



DefiniGEN
DISEASE MODEL INNOVATION

Case study

Generating human preclinical data
for candidate therapeutics against
Alpha-1-antitrypsin deficiency

Alpha-1-antitrypsin deficiency (A1ATD) is a disease which manifests in the liver as a result of an inherited mutation in the SERPINA1 gene (E342K ZZ being the most prevalent) and results in the misfolding of the A1AT protein, forming aggregates in the endoplasmic reticulum of the hepatocytes thereby causing liver damage. In addition, the lack of active A1AT protein in the bloodstream causes a failure to inhibit neutrophil elastase in the lungs, leading to chronic and life-threatening lung damage.

Background

A1ATD is a multifocal disease affecting the lungs and the liver. Efficacious treatments have been developed to alleviate disease progression in the lungs, but no equivalent treatments exist for the liver. Currently the only curative option is a liver transplant. Therefore, there is a clinical need to develop novel treatments for A1ATD-induced liver disease.

Presently there are a number of therapeutic techniques at various stages of clinical evaluation. These include the modulation of A1AT polymer folding or transport through the targeting of chaperone proteins and reducing A1AT polymer accumulation in hepatocytes via siRNA-mediated mRNA degradation or induction of autophagy-dependent protein polymer clearance.

As yet, no treatment has proven to be both safe and efficacious in the treatment of A1ATD-induced liver disease, meaning that the clinical need remains unmet.

Through our unique and innovative differentiation platform, DefiniGEN provides a unique service offering for the profiling and evaluation of therapeutic candidates being developed for A1ATD. Human hepatocyte-like cells are generated from induced pluripotent stem cells (iPSCs) using a proprietary differentiation protocol and display relevant key hepatocyte markers. The Def-HEP A1ATD cells recapitulate observations as seen in human A1ATD livers, thus making it a phenotypically relevant model.

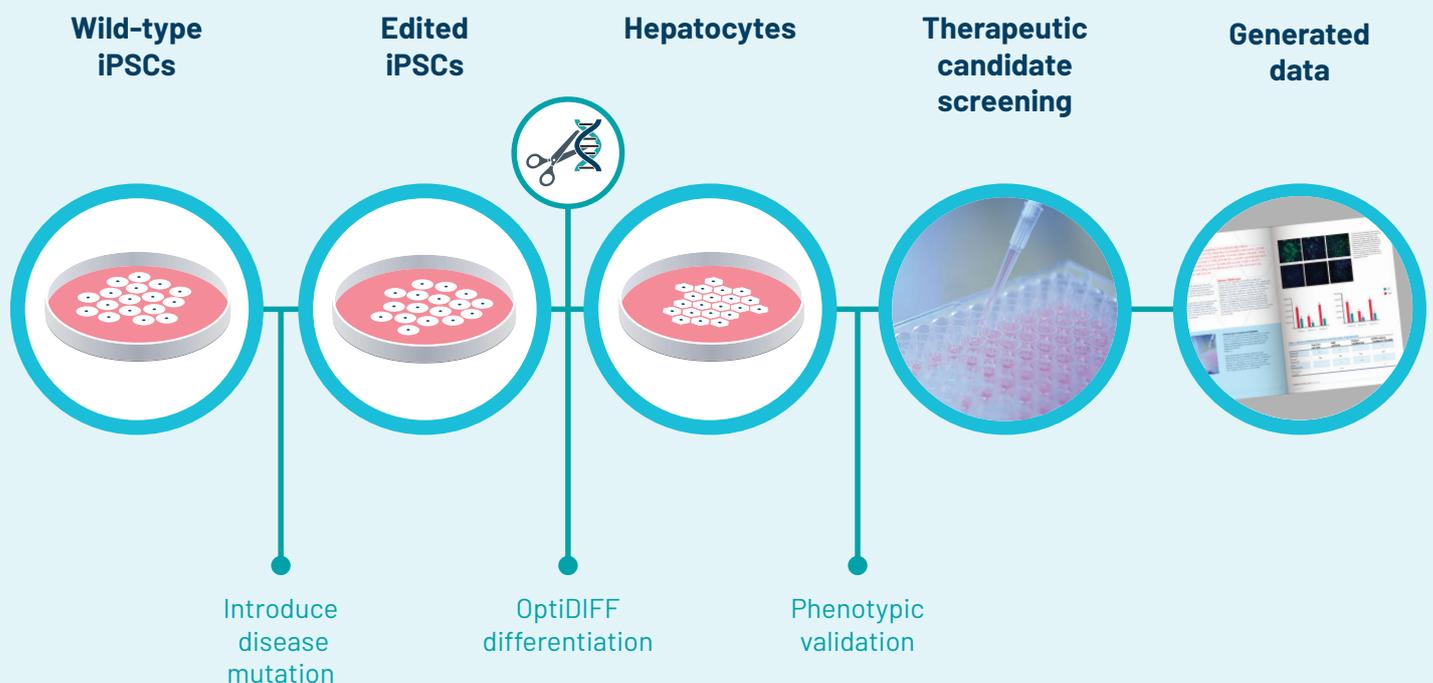


Figure 1, Schematic overview of DefiniGEN's disease modelling platform: introduction of relevant disease mutation, iPSC differentiation into hepatocytes, phenotypic validation with bespoke bioassay development.

Methodology

To initially validate hepatocyte function in DefiniGEN's iPSC-derived HLCs, our cells were differentiated under DefiniGEN's OptiDIFF platform, and differentiation was assessed by measuring mature hepatocyte marker expression (albumin, A1AT) as well as presence of active gluconeogenesis/glycogenolysis. Albumin and A1AT expression levels were determined at mRNA and protein level by qPCR and ICC, respectively, and compared to levels seen in primary human hepatocytes (PHH). Active gluconeogenesis/glycogenolysis was confirmed by measuring glucose secretion following glucagon stimulation in a time-dependent manner using a commercially available glucose kit.

Following successful validation of hepatocyte functionality, the SERPINA1 mutation E342K (Glu342Lys) was introduced in DefiniGEN's wild-type iPSCs by CRISPR gene editing. iPSC pluripotency was assessed by qPCR (NANOG, SOX2, OCT4 expression), and genotype confirmation by Sanger sequencing. Finally, accumulation of polymeric A1AT and response to autophagy-inducing compounds and nucleotide-based therapeutics was determined by ICC using a high-content imaging analyzer (CellInsight CX7).

Results

1) Characterization of our hepatocyte-like cells

Using our proprietary differentiation protocol, which derives a very pure definitive endoderm population early in the cell generation process, DefiniGEN's terminally differentiated hepatocytes have comparable levels of key markers (albumin, A1AT, HNF4a and AFP) to primary human hepatocytes (PHH).

They display low density lipoprotein (LDL) incorporation, a demonstrably functional urea cycle, gluconeogenesis/glycogenolysis and have also been characterized to show the conservation of many cellular processes which are vital for in the *in-vitro* modeling of monogenic liver conditions.

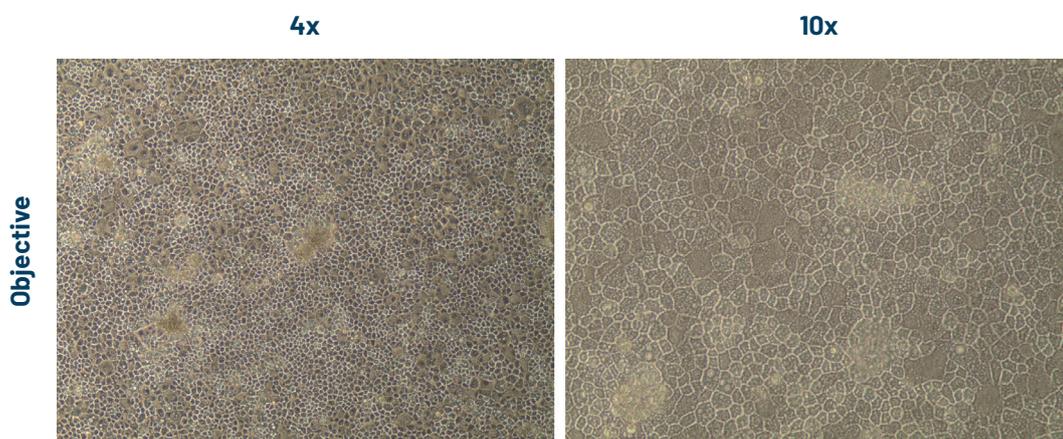


Figure 2. DefiniGEN hepatocyte-like cells (HLCs) demonstrate the characteristic cobblestone morphology, and the presence of a uniformed monolayer following >3 weeks of iPSC differentiation.

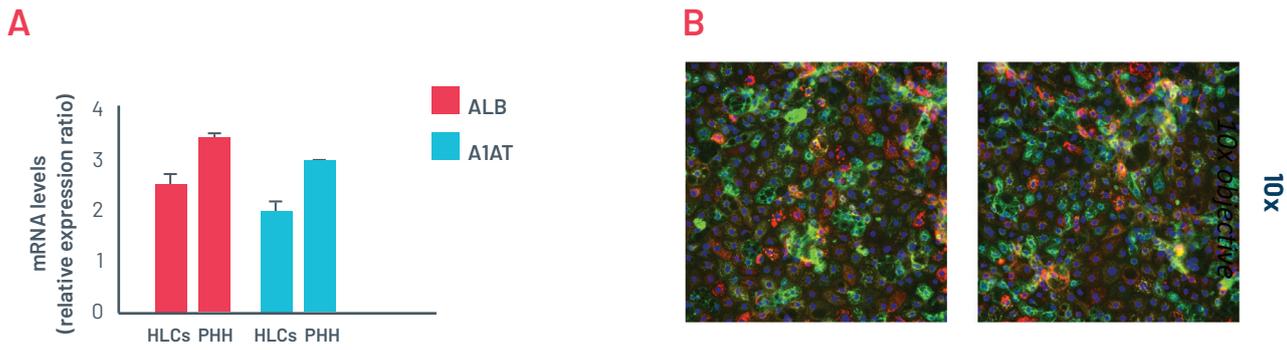


Figure 3. A) mRNA expression levels of albumin (ALB) and alpha-1-antitrypsin (A1AT) in DefiniGEN mature hepatocyte-like cells (HLCs) and primary human hepatocytes (PHH). B) Representative pictures of mature HLCs revealing protein expression of the hepatocyte maturity markers albumin (green) and alpha-1-antitrypsin (red). Data are presented as mean±SD of n=3 biological replicates. mRNA expression data were normalised to PPIA.

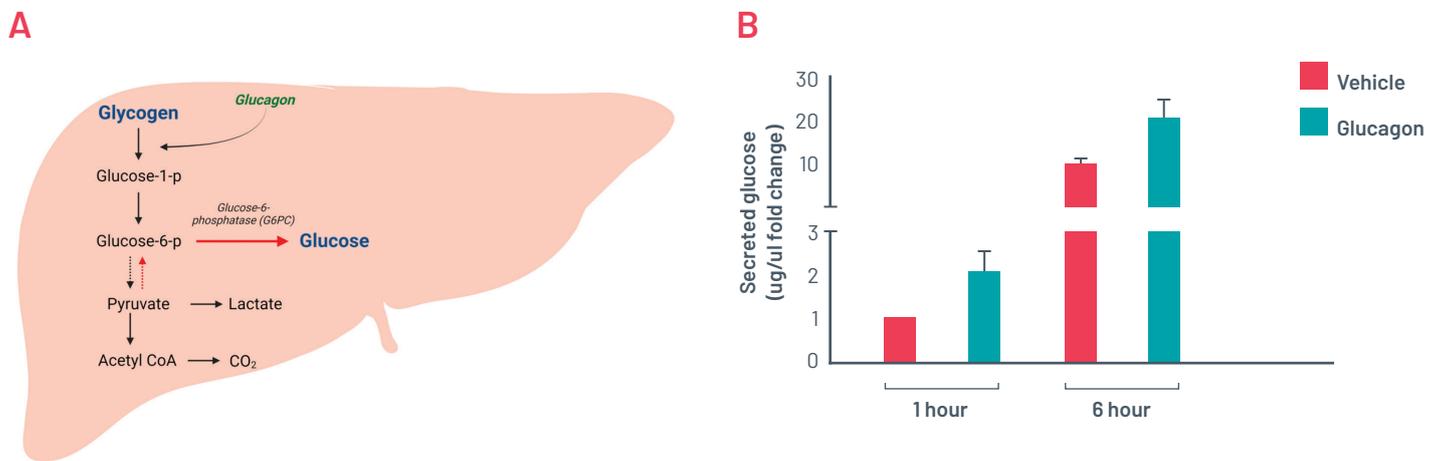


Figure 4. A) Simplified schematic on the gluconeogenesis/glycogenolysis pathways within human liver. B) Media glucose levels from mature HLCs treated with glucose-free media for 1h and 6h with or without glucagon, suggesting the de novo synthesis of glucose from non-lipid precursors, accompanied by functional glycogenolysis pathway additionally contributing to total glucose levels. Data are presented as mean±SD of n=2 technical replicates.

2) CRISPR Alpha-1 Antitrypsin Deficiency Model

DefiniGEN have an iPSC line which has been selected out of many hundreds of lines for its robust differentiation characteristics, and, by using CRISPR gene editing, have generated a ZZ mutant with an otherwise healthy genetic background for primary screening activities.

CTCCCCTCCAGGCCGTGCATAAGGCTGTGCTGACCATCGACGAGAAAGGGACTGAAGCTGCTGGGGC
 CTCCCCTCCAGGCCGTGCATAAGGCTGTGCTGACCATCGACAAGAAAGGGACTGAAGCTGCTGGGGC

Figure 5. The area highlighted in yellow shows homozygous E342K mutation (GAG > AAG) in the clone sequence.

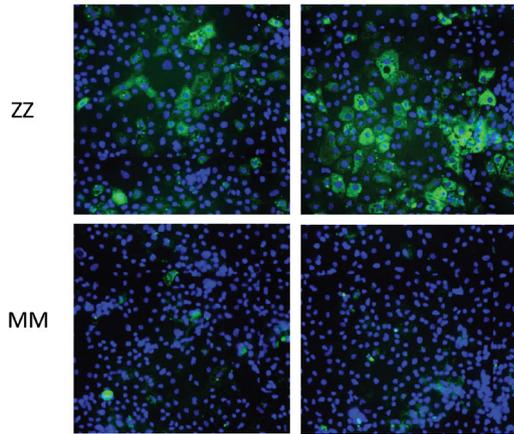
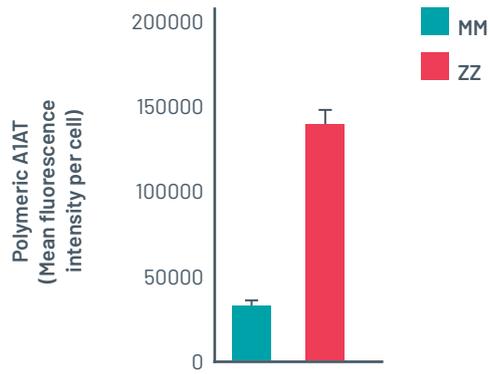
A**B**

Figure 6. Immunostaining with polymer-specific A1AT antibody shows intracellular accumulation of polymeric A1AT in ZZ hepatocyte-like cells (HLCs) vs. isogenic MM HLCs. B) Quantification of the intracellular levels of polymeric A1AT in ZZ hepatocyte-like cells (HLCs) vs. isogenic MM HLCs. Mean fluorescence intensity (MFI) was normalised to number of nuclei. Results presented as mean \pm SEM of n=3 independent experiments.

3) Phenotypic Validation

Polymer build up in the livers of A1ATD patients, and DefiniGEN's model directly measures hepatic polymer accumulation which is also used as a clinical endpoint for the in-vivo testing of candidate therapeutics.

An optimized immunofluorescent bioassay for polymeric A1AT gives a reliable endpoint, and treatment windows for different molecular entities have been established.

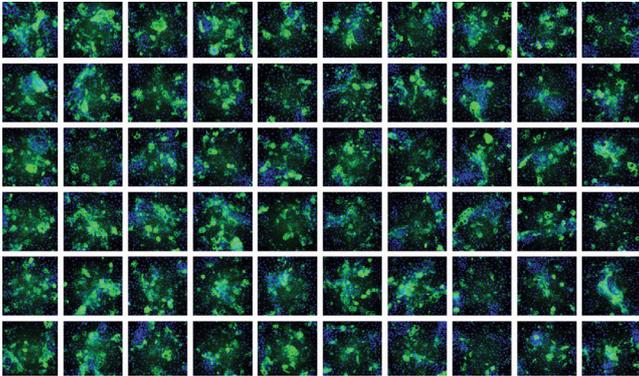
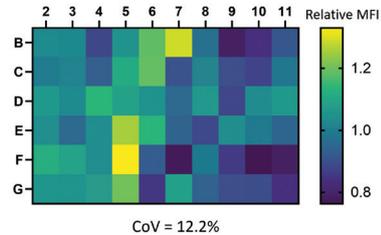
A**B**

Figure 7. Immunostaining with polymer-specific A1AT antibody shows levels of polymeric A1AT in ZZ-HLCs across the central wells of a 96 well plate. Representative images of 3 independent experiments. B) Quantification of the intracellular levels of polymeric A1AT in in ZZ-HLCs across the central wells of a 96 well plate relative to the average of the plate set as 1. Results expressed as average from n=3 independent experiments.

4) Phenotypic Recovery

A1ATD HLCs respond to reference drug carbamazepine (CBZ)

Autophagy-inducing compounds including carbamazepine have been shown to accelerate the clearance of polymeric A1AT in hepatocytes, and DefiniGEN have used this compound as our standard positive control in every plate run for small molecule screens.

Recent advances in gene therapies and innovative small molecule approaches offer new hope for curative treatments, however there is a paucity of pre-clinical human models available to assess efficacy. DefiniGEN offer screening services on metabolically competent hepatocytes with a mature phenotype, to help support drug development for this indication and generate valuable human data for your candidate therapeutics. Our cells give robust responses to carbamazepine and other autophagy inducing small molecules.

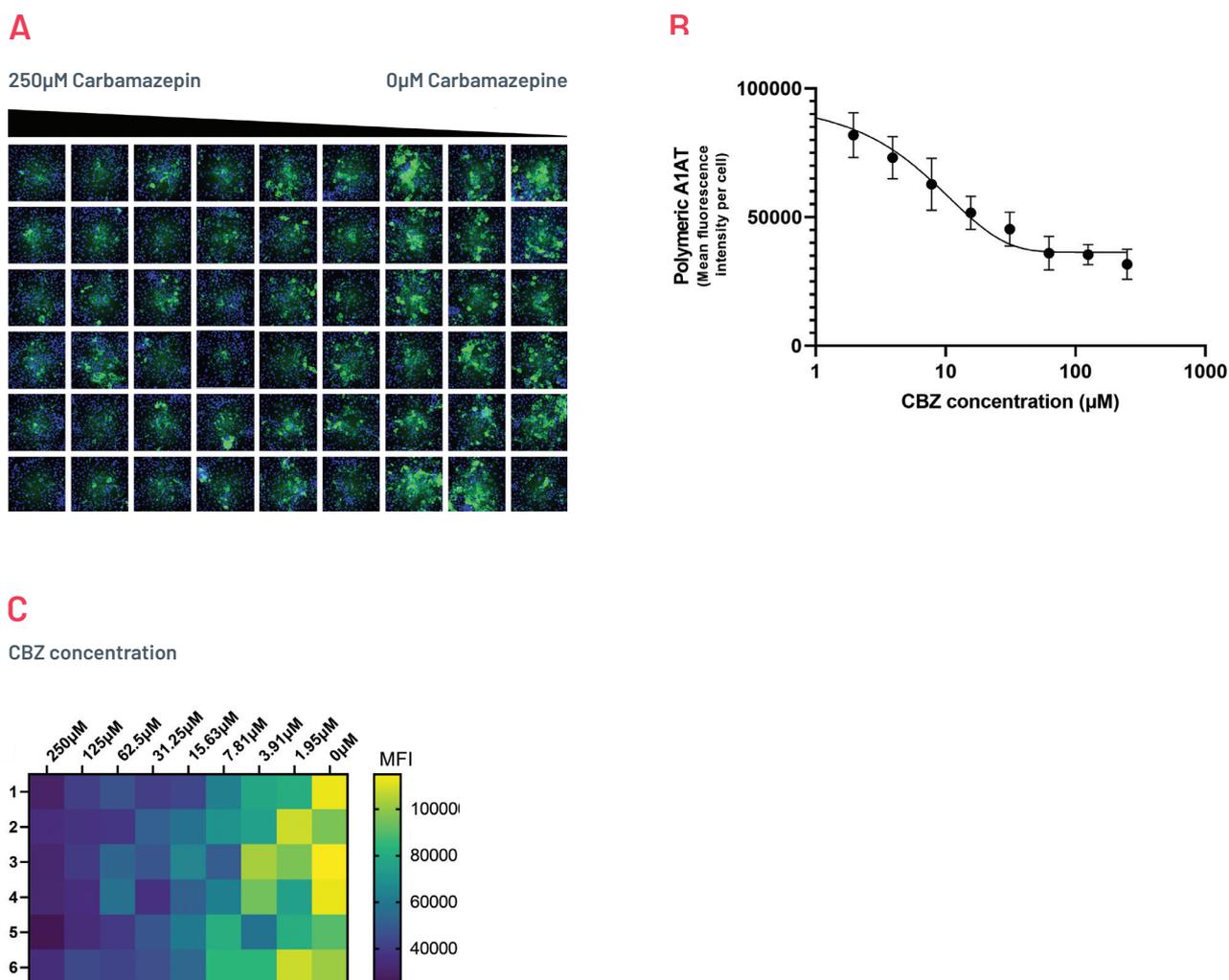


Figure 8. A) Intracellular accumulation of polymeric A1AT in ZZ-HLCs in response to treatment with different concentrations of Carbamazepine following immunostaining with polymer-specific A1AT antibody. B) Quantification of the intracellular levels of polymeric A1AT showing MFI per cell upon treatment with CBZ. Results expressed as mean±SEM of n=4 independent experiments. C) Heatmap showing quantification of polymeric A1AT across a 96 well plate upon treatment with decreasing concentrations of CBZ, representative of 4 independent experiments.

A1ATD HLCs show little plate to plate variability in their response to CBZ

A

Concentration CBZ (μM)	<i>n</i>	Range (MFI)	Average	SD	CoV
250	3	25653-31229	27868.88	2092.384	7.5%
125	3	28207-38516	32909.47	3686.746	11.2%
62.5	3	32472-46180	37477.22	5349.25	14.3%
31.25	3	34072-45487	41553.91	4583.86	11%
15.625	3	46412-55312	49825.14	3392.976	6.8%
7.8125	3	50119-68717	61781.53	7184.113	11.6%
3.90625	3	67485-82122	72726.24	5766.531	7.9%
1.953125	3	68676-91419	82030.9	8399.951	10.2%
0	3	70655-104489	87771.57	11964.4	13.6%

B

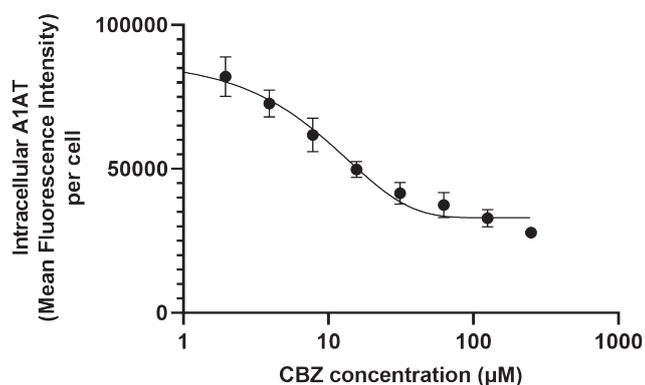


Figure 9. A) Quantification of polymeric A1AT in ZZ-HLCs in response to increasing concentrations of Carbamazepine (CBZ) across several plates. B) Quantification of the intracellular levels of polymeric A1AT showing mean fluorescence intensity (MFI) per cell. An average of 3 plates run in parallel.

4.1 Phenotype Recovery using autophagy compounds

A1ATD HLCs respond to novel autophagy-inducing compounds

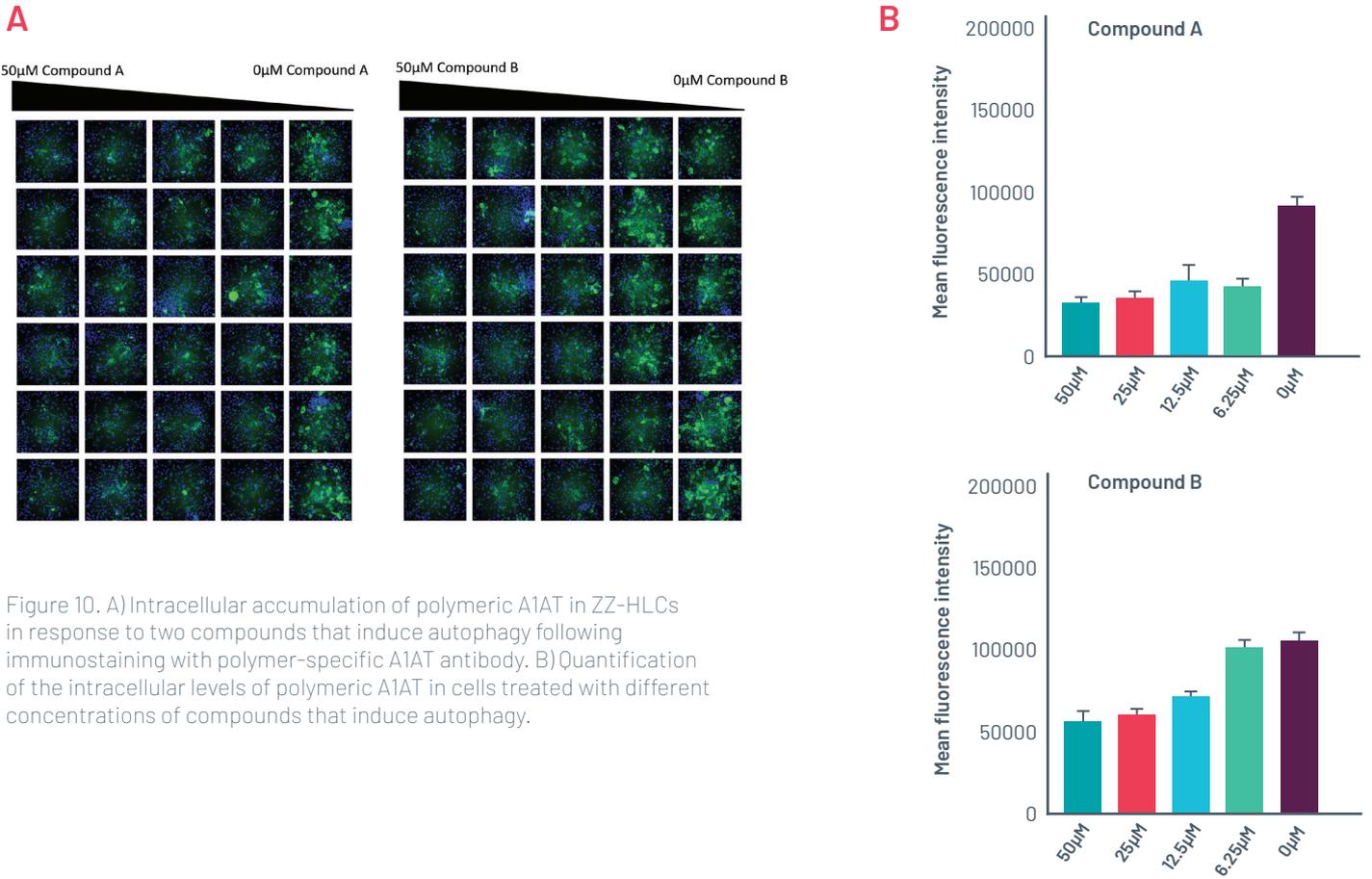


Figure 10. A) Intracellular accumulation of polymeric A1AT in ZZ-HLCs in response to two compounds that induce autophagy following immunostaining with polymer-specific A1AT antibody. B) Quantification of the intracellular levels of polymeric A1AT in cells treated with different concentrations of compounds that induce autophagy.

4.2 Phenotypic recovery using Nucleotide based therapeutics

Def-HEP A1ATD cells are readily transducible or transfectable, and lend themselves to the evaluation of gene therapy approaches, from siRNA to viral vectors or base-editing approaches. Our clients can send their compounds or reagents directly to our facility in Cambridge UK and data can typically be generated in 6-8 weeks.

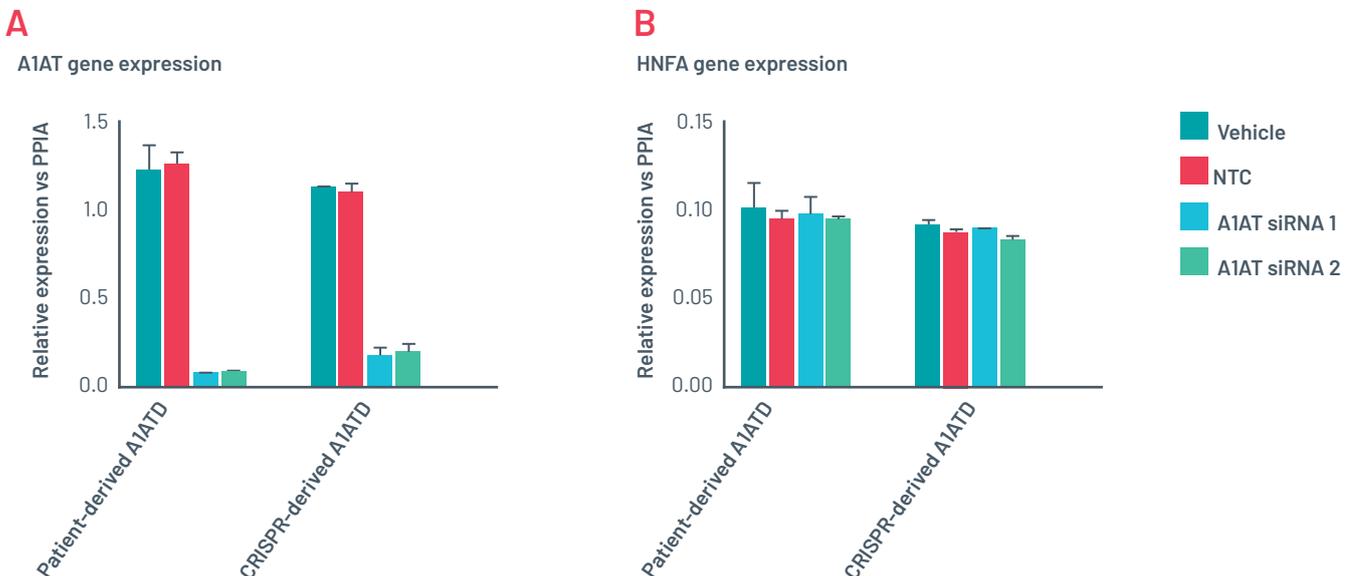


Figure 11. Gene expression of Def-HEP A1ATD 48h after transfection with scramble siRNA (NTC) and two different combinations of siRNA targeting SERPINA1(A1AT). Gene expression of A1AT dramatically decreases with both sets of siRNAs(A) but other genes such as HNF4a (B) remain consistently expressed.

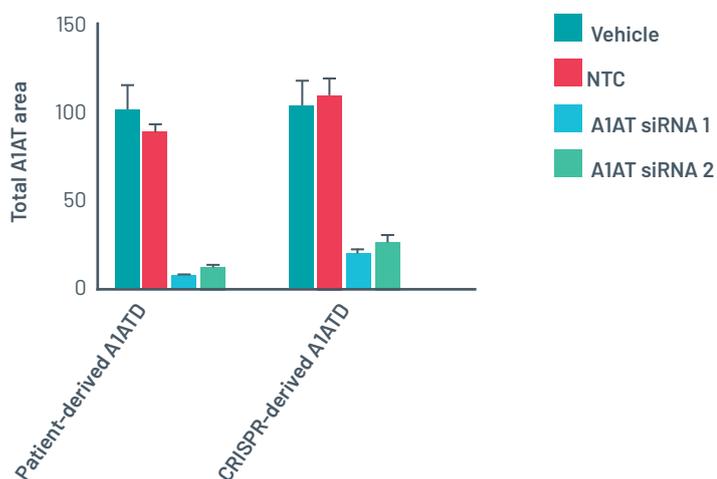
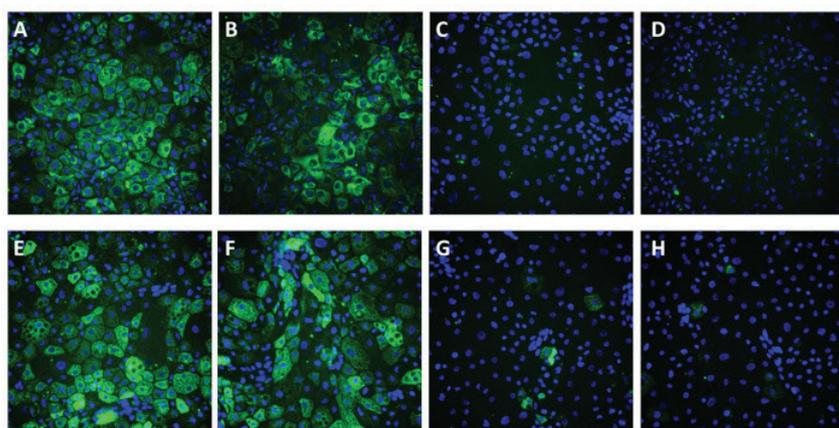
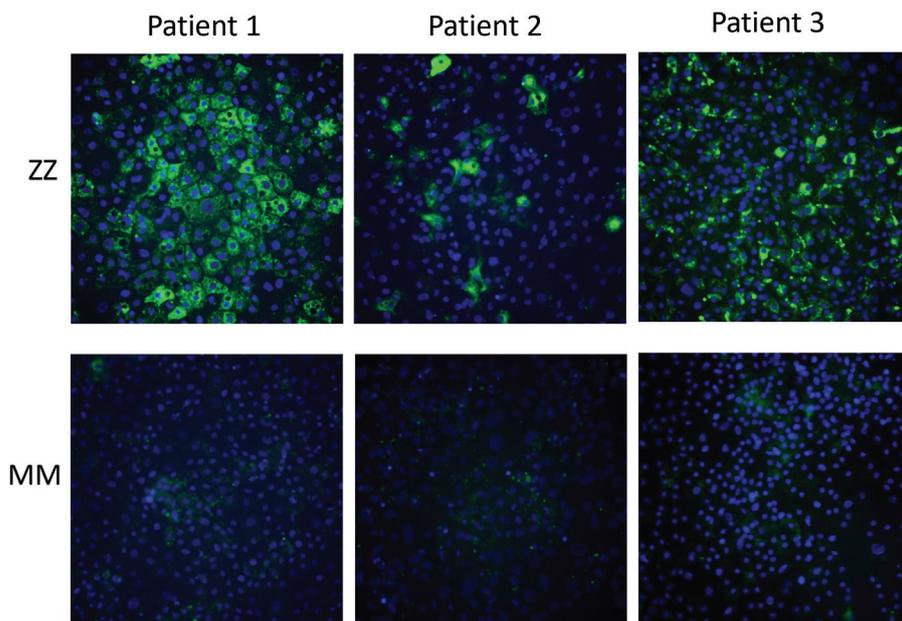


Figure 12. Immunofluorescence for polymeric A1AT. Representative pictures of polymeric A1AT staining for patient derived A1ATD hepatocytes transfected for 72h with Vehicle (A), Scrambled siRNA (B), and two different combinations of siRNA targeting SERPINA1 (C, D). Representative pictures of A1AT staining for CRISPR-derived A1ATD hepatocytes transfected for 72h with Vehicle (E), Scrambled siRNA (F), and two different combinations of siRNA targeting SERPINA1 (G, H). Quantification of polymeric inclusions of A1AT per cell in different conditions.

5) Alpha-1 Antitrypsin Deficiency screening in a panel of patient lines

iPSC derived models used in combination with well characterised bioassays can be used to screen drug candidates for efficacy, examine the effects of donor variance and perhaps ultimately inform the make-up of clinical cohorts. Alpha-1 antitrypsin deficiency can be modelled using immunocytochemistry as an endpoint in DefiniGEN's HLCs from a panel of patients.

A



B

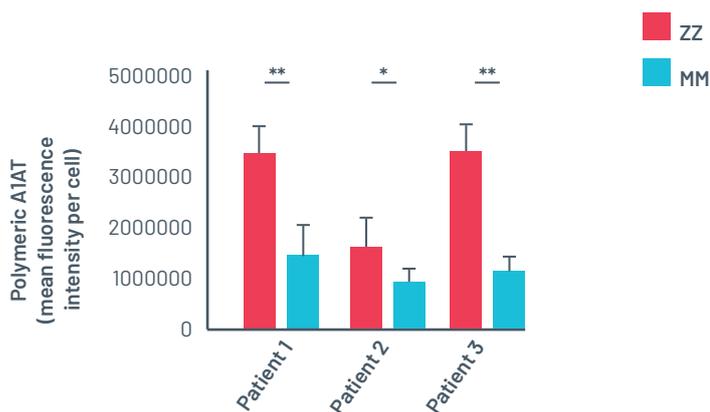


Figure 13. A) Representative images of untreated ZZ-HLCs from a panel of A1ATD patients stained for the polymeric form of A1AT compared to their corrected MM-HLCs. B) Quantification of polymeric A1AT in ZZ-HLCs vs. their corrected MM-HLCs. Data presented as mean \pm SEM of n=3 biological replicates. *p<0.05 **p<0.01

Patient A1ATD HLCs show A1AT polymer accumulation consistently

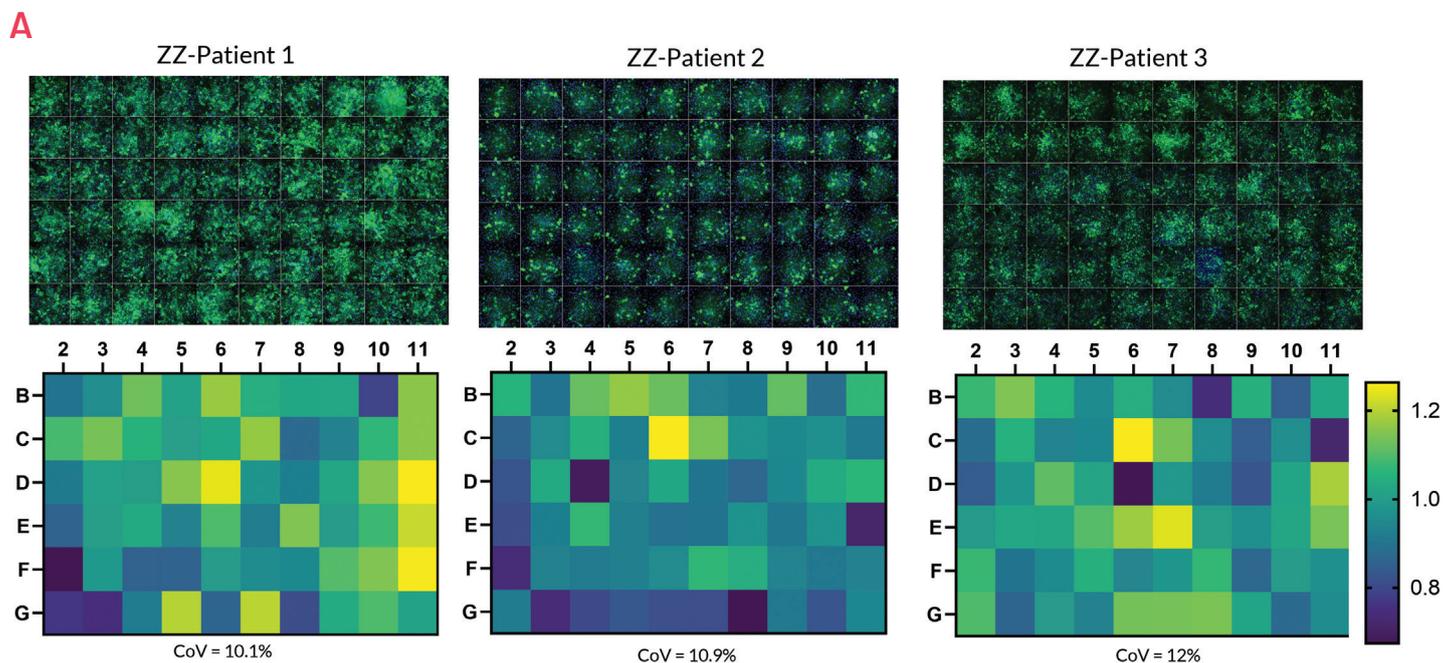


Figure 14. Intracellular accumulation of polymeric A1AT in patient-derived A1ATD HLCs across the central wells of a 96 well plate following immunostaining with polymer-specific A1AT antibody. Representative images of 3 independent experiments. B) Quantification of the intracellular levels of polymeric A1AT in A1ATD HLCs across the central wells of a 96 well plate (n=3).

Patient A1ATD HLCs respond to reference drug carbamazepine (CBZ)

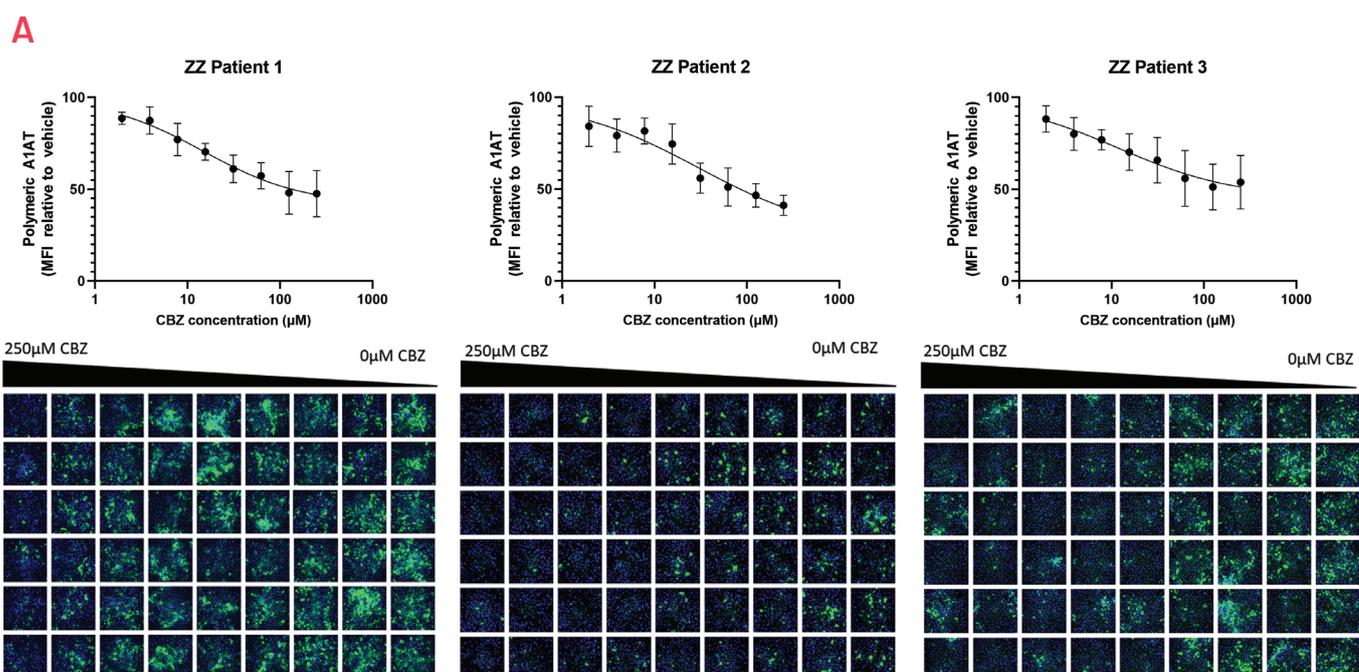


Figure 15. A) Quantification of intracellular polymeric A1AT in 3 different patient-derived ZZ-A1AT HLCs in response to carbamazepine (CBZ) (n=3). Results expressed relative to vehicle set as 100. B) Representative images of the polymeric staining.

Conclusion

Our data demonstrates, for the first time, a robust, highly predictive, and tangible alpha-1-antitrypsin *in vitro* model for large-scale drug screening and efficacy. Unlike other commercially available products and services, our CRISPR-derived A1AT cells can reproducibly recapitulate the disease phenotype in-a-dish, revealing increased polymeric alpha-1-antitrypsin formation and accumulation compared to their isogenic controls. Crucially, the observed phenotype can be pharmacologically reversed, making our model a unique platform for the screening of novel chemical compounds and nucleotide-based therapeutics with quick turnaround, and the accurate identification of potential drug candidates that can move to next-phase clinical trials with significant success. Last, but not least, the superiority of our iPSC-derived HLCs in the demonstration and long-term maintenance of gold-standard physiological hepatocyte functions provide a great opportunity to investigate additional key aspects of drug screening, including drug tolerance and cytotoxicity, opening promising scientific avenues in the field of drug-induced liver injury.

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