated organoids. Notably, the multiple cell types that constitute organoids also allow the simultaneous evaluation of potential off-target effects. Gene therapies are being explored for a growing range of neuromuscular (Cavalcante Jr. et al., 2024) and cardiovascular (Cannatà et al., 2020) disorders.

In conclusion, while organoids and assembloids are currently still complementary to *in vivo* models, they hold a tremendous promise. One day they might reliably mimic the complexity of organs such as skeletal or cardiac muscle and the most challenging neural systems. The booming progress of biotechnologies is indeed providing scientists with tools for the construction of biological structures that were unimaginable even a few years ago.

CRediT authorship contribution statement

Lorenzo Fontanelli: Writing – review & editing, Writing – original draft, Visualization, Conceptualization. **Noemi Nisini:** Writing – review & editing. **Sergio Pirola:** Writing – review & editing. **Fabio A. Recchia:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition, Conceptualization.

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Declaration of competing interest

None.

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