Precision medicine and nucleotide-based therapeutics to treat MASH

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Abbreviations:

AI: artificial intelligence
ALT: alanine transaminase
ASGPR: asialo-glycoprotein receptor
ASO: antisense oligonucleotide
ATRA: all trans retinoic acid
BFAR: bifunctional apoptosis regulator
CIDEB: Cell death-inducing DNA fragmentation factor-like effector b

ER: endoplasmic reticulum

ERLIN: ER lipid raft proteins

GalNAc: N-acetylgalactosamine

GAN: Gubra Amylin NASH diet

GWAS: genome-wide association study

HCC: hepatocellular carcinoma

HSD17B13: 17-beta hydroxysteroid dehydrogenase 13

IHH: Indian hedgehog

LD: lipid droplet

LPIAT1: lysophosphatidylinositol acyltransferase 1

LRS: liver risk score

MASH: metabolic dysfunction-associated steatohepatitis

MASLD: Metabolic dysfunction–associated steatotic liver disease

MBOAT7: Membrane bound O-acyltransferase domain-containing 7

MTARC1: Mitochondrial amidoxime-reducing component 1

NAFLD: non-alcoholic fatty liver disease

PCSK9: proprotein convertase subtilisin/kexin type 9

PNPLA3: Patatin-like phospholipase domain-containing protein 3

PS: phosphorothioate
PSD3: Pleckstrin and sec7 domain-containing 3

PUFA: polyunsaturated fatty acid

RISC: RNA-induced silencing complex

RNAi: RNA interference

siRNA: small interfering RNA

SLD: steatotic liver disease

SNP: single nucleotide polymorphism

TAZ: Transcriptional Coactivator With PDZ-Binding Motif

T2DM: type 2 diabetes mellitus

TM6SF2: Transmembrane 6 superfamily member 2

TCF7L2: transcription factor 7-like 2

VLDL: very low density lipoprotein
Abstract:

Metabolic dysfunction-associated steatotic liver disease (MASLD) is a complex multifactorial disease and becoming the leading cause of liver-related morbidity and mortality. MASLD spans from isolated steatosis to metabolic dysfunction-associated steatohepatitis (MASH), that may progress to cirrhosis and hepatocellular carcinoma (HCC). Genetic, metabolic, and environmental factors strongly contribute to the heterogeneity of MASLD. Lifestyle intervention and weight loss represent a viable treatment for MASLD. Moreover, Resmetirom, a thyroid hormone beta receptor agonist, has recently been approved for MASLD treatment. However, most individuals treated did not respond to this therapeutic suggesting the need for a more tailored approach to treat MASLD.

Oligonucleotide-based therapies, namely small-interfering RNA (siRNA) and antisense oligonucleotide (ASO), have been recently developed to tackle MASLD by reducing the expression of genes influencing MASH progression, such as PNPLA3 and HSD17B13. Here, we review the latest progress made in the synthesis and development of oligonucleotide-based agents targeting genetic determinants of MASH.

Keywords: MASLD, MAFLD, human genetics, drug
Introduction

Metabolic dysfunction–associated steatotic liver disease (MASLD), previously known as non-alcoholic fatty liver disease (NAFLD), ranges from simple hepatic steatosis to metabolic dysfunction-associated steatohepatitis (MASH), leading to fibrosis, and ultimately to cirrhosis and hepatocellular carcinoma (HCC) \(^1\). While the early stage of MASLD can be treated by weight loss, the late stage of MASLD can only be tackled by liver transplant \(^2\). MASLD coexists with other metabolic derangements, namely obesity and hypertriglyceridemia, the two strongest independent predictors of this disease, along with insulin resistance and type 2 diabetes mellitus (T2DM) \(^3\). Hence, individuals with MASLD have also an intrinsic risk for developing cardio-metabolic syndrome caused by these risk factors \(^4\). However, it is not clear why an individual enters a specific clinical trajectory leading to liver related as opposed to cardio-renal-vascular related events and \textit{vice versa}. The presence of different clinical trajectories spanning from liver to extrahepatic complications suggests the existence of different types of MASLD with specific disease-causing mechanisms.

In the context of disease heterogeneity, precision medicine aims to find the most efficacious treatment with no side effects by exploiting individual variability in genes, environment, and lifestyle \(^5\). In a framework of precision medicine, clinical features, genetic background, and omics forge the molecular understanding of diseases offering the means to effectively treat them. In addition, the application of novel techniques and technologies to ensure specific organ and gene targeting may also be considered a form of precision medicine.

In this review, we start by reasoning on the different strategies to implement precision medicine and continue by examining precision medicine based on inborn genetic variants and the use of nucleotide-based therapeutics to treat MASLD.

Using acquired genetic changes to implement precision medicine

In the field of oncology, understanding the consequences of acquired DNA changes has been used to implement precision medicine for decades. For example, retinoic acid was demonstrated as an effective treatment for individuals with acute promyelocytic leukaemia for the first time in 1988 \(^6\). Acute promyelocytic leukaemia is caused by a (15;17) (q24;q21) translocation generating a \textit{PML-RARA} fusion gene. This fusion gene binds to the \textit{RAR/RXR} target genes and
acts as a potent transcriptional repressor hindering its activation by physiological concentrations of ligands, with the ultimate effect of blocking differentiation of granulocytes. Hence, treatment with all trans retinoic acid (ATRA), a potent derivate of retinoids, overcomes this repression with large and unprecedented beneficial effects on survival for this cancer. As one may anticipate, ATRA is still the backbone treatment for this disease.

A precision medicine journey starts with the identification of recurrent genetic changes in individuals with cancer. After their identification, understanding the molecular defect caused by the genetic change is paramount to identify drugs overcoming this disorder. Oncologists forged precision medicine roadmaps with several therapeutic regimes today approved when specific genetic variants/rearrangements arise in determined genes. In the future, one may envisage classification and treatment of cancer not based on the organ/cell type the cancerous cells belong to, but on the mutations the cancerous cells harbour.

### Using inborn genetic variants to implement precision medicine

Inborn, also known as germline, genetic variants are inherited from each biological parent and are stable over a lifetime. MASLD has a strong genetic component with common genetic variants in the *PNPLA3* and *TM6SF2* having collectively the largest effect sizes. This contrasts other multifactorial diseases, including diabetes and obesity, where common genetic variants have less pronounced effect size in determining these traits. However, a common genetic variant in the *transcription factor 7-like 2 (TCF7L2)* gene, the strongest genetic risk contributor of T2DM progression, has a sizeable allelic relative risk of ~1.4 that is comparable to *PNPLA3* and *TM6SF2* variants.

Another specific feature of common variants increasing the risk of MASLD is the interaction with excess in body weight, insulin resistance and body fat distribution. Due to this interaction, in the absence of metabolic stressors genetic variants have a low and sometimes even negligible effect on increasing the risk of MASLD. However, due to the obesity epidemics, these metabolic stressors become every year more present in the general population.

Among treating physicians in Hepatology among other disciplines, there is always the argument on the lack of clinical value of routinely assessing the presence of genetic variants in individuals with MASLD based on the fact that genetic
background cannot be changed. On the other hand, there are those in favour arguing that lifestyle changes due to awareness of the potential risk of MASLD will mitigate the risk itself in virtue of the gene-environment interaction. Indeed, the identification of these variants early in life may encourage individuals to strive for reducing weight excess and being more active. Interestingly, Harris et al. showed that the presence of a specific genotype makes less effective the beneficial effect of exercising in MASLD individuals, and that reduction of serum biomarkers of liver fibro-inflammation after weight loss was more pronounced in carriers of a specific genotype 17.

A case of interest is when a genetic variant minor allele, as for example the HSD17B13 rs72613567:TA variant, results in a loss of function and associates with a protection against MASLD. This event opens the avenue to the identification of therapeutic targets to treat MASLD. Indeed, targeted downregulation/ablation of specific genes is relatively easy now days due to the application of nucleotide-based therapeutics to pharmacology. Over time, all this is leading to a precision medicine approach by giving tailored-lifestyle counselling and targeting specific genetic variants in individuals with MASLD (Figure 1).
Figure 1. **Differences between traditional and precision medicine.**

A) Traditional medicine is based on a one-size-fits-all approach that involves the use of only rigid, standardized, already defined methods for clinical care, regardless the background of each individual. B) Precision medicine is based on the concept that one drug fits a specific subset of individuals stratified based on their (epi)genetic, clinical, environmental, and ethnic/sex background. Created with BioRender.com.

**Using machine learning to implement precision medicine**

The development of precision medicine strategies involves the integration of data deriving from clinical health-related records, computational analyses, artificial intelligence (AI), biobanks, and multi-omics analyses. Indeed, AI, machine learning, and deep learning are used to generate accurate *in silico* predictive models aiming to implement
precision medicine in hepatology. Multiparametric data sets generated by using clinical health-related records, imaging techniques and multi-omics, can be integrated by validated algorithms. These algorithms can predict biological processes, functional associations, chemical interactions of a disease-causing protein, diagnosis, clinical outcomes, or the effect of lifestyle interventions by clustering individuals into more homogenous categories.

One of the greatest advantages of AI is the possibility to account for complex and collinear relationships among traits in the identification of meaningful patterns to predict disease progression. In 2023, Serra-Burriel et al. generated the Liver Risk Score (LRS) and effectively predicted liver fibrosis at a population level by applying machine learning, namely a recursive feature elimination algorithm, to clinically available data. Indeed, the LRS outperformed the other non-invasive tests in predicting fibrosis resolution in individuals after metabolic surgery.

Machine learning can also be used to detect independent predictors of disease that may be clinically useful to identify individuals with MASLD. For example, in this study hypertriglyceridemia is, after body mass index, the second strongest independent predictors of liver triglyceride content, while T2DM, contrarily to the epidemiological evidence, is not. Noteworthily, studies on PNPLA3 rs738409 p.I148M, the strongest genetic determinant of MASLD, are leading to the concept of life-style recommendation tailored on this genetic feature. For instance, Davis et al. showed that overweight Hispanic children carrying the PNPLA3 148M/M variant had lower capacity to hydrolyse hepatic triglycerides and, therefore, increased hepatic triglyceride accumulation as compared to wild type, proposing that the use of a personalized low carbohydrate diet in this group would lead to more beneficial results. Moreover, one year-long lifestyle modification in MASLD subjects led to a three-fold higher reduction of intrahepatic triglyceride content in the PNPLA3 148M/M group compared to wild type carriers, suggesting a higher sensitivity of these individuals to lifestyle modification. Indeed, dietary interventions, based on genetic and clinical data, are pivotal to obtain beneficial therapeutic outcomes for MASLD. In addition, AI can be used for treatment optimization, risk assessment, and cost-effectiveness analyses to reduce costs and measure the impact of interventions, as the cost of remedying adverse drug reactions is usually higher than the cost of medication itself. Overall, the application of medical AI models in hepatology has the potential to help clinicians in the medical practice from diagnosis to outcome prediction.
Using nucleotide-based therapeutics to implement precision medicine

Nucleotide-based therapeutics, namely small interfering RNA (siRNA) and antisense oligonucleotides (ASO), are techniques capable of selectively ablating a specific gene in a determined disease pathway, thus improving disease phenotypes. A potential drawback of this treatment is the unwanted ablation of off-target genes. However, until now these treatment modalities have been shown well tolerated as they can be administered in very low doses. In the pharmacokinetics, the conjugation of these molecules with N-acetylgalactosamine (GalNAc) moieties played a pivotal role. This chemical modification dramatically increases the uptake of the molecule in the hepatocyte due to its binding with the hepatocyte-specific transmembrane asialo-glycoprotein receptor (Ashwell–Morell receptor, ASGPR) that is present specifically on this cell type. This conjugation has allowed unprecedented specificity in targeting the liver and has reduced the drug dose avoiding side effects deriving by the downregulation of the gene in organs other than the liver.

Fundamental molecular mechanism of siRNA

RNA interference (RNAi) is an ancient intracellular defence mechanism through which target mRNAs are degraded before translation as a defence against exogenous genetic material. Small interfering RNAs (also known as short interfering RNA, and silencing RNA, siRNA) have been identified in 1998 by Andrew Fire and Craig Mello in Caenorhabditis elegans granting these basic scientists the 2006 Nobel Prize in Physiology or Medicine award for their discovery.

siRNAs are 20–24-long base pairs double-stranded non-coding RNA molecules composed of a passenger strand (sense strand), used as a drug delivery device, and a guide strand (antisense strand) responsible for triggering RNAi-based gene silencing.

siRNA-induced post transcriptional gene silencing starts with the interaction of siRNA with the multidomain type III ribonuclease DICER. DICER, by binding the double-stranded siRNA, cleaves the putative sense strand to generate a
mature siRNA that is then loaded onto the RNA-induced silencing complex (RISC) \(^{33}\). Alternatively, gene silencing may start with the direct incorporation of siRNA into RISC where its Argonaute-2 component, a double-stranded RNase, degrades the sense strand to form a mature siRNA-RISC complex. The remaining antisense strand, complementary to the mRNA of interest, drives the RISC complex to the mRNA target sequence, thus leading to its cleavage and degradation through the activity of intracellular exonucleases (Figure 2A). Sequence complementarity between the guide strand and the target mRNA is crucial to reduce unwanted sequence-dependent off-target transcript silencing due to partial homology with the mRNA of interest \(^{34}\). Indeed, partial complementary pairing can cause the unpredictable degradation of similar mRNAs, thence inducing the downregulation of untargeted genes.

**Fundamental ASO molecular mode of action**

ASOs are single-stranded nucleic acids (synthetic RNA or DNA sequences) designed to bind the pre- or mature mRNA target through complementary base-pairing. The binding degrades the target transcript and alters the splicing, therefore blocking endogenous or exogenous protein translation \(^{35}\). ASOs exert their activity through two mechanisms: a) occupancy-mediated RNA degradation that triggers the cleavage of the target transcript either by Argonaute-2, specific for RNA-RNA-like duplexes, or RNase-H1, that specifically cleaves the RNA–DNA-like heteroduplexes formed by ASOs and the complementary RNA target sequence; and b) occupancy-only mechanism that causes steric interference without any cleavage of the RNA of interest by triggering the alternative splicing of pre-mRNA, and therefore affecting mRNA stability and maturation through the inhibition of the 5’ end CAP formation or the 3’ end polyadenylation, or preventing protein translation by blocking the interaction mRNA-ribosome via steric hindrance \(^{36,37}\) (Figure 2B). Notably, short methylated oligonucleotides modulate gene expression by inducing DNA hypermethylation at specific promoters, thus inhibiting transcription and protein translation for longer time. Interestingly, DNA methylation is stable even after cell division, leading to a longer-lasting silencing when compared to standard unmethylated oligonucleotides \(^{38}\).

Depending on the disease molecular mechanism, these therapeutics can downregulate or upregulate the expression of the gene of interest, resulting in lower or higher protein levels. In this regard, ASOS may be used to regulate the
methylation of promoter regions of survival motor neuron gene 2 (SMN2) and, therefore, to enhance its gene and protein expression. Indeed, homozygous deletions or mutations in SMN1, a gene highly homologous to SMN2, are responsible for the development of spinal muscular atrophy (SMA) as they lead to the synthesis of a truncated and unstable SMN protein. By boosting the transcription of SMN2 and, consequently, the expression of functional SMN protein, mice improved motor function and increased their survival rate. Noteworthily, ASOs activities depend on the intracellular availability of RNase-H1 or Argonate-2. To exert its enzymatic activity, RNAse-H1 needs a very high level of complementarity between the single-strand sequence of ASOs and the target mRNA, thus reducing off-target transcript silencing. Contrarywise, Argonaute-2 does not require the same level of sequence complementarity, and therefore more likely to result in off target effects. In the last years, several mRNA-targeting ASOs entered clinical trials, as for example Mipomersen and Volanesorsen for homozygous familial hypercholesterolaemia and familial chylomicronaemia syndrome, respectively.

**Chemical modifications of nucleotide-based therapeutics to increase stability**

Unmodified oligonucleotides are susceptible to a rapid nuclease-mediated degradation within minutes, they induce the activation of the innate immune system and off-target toxicity due to their intracellular accumulation causing RNAi-dysfunction, and therefore they did not go through in clinical practice. However, low stability, specificity, bioavailability, cellular uptake, and the induction of unwanted off-target effects have been mitigated by introducing chemical modifications in three specific sites of the oligonucleotides: phosphate backbone, ribose moiety or nucleobase. These chemical modifications allowed scientists to better understand the gene silencing machinery and to evaluate new modalities aimed to reduce off-target effects and unintended mRNA silencing.

Among the phosphates backbone modifications, the phosphorothioate (PS) linkage is one of the most used in oligonucleotides. On the one hand, moderate degree of PS linkage renders oligonucleotides more hydrophobic, resistant to nuclease digestion and easily bound to albumin allowing a longer half-life and higher cellular uptake. On the other hand, high degree of PS modification reduces the specificity of the oligonucleotide for its target, thus
inducing severe toxic effects \(^4^8\). Alternative modifications at the phosphate backbone include 5′-(E)-vinyl phosphonate, phosphorodithioate, methylphosphonate and methoxypropylphosphonate. All these modifications enhance the activity of the oligonucleotide-based drug \(^2^8\).

Ribose is usually modified by a 2′-O-methyl group, thus improving stability, binding affinity, nuclease resistance and reducing immune reactions \(^4^9,5^0\). Other 2′-sugar-modified oligonucleotides already in clinical trial are characterized by the addition of 2′-methoxyethyl or 2′-fluoro chemical modifications \(^5^1\).

Nucleobase modifications with uridine analogous and cytidine stabilize the base pair making oligonucleotides more resistant to serum nucleases, and reduce innate immune activation usually triggered by Toll-like receptor (TLR)-dependent or independent pathways \(^5^2\).

**Chemical modifications of nucleotide-based therapeutics to allow targeted organ delivery**

The conjugation of multivalent-GalNAc moieties to the 3′- and 5′-terminal of oligonucleotides allows targeting hepatocyte-specific mRNAs as GalNAc selectively binds the ASGPR, a protein highly expressed in the membrane of hepatocytes while poorly expressed on the surface of other cell types \(^5^3\). ASGPR allows the internalization of circulating glycoproteins exposing GalNAc glycans via a clathrin-mediated endocytosis \(^5^4\). Trivalent-GalNAc conjugation requires the integration of modified ribose and 5′ phosphate resulting in a metabolically stabilized oligonucleotide scaffold with a higher delivery capability \(^5^5\). These minor chemical changes and optimized structures have a large impact on the metabolic stability of GalNAc-oligonucleotides conjugates, as they show high stability, specificity, long-term safety by attenuating the activation of innate immune system, liver-specific uptake, and robust gene silencing of the targeted mRNA in the liver \(^5^4\). Importantly, they can be administered subcutaneously monthly or even twice a year, thus potentially increasing compliance \(^3^0,5^6\). For example, Inclisiran, a cholesterol-lowering siRNA conjugated to a triantennary GalNAc targeting *proprotein convertase subtilisin/kexin type 9 (PCSK9)* mRNA, is currently approved for the treatment of familial hypercholesterolaemia and mixed hyperlipidaemia in adults \(^5^7\). Moreover, siRNAs therapies against liver disease are now in clinical studies with some close to be approved as therapies (Table 1).
Oligonucleotide-based therapeutics delivery methods

Naked oligonucleotides are hardly up taken by cells due to their size, high hydrophilicity, and negative charge. Therefore, delivery systems, such as nanoparticles, aptamers, and exosomes, are required to guarantee cellular uptake by energy-dependent endocytosis or pinocytosis. These delivery systems are also employed to increase the molecule half-life by avoiding serum RNase A-like nucleases degradation and renal clearance, and to escape immune response. Once inside the cell, the delivery system must release the oligonucleotide-based molecule either in the cytoplasm or inside the nucleus where they exert their activity by activating the RISC complex or RNase-H1.
Figure 2. Molecular mechanism of action of nucleotide-based therapeutics. A) ds-siRNA, once introduced into hepatocytes by endocytosis, interacts with the enzyme DICER or with the RNA-induced silencing complex (RISC) that, by degrading its passenger strand, produce a mature ss-siRNA complementary to the mRNA target. B) ss-ASO either interacts with RNase-H1 or the RISC complex to bind and cleave the complementary mRNA target sequence, or directly interacts with the mRNA or pre-RNA of interest affecting its stability, maturation or causing alternative splicing, thus preventing its translation to protein. Created with BioRender.com.

Genetic therapeutic targets in MASLD

Human genetics strongly contributes to the onset and progression of steatotic liver disease (SLD) \textsuperscript{60,61}. In the last decades, Genome-wide association studies (GWAS) have been used to associate genetic variants in genes involved in lipid metabolism, such as \textit{PNPLA3, TM6SF2, MBOAT7, GCKR, HSD17B13, MARC1, APOE} and \textit{GPAM}, to SLD progression or protection \textsuperscript{61}. Genotyping may also be used to predict liver disease progression. Indeed, the weighted sum of these single nucleotide polymorphisms (SNPs), namely polygenic risk scores (PRS), allows the identification
of individuals that will not develop SLD $^{16,18}$. PRS can also be used to re-stratify individuals in fibrosis classes measured by non-invasive predictive tests $^{15}$ potentially facilitating early screening, interventions, prioritization of follow up and treatments of high-risk groups. Furthermore, the identification of these genetic variants and the understanding of their fundamental mechanisms has led to several genetic validated therapeutic targets to treat MASLD (Table 1). However, routine genetic screening is available only in tertiary level hospitals.

**Patatin-like phospholipase domain-containing protein 3 (PNPLA3)** is a triacylglycerol lipase that localizes in lipid droplets (LD), endoplasmic reticulum (ER) and Golgi apparatus that hydrolyses triglycerides, phospholipids and retinyl esters, mostly in adipocytes and hepatocytes $^{62}$. *PNPLA3* and its protein synthesis are induced during obesity $^{14}$, while the *PNPLA3* rs738409 variant encodes for a p.I148M substitution that results in a loss of function of the protein enzymatic activity $^{63}$. In addition, the I148M variant compromises protein-mediated retinol release from hepatic stellate cells promoting their activation and thus inflammation, fibrosis and carcinogenesis $^{64,65}$.

The PNPLA3 rs738409 polymorphism has been robustly associated with an increased susceptibility to the entire spectrum of hepatic injury development (to MASH and cirrhosis) and increased risk of HCC, by impairing mitochondrial function and antioxidant response, and by hindering lipid and retinol metabolism in hepatocyte and hepatic stellate cells, respectively $^{66}$. Luukkonen et al. showed that PNPLA3 rs738409 carriers have hepatic mitochondrial dysfunction that causes increased hepatic β-oxidation/ketogenesis, impaired de novo lipogenesis and mitochondrial citrate synthase flux that might predispose to the progression from early-stage to late-stage MASLD $^{67}$.

In addition, these individuals seem to be protected from cardiovascular disease as they exhibit an antiatherogenic lipid profile, and a better response to lifestyle modifications $^{68-71}$.

Interestingly, PNPLA3 turnover in hepatocytes is controlled by the bifunctional apoptosis regulator (BFAR) which promotes PNPLA3 degradation by inducing its ubiquitylation $^{72}$. On the contrary, the PNPLA3 I148M mutant isoform eludes ubiquitylation and proteasomal degradation and accumulates in the LDs where it hampers triglyceride breakdown causing impaired very low density lipoprotein (VLDL) secretion $^{73,74}$. 


Noteworthily, the effect of carrying the 148M mutant protein is larger in women because PNPLA3 expression is regulated by estrogens \(^{75}\). From this perspective, a potential PNPLA3 inhibitor may be more effective for the treatment of women with MASLD and carrying the 148M mutant protein.

*Pnpla3* silencing by GalNAc-conjugated ASO ameliorated MASLD in human *PNPLA3 I148M* mutant knock-in mice fed a NASH-inducing diet \(^{76}\). Besides, administration of *PNPLA3 I148M* siRNA in hepatocyte-specific human *PNPLA3 I148M* knock-in mice re-established PNPLA3 WT phenotype \(^{77}\). It is critical to consider that the expression and enzymatic activity of PNPLA3 would not be recovered following the silencing of the I148M variant \(^{61}\). To date, several oligonucleotide-based drugs targeting *PNPLA3* are in early phase clinical trials to treat SLD (Table 1).

**17-beta hydroxysteroid dehydrogenase 13 (HSD17B13)** is a LD-associated protein expressed only in hepatocytes with a yet to define function. The *rs72613567:TA* polymorphism encodes for a frameshift resulting in a truncated HSD17B13 protein that is present at a low level in the hepatocytes. The minor allele of this variant associates with lower transaminases and with prevention of MASLD progression that is consistent among several ethnic groups \(^{78}-^{81}\). However, the variant protective effect on liver disease without influencing liver steatosis may be elicited through mitigation of inflammation \(^{82,83}\). Recently, Ma et al. showed that therapeutic administration of *Hsd17b13* ASO induced a dose-dependent inhibition of the hepatic expression of Hsd17b13 and a significant reduction of liver steatosis in a NASH-like hepatic fibrosis mice model, but it did not affect the hepatic fibrosis phenotype \(^{84}\).

Currently, various oligonucleotide-based drugs targeting *HSD17B13* are in early phase clinical trials (Table 1) with some promising findings. Indeed, subcutaneous administration of ARO-HSD was well tolerated and led to a dose-dependent depletion of the *HSD17B13* mRNA and protein levels, followed by a reduction of circulating alanine transaminase (ALT) levels in healthy adult volunteers and individuals with MASH \(^{85}\).

**Mitochondrial amidoxime-reducing component 1 (MTARC1)** is a reductase that localizes in the outer membrane of mitochondria and catalyses the reduction of N-oxygenated compounds, thus mediating detoxification reactions
under acidic and hypoxic conditions. The MTARC1 rs2642438 minor and protective allele encodes for an alanine to threonine substitution at position 165 of the protein (p.A165T) that results in lower intracellular protein levels due to a higher ubiquitin-mediated proteosome degradation. The MTARC1 p.A165T variant does not affect the enzymatic activity of the protein, but its stability. This variant associates with lower hepatic triglyceride content, lobular inflammation, serum ALT, alkaline phosphatase and cholesterol levels, and with protection against MASLD severity and HCC. Indeed, MTARC1 deficiency seems to increase intracellular fatty acid oxidation and substrate utilization. Recently, Lewis et al. showed that the administration of GalNAc-siMtarc1 to mice fed the Gubra Amylin NASH (GAN) diet reduced liver fat content whereas did not cause phenotypic change in fibrosis despite markers of fibrosis were improved. Importantly, circulating triglyceride levels increased following the GalNAc-siMtarc1 administration. Moreover, Mtarc1 hepatocyte-specific knockdown by GalNAc-Mtarc1 siRNA improved lipid profiles and protects against MASH progression in obese and diet-induced MASH mouse models. To date, the NN6581-4860 siRNA-based drug inhibitor of MTARC1 is currently in a phase 1 clinical trial (Table 1). Interestingly, MTARC1 inhibition may work with the same mechanism of thyroid hormone receptor beta agonist Resmetirom, the first drug approved by the U.S. FDA for treating subjects with noncirrhotic-NASH with moderate to advanced hepatic fibrosis (NCT03900429), namely increase in beta-energy substrate utilization.

Membrane bound O-acyltransferase domain-containing 7 (MBOAT7) is a lysophosphatidylinositol acyltransferase (LPIAT1) that localizes in ER, mitochondria-associated membranes and LD. MBOAT7 is a multi-transmembrane protein catalysing the acyl-chain remodelling of phospholipids, preferentially transferring polyunsaturated fatty acids (PUFAs), such as arachidonic acid, to phosphatidylinositol. MBOAT7, by finely regulating the availability of acyl-CoA and lysophospholipids, can predispose to the onset and progression of MASLD-driven liver damage. Indeed, the rs641738 C>T variant in the locus containing the MBOAT7 gene has been linked to reduced gene and protein expression, and to increased risk of hepatic steatosis and progressive MASLD, characterized by higher levels of serum ALT, inflammation, fibrosis, and HCC. On the other hand, the rs641738 C>T variant has been associated with lower serum triglycerides. Recently, Sharpe et al. showed that hepatic MBOAT7 overexpression in murine NASH models significantly improved hepatic lipid content and serum transaminases levels, but no change in NASH
Histology was detected. Moreover, hepatocyte-specific MBOAT7 knockout in mice worsened liver steatosis and fibrosis by inducing triglyceride synthesis and the expression of TAZ, a master regulator of hepatic fibrosis. Indeed, Moore et al. proved for the first time that MBOAT7 depletion in hepatocytes induced changes in phospholipids profile by promoting a cholesterol trafficking pathway able to induce the expression and activity of TAZ and Indian hedgehog (IHH), a known TAZ-induced pro-fibrotic factor. Indeed, carriers of the rs641738 C>T variant with MASH have higher TAZ and IHH levels. Conversely, hepatocyte-MBOAT7 restoration in MASH mice reduced the expression of TAZ, thus slowing down fibrosis development.

Noteworthily, homozygous carriers of loss of function mutations in MBOAT7 have severe neuronal defects, intellectual disability, autism and epilepsy due to the involvement of MBOAT7 in brain development.

Transmembrane 6 superfamily member 2 (TM6SF2) is a multi-transmembrane protein that localizes in the ER and LD of hepatocytes and intestinal cells. The TM6SF2 rs58542926 (p.E167K) minor allele associates with increased MASLD and fibrosis but reduces risk of cardiovascular events. The aminoacidic change results in a protein with reduced stability and, therefore, the loss of function causes liver fat accumulation by retention of lipoproteins. Indeed, carriers of the TM6SF2 rs58542926 genetic variant have MASLD due to increased lipid biosynthesis and defective VLDL assembly and secretion. This induces hepatic lipid entrapment resulting in lower serum lipid levels and reduced risk of myocardial infarction.

The TM6SF2 protein is stabilized by ER lipid raft proteins (ERLINS) and in turns stabilizes apolipoprotein B by two intraluminal loops. Interestingly, the effect of TM6SF2 E167K on MASLD is weakened by the protective effect of the ERLIN1 rs2862954 (p.I291V) loss of function variant against hepatic fat accumulation.

Pleckstrin and sec7 domain-containing 3 (PSD3) is a member of the Guanine nucleotide exchange factor for ARF-6 (EA6) family. The rs71519934 variant in the PSD3 gene, resulting in the p.L186T amino acid substitution, has been associated, although with some inconsistencies, with protection against MASLD development. Moreover, PSD3
downregulation by siRNAs in primary and immortalized hepatocytes in vitro, as well as Psd3 silencing by GalNAc-conjugated ASO in mice fed a NASH-inducing diet, ameliorated MASLD \(^{119}\).

**Cell death-inducing DNA fragmentation factor-like effector b (CIDEB)** localizes in hepatic ER and LD \(^{120}\). CIDEB is an important regulator of the VLDL pathway as it interacts with apolipoprotein B (APOB) to allow triglyceride-enriched VLDL particles biogenesis, lipidation and maturation \(^{120,121}\). On the one hand, Cideb-deficient mice showed smaller hepatic LD, decreased de novo lipogenesis and lower lipid exchange activities \(^{122}\). On the other hand, they displayed higher hepatic triglyceride content due to reduced VLDL secretion, and lower levels of circulating free fatty acids and triglycerides \(^{120}\). Recently, Chen et al. showed that under ER stress the expression of Cideb is suppressed by C/EBP homologous protein (Chop), thus causing the inhibition of VLDL maturation and transport, and inducing hepatic oxidative stress, triglyceride accumulation and inflammation \(^{123}\). Strikingly, in a multistage analysis including more than one million individuals, loss-of-function variants plus missense variants in CIDEB have been associated with lower ALT levels and reduced risk of SLD development \(^{124}\).

**Other potential genetic therapeutic targets in MASLD**

The apolipoprotein E (APOE) rs429358 \(^{125}\), glucokinase regulator (GCKR) rs1260326 \(^{126}\) and the glycerol-3-phosphate acyltransferase, mitochondrial (GPAM) rs2792751 \(^{125}\) genetic variants contribute to MASLD susceptibility. However, these variants are very pleiotropic with effects on multiple traits that make problematic the manipulation of these genes for therapeutic purposes. For example, the GCKR rs1260326 minor allele increases liver triglyceride content by modulating the handling of glucose and triglycerides in hepatocytes. Indeed, carriers of the rs1260326 minor allele show lower serum glucose and insulin levels, have higher LDL cholesterol and circulating triglycerides and lower intrahepatic triglyceride levels\(^{127,128}\). The dissociation between intrahepatic and circulating triglycerides makes manipulation of this gene problematic. The APOE rs429358 variant is strongly associated with an increased risk of Alzheimer disease \(^{129}\), whilst the same minor allele protects against MASLD making this gene unattractive to target.
Finally, the glycerol-3-phosphate acyltransferase, mitochondrial (GPAM) rs2792751 minor allele associates with higher hepatic triglyceride content and transaminases. However, while some data suggest that downregulation of this gene would be effective in reducing liver triglyceride content, to the best of our knowledge, targeting this gene for MASLD it is not in any current pharmaceutical pipeline.

Figure 3. Representative figure of the main validated hepatic gene targets involved in SLD. Abbreviations: APOB100, Apolipoprotein B; ATGL (PNPLA2), Patatin like phospholipase domain containing 2; CD36, CD36 Molecule (CD36 Blood Group); CGI-58, comparative gene identification-58 (Abhydrolase domain containing 5 (ABHD5), Lysophosphatidic acid acyltransferase); CIDEH, Cell death-inducing DNA fragmentation factor-like effector b; DGTA2, Diacylglycerol O-acyltransferase 2; FFA, free fatty acid; GLUT, Glucose transporter; GPAM, Glycerol-3-phosphate acyltransferase 1, Mitochondrial; HSD17B13, 17-beta hydroxysteroid dehydrogenase 1; LD, lipid droplet; MBOAT7, Membrane bound O-acyltransferase domain-containing 7; MTARC1, Mitochondrial amidoxime-reducing component 1; MTTP, Microsomal triglyceride transfer protein; PLA2, Phospholipase A2; PNPLA3, Patatin-like phospholipase domain-containing protein.
Conclusions

Significant progress have been made in the last decade in designing and synthetizing chemically modified oligonucleotides with an important metabolic stability, specificity and efficacy capable of post-transcriptional hepatic gene silencing for clinical purposes. One of the main advantages of nucleotide-based therapies is that the target is not a protein with its own three-dimensional structure, stability and specificity that can move into the cells, but they only need to bind with their antisense strand the right nucleotide sequence of the target mRNA. Moreover, they can only have antagonist effects, they are characterized by a very high selectivity, and their synthesis is easier than other tools, such as antibodies \(^{32}\). Another significant advantage of modern oligonucleotides is that they can target “undruggable targets” without off target effects due to the interaction with unspecific mRNAs. To date, oligonucleotides technology is rapidly advancing, and it already represents an encouraging novel method for the treatment of rare disease as well as SLD, with promising studies in late-stage clinical development. Thanks to their efficacy, low toxicity, high specificity, and high tissue distribution, oligonucleotides-based drugs will soon represent a pivotal tool for personalized therapeutic strategies for the treatment of both the early and late stages of hepatic disorders \(^{53}\).

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Authors’ contribution

Drafting of the manuscript: AC; Critical revision and finalization of the manuscript: SR. Final approval of the manuscript: AC, SR.
**Conflicts of Interest**

The authors have no conflicts to disclose.


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Table 1. A summary of nucleotide-based therapeutics (siRNA or ASO) targeting the main genetic therapeutic targets in MASLD currently undergoing clinical trials.