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ADVANCED REVIEW

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iPSCs as a groundbreaking tool for the study of adverse drug reactions: A new avenue for personalized therapy

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Abstract

Induced pluripotent stem cells (iPSCs), obtained by reprogramming different somatic cell types, represent a promising tool for the study of drug toxicities, especially in the context of personalized medicine. Indeed, these cells retain the same genetic heritage of the donor, allowing the development of personalized models. In addition, they represent a useful tool for the study of adverse drug reactions (ADRs) in special populations, such as pediatric patients, which are often poorly represented in clinical trials due to ethical issues. Particularly, iPSCs can be differentiated into any tissue of the human body, following several protocols which use different stimuli to induce specific differentiation processes. Differentiated cells also maintain the genetic heritage of the donor, and therefore are suitable for personalized pharmacological studies; moreover, iPSC-derived differentiated cells are a valuable tool for the investigation of the mechanisms underlying the physiological differentiation processes. iPSCs-derived organoids represent another important tool for the study of ADRs. Precisely, organoids are in vitro 3D models which better represent the native organ, both from a structural and a functional point of view. Moreover, in the same way as iPSC-derived 2D models, iPSC-derived organoids are appropriate personalized models since they retain the genetic heritage of the donor.

Abbreviations: 4BPPNit, 4-(4-bromophenyl)-6-ethylsulfanyl-2-oxo-3,4-dihydro-1H-pyridine-5-carbonitrile; 4-HPR, N-(4-hydroxyphenyl)retinamide; ADRs, adverse drug reactions; AECs, alveolar epithelial cells; ALT, alanine aminotransferase; AMP, adenosine monophosphate; Ang-II, angiotensin II; AOs, alveolar organoids; AST, aspartate aminotransferase; BMPR1A, BMP receptor type 1A; BMPs, bone morphogenetic proteins; BPA, bisphenol A; BUN, blood urea nitrogen; CA2, carbonic anhydrase II; CFTR, cystic fibrosis transmembrane conductance regulator; CMs, cardiomyocytes; CYP, cytochrome P; DILD, drug-induced interstitial disease; DILI, drug induced liver injury; DIRI, drug-induced renal injury; DMSO, dimethyl sulfoxide; DOX, doxorubicin; EGF, epidermal growth factor; EPCs, endothelial progenitor cells; ER, endoplasmic reticulum; FCS, fetal calf serum; FGF, fibroblast growth factor; FKRP, fukutin-related protein gene; GLP-1, glucagon-like peptide 1; HDFs, human dermal fibroblasts; HGF, hepatocyte growth factor; HIF-1α, hypoxia-inducible factor-1α; hiPSC-CMs, human induced pluripotent stem cell-derived cardiomyocytes; HLCs, hepatocyte-like cells; HUVECs, human umbilical cord vein endothelial cells; IBD, inflammatory bowel disease; IFN-γ, interferon γ; iMeLCs, incipient mesoderm-like cells; iPSCs, induced pluripotent stem cells; KRT19, cytokeratin 19; LC, light chain; LDH, lactate dehydrogenase; MEF, mouse embryonic fibroblast; MFG-E8, milk fat globule-epidermal growth factor; miRNA, microRNA; MLC, myosin light chain; mTOR, mammalian target of rapamycin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; MUC1, mucin 1; NE, norepinephrine; OSM, oncostatin M; PBMCs, peripheral blood mononuclear cells; PDLOs, pancreatic duct-like organoids; PGCLCs, primordial germ-cell like cells; PHH, primary human hepatocytes; RA, retinoic acid; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase; SLC, solute carrier; T1D, type I diabetes; TEM, transmission electron microscopy; TGF-β, transforming growth factor-β; TIP, thiopurine-induced pancreatitis; TPMT, thiopurine S-methyltransferase; VD3, vitamin D3; VEGF, vascular endothelial growth factor; WD, Wilson's disease; XIAP, X-linked inhibitor of apoptosis; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole.

In comparison to other in vitro models, iPSC-derived organoids present advantages in terms of versatility, patient-specificity, and ethical issues. This review aims to provide an updated report of the employment of iPSCs, and 2D and 3D models derived from these, for the study of ADRs.

This article is categorized under:

Cancer > Stem Cells and Development

KEYWORDS

adverse drug reactions, induced pluripotent stem cells, medicine, organoids, personalized, pharmacology

1 | INTRODUCTION

Adverse drug reactions (ADRs) are major clinical problems (Tissot et al., 2022) which can lead to therapy interruption, can cause suffering to patients, and could be more difficult to manage in fragile populations such as elderly or children (Neininger et al., 2022). ADRs are one of the most limiting factors in drug approval and often cause drug withdrawal from the market (Tosca et al., 2021). ADRs management requires important clinical and economical efforts; as a matter of fact, it is necessary to develop innovative drug toxicity in vitro models to study ADRs, offering the possibility to avoid them and their clinical risks (Cacabelos et al., 2021).

In the last decade, several steps have been made toward the establishment of more sensitive assays for a better management of ADRs (Shi et al., 2017), and the most ground-breaking advance has been in the set-up of patient-specific assays (Lynch et al., 2019). Indeed, ADRs are strictly related to individual factors, including genetics, and this interindividual variability can cause a wide range of drug toxicities (Cacabelos et al., 2021). Many factors affect pharmacokinetics and pharmacodynamics, such as genetic polymorphisms for drug-metabolizing enzymes and transporters, ion channels and receptors, epigenetic alterations, environmental factors, and concomitant diseases. A powerful strategy to overcome this issue is represented by induced pluripotent stem cells (iPSCs), discovered by K. Takahashi and Yamanaka (2006), which offer a versatile tool to study ADRs in personalized assays (Inoue et al., 2020; Wills & Rajagopalan, 2020). iPSCs are stem cells generated by reprogramming differentiated cells, usually adult somatic cells, which can be easily obtained from peripheral blood samples. iPSCs, in turn, can be differentiated into any tissue of the human body (Genova et al., 2019). Reprogramming methods for iPSCs obtainment, with their advantages and disadvantages, are reported in Figure 1. Schematic differentiation of iPSCs into the different tissues of the human body is represented in Figure 2; all the stimuli, and their molecular targets/mechanisms, used during the differentiation protocols mentioned in this article are listed in Table 1. Table 2 provides a schematic overview of the usage of the stimuli for proper iPSCs differentiation in the studies reviewed in this article. iPSCs can also give rise to organoids, models presenting a 3D microarchitecture resembling human native tissues (Rauth et al., 2021). In the same way of iPSC-derived 2D models, iPSC-derived organoids present advantages in terms of versatility, patient specificity, and ethical issues in comparison to other in vitro models (Driehuis et al., 2020; Harrison et al., 2021), and therefore are powerful tools for toxicity screening, disease modeling and personalized medicine (Lancaster & Huch, 2019; T. Takahashi, 2019). These models may be accurate in predicting drug toxicity, especially when it is related to individual genetic differences, which are preserved in iPSC-based patient-specific models (Azar et al., 2021).

The set-up of sensitive and personalized assays to understand and prevent ADRs is strongly needed, especially when it comes to pediatric patients, more likely to develop different and more severe kinds of ADRs, leading, in some cases, to hospitalization, disability, or even death (Genova et al., 2019). Clinical experience on ADRs in children is limited, and clinical trials on pediatric population often have ethical concerns. Consequently, most drugs have been analyzed in clinical trials only in adults and most pediatric patients are treated with off-label medicines, with a higher risk to develop ADRs (Gore et al., 2017). Also, there are some differences in pharmacokinetic and pharmacodynamic profiles between children and adults: absorption, plasma protein binding, biotransformation, and excretion in children are reduced, whereas the volume of distribution is increased. Moreover, children have a different expression of cytochromes; for instance, CYP3A4 is the major CYP expressed in adult liver, whereas CYP3A7 is the major CYP expressed in the fetal liver. In this context, iPSCs derived from pediatric patients, which could better recapitulate children metabolic features, such as CYP expression, represent a suitable model for drug safety assessment. As a matter of fact,

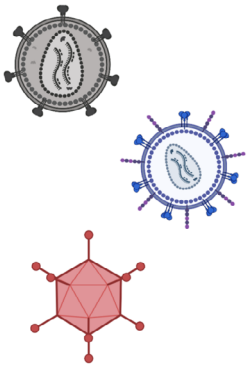
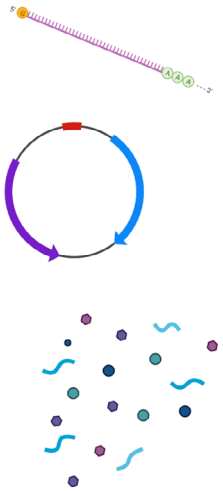
Method	Type	Subtype	Advantages	Disadvantages
VIRAL 	Integrating	Retrovirus (Omole & Fakoya, 2018; Zhou & Zeng, 2013)	Highly efficient	Permanent integration into host genome
		Lentivirus (Omole & Fakoya, 2018; Somers et al., 2010)	Infection of non-proliferative cells Possible excision from host genome	Integration into host genome
	Non-integrating	Adenovirus (Hu, 2014)	No integration in host genome	Low efficiency in comparison to lentivirus
		Sendai virus (Bernal, 2013; Q. Wang et al., 2019)	The virus does not enter the nucleus	Need of repeated passaging for virus remotion
NON-VIRAL 	Integrating	PiggyBac transposons (Omole & Fakoya, 2018; Yusa, 2015)	Highly active in mammalian cells Possible excision from host genome	Low efficiency
	Non-integrating	mRNA transfection (Warren & Lin, 2019)	No integration in host genome	Labor intensive
		miRNA transfection (Miyoshi et al., 2011)	Absence of breaks in existing genes	Labor intensive
		Episomal plasmids (Hu, 2014; Yu et al., 2009)	More stable expression in comparison to standard plasmids	Low efficiency
		Recombinant proteins (Nordin et al., 2017)	Absence of breaks in existing genes	Low efficiency
		Small molecules (G. Chen et al., 2020)	Non immunogenic	Low efficiency

FIGURE 1 Overview of the reprogramming methods for iPSCs obtainment, with their advantages and disadvantages.

iPSC-based in vitro models could bypass the concerns due to the lack of clinical trials in the pediatric setting (Freel et al., 2020), constituting a valuable model for therapy personalization in these patients (Masui et al., 2022).

This review aims to provide an updated report of the employment of iPSCs as a model to study ADRs; a peculiar focus has been made on adverse effects occurring at the intestinal, hepatic, pancreatic, renal, cardiac, neuronal, and pulmonary levels, also discussing progress in organoid arrangement. Studies merging other innovative approaches, such as organs-on-chip, assembloids, and CRISPR/Cas9 based genome-editing, with iPSCs technology are also reviewed in this article.

2 | IPSCS AND DRUG-INDUCED TOXICITIES

2.1 | iPSCs and intestinal ADRs

The intestine is one of the organs most affected by drug-induced toxicity. The mechanisms of damage can be related to topical effects, pharmacological effects of the drugs on intestinal secretion or motility, or drug-mediated effects on the immune system (Kwak & Hart, 2017). Also, intestinal transporters, important for drug absorption, can be related to

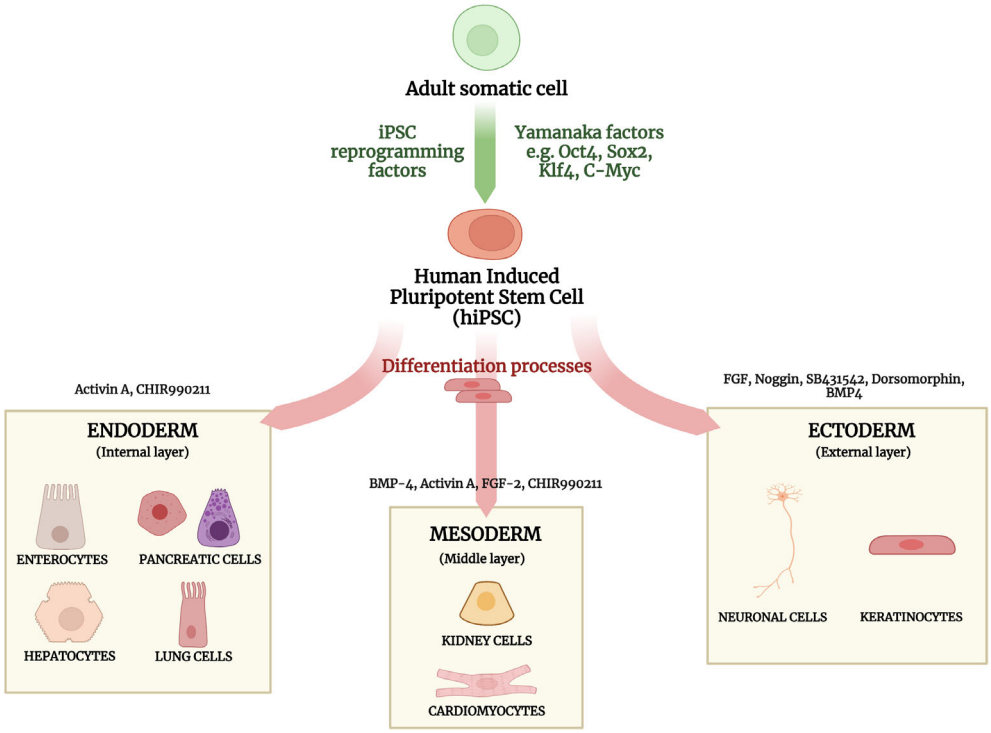


FIGURE 2 Schematic representation of the possibility to differentiate iPSCs into the three different germ layers, subsequently obtaining different cell types.

intestinal drug toxicities (Xue et al., 2019). Intestinal ADRs can result in malabsorption, mucosal damage, hemorrhagic phenomena, or alterations in the motility (Srisajjakul et al., 2022). To manage intestinal ADRs, many scientists developed in-vitro models based on immortalized intestinal cell lines like Caco-2 (Volpe, 2011; Voss et al., 2021), or LS-180 (Zudeh et al., 2023); nonetheless, such models do not represent the genetic differences existing between different patients. In this regard, iPSC-derived intestinal cells and organoids can help to overcome these issues.

Recently, Yamada et al. (2022) investigated the effects of a synthetic lithocholic acid derivative, developed to have the same effects as vitamin D and referred to as Dcha-20, on gene expression in iPSC-derived intestinal organoids. Precisely, the authors evaluated the effects of Dcha-20 on the gene expression of metabolizing enzymes and proteins involved in drug transport and excretion, and made a comparison with those of vitamin D. Briefly, starting from 253G1 iPSCs, definitive endoderm cells were obtained by adding activin A and CHIR99021 to the culture medium. Subsequently, CHIR99021, activin A, and FGF4 were supplemented to the medium for 4 days, followed by the addition of EGF, R-spondin, and noggin until day 21 of differentiation. From day 21 onward, the medium composed by EGF, R-spondin, and noggin was supplemented with Dcha-20, in order to assess its activity on iPSC-derived intestinal organoids. Since it has been reported that vitamin D3 (VD3) acts as a powerful inducer of CYP3A4 in human intestinal cells (Qin & Wang, 2019), the authors investigated its effects on iPSC-derived intestinal organoids making a comparison with those induced by different concentrations of Dcha-20. As a result, RT-qPCR analyses revealed that Dcha-20 treatment at the concentrations 0.1–10 μM induced a higher CYP3A4 expression in comparison to VD3 treatment. To further characterize the effect of Dcha-20 on intestinal metabolism, the expression of other drug-metabolizing enzymes, UGT1A1, and UGT1A3, was assessed. Particularly, an induction of UGT1A1, and UGT1A3, normally responsible for drug detoxification via glucuronidation, was noticed after Dcha-20 treatment; on the other hand, this upregulation was absent after VD3 treatment, demonstrating the specificity of this response after Dcha-20 treatment. Moreover, an investigation of the impact of Dcha-20 on the intestinal absorptive system was carried out, and a significant increase in the expression of ABCB1 and MRP2 genes, encoding for proteins normally involved in intestinal drug transport and excretion, was noticed after Dcha-20 treatment. Furthermore, ABCB1 gene was more induced in response to Dcha-20 in comparison to VD3 treatment; on the other hand, the induction of MRP2 gene seemed to be specifically related to Dcha-20 treatment, due to its absence after VD3 exposure. In summary, this study further confirms the feasibility of iPSC-derived intestinal organoids as a tool for pharmacokinetic studies, and thus for drug toxicity evaluations.

TABLE 1 Stimuli and their molecular targets/mechanisms used in the studies mentioned in this article.

Stimulus	Molecular target/mechanism
8-Br-cAMP	Protein kinase A (PKA) activator
A-8301	ALK 4, 5, 7 inhibitor
Alk5i-II	Inhibitor of the TGF-β type I receptor
Ascorbic acid	Antioxidant properties
BDNF	Activator of tropomyosin receptor kinase B (TrkB)
Betacellulin	Ligand of the EGF receptor
BMP4	Member of TGFβ superfamily; activation of serine/threonine kinase receptors
CHIR99021	GSK-3β/3α inhibitor; Wnt pathway activator
Cyclopamine	Sonic hedgehog pathway inhibitor
DAPT	γ-secretase inhibitor
Dexamethasone	Glucocorticoid drug; activator of intracellular glucocorticoid receptors
DMH1	ALK 2 inhibitor
Dorsomorphin	AMPK inhibitor
Doxycycline	Tetracycline class drug; downregulation of PI3K/Akt pathway
EGF	EGF receptor activator
FGF1	FGF receptors activator
FGF2	FGF receptors activator
FGF4	FGF receptors activator
FGF7	FGF receptors activator
FGF10	FGF receptors activator
GDNF	Activator of tyrosine kinase GDNF receptor
GLP-1	Activator of GLP-1 receptor; increase of the release of pancreatic enzymes and insulin secretion
HGF	HGF receptor activator
IBMX	Phosphodiesterase inhibitor increasing intracellular cAMP
IGF1	Activator of IGF1 receptor and insulin receptor
Insulin	Activator of tyrosine kinase insulin receptor
IWP2	Wnt signaling inhibitor
IWP4	Wnt signaling inhibitor
LDN193189	BMP signaling inhibitor
Monothioglycerol	Inhibitor of glycerol kinase; antioxidant properties
Nicotinamide	PARP-1 inhibitor; inhibitor of Casein kinase 1 and rock kinases
Noggin	Bone morphogenetic protein inhibitor
NT-3	Tropomyosin receptor kinase (Trk) C, TrkB, and TrkA activator
Oncostatin M	Member of the IL-6 family cytokines; activator of cytokines receptors
PBDU	Protein kinase C (PKC) activator
Retinoic acid	Nuclear retinoic acid receptor activator
R-spondin	Wnt signaling enhancer
Rock inhibitor	Rho-kinase inhibitor
SAG	Smoothened receptor agonist
SANT1	Sonic hedgehog pathway inhibitor
SB431542	ALK4, ALK5, and ALK7 inhibitor
VEGF	VEGF tyrosine-kinase receptors activator
Vitamin A	Nuclear retinoic acid receptor activator

(Continues)

TABLE 1 (Continued)

Stimulus	Molecular target/mechanism
Wnt3A	Wnt/β-catenin signaling pathway activator
Wnt-C59	Wnt inhibitor
XAV-939	Tankyrase inhibitor; Wnt/β-catenin signaling inhibitor

Indeed, in some cases, drug toxicity could be related to altered expression or functionality of drug transporters and drug metabolizing enzymes, and therefore it becomes important to have feasible models to investigate these aspects.

An interesting study focusing on large-scale generation and utilization of iPSC-derived organoids for drug screening was made by Y. Takahashi et al. (2023), who used high-throughput approaches to assess the cytotoxicity of 3500 compounds on iPSC-derived intestinal organoids, comparing the results with those obtained in Caco-2 cell line. As a result, 62 compounds resulted selectively active in iPSC-derived intestinal organoids, and not in Caco-2 cells. Of these compounds, ABT-737, N-(4-hydroxyphenyl)retinamide (4-HPR), ivermectin, N-oleoyldopamine, and 3-(5′-hydroxymethyl-2′-furyl)-1-benzyl indazole (YC-1) showed a dose-dependent cytotoxicity. YC-1, also called lificiguat, is an activator of the soluble guanylyl cyclase (sGC) and an inhibitor of hypoxia-inducible factor-1α (HIF-1α). To assess if YC-1 cytotoxicity was mediated by sGC activation or HIF-1α inhibition, compounds with the same actions as YC-1 were used; among these, vericiguat is an sGC activator, while LW6, PX-478, and PT-2385 act as HIF-1α inhibitors. All these compounds caused a little cytotoxicity against intestinal epithelial cells derived from the dissociation of iPSC-derived intestinal organoids; this result suggested that YC-1 cytotoxicity does not seem to be related to sGC activation nor to HIF-1α inhibition. Moreover, YC-1 was showed to dose-dependently induce the cleavage of caspase-8 and its downstream pro-apoptotic proteins and lead to a decrease of the anti-apoptotic protein X-linked inhibitor of apoptosis (XIAP). YC-1-related apoptosis was prevented by PD98059, a MEK/ERK inhibitor, which was instead not able to prevent cytotoxicity caused by ABT-737, 4-HPR, ivermectin, and N-oleoyldopamine, suggesting a unique molecular mechanism of cytotoxicity of YC-1.

2.2 | iPSCs and hepatic ADRs

Drug induced liver injury (DILI), one of the most dangerous and difficult to manage, is generally divided into intrinsic (dose-dependent) and idiosyncratic. The first type is related to the chemical properties of a drug or its metabolites; it is related to overdose, and it is highly reproducible. Instead, idiosyncratic DILI manifests at therapeutic dosages, with a lower incidence compared to the intrinsic type (McGill & Jaeschke, 2019). DILI can manifest as acute or chronic condition such as acute liver failure and hepatitis (Alempijevic et al., 2017). Given the clinical impact of DILI, managing and preventing this adverse effect is necessary. Nonetheless, current in vitro models, especially hepatocarcinoma-derived cell lines, have some limitations (Kuna et al., 2018), which can be overcome by iPSC-derived hepatocytes. In this regard, Ghosh et al. (2021) differentiated iPSCs into hepatocyte-like cells (HLCs) and used these cells for toxicity studies. The authors first added Wnt, activin A, and BMP4 to the culture medium, and used increasing concentrations of DMSO (from 0.6% to 2%), followed by an omission of DMSO starting from day 14. Moreover, doxycycline was added to the culture medium from day 4 to the end of the differentiation process, and FGF1 and HGF were also added. The effective differentiation was confirmed by real-time PCR and immunostaining: HLCs expressed hepatocyte markers such as albumin, CYP3A4, CYP2C9, and HNF4A. HLCs produced about 40% as much albumin as secreted by the primary human hepatocytes (PHH), a primary cell line obtained from two cadaveric donors. To date, PHH are the gold standard for in vitro pharmacological studies, and different protocols are available for PHH obtainment (Kegel et al., 2016). Furthermore, more HLCs stained positive for CYP3A4 in comparison to the stable cell line of hepatic origin HepG2. Subsequently, iPSC-derived HLCs were used to assess the toxicity of 14 drugs, after an exposure of 48 h, using lactate dehydrogenase (LDH) assay to detect cytotoxicity. Tested substances included drugs not known to induce acute hepatic toxicity at therapeutic doses, such as doxorubicin, olanzapine, or cyclosporine A, and also notoriously hepatotoxic drugs, like amiodarone, diclofenac, and clozapine. Dose-dependent cytotoxicity was observed for the aforementioned hepatotoxic drugs, as indicated by the dose-dependent increase in LDH activity after the treatments in comparison to untreated controls; instead, as expected, no toxic effects were detected after the treatment with those drugs not known to induce acute hepatotoxicity, as demonstrated by the absence of changes in LDH levels.

TABLE 2 Schematic overview of the usage of different stimuli in the context of iPSC-differentiation into bidimensional and tridimensional intestinal, hepatic, pancreatic, renal, cardiac, cerebral, and pulmonary organoids.

Cell type of origin	Stimuli for differentiation in the appropriate germ layer	Germ layer	Other stimuli used for proper differentiation in target organs	Differentiated 2D and 3D models obtained in the studies mentioned in this article	References
iPSCs	Activin A CHIR99021 BMP4 Wnt3A	Endoderm	CHIR99021 EGF FGF4 HGF Noggin R-spondin Wnt3a	Intestinal 2D and 3D models	(Y. Takahashi et al., 2023; Yamada et al., 2022)
iPSCs	Activin A CHIR99021 BMP4 Wnt3A	Endoderm	A-8301 Ascorbic acid CHIR99021 DAPT Dexamethasone Doxycycline EGF FGF1 FGF2 FGF4 HGF Oncostatin M Retinoic acid VEGF	Hepatic 2D and 3D models	(Bircsak et al., 2021; Ghosh et al., 2021; Shinozawa et al., 2021; Song et al., 2022; C. J. Zhang et al., 2023)
iPSCs	Activin A CHIR99021 BMP4 Wnt3A	Endoderm	Alk5i-II Ascorbic acid Betacellulin Cyclopamine Dorsomorphin EGF FGF2 FGF7 FGF10 GLP-1 LDN193189 Nicotinamide Noggin PBDU Retinoic acid SANT1 Wnt3A	Pancreatic 2D and 3D models	(Genova et al., 2021; Leite et al., 2020; Peterson et al., 2020; Takizawa-Shirasawa et al., 2013; Wiedenmann et al., 2021)
iPSCs	Activin A CHIR99021 BMP4 Wnt3A	Endoderm	8-Br-cAMP CHIR99021 DAPT Dexamethasone FGF7 FGF10 IBMX Monothioglycerol Noggin Retinoic acid SB431452	Pulmonary 2D and 3D models	(Heo et al., 2019; J.-H. Kim, An, et al., 2021; J. Lee et al., 2023)

(Continues)

TABLE 2 (Continued)

Cell type of origin	Stimuli for differentiation in the appropriate germ layer	Germ layer	Other stimuli used for proper differentiation in target organs	Differentiated 2D and 3D models obtained in the studies mentioned in this article	References
iPSCs	Activin A CHIR99021 BMP4 FGF2	Mesoderm	CHIR99021 Rock-inhibitor	Renal 2D and 3D models	(J. W. Kim, Nam, et al., 2021; Q. Wang et al., 2022)
iPSCs	Activin A CHIR99021 BMP4 FGF2	Mesoderm	ACTIVIN A BMP4 CHIR99021 DMH1 Doxycycline IWP2 IWP4 SB431542 Wnt-C59	Cardiac 2D and 3D models	(Hoang et al., 2021; Magdy et al., 2022; E.Y. Wang et al., 2021)
iPSCs	FGFs Noggin SB431542 Dorsomorphin BMP4	Ectoderm	Ascorbic acid BDNF CHIR99021 DAPT Dorsomorphin EGF FGF2 GDNF IGF1 Insulin IWP2 NT-3 Retinoic acid SAG SB431542 Vitamin A Wnt3A XAV-939	Neuronal 2D and 3D models	(Andersen et al., 2020; Birey et al., 2022; Ho et al., 2022; J. Kim et al., 2019; Liu et al., 2019; Scholz et al., 2022)

Recently, Shinozawa et al. (2021) developed a reproducible protocol for the generation of liver organoids starting from iPSC-derived foregut cells, and then used this model to perform a liver organoid-based toxicity screen on 238 different drugs including 32 negative controls, such as ascorbic acid, metformin hydrochloride, or theophylline, and 206 reported DILI compounds, among which pyrimethamine, furosemide, nimesulide, and sulpiride. Briefly, activin A and BMP4 were added to iPSC-culture medium during the first day of differentiation, followed by the addition of activin A and 0.2% and 2% of fetal calf serum (FCS), during the second and the third day of differentiation, respectively, to obtain definitive endoderm cells. Then, FGF4 and CHIR99021 were added to the culture medium, and the obtained foregut cells were subsequently resuspended and embedded in matrigel drop in organoid formation media, formed by FGF2, VEGF, EGF, CHIR99021, A-8301, and ascorbic acid. Once organoids formed, retinoic acid (RA) was added to the medium, followed by the subsequent addition of HGF, oncostatin M (OSM), and dexamethasone. Quantitative PCR analysis revealed that the expression of hepatic marker genes, such as albumin, and CYP2C9 was upregulated in the organoids. From the functional point of view, CYP3A4 and CYP1A2 were induced after the treatment with rifampicin and omeprazole, respectively, demonstrating the activity of these enzymes in the 3D liver model generated. For the subsequent toxicity screen, each drug was diluted into 4 doses and liver organoids were seeded into 384-well plates containing the compounds under examination; cell viability was tested using the CellTiter-Glo luminescent cell viability assay. After the treatment with tolcapone, diclofenac, and nefazodone, dose-dependent increases in mitochondrial membrane potential were observed; troglitazone also increased mitochondrial membrane potential, but no

dose-dependence was observed. Lastly, the authors investigated if N-acetylcysteine, a well-known antioxidant, could help organoids in the recovering from DILI-like conditions, like those induced by troglitazone; as expected, N-acetylcysteine highly increased cell viability.

2.3 | iPSCs and pancreatic ADRs

The pancreas is another organ often affected by drug-induced adverse effects. Most drug-induced pancreatitis cases manifest as mild or moderate events, but they sometimes evolve into more severe episodes, which can lead to hospitalization or death (Weissman et al., 2020). Moreover, there is a lack of a precise knowledge of the mechanisms of drug-induced pancreatitis, making it difficult to associate specific pharmacological treatments with the development of acute pancreatitis (Genova et al., 2021); in this regard, iPSCs-derived pancreatic cells represent a useful tool for toxicological studies. To date, most of the protocols available focus on the differentiation of iPSCs into endocrine pancreatic lineage, for studying type I diabetes (T1D). For instance, Leite et al. (2020) used two different protocols for the differentiation of patient-derived iPSCs in both endocrine α and β cells, for modeling T1D. Precisely, the authors used iPSCs derived from three donors affected by type I diabetes, and three donors without this pathology. To obtain pancreatic β cells, at first activin A, and CHIR99021 were added to the culture medium, followed by the supplementation of activin A only; in this way, they obtained definitive endoderm cells. Then, FGF7, LDN193189, SANT1, retinoic acid, and PBDU were added to the medium, followed by the addition of betacellulin, and Alk5i-II, to obtain β cells. To generate pancreatic endocrine α cells, the authors referred to another protocol, developed by Peterson et al. (2020). Briefly, definitive endoderm cells were obtained as described above, and then LDN193189 and retinoic acid were added to the medium, followed by the supplementation with Alk5i-II, and PBDU for 7 and 28 days, respectively. Obtained β cells were co-cultured with peripheral blood mononuclear cells (PBMCs) isolated from the corresponding donors, in order to assess the expression of molecules relevant for immune system activation. To this purpose, iPSC-derived α and β cells were initially treated with IFN-γ, to mimic inflammatory stress, and thapsigargin, to induce endoplasmic reticulum (ER) stress, which is a major contributor of β cell failure in diabetes. After the treatment with IFN-γ, but not with thapsigargin, HLA class I molecules, notorious risk alleles in type I diabetes, were upregulated in both iPSC-derived α and β cells, and no relevant differences were found between cells derived from T1D patients and patients without the pathology. Co-culturing experiments showed no T cell activation when autologous PBMCs were co-cultured with the corresponding untreated iPSC-endocrine cells; in the same way, no immune response was detected after the pre-stimulation of iPSC-derived β cells with IFN-γ. To assess the role of ER stress, iPSC-derived β cells were pre-treated with thapsigargin, and subsequently co-cultured with autologous PBMCs; this resulted in upregulated immune cell activation markers CD25 and CD69 on T cell populations, and in increased levels of IL-2, and IL-17 in autologous co-cultures of both T1D donors and donors without the pathology. A decrease in viable iPSC-derived β cells in the thapsigargin-treated co-culture experiments was also observed, supporting the hypothesis of a causal role of ER stress in eliciting the immune response related to β-cell destruction in T1D.

Regarding the differentiation of iPSCs into exocrine pancreatic cells, the most efficient protocol for the generation of iPSC-derived acinar cells was developed by Takizawa-Shirasawa et al. (2013). Our group also used this protocol to obtain, starting from iPSCs derived from pediatric inflammatory bowel disease (IBD) patients, patient-specific exocrine pancreatic cells to study thiopurine-induced pancreatitis (TIP) (Genova et al., 2021). TIP is a severe side effect affecting about 3% of IBD patients treated with thiopurines (Ledder et al., 2015); it often requires therapy interruption (Genova et al., 2021) and it manifests with more severe anatomic involvement in pediatric patients. The mechanism of TIP is currently unknown, possibly involving genetic predisposition (Wilson et al., 2018), abnormalities in the immune response (Stocco et al., 2015; Weersma et al., 2008), or alteration in thiopurine biotransformation (Stocco et al., 2015). Briefly, the differentiation of iPSCs into exocrine pancreatic cells required the initial supplementation of activin A, and CHIR99021 to the culture medium, necessary to obtain definitive endoderm cells. Primitive gut tube cells are subsequently obtained by adding FGF7; then, cyclopamine, noggin, and retinoic acid lead to the differentiation of pancreatic progenitors. Final differentiation into pancreatic exocrine cells is obtained with the addition of FGF7, nicotinamide, and GLP-1 (glucagon-like peptide 1) to the culture medium. Real-time PCR and immunofluorescence analyses confirmed the identity of the cells in the different stages of the differentiation: FOXA2, and SOX17 were found in definitive endoderm cells, pancreatic progenitors were positive for PDX1, and differentiated pancreatic exocrine cells showed the expression of α-amylase and carboxypeptidase A. Cytotoxicity assayed by MTT showed a major sensitivity of TIP in comparison to no-TIP, after the treatment with mercaptopurine and thioguanine, for both iPSCs and differentiated

exocrine pancreatic cells, even if it was more marked in iPSCs rather than in the differentiated cells. TPMT polymorphisms were excluded from the possible causes of the differences noticed since all patients resulted wild type for three important variants (rs1142345, rs1800460, and rs1800462). These preliminary evidences, despite requiring confirmation on larger patient population, support that iPSC-derived pancreatic models are valuable tools for personalized pharmacological studies, able to take into account the existing differences between patients and their impact on drug toxicities (Genova et al., 2021).

Recently, many scientists focused on the generation of stable iPSC-derived pancreatic organoids. For instance, Wiedenmann et al. (2021) developed a protocol to generate pancreatic duct-like organoids (PDLOs) from human iPSCs, using a microwell chip helping in the uniform aggregation of iPSC-derived pancreatic progenitors. Activin A, and CHIR99021 were added to the culture medium, followed by the addition of FGF2, and subsequently of FGF10, dorsomorphin, and Wnt3A. Once definitive endoderm cells were obtained, the medium was supplemented with FGF10, LDN193189, SANT1, retinoic acid, and L-ascorbic acid, and then with EGF and nicotinamide, to obtain pancreatic progenitors, characterized by the markers PDX1 and NKX6-1. Pancreatic progenitors were transferred on a microwell chip to generate PDLOs; in addition, the medium was supplemented with nicotinamide, EGF, FGF10, and FGF7, to obtain the proper differentiation into PDLOs. The epithelial nature and pancreatic ductal identity of PDLOs were confirmed by the upregulation of cytokeratin 19 (KRT19), mucin 1 (MUC1), cystic fibrosis transmembrane conductance regulator (CFTR), and carbonic anhydrase II (CA2). Lastly, the obtained PDLOs were transplanted orthotopically into the pancreas of immunocompromised mice, and, after 8 weeks, the formation of tubular duct-like tissue, characterized by ductal markers like SOX9, and KRT19, was detected.

2.4 | iPSCs and renal ADRs

Another relevant drug-induced toxicity is drug-induced renal injury (DIRI), particularly frequent in critical patients receiving complex pharmacological treatments (Wu & Huang, 2018). Kidneys play a pivotal role in the excretion of metabolites, drugs, and toxins from the blood: the glomerulus is responsible for the filtration of the blood, and the renal tubules for the reabsorption and secretion of substances (Huang et al., 2016; Wu & Huang, 2018). DIRI can manifest as acute kidney injury, or chronic kidney disease (Unwin, 2022; Wu & Huang, 2018); furthermore, DIRI may be the result of dose-dependent toxicity or dose-independent idiosyncratic toxicity. So far, blood urea nitrogen (BUN) and serum creatinine have been considered useful DIRI markers, despite many renal and non-renal factors influencing their levels. Therefore, it is necessary to identify better markers, possibly released by the kidneys directly into urine or blood in response to renal injury (Griffin et al., 2019). Another issue in DIRI management is that validated in vitro models for the prediction of nephrotoxicity are currently not available (Hall et al., 2022). Furthermore, immortalized kidney cells have some limitations, such as issues for successful isolation, or restricted morphological and/or functional characteristics in comparison to renal cells of the human body (Lechner et al., 2021). Renal organoids derived from iPSCs gained a great attention for their suitability for drug screening and drug toxicity evaluation. For instance, Kim, et al. (2021) investigated tacrolimus-induced nephrotoxicity using iPSC-derived kidney organoids. Briefly, iPSCs culture medium was supplemented with CHIR99021 and cells were fed every 2 or 3 days to obtain kidney organoids. Kidney organoids were treated with different concentrations of tacrolimus for 24 h; cell viability was assessed using the CellTiter-Glo Assay. In addition, live/dead cell staining revealed a dose-dependent increase of the proportion of dead cells. Nephrotoxicity was also investigated using the CCK-8 assay, which revealed a decrease of cell viability after the treatment with tacrolimus 60 μM in comparison to untreated cells. Furthermore, a comparison of the structural changes induced by tacrolimus in iPSC-derived kidney organoids and in a mouse model of tacrolimus nephrotoxicity, developed in the same work, indicated that iPSC-derived kidney organoids recapitulate the structural changes in nephrons observed in tacrolimus nephrotoxicity. Indeed, in both in vivo and in vitro models, a decrease of proximal and distal tubular cells was observed after the treatment with tacrolimus. TEM analysis showed a disorganized arrangement of the podocytes along the basement membrane, and a reduction of the number of microvilli, after tacrolimus treatment, with podocyte detachment at a concentration of 60 μM. Moreover, the examination of the podocyte injury in the mouse model showed similar results to those obtained in the iPSC-derived kidney organoids. Lastly, the investigation of the induction of autophagy after tacrolimus treatment showed a dose-dependent increase of the light chain (LC_3)-II/LC_3-I ratio, a classic autophagy marker; also, the number of lysosomes in kidney organoids increased after tacrolimus treatment. Taken together, these findings indicate an enhancement of autophagy in tacrolimus nephrotoxicity, but it remains unclear if autophagy induction has a protective or enhancer role in tacrolimus nephrotoxicity. To this purpose, iPSC-derived

kidney organoids were treated with tacrolimus 60 μM and/or different concentrations of rapamycin, a well-known autophagy inducer; a dose-dependent decrease of cell viability after the treatment with rapamycin was noticed, and structural alterations of the organoids were observed. These findings suggest that the augmentation of autophagy due to the treatment with rapamycin may accelerate nephrotoxicity.

Wang et al. (2022) used kidney organoids derived from human iPSCs produced from urine-derived cells from healthy donors to assess drug nephrotoxicity. Merging organoid technology with a high-throughput "read-on-ski" automated imaging and label-free detection system, the authors investigated the effects of cisplatin, celastrol, and vitamin C; the obtained automated algorithm-computed results were verified by hematoxylin and eosin staining, real-time PCR and immunofluorescence techniques. Organoids were continuously imaged for 96 h after the treatment with cisplatin, in order to let the computer vision system detect the changes in organoid texture, as tubule formation: minor changes were noticed in untreated organoids in comparison to those treated. Subsequent immunofluorescence analysis indicated higher expression levels of the proximal tubule marker KIM1 and pro-inflammatory cytokine TNF-α in treated organoids in comparison to the untreated group. About celastrol and vitamin C treatments, the automated computer vision system provided results consistent with those previously obtained with conventional biological analyses, with similar IC_{50} values obtained with the two models.

2.5 | iPSCs and cardiac ADRs

Drug-induced cardiotoxicity is one of the most challenging ADRs in drug toxicity management; for instance, it is related to many cancer drugs, which cause serious and sometimes fatal adverse effects (Huang et al., 2021). One of the more critical problems during drug development is represented by the potential cardiotoxicity of the therapeutic agent; moreover, different drugs have been withdrawn from the market due to cardiotoxicity. For instance, sibutramine, used in the treatment of obesity, was revoked in 2010 because of increased risk of nonfatal myocardial infarction and nonfatal stroke in patients with pre-existing cardiovascular disease (James et al., 2010). A promising approach for cardiotoxicity assessment could be represented by human iPSC-derived cardiomyocytes (hiPSC-CMs), and especially iPSC-derived cardiac organoids, which allow patient-specific studies.

Richards et al. (2020) generated human cardiac organoids that, under the effects of norepinephrine, were able to incorporate an oxygen-diffusion gradient. These organoids resembled the human heart after myocardial infarction (with organoid infarction protocol timeline using altered oxygen (O_2) and norepinephrine (NE) in culture for 10 days), and therefore are valuable tools for toxicological studies. The authors used infarct organoids as a model to study the non-genetic basis of drug-induced exacerbation of anthracycline cardiotoxicity. Hypoxic culture of the organoids with norepinephrine, in which microtissues were placed in a hypoxic chamber within the incubator at 10% O_2 with 1 μM of norepinephrine for 10 days, before doxorubicin (DOX) treatment, led to an increase of DOX-induced reduction in viability and to a reduction in contractile structures/organization. 2D human iPSC-CMs were used as a control. Treatment with DOX led to functional toxicity, such as cessation of beating in infarct and control organoids, in concentrations 1 and 50 μM, respectively. Furthermore, in infarct organoids, DOX treatment induced an increase in vimentin density, and this effect was noticed at a lower dose in comparison to control organoids. Overall, this study demonstrates that DOX cardiotoxicity is exacerbated by hypoxic cardiac injury.

Another study in this field was performed by Hoang et al. (2021), who engineered spatially organized cardiac organoids by micropatterning and differentiating human iPSCs. Cardiac organoids were characterized by contracting cardiomyocytes surrounded by stromal cells, and served as a platform to assess the embryotoxic potential of nine drugs ranking from safe (class A) to toxic (class X) in the past FDA pregnancy category system. The comparison between the effects of doxylamine succinate (category A drug) and thalidomide (class X drug) on cardiac differentiation showed a dose-dependent decrease after thalidomide treatment, which also leads to the generation of abnormal cardiac organoids with less cardiac tissue. On the other hand, doxylamine succinate lowered contraction velocity, effect not noticed after thalidomide treatment, which only impaired beat rate at high dosages. Amoxicillin (category B antibiotic) treatment showed no toxic effect on either function or structure of cardiac organoids, while high doses of rifampicin (class C antibiotic) contrasted organoid formation, and doxycicline (category D antibiotic) severely impaired cardiac differentiation and organoid formation even at a moderate concentration. Three other category D drugs, lithium carbonate, phenytoin, and tretinoin (all-trans-retinoic acid), were tested: lithium carbonate exhibited mild toxicity in organoid formation, phenytoin treatment produced smaller organoids compared to controls, and tretinoin produced large organoids similar to

controls, but completely abolished the cardiac differentiation. Lastly, isotretinoin (13-cis-retinoic acid, category X) showed similar effect to tretinoin, but abolished cardiac differentiation at lower concentrations.

Ma et al. (2023) studied the effects of bisphenol A (BPA), a common environmental chemical, and its analogues, in human iPSC-derived cardiac organoids by patch-clamp and confocal fluorescence imaging. Acute exposure to BPA led to a delayed repolarization and prolonged the duration of action potential, related to the inhibition of hERG K^+ channels. No ectopic excitation was found in the baseline condition, but tachycardia-like events were noticed in myocytes having drug-induced QT prolongation. Also, chemical analogues of BPA showed the same effects on cardiac excitation, even if the most pronounced effects were related to BPA exposure. This study demonstrates the pro-arrhythmic effects of BPA, which may be more pronounced in susceptible individuals, for instance, subjects with genetic mutations responsible for long QT syndrome.

2.6 | iPSCs and neuronal ADRs

Drug-induced neurotoxicity, an issue related to many drugs, can present with manifestations related to the central nervous system, or to the peripheral nervous system. For instance, chemotherapeutic agents, such as vincristine or cisplatin, are often related to peripheral toxicity, especially peripheral neuropathy (Cavaletti et al., 2019); instead, some antiepileptic drugs, such as valproic acid, are mostly related to central neurotoxicity. Drug-induced neurotoxicity can lead to states of suffering for patients, which might experience neuropathic pain, difficult to manage. Since neurotoxicity is one of the most common adverse effects of chemotherapeutic agents, to comprehend this clinical issue is necessary. To date, most of the models used for the assessment of drug-induced neurotoxicity are constituted by immortalized cell lines, such as SH-SY5Y, a cell lineage derived from human neuroblastoma. Apart from the limitations of this cell line related to its tumoral derivation, there is a lack of clearly defined neuronal subtypes obtained after the differentiation process of SH-SY5Y, constituting another issue (Bell & Zempel, 2022). Therefore, iPSC-derived neurons, which do not have tumoral derivation and better resemble human neurons (Snyder et al., 2018), and especially iPSC-derived brain organoids, offer an innovative tool for the investigation of drug-induced neurotoxicity, better resembling human neurons.

Liu et al. (2019) generated iPSC-derived cerebral organoids and used these 3D models to evaluate neurotoxic mechanisms of vincristine. Briefly, WNT-3a, CHIR99021, and SB431542 were used for the initial 2D differentiation; for the subsequent generation of the cerebral organoids, the differentiation medium was supplemented with insulin, and, for organoids maintenance, medium was supplemented with vitamin A. Immunofluorescence and RT-PCR showed the presence of forebrain markers, like FOXG1/EMX1, hippocampal markers, such as Isl1, prefrontal cortex markers, like Auts2. The cytotoxic effect of vincristine on cerebral organoids was evaluated by MTT assay: a dose-dependent growth inhibition was noticed, with an absence of toxicity for low concentrations and a subsequent blockage of neuroepithelium outgrowth with cell apoptosis for higher concentrations. Immunofluorescence techniques were used to investigate the levels of Ki-67, and cleaved caspase 3, in order to investigate the impact of vincristine on cellular proliferation and apoptosis, respectively. After exposure to vincristine, the iPSC-derived brain organoids exhibited decreased expression of Ki-67, while the expression of caspase 3 was increased. Furthermore, this analysis showed that cerebral organoids treated with high doses of vincristine displayed structural damage.

Another study in the field of brain organoids was performed by Scholz et al. (2022), who generated brain organoids from iPSC-derived neuronal precursor cells to study post-chemotherapy cognitive impairment. Brain organoids were composed by ventricle like structures, matured neurons, and glial cells near the surface, and showed functional Ca^{2+} signaling upon stimulation with glutamate. Brain organoids were treated with increasing concentrations of paclitaxel, and apoptosis was assessed at different time points after the exposure period of 14 h. The first relevant effects on apoptosis induction after drug treatment were noticed 8 h after the 14 h paclitaxel exposure, and the effects increased over time. Paclitaxel cytotoxicity was also dose-dependent, and the EC_{50} value detected was consistent with a previously published murine system (Huehnchen et al., 2017). Moreover, paclitaxel exposure negatively impacted the pool of neuronal and astrocyte precursor cells and also mature neurons.

Ho et al. (2022) performed single nucleus RNA-seq on iPSC-derived forebrain organoids from three subjects with opioid use disorder consequent to oxycodone and buprenorphine exposure, aiming to investigate drug-specific transcriptional responses. Oxycodone, a full opioid receptor agonist, is one of the most prescribed opioid drugs in the United States, while buprenorphine, acting as a partial agonist, is the most prescribed substitution treatment for opioid use disorder. Buprenorphine influenced transcription regulation only in glial cells, while oxycodone had effects in different cell types, including neurons and astrocytes. Specifically, oxycodone induced IFNγ signaling pathway, as

demonstrated by the elevation of STAT1 expression, a downstream protein in IFNγ pathway. Definitely, this study indicates that STAT1 expression in opioid use disorder might play a role in transcriptional regulation after oxycodone treatment.

2.7 | iPSCs and pulmonary ADRs

Another possible manifestation of drug-induced toxicity is lung disease, which has been described with over 300 different agents (Taylor et al., 2016). Drug-induced interstitial disease (DILD) is a common manifestation associated with several medications, and its diagnosis is often delayed because of physician and patient factors (Cosgrove et al., 2018). Interstitial lung disease comprises different lung disorders characterized by alveolar inflammation, and fibrosis (Richeldi et al., 2018; Spagnolo et al., 2021), related to fibroblast proliferation and collagen deposition. Among drugs responsible for lung interstitial disease, there are chemotherapeutic agents, such as taxanes (Long & Suresh, 2020), or antiarrhythmic drugs, such as amiodarone (Skeoch et al., 2018). Also, mTOR inhibitors, such as everolimus, are known to be responsible for severe pulmonary toxicity in the form of interstitial lung disease (Yamamoto et al., 2022). Recently many scientists focused on differentiating iPSCs into different types of lung cells and into lung organoids to study drug-induced pulmonary toxicity. For instance, Lee et al. (2023) differentiated bidimensional alveolar epithelial cells (AECs) from human iPSCs, and then obtained alveolar organoids from iPSC-derived AECs for testing chemical substances cytotoxicity. Alveolar organoids showed higher expression levels of alveolar epithelium-related genes in comparison to 2D models, and were also characterized by the presence of sac-like structures surrounded by thin-walls resembling type I pneumocytes. MTT assays after 24 h of exposure to nine chemical substances classified as "acute toxicity, inhalation," were used to assess cytotoxicity in 2D and 3D models. Specifically, the compounds investigated were chromium VI trioxide, sodium chromate, potassium dichromate, chloroacetic acid, glycolic acid, ethephon, guanidine monohydrochloride, sodium nitrite, and o-phenylenediamine. In both models taken into account, a dose-dependent decrease in cell viability was noticed. Moreover, the correlation between 2D and 3D models based on cytotoxicity was investigated calculating Pearson's correlation coefficient. The correlation between 2D and 3D models was evident for all the chemical tested, except for chloroacetic acid, glycolic acid, and guanidine monohydrochloride. Lastly, since macrophages are generally present in lung system under pathophysiologic conditions, their co-culture with iPSC-derived alveolar organoids was carried out. Co-culture system was exposed to sodium chromate for 24 h and a consequent reduction in cell viability was noticed. Moreover, organoids co-cultured with macrophages revealed to be more sensitive to sodium chromate in comparison to iPSC-derived alveolar organoids alone. Lastly, nitric oxide macrophages production, which normally occurs in response to viruses or bacteria, was assessed, and an increase in its release after high doses (50 and 100 μg/mL) of sodium chromate was detected. Therefore, nitric oxide production might be a good biomarker to predict chemicals or drugs susceptibility.

Recently, Kim, et al. (2021) generated human iPSC-derived alveolar organoids (AOs) to study pulmonary fibrosis and for drug efficacy evaluation. To obtain AOs, the protocol provided for the obtainment of alveolar epithelial cells, with a protocol previously developed by the same authors (Heo et al., 2019). On day 21 of differentiation, alveolar epithelial cells were cultured for 6 days in the presence of FGF10, IBMX, dexamethasone, FGF7, and 8-Br-cAMP. The obtained AOs exhibited an alveolar sac-like structure characterized by multiple alveoli and layers of epithelial cells. Immunofluorescence staining showed the presence of typical markers of the alveolar progenitor cells, such as EPCAM; also typical markers of alveolar epithelial type 1 and type 2 cells were found. Since TGF-1β acts as a known pro-fibrotic agent (Inui et al., 2021), AOs were treated with 25 ng/mL of TGF-1β for 72 h to evaluate their ability to respond to fibrogenic stimuli. Transcript and protein levels analyses indicated that TGF-1β significantly induced extracellular matrix-related genes, such as COL1A1, and COL1A2, and mesenchymal-related genes, such as vimentin. Furthermore, immunofluorescence staining showed an increase in the fibrotic area in comparison to untreated controls. A recent study, Fujiwara et al. (2019) indicates that milk fat globule-epidermal growth factor (MFG-E8) has anti-fibrotic properties; by chemical modification of this compound, the truncated form NP-011 was obtained. The therapeutic efficacy and the anti-fibrotic properties of NP-011 were tested in organoids in which fibrosis was previously induced with TGF-1β; as a result, qPCR showed a reduction in the levels of the ECM markers COL1A1, and COL1A2 in fibrotic AOs after the treatment with NP-011500 ng/mL. Also, western blot analysis detected a reduction of fibronectin, and collagen in organoids treated with NP-011 (Kim et al., 2021).

3 | MERGING ORGANS-ON-CHIP AND iPSCs TECHNOLOGIES

Since the clinical relevance of ADRs, there is a strong need to improve technologies for drug testing. In this regard, organs-on-chip represents a recently developed technology able to emulate the functionality and the physiological environment of human organs (Ma et al., 2021), being in this way suitable for disease modeling and drug screening. These models are normally fabricated with the organic polymer polydimethylsiloxane and present microchannels useful to precisely pattern cells; moreover, flow rate, pressure, oxygen, and pH can be controlled, allowing reproducible culture conditions (Wu et al., 2020). The combination of the usage of iPSCs and organs-on-chip technologies could revolutionize the evaluation of drug-induced toxicities in a personalized way, putting together the patient-specificity of iPSCs with a 3D model mimicking in vivo tissue characteristics. In this context, Bircsak et al. (2021) developed and validated an automatized 3D microfluidic liver-on-a-chip, called OrganoPlate LiverTox, for high throughput hepatotoxicity screening. Precisely, aggregates of iPSC-derived hepatocytes were seeded in an extracellular matrix in the organ channel and subsequently co-cultured with THP-1 cell line, able to differentiate into macrophages, and HMEC-1 endothelial cells. After confirming the proper differentiation of iPSCs into hepatocytes, a toxicologic screening of 159 compounds with known liver effects was performed, using the hepatotoxic troglitazone as positive control. Then, 21 of the analyzed compounds were chosen for dose–response evaluation: OrganoPlate LiverTox cultures were treated every 24 h, for a total of 72 h, with seven concentrations of the compounds under examination. As a result, nimesulide, azathioprine, and tolcapone induced hepatotoxicity, which was not induced after iproniazid, dexamethasone, and theophylline treatment. Similarly, Wang et al. (2021) put together iPSCs and organs-on-chip technologies to obtain a model for pre-clinical drug evaluation in progressive non-genetic angiotensin II (Ang II)-related cardiomyopathy. Cardiomyocytes derived from human iPSCs were co-cultured with human ventricular cardiac fibroblasts in an array of microchambers on a polystyrene chip. Cells were encapsulated in a fibrin based-hydrogel, enabling the generation of functional beating myocardium. On day 7 of culture, a combined treatment with Ang II and losartan, relaxin, or saracatinib was performed; the substances tested were chosen based on their efficacy in reversing Ang-II induced pathological cardiac remodeling. After a 2 weeks treatment, all three drugs alleviated passive tension elevated by Ang-II, even if a diminished active force, indicating compromised tissue contractility, was noticed after the treatment with saracatinib. The active force was instead increased by relaxin. Moreover, the system used allowed the assessment of electrical excitability: relaxin led to a significant reduction of the excitation threshold and improved the maximum capture rate. Overall, relaxin restored contractile function and reduced fibrotic remodeling induced by Ang-II.

Other scientists (Skardal et al., 2020) developed an innovative approach using human iPSC- and primary cell-derived 3D organoid technology together with organ-on-a chip systems to screen a panel of drugs withdrawn from the market by the FDA. The platform was formed by multiple tissue organoid types, comprising liver, cardiac, testis, vascular, lung, colon, and brain, able to remain viable in culture for at least 28 days. At first, the cardiotoxic and hepatotoxic effects of bromfenac, tienilic acid, troglitazone, astemizole, cisapride, mibefradil, pergolide, rofecoxib, terodiline, and valdecoxib were tested in the model developed and compared with 2D models composed by the same cells. As a result, for some of the drugs tested, differences in the cytotoxic responses were noticed between 2D and 3D models, indicating that the relationships between drug toxicity in the 3D models versus 2D models are complex and that the differences in the responses in the different models might be partly related to toxicity mechanisms. Moreover, the development of the model composed by multiple tissue organoids revealed that the presence of higher numbers of tissue types resulted in the conditioning of the media, as indicated by the presence over time in the medium of soluble biomarkers such as IL-8. This, in turn, highlights the importance of creating an environment able to mimic the complex relationships existing between the different organs of the human body, in order to set-up more representative models for drug assessment. In this context, the authors used microfluidic platforms with 6- or 5-organoid systems containing liver, lung, cardiac, endothelia, testis, and brain organoids to assess drug cytotoxicity evaluating the viability by LIVE/DEAD staining after 7 days of exposure to drugs. After 7 days of culture, the 6- or 5-organoid platforms were treated with capecitabine and cyclophosphamide; both drugs, used to treat different types of cancer, are prodrugs needing hepatic metabolism to be activated. As expected, viability was not affected in the organoid platform without the liver since the drugs cannot be converted into active metabolites. Conversely, in the organoid platforms presenting the liver, both drugs could be metabolized in the active forms and toxicity was observed in lung and cardiac organoids.

Zhang et al. (2023) developed hepatic organoids from different iPSCs and merged this technology with organ-on-chip to study DILI, creating a system suitable for large-scale testing of drugs. Human hepatic organoids were transferred on a specific chip allowing the collection of media from the different reservoirs for AST (aspartate aminotransferase), ALT (alanine aminotransferase), and albumin measurements, used to evaluate drugs' cytotoxicity. The drugs tested

were acetaminophen, fialuridine, tenofovir, inarigivir soproxil or tenofovir, and inarigivir soproxil. For all the different iPSC-derived organoids, acetaminophen increased ALT levels, while fialuridine led to an increase of both ALT and AST; albumin levels were instead reduced after the treatment with both drugs. The toxicity of the combination of tenofovir and inarigivir was assessed in 384-well plates, using a 16-point dose of tenofovir, inarigivir, or the combination of the two drugs. After 120 h of treatment, confocal microscopy revealed 100% of cytotoxicity in the organoids treated with the combination of the drugs, while a slight toxicity was noticed after the treatments with both drugs alone. This study demonstrates the suitability of iPSC-derived liver organoids, especially when associated to organ-on-chip technology, for large-scale and high throughput screening.

4 | ASSEMBLOIDS: A NEW POWERFUL TOOL FOR DISEASE MODELING AND DRUG SCREENING

Another recent and innovative technology is represented by assembloids, 3D structures formed by the fusion and functional integration of multiple cell types, able to faithfully mimic the cellular interactions normally needed for organogenesis (Makrygianni & Chrousos, 2021; Sloan et al., 2018). Firstly coined by Sergiu Paşca (Vogt, 2021), assembloids overcome the limitations of organoids, especially in the field of brain models. Indeed, brain organoids often do not fully recapitulate the native organ due to their inability to mimic the different cortical layers or to the lack of functional neuronal circuitry (Makrygianni & Chrousos, 2021). Using assembloid technology, it is instead possible to shed light and mimic the interactions between neuronal circuits, developing even more representative models of the human brain (Paşca, 2018). In addition to brain system, other organs are not completely represented by organoids, and therefore assembloids constitute a more powerful approach. Furthermore, organoids often have characteristics of fetal organs, but they do not properly represent adult organs; also this issue could be overcome with 3D assembloids, where the presence of different cell types provide the stimuli and the interactions necessary for the development of the mature organs (Makrygianni & Chrousos, 2021). To date, assembloids are used to study early events in organogenesis and as tools for the investigation of different types of clinical disorders. Moreover, creating assembloids from cells of people with different pathologies allows the development of personalized models. Furthermore, assembloids could be a powerful tool for high-throughput screening of drugs, and could potentially replace the usage of animal models. To date, most of the applications of assembloids reported in literature regard disease investigation and modeling, rather than drug toxicity evaluation. For instance, Andersen et al. (2020) generated 3D cortico-motor assembloids by putting together cerebral organoids, or hindbrain/spinal cord organoids, and human skeletal muscle spheroids. This model was used to investigate connections between corticofugal neurons and spinal spheroids, and between spinal-derived motor neurons and muscles, with the ultimate aim to set-up a model suitable for development and disease studies. Moreover, this system overcame the limits of the conventional co-cultures of motor neurons and muscular structures, such as the impossibility to maintain these cells for long-term culture. Conversely, 3D cortico-motor assembloids could be kept in culture for up to 10 weeks without structural alterations, as demonstrated by the maintenance over time of intact axons and dendrites, and by the presence of mature synapses and organized skeletal muscle fibers during long-term culture. The possibility of long-term culturing, in turn, would allow the maturation of neuronal connections, as well as myelination, thus facilitating the study of neurodegenerative disorders. Apart from brain, other scientist tried to develop assembloids of different human organs. This is the case of Kim et al. (2020), who developed bladder assembloids able to recapitulate tissue regeneration and cancer. Their model, formed by reconstituting bladder stem cells with stromal components, was composed by different layers and presented an organized architecture with an epithelium surrounding stroma and an outer muscle layer. This system faithfully resembled mature adult bladder in cell composition and gene expression, and recapitulated the dynamics of regenerative responses to injury in vivo. The authors also generated tumoral bladder assembloids to study pathophysiological features of this type of cancer. As a result, it emerged that stromal bone morphogenetic proteins (BMPs) induced tumoral FOXA1 expression and this, in turn, directs the tumor towards a specific phenotype. Another example in literature is provided by Lin et al. (2023), who investigated the interplay between epithelial crypts and their mesenchymal niche using a colon assembloid system, formed by the epithelium and different stromal cell subtypes, recapitulating mature crypts. This system also presented stem/progenitor cells able to differentiate into secretory/absorptive cells. As well as Kim et al. (2020) highlighted the role of BMPs in bladder tumors, the data obtained by Lin et al. (2023) indicated the importance of stromal BMP signaling for gastrointestinal and colonic homeostasis. Indeed, assembloids from wild-type stromal cells and BMP receptor type 1A (BMPR1A) knock-out were generated, but those knock-out for BMPR1A failed to compartmentalize remaining as spheroids, and epithelial cells did not

differentiate; this confirmed the pivotal role of BMP signaling for epithelial differentiation and crypt formation. Definitely, assembloid technology is the next generation of 3D multicellular systems, combining together different cell lines or organoids, useful for disease modeling and potentially for pharmacological studies. In this regard, the study performed by Birey et al. (2022) opens the way for assembloids as powerful tools for drug-screening. The authors obtained forebrain patient-specific assembloids from subjects affected by Timothy syndrome, a neurodevelopmental disorder related to mutations in the $Ca_V1.2$ calcium channel, and demonstrated defects in interneuron migration that could be modulated by acute pharmacological treatment with 1,4-dihydropyridine drugs, such as nimodipine and isradipine, acting as L-type calcium channel blockers.

5 | INDUCED PLURIPOTENT STEM CELLS AND CRISPR/Cas9 TECHNOLOGY

CRISPR/Cas9 is a powerful gene editing-tool widely used (Li et al., 2023) for instance to correct deleterious base mutations. Over the years, different Cas9 variants have been developed to face the complex genomic changes occurring during diseases. Indeed, Cas9 is able to cleave double-stranded DNA, but the single guide RNA often mismatches with double-stranded DNA with consequent off-target effects (Chen, 2019). Gene-editing techniques, and particularly CRISPR/Cas9 technology, have different applications, from utility as tools for disease treatment to diagnostic. In the context of disease treatment, for instance, CRISP/Cas9 gene-editing can silence aberrantly expressed genes found in many tumors (Li et al., 2023). To date, merging CRISPR/Cas9 and iPSCs technologies leads to multiple advantages, and different studies are available in the literature. For instance, Magdy et al. (2022) used iPSC-derived cardiomyocytes from pediatric patients affected by different types of cancer to investigate the role of SLC28A3 variants rs7853758 and rs885004 in anthracycline-induced cardiotoxicity. Previous studies associated rs7853758 and rs885004 SLC28A3 variants with a lower incidence of anthracycline-induced cardiotoxicity (Aminkeng et al., 2016), and the study by Magdy et al. (2022) focused on confirming these results assessing doxorubicin-induced cardiotoxicity. The role of SLC28A3 in anthracycline-induced cardiotoxicity was evaluated using CRISPR/Cas9-mediated knockout and overexpression in isogenic human iPSCs, and doxorubicin-induced cardiotoxicity was assessed by cell viability assays, activated caspase 3/7 assay, and doxorubicin uptake. The results obtained confirmed the protective effects of rs7853758 and rs885004 SLC28A3 variants, and detected a novel cardioprotective SNP, rs11140490, in the SLC28A3 locus. Moreover, high-throughput screening in patient-derived cardiomyocytes identified the SLC (solute carrier) inhibitor desipramine as a protective agent against doxorubicin-induced cardiotoxicity. Apart from the result obtained, this work exploits the possibility to use CRISPR/Cas9 technology to obtain the so-called "isogenic controls." Indeed, a major inconvenience in the field of iPSC-based disease modeling regards the inability to discriminate between the effects of the causative mutation and the genetic background of the cells (Ben Jehuda et al., 2018). Using CRISPR/Cas9 technology allows to obtain a genetically homogeneous population, only differing for the causative variant responsible for the pathology under examination. In this regard, Kim et al. (2019) used patient-derived iPSCs and CRISPR/Cas9-based technologies to study dystroglycanopathies, a group of congenital muscular dystrophies characterized by a defective glycosylation of α-dystroglycan and often related to brain malformations. Since dystroglycanopathies investigation is often limited by the lack of isogenic human cellular models of this pathology, this study aimed to create isogenic human iPSC-based models useful for drug discovery. Human iPSCs were generated from a dystroglycanopathy patient with homozygous FKRP (fukutin-related protein gene) mutation; this mutation is related to impaired α-dystroglycan glycosylation. iPSCs were corrected for FKRP mutation with CRISPR/Cas9 technology, and cortical neurons were generated from both FKRP- and CRISPR/Cas9 corrected-iPSCs. As in CRISPR/Cas9 corrected-iPSCs, also in cortical neurons derived from these cells α-dystroglycan glycosylation was restored. After a previous screening of 31,954 small compounds in a mouse myoblast line, FKRP-iPSC-derived neural cells were further used for compound validation; as a result, 4-(4-bromophenyl)-6-ethylsulfanyl-2-oxo-3,4-dihydro-1H-pyridine-5-carbonitrile (4BPPNit) significantly increased α-dystroglycan glycosylation. Chang et al. (2018) studied the myeloid malignancy and the individual contributions of SRSF2 P95L mutation and chromosome 7q deletion to the development of this pathology using patient-derived iPSCs and CRISPR/Cas9 genome editing. A panel of isogenic iPSCs, with one, none or both genetic mutations, was used for drug screening and drug sensitivity testing. The SRSF2 P95L mutation was found to be related to splicing alterations and to confer selective susceptibility to splicing inhibitors. Since SRSF2-mutant primary human leukemias respond to treatment with the splicing inhibitor E7107 (Lee et al., 2016), the effect of this drug was tested in iPSC-derived hematopoietic progenitor cells with isolated SRSF2 and isolated chromosome 7q mutations. Selective growth inhibition was

noticed in SRSF2-mutant cells, but not in isolated chromosome 7q-mutant cells or isogenic normal cells. Also, Cpd-1, Cpd-2, and Cpd-3, small molecules modulating splicing process, had inhibition effects only on SRSF2-mutant cell line and not on the isogenic normal or 7q-mutant cells. Another interesting study in this field was performed by Song et al. (2022), who focused on Wilson's disease (WD), a copper metabolic disorder caused by a defective ATP7B function. iPSCs were generated from four WD patients carrying heterozygous mutations in ATP7B gene; CRISPR/Cas9-based genome editing was used to obtain ATP7B-deficient iPSCs and heterozigously correct R778L WD patient-derived iPSCs. In this context, R778L is a common mutation in ATP7B gene in WD patients (Czlonkowska et al., 2018). Moreover, iPSCs were differentiated into iPSC-derived hepatocytes. An analysis of the expression and secretion of ceruloplasmin, a downstream copper carrier in plasma, showed decreased levels in WD patient-derived and ATP7B-deficient hepatocytes. Drug screening in WD patient-derived hepatocytes indicated retinoids as potentially useful for rescuing ceruloplasmin secretion, and abnormalities in retinoid signaling pathway were noticed by a previous transcriptome analysis. This analysis also revealed alteration in lipid metabolism in WD-specific hepatocytes, responsible for reactive oxygen species production. All-trans retinoic acid was also able to alleviate ROS production in oleic acid-treated WD-specific hepatocytes.

6 | LIMITS OF iPSCs

Despite all the benefits, iPSCs technology presents some limitations, principally due to the difficulties related to reprogramming processes, to clonal variability, and epigenetic memory. Reprogramming processes were classically performed using integrating viral vectors, such as lentivirus; nevertheless, there were worries about the incorporation of the lentiviral vector sequences into iPSCs genome (Omole & Fakoya, 2018). Therefore, other technologies using non-integrating viruses have been developed. In this context, reprogramming with Sendai virus is one of the most commonly used, due to the advantageous property of this RNA virus to not enter the nucleus (Haridhasapavalan et al., 2019) and to the high efficiency of the reprogramming process. On the other hand, this technology presents some disadvantages like the need of about 10 or more passages for a complete elimination of the virus from reprogrammed iPSCs (Borgohain et al., 2019). Therefore, some nonviral reprogramming methods have been developed, such as transfection using mRNAs or miRNAs, in an attempt to overcome limitations of virus-based reprogramming. In this regard, recently Annand (2021) described a method for the reprogramming of human fibroblasts using a cocktail of mRNAs without any base modification. In the past, mRNAs used for transfection contained modified bases, such as pseudouridine, to minimize the toxic responses. Particularly, the higher reprogramming efficiency in comparison to methods using base modified mRNAs was obtained including a cocktail of microRNAs (miRNAs). In this context, miRNAs have gained attention for their roles as modulators of embryonic stem cells gene regulatory networks, and, therefore, for the capacity of some miRNAs to influence cell reprogramming towards iPSCs (Divisato et al., 2020; Pascale et al., 2022). For instance, miR-302s, miR-369s, and miR200c are highly expressed in iPSCs and participate in the process of reprogramming to pluripotency (Balzano et al., 2018). As a matter of fact, expression of members of the miR-200 family is enhanced by Oct4 and Sox2, two key pluripotency-associated transcription factors (Medeiros et al., 2011). In addition, the miR-302/367 family, the most abundant miRNA family in human embryonic stem cells, is known to promote somatic cell reprogramming (Balzano et al., 2018). Despite many efforts in this field, the low efficiency of the reprogramming techniques employing miRNAs constitutes a limitation in iPSCs technology; thus, an implementation of this method is necessary to increase its efficiency, extending the field of application.

Another limitation related to iPSCs technology is the intra-variability of clones of the same patient. In this context, Yokobayashi et al. (2017) noticed differences among different iPSCs clones in the efficiency of the induction of iPSC-derived primordial germ-cell like cells (PGCLCs). Indeed, the PGCLCs induction efficiencies of iPSCs clones were correlated with their gene expression states in the incipient mesoderm-like cells (iMeLCs), the intermediate state between iPSCs and PGCLCs. For instance, the levels of genes such as MIXL1, or EOMES, in the iMeLCs positively correlated with the efficiency of PGCLCs generation; conversely, the expression levels of SOX3, or FGF2 were negatively correlated.

Another issue is the maintenance of a residual epigenetic memory, which can influences the iPSCs phenotype (Poetsch et al., 2022). It is known that during reprogramming of somatic cells into iPSCs, modifications in the epigenetic landscape of the cells allow the silencing of somatic cell-specific genes and the activation of pluripotency-associated genes (Guo et al., 2017; Zhao et al., 2021). In most iPSCs lines, epigenetic landscape is correctly reprogrammed; nonetheless, it can be incompletely reset in partially reprogrammed iPSCs, constituting a problem

(Poetsch et al., 2022). For instance, Phetfong et al. (2016) reprogrammed into iPSCs three different primary cell lines, human umbilical cord vein endothelial cells (HUVECs), endothelial progenitor cells (EPCs), and human dermal fibroblasts (HDFs), comparing their capacity to differentiate into hematoendothelial cells. Their results showed that HUVECs and EPCs were more efficient in iPSC reprogramming in comparison to HDFs. Also, HUVEC and EPC-derived iPSCs differentiated more efficiently into endothelial cells in comparison to HDF-derived iPSCs. On the other hand, only HUVEC-derived iPSCs could be efficiently differentiated into hematopoietic stem/progenitor cells. DNA methylation analyses indicated the existence of epigenetic memory at the endothelial genes, but not at the hematopoietic genes, in HUVEC and EPC-derived iPSCs.

In addition, there are also issues related to organoid technology. For instance, the matrices used to facilitate the three dimensional growth of organoids, such as Matrigel, could interfere with drug penetration (Genova et al., 2019). Other limitations regard the variability in the efficiency between and within the same protocols, which is sometimes also observed in monolayer culture. Moreover, many organoids present a heterogenicity of cell types, limited maturity, and limited size. The last point is due to the lack of blood vessels and vascular structures, which limits an appropriate nutrient absorption (He et al., 2020). Lastly, currently used technologies are not fully standardized, thus resulting in varying degrees of reproducibility. Moreover, many organoids did not present the cells of immune system, normally present in many native organs, where they perform several biological functions and often play a role in many diseases.

7 | CONCLUSION

Since ADRs constitute a relevant clinical issue, there is a strong need to develop innovative reliable cellular models to conduct more appropriate studies. Particularly, it is necessary to set-up personalized patient-specific models to study ADRs and to better comprehend them, allowing an investigation of any component related to the genetic background of the patients. In this context, patient-derived iPSCs represent a useful tool that revolutionized the studies in the field of ADRs, since these cells retain the same genetic heritage of the donor and can be differentiated into each tissue of the human body. Also, another recent advance in this field is the possibility to develop human organoids starting from patient-derived iPSCs. Organoids, in comparison to iPSC-derived 2D models, are 3D multicellular models which better resemble and recapitulate the native organ, allowing a more reliable study of ADRs. In addition, recently many scientists focused on the optimization of models combining 3D organoids technology with microphysiological systems based on microfluidics technology, which can mimic physiological conditions of the native organs, such as perfusion. Therefore, the implementation of these models and the development of other systems, such as assembloids, could offer the possibility to generate ever more representative 3D models of the native organs.

The advantages linked to iPSC and organoid technologies are evident especially for fragile patients, such as pediatric or elderly populations. The elderly are often affected by multiple pathologies, making difficult to perform clinical studies, while clinical trials in children are limited by ethical problems and issues in the obtainment of biological materials. Because of all these difficulties, so far most of the studies in the field of ADRs have been performed using adult-derived iPSCs; nonetheless, there are many differences in the pharmacokinetics of drugs in adults and children, and this point leads to the necessity to perform studies on children-derived iPSCs. Moreover, iPSC-derived 2D and 3D models offer the opportunity to study ADRs in organs that cannot be directly analyzed, or for which it is difficult to collect biological material (such as pancreas, or nervous system). In summary, iPSCs and 3D organoids represent more reliable models in comparison to immortalized cell lines, able to model interindividual variability, since they retain the same genetic heritage of the patients and, especially for organoids, better resemble the native organ. In this way, it is possible to study physiological processes, and mechanisms related to ADRs, in models more representative of the human physiopathology and pharmacology, obtaining more indicative results. Reported studies are examples of the potential of the use of iPSCs for the evaluation of pharmacological and toxicological effects of different drugs in several tissues of the human body. These studies highlight the reliability of iPSC-derived 2D and 3D models as tools for personalized medicine, and provide a concrete proof of the suitability of these models for ADRs assessment in the context of gastrointestinal, renal, cardiac, pulmonary and nervous systems.

AUTHOR CONTRIBUTIONS

Paola Rispoli: Data curation (equal); investigation (equal); writing – original draft (lead). **Tatiana Scandiuzzi Piovesan:** Investigation (equal); writing – original draft (equal). **Giuliana Decorti:** Conceptualization (equal); funding

acquisition (equal); supervision (equal); writing – review and editing (equal). **Gabriele Stocco:** Conceptualization (equal); funding acquisition (equal); project administration (equal); supervision (equal); writing – review and editing (equal). **Marianna Lucafò:** Conceptualization (equal); funding acquisition (equal); supervision (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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