Functional Insights Into Novel Regulators of Plasma Lipids

Loaiza, Natalia

DOI:
10.33612/diss.508413229

7

General Discussion

GENERAL DISCUSSION

Atherosclerotic cardiovascular disease (ASCVD) remains a leading cause of death worldwide despite the great advances in understanding the pathophysiology and risk factors of the disease. This, all together, has made the development of effective evidence-based therapies and preventive measures possible. Making biological sense out of the wealth of statistical associations emerging from human genetic data currently available on plasma lipids and atherosclerosis, even promises the realization of personalized diagnosis and tailored treatments to further alleviate this burden in the future. Aiming to contribute to this general goal, we have studied here the molecular mechanisms and biological function of two novel genes that have been proposed to be implicated in plasma lipid metabolism: *STAP1* and *GPR146*. Using a combination of experimental approaches, including *in vitro* systems, mouse models, as well human genetic data, we have shed new light on functions of these proteins on plasma lipids and atherosclerosis development and provided clues to guide further research efforts. In this section, we summarize our findings and place them in the larger context of the cardiovascular research field.

STAP1 does not regulate plasma LDL-c in mice or humans

Emerging from studying families with unexplained autosomal dominant hypercholesterolemia or familial hypercholesterolemia (FH), *STAP1* was proposed in 2014 to be a novel FH gene but without experimental evidence of the underlying biological mechanisms (1). The fact that *STAP1* is mainly expressed in B cells was puzzling and suggested a novel regulatory pathway connecting immune cell function with lipid regulation in patients with FH, an attractive hypothetical scenario to expand the notion of ASCVD as an inflammatory as well as lipid-driven disease (2–4). This identification of defective *STAP1* as a novel cause of hypercholesterolemia generated significant interest in the FH research field, as illustrated by the following subsequent instances:

1. *STAP1* became annotated as the familial hypercholesterolemia locus 4 (FH4) in subsequent opinion articles, which *'essentially informally certified its role in the disease for the scientific community'* (5,6).

2. *STAP1* was added to the updated lists of FH causal genes in scientific reviews (7).

3. *STAP1* was included in FH targeted sequencing panels for molecular patient diagnosis and research purposes (8,9), as it was expected that the expanded screening for *STAP1* variants in FH patients and healthy individuals would

eventually lead to the identification of additional loss of function variants to confirm its nomination as causal FH gene (6).

In spite of this initial enthusiasm, follow up clinical studies failed to validate a role for STAP1 in FH (10–13). However, these studies focused on only a few patients while they did not present experimental evidence to allow drawing firm conclusions. In fact, experimental approaches were absent from the literature until the publication of investigations described in Chapter 2. Here, we present our work on the development and use models to study the molecular mechanisms underlying the association between STAP1 and FH. The lack of positive findings with *Stap1*$^{-/-}$ mice and *Ldlr*$^{-/-}$ mice transplanted with *Stap1*$^{-/-}$ bone marrow, prompted us to contact our colleagues in Amsterdam to study the discrepancy of our study outcome. Our colleagues subsequently invited the subjects of their initial study for a blood withdrawal to measure plasma lipids for a second time. In this effort, they did not find statistically significant differences in levels of plasma TC or LDL-c between carriers of *STAP1* gene variants and controls. These findings combined with our animal studies show that STAP1 does not contribute to LDL-c regulation and should be delisted as an FH candidate gene. Our findings on *Stap1*$^{-/-}$ mice have also been independently validated in a murine knockout model, developed by targeted homologous recombination of embryonic stem cells (instead of CRISPR/Cas9 technology used by us) and after a longer diet challenge with Western-type diet (14). In an editorial comment accompanying the publication of our study, Hegele and coauthors (2020) remarked that this work was "*the final nail in the coffin for STAP1 as a causative gene for FH*"(6) .

In the same editorial the authors proposed the removal of *STAP1* from FH sequencing panels and underscored the need of stringent supportive evidence for the endorsement of future emerging FH genes. This includes providing strong statistical data, evolutionary conservation based on predictive bioinformatic tools, expression and structural modeling, and functional/experimental assessments *in vitro*, *ex vivo*, and *in vivo* (6), in accordance to the proposed analogous principles of the Koch postulates for genetics (15,16).

Table 1. Comparison between the original Koch postulates and the analogous principles proposed to demonstrate causality in genetics, as proposed by Marian (2014) (15)

	Original Koch postulates	The analogous components of the Koch's postulates for establishing causality in genetics (extracted from Marian, 2014)
1.	The microorganism must be found in diseased but not healthy individuals	Causal variants must be found and enriched in the families or subjects with the phenotype
2.	The microorganism must be cultured from the diseased individual	The candidate causal variants must be functional and pathogenic (novel or rare, conserved, and protein-altering).
3.	Inoculation of a healthy individual with the cultured microorganism must recapitulated the disease	The introduction of the variants into an experimental model should cause a phenotype that resembles the phenotype in humans.
4.	The microorganism must be re-isolated from the inoculated, diseased individual and matched to the original microorganism.	The removal (deletion or silencing) of the candidate causal variants should reverse the phenotype.

In retrospect, it has become clear that the inclusion of *STAP1* in the family of FH genes in 2014 precipitated due to the lack of key evidence to support the original association reported in 2021. Had that study included the experimental work provided in Chapter 2, the conclusion of the study would have been different, preventing the follow up 6 years of research efforts to demonstrate that *STAP1* does not meet the scientific criteria as a genetic disease causal gene (15,16). It is also likely that many research efforts lacking support for *STAP1* as an FH gene, did take place but were not published due to the prevailing publication bias in favor of positive results (17–19). This situation is aggravated as the original publication was published in a high-profile journal. Our work with *STAP1* is an example that this practice needs attention, and it is also our hope that our study contributes to prevent similar situations in the future.

Although the lipoprotein field had relied on family studies for years as a major tool to discover new lipid and lipoprotein regulators, other recent genetic approaches have emerged in use, of which Genome Wide Association Studies (GWAS) have drawn most attention. Several major studies were published between 2008 and 2018 which were brought to the lipoprotein community a "treasure trove" of more than 150 annotated genes and loci, many of which had previously unidentified links to plasma lipid regulation. These studies inspired a wave of efforts to validate the biological relevance of these hits and searches for new potential therapeutic opportunities (20). Despite large and ongoing efforts, only very few novel GWAS hits have fulfilled the analogous Koch principles to demonstrate causality for their associated plasma lipid phenotypes,

such as *GALNT2* (21), *SORT1* (22), and *TTC39B* (23). In 2013, *GPR146* appeared for the first time on these lists (24). In other cases, such as *ABCA6* (25), attempts the work on *in vivo* models did not lead to validation of the GWAS hit (26), highlighting the absolute necessity of experimental confirmation for genetic associations.

Orphan G-protein-coupled receptor 146, GPR146, influences plasma total cholesterol mainly through its effects on HDL-c

GPR146 belongs to the list of novel GWAS hits reported in 2013 by Willer *et al*., (2013), making entrance into the lipid research field with the promise that elucidation of its statistical association with total plasma cholesterol levels could shed light on novel regulatory pathways. This was accomplished by an experimental animal study published six years later describing that hepatic *GPR146* expression modulates the VLDL production and SREBP2 pathway via ERK1/2 signaling (27). It was also shown that GPR146 deficiency reduces plasma lipid levels and protects against atherosclerosis independent of the LDL receptor.

While the initial GWAS showed that a common genetic *GPR146* variant was associated with total cholesterol levels, a later study showed that this SNP is a causal variant which increases *GPR146* mRNA expression (27,28). Later larger GWAS showed that the association of this same SNP (rs1997243) with HDL-c was stronger than its associations with LDL-c levels. In other words, increased *GPR146* expression is associated with elevated LDL-c and HDL-c levels. This observation is quite unusual as changes in LDL-c are normally not accompanied by changes in HDL-c or *vice versa* (29).

In Chapter 4, we show that in humans, a second SNP (rs2362529), negatively affecting *GPR146* at the mRNA level and, opposite to the previously reported SNP, is instead associated with lower LDL-c and HDL-c. These relationships were found to be gene-dose dependent, suggesting the involvement of a biological relationship. *Gpr146*$^{-/-}$ mice also display reduced HDL-c, which has thus far been left unexplained. Research in this direction is warranted to improve our understanding of the regulation of the bad (LDL) and good (HDL) cholesterol but also because reductions in HDL-c are unwanted (30–34) although it may not pose a threat for the development of GPR146 inhibitors (35). Recent advances in the HDL field have emphasized that HDL function may be a better predictor of ASCVD risk than the cholesterol content reflected by HDL-c plasma levels (36). This may indeed be true when considering that ANGPTL3 loss-of-function mutations have been shown to be associated with reductions in both LDL-c and HDL-c while also protecting against CVD (33,37–39).

To study the role of GRP146 in HDL metabolism, we tested the hypothesis that SR-B1, the main HDL receptor, may be involved in explaining the lower HDL-c phenotype in humans as well as mouse models (Chapter 5). Carriers of common variants affecting *GPR146* expression levels showed changes in HDL-c without affecting triglycerides, which is similar to what has been reported for carriers of mutations in *SCARB1*, encoding for SR-B1 (40–42). Moreover, we found an inverse and consistent correlation between GPR146 expression and SR-B1 protein levels in cellular as well as animal studies. To study a possibly causal contribution of SR-B1 to reduced HDL-c levels in *Gpr146*$^{-/-}$ mice, we overexpressed the PDZ1 domain of PDZK1, which is known to block SR-B1 protein function and trigger a phenotype similar to the one observed in *Scarb1*$^{-/-}$ mice (43). The results led to the conclusion that SR-B1 may not be the main driver of the low HDL-c phenotype in mice and other molecular players remain to be uncovered.

It is, however, necessary to confirm the robustness of these findings with a better experimental set-up. Although overexpression of PDZ1 significantly reduced SR-B1 protein levels in the liver of treated mice as well as its intracellular mislocalization (43), it is unclear whether the remaining SR-B1 protein could have differential activity in *Gpr146*$^{-/-}$ mice compared to *Gpr146*$^{+/+}$, which could explain the persisting differences between genotypes. It has also been reported that as a scaffold protein, PDKZ1 is also involved in the plasma membrane expression of other G-protein coupled receptors (GPCR), thus potentially introducing direct or indirect bystander effects in our experimental system (44). It would be better to backcross *Gpr146*$^{-/-}$ mice to *Scarb1*$^{-/-}$ mice, mimicking the study of Yu et al (2019) to demonstrate that the hypolipidemic phenotype of *Gpr146*$^{-/-}$ mice is independent of the LDLR. Alternatively, simultaneous somatic gene editing of the *Scarb1* and *Gpr146* genes could be an eligible set up to test our hypothesis. Last but not least, functional assays such as selective HDL cholesterol uptake assays (45), will allow to compare SR-B1 activity between genotypes and offer increased insight into the contribution of SR-B1 to changes in HDL-c in *Gpr146*$^{-/-}$ mice.

Alternative to SR-B1, we also propose additional candidates for further exploration. Using targeted mass-spectrometry-based proteomics, we found significant reductions in other HDL-c related proteins in the plasma of *Gpr146*$^{-/-}$ mice, including ApoA-I, ApoE, phospholipid transfer protein (PLTP), ANGPTL3, ApoC-III, lecithin: cholesterol acyltransferase (LCAT) and endothelial lipase (EL; Figure 1), all well-known regulators of HDL-c plasma levels in mice and humans (46–49). However, as with SR-B1, we have at this point only observational data and actual functional contributions needs to be addressed. As summarized in Table 2, the effects of the loss of PLTP, ApoC-III, LCAT and EL in mouse models on plasma lipids is different from

Gpr146$^{-/-}$ (46,49,50). The plasma reductions for these proteins shown in Figure 1 is probably a consequence of an overall decrease in HDL particles in *Gpr146*$^{-/-}$ mice. On the other hand, the loss of ApoA-I (48) and ANGPTL3 (Fujimoto et al., 2006; Wang et al., 2015) appear to mimic the loss of GPR146 (Table 2).

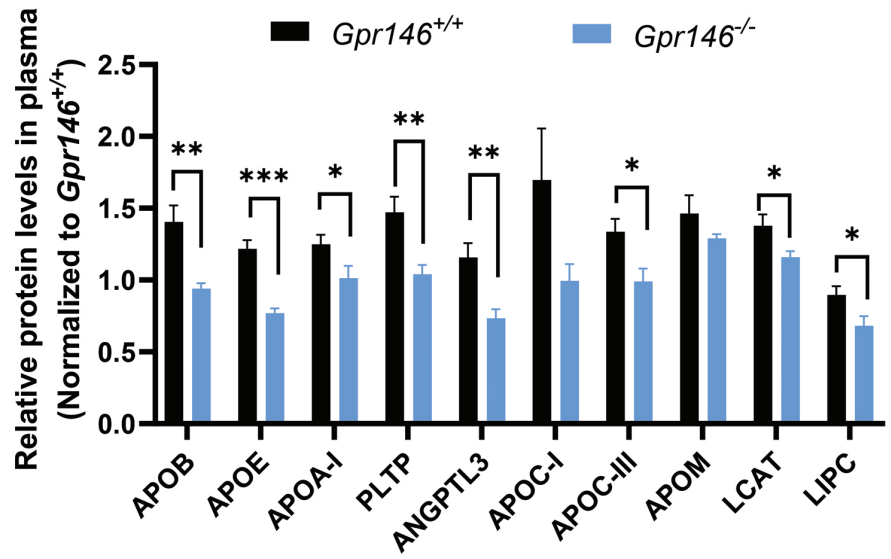

Figure 1. Plasma proteins found to be reduced in chow fed male *Gpr146*$^{-/-}$ mice compared to controls, as measured with targeted mass-spectrometry based proteomics.

Table 2. Comparison of the expected phenotypes observed in knockout mouse models compared to *Gpr146*$^{-/-}$ for known regulators of HDL-c found decreased in the plasma of *Gpr146*$^{-/-}$ mouse

Gene	*Plasma lipid profile in knockout mouse*	*Compatible with Gpr146*$^{-/-}$ *mouse phenotype (↓HDL, ↓LDL-c ↓TG)*	*References*
APOA1	↓HDL, ↓LDL-c ↓TG	Yes	(48)
PLTP	↓HDL, ↑LDL-c ↑TG	No	(50)
ANGPTL3	↓HDL, ↓LDL-c ↓TG ↓ VLDL production	Yes	(47, 51)
APOC-III	↔HDL↓LDL-c ↓TG	No	(52)
LCAT	↓HDL, ↓LDL-c ↑TG	No	(49)
LIPC	↑HDL, ↑LDL-c ↑TG	No	(46)

↔ no change ↓Decrease ↑increase

Of these candidates, ANGPTL3 is of special interest as it shares three important similarities with GPR146: 1) its mechanism of action has been shown to be independent of the LDL receptor (37,51,53), which is also reported for GPR146 (27), and 2) a recent Mendelian randomization analysis shows that loss of ANGPTL3 is also associated with reduced CRP levels (54), similar to what we observed in our study of common *GPR146* variants in Chapter 4.

ANGTPL3 inhibitors have proven to be successful in reducing atherogenic plasma lipids in clinical trials (51,53,55,56) while ANGPTL3 genetic efficiency is associated with protection from ASCVD (39,57). If GPR146 is causally associated with ANGPTL3 plasma levels, GPR146 antagonism using small molecules could constitute a new means to reduce ANGPTL3. Apart from these pharmaceutical considerations, further exploring the association between *GPR146* and ANGPTL3 may provide new scientific insight.

Despite the similarities between loss of GPR146 and ANGPTL3, there is one remarkable difference: ANGPTL3 deficiency is also associated with a marked reduction in plasma triglycerides in humans (22,47,58,59) while for GPR146, this is observed in mice but genetic variation in GPR146 has thus far not been shown to affect plasma triglycerides (27,28).

Another recent promising lipid-lowering drug that may offer clues to better understand the role of GPR146 in lipid metabolism is bempedoic acid because the effects on plasma lipids are similar to those observed in *Gpr146*$^{-/-}$ mice (28). Bempedoic acid is an inhibitor of the ATP-citrate lyase (ACL), blocking the lipid biosynthesis pathway upstream of HMG-CoA reductase (the target of statins), and reducing LDL-c and atherosclerosis in experimental settings (60–62) as well as recent clinical trials (63,64). Similar to the phenotype of carriers of the possibly functional GPR146-p.Pro62Leu mutation, bempedoic acid reduces not only LDL-c but also on HDL-c and CRP (65). In addition, studies in *Ldlr*$^{-/-}$ mice and Yucatan miniature pigs indicate that its effect is independent of the LDLR pathway (60,62) while it also shows decreased activation of pERK1/2 (60). In addition, both bempedoic acid and GPR146 inactivation are known to have effects on cholesterol synthesis genes (27,60). These data suggest that interventions in the cholesterol synthesis pathway can have more complex consequences than boosting the SREBP2 pathway and increasing LDL receptor protein as currently assumed. Studying the lipid-lowering mechanisms of GPR146 and bempedoic acid in *Ldlr*$^{-/-}$ models may help elucidating complementary mechanisms. In addition, it would be interesting to compare the response to bempedoic acid treatment between *Gpr146*$^{-/-}$ mice and controls, and to also compare the effects of common

variants in the *ACLY* and *GPR146* genes in plasma lipid traits and the risk of ASCVD to see if how much their effects are similar in magnitude and direction, following a Mendelian Randomization approach (66).

An experimental system to test the functional impact of rare genetic variants in *GPR146*

To increase our understanding of the function of *GPR146* in humans, we sequenced the *GPR146* gene in subjects with the highest and lowest LDL-c for their age and gender in two general population studies as described in Chapter 4. The p.Pro62Leu variant emerged as an interesting variant taken an expected impact of a loss of a proline residue that has been shown to be evolutionary conserved across species, and it is predicted to be damaging based on different established algorithms. We later found that this variation is present in approximately 1 in a 1000 in the general population, which made more extensive studies possible. The association of this variant with changes in lipid levels are considerably stronger compared to a common variant (rs2362529) associated with lower *GPR146* expression. These findings led us to hypothesize that the p.Pro62Leu variant confers loss-of-function, which we have tried to validate in *in vitro* experiments.

We were, however, not able to identify significant differences between wild-type and mutant p.Pro62Leu GPR146 on ERK1/2 activation and were confronted with technical shortcomings of our experimental set up. First, we observed that regular GPR146 overexpression caused cell death after a few days of culture, which made it impossible to establish a stable overexpression cell line. This indicated the need of an inducible/titratable system to offer more control on the timing and levels of GPR146 overexpression. Although not discussed in Chapter 4, we attempted to do this using the fU-tetO-gateway lentiviral system, which is inducible with doxycycline. However, in our hands, this system showed large variability between experiments and proved to be not sensitive enough to assess the functionality of the p.Pro62Leu variant based on ERK activation. This system also lacked a selection marker and thus did not allow to generate a stable cell line. We speculate that this could have contributed to the large variability observed between experiments, which could be addressed thought e.g., implementing a FACS selection cassette or an antibiotic marker independent of an inducible GPR146 expression. This way we could generate a more homogeneous and controlled system. But even with a trustworthy overexpression system in place, the actual readout of GPR146 activation may also need revisiting. Although pERK activation through western blotting has been the focus of our studies thus far, it would be useful to implement an assay that allows for better quantitative results. It would in this regard good to test assays based on Ca2+ release, cAMP or B-arrestin,

as generally performed for GCPRs (67). Such a tool will not only help the envisioned functional studies (testing GPR146 variants) but also help us to decipher how activation of this receptor affects metabolism.

To overcome inherent problems with *in vitro* models, we also studied the impact of the p.Pro62Leu variant *in vivo* (Chapter 4). Unfortunately, overexpression of human GPR146 or its mutant form p.Pro62Leu in *Gpr146*$^{-/-}$ mice did not induce changes in plasma lipids. This could be due to species-specific differences or protein folding problems related to overexpressing of G-protein coupled receptors *in vivo*. It would not be the first time when overexpression of a human homolog protein fails to rescue a mouse phenotype (68,69). In this light, there are ongoing efforts to develop a *Gpr146*-p.Pro62Leu knock-in mouse model.

Deorphanization of GPR146

Finding the endogenous ligand of GPR146 will be instrumental to understand its physiological role in lipid metabolism and provide insight into its potential as a drug target. The first published study attempting to elucidate this, reported that Proinsulin C-peptide was likely the endogenous ligand of GPR146 (70). However, a recent study could not validate this interaction in CHO-K1 cells expressing human GPR146 stimulated over a wide range of concentrations of C-peptide. In fact, these investigators did not show any of the expected intracellular responses with ligand binding which was based on multiple readouts, including dynamic mass redistribution and GPCR β-arrestin assays, as well as with fluorescence confocal microscopy (71). Thus, in the public domain, GPR146 remains an orphan GPCR, which limits the development of tools and assays to understand its function in health and disease.

In Chapter 4, we show that no pERK1/2 activation is observed in cells overexpressing GPR146 when these are starved and only show a difference after stimulation with FCS following starvation. In other words, it seems that FCS contains the natural ligand(s) necessary to activate GPR146 and trigger ERK1/2 signaling. Apparently, in plasma from fasted-refed mice, GPR146 activators are also more abundantly or exclusively present compared to the fed state. It is possible, that these compounds are present in the food, but it could also concern an intrinsic component related to the refeeding response that is produced by the mice as e.g., insulin. While the C-peptide, a protein domain of insulin, has been discarded as the ligand of GPR146 (71), it is possible that insulin signaling is linked to GPR146 via the ERK1/2 pathway (72). Tests with plasma of starved or fasted mice could be an interesting option to test this hypothesis. One could alternatively explore whether the phenotype of *Gpr146*$^{-/-}$ mice is dependent on

insulin signaling through blunting insulin production with streptozotocin followed by insulin administration (73).

GPR146 downregulation as novel lipid lowering approach and its effect on atherosclerosis development

The finding that the effects of GPR146 are independent of (27) is of potential relevance to patients with homozygous FH with a complete lack of LDL receptor activity. This especially relevant because registered drugs such as statins and PCSK9 inhibitors are dependent on LDL receptor function. While the development of small molecule inhibitors for GPR146 has not been reported so far, inhibition of GPR146 in only liver is also feasible with RNAi and antisense oligonucleotide (ASO) technologies, which have been already shown successful results inhibiting hepatic expression of PCSK9, ANGPTL3 and Lp(a) (Chaudhary et al., 2017; Gaudet et al., 2020; Graham et al., 2017; Tsimikas et al., 2020). Furthermore, newer chemical modification even allow oral formulation instead of subcutaneous delivery of antisense oligonucleotide (77) and even CRISPR based editing targeting with promising results in primates (78,79).

In Chapter 6, we set out to study the atheroprotective effects of liver-specific loss of GPR146 with a shRNA approach in a murine model with intact LDL receptor function and resembling the human lipoprotein profile: the apoE*3-Leiden-CETP mouse model. With this study we aimed to demonstrate the feasibility of hepatic downregulation of GPR146 to reduced atherosclerosis. Although our experimental model recapitulated the reduction in total cholesterol and triglycerides as seen in whole body knockout mice, we did not find differences in plaque size or severity compared to controls. This may be related to the fact that the shRNA tool used did not sustain downregulation of the GPR146 for the duration of the experiment but it is also possible that the high dose of AAV8-shRNA used together with a strong promoter may have led to hepatotoxic effects (80). This might have also triggered systemic or hepatic inflammatory pathways that cancelled out the beneficial effect of a lower cholesterol exposure. Since the U6 promoter is known to not be fully liver specific (81), it also possible that GPR146 downregulation outside the liver might have pro-atherogenic effects. Since GPR146 is ubiquitously expressed, downregulation in the endothelium may have detrimental effects on vascular function. With the technical constrains of our experiment, it remains to be established whether hepatic downregulation of GPR146 will protect against atherosclerosis.

Final remarks on GPR146 and its molecular mechanisms of action

From our observations with *Gpr146*$^{-/-}$ mice and based on the work from Yu et al (2019), we would like to highlight that GPR146 seems to enhance ERK1/2 signaling only

after 6h refeeding after a 16h fast. It is puzzling that *ad libitum* fed *Gpr146*$^{-/-}$ mice exhibit a hypocholesterolemic phenotype and that no differences in gene expression of cholesterol synthesis genes are observed in the fasted state (unpublished data from our lab and confirmed by personal communication with Dr. Haojie Yu). Artificial, prolonged fasting and refeeding known to induce a "enzyme overshoot", is a recognized as a means to dramatically stimulate fatty acid (82) and cholesterol synthesis pathways (83,84), which increases chances to find small differences in gene expression. The gene expression changes affecting cholesterol synthesis genes in *Gpr146*$^{-/-}$ mice, undetectable in the postprandial state, may still drive the phenotype in *ad libitum* fed mice based on the proposed mechanistic model for GPR146 proposed by Yu *et. al* (2019). However, in our opinion, inhibition of ERK1/2/SREBP2 pathway under fasting-refeeding conditions is insufficient to clarify the lipoprotein profile of *Gpr146*$^{-/-}$ mice**.** It also remains poorly understood how this mechanism, acting at the level of cholesterol synthesis and via de SREPB2 pathway can work independently of the LDL receptor. In addition, our studies suggest that another main lipoprotein receptor, SR-B1 is unlikely to explain the effects on HDL-c. A remarkably message emerges from the data obtained thus far: GPR146 modulation of plasma lipids seems to be independent of the known major molecular determinants of plasma concentrations of LDL-c and HDL-c, i.e., LDLR and SR-B1, which highlights our incomplete understanding of plasma lipid homeostasis. What seems clear is that the mechanisms underlying the phenotypes observed upon loss of GPR146 are responsive to a signal coming from one single plasma membrane GPCR leading to a peculiar plasma lipid phenotype, which seems to induce a rearrangement of the plasma cholesterol steady-state without hepatic side effects. As discussed above, comparative analysis of GPR146, ANGPTL3 and ACLY may be warranted. It is also possible that GPR146 mediates its effects through multiple pathways in the fed and feeding states.

The GPR146-associated plasma lipid phenotypes studied in mice thus far are of a relatively small magnitude (e.g. 20% difference in total cholesterol with WT mice), which leaves space for questioning the potential of this target and the availability of promising lipid-lowering drugs targets already available or under development (85). While this is true for general dyslipidemia, FH caused by complete impairment of LDL receptor function continues to hold the unmet medical need for affordable drugs acting independently of the LDLR in spite of recent developments (86). Although ANGPTL3 inhibitors have been recently approved by the Food and Drug Administration in the US to treat these FH patients, the estimated cost of $450,000 per year on average per patient (87) indicates a need for more affordable alternatives. In this regard, small molecule inhibitors of GPR146 may be welcomed as a possible cheaper alternative. Although we have only started the unraveling of the mechanisms

by which GPR146 acts, this does not stop pharmaceutical companies to start exploring GPR146 antagonism (Alnylam Pharmaceuticals filed a patent to silence GPR146 through RNAi; Patent Application Number US2021019987).

On the other hand, further characterization of Gpr146$^{-/-}$ mice performed by the International Mouse Phenotyping Consortium (www.mousephenotype.org) already warns of possible safety concerns of GPR146 inhibition at the level of red blood cells and platelets, among others (88), which will require close examination and understanding to clarify the true potential for the development of GPR146 inhibitors in humans. Our work on common and rare variants affecting GPR146 gene expression can already offer initial tools to explore whether these extra hepatic phenotypes could also be a concern in humans.

Final considerations and future perspectives

In the new age of omics data and precision medicine, is there still space and need for the "one gene at the time" style of research followed in this thesis? It is time consuming, costly, risky, and inefficient. However, it drives translational advances to enable more effective prevention and/or treatment of disease as one of the ultimate goals of genetic research (89). Diving into the biological mechanisms remains an unavoidable task for the lists of new genetic targets. Unfortunately, current prioritization tools have very limited strengths, and there are no viable systems available to comprehensively assess the effects of genetic variants on the human health and disease. It thus remains a challenge to make biological sense of genome-wide significant p-values. GWAS are an effective means to discover potential new drug targets, but this in our view the start of the hard experimental work starts. Only after delivering molecular understanding of how candidate genes affect metabolism, regulatory agencies will allow the testing of new drugs in the clinic (90,91). It is in this regard of note that, to our knowledge, no new lipid-lowering therapies are being explored through GWAS findings.

On the other hand, given the multiple existing and upcoming drug targets to tackle cardiovascular disease, one can question whether we should continue studies into "lower-level" candidates, meaning genes for which the common variants known show only small effects sizes in the general population. The answer can always be yes, not only out of basic scientific interest and the desire to better understand cellular and physiological function of unknown genes but also to drive the health improvements of the future, for example, by enabling more personalized diagnosis and treatments (92). In depth insight will hopefully help for better diagnosis and stratification of patients to optimize care. Good knowledge of molecular of cellular mechanisms may not seem

mandatory, but it can provide a basis for fast pharmaceutical and medical progress. A good example of this is PCSK9, a target that was long studied before acknowledging its effects on plasma lipids. This *a priori* insight greatly facilitated the major advances to generate effective inhibitors (93–96) to treat patients at very high risk of ASCVD. Whether a similar tail would be told for GPR146 in the future, remain to be uncovered.

REFERENCES

1. Fouchier Sigrid W., Dallinga-Thie Geesje M., Meijers Joost C.M., Zelcer Noam, Kastelein John J.P., Defesche Joep C., et al. Mutations in STAP1 Are Associated With Autosomal Dominant Hypercholesterolemia. Circ Res. 2014 Aug 29;115(6):552–5.
2. Galkina E, Ley K. Immune and Inflammatory Mechanisms of Atherosclerosis. Annu Rev Immunol. 2009;27:165–97.
3. Ketelhuth DFJ, Lutgens E, Bäck M, Binder CJ, Van den Bossche J, Daniel C, et al. Immunometabolism and atherosclerosis: perspectives and clinical significance: a position paper from the Working Group on Atherosclerosis and Vascular Biology of the European Society of Cardiology. Cardiovasc Res. 2019 Jul 1;115(9):1385–92.
4. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med. 2011 Nov 7;17(11):1410–22.
5. Day Ian N.M. FH4=STAP1. Another Gene for Familial Hypercholesterolemia? Circ Res. 2014 Aug 29;115(6):534–6.
6. Hegele RA, Knowles JW, Horton JD. Delisting STAP1. Arterioscler Thromb Vasc Biol. 2020 Apr 1;40(4):847–9.
7. Berberich AJ, Hegele RA. The complex molecular genetics of familial hypercholesterolaemia. Nat Rev Cardiol. 2018 Jul 4;1.
8. Balder JW, Rimbert A, Zhang X, Viel M, Kanninga R, van Dijk F, et al. Genetics, Lifestyle, and Low-Density Lipoprotein Cholesterol in Young and Apparently Healthy Women. Circulation. 2018 Feb 20;137(8):820–31.
9. Hegele RA. Editorial: designing targeted sequencing panels for dyslipidemia. Curr Opin Lipidol. 2019 Apr;30(2):53–5.
10. Blanco-Vaca F, Martín-Campos JM, Pérez A, Fuentes-Prior P. A rare STAP1 mutation incompletely associated with familial hypercholesterolemia. Clin Chim Acta Int J Clin Chem. 2018 Dec;487:270–4.
11. Brænne I, Kleinecke M, Reiz B, Graf E, Strom T, Wieland T, et al. Systematic analysis of variants related to familial hypercholesterolemia in families with premature myocardial infarction. Eur J Hum Genet. 2016 Feb;24(2):191–7.
12. Danyel M, Ott CE, Grenkowitz T, Salewsky B, Hicks AA, Fuchsberger C, et al. Evaluation of the role of STAP1 in Familial Hypercholesterolemia. Sci Rep. 2019 Aug 19;9(1):1–8.
13. Lamiquiz-Moneo I, Restrepo-Córdoba MA, Mateo-Gallego R, Bea AM, Del Pino Alberiche-Ruano M, García-Pavía P, et al. Predicted pathogenic mutations in STAP1 are not associated with clinically defined familial hypercholesterolemia. Atherosclerosis. 2020 Jan;292:143–51.
14. Kanuri B, Fong V, Haller A, Hui DY, Patel SB. Mice lacking global Stap1 expression do not manifest hypercholesterolemia. BMC Med Genet. 2020 Nov 23;21(1):234.
15. Marian AJ. Causality in Genetics. Circ Res. 2014 Jan 17;114(2):e18–21.
16. Marian AJ, Roberts R. On Koch's postulates, causality and genetics of cardiomyopathies. J Mol Cell Cardiol. 2002 Aug;34(8):971–4.
17. Begg CB, Berlin JA. Publication Bias and Dissemination of Clinical Research. JNCI J Natl Cancer Inst. 1989 Jan 18;81(2):107–15.
18. Fanelli D. Negative results are disappearing from most disciplines and countries. Scientometrics. 2012 Mar 1;90(3):891–904.
19. Joober R, Schmitz N, Annable L, Boksa P. Publication bias: What are the challenges and can they be overcome? J Psychiatry Neurosci JPN. 2012 May;37(3):149–52.
20. Lin J, Musunuru K. From Genotype to Phenotype: A Primer on the Functional Follow-up of Genome-Wide Association Studies in Cardiovascular Disease. Circ Genomic Precis Med. 2018 Feb;11(2):e001946.

21. Khetarpal SA, Schjoldager KT, Christoffersen C, Raghavan A, Edmondson AC, Reutter HM, et al. Loss of Function of GALNT2 Lowers High-Density Lipoproteins in Humans, Nonhuman Primates, and Rodents. Cell Metab. 2016 09;24(2):234–45.
22. Musunuru K, Strong A, Frank-Kamenetsky M, Lee NE, Ahfeldt T, Sachs KV, et al. From noncoding variant to phenotype via SORT1 at the 1p13 cholesterol locus. Nature. 2010 Aug 5;466(7307):714–9.
23. Hsieh J, Koseki M, Molusky MM, Yakushiji E, Ichi I, Westerterp M, et al. TTC39B deficiency stabilizes LXR reducing both atherosclerosis and steatohepatitis. Nature. 2016 Jul 14;535(7611):303–7.
24. Willer CJ, Schmidt EM, Sengupta S, Peloso GM, Gustafsson S, Kanoni S, et al. Discovery and refinement of loci associated with lipid levels. Nat Genet. 2013 Nov;45(11):1274–83.
25. Klarin D, Damrauer SM, Cho K, Sun YV, Teslovich TM, Honerlaw J, et al. Genetics of blood lipids among ~300,000 multi-ethnic participants of the Million Veteran Program. Nat Genet. 2018 Nov;50(11):1514.
26. He B, Kang S, Chen Z, Liu X, Wang J, Li X, et al. Hypercholesterolemia risk associated Abca6 does not regulate lipoprotein metabolism in mice or hamster. Biochim Biophys Acta BBA - Mol Cell Biol Lipids. 2021 Nov 1;1866(11):159006.
27. Yu H, Rimbert A, Palmer AE, Toyohara T, Xia Y, Xia F, et al. GPR146 Deficiency Protects against Hypercholesterolemia and Atherosclerosis. Cell. 2019 Nov 27;179(6):1276-1288.e14.
28. Han F, Liu X, Chen C, Liu Y, Du M, Zhou Y, et al. Hypercholesterolemia risk-associated GPR146 is an orphan G-protein coupled receptor that regulates blood cholesterol levels in humans and mice. Cell Res. 2020 Apr;30(4):363–5.
29. Grover SA, Dorais M, Coupal L. Improving the Prediction of Cardiovascular Risk: Interaction Between LDL and HDL Cholesterol. Epidemiology. 2003 May;14(3):315–20.
30. von Eckardstein A. High Density Lipoproteins: Is There a Comeback as a Therapeutic Target? Handb Exp Pharmacol. 2022;270:157–200.
31. Madsen CM, Varbo A, Nordestgaard BG. Novel Insights From Human Studies on the Role of High-Density Lipoprotein in Mortality and Noncardiovascular Disease. Arterioscler Thromb Vasc Biol. 2021 Jan;41(1):128–40.
32. Miller GJ, Miller NE. Plasma-high-density-lipoprotein concentration and development of ischaemic heart-disease. Lancet Lond Engl. 1975 Jan 4;1(7897):16–9.
33. Tall AR. Increasing Lipolysis and Reducing Atherosclerosis. N Engl J Med. 2017 Jul 20;377(3):280–3.
34. Toth PP, Barter PJ, Rosenson RS, Boden WE, Chapman MJ, Cuchel M, et al. High-density lipoproteins: a consensus statement from the National Lipid Association. J Clin Lipidol. 2013 Oct;7(5):484–525.
35. Bartlett J, Predazzi IM, Williams SM, Bush WS, Kim Y, Havas S, et al. Is Isolated Low HDL-C a CVD Risk Factor?: New Insights from the Framingham Offspring Study. Circ Cardiovasc Qual Outcomes. 2016 May;9(3):206–12.
36. Khera AV, Cuchel M, de la Llera-Moya M, Rodrigues A, Burke MF, Jafri K, et al. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. N Engl J Med. 2011 Jan 13;364(2):127–35.
37. Dewey FE, Gusarova V, Dunbar RL, O'Dushlaine C, Schurmann C, Gottesman O, et al. Genetic and Pharmacologic Inactivation of ANGPTL3 and Cardiovascular Disease. N Engl J Med. 2017 Jul 20;377(3):211–21.
38. Musunuru K, Pirruccello JP, Do R, Peloso GM, Guiducci C, Sougnez C, et al. Exome Sequencing, ANGPTL3 Mutations, and Familial Combined Hypolipidemia. N Engl J Med. 2010 Dec 2;363(23):2220–7.
39. Stitziel NO, Khera AV, Wang X, Bierhals AJ, Vourakis AC, Sperry AE, et al. ANGPTL3 Deficiency and Protection Against Coronary Artery Disease. J Am Coll Cardiol. 2017 Apr 25;69(16):2054–63.

40. Vergeer M, Korporaal SJA, Franssen R, Meurs I, Out R, Hovingh GK, et al. Genetic Variant of the Scavenger Receptor BI in Humans. N Engl J Med. 2011 Jan 13;364(2):136–45.
41. Yang X, Sethi A, Yanek LR, Knapper C, Nordestgaard BG, Tybjærg-Hansen A, et al. SCARB1 Gene Variants Are Associated With the Phenotype of Combined High High-Density Lipoprotein Cholesterol and High Lipoprotein (a). Circ Cardiovasc Genet. 2016 Oct;9(5):408–18.
42. Zanoni P, Khetarpal SA, Larach DB, Hancock-Cerutti WF, Millar JS, Cuchel M, et al. Rare variant in scavenger receptor BI raises HDL cholesterol and increases risk of coronary heart disease. Science. 2016 Mar 11;351(6278):1166–71.
43. Fenske SA, Yesilaltay A, Pal R, Daniels K, Rigotti A, Krieger M, et al. Overexpression of the PDZ1 Domain of PDZK1 Blocks the Activity of Hepatic Scavenger Receptor, Class B, Type I by Altering Its Abundance and Cellular Localization. J Biol Chem. 2008 Aug 8;283(32):22097–104.
44. Walther C, Caetano FA, Dunn HA, Ferguson SSG. PDZK1/NHERF3 differentially regulates corticotropin-releasing factor receptor 1 and serotonin 2A receptor signaling and endocytosis. Cell Signal. 2015 Mar;27(3):519–31.
45. Wijers M, Zanoni P, Liv N, Vos DY, Jäckstein MY, Smit M, et al. The hepatic WASH complex is required for efficient plasma LDL and HDL cholesterol clearance. JCI Insight [Internet]. 2019 Jun 8 [cited 2019 Jul 8];4(11). Available from: https://insight.jci.org/articles/view/126462
46. Brown RJ, Lagor WR, Sankaranaravanan S, Yasuda T, Quertermous T, Rothblat GH, et al. Impact of Combined Deficiency of Hepatic Lipase and Endothelial Lipase on the Metabolism of Both High-Density Lipoproteins and Apolipoprotein B–Containing Lipoproteins. Circ Res. 2010 Aug 6;107(3):357–64.
47. Fujimoto K, Koishi R, Shimizugawa T, Ando Y. Angptl3-null mice show low plasma lipid concentrations by enhanced lipoprotein lipase activity. Exp Anim. 2006 Jan;55(1):27–34.
48. Plump AS, Azrolan N, Odaka H, Wu L, Jiang X, Tall A, et al. ApoA-I knockout mice: characterization of HDL metabolism in homozygotes and identification of a post-RNA mechanism of apoA-I up-regulation in heterozygotes. J Lipid Res. 1997 May;38(5):1033–47.
49. Sakai N, Vaisman BL, Koch CA, Hoyt RF, Meyn SM, Talley GD, et al. Targeted Disruption of the Mouse Lecithin:Cholesterol Acyltransferase (LCAT) Gene: GENERATION OF A NEW ANIMAL MODEL FOR HUMAN LCAT DEFICIENCY *. J Biol Chem. 1997 Mar 14;272(11):7506–10.
50. Qin S, Kawano K, Bruce C, Lin M, Bisgaier C, Tall AR, et al. Phospholipid transfer protein gene knock-out mice have low high density lipoprotein levels, due to hypercatabolism, and accumulate apoA-IV-rich lamellar lipoproteins. J Lipid Res. 2000 Feb 1;41(2):269–76.
51. Wang Y, Gusarova V, Banfi S, Gromada J, Cohen JC, Hobbs HH. Inactivation of ANGPTL3 reduces hepatic VLDL-triglyceride secretion. J Lipid Res. 2015 Jul 1;56(7):1296–307.
52. Jong MC, Rensen PC, Dahlmans VE, van der Boom H, van Berkel TJ, Havekes LM. Apolipoprotein C-III deficiency accelerates triglyceride hydrolysis by lipoprotein lipase in wild-type and apoE knockout mice. J Lipid Res. 2001 Oct;42(10):1578–85.
53. Gaudet D, Gipe DA, Pordy R, Ahmad Z, Cuchel M, Shah PK, et al. ANGPTL3 Inhibition in Homozygous Familial Hypercholesterolemia. N Engl J Med. 2017 Jul 20;377(3):296–7.
54. Wang Q, Oliver-Williams C, Raitakari OT, Viikari J, Lehtimäki T, Kähönen M, et al. Metabolic profiling of angiopoietin-like protein 3 and 4 inhibition: a drug-target Mendelian randomization analysis. Eur Heart J. 2021 Mar 21;42(12):1160–9.
55. Gaudet D, Karwatowska-Prokopczuk E, Baum SJ, Hurh E, Kingsbury J, Bartlett VJ, et al. Vupanorsen, an N-acetyl galactosamine-conjugated antisense drug to ANGPTL3 mRNA, lowers triglycerides and atherogenic lipoproteins in patients with diabetes, hepatic steatosis, and hypertriglyceridaemia. Eur Heart J. 2020 Oct 21;41(40):3936–45.
56. Graham MJ, Lee RG, Brandt TA, Tai LJ, Fu W, Peralta R, et al. Cardiovascular and Metabolic Effects of ANGPTL3 Antisense Oligonucleotides. N Engl J Med. 2017 Jul 20;377(3):222–32.

57. Dewey FE, Gusarova V, Dunbar RL, O'Dushlaine C, Schurmann C, Gottesman O, et al. Genetic and Pharmacologic Inactivation of ANGPTL3 and Cardiovascular Disease. N Engl J Med. 2017 Jul 20;377(3):211–21.

58. Koishi R, Ando Y, Ono M, Shimamura M, Yasumo H, Fujiwara T, et al. Angptl3 regulates lipid metabolism in mice. Nat Genet. 2002 Feb;30(2):151–7.

59. Minicocci I, Montali A, Robciuc MR, Quagliarini F, Censi V, Labbadia G, et al. Mutations in the ANGPTL3 Gene and Familial Combined Hypolipidemia: A Clinical and Biochemical Characterization. J Clin Endocrinol Metab. 2012 Jul;97(7):E1266–75.

60. Burke AC, Telford DE, Sutherland BG, Edwards JY, Sawyez CG, Barrett PHR, et al. Bempedoic Acid Lowers Low-Density Lipoprotein Cholesterol and Attenuates Atherosclerosis in Low-Density Lipoprotein Receptor–Deficient (LDLR+/- and LDLR-/-) Yucatan Miniature Pigs. Arterioscler Thromb Vasc Biol. 2018 May;38(5):1178–90.

61. Pinkosky SL, Newton RS, Day EA, Ford RJ, Lhotak S, Austin RC, et al. Liver-specific ATP-citrate lyase inhibition by bempedoic acid decreases LDL-C and attenuates atherosclerosis. Nat Commun. 2016 Nov 28;7(1):13457.

62. Samsoondar JP, Burke AC, Sutherland BG, Telford DE, Sawyez CG, Edwards JY, et al. Prevention of Diet-Induced Metabolic Dysregulation, Inflammation, and Atherosclerosis in Ldlr-/- Mice by Treatment With the ATP-Citrate Lyase Inhibitor Bempedoic Acid. Arterioscler Thromb Vasc Biol. 2017 Apr;37(4):647–56.

63. Banach M, Duell PB, Gotto AM Jr, Laufs U, Leiter LA, Mancini GBJ, et al. Association of Bempedoic Acid Administration With Atherogenic Lipid Levels in Phase 3 Randomized Clinical Trials of Patients With Hypercholesterolemia. JAMA Cardiol. 2020 Oct 1;5(10):1124–35.

64. Goldberg AC, Leiter LA, Stroes ESG, Baum SJ, Hanselman JC, Bloedon LT, et al. Effect of Bempedoic Acid vs Placebo Added to Maximally Tolerated Statins on Low-Density Lipoprotein Cholesterol in Patients at High Risk for Cardiovascular Disease: The CLEAR Wisdom Randomized Clinical Trial. JAMA. 2019 Nov 12;322(18):1780–8.

65. Cicero AFG, Fogacci F, Hernandez AV, Banach M, Panel (ILEP) on behalf of the L and BPMAC (LBPMC) G and the ILE. Efficacy and safety of bempedoic acid for the treatment of hypercholesterolemia: A systematic review and meta-analysis. PLOS Med. 2020 Jul 16;17(7):e1003121.

66. Ference BA, Ray KK, Catapano AL, Ference TB, Burgess S, Neff DR, et al. Mendelian Randomization Study of ACLY and Cardiovascular Disease. N Engl J Med. 2019 Mar 14;380(11):1033–42.

67. Ngo T, Kufareva I, Coleman JL, Graham RM, Abagyan R, Smith NJ. Identifying ligands at orphan GPCRs: current status using structure-based approaches. Br J Pharmacol. 2016 Oct;173(20):2934–51.

68. Liu S, Guo R, Tu Q, Quarles LD. Overexpression of Phex in Osteoblasts Fails to Rescue the Hyp Mouse Phenotype*. J Biol Chem. 2002 Feb 1;277(5):3686–97.

69. Yadava RS, Kim YK, Mandal M, Mahadevan K, Gladman JT, Yu Q, et al. MBNL1 overexpression is not sufficient to rescue the phenotypes in a mouse model of RNA toxicity. Hum Mol Genet. 2019 Jul 15;28(14):2330–8.

70. Yosten GLC, Kolar GR, Redlinger LJ, Samson WK. Evidence for an interaction between proinsulin C-peptide and GPR146. J Endocrinol. 2013;218(2):B1-8.

71. Lindfors L, Sundström L, Fröderberg Roth L, Meuller J, Andersson S, Kihlberg J. Is GPR146 really the receptor for proinsulin C-peptide? Bioorg Med Chem Lett. 2020 Jul 1;30(13):127208.

72. Ozaki KI, Awazu M, Tamiya M, Iwasaki Y, Harada A, Kugisaki S, et al. Targeting the ERK signaling pathway as a potential treatment for insulin resistance and type 2 diabetes. Am J Physiol Endocrinol Metab. 2016 Apr 15;310(8):E643–51.

73. Furman BL. Streptozotocin-Induced Diabetic Models in Mice and Rats. Curr Protoc Pharmacol. 2015 Sep 1;70:5.47.1-5.47.20.

74. Chaudhary R, Garg J, Shah N, Sumner A. PCSK9 inhibitors: A new era of lipid lowering therapy. World J Cardiol. 2017 Feb 26;9(2):76–91.

75. Gaudet D, Karwatowska-Prokopczuk E, Baum SJ, Hurh E, Kingsbury J, Bartlett VJ, et al. Vupanorsen, an N-acetyl galactosamine-conjugated antisense drug to ANGPTL3 mRNA, lowers triglycerides and atherogenic lipoproteins in patients with diabetes, hepatic steatosis, and hypertriglyceridaemia. Eur Heart J. 2020 Oct 21;41(40):3936–45.

76. Tsimikas S, Karwatowska-Prokopczuk E, Gouni-Berthold I, Tardif JC, Baum SJ, Steinhagen-Thiessen E, et al. Lipoprotein(a) Reduction in Persons with Cardiovascular Disease. N Engl J Med. 2020 Jan 16;382(3):244–55.

77. Gennemark P, Walter K, Clemmensen N, Rekić D, Nilsson CAM, Knöchel J, et al. An oral antisense oligonucleotide for PCSK9 inhibition. Sci Transl Med [Internet]. 2021 May 12 [cited 2021 Aug 9];13(593). Available from: https://stm.sciencemag.org/content/13/593/eabe9117

78. Musunuru K, Chadwick AC, Mizoguchi T, Garcia SP, DeNizio JE, Reiss CW, et al. In vivo CRISPR base editing of PCSK9 durably lowers cholesterol in primates. Nature. 2021 May;593(7859):429–34.

79. Rothgangl T, Dennis MK, Lin PJC, Oka R, Witzigmann D, Villiger L, et al. In vivo adenine base editing of PCSK9 in macaques reduces LDL cholesterol levels. Nat Biotechnol. 2021 Aug;39(8):949–57.

80. Sun CP, Wu TH, Chen CC, Wu PY, Shih YM, Tsuneyama K, et al. Studies of Efficacy and Liver Toxicity Related to Adeno-Associated Virus–Mediated RNA Interference. Hum Gene Ther. 2013 Aug;24(8):739–50.

81. An DS, Qin FXF, Auyeung VC, Mao SH, Kung SKP, Baltimore D, et al. Optimization and Functional Effects of Stable Short Hairpin RNA Expression in Primary Human Lymphocytes via Lentiviral Vectors. Mol Ther J Am Soc Gene Ther. 2006 Oct;14(4):494–504.

82. Hillgartner FB, Salati LM, Goodridge AG. Physiological and molecular mechanisms involved in nutritional regulation of fatty acid synthesis. Physiol Rev. 1995 Jan;75(1):47–76.

83. Horton JD, Bashmakov Y, Shimomura I, Shimano H. Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. Proc Natl Acad Sci U S A. 1998 May 26;95(11):5987–92.

84. Tomkins GM, Chaikoff IL. Cholesterol synthesis by liver. I. Influence of fasting and of diet. J Biol Chem. 1952 May;196(2):569–73.

85. Nurmohamed NS, Navar AM, Kastelein JJP. New and Emerging Therapies for Reduction of LDL-Cholesterol and Apolipoprotein B: JACC Focus Seminar 1/4. J Am Coll Cardiol. 2021 Mar 30;77(12):1564–75.

86. Warden BA, Duell PB. Evinacumab for treatment of familial hypercholesterolemia. Expert Rev Cardiovasc Ther. 2021 Aug;19(8):739–51.

87. Armstrong M. Regeneron's Evkeeza leads a new class of lipid lowerers [Internet]. Evaluate.com. 2021 [cited 2022 May 4]. Available from: https://www.evaluate.com/vantage/articles/news/snippets/regenerons-evkeeza-leads-new-class-lipid-lowerers

88. Dickinson ME, Flenniken AM, Ji X, Teboul L, Wong MD, White JK, et al. High-throughput discovery of novel developmental phenotypes. Nature. 2016 Sep;537(7621):508–14.

89. Visscher PM, Wray NR, Zhang Q, Sklar P, McCarthy MI, Brown MA, et al. 10 Years of GWAS Discovery: Biology, Function, and Translation. Am J Hum Genet. 2017 Jul 6;101(1):5–22.

90. Davis RL. Mechanism of Action and Target Identification: A Matter of Timing in Drug Discovery. iScience. 2020 Aug 21;23(9):101487.

91. Moffat JG, Vincent F, Lee JA, Eder J, Prunotto M. Opportunities and challenges in phenotypic drug discovery: an industry perspective. Nat Rev Drug Discov. 2017 Aug;16(8):531–43.

92. Tada H, Fujino N, Nomura A, Nakanishi C, Hayashi K, Takamura M, et al. Personalized medicine for cardiovascular diseases. J Hum Genet. 2021 Jan;66(1):67–74.

93. Abifadel M, Varret M, Rabès JP, Allard D, Ouguerram K, Devillers M, et al. Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet. 2003 Jun;34(2):154–6.

94. Brown MS, Goldstein JL. A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood. Proc Natl Acad Sci U S A. 1999 Sep 28;96(20):11041–8.

95. Elagoz A, Benjannet S, Mammarbassi A, Wickham L, Seidah NG. Biosynthesis and cellular trafficking of the convertase SKI-1/S1P: ectodomain shedding requires SKI-1 activity. J Biol Chem. 2002 Mar 29;277(13):11265–75.

96. Seidah NG, Prat A. The biology and therapeutic targeting of the proprotein convertases. Nat Rev Drug Discov. 2012 May;11(5):367–83.