Modelling urea cycle disorders using iPSCs

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The urea cycle is a liver-based pathway enabling disposal of nitrogen waste. Urea cycle disorders (UCDs) are inherited metabolic diseases caused by deficiency of enzymes or transporters involved in the urea cycle and have a prevalence of 1:35,000 live births. Patients present recurrent acute hyperammonaemia, which causes high rate of death and neurological sequelae. Long-term therapy relies on a protein-restricted diet and ammonia scavenger drugs. Currently, liver transplantation is the only cure. Hence, high unmet needs require the identification of effective methods to model these diseases to generate innovative therapeutics. Advances in both induced pluripotent stem cells (iPSCs) and genome editing technologies have provided an invaluable opportunity to model patient-specific phenotypes in vitro by creating patients’ avatar models, to investigate the pathophysiology, uncover novel therapeutic targets and provide a platform for drug discovery. This review summarises the progress made thus far in generating 2- and 3-dimensional iPSCs models for UCDs, the challenges encountered and how iPSCs offer future avenues for innovation in developing the next-generation of therapies for UCDs.

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INTRODUCTION

Urea cycle disorders (UCDs) encompass a set of rare inherited disorders characterised by defects in one of the urea cycle enzymes or transporters that contribute to detoxify nitrogen waste i.e., ammonia. Ammonia is produced by the deamination of amino acids and is a highly neurotoxic molecule. The urea cycle pathway is fully expressed in the liver (Fig. 1), precisely in periportal hepatocytes following a specific metabolic zonation. Six enzymes and two transporters have been identified as causing primary UCDs (Table 1), with an estimated incidence of 1:35,000 births in the USA and Western Europe. UCDs cause hyperammonaemic decompensation with cerebral oedema, resulting in a range of specific symptoms from loss of appetite, vomiting, dizziness, lethargy and seizures, to coma leading to death. Unrecognised chronic hyperammonaemia causes severe developmental delay and behavioural difficulties. Gold standard management relies on protein-restricted diet, ammonia scavenger drugs, arginine supplementation and close monitoring of plasma ammonia, amino acids and nutritional status. Liver transplantation is currently the only curative option. Gene therapy was developed targeting ornithine transcarbamylase deficiency in adults has recently shown promise as an alternative disease-modifying therapy. Some mouse models fail to recapitulate the human phenotype, such as the hypomorphic Spf6th mouse modelling ornithine transcarbamylase deficiency (OTCD), where a high residual ureagenesis capacity prevents hyperammonaemia, UCDs’ cardinal feature, under standard diet. In the Spf623 mouse, hyperammonaemia is observed with a protein-enriched diet meaning that only partial OTCD is recapitulated. In addition, traditional cell lines usually derived from immortalised cancer cell lines, have their own limitations. In vitro is often the lack of reliable models. Whilst immortalised hepatocyte-derived Huh7 or HepG2 cell lines as surrogates of human primary hepatocytes, they differ from them in many aspects. Metabolic enzymes are invariably expressed at suboptimal levels when compared to primary hepatocytes. Primary hepatocytes are considered the gold standard and provide a more biologically relevant model.

UCD-DERIVED IPSCS: PURPOSE AND ADVANTAGES

One of the challenges in modelling liver monogenic diseases in vitro is often the lack of reliable models. Whilst immortalised human liver cancer cell lines are commonly used, such as human hepatocyte-derived Huh7 or HepG2 cell lines as surrogates of human primary hepatocytes, they differ from them in many aspects. Metabolic enzymes are invariably expressed at suboptimal levels when compared to primary hepatocytes. In addition, immortalised cancer cell lines are often immature, have dysfunctional apoptotic pathways and have limited genotypic variability. Primary hepatocytes are considered the gold standard and provide a more biologically relevant model.
Fig. 1  The urea and citrulline-nitric oxide cycles. Ammonia is converted to urea via five consecutive enzymatic reactions within the liver. The urea cycle begins in the mitochondria (blue box), where ammonia and bicarbonate are converted into carbamoyl phosphate. Carbamoyl phosphate requires allosteric activation by N-acetylglucosamine (NAG). Ornithine transcarbamylase (OTC) then catalyses formation of citrulline from carbamoyl phosphate and ornithine. Citrulline is channelled out of the mitochondria via the ornithine transporter (ORNT1), whilst citrin transports aspartate from the mitochondria to the cytoplasm. Argininosuccinate synthetase then enables the synthesis of argininosuccinate from citrulline and aspartate. Argininosuccinate is subsequently broken down into arginine and fumarate by argininosuccinate lyase, with fumarate directed to the Krebs cycle. Arginine is broken down into urea and ornithine by arginase. Ornithine is transported into the mitochondria by the ORNT1 transporter. CASA carbonic anhydrase VA, CPS1 carbamoyl phosphate synthetase 1, NAG N-acetyl glutamate, NAGS N-acetyl glutamate synthase, OTC ornithine transcarbamylase, OTC1 ornithine transporter, ASS argininosuccinate synthetase, ASL argininosuccinate lyase, ARG arginase, NOS nitric oxide synthase.

recapitulating several features of a healthy liver cell in vivo. However, these cells are challenging to isolate, have limited proliferative capacity, limited viability in vitro and rapidly lose hepatic function, preventing them being maintained in culture long-term. Foetal and adult human stem cells can as well be used although they can be technically challenging to isolate and culture. Therefore, in the last few years, iPSCs have provided an alternative strategy to model inherited metabolic diseases, particularly UCDs (Table 2). These cells have the capacity to differentiate into all three germ layers, enabling the prospect of generating any cell type in vitro. In addition to averting the ethical issues associated with embryonic stem cells, one major benefit of iPSCs is the opportunity to derive cells from individuals encompassing a spectrum of genetic backgrounds, generating patients’ avatars. Protocols are constantly being optimised with the aim to produce fully mature and functional cells. These protocols utilise growth factors and small molecules which replicate in vivo development. Differentiated iPSCs can be used for an organ-on-a-chip approach. Previous work in other fields, such as cancer, has demonstrated that organ-on-a-chip approaches can yield organ-level function superior to other 2D/3D in vitro cell models. In addition, unlike 2D culture systems, organ-on-a-chip can elicit more physiologically relevant pharmacological responses, allowing for more accurate predictions of drug efficacy, toxicity and pharmacokinetics. Further, recent studies have produced multi-organ platforms, where multiple organs are integrated on a single device, which allows modelling of the more complex in vivo environment.

Utilising iPSCs can also have advantages over in vivo models, particularly when considering the ethical principles of the three Rs (replacement, reduction and refinement), which oversee animal research. In vivo animal models can be labour intensive and expensive, and the resultant animal may fail to recapitulate several features of the human phenotype. In addition, the translation of drug trials to humans from animal models can be unsuccessful due to species-specific biological responses, contributing to the translational “valley of death” and high failure rates in the approval of novel therapies. With iPSCs, multiple cell lines derived from the same patient can be cultured simultaneously in a much smaller space, with each line having the capacity to be differentiated into several tissue types, which can also reduce the time and labour required. iPSCs are therefore more easily scalable and accessible than animal models. Experiments can be compared across different tissue types to cells that are all derived from the same genetic background. Furthermore, pharmacology and toxicology assessments can be made patient-specific to avoid possible adverse side effects.

UCD-DERIVED IPSCS: STATE OF THE ART

In the last decade, iPSCs have been increasingly used as experimental models to (i) identify pathogenicity of variant of uncertain significance (VUS), (ii) study disease mechanisms, (iii) develop and screen therapeutic strategies in vitro, with a potential for in vivo applications and iPSC transplantation to treat animal models (Table 3). As the urea cycle is only able to express in its entirety in vivo, UCDs-derived iPSCs are frequently differentiated into hepatocyte-like cells. These cells are functionally assessed and compared to isogenic control lines, often generated via CRIPSR/Cas9 editing.

CPS1 deficiency

This disease has been investigated using patient-derived iPSCs, enabling the results to be compared to the clinical phenotype of the affected individuals. In this study, patient iPSC lines were genetically modified via CRISPR/Cas9 to constitutively express human codon optimised CPS1 from the AAVS1 safe harbour site and then differentiated into hepatocytes. The study aimed to assess the therapeutic potential of this approach. Despite successful differentiation, interestingly, the corrected cells failed to metabolise ammonia any more effectively than the patient cell.
### Table 1. Summary of the Urea Cycle Disorders.  

| Inheritance | Mutation | Gene | Defect | Prevalence | Main presentation |
|-------------|----------|------|--------|------------|------------------|
| Recessive   | CA5A     | Carbonic anhydrase VA | CAVA deficiency | <1/2,000,000 | Acute encephalopathy |
| Recessive   | N-acetylglutamate synthase | CPS1 | CPS deficiency | <1/2,000,000 | Hyperammonaemia |
| Recessive   | Carbamoyl phosphate synthase | OTC | OTC deficiency | <1/2,000,000 | Hyperammonaemia, chronic liver disease |
| X-linked    | Argininosuccinate lyase | ASS | Citrullinemia 1 | 1/250,000 | Hyperammonaemia |
| Recessive   | Argininosuccinate lyase | ASS | Citrullinemia 1 | 1/250,000 | Hyperammonaemia |
| Recessive   | Ornithine-citrulline antiporter | SLC25A13 | Lysinuric protein intolerance (LPI) | <1/2,000,000 | Hyperammonaemia, diplegic spasticity |

### Ornithine transcarbamylase deficiency

Some studies develop iPSC lines from peripheral blood mononuclear cells (PBMCs), such as with neonatal-onset OTCD patients. These studies show proof of concept that iPSCs can be generated from newly diagnosed patients and thus provide a model to develop a personalised therapeutic approach. Zabulica et al. functionally characterised iPSC-derived hepatocytes compared to isogenic controls and showed an isolated reduced OTC expression from day 8 of differentiation onwards and a significant reduction in ureagenesis. Importantly, the study identified only three potential off-target modifications of CRISPR/Cas9 editing, located in intronic or intergenic regions and highly likely to be biologically inactive. Laemmle et al. investigated the role of aquaporin 9 (AQ9), a membrane channel protein that facilitates transit of water, glycerol, and urea, in OTCD iPSC-derived hepatocytes. In healthy control, AQ9 overexpression enabled increased ureagenesis and a phenotype closer to the in vivo environment. OTCD iPSC-derived hepatocytes recapitulated the hepatic manifestation of the patient phenotype with reduced ureagenesis and OTC activity. This phenotype was corrected after lentiviral gene therapy.

### Citrullinemia type I

More than 130 private mutations of the ASS1 gene have been described. The identification of variant of uncertain significance (VUS) is a large-scale problem in the era of high-throughput genomics. The use of patients’ iPSCs can provide a unique model to perform functional studies to assess pathogenicity of a mutation with the additional possibility of screening personalised therapeutics. iPSCs carrying the disease-specific mutations can be artificially created and then used for functional studies as highlighted in citrullinemia type I (CTLN1). Yoshitoshi-Uebayashi et al. differentiated iPSCs from a citrullinemia patient and isogenic controls into functional hepatocytes, but failed to fully recapitulate a UCD phenotype, potentially due to incomplete hepatocyte differentiation. Baseline ammonia and urea levels between patient and control lines were not different but an ammonia challenge assay exhibited significantly reduced ureagenesis in ASS1-/- cells. Interestingly, the study highlighted significantly increased levels of several tricarboxylic acid (TCA) cycle-related metabolites such as pyruvate, succinate and aspartate, shedding light on aspartate accumulation caused by ASS deficiency and diversion towards TCA cycle.

Akbari et al. differentiated iPSCs from a CTLN1 patient into endoderm-derived hepatic organoids, following a 2-week differentiation protocol and prolonged expansion over a year. The liver organoids for both wild-type and patient cells demonstrated similar architecture and appropriate hepatocyte differentiation confirmed by expression of hepatocyte markers, e.g., HNF4A and CK19 and functional assays, e.g., albumin production, low density lipoprotein (LDL) uptake and glycogen storage. Transcriptomic analysis was similar between wild-type and patient 3D cultures indicating that ASS1 mutation did not interfere with differentiation ability. Functionally, the CTLN1 organoids had significantly increased ammonia, with overexpression of wild-type ASS1 restoring arginine levels to wild-type levels and partially rescuing ureagenesis. These data demonstrated that hepatic organoids can recapitulate several features of the disease phenotype that can be alleviated with therapies targeting gene function.
Argininosuccinate lyase deficiency

Argininosuccinate lyase deficiency (ASLD) is an atypical UCD due to a systemic phenotype affecting multiple organs. It is a challenging disease to treat as liver-targeted gene therapy is limited by its capacity, significantly reduced expression of glycolytic genes such as SLC2A1, PKM and LDHA, or GLUT1 protein suggesting that ASLD leads to the deficient glycolysis. Treatment with NO donor S-nitroso-N-acetylpenicillamine (SNAP) during osteoblast differentiation led to an upregulation of the glycolytic genes. This finding highlights the reliability of iPSCs in investigating pathophysiology and identifying new therapeutic targets.

Arginase-1 deficiency

Hui et al. studied CRISPR/Cas9-edited iPSCs lines from three patients with arginase-1 deficiency, in an attempt to restore arginase-1 activity. Corrected iPSCs were compared to unedited controls and exhibited restoration of arginase expression and ureagenesis from functional arginase. The three corrected lines could be restored. Successful gene repair was confirmed in silico being at risk of off-target effect. Neither insertion nor deletion had occurred. Such studies provide a strategy for restoring gene function by editing paving the way for genetically modified cell therapies to treat UCDs. Arginase-1 deficiency has also been studied using iPSC-derived hepatocyte-like cells, as well as iPSC-derived macrophages. Utilising PiggyBac technology alongside CRISPR/Cas9, exons 7 and 8 of ARG1 were incorporated into iPSCs derived from an arginase-1 deficient mouse model, a knockout model where mutant animals die at about 2 weeks of age, in order to assess whether ARG1 function could be restored. Successful gene repair was confirmed, but ureagenesis remained suboptimal compared to adult hepatocytes, probably caused by insufficient hepatocyte differentiation. In comparison, iPSC-derived macrophages showed corrected arginase function that could be explained by a more ready differentiation into a functionally mature state. The study demonstrated how in vitro research can be developed using iPSCs and CRISPR/Cas9 to conduct follow-up investigations in various differentiated cell types. Differentiated cells can then be transplanted in vivo to enable further maturation and long-term repopulation of hepatocytes, for instance. The same group

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### Table 2. Advantages and Pitfalls of Experimental Approaches in Disease Modelling.

| Model                                      | Pros                                                                 | Cons                                                                 |
|--------------------------------------------|----------------------------------------------------------------------|---------------------------------------------------------------------|
| Primary cells121–123                        | • Conserved functional properties                                   | • Limited availability / limited proliferation in vitro (cell type-dependent) |
|                                            | • Incorporate all cell types in heterogeneous tissue                | • Different genotype depending on donor – lack of reproducibility   |
|                                            |                                                                      | • Large variation in quality/viability                              |
|                                            |                                                                      | • Difficult to maintain cell functionality in vitro                 |
| Human cell lines122–124                     | • Expandable                                                        | • Limited genetic manipulation                                     |
|                                            | • Similar transcriptional markers and functional                    |                                                                      |
|                                            |   properties to human primary cells                                 |                                                                      |
| Human adult stem cells and human foetal liver124,125 | • Share similar properties to human primary cells                   | • Immortalised/proliferative                                        |
| Human induced pluripotent stem cells79,126,127 | • Can differentiate into any cell type                             | • Low clonal efficiency                                             |
| Organ-on-a-clip44–46,49                     | • More physiologically relevant pharmacological responses           | • Genetic manipulation can be limited                              |
| Genetically modified models128,129          | • In vivo                                                           | • Batch-to-batch variations                                         |
|                                            | • Some anatomical and physiological similarities                    | • Reduced functional resolution                                     |
| Xenograft models15,130,131                  | • In vivo                                                           | • No disease-enabling genetic background                            |
|                                            | • Interventions can be implemented at optimal time                  |                                                                      |
|                                            | • Multiple therapies can be tested on the same biopsy               |                                                                      |
| Donor Information | Cell source | Genotype | Age | Sex | Clinical severity | Purpose of study | Experimental Design |
|-------------------|-------------|----------|-----|-----|-------------------|-----------------|---------------------|
| Ornithine transcarbamylase deficiency | PBMCs | Deletion of 3 to 9 exons in OTC gene (c.366 G > A; XXY); | 4 days | M | Hyperammonaemia | Generation of stem cell line from patient | Embryoid bodies |
| Fibroblasts | PBMCs | Fibroblasts | 3 days | M | Multiple organ failure | Generation of stem cell line from patient | Embryoid bodies |
| | Fibroblasts | | 9 months | M | Hyperammonaemia | Assessment of genetic and phenotypic markers in both patient and corrected differentiated cells | Hepatocyte-like cells |
| | | | Neonatal; | M, F | Fatal neonatal hyperammonaemia; fatal acute liver failure | In vitro model of liver disease | Healthy donors |
| Argininosuccinate synthetase deficiency (Citrullinemia type 1) | Fibroblasts | p.G390R (c.1168 G > A); Exon 6, c.364-2 A > G, p. G259*; Exon 13, c.910 C > T, p. R304W | N/S | N/S | Neonatal hyperammonaemia | Generating and characterising the hepatic organoid (eHEPO) culture system | Healthy donors |
| Argininosuccinate lyase deficiency | Fibroblasts | Compound heterozygous at exon 7 and 11 | N/S | N/S | N/S | Assessment of effect of ASL loss on endothelial cells | Endothelial cells |
| | | Compound heterozygous at exon 7 and 11 | N/S | N/S | N/S | Assessment of loss of ASL on osteoblast differentiation | Osteogenic |
| Arginase deficiency | Fibroblasts | N/S | 1 F – 5 years | 1 M, 2 F | Developmental delay, microcephaly, spasticity (1 F, 5 years), other lines N/S | Implement a CRISPR/Cas9-based strategy to genetically modify and restore arginase activity | Healthy donor |
| Citrin or aspartate/glutamate carrier | Fibroblasts | Compound heterozygous (851del4; IVS16ins3kb) | 1-year old | M | Jaundice and liver failure | Assessment of differentiated patient-derived iPSCs – show that aberrant mitochondrial b-oxidation may give rise to fatty liver in citrin deficient patients | Healthy donor |
| Carbamoyl phosphate synthetase 1 (CPST) deficiency | Fibroblasts | E832X; D914H (c.4162-2 A > G); V204F | Neonatal | M, M, F | Neonatal hyperammonaemia | Assessment of differentiated patient-derived iPSCs in comparison to CRISPR/Cas9 corrected controls. | Healthy donor |

* Table 3. Summary of studies performed using iPSCs to model UCDs. *
also used transcription activator-like effector nucleases (TALENs) to correct the same defect and again, differentiated these cells into hepatocyte-like cells. These cells were then transplanted back via intravenous infusion into 12-week-old arginase-1 deficient mice, in a tamoxifen-induced ARGI knock-out mouse model. iPSC-transplanted arginase-1 deficient mice survived an additional week compared to untreated knock-out littersmates without reaching statistically significant increase of survival. The treated mice had increased arginase-1 expression but ureagenesis was not comparable to that of wild-type controls. The inability to fully rescue the phenotype may be caused by the inability for transplanted cells to repopulate the periportal hepatocytes where the urea cycle is expressed, suboptimal hepatocyte differentiation, low engraftment or immunological rejection of transplanted cells64.

Citrin deficiency

Sin et al. differentiated iPSC from a patient with citrin deficiency into hepatocytes. Functional assessment confirmed that patient cells did not exhibit ureagenesis. Genes involved in mitochondrial beta-oxidation were downregulated in the patient cells, with abnormal mitochondrial structure, leading to significantly higher levels of cellular triglycerides and lipid granule levels compared to the wild-type cells. The work was further validated by a complementary Slc25a13 knockout mouse model, which recapitulated some, although not all, of the patient phenotype75.

LIMITATIONS OF IPSCS FOR UCD RESEARCH

iPSCs models present intrinsic limitations, particularly for therapeutic application and clinical translation.

One of the significant challenges faced by iPSCs research is, despite ongoing improvements in differentiation protocols, the immaturity of iPSC-derived cells, which remain closer to foetal rather than their fully differentiated counterparts76. This has been demonstrated in modelling UCDs, where both wild-type and mutated immature differentiated hepatocytes failed to achieve equivalent functionality with that of in vivo hepatocytes53,63,74. This means that results must be interpreted with caution for UCDs and particularly when modelling disease specificities associated with age. Furthermore, differentiation efficiency and reproducibility can vary due to differing protocols, genetic backgrounds, passage number and epigenetic memory77-79. iPSCs can also acquire mutations that confer a growth advantage, particularly p53 mutations, advocating for thorough genetic characterisation prior to translation to the clinic80. With CRISPR/Cas9 mediated reprogramming, off-target effects that could adversely alter the cell function need to be carefully assessed81. However, efforts have been made to improve identification of off-targets using in vitro assays, including circularisation for in vitro reporting of cleavage effects by sequencing (CIRCLE-seq)82 and selective enrichment and identification of tagged genomic DNA ends by sequencing (SITE-seq)83, as well as improving the computational platforms used to design sgRNAs84. Another approach that can reduce the number of off-target effects is base editing, whereby a catalytically inactive Cas9 is employed together with cytidine deaminases to introduce site specific DNA mutations/corrections without the requirement for a double-strand break85.

Another iPSC limitation of use in research is that the cell population produced may not be representative of more complex in vivo architecture and morphology. Differentiation protocols often make a single cell type, whereas in vivo hepatocytes, for instance, are in close proximity to non-parenchymal cells such as stellate cells, Kupffer cells and endothelial cells, facilitating crosstalk between parenchymal and non-parenchymal cells. This heterogeneous population, in addition to nutrients, growth factors and vasculature all work together to support the genetic expression and viability of the hepatocytes86. However, there are steps to overcome this limitation with the use of co-cultures – for instance, co-culturing hepatocytes with endothelial and stromal cells aids maturation of hepatocytes87. In the context of UCDs, urea cycle activity and ammonia metabolism are confined to zone 1 located hepatocytes nearest the portal vein, as part of the regulated compartmentalisation of the hepatic lobule88. However, at present, only partial zonation is able to be achieved, which may influence how well ammonia metabolism can be modelled89. Nevertheless, these co-cultures cannot completely recapture the complexity of the in vivo environment.

A safety issue for clinical translation is the risk of teratoma formation90. This is particularly a consideration for UCDs as these diseases have occasionally been associated with hepatocellular carcinoma91. There is a risk of potential tumorigenicity and malignant tumours if transplanted cells are contaminated with undifferentiated iPSCs. However, there are treatments that could potentially treat these teratomas if they occur93. There are also purification methods such as flow cytometry sorting and small chemical molecules that initiate death of undifferentiated iPSCs which could help lower the risk of tumorigenicity. However, the effectiveness of such methods has yet to be fully elucidated94. Autologous iPSC therapies effectively eliminate the potential for immune rejection. The generation of clinical grade autologous iPSC-derived cells entails high production costs associated with a rigorous quality control system. As the generation of the patient-specific tissue of interest can take several months, this timeframe might not be suitable for neonatal-onset UCD patients, which require an urgent liver replacement strategy95. Therefore, an alternative approach is to use allogeneic iPSC-derived cell sources which would enable biobanking a limited number of approved iPSCs from assorted human leukocyte antigen (HLA)-homozygous donors that would match the bulk of the population96. These cells would have all undergone regulatory clearance and have been thoroughly tested meaning that the cells could be more readily used to treat patients who require timely intervention. However, patients would be required to undergo lifelong immunosuppression treatment.

For clinical trial applications, ethical consent should be obtained freely. For children, eligibility should be considered only if there is minimal or low risk with potential for direct benefit, and the procedure is as favourable as available alternatives97. Regulatory guidelines such as the US Food and Drug Administration (FDA)98, the European Medicines Agency (EMA) (https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-human-cell-based-medicinal-products_en.pdf) and the International Society for Stem Cell Research are in place to ensure that patient safety is at the forefront99-101. Adhering to these guidelines, alongside potential intellectual property and patent considerations, can add substantial time and effort to the setting up and delivery of iPSCs studies in accordance with Good Clinical Practice. Additionally, scalable technology to meet regulatory standards remains a challenge for generating genetically modified iPSCs for cell therapy, as it requires special equipment alongside skilled operators102-104. Thus, these limitations may prevent potential therapeutic treatments from reaching patients due to prohibitive cost and time requirements.

FUTURE DIRECTIONS

iPSC differentiation provides a unique opportunity to study a genetic disease in a specific genetic and epigenetic background in vitro and screen for therapeutic candidates. iPSCs have improved our knowledge of the pathophysiological mechanisms of several diseases, alongside accelerating progress in our understanding of human physiology at the cellular level. In targeting UCDs, iPSC differentiation into hepatocytes has been the
primary focus as the urea cycle is only expressed in totality in this cell type. Optimisation of the hepatocyte differentiation is key in reproducing a defective ureagenesis. Differentiation in additional cell subtypes has enabled to study novel mechanistic approaches and pathophysiology, especially in endothelial cells and osteoblasts in ASLD. In the future, iPSC differentiation towards other cell types, particularly involved in the specific pathogenesis of the central nervous system, will be of utmost interest.

iPSCs can be used as a platform for drug discovery, testing different pathways of UCDs for potential therapeutic treatment. For example, induced autophagy has recently been shown to increase ureagenesis, via the cell-penetrating autophagy-inducing Tat-Beclin-1 (TB-1) peptide in OTC and ASL deficiencies when tested in an animal model. This approach could be translated to drug screening using iPSCs for other compounds. Drug screening via this approach has been tried in different contexts, including using Sendai virus, and would be amenable to discover novel therapies to restore ureagenesis in UCD or to protect the brain in the context of hyperammonaemia.

Advancements in both iPSCs differentiation protocols and CRISPR/Cas9 techniques are continually emerging. For instance, recently researchers have managed to cultivate four organs in a microbioreactor from iPSCs from a single donor. The organ models were positioned in separate compartments of the bioreactor and connected by a microfluidic network. In addition, another study has generated an in vitro whole-organ “Bioreactor grown Artificial Liver Model” (BALM) which enables the long-term 3D culture of iPSC-derived hepatocyte-like cells. This model also allows the transduction of adeno-associated viral and lentiviral vectors, meaning this model could provide a more representative preclinical therapy testing environment. There are also numerous techniques to help identify off-target effects associated with CRISPR/Cas9 editing, such as in vitro and cell-based assays. These allow off-target effects to be more readily assessed and thus can be taken into account when performing differentiation experiments. Alongside this, the rapid evolution of next-generation sequencing techniques provides means to analyse transcriptomics and chromatin accessibility of both iPSCs and differentiated cell populations. Utilising different multi-omics approaches allows refinement of in-depth analysis and the discovery of novel pathophysiological mechanisms.

CONCLUSION
UCD-derived iPSCs are progressively expanding their applications in several areas, from supporting a genetic diagnosis in the genomic era, studying pathophysiology to drug development and validation. Differentiated iPSCs creating patients’ avatars are of particular interest in proof-of-concept studies where they recapitulate key aspects of the disease in a specific disease-enabling genetic and epigenetic background, allowing novel pathophysiological insights. By providing this invaluable opportunity to analyse a genotype of interest within the genetic background of the patient, this allows functional studies in a physiologically relevant model. iPSCs offer the unique opportunity to implement personalised medicine and perform drug screens. The field still faces several challenges, particularly with optimising iPSCs differentiation and successfully translating proof-of-concept studies to effective long-term treatments whilst minimising the risk of adverse effects. While considerable efforts are in place to overcome these challenges, iPSCs present a promising avenue for UCD research and therapy development.

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C.D. and J.B. designed the study. C.D. wrote the manuscript. All authors reviewed and approved the manuscript.

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Competing interests
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npj Regenerative Medicine (2022) 56