PNPLA3 I148M RNA Editing Outperforms *PNPLA3* Silencing in Improving Hepatocyte Lipid Metabolism *in vitro* and *ex vivo*

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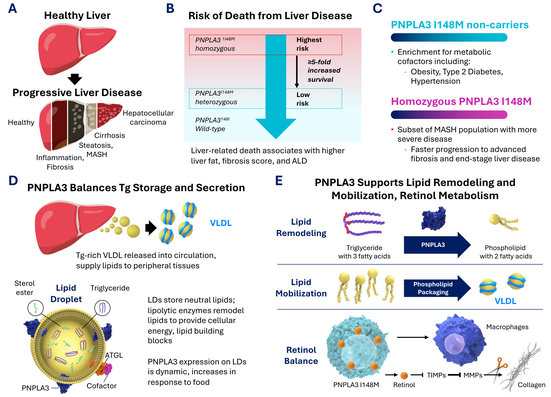
SUMMARY

- *PNPLA3 I148M* is both a gain- and loss-of function variant that disrupts PNPLA3 function, hepatic lipid homeostasis, fibrosis, and retinol metabolism, and is associated with substantially increased risk of liver-related deaths, including metabolic dysfunction-associated steatohepatitis (MASH).[1-4]
- We developed RNA editing oligonucleotides, or AIMers, to edit the base sequence of *PNPLA3 I148M* transcripts to restore functional protein in hepatocytes and hepatic stellate cells (HSCs), which play a key role in fibrogenesis associated with chronic liver disease.
- An RNA editing approach that restores wild-type protein activity may be preferable to silencing, which may lead to the loss of physiological PNPLA3 functions. Emerging evidence shows that PNPLA3 plays an important role in hepatic lipid metabolism.
- In primary human hepatocytes homozygous for the PNPLA3 I148M variant in monolayer culture, HEPATOPAC® culture, or long-term culture, PNPLA3 AIMers were potent and supported substantial editing of the PNPLA3 I148M transcript, exceeding the ~50% threshold expected to lower risk for liver disease by restoring a heterozygous PNPLA3 I148M phenotype.
- RNA editing with PNPLA3 AIMers significantly decreased lipid droplet density in human hepatocytes in HEPATOPAC® and long-term cultures. RNA editing decreased lipid droplet density more than a small interfering RNA (siRNA), which decreased the expression of the PNPLA3 I148M transcript.
- In wild-type mice that do not express the human PNPLA3 gene, a single dose of multiple PNPLA3 AIMers achieved concentrations in the liver that are expected to support substantial editing based on pharmacokinetic-pharmacodynamic (PK-PD) observations in primary human hepatocytes.
- Treatment with a surrogate site AIMer-3 in wild-type mice demonstrated that (i) PNPLA3 AIMer was distributed in all three zones of the liver (ii) PNPLA3 AIMer colocalizes with HSCs and shows dose-dependent detection in HSCs; and (iii) PNPLA3 AIMer editing was similar between hepatocytes and HSCs in mice dosed biweekly.
- These data demonstrate that AIMers supporting *PNPLA3 I148M* editing to restore functional PNPLA3 protein decrease fat content in hepatocytes, and outperform *PNPLA3* silencing in lowering lipid density. Successful PNPLA3 AIMer uptake and editing directly in HSCs demonstrates a promising role for PNPLA3 editing to address fibrotic disease mechanisms underlying MASH progression.
- Overall, these data support continued advancement of AIMers for the treatment of *PNPLA3 I148M* liver disease, and we expect to file a CTA to advance this program in 2026.

INTRODUCTION TO MASH AND PNPLA3

- *PNPLA3 I148M* is the strongest genetic risk factor for liver disease, including MASH (**Figure 1A**).[1-4]
- The *PNPLA3 I148M* variant (rs738409 G) is common worldwide, with >9 million homozygous individuals in US and Europe. Heterozygous individuals have lower risk of liver disease, and >5-fold increase in survival compared to homozygotes (**Figure 1B**).[1-4]
- Up to 25% of all MASH cases are homozygous for *PNPLA3 I148M* (**Figure 1C**).[5]
- In hepatocytes of the liver, PNPLA3 aides in balancing triglyceride and phospholipid storage and secretion by supporting lipid remodeling and mobilization, and retinol metabolism (**Figure 1D,E**).[6]
- The *PNPLA3 I148M* allele disrupts PNPLA3 function and hepatic lipid metabolism.[6]

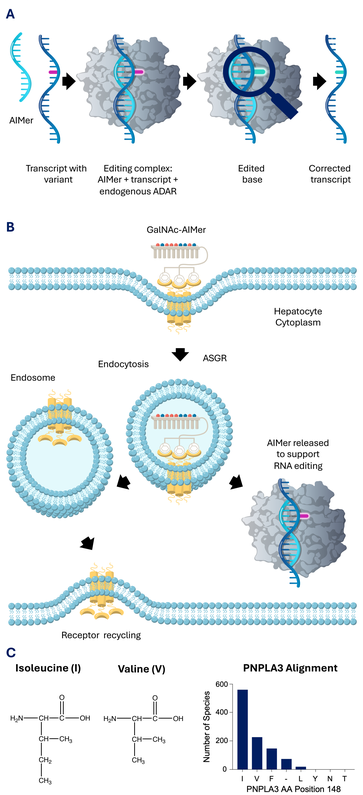
Figure 1. The *PNPLA3 I148M* variant increases the risk for liver disease



INTRODUCTION TO RNA EDITING

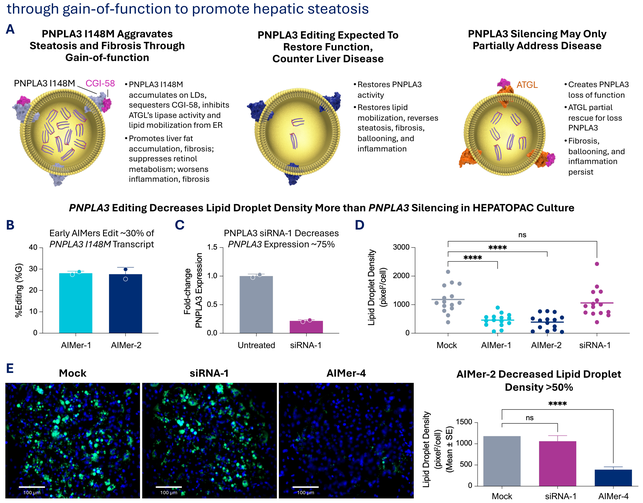
- We have developed RNA editing oligonucleotides, called AIMers, that direct efficient and specific A-to-I RNA editing of mRNA with endogenous ADAR (adenosine deaminase acting on RNA) enzymes (**Figure 2A**).[7,8]
- These AIMers are conjugated to *N*-Acetylgalactosamine (GalNAc) to facilitate delivery to liver hepatocytes (**Figure 2B**).
- PNPLA3 GalNAc-AIMers are designed to convert the methionine in PNPLA3 I148M, which suppresses enzymatic activity by 80%, to valine, which is expected to restore access to the active site and PNPLA3 enzymatic activity (**Figure 2C**). Bioinformatic analysis shows that this amino acid in PNPLA3 is valine in over 200 species, which further supports the notion that an I148V substitution will be functional (**Figure 2C**).
- Emerging evidence showing that PNPLA3 plays an important role in hepatic lipid metabolism[6] suggests that an RNA editing approach that restores PNPLA3 protein function may be preferable to silencing *PNPLA3* expression for the treatment of liver disease driven by this variant.

Figure 2. RNA editing and delivery to liver hepatocytes



RESULTS

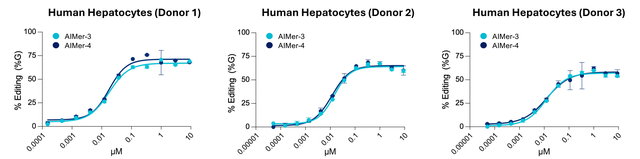
Figure 3. PNPLA3 I148M variant impairs lipase activity, inhibits triacylglycerol hydrolysis through gain-of-function to promote hepatic steatosis



GalNAc-AIMers or GalNAc-siRNAs were delivered gymnotically (by free uptake) to human hepatocytes in monolayer or in HEPATOPAC® cultures incorporating human hepatocytes homozygous for the PNPLA3 I148M variant. RNA editing was quantified by Sanger sequencing. RNA silencing was quantified by qPCR. Lipid droplet density was quantified by ImageJ. ns non-significant; **** p<0.0001 One-way ANOVA.

- To compare RNA editing and RNA silencing as an approach to address liver disease caused by homozygous *PNPLA3 I148M* allele (**Figure 3A**), we evaluated PNPLA3 AIMers and a PNPLA3 (siRNA-1), which was designed to silence expression of *PNPLA3* transcripts regardless of genotype, in HEPATOPAC® cultures incorporating human hepatocytes homozygous for the *PNPLA3 I148M* allele. HEPATOPAC® cultures better replicate the physiological environment of the liver than monolayer hepatocytes in primary culture.
- AIMer-1 and AIMer-2 are early, non-optimized PNPLA3 GalNAc-AIMers. AIMer-1 and AIMer-2 directed ~30% editing of the *PNPLA3* transcript (**Figure 3B**). siRNA-1 decreased expression of *PNPLA3* transcripts by ~75% (**Figure 3C**).
- AIMer-1 and AIMer-2 significantly decreased lipid droplet density (>50%, p<0.0001) compared to mock treatment, whereas siRNA-1 did not (**Figure 3D**).
- AIMer-4, a more advanced PNPLA3 GalNAc-AIMer, significantly decreased lipid droplet density (>50%, p<0.0001) compared to mock treatment, whereas siRNA-1 did not (**Figure 3E**).
- These data indicate that RNA editing may be preferable to *PNPLA3* silencing to restore healthy lipid metabolism in hepatocytes.

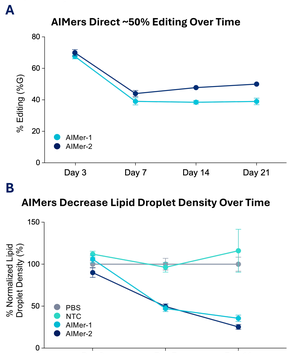
Figure 4. PNPLA3 AIMers support efficient RNA editing in hepatocytes from multiple human donors with homozygous *PNPLA3 I148M* variants



GalNAc-AIMers were delivered gymnotically (by free uptake) to human hepatocytes from three different donors at concentrations ranging from 50 pM to 9 μM for 72 hrs. RNA editing was quantified by Sanger sequencing.

- We evaluated PNPLA3 GalNAc-AIMers, AIMer-3 and AIMer-4, in hepatocytes derived from multiple human donors who were homozygous for the *PNPLA3 I148M* allele.
- *In vitro* half maximal effective concentrations (EC_{50}s) for the AIMers approximated 12 nM, with 60%-70% of the *PNPLA3* transcripts edited at the maximum (**Figure 4**), indicating that the PNPLA3 AIMers are both potent and efficient.

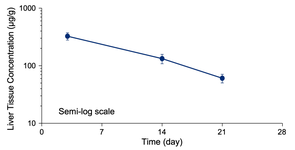
Figure 5. *PNPLA3* RNA editing improves lipid phenotypes in long-term human hepatocyte cultures



GalNAc-AIMers (1 μM) were delivered gymnotically (by free uptake) to long-term human hepatocytes cultures. RNA editing (Sanger sequencing), and lipid droplet densities (ImageJ) were quantified over time in separate experiments.

- We evaluated PNPLA3 AIMers, AIMer-1 and AIMer-2, in long-term cultures in human hepatocytes homozygous for the *PNPLA3 I148M* allele.
- In long-term cultures, RNA editing peaked by day 3 between 65%-70%, and editing levels persisted at ~40%-50% through day 21 (**Figure 5A**).
- In the same cultures, AIMer-1 and AIMer-2 decreased lipid droplet density by 47% and 50% (day 7) and by 65% and 75% (day 14), respectively, with the AIMer (AIMer-2) that supported the most editing having the largest lowering effect on lipid density (**Figure 5B**).
- Together, these data support the hypothesis that *PNPLA3* RNA editing will restore healthy lipid metabolism in hepatocytes.

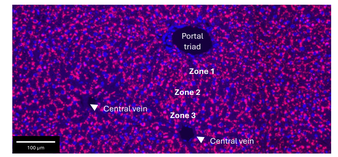
Figure 6. PNPLA3 AIMers achieve tissue concentrations expected to support *PNPLA3* RNA editing in mice



Wild-type mice were dosed subcutaneously with AIMer-4, and tissue concentrations in liver were quantified by ELISA.

- In wild-type mice, which lack the *PNPLA3 I148M* variant, we assessed concentrations of AIMer in the liver after dosing.
- Based on experiments in hepatocytes, the concentrations of AIMer-4 detected in the liver (**Figure 6**) are expected to yield substantial RNA editing.
- Overall, these data support continued advancement of PNPLA3 AIMers for the treatment of liver disease caused by homozygous *PNPLA3 I148M* variant.

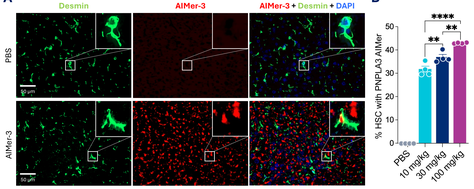
Figure 7. PNPLA3 AIMers distribute evenly across all three zones of liver



Wild-type mice were dosed (10, 30 and 100 mg/kg) subcutaneously with a surrogate site AIMer-3, and PNPLA3 expression was detected in liver tissue by in situ hybridization (ISH) at Day 7. Red signal: RNAscope ISH probe for detecting PNPLA3 AIMer; Blue signal: DAPI nuclear counterstain.

- Data demonstrate that PNPLA3 AIMer is detected in all three liver zones, primarily within the cytosolic compartment of hepatocytes (**Figure 7**).
- These data support successful even distribution of PNPLA3 AIMers to liver.

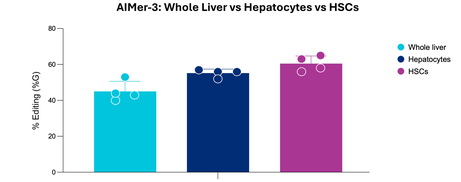
Figure 8. Representative images show colocalization of AIMer-3 and Desmin in AIMer-treated mice



Wild-type mice were dosed (10, 30 and 100mg/kg) subcutaneously with a surrogate site AIMer-3 or PBS and immunofluorescence was performed in liver collected at Day 7 for PNPLA3 AIMer (red), DAPI (blue), desmin to mark HSCs (green) and colocalization (yellow) of PNPLA3 AIMer and desmin. Uptake of PNPLA3 AIMer in HSCs was quantified in wild-type mice dosed with 10, 30 and 100 mg/kg subcutaneously with AIMer-3. Representative images (20x) from mice with values similar to the group mean. AIMer-3: Animal no 26. Data are means ± SEM of n=4 mice. Each dot represents an individual mouse. One-way ANOVA with Tukey multiple comparison test. **p<0.01, ****P<0.0001.

- In wild-type mice treated with AIMer-3, desmin-positive HSCs express PNPLA3 AIMer demonstrating HSC uptake of PNPLA3 AIMer (**Figure 8A**).
- There was a significant dose-dependent increase in HSC uptake in wild-type mice treated with 10, 30 and 100 mg/kg AIMer-3 compared to PBS treatment (**Figure 8B**).
- These data demonstrate successful uptake of PNPLA3 AIMer in HSCs supporting the role for PNPLA3 editing in addressing fibrotic disease.

Figure 9. PNPLA3 AIMer editing was similar between hepatocytes and HSCs in mice treated with PNPLA3 AIMer-3



Mice were dosed subcutaneously biweekly with AIMer-3, and RNA editing was quantified by Sanger sequencing in whole liver, hepatocytes and HSCs collected at Day 77 after the initial dose.

- In human PNPLA3 I148M transgenic mice (SMOC) treated with AIMer-3 RNA editing was similar between hepatocytes and HSCs in mice dosed biweekly (**Figure 9**).
- These data demonstrate successful *in vivo* editing to both hepatocytes and HSCs supporting PNPLA3 AIMer editing as a promising tool to target both lipid metabolism and fibrosis in MASH.

References: 1. Unalp-Arida, A. and Ruhl, C.E. 2020. *Hepatology* doi: 10.1002/hep.31033; 2. Xiang, H. 2021. *J Cancer* doi: 10.7150/jca.57951; 3. Kozlitina, J. and Sookoian, S. 2025. *Liver Int.* doi: 10.1111/liv.16123; 4. Carlsson, B., et al., 2020. *Aliment Pharmacol Ther.* doi: 10.1111/apt.15738; 5. Petta, S., et al., 2025. *Liver Int.* doi: 10.1111/liv.16133; 6. Johnson, S.M. et al., 2024. *Nature Commun.* doi: 10.1038/s41467-024-49224-x; 7. Monian, P. et al., 2022. *Nat. Biotechnol.* doi: 10.1038/ s41587-022-01225-1; 8. Lu, G. et al., 2024, *Nucleic Acids Research* doi: 10.1093/nar/gkae681. **Acknowledgment:** The authors are grateful to Heather Francis (Wave Life Sciences) and Eric Smith for editorial and graphical support, respectively. This work was funded by Wave Life Sciences.