ADAR-Mediated RNA Editing-Based Correction of PNPLA3 I148M Functionality to Address Hepatic Steatosis

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Introduction

PNPLA3 I148M a variant commonly reported in the MASH population worldwide

Metabolic dysfunction–associated steatotic liver disease and steatohepatitis (MASLD/MASH) and subsequent stages of liver disease are very prevalent and still on the rise worldwide.

MASH individuals have a high unmet medical need due to the progressive nature of the disease (hepatic steatosis, cirrhosis, and hepatocellular carcinoma) and the limited therapeutic options available, making it the second cause of liver transplantation in the US.[1]

Patatin-like phospholipase domain-containing protein 3 (*PNPLA3*), a lipase expressed in hepatocytes and liver stellate cells, is involved in lipid homeostasis and metabolism. PNPLA3 I148M (rs738409) is a variant commonly reported in the MASH population worldwide (20-60% of the patients) and is known as an associated risk factor for hepatic steatosis and an increased risk of developing MASH.[2,3] Approximately 8 million individuals in US and EU are homozygous for the 148M variant. PNPLA3 I148M variant induces triglyceride (TG) accumulation in hepatocytes, leading to increased lipid droplet (LD) size and number, which in turn promotes cellular stress, fibrosis, and disease progression to cirrhosis.

Axiomer™ editing oligonucleotides (EONs) as a potential therapeutic approach (1/2)

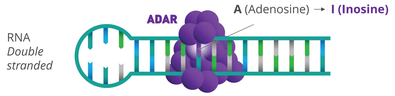
Adenosine deaminase acting on RNA or ADAR is an RNA editing machinery naturally present in all human cells, which can edit Adenosine (A) to Inosine (I) in double stranded RNA. (**Fig. 1**, top panel) This editing is a very frequently occurring biological process and has important functions such recoding transcript during the maturation of neurons, for immunity and others.

ProQR researchers have developed a therapeutic RNA platform called **Axiomer** using editing oligonucleotides, or "EONs", learning from ADAR natural process. EONs are designed to bind to single stranded RNA with high specificity. (**Fig. 1**, bottom panel) This forms a double stranded structure which recruits and directs endogenously expressed ADARs to change an Adenosine (A) to an Inosine (I) in the RNA with high specificity.

Introduction (continued)

The Inosine (I) is then translated as a Guanosine (G) – with the potential to correct an RNA with a disease-causing mutation back to a normal (wild type) RNA or to modulate proteins, so that it will have an improved new function or wild type like function that helps prevent or treat a disease.

Natural ADAR editing *(A-to-I)*



Editing Oligonucleotide (EON)-directed (Axiomer) therapeutic editing *(A-to-I)*

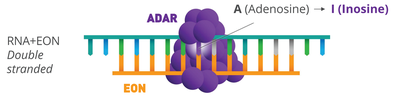


Figure 1. *On the top*, ADAR enzymes naturally act on double stranded RNAs and perform a specific A-to-I editing, changing Adenosine (A) to Inosine (I). *On the bottom*, by learning from this natural process, ProQR has developed editing oligonucleotides (EONs) which consist of short single stranded RNA molecules that are complementary to a target RNA. The target RNA is also a single strand. By binding to it, editing oligonucleotides create a double stranded structure which will attracts ADARs and allow the specific A-to-I edit to be performed.

Axiomer editing oligonucleotides (EONs) as a potential therapeutic approach (2/2)

Axiomer program AX-2911 using EONs as a therapeutic approach could convert the PNPLA3 148 Methionine into a Valine, restoring wild-type like function of PNPLA3 alleviating the induction of hepatic steatosis and the subsequent formation of MASH. (**Fig. 2**)

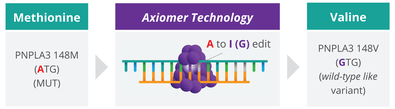


Figure 2. Schematic representation of PNPLA3 148M mutant sequence edited via ADAR leading to PNPLA3 148V wild-type like variant.

Objectives

- **To investigate how Axiomer technology can become a potential therapeutic approach for MASLD/MASH by converting PNPLA3 I148M mutant to 148V wild-type like variant.**
- **To validate that PNPLA3 148V wild-type like variant has similar characteristics to WT PNPLA3 (148I).**
- **To assess Axiomer EON editing efficiency *in vitro* and its capacity to restore wild-type like function of the PNPLA3.**

Results

PNPLA3 148V Axiomer wild-type like variant has similar characteristics to WT PNPLA3 148I (1/2)

In-silico 3D Protein structure modeling and analysis (ChimeraX) revealed that PNPLA3 148M (ATG) shows a non-conservative substitution with predicted functional consequences, leading to a change in binding cavity volume (**Fig. 3**, red circles) identified as PNPLA3's active pocket. This potentially limits substrate access to the active site, impairing PNPLA3's-metabolic function. (**Fig. 3B**) On the contrary, PNPLA3 148V (GTG) variant showed similar structure to WT PNPLA3 (148I, ATC), (**Fig. 3A and 3C**) with Valine expected to exhibit similar properties mimicking isoleucine, leading to minimal impact on protein activity/functionality and substrate interaction.

Results (continued)

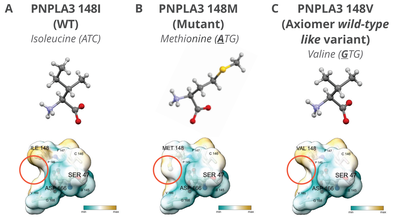
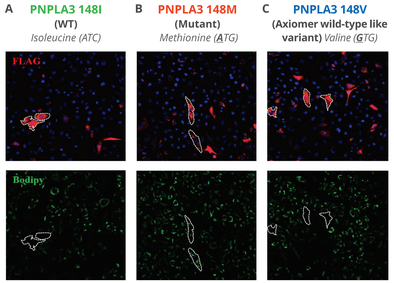


Figure 3. Schematic representation of PNPLA3 148I wildtype (WT) (**A**), PNPLA3 148M mutant sequence (**B**) and Axiomer generated PNPLA3 148V wild-type like variant (**C**). Red circles highlight the location of PNPLA3's ligand binding pocket or absence thereof in case of PNPLA3 148M

PNPLA3 148V Axiomer wild-type like variant has similar characteristics to WT PNPLA3 148I (2/2)

To investigate experimentally if PNPLA3 148V variant can function as WT, PNPLA3 148V (**Fig. 4C**), 148M (**Fig. 4B**) and WT (**Fig. 4A**) variants were expressed in HeLa cells loaded with 250µM of Linoleic acid for 24h. Confocal imaging revealed that PNPLA3 148V (Bodipy, mean area per cell of 3.65 µm^2) showed similar levels of triglycerides and lipid droplet sizes inside the cell compared to PNPLA3 WT (1.33 µm^2 per cell, P<0.0001, one-way ANOVA) versus *PNPLA3* 148M (34.00µm^2 per cell). (**Fig. 4D**).



Hoechst (nuclei), Bodipy (Triglycerides), M2 anti-flag (PNPLA3)

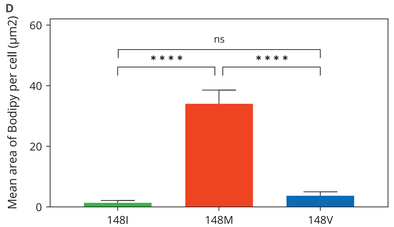


Figure 4. Fluorescent staining (Bodipy; green) of TG in HeLa cells transfected with different PNPLA3 (red) variants exposed to Linoleic acid (24h). And total cells staining using DAPI (blue). (**A**, **B**, **C**) Comparative analysis of TG and LD content is visualized in (**D**). Cell lipase activity by IF One-way ANOVA, ****, P<0.0001; Mean, SEM)

Axiomer mediated PNPLA3 editing leads to strong editing and change in lipid droplet (1/2)

Following these preliminary data confirming the characteristics of PNPLA3 148V Axiomer wild-type like variant, EONs have been designed with different lengths and chemical modifications in a proof-of-concept screening based on editing of PNPLA3 I148M to PNPLA3 148V (**Fig. 5**) in mutant donor PHH. Over 25% PNPLA3 editing was observed.

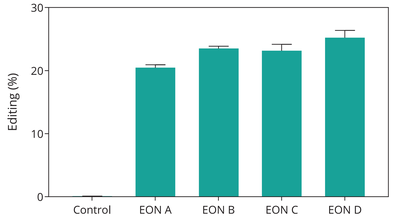


Figure 5. Editing of PNPLA3 148M to148V in Mutant-PHHs. Upon ASGPR-mediated uptake (24h treatment) of 5µM GalNAc conjugated EON. Editing evaluated 72h after treatment with dPCR. Data represented are mean, SD.

Axiomer EON mediated PNPLA3 editing leads to strong editing and change in lipid droplet (2/2)

Subsequently, in a homozygous 148M HepG2 cell model exposed to linoleic acid, Axiomer editing resulted in a significant decrease in LD size, ranging from -17% to -26% compared to control.

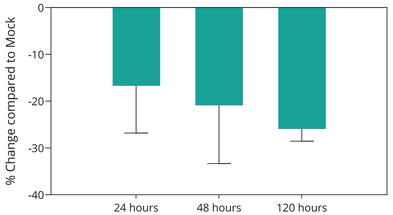


Figure 6. Change in intracellular LDs post PNPLA3 148V EON treatment . Bodipy/DAPI stainings, 5µM EON, transfection, exposure to linoleic acid, mean, SEM, n=2.

Conclusion

- ***In silico* analysis and *in vitro* protein functionality assays of PNPLA3 148V demonstrated similarity to PNPLA3 WT 148I.**
- **Axiomer mediated ADAR RNA editing technology converts PNPLA3 148M into a WT-like functional PNPLA3 148V protein.**
- **The AX-2911 program could become a promising therapeutic opportunity for homozygous and heterozygous MASLD/MASH individuals, limiting changes only to mutant allele(s).**

Literature

1. Sandireddy R, et al. Front Cell Dev Biol. 2024 Jul 16;12:1433857;
2. Romeo S, et al. Nat Genet. 2008 Dec;40(12):1461-5;
3. Salari N, et al. BMC Endocr Disord. 2021 Jun 19;21(1):125.