

APPLICATION NOTE

**Developing a translational  
biomarker panel for use  
in pre-clinical advanced  
*in vitro* studies of  
Non-alcoholic  
steatohepatitis**

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# Introduction



Significant efforts to identify non-invasive clinical biomarkers, and profile proteomic and transcriptomic changes at different stages of non-alcoholic steatohepatitis (NASH)\* disease progression, have recently been made. Despite these insights there is still much to learn about this multi-faceted disease and, after years of R&D, only one regulatory approved therapeutic is currently available for its treatment.

A pressing need for more human relevant pre-clinical tools to support therapeutic advancement has led to the development of Microphysiological systems (MPS) that more closely model the human liver and NASH<sup>1</sup>. To predict novel therapeutic efficacy and aid data translatability between the laboratory and clinic, MPS models of NASH must enable progression into an advanced phenotype and express a wide range of clinically relevant biomarkers of inflammation and steatosis.

Here, we describe a NASH MPS model comprised of primary human hepatocytes (PHHs) and non-parenchymal cells (human Kupffer cells and human stellate cells) that form 3D microtissue structures when cultured together using our PhysioMimix<sup>®</sup> Organ-on-a-chip (OOC) System. We explore the model's ability to recapitulate an advanced NASH phenotype, characterized by fibrosis, and quantify the expression of biomarkers.

The results of this study demonstrate that the PhysioMimix<sup>®</sup> NASH MPS delivers a more advanced phenotype versus traditional *in vitro* approaches and expresses quantifiable levels of biomarkers that have shown promise in clinical studies<sup>3-6</sup>. We also confirm clinical findings for the biomarker thrombospondin- 2, which was shown to be differentially expressed in 191 patients, stratified by fibrosis stage, in a NASH proteomic biomarker study by the 'Litmus Partners'<sup>2</sup>.

The PhysioMimix NASH MPS was challenged by four anti-NASH compounds that entered human clinical trials to assess their efficacy. A panel of inflammatory, fibrosis and steatosis biomarkers were used to report each compound's ability to resolve the disease phenotype and aid data translatability between laboratory and clinic.

\*NASH is also known as Metabolic dysfunction-associated steatohepatitis (MASH) following proposed nomenclature changes in 2023.

# Materials and Methods



The PhysioMimix® NASH MPS combines in-house 3D-validated tri-cultures of primary human hepatocytes (PHHs), and non-parenchymal cells (human Kupffer cells and human stellate cells). The model is established using the PhysioMimix® Multi-chip Liver-12 plate (**Fig 1**) in medium containing free fatty acids (FFAs) and sugar (referred to as HEP-Fat media) for 14 days (**Fig 2**).

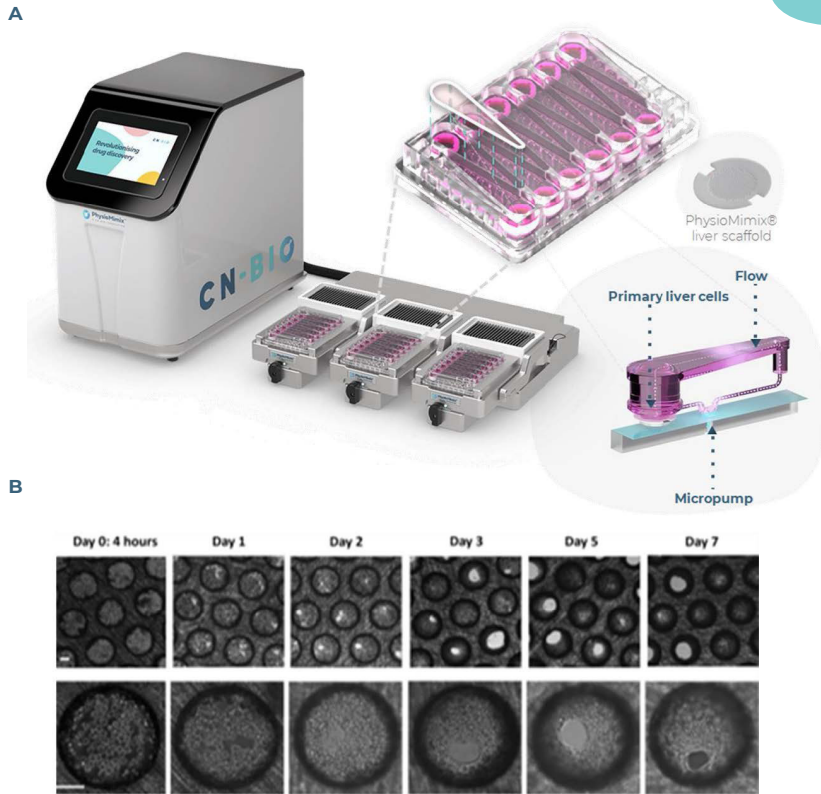
For profiling disease progression and to confirm an advanced diseased phenotype, we induced NASH in six different donor cell combinations. TGF- $\beta$  was added every 2-3 days from day 4 onwards. With the experiments completed on Day 14.

Having conducted a literature search to identify disease-associated biomarkers, selecting the following to assay:

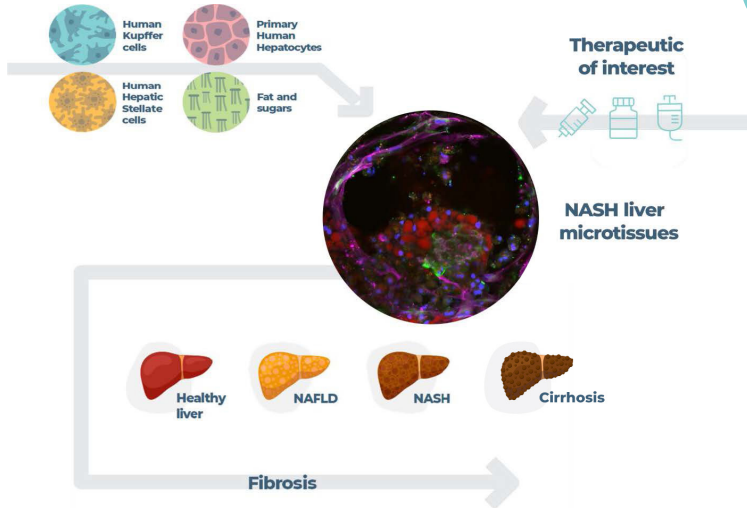
- Matrix metalloproteinase-1 (MMP-1)<sup>3</sup>
- A ratio of MMP-9/TIMP-1<sup>4</sup>
- Lipocalin-2<sup>5</sup>
- Thrombospondin-2<sup>6</sup>

Biomarkers for fibrosis and inflammation were quantified using a Luminex assay or standard ELISA assays. We compared changes in biomarker levels in the PhysioMimix NASH MPS to the clinical proteomic biomarker study by the 'Litmus Partners'<sup>2</sup>.

For compound screening, we evaluated four anti-NASH compounds: Aramchol (Stearoyl-CoA desaturase-1 (SCD-1) inhibitor); Cenicriviroc (Chemokine receptor type-2/5 (CCR2/5) antagonist); SB-431542 (Activin-like kinase 4/5/7 (ALK-4/5/7) inhibitor); Selonsertib (Apoptosis signal-regulating kinase-1 (ASK-1) inhibitor); with Elafibranor (Peroxisome proliferator-activated receptor-alpha/delta (PPAR- $\alpha/\delta$ ) agonist) as the positive control. Here, the highest concentration used matched the clinical trial dose, with dosing occurring every 2-3 days over a 10-day time course.



**Figure 1.** The formation of 3D human liver microtissues using PhysioMimix® OOC. **A)** Schematic representation of the PhysioMimix® Multi-chip Liver-12 plate, including a cross-section of one open cell culture well indicating the 3D scaffold and the microfluidic flow. **B)** Morphogenesis from single cells to 3D human liver microtissues over a 7-day time course.



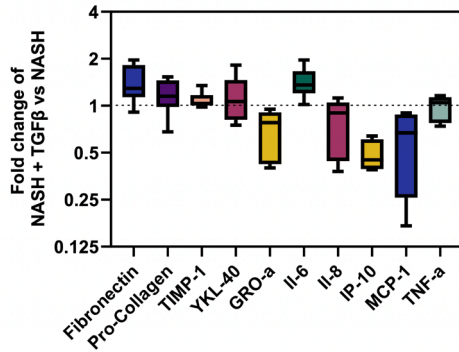
**Figure 2.** Our pre-clinical NASH MPS. Pre-validated primary human hepatocytes (PHHs) are seeded alongside non-parenchymal cells (human Kupffer cells, and human stellate cells) and cultured by the PhysioMimix® OOC System. In the presence of HEP-Fat media, microtissues form and progress from healthy liver to a NASH-like phenotype. Inclusion of therapeutics of interest over the 10 day time course can test anti-NASH compound efficacy as well as optimum dosing and concentrations.

## Results

NASH phenotype was induced in six different donor combinations of PHHs, human Kupffer cells and human stellate cells with addition of TGF- $\beta$  to promote an advanced disease phenotype. Fold change is the ratio of NASH + TGF- $\beta$  to NASH at Day 14. N=3 for each condition (**Fig 3**).

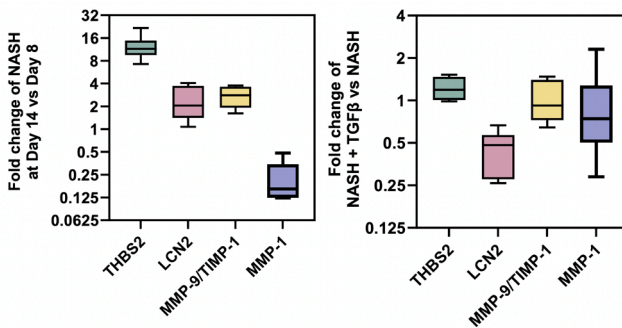
When compared with our NASH only model, NASH + TGF- $\beta$  shows increased expression of soluble biomarkers for fibrosis (fibronectin, pro-collagen, and TIMP-1) and inflammation (IL-6, YKL-40, and TNF- $\alpha$ ; **Fig 4**).

Fig 3



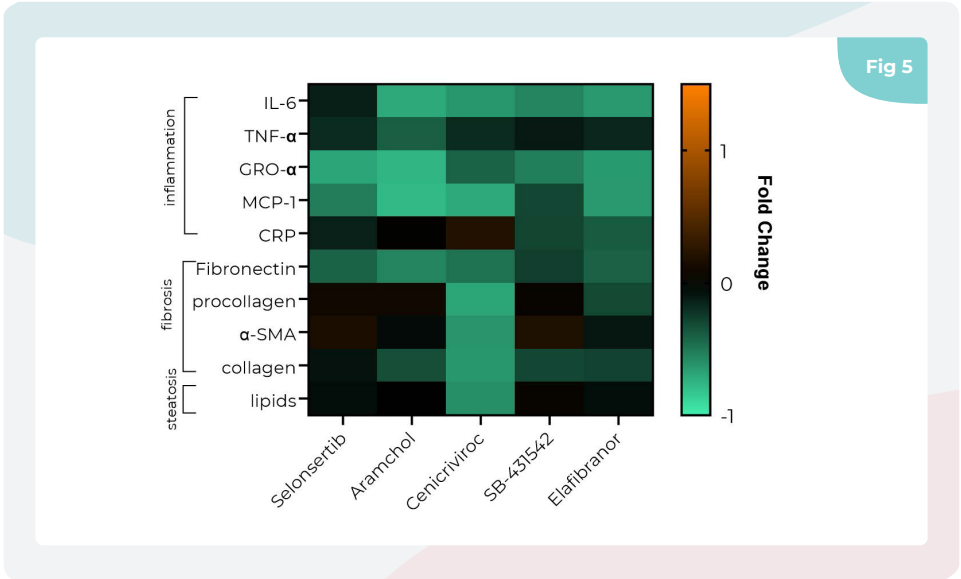
**Figure 3.** TGF- $\beta$  promotes fibrosis in the PhysioMimix<sup>®</sup> NASH MPS. Addition of TGF- $\beta$  in parallel with HEP-Fat media drives an advanced NASH phenotype in our model at day 14.

Fig 4



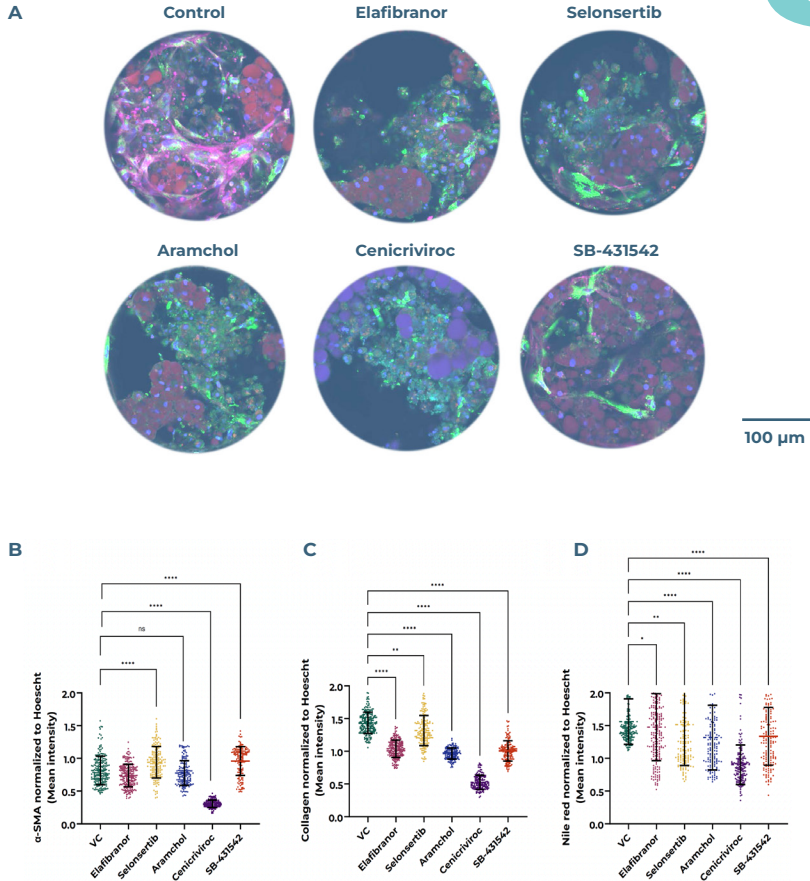
**Figure 4.** The NASH MPS confirms clinical findings with expression of all four biomarkers. The increase in production of thrombospondin-2 (THBS2) as the NASH phenotype progresses, correlates with the NASH proteomic study published by the Litmus partners<sup>2</sup>.

The heatmap depicts fold change relative to vehicle control at Day 14 (or 10-day exposure) from soluble biomarkers (**Fig 5**). Confocal microscopy image analysis quantification of fibrosis ( $\alpha$ -SMA and collagen type I), and lipid accumulation (Nile red) in the liver microtissues, demonstrating their overall effect in resolving NASH (**Fig 6**).



**Figure 5.** Testing efficacy of anti-NASH compounds using a panel of biomarkers in the NASH MPS focusing on inflammation, fibrosis, and steatosis.





**Figure 6.** Reduction in NASH phenotype with addition of anti-NASH compounds in our NASH MPS. **(A)** Confocal microscopy imaging of NASH microtissues collected at Day 14 from samples treated with vehicle, or anti-NASH compounds (at highest dose). The samples were fixed in 4% paraformaldehyde, and stained for alpha-smooth muscle actin ( $\alpha$ -SMA, magenta), collagen type I (green), Nile red (lipid accumulation, red), and Hoechst 33342 (blue). Image analysis quantification of  $\alpha$ -SMA **(B)** and collagen type 1 **(C)**, both markers of fibrosis, as well as lipid accumulation **(D)** in liver microtissues treated with anti-NASH compounds.

# Discussion



Here, we present a range of biomarkers associated with fibrosis, inflammation and steatosis which we used to assess drug efficacy using our PhysioMimix NASH MPS. Using clinically relevant dosing we show clear effect on disease phenotype with a reduction in disease-associated markers seen most greatly with Aramchol and Cenicriviroc treatment.

The NASH MPS expresses a range of clinically relevant biomarkers, including thrombospondin-2, one of only 117 circulating proteins out of 4,730 to be differentially expressed in the NASH proteomic study published by the Litmus partners.

Our NASH MPS provides translatable insights into drug efficacy and, by combining biomarkers shown to be clinically significant, offers a sensitive approach for pre-clinical NASH screening to provide drug developers with greater confidence in decision making.

# References



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