PCSK9 inhibitor cooperatively with high triglyceride induces adverse pregnancy outcomes by impairing mitochondrial function

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Research Article

**Keywords:** PCSK9 inhibitor, adverse pregnancy outcomes, neural tube defects, high triglyceride, mitochondrial dysfunction

**Posted Date:** January 11th, 2023

**DOI:** https://doi.org/10.21203/rs.3.rs-2453552/v1

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Abstract

The two monoclonal antibodies targeting proprotein convertase subtilisin/kexin type 9 (PCSK9), namely evolocumab and alirocumab, were first approved by the U.S. Food and Drug Administration (FDA) in 2015. PCSK9 inhibitor (PCSK9i) has served as a viable new therapeutic option to lower cholesterol levels and associated cardiovascular events. However, a better understanding of safety issue of PCSK9i is necessarily needed. We present here an increased risk of adverse pregnancy outcomes, including stillbirths and embryonic defects, particularly neural tube defects, following PCSK9i treatment harnessing a high-fat diet-based mouse model. Proteomics of PCSK9i-treated neural stem cells uncovered that PCSK9i may disrupt mitochondrial function via succinic dehydrogenase complex subunit A (SDHA), which is a key enzyme in the mitochondrial respiratory chain. Furthermore, PCSK9i-induced SDHA deficiency combines with high triglyceride to drive the production of reactive oxygen species and subsequent apoptosis during embryonic development, which may account for the occurrence of adverse pregnancy outcomes. Our findings reveal the existence of a hitherto unknown risk of PCSK9i in pregnant women, as well as novel gene-environment interaction.

Introduction

Hypercholesterolaemia is an important risk factor for atherosclerosis and a leading cause of cardiovascular disease (CVD) (1). Since proprotein convertase subtilisin/kexin type 9 (PCSK9) was first discovered in 2003 (2), it has attracted widespread attention for its clinical relevance to hypercholesterolaemia and associated CVD (3). PCSK9 downregulates low-density lipoprotein receptor (LDLR) by binding and directing to the lysosome for degradation. Accordingly, PCSK9 inhibition contributes to increased LDLR density, thereby decreasing circulating levels of low-density lipoprotein cholesterol (LDL-C) (4).

In the last decade, the development of PCSK9 inhibitors (PCSK9i) has progressed rapidly, especially following the first two monoclonal antibodies targeting PCSK9, namely evolocumab and alirocumab, being approved by the U.S. Food and Drug Administration (FDA) in mid-2015 (5, 6). Currently, PCSK9i could be a valuable new therapeutic option for patients with familial hypercholesterolaemia, statin intolerance, or those at high-risk of atherosclerotic CVD, due to reducing serum LDL-C levels by 50–60% (7, 8). PCSK9i has been recently approved by the European Medicines Agency, Health Canada and China National Medical Products Administration to reduce cardiovascular events in patients with established atherosclerotic CVD (9). However, it is virtually a given that when a new medicine is introduced to the market, the list of potential adverse effects is often not exhaustive. Some studies have reported an increased rate of adverse neurocognitive, plaque progression, and ophthalmologic events associated with the use of PCSK9i compared with a placebo (6, 10, 11). Considering the novel nature of PCSK9i, it is likely that the full array of adverse effects is yet to be uncovered, thus early detection of such safety issues can aid in their prevention.
PCSK9 is a useful biomarker of human neural tube defects (NTDs) as reported in our previous study (12). NTDs, which occur following failure of neural tube closure during the fourth week of embryonic development, are among the most common and devastating congenital anomalies of the central nervous system (13). Typical NTDs include spina bifida, anencephaly, and exencephaly, according to the location and severity of the lesion, which pose huge medical, financial, and psychological costs on affected families (14). A more recent study confirmed that reduced PCSK9 in maternal serum is a potential biomarker for human pregnancies with open NTDs (15). PCSK9 is expressed in the brain at certain crucial stage during embryonic neurulation, which is a key time point in NTD formation (2, 16, 17). Knockdown of PCSK9 mRNA in zebrafish eggs resulted in embryonic death due to defective neurogenesis (17). Moreover, a phenome-wide association study of 29,722 human patients showed a significant relationship between R46L loss-of-function variants in PCSK9 and an increased risk of NTDs (18). Therefore, it is plausible that reduced PCSK9 may lead to an increased incidence of foetal NTDs. However, clear evidence for this hypothesis is still lacking, thus warranting further investigation of the latent connections between PCSK9i administration in pregnant women and foetal NTD development.

Maternal obesity is thought to represent an increased risk of NTD outcomes in foetuses, particularly exencephaly and spina bifida (19, 20). Similarly, female mice fed a high-fat diet (HFD) exhibited increased NTD formation in their offspring (21). However, the mechanism underlying this relationship remains unclear. Obesity is often linked to dyslipidaemia, which is significantly associated with foetal NTDs (22, 23). Thus, dyslipidaemia and PCSK9i are both associated with an increased risk of NTD outcomes and both related to lipid metabolism. Noticeably, mounting evidence indicates that PCSK9 is independently associated with serum triglyceride (TG) levels (24, 25). PCSK9i predominantly exhibits good effects on high cholesterol; however, it is highly uncertain whether PCSK9i can efficiently reduce elevated TG levels caused by the LDLR-independent effects of PCSK9 on TG-rich lipoprotein metabolism (26, 27). As such, it is unknown whether the use of PCSK9i in pregnant women with dyslipidaemia has a synergistic effect with high triglyceride (HTG) on the reproductive risk of NTDs. Because obese pregnant women with dyslipidaemia are not included in clinical trials, the potential of this adverse effect will not be determined until the medicine is available in the market.

Therefore, to unravel this doubt, we explore the synergistic effect of PCSK9i and high fat on adverse pregnancy outcomes for the first time by administering PCSK9i to an HFD-based female mouse model. Notably, HFD embryos exhibit higher incidences of stillbirths and defects, including exencephaly, spina bifida, and cleft lip palate, following PCSK9i treatment. Moreover, PCSK9i-induced succinic dehydrogenase complex subunit A (SDHA) deficiency and TG accumulation cooperatively drive the production of reactive oxygen species (ROS) and subsequent apoptosis during embryonic development, which may account for the occurrence of NTDs. Our findings highlight the potential safety challenges associated with PCSK9i treatment in pregnant women with dyslipidaemia.

**Methods**

**Experimental animals**
All animal experiments followed the National Institutes of Health guidelines for the care and use of laboratory animals. The study protocol was approved by the Committee for Animal Care of Shengjing Hospital, China Medical University (2021PS238K). Wistar rats and C57BL/6J mice were purchased from the Animal Centre of China Medical University. PCSK9 knockout mice were purchased from the Jackson Laboratory. In our previous work (28, 29) and other studies (30), a rodent model of ATRA-induced NTDs was employed for research on NTD embryopathy. Briefly, at E10.5/E8.5, pregnant rats/mice were randomised into two groups: the experimental group was treated with ATRA (140 mg/kg body weight/70 mg/kg body weight; Sigma-Aldrich, St. Louis, MO, USA) by gavage to develop NTD, whereas the control group received the same volume of olive oil.

For the HFD-based female mouse model, four-week-old female C57BL/6J mice were assigned to two diet groups: the experimental group received an HFD with 20% protein, 20% carbohydrates, and 60% fat (H10060, HFK Bio-Technology, Beijing, China), whereas the normal diet (ND) group received a general diet with 20% protein, 70% carbohydrates, and 10% fat for 12 weeks. Body weights were recorded weekly. After 12 weeks, the BMI of mice was determined using the Lee’s Index. HFD-fed mice were intraperitoneally administered 10 mg/kg/day of small-molecule inhibitor of PCSK9 (SBC-115076; APE×BIO, Boston, USA) or subcutaneously injected with 10 mg/kg/day of monoclonal antibodies targeting PCSK9 (evolocumab; Amgen Manufacturing Limited, Juncos, PR, USA) daily from E5.5 to E9.5. Thus, the developing embryos were exposed to PCSK9i during neurulation (E8–10.5). At E11.5, and E18.5, the mice were euthanised and the embryos were collected. At E11.5, and E18.5, the mice were euthanized by intraperitoneal injection of pentobarbitone sodium (60 mg/kg body weight) and the embryos were collected.

**Lipidomic assay**

The serum of pregnant rats with NTD foetuses or normal foetuses, as well as the neural tubes (from the inferior margin of the forelimb bud to the tail bud) of the corresponding embryos, were collected at E11.5 for lipidomics analysis. Lipid separation and lipidomics analysis were performed using a Nexera LC-30A ultra-high-performance liquid chromatography system (SHIMADZU, Japan) coupled to Q Exactive Plus (Thermo Fisher Scientific, Waltham, MA, USA), as previously described (31). Lipid identification, peak alignment, retention time correction, and peak area extraction were assessed using Lipid Search software version 4.2 (Thermo Fisher Scientific).

**Cell culture, transfection, and reagent treatment**

The mouse neural stem cells (C17.2) used in this study were purchased from Beina Chuanglian Biology Research Institute and cultured as previously described (29). C17.2 cells were cultured in six-well plates at a density of 1 × 10^5 cells/well for 24 h before treatment. The PCSK9 plasmid (Addgene, Cambridge, MA, USA) was transfected into C17.2 cells according to the manufacturer’s protocols using JetPRIME (Polyplus Transfection, Illkirch, France). Cells were collected 48 h after transfection and used in subsequent experiments. C17.2 were treated with 0.3 mM of OA (Sigma-Aldrich) and 30 µM of PCSK9i (SBC-115076) for 12 h. DMSO was used as the solvent for PCSK9i, which served as the control.
Proteomics

C17.2 cells were treated with 30 µM of PCSK9i (SBC-115076) or the same volume of DMSO for proteomic analysis on tandem mass tag (TMT)-based liquid chromatography tandem mass spectrometry (LC-MS/MS). After trypsin digestion, the peptides were labelled according to the manufacturer’s protocol for the TMT kit (Thermo Fisher Scientific) and analysed by LC-MS/MS in Q Exactive™ Plus (Thermo Fisher Scientific), as previously specified. The raw MS data were analysed using Proteome Discoverer™ Software 1.4.

Mitochondrial respiration

An Agilent cell mitochondrial stress test kit (Agilent Technologies) and Seahorse XFe96 Analyser were used to evaluate the oxygen consumption rate (OCR) (Seahorse Bioscience, Billerica, MA, USA). Cells were seeded into a Seahorse XF cell culture plate and treated with the indicated agents for 12 h. C17.2 cells were rinsed, and the media was changed with pre-warmed XF assay medium supplemented with 1 mM of pyruvate, 10 mM of glucose, and 2 mM of glutamine, then incubated for 1 h in a CO₂-free incubator at 37°C. After recording the basic measurements, OCR was calculated by sequential measurement cycles following each reagent addition in dilutions according to the manufacturer’s recommendations (2 µM oligomycin, 1 µM FCCP, 0.5 µM Rotenone/antimycin A) using a SeahorseXFe96 FluxPak. OCR was calculated using the Seahorse XFe96 program and expressed as basal, maximal, ATP generation, and non-mitochondrial respiration.

Transmission electron microscopy analysis (TEM)

The treated C17.2 cells were carefully collected, fixed in 2.5% glutaraldehyde, and post-fixed in 1% osmium tetraoxide. The cells were then dissected, and TEM images were acquired at 30,000× magnification in randomly selected fields under an 80-kV transmission electron microscope (JTM-1400Flash, JEOL, Japan).

Reactive oxygen specie (ROS) generation

ROS quantification was performed using the ROS Assay Kit (Beyotime, Shanghai, China) after seeding C17.2 cells in 12-well plates and treating them with the indicated agents for 12 h. After three washes with cell medium, cells were incubated with 2, 7-dichlorofluorescein diacetate at 37°C for 25 min. The distribution of dichlorofluorescein fluorescence was measured using a fluorescence microscope (Nikon Eclipse Ti, Kyoto, Japan) and a fluorescence microplate reader (Tecan Infinite M200Pro, Mannedorf, Switzerland).

Mitochondrial permeability transition pore and JC-1 staining

The MPTP Staining Kit was used to measure mitochondrial permeability (Beyotime), and the mitochondrial membrane potential (MMP) was detected using a JC-1 Staining Kit (Beyotime). A decrease in the green fluorescence intensity of calcein flowing out of the mitochondria indicates MPTP opening. The ratio of red fluorescence intensity to green fluorescence intensity represents the MMP. Fluorescence
intensity was determined using a microplate reader (Tecan Infinite M200 Pro) or fluorescence microscope (Olympus IX73, Tokyo, Japan).

**Detection of apoptosis**

A terminal deoxynucleotidyl transferase dUTP nick-end labelling (TUNEL) assay was carried out to evaluate the apoptosis rate of cells using the One-Step TUNEL Apoptosis Assay Kit (Roche, Mannheim, Germany). Nuclei were stained with DAPI to categorise the cells, and the staining was evaluated using a fluorescence microscope (Nikon ECLIPSE 80i). The activity of caspase 3 in the cells was analysed using a GreenNuc kit (Beyotime) according to the manufacturer’s instructions. Images were obtained using a fluorescence microscope (Nikon Eclipse Ti).

**Quantitative reverse transcription polymerase chain reaction (qPCR)**

Total RNA was extracted from the neural tube tissues of embryos and C17.2 cells using TRIzol reagent (TakaRa, Ohtsu, Japan), then reverse transcribed into cDNA using PrimeScript RT Master Mix (TaKaRa, Tokyo, Japan). qPCR was performed using the SYBR Premix Ex Taq kit (TaKaRa) on a 7500 Real-Time PCR system (StepOnePlus, ABI Company, Oyster Bay, NY, USA). The results were normalised to GAPDH levels. The primer sequences for PCSK9 and GAPDH were as follows: PCSK9-F TGGCTGACATGACATTGCTTCTC, PCSK9-R GCACTGGAGAACCACACAGG; GAPDH-F 5'-TGCCGCCTGGAGAAACCTGC-3', GAPDH-R 5'-AGCAATGCCAGCCCCAGCAT-3'.

**Immunoblotting**

Neural tube tissues in embryos and C17.2 cells were lysed using RIPA lysis buffer (Beyotime) to extract the protein, and protein concentrations were measured with a BCA kit (Takara, Ohtsu, Japan). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis then transferred to nitrocellulose membranes, as previously described. After blocking with 5% milk, the membranes were incubated overnight at 4°C with primary antibodies against PCSK9 (Abcam, 1:1000), SDHA (Proteintech, 1:1000), caspase 9 (Proteintech, 1:1000), caspase 3 (Cell Signalling Technology, 1:1000), GAPDH (Invitrogen, 1:5000), and β-actin (Proteintech, 1:5000). Horseradish peroxidase-conjugated secondary antibodies (Jackson, 1:5000) were incubated after washing the membranes. Chemiluminescent substrate (Millipore, MA, USA) was used to visualise the signals.

**Statistical analysis**

Graph Pad-Prism8 software was used for graphic presentation and statistical analysis. The results are expressed as the mean per group ± s.e.m. The data were analysed as reported in the figure legends and *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001 were considered statistically significant. We employed a t-test approach for comparisons between two groups and one-way ANOVA test for multiple comparisons. Significant differences in NTD incidence between groups, expressed by the number of embryos, were analysed by the Chi-square test. All experiments were completely randomised and repeated in triplicate.
Results

PCSK9 deficiency increased the risk of adverse pregnancy outcomes during embryonic development

Our previous studies showed that maternal plasma levels of PCSK9 are associated with the incidence of human NTDs (12). Moreover, we explored the expression of the PCSK9 protein and mRNA in the neural tubes during embryonic development from E11 to E20. The mRNA levels of PCSK9 in the normal foetal rats are gradually elevated with embryonic neurulation (Fig. 1A). Compared with normal controls, both the mRNA (Fig. 1A) and protein (Fig. 1, B and C) levels of PCSK9 decreased significantly in the ATRA-induced foetal NTD group on each embryonic day.

However, the definitive effects of PCSK9 deficiency on neural tube closure remains to be defined. To this end, we investigated the role of PCSK9 deficiency in NTD formation using PCSK9 knockout mice, in which, deletion of the PCSK9 gene evidently increased the incidence of all-trans retinoic acid (ATRA)-induced adverse pregnancy outcomes, especially NTDs, albeit PCSK9 knockout embryos alone did not exhibit any NTD phenotypes (Table S1). Thus, PCSK9 may play a vital role in embryonic neural development and PCSK9 deficiency may contribute to foetal NTDs.

Lipidomic profiling of maternal and foetal samples from NTD model

PCSK9 and NTDs have been known to be implicated in lipid metabolism (3, 32). However, to the best of our knowledge, the overall lipid composition associated with NTDs is lacking. Therefore, we performed mass spectrometry-based lipidomics analysis of the plasma of pregnant rats with NTD or normal foetuses, as well as the neural tubes of the corresponding embryos. According to the principal component analysis, we observed clearly distinguished clusters of NTD and control groups in the maternal plasma (Fig. S1A) and foetal neural tubes, respectively (Fig. S1B). Moreover, the orthogonal projections to latent structures discriminant analysis (OPLS-DA) plot also showed a clear separation of NTD and control groups in the maternal plasma (Fig. 2A) and foetal neural tubes (Fig. 2B). The deregulated lipid species were screened with the OPLS-DA model, using a variable importance in projection (VIP) score > 1 and a p value < 0.05 as selection criteria. The results showed that 57 lipid species in the plasma and 27 lipid species in the neural tube were significantly altered. The samples were clustered into two different phenotypic groups, NTDs and controls, in an unsupervised manner in the maternal plasma (Fig. 2C) and foetal neural tubes (Fig. 2D). Impressively, compared to the control group, the TG levels in lipid species (Fig. 2, E and F) and lipid classes (Fig. 2, G and H) were robustly upregulated in both the maternal plasma and neural tubes of the corresponding embryos from the NTD group. These results indicate a critical role of TG in the occurrence of foetal NTDs.
**PCSK9i increased the incidence of adverse pregnancy outcomes in HFD mice**

Now that PCSK9i and high fat are both disadvantageous factors for NTDs in offspring, we investigated their synergistic effect on foetal outcomes. To this end, we utilised an HFD-based female mouse model. Female mice were continuously fed with an HFD or normal diet (ND) for 12 weeks beginning at 4 weeks of age (Fig. S2) (we refer to the mothers as ‘HFD mice’ and ‘ND mice’ and to their embryos as ‘HFD embryos’ and ‘ND embryos’, respectively). HFD embryos appeared higher incidences of stillbirths and defects, including exencephaly, spina bifida, and cleft lip palate, after administration of a small-molecule inhibitor of PCSK9 (SBC-115076) in the first-trimester (from E5.5 to E9.5) (Table 1). Moreover, we applied evolocumab, a PCSK9i already approved for clinical use, to confirm the reliability of the results of SBC-115076. Similarly, the percentage of total abnormal embryos, including those exhibiting NTDs, stillbirth, and developmental delay, increased when HFD mice were subcutaneously injected with 10 mg/kg/day of evolocumab from E5.5 to E9.5 (Table 1). Typical images of the neural tubes of embryos are shown in Fig. S3. The above observations suggest that PCSK9i-exposed HFD mice have higher incidences of foetal NTDs.

**Table 1**

<table>
<thead>
<tr>
<th></th>
<th>HFD Control</th>
<th>HFD SBC-115076</th>
<th>HFD evolocumab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Litters (n)</td>
<td>8</td>
<td>11</td>
<td>6</td>
</tr>
<tr>
<td>Embryos (n)</td>
<td>65</td>
<td>74</td>
<td>51</td>
</tr>
<tr>
<td>Normal embryos [n (%)]</td>
<td>57 (87.69)</td>
<td>48 (64.86)*</td>
<td>34 (66.67)*</td>
</tr>
<tr>
<td>NTDs [n (%)]</td>
<td>0 (0.00)</td>
<td>5 (6.76)*</td>
<td>4 (7.84)*</td>
</tr>
<tr>
<td>Other Defects [n (%)]</td>
<td>3 (4.62)</td>
<td>6 (8.11)</td>
<td>5 (9.80)</td>
</tr>
<tr>
<td>Stillbirth [n (%)]</td>
<td>5 (7.69)</td>
<td>17 (22.97)*</td>
<td>10 (19.61)*</td>
</tr>
</tbody>
</table>

*Other defects include developmental delay, cleft lip palate, craniofacial malformation, and ocular malformation.

*p < 0.05 vs. control group using Chi-square test.

**Proteomic analysis revealed mitochondrial dysfunction following PCSK9i administration**

To understand the pathway mediating the role of PCSK9i in embryonic development, proteomic analysis of PCSK9i-treated C17.2 cells were carried out. Clustering analysis of the overall protein expression heat map indicated that cells treated with or without PCSK9i were completely divided into two groups with
different protein expression profiles (Fig. 3A). A total of 426 proteins were identified as differentially expressed (fold-change > 1.2; p < 0.05). Strikingly, KEGG pathway analysis and Gene Ontology (GO) analysis revealed three pathways (Fig. 3B) and four biological processes (Fig. S4A) enriched in the PCSK9i group, all of which were associated with mitochondrial function. Specifically, the proteins in the pathways of thermogenesis, oxidative phosphorylation, the tricarboxylic acid (TCA) cycle, and the biological process of succinate metabolic process all experienced downregulation, whereas all proteins in the biological processes (BP) of the cellular/response to ROS and oxidative stress were upregulated in the PCSK9i group compared to the PCSK9i group. Moreover, the cellular components (CC) of GO analysis indicated that the participation of differentially expressed proteins mainly occurred in the mitochondria (Fig. S4B). Detailed proteomic analysis revealed that impaired mitochondrial function arose from the proteins of the key structural components of electron transport chain (ETC) mitochondrial complexes (complex I–V), which were affected in the PCSK9i group but not in the control group (Fig. 3C). Furthermore, gene set enrichment analysis (GSEA) of differentially expressed proteins identified the top five enriched terms related to PCSK9i, including oxidative phosphorylation and fatty acid metabolism (Fig. 3D). In particular, oxidative phosphorylation was dramatically enriched in response to PCSK9i treatment (false discovery rate [FDR] = 0.011) (Fig. 3D). These findings imply that mitochondrial dysfunction is the predominant process following PCSK9i treatment.

**PCSK9i resulted in SDHA deficiency and mitochondrial abnormalities**

We further compared the three pathways related to mitochondrial function derived from KEGG analysis, which included thermogenesis, oxidative phosphorylation, and the citrate cycle. Among these pathways, the only protein in the overlap is SDHA, a key enzyme of the mitochondrial respiratory chain (Fig. 4A). Based on the above data, we consider SDHA to be a valuable target gene of PCSK9i. We then verified the modulation of PCSK9i on SDHA by western blot analysis. Suppressed expression of PCSK9, a higher level of LDLR was observed (Fig. S5A). Consistent with the results of proteomic analysis, the administration of PCSK9i decreased the expression of SDHA in C17.2 cells (Fig. 4B). In contrast, PCSK9 overexpression improved the protein levels of SDHA in vitro (Fig. 4C). In addition, downregulation of PCSK9i on SDHA was confirmed in vivo. Reduced expression of SDHA protein, as well as a higher level of LDLR, was observed in embryos with PCSK9i treatment compared to those without PCSK9i treatment (Fig. 4D and Fig. S5B). Given the potential role of PCSK9 in SDHA-mediated mitochondrial function, we examined the impact of PCSK9i on energy metabolism. PCSK9i-treated C17.2 cells exhibited a distinct reduction in the mitochondrial OCR compared to control cells under maximal coupled and uncoupled respiration (Fig. 4E). Accordingly, TEM analysis of mitochondria showed less dense, less organised, or even absent mitochondrial cristae in cells treated with PCSK9i than in the control (Fig. 4F). Collectively, these data suggest that PCSK9i disrupts the expression of SDHA both in vitro and in vivo and impairs mitochondrial function.

**PCSK9i cooperatively HTG drives mitochondrial damage**
At the molecular level, on the one hand, TG accumulation and fatty acid overload impair mitochondrial function (33) and on the other hand, PCSK9 deficiency reduces the expression of SDHA, a key enzyme in the mitochondrial respiratory chain. Therefore, we explored the cooperative effects of PCSK9 deficiency and HTG treatment on mitochondrial damage. Oleic acid (OA), the most prevalent free FA was used to induce TG accumulation. Following 12 h of OA treatment drives ROS production compared to the control group (Fig. 5, A and B). Intriguingly, under the joint effect of PCSK9i and OA, the increase of ROS activity was much greater than that in the OA group (Fig. 5, A and B). Furthermore, a significant reduction in mitochondrial membrane potential according to JC-1 staining (Fig. 5, C and D), as well as an obvious increase in mitochondrial permeability transition pore (MPTP) opening (Fig. 5, E and F), were observed in the PCSK9i and OA treatment group compared to the OA group. These findings support the cooperative effects of PCSK9i and HTG on mitochondrial damage.

Cooperative effects of PCSK9i and HTG on apoptosis

Although the knockdown of PCSK9 in zebrafish eggs can lead to defective neurogenesis (17), there is currently no evidence to detect the influence of PCSK9 on apoptosis in embryonic neurulation, which accounts for NTD formation. Given the effect of PCSK9i and HTG on mitochondrial damage, we assessed the induction of apoptosis by PCSK9i and HTG treatment. The OA group in the C17.2 neural stem cell line exhibited a higher percentage of apoptotic cells compared to the control group according to TUNEL (Fig. 6A) and active caspase 3 assays (Fig. 6B). Moreover, the combined effects of PCSK9i and OA evidently exacerbated cell apoptosis compared with OA treatment alone (Fig. 6, A and B). In addition, analysis of the apoptosis-related pivotal proteins cleaved caspase 9 and cleaved caspase 3 in C17.2 cells further confirmed the induction of apoptosis by PCSK9i and OA (Fig. 6C). Similarly, cleaved caspase 9 and cleaved caspase 3 were more abundant in HFD embryos with PCSK9i treatment than in those without PCSK9i treatment and ND embryos (Fig. 6D). These results suggest the important role of PCSK9 deficiency combined with HTG in triggering apoptosis during embryonic neurulation, which further infers their cooperative effects on neural tube closure through mitochondrial damage and subsequent apoptosis.

Discussion

It is worth mentioning that from preclinical discovery to clinical development, PCSK9i has been authorised for clinical application for only a few years, potential adverse outcomes, and yet requires extra investigation. In addition to the most frequently reported side effects, including injection-site reactions and upper respiratory tract infections (34), accumulating evidence also indicates that PCSK9i increases the rate of adverse events such as diabetes and neurocognitive deficits. Specifically, participants receiving PCSK9i reported an absolute increase in fasting blood glucose and HbA1c as well as an increased risk of incident diabetes (35). Additionally, both alirocumab and evolocumab are reportedly associated with an increased risk of adverse neurocognitive events compared to a placebo (6, 11). Notably, the safety of using PCSK9i during pregnancy is yet to be clearly established, particularly as
PCSK9i has been shown to cross the placental barrier (18). Our prior research as well as a recent study identified lower PCSK9 expression in the serum of pregnant women with NTD fetuses (12, 15), assuming that NTDs could be possible side effects among pregnant women exposed to PCKS9i. However, there is no evidence to manifest the safety of PCSK9i with regard to foetal NTDs.

In this work, we demonstrated that PCSK9 knockout mice noticeably increased the incidence of ATRA-induced adverse pregnancy outcomes, particularly NTDs, although PCSK9 knockout embryos alone did not exhibit any NTD phenotypes. Nevertheless, whether other risk factors interacting with PCSK9 deficiency generate rising incidence of adverse pregnancy outcomes remains unclear. Lipids known to be involved in the formation of NTDs (32) notwithstanding, lipidomic profiling of NTDs has never before been surveyed. This study utilised lipidomics to investigate the global pattern of lipid profiles in the serum of pregnant rats with NTD, as well as in the neural tubes of NTD embryos. Interestingly, we observed higher TG levels, both in lipid species and lipid classes, in the NTD group than in the control group. In further animal experiments, our data showed that PCSK9i treatment (SBC-115076 or evolocumab) raised the occurrence of stillbirths and defects, including exencephaly, spina bifida, and cleft lip palate, in HFD embryos, indicating the risk of PCSK9i and HTG interaction for adverse pregnancy outcomes, especially NTDs. Thus, a vital issue arose: might this novel medicine is safe and beneficial for all patients?

Despite the potent clinical benefits of PCSK9i, large gaps remain in our understanding of PCSK9i effects. PCSK9i causes an increase in LDLR by disrupting the binding and degradation of PCSK9 to LDLR, thereby decreasing circulating cholesterol levels, which have been widely described in the liver (4). PCSK9, however, has less well-known functions and target proteins in extrahepatic tissues, which will aid to determine the hidden adverse effects of targeting PCSK9. PCSK9 has been reported to cause a reduction in ABCA1, which facilitates the pro-atherosclerotic status of macrophages in the vasculature (36). Here, we provide evidence that PCSK9 expression in the neural tube of normal foetal rats gradually elevated with embryonic neurulation; meanwhile, decreased significantly in NTD foetuses at each embryonic day.

Furthermore, detailed profiling was performed under PCSK9-deficient conditions in neural stem cells by proteomic analysis. Impaired mitochondrial function, consisting of thermogenesis, oxidative phosphorylation, the TCA cycle, and the succinate metabolic process, paralleled by the enhanced biological processes of response to ROS and oxidative stress, are revealed to be associated with PCSK9 deficiency. As further assessed by biological functions as well as mitochondrial morphology, the current work firstly provides firm evidence for the impact of PCSK9 deficiency on mitochondrial dysfunction, with subsequent ROS production and apoptosis in the nervous system, which are pivotal causes of NTD formation. Overwhelming evidence, including our previous studies, has documented that impaired mitochondrial function, followed by ROS production and apoptosis, causes an insufficient number of neuroepithelial cells within the neural folds, which is a pivotal cause of NTD formation (29, 37, 38). Although some existing studies have shown the dual behaviour of PCSK9 in the regulation of apoptosis in neurodegenerative diseases (39), increasing evidence suggests that PCSK9 regulates apoptosis in CVD and tumourigenesis (40–44).
Among the related proteins in functional pathway analysis of the proteome following PCSK9i treatment, SDHA is of utmost concern. We further confirmed the regulation of PCSK9 on SDHA both in vitro and in vivo, which has not been previously elucidated. SDHA, the most important catalytic subunit of complex II, serves as a key enzyme involved in both oxidative phosphorylation and TCA cycle in mitochondrial respiration, and is responsible for catalysing succinate to fumarate and maintaining ROS homeostasis (45). SDHA deficiency-induced succinate accumulation drives ROS production and subsequent apoptosis (46, 47). However, no previous research has characterised the critical role of SDHA in NTD development. Herein, we discovered that PCSK9 deficiency mediates NTD induction by causing mitochondrial damage followed by ROS production and apoptosis, probably via the inhibition of SDHA.

Despite the reported connection between hypertriglyceridaemia in pregnant women and foetal NTDs (22, 23), to date, the underlying mechanisms remain undefined. Our findings from both animal models and cell experiments support the hypothesis that mitochondrial dysfunction arises from both TG accumulation and PCSK9i-induced SDHA deficiency, is responsible for ROS production and subsequent apoptosis that disrupts closure of the neural tube. Despite compelling evidence that susceptibility to NTDs is determined by genetic and environmental factors (48), the precise nature of the link is poorly understood. Our findings revealed that PCSK9 deficiency did not cause NTDs in ND-fed mice but caused a significant rising in NTD occurrence among HFD-fed embryos, demonstrating a gene-environment interaction between the loss of PCSK9 and HTG. Succinate accumulation from fatty acid overload due to HTG coupled with PCSK9i-induced SDHA deficiency, which drives ROS production and subsequent apoptosis, may provide an explanation for the synergistic effects of the environment and genes. However, further clinical investigations based on large-scale, multicentre population studies will be required to support the results from animal experiments. Additionally, continued research into the exact molecular mechanisms should address the regulatory effect of PCSK9 on SDHA.

With the rapid progress of PCSK9i agents, more attention should be directed to the safety issues associated with this novel therapeutic agent. Here, we identified an increased risk of adverse pregnancy outcomes following PCSK9i treatment harnessing an HFD-based female mouse model, which revealed a previously unknown side effect of PCSK9i. We also defined a mechanism whereby PCSK9i-induced SDHA deficiency and hypertriglyceridemia-induced TG accumulation cooperatively give rise to ROS production and subsequent apoptosis, which in turn leads to adverse pregnancy outcomes (Fig. 7). These findings highlight the potential safety challenges of PCSK9i therapy for hypercholesterolaemia and CVD in pregnant women.

Declarations

Data sharing statement

The related data and materials are available for sharing upon request to Prof. Hui Gu and Prof. Zhengwei Yuan.

Acknowledgement
This work was supported by the National Key R&D Program of China (2021YFC2701003, 2021YFC2701104), the National Natural Science Foundation of China (Grant numbers: 82271730, 81771595, 82171649, 81901565, 82001643, 82101740) Shenyang Science and Technology Planning Project (Grant numbers: 22-321-33-22) and the 345 Talent Project.

Declaration of Interests

The authors have declared that no competing interest exists

Contributors:

H.G., and Z.Y. conceived the study, designed the experiments, and wrote the manuscript. W.H., J.F., Y.L., and T.H. performed the experiments and data analyses. X.W., D.L., W.L., W.M., S.C., and S.J. designed experiments and/or oversaw the research. Y.H., and S.D. participated in data analyses. All authors reviewed the manuscript and approved the submitted version.

References


Figures
Figure 1

Temporal expression pattern of PCSK9 in ATRA-induced NTD models.

(A) PCSK9 mRNA levels in control and ATRA-induced rat NTD neural tubes from E11 to E20 determined using qPCR. All experiments were performed using embryos from five dams (n = 5). (B, C) PCSK9 protein abundance in neural tubes from control and NTD groups from E11 to E20. Experiments were performed
using embryos from four different dams (n = 4). Bar graphs show quantitative data (mean ± s.e.m). *p < 0.05.

Figure 2

Lipidomic profiling of foetal neural tubes and maternal plasma from the NTD model.
OPLS-DA score plot from lipidomic profiles of maternal plasma (A) and foetal neural tubes (B) from control and NTD groups (control group, n = 9; NTD group, n = 9). Heatmap visualisation of dysregulated lipids between control and NTD groups in maternal plasma (C) and foetal neural tubes (D), as identified by VIP > 1 and p value < 0.05. 57 lipids in maternal plasma (E) and 27 lipids in the foetal neural tube (F) based on VIP scores in OPLS-DA and FC between control and NTD groups. Columns are coloured by VIP and the size of the circle represents log₂FC. VIP: variable importance in projection; FC: fold-change. Difference analysis of TG levels in lipid classes between control and NTD groups in maternal plasma (G) and foetal neural tubes (H).
Figure 3

Proteomic analysis of differential proteins in PCSK9i-treated C17.2 cells.

(A) Unsupervised hierarchical clustering of 426 differentially expressed proteins in PCSK9i (SBC-115076)-treated C17.2 cells compared with the control. (B) KEGG enrichment analysis corresponding to upregulated and downregulated proteins with the top 20 enrichment scores. (C) Relevant proteins of ETC
mitochondrial complexes significantly modulated after proteomic analysis are displayed as coloured dots. Colour gradient panel represents the relative expression levels of proteins: blue indicates low expression and red indicates high expression. **(D)** Bubble chart of the top five enriched terms across core enrichment proteins (left) and enrichment plot (right) derived by the GSEA for potential pathways related to PCSK9.

**Figure 4**
PCSK9i leads to SDHA deficiency and mitochondrial damage.

(A) Venn diagram of differentially expressed proteins from three KEGG pathways related to mitochondrial function (thermogenesis, oxidative phosphorylation, and citrate cycle). (B) Alteration of SDHA protein expression after treating C17.2 cells with PCSK9i or DMSO. (C) Levels of SDHA protein after transfection with the PCSK9 plasmid or control into C17.2 cells. (D) Protein abundance of SDHA in E11.5 mouse neural tubes treated with PCSK9i. (E) Mitochondrial OCR measured by Seahorse XF96 after treating C17.2 cells with PCSK9i or DMSO. (F) Morphology of mitochondria in C17.2 cells treated with PCSK9i or DMSO visualised by TEM; scale bar = 200 nm. Data from three replicate experiments are shown as bar graphs (mean ± s.e.m). PCSK9i: SBC-115076. *p < 0.05.
Figure 5

PCSK9i cooperated with HTG to drive mitochondrial disfunction.

(A, B) Fluorescence images and relative levels of ROS production in C17.2 cells treated with OA or OA plus PCSK9i treatment detected by the 2, 7-dichlorofluorescein diacetate probe. Scale bar = 100 μm. Fluorescence intensity was quantified using a microplate reader. (C, D) Effects of OA and PCSK9i on...
MMP measured via JC-1 staining in C17.2 cells. Scale bar = 50 μm. Ratio of red (aggregates) to green (monomers) fluorescence intensity, indicating the relative MMP, was quantified using a microplate reader, as shown in the bar graph from three replicate experiments. MMP: mitochondrial membrane potential. (E, F) Representative images of calcein AM staining used to evaluate the effects of OA or PCSK9i on MPTP opening of C17.2 cells. Scale bar = 50 μm. Bar graph represents quantification data of fluorescence intensity from three replicate experiments using ImageJ software. MPTP: mitochondrial permeability transition pore. PCSK9i: SBC-115076. *p < 0.05.
**Figure 6**

**PCSK9i cooperated with HTG to trigger apoptosis.**

**(A)** Apoptotic cells in representative microscopic images of C17.2 cells treated with OA or PCSK9i determined by TUNEL assay and quantification of apoptotic cells. Scale bar = 50 μm. **(B)** Representative images of active caspase 3 assay in C17.2 cells treated with OA or PCSK9i using the GreenNuc kit. Green fluorescence indicates active caspase 3. Scale bar = 100 μm. **(C)** Protein expression of cleaved caspase 9 and cleaved caspase 3 estimated by western blot analysis in C17.2 cells treated with OA or PCSK9i. **(D)** Protein levels of cleaved caspase 9 and cleaved caspase 3 in ND and HFD embryos treated with or without PCSK9i. Data from three replicate experiments are shown as bar graphs (mean ± s.e.m). PCSK9i: SBC-115076. ND: normal diet. HFD: high-fat diet. *p < 0.05.
Figure 7

PCSK9i cooperated with HTG to impair mitochondrial function and trigger apoptosis in NTDs.

A schematic diagram depicting PCSK9i-induced SDHA deficiency and hypertriglyceridemia-induced TG accumulation collaboratively drive the production of ROS and subsequent apoptosis, ultimately lead to the formation of NTDs.
Supplementary Files

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