patient-oriented and epidemiological research

The apolipoprotein C-III (Gln38Lys) variant associated with human hypertriglyceridemia is a gain-of-function mutation[S]

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Abstract **Recent cell culture and animal studies have suggested that expression of human apo C-III in the liver has a profound impact on the triacylglycerol (TAG)-rich $VLDL_1$ production under lipid-rich conditions. The apoC-III Gln38Lys variant was identified in subjects of Mexican origin with moderate hypertriglyceridemia. We postulated that Gln38Lys ($C3^{QK}$), being a gain-of-function mutation, promotes hepatic $VLDL_1$ assembly/secretion. To test this hypothesis, we expressed $C3^{QK}$ in McA-RH7777 cells and *apoc3*-null mice to contrast its effect with WT apoC-III ($C3^{WT}$). In both model systems, $C3^{QK}$ expression increased the secretion of $VLDL_1$-TAG (by 230%) under lipid-rich conditions. Metabolic labeling experiments with $C3^{QK}$ cells showed an increase in de novo lipogenesis (DNL). Fasting plasma concentration of TAG, cholesterol, cholesteryl ester, and FA were increased in $C3^{QK}$ mice as compared with $C3^{WT}$ mice. Liver of $C3^{QK}$ mice also displayed an increase in DNL and expression of lipogenic genes as compared with that in $C3^{WT}$ mice. These results suggest that $C3^{QK}$ variant is a gain-of-function mutation that can stimulate $VLDL_1$ production, through enhanced DNL.**—Sundaram, M., K. R. Curtis, M. Amir Alipour, N. D. LeBlond, K. D. Margison, R. A. Yaworski, R. J. Parks, A. D. McIntyre, R. A. Hegele, M. D. Fullerton, and Z. Yao. **The apolipoprotein C-III (Gln38Lys) variant associated with human hypertriglyceridemia is a gain-of-function mutation.** ***J. Lipid Res.*** **2017.** 58: **2188–2196.**

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Human apo C-III is a small (79 amino acids) exchangeable apolipoprotein that is composed mainly of amphipathic α-helices (1). The majority of apoC-III in the plasma is secreted from the liver and intestine as a constituent of VLDL and HDL (2). The plasma apoC-III concentration is positively correlated with the plasma triacylglycerol (TAG) concentrations, and elevated plasma apoC-III is invariably associated with hypertriglyceridemia (3). On the other hand, loss-of-function apoC-III mutations are associated with lowered plasma TAG and reduced risk of coronary disease (4) and ischemic vascular disease (5). Recent studies have shown that inhibition of apoC-III expression, using antisense oligonucleotides, results in lowered plasma TAG in rodents, nonhuman primates, and humans (6). Thus, attenuating apoC-III expression in the liver may offer a therapeutic advantage in the prevention and treatment of hypertriglyceridemia.

The hypertriglyceridemic effect of apoC-III expression has been attributed to at least three underlying mechanisms. First, plasma apoC-III attenuates TAG hydrolysis catalyzed by lipoprotein lipase, resulting in elevated plasma TAG (7). Second, high concentrations of apoC-III interfere with the clearance of TAG-rich lipoprotein remnants, through receptor-dependent or receptor-independent pathways, leading to hypertriglyceridemia (8). Third, overexpression of apoC-III in the liver promotes hepatic TAG-rich $VLDL_1$ assembly and secretion under lipid-rich conditions (9), thus exacerbating hypertriglyceridemia. Cell culture and mouse studies of two loss-of-function apoC-III variants, Ala23Thr (10) and Lys58Glu (11), originally identified in subjects of hypotriglyceridemia, showed that the two mutations entirely abolished apoC-III ability in promoting $VLDL_1$ assembly/secretion (12, 13). The Lys58Glu mutation

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Abbreviations: CE, cholesteryl ester, Chol, cholesterol; DAG, diacylglycerol; DNL, de novo lipogenesis; FPLC, fast protein LC; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TAG, triacylglycerol.

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apparently renders a lowered lipid-binding activity, thus resulting in diminished formation of lipid substrates, termed microsome-associated lipid droplets (MALDs), that presumably are utilized during $VLDL_1$ assembly/secretion (13). On the other hand, the Ala23Thr mutation exhibits no impairment in lipid-binding, yet it fails to facilitate recruitment of lipid substrates into VLDL (12).

The apoC-III (Gln38Lys) variant was originally identified in large kindred of Mexican origin with moderate hypertriglyceridemia (14). Compared with unaffected relatives, individual heterozygous carriers for the Gln38Lys ($C3^{QK}$) variant had ~30% elevation in plasma TAG and normal levels of other lipids or lipoproteins (14). In the present study, we determined the functionality of the $C3^{QK}$ variant in McA-RH7777 cells and *apoc3*-null mice. Data obtained suggest that $C3^{QK}$ is a gain-of-function mutation that stimulates $VLDL_1$ assembly/secretion as compared with $C3^{WT}$, and the stimulatory effect is associated with increased de novo lipogenesis (DNL).

MATERIALS AND METHODS

Materials

Reagents and medium for cell cultures used in the studies were obtained from Invitrogen (Burlington, ON, Canada). The [2-^{3}H] glycerol (9.6 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO); [^{3}H]acetic acid (0.1 Ci/mmol), [^{14}C]2-deoxyglucose (2-DG) (60 mCi/mmol), and [^{35}S]methionine/cysteine (1,000 Ci/mmol) were obtained from PerkinElmer (Woodbridge, ON, Canada); protein A-Sepharose™ CL-4B beads and HRP-linked anti-mouse and anti-rabbit IgG antibodies were obtained from GE Healthcare (Mississauga, ON); and HRP-linked anti-goat antibody was obtained from Sigma-Aldrich (Oakville, ON, Canada). Oleate, TAG, and phospholipid standards were from Avanti Polar Lipids (Albaster, AL). For Western blots, antibody against human apoC-III was obtained from Academy Biomedical Co., Inc. (Houston, TX); polyclonal goat anti-mouse apoB was obtained from Millipore (Billerica, MA); and polyclonal anti-mouse apoA-I and anti-mouse apoE antisera were obtained from BioDesign International (Saco, ME). Protease inhibitor cocktail and chemiluminescent substrates were obtained from Roche Diagnostics (Laval, PQ, Canada). Poloxamer 407 (P407) was a gift from BASF Corp. (Florham Park, NJ).

Preparation of expression plasmids and transfection

The coding sequence of human $C3^{QK}$ variant protein was synthesized (Life Technologies) flanked with 5′-*Eco*RI and 3′-*Hind*III restriction sites, and inserted into the pCMV5 vector predigested with *Eco*RI and *Hind*III restriction enzymes. The coding sequences of the cDNA constructs were verified by sequencing. Stably transfected McA-RH7777 cells expressing $C3^{WT}$ or $C3^{QK}$ were generated, and the cells were maintained in DMEM containing 10% FBS, 10% horse serum, and 200 μg/ml G418 as previously described (9).

Metabolic labeling of lipids

Cells (~1.8×10^6 cells per 60 mm dish, in triplicate) were labeled with [^{3}H]glycerol (5 μCi/ml) in DMEM supplemented with 20% FBS and 0.4 mM oleate for up to 2 h. For DNL experiments, cells were labeled with [^{3}H]acetic acid (25 μCi/ml) for up to 2 h in DMEM supplemented with 20% FBS and either with 0.4 mM oleate (lipid-rich conditions) or without oleate (lipid-poor conditions). At the end of labeling, lipids were extracted from cell and conditioned media, respectively, and resolved by TLC, and radioactivity was quantified by scintillation counting as previously described (9).

Lipoprotein fractionation

Cells were labeled for 2 h with [^{35}S]methionine/cysteine (100 μCi/ml in 100 mm dishes) in methionine/cysteine-free DMEM supplemented with 20% FBS and 0.4 mM oleate. The lipoproteins secreted into the medium were fractionated into $VLDL_1$ ($S_f > 100$), $VLDL_2$ (S_f 20–100), and other lipoproteins by cumulative rate flotation ultracentrifugation as previously described (9). The ^{35}S-labeled apolipoproteins were resolved by using SDS-PAGE, and radioactivity was quantified by scintillation counting as previously described (15).

Mouse studies

Apoc3-null mice (B6.129-Apoc3^{tm1Unc}/J), obtained from Jackson Laboratory (Bar Harbor, ME), were bred and maintained on chow diet at the University of Ottawa animal care facility according to the institutional animal care committee-approved protocol. For experimental purposes, male *apoc3*-null mice (12 weeks old) were fed a high-fat diet (TD.88137; Harlan Laboratories, Madison, WI) for 7 days with unlimited access to food. The mice were then injected, via tail vein, with adenovirus encoding $C3^{WT}$, $C3^{QK}$, or empty vector (10^9 pfu/mouse, mixed in 150 μl sterile saline). Forty-eight hours later, blood samples (about 50 μl) were collected from the saphenous vein, and apoC-III expression/secretion was determined by Western blot analysis. Mice that expressed the desired human apoC-III were fasted for 16 h and injected intraperitoneally with P407 (1 mg/g diluted in sterile saline). Blood samples were collected before (zero time point) and at 1 and 2 h after P407 injection. Plasma lipoproteins were fractionated by either ultracentrifugation (16) or size-exclusion chromatography (fast protein LC; FPLC) before lipid and apo determination as previously described (13).

Plasma biochemistry

Mice fed with normal chow diet were injected, via tail vein, with adenovirus (10^9 pfu per mouse) encoding apoC-III variants or empty vector. Three days later, mice were fasted for 8 h, and blood from the saphenous vein was collected into lithium heparin-coated tubes. Plasma separated from the blood was used in the colorimetric analysis to quantify TAG (BioVision K622-100), FA (BioVision K612-100), cholesterol (Chol), and cholesteryl ester (CE) (BioVision K623-100).

Size-exclusion chromatography

Plasma of three mice from each group ($C3^{WT}$, $C3^{QK}$, or vector) collected at 2 h after P407 injection were pooled and filtered by using Costar Spin-X columns (Fisher Scientific, Nepean, Canada) by centrifugation (12,000 *g*, 5 min). This filtered plasma (65 μl aliquot) was applied to a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech Inc., Piscataway, NJ) on FPLC system (Gilson, Middleton, WI). Samples were eluted with a buffer containing 150 mM NaCl, 10 mM Na_2HPO_4, and 100 μM EDTA (pH 7.5), at a flow rate of 0.5 ml/min. Thirty 0.5 ml fractions (fraction numbers 11–40) were collected, each successive two fractions were combined, and lipoproteins in the combined fractions were concentrated by using hydrated fumed silica (Cab-O-Sil) as previously described (15). The silica-bound proteins were eluted into 100 μl of SDS-PAGE sample buffer (8 M urea, 2% SDS, and 10% β-mercaptoethanol) and resolved on SDS-PAGE, and then apoB-100, apoB-48, apoC-III, apoE, and apoA-I were detected by immunoblot analysis using appropriate antibodies.

Histology of mouse liver sections

Liver samples collected from the mice fed with high-fat diet were frozen fresh in Cryomatrix polymer complex (Thermo Scientific). The frozen blocks of liver were cut into thin sections by using a Cryostat (HM525 NX, Thermo Scientific) and then mounted on glass slides, followed by Oil Red O staining and covered with a glass coverslip. Intrahepatic lipid droplets stained by Oil Red O were visualized under a Zeiss AxioImager M2 microscope.

In vivo hepatic DNL assay

Mice fed chow diet were injected, via tail vein, with adenovirus (10^9 pfu per mouse) encoding apoC-III variants or empty vector. Three days later, mice were fasted for 8 h, and [^{3}H]acetic acid (10 μCi/g body weight) was injected via tail vein. Three hours later, liver samples were collected, snap-frozen in liquid nitrogen, and stored at −80°C. Lipids extracted from the frozen liver samples were separated by TLC, and the ^{3}H-labeld lipids was quantified by scintillation counting as previously described (9).

Hepatic lipogenesis gene expression analysis

Total liver RNA from mice injected with adenovirus (10^9 pfu per mouse) encoding apoC-III variants or empty vector was isolated with TriPure reagent (Roche), as per the manufacturer's instructions. After removal of genomic DNA, the first-strand cDNA synthesis was performed by using a QuantiNova reverse transcription kit (Qiagen), and the template cDNA was diluted 1:20 with nuclease-free water. Relative mRNA expression was determined by quantitative RT-PCR using inventoried TaqMan assays (*Srebf1*; Mm00550338_m1, *Srebf2*; Mm01306292_m1, *Fas*; Mm00662319_m1, *Scd1*; Mm00772290_m1, *Acc1*; Mm00728460_s1, *Acc2*; Mm00624282_m1, *Cd36*; Mm00432403_m1, *Hmgcr*; Mm01282499_m1, *Ldlr*; Mm01177349_m1, *Tbp*; Mm00446973_m1, *Pparg*; Mm00440940_m1, Nr1h3 [liver X receptor a (LXRa)]; Mm00443451_m1, and β*actin*; Mm00607939_s1) combined with QuantiNova Probe PCR mix (Qiagen) on a Rotor-Gene-Q instrument (Qiagen). Relative expression was determined by using the $^{\Delta\Delta}$Ct method (17), normalized to the average of both *Tbp* and β*actin*, and data were presented relative to vector control samples.

Next-generation sequencing and bioinformatics analysis

Genomic DNAs were isolated from whole blood of 1,557 samples of dyslipidemia phenotypes, and target-enriched genomic libraries of indexed and pooled samples were generated for 69 target candidate genes in lipid metabolism, including *APOC3* on the LipidSeq Panel, as described (18, 19). Prepared sample libraries were assayed in the Illumina MiSeq personal sequencer (Illumina Inc., San Diego, CA) as described (18). FASTQ files from the MiSeq platform were processed individually by using a custom automated workflow in CLC Genomics Workbench version 8.5.1 (CLCbio, Aarhus, Denmark) for sequence mapping, variant calling, and target region coverage statistics. The variant annotation was performed by using ANNOVAR (https://www.qiagenbioinformatics.com/) with customized scripts. The frequency of the *APOC3* p.Q58K variant was also assessed in the ExAC database totaling 60,706 sequenced samples from a wide range of ethnic groups and clinical phenotypes (http://exac.broadinstitute.org/).

Other assays

The Fat Western Lipid-Protein Overlay Assay to determine apoC-III-lipid binding was performed as previously described (13). Briefly, lipid samples (up to 80 μg) were spotted onto nitrocellulose membrane strips and incubated with conditioned medium (collected from cells expressing apoC-III proteins) overnight at 4°C. The apoC-III protein bound to lipid spots was detected by immunoblotting using an anti-hapoC-III antibody. Cell protein concentration was quantified by using the Bradford method (20).

Statistics

Student's *t*-test and ANOVA statistical analysis of the data were performed by using GraphPad Prism program (GraphPad Software, Inc., La Jolla, CA).

RESULTS

Expression of C3^{QK} variant in McA-RH7777 cells results in increased VLDL$_1$ secretion

Human apoC-III is composed of six α-helices (1); the Gln38Lys mutation occurs within helix 3 (**Fig. 1A**). To determine the effect of Gln38Lys mutation on VLDL$_1$ assembly/secretion, we contrasted expression of C3^{QK} with that of C3^{WT}. Stable cell lines expressing similar levels of C3^{QK} or C3^{WT} (Fig. 1B), when metabolically labeled with [^{3}H]glycerol in the presence of 0.4 mM oleate for 2 h, showed that secretion of [^{3}H]TAG, [^{3}H]diacylglycerol, and [^{3}H]phosphatidylcholine ([^{3}H]PC) from C3^{QK} cells was significantly higher (by 60, 60, and 190% respectively) than that from C3^{WT} cells (Fig. 1C). Ultracentrifugal fractionation of lipoproteins secreted from the cells revealed that C3^{QK} expression markedly stimulated [^{3}H]TAG and [^{3}H]PC secretion as VLDL$_1$ (by 230%) (Fig. 1D). Ultracentrifugal fractionation of medium lipoproteins at 2 h postmetabolic labeling with [^{35}S]methionine/cysteine (in the presence of 0.4 mM oleate) showed that the secreted [^{35}S]apoB100 associated with VLDL$_1$ to a similar extent in both C3^{QK} cells and C3^{WT} cells (Fig. 1E, F). However, C3^{WT} cells secreted 30% more [^{35}S]apoB100 that were associated with VLDL$_2$ particles (Fig. 1E). The secreted [^{35}S]apoE and [^{35}S]apoA-I were mainly associated with HDL under density ultracentrifugal fractionation conditions (Fig. 1F). The combined [^{3}H]lipid and [^{35}S]apoB data suggest that expression of C3^{QK} variant in McA-RH7777 cells results in secretion of TAG-rich VLDL$_1$ particle without an increase in apoB100 secretion suggesting late addition of TAG posttranslocationally to apoB-100 during the assembly of lipid-rich VLDL$_1$ particles.

Expression of C3^{QK} variant in McA-RH7777 cells enhances DNL

We next wanted to know the source of increased TAG in driving the TAG-rich VLDL$_1$ secretion in cells expressing C3^{QK}. Hence, we determined the effect of C3^{QK} expression on the intracellular DNL by metabolic labeling with [^{3}H]acetate under lipid-poor (medium devoid of exogenous FAs) or lipid-rich (medium supplemented with 0.4 mM oleate) conditions. Results showed that significantly increased incorporation of [^{3}H]acetate into FAs, TAG, diacylglycerol (DAG), and phosphatidylcholine (PC) [but not phosphatidylethanolamine (PE)] occurred in C3^{QK} cells under both lipid-poor (**Fig. 2A**) and lipid-rich (Fig. 2B)

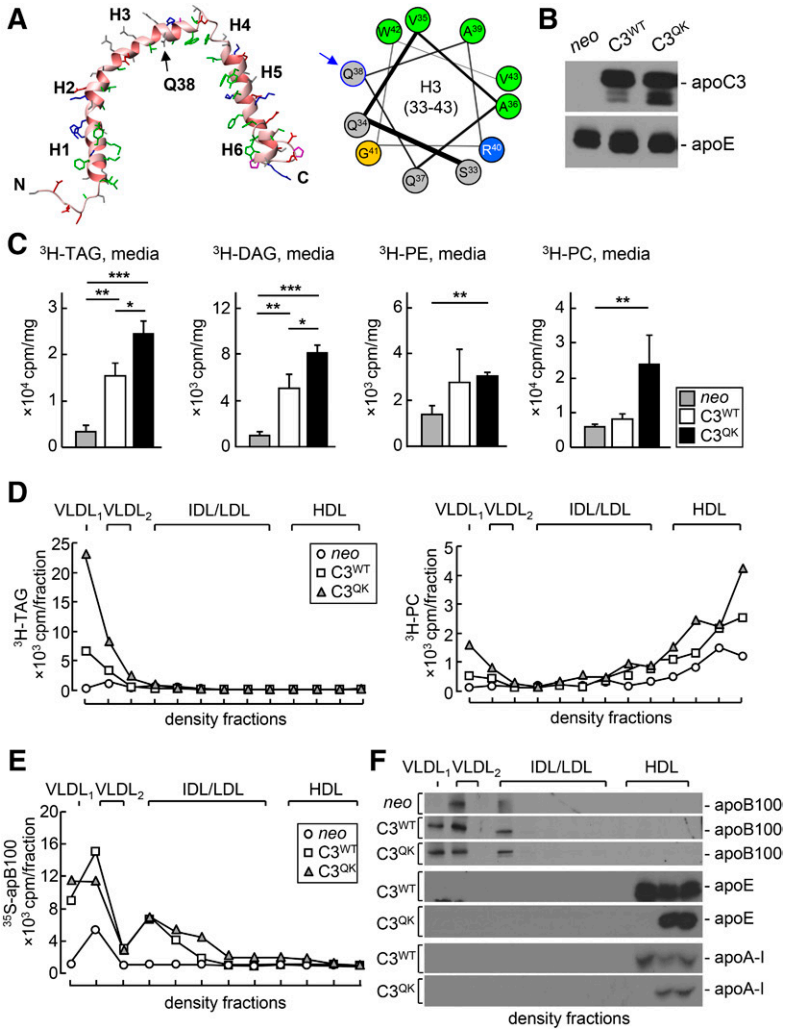

Fig. 1. ApoC-III Q38K variant is a gain-of-function mutation that enhances secretion of $VLDL_1$-associated TAG and apoB-100. A: Six-helix (H1–H6) structure of human apoC-III (left) and position of Gln^{38} at the putative water-lipid interface of helix 3 (right). Apolar residues are colored in green, positively charged residues in blue, polar residues in gray, and Gly residue in yellow. The Q38K mutation introduces a positively charged Arg residue at the putative water-lipid interface, thus presumably rendering helix 3 a type A amphipathic helix (26). B: Immunoblots of human apoC-III and endogenous rat apoE in the medium of *neo*, $C3^{WT}$, or $C3^{QK}$ cells. C: [^{3}H]TAG in the medium of *neo*, $C3^{WT}$, or $C3^{QK}$ cells labeled with [^{3}H]glycerol for 2 h under lipid-rich conditions. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (n = 3 dishes per cell line). D: [^{3}H]TAG (left) and [^{3}H]PC (right) in fractionated lipoproteins secreted from cells 2 h after labeling with [^{3}H]glycerol. E: [^{35}S]ApoB-100 in fractionated lipoproteins secreted from cells 3 h after labeling with [^{35}S]methionine/cysteine. F: Fluorography of [^{35}S]apoB-100, [^{35}S]apoE, and [^{35}S]apoA-I in fractionated lipoproteins.

conditions. There was also a trend of increased incorporation of [^{3}H]acetate into CE in $C3^{QK}$ cells, but the increase was not statistically significant as compared with that in $C3^{WT}$ cells (Fig. 2A, B). These results suggest that $C3^{QK}$ expression results in enhanced hepatic DNL. We also performed metabolic labeling, using [^{3}H]glycerol under lipid-rich conditions (Fig. 2C), to assess the effect of $C3^{QK}$ expression on glycerolipid biosynthesis. Results from these experiments showed increased incorporation of [^{3}H]glycerol into DAG and PC in $C3^{QK}$ cells as compared with that in $C3^{WT}$ cells, but the incorporation of [^{3}H]glycerol into intracellular TAG was similar, and the incorporation of [^{3}H]glycerol into PE was decreased (Fig. 2C). Together, these data suggest strongly that the enhanced $VLDL_1$-TAG secretion (shown in Fig. 1) observed in $C3^{QK}$ cells is associated with enhanced hepatic lipogenesis.

Expression of $C3^{QK}$ variant in *apoc3*-null mice results in hypertriglyceridemia

We next determined the effect of $C3^{QK}$ expression on lipoprotein production in vivo, using adenovirus-mediated gene transfer into *apoc3*-null mice fed with a high-fat diet for 7 days. Expression of $C3^{WT}$ and $C3^{QK}$ was confirmed by immunoblot analysis of the mouse plasma (**Fig. 3A**). Quantification of TAG and CE mass from the plasma collected from mice before injecting P407 (time zero) and at 1 and 2 h after P407 injection was carried out (Fig. 3B, C). Lipid analysis results showed that at the initial time point (time zero), there was no significant differences in TAG content among plasma from all three mice groups. But at 1 and 2 h time points, plasma from $C3^{QK}$ mice accumulated more TAG than plasma from $C3^{WT}$ or vector control mice (Fig. 3B). On the other hand, the plasma CE content was slightly higher in $C3^{QK}$ mice, even at time zero, and continued to accumulate more during the next 2 h of post-P407 injection time points than that in $C3^{WT}$ or vector control mice (Fig. 3C). Fractionation of plasma collected before P407 injection (at time zero) (Fig. 3D), at 1 h (Fig. 3E), and 2 h (Fig. 3F) after P407 injection into various lipoprotein particles using cumulative rate floatation centrifugation was carried out, followed by mass measurement of TAG, CE, and PC in various lipoprotein fractions. Lipid analysis confirmed increased $VLDL_1$-TAG (by ~2-fold) (Fig. 3D–F, left) and $VLDL_1$-CE (by >2-fold) (Fig. 3D–F, middle) in $C3^{QK}$ plasma than that in $C3^{WT}$ plasma. There was also increased $VLDL_1$-PC (by ~40%) in $C3^{QK}$ plasma (Fig. 3D–F, right). At 2 h after P407 injection, the accumulation of lipid-rich $VLDL_1$ was strikingly visible in $C3^{QK}$ mice plasma as compared with $C3^{WT}$ mice plasma (Fig. 3F, left, inset).

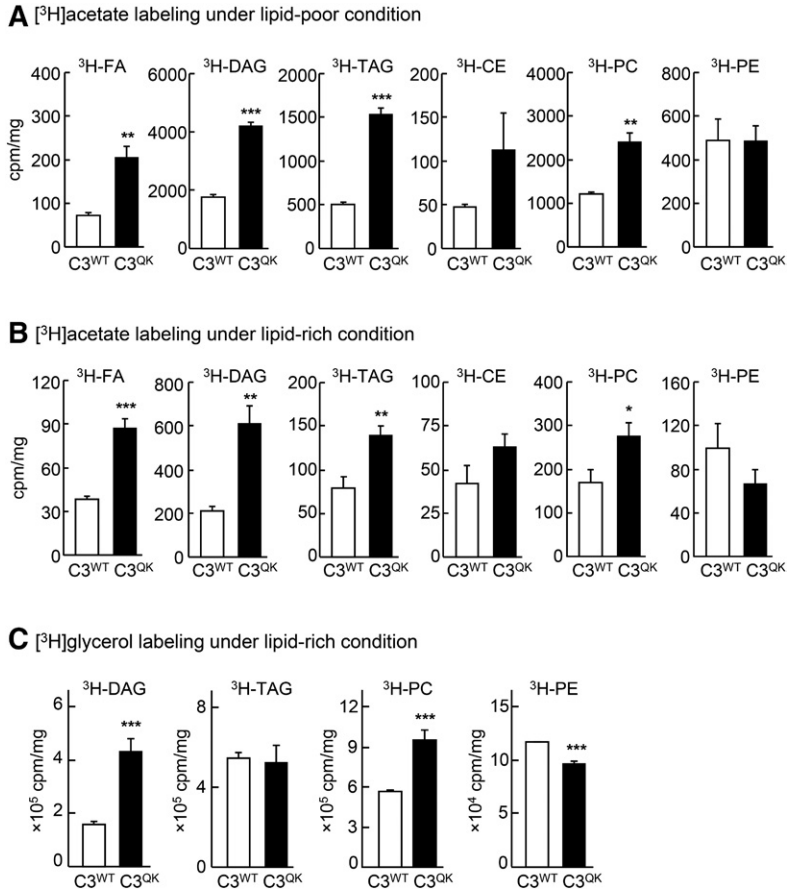

Fig. 2. Expression of $C3^{QK}$ mutant increases DNL. A, B: Cells were labeled with [^{3}H]acetic acid (25 μCi/ml) for up to 2 h under lipid-poor (A) or lipid-rich (B) conditions. C: Cells were labeled with [^{3}H]glycerol for up to 2 h under lipid-rich conditions. At the end of labeling, cell-associated lipids were quantified. Data are expressed as mean ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (n = 3 dishes per cell line).

Expression of $C3^{QK}$ variant in *apoc3*-null mice increases the association of apoB-100 and apoE with large lipoprotein particles

The plasma lipoproteins were also fractionated by using size exclusion chromatography. Immunoblots of the fractionated plasma at 2 h after P407 injection of respective mouse apoB-100, apoB-48, apoE, and apoA-I are shown in **Fig. 4A**. Although size exclusion chromatography was unable to resolve $VLDL_1$ from $VLDL_2$ as effectively as density ultracentrifugation, a slight increase in apoB-100 signal associated with larger lipoproteins (VLDL and IDL) in $C3^{QK}$ plasma was discernible (e.g., FPLC fractions 19–20 in the apoB-100 blot in $C3^{QK}$ compared with $C3^{WT}$). Notably, VLDL and IDL particles in $C3^{QK}$ plasma were also enriched with apoE (Fig. 4A, right). These in vivo data are the first indication that expression of $C3^{QK}$ in mice causes hypertriglyceridemia, which may explain the observed phenotypes in heterozygotes carrying the Gln38Lys variant in humans (14). Furthermore, fractionation of 2 h post-P407-injected mice plasma lipoproteins using size exclusion chromatography showed that apoC-III protein itself was almost exclusively found in association with VLDL in the $C3^{QK}$ mutant, whereas in $C3^{WT}$, apoC-III protein was associated with both VLDL and HDL (Fig. 4B), indicative of an enhanced affinity of apoC-III from $C3^{QK}$ toward VLDL particles. We then determined in vitro lipid-binding properties of apoC-III using fat Western lipid-protein overlay assay (Fig. 4C). Compared with $C3^{WT}$, $C3^{QK}$ bound to PC, PE, and SM in a similar manner (Fig. 4C). Neither $C3^{WT}$ nor $C3^{QK}$ bound to neutral lipids such as CE, TAG, or DG (Fig. 4C). However, $C3^{QK}$ showed a noticeable increase in binding to Chol as compared with $C3^{WT}$ (Fig. 4C). Additional fat Western lipid-protein overlay assay was performed by using a commercial membrane (Echelon Biosciences Inc.) bearing various phosphatidylinositides in addition to the major phospholipid species (supplemental Fig. S1). Overall, there was no major difference in lipid-binding properties between $C3^{WT}$ and $C3^{QK}$.

Expression of $C3^{QK}$ variant in *apoc3*-null mice results in steatosis

Under high-fat diet conditions, $C3^{QK}$ expression in mice resulted in steatosis as compared with $C3^{WT}$. Accumulation of lipids in the $C3^{QK}$ liver specimens was apparent by Oil Red O staining of liver sections (**Fig. 5A**). Representative liver sections from three $C3^{WT}$ mice (Fig. 5A, left) and three $C3^{QK}$ mice (Fig. 5A, right) stained with Oil Red O are shown. Quantification of hepatic lipid mass showed that $C3^{QK}$ livers accumulated significantly more TAG (by 20%) (Fig. 5B) and CE (by 110%) (Fig. 5C), when compared with $C3^{WT}$ livers. These in vivo data offer the first indication that $C3^{QK}$ expression could also lead to a certain degree of hepatic steatosis under high fat diet condition.

Expression of $C3^{QK}$ variant in *apoc3*-null mice increases hepatic lipogenic gene expression and hepatic DNL

We determined the expression of mRNA of various lipogenic genes using quantitative RT-PCR. The level of SREBP-1, SREBP-2, FAS, stearoyl-CoA desaturase, ACC1,

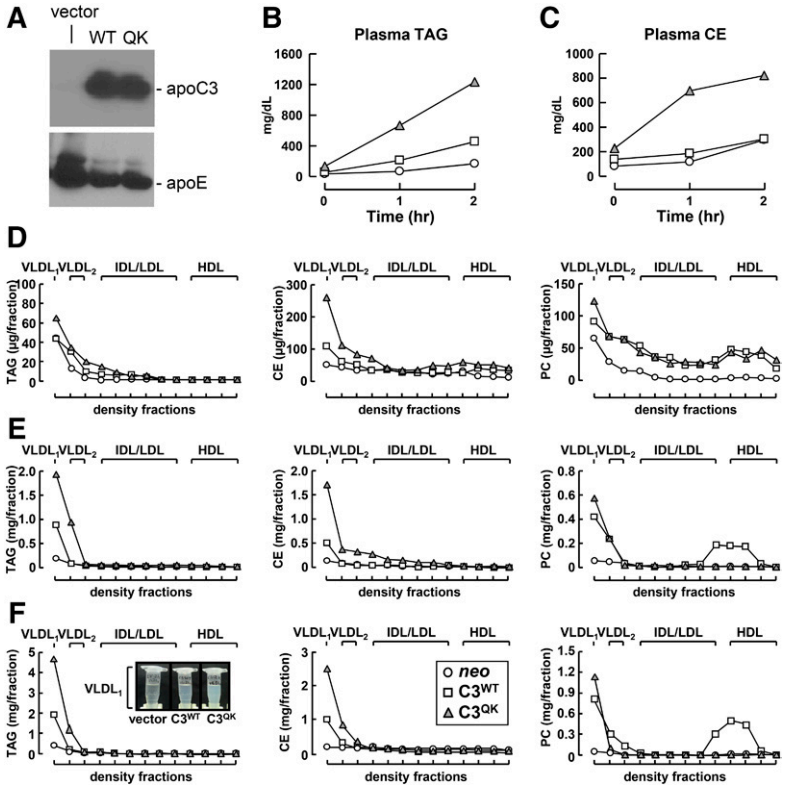

Fig. 3. Expression of $C3^{QK}$ mutant in *apoc3*-null mice promotes TAG-rich $VLDL_1$ production. *Apoc3*-null mice were fed with a high-fat diet for a week and then injected with adenovirus constructs encoding $C3^{WT}$, $C3^{QK}$, or empty vector. At 48 h after injection, mice were fasted for 12 h, and blood samples were collected (time zero) and then injected with P407 intraperitoneally. At 1 and 2 h after P407 injection, plasma samples from three mice per time point per group were collected, pooled, and analyzed for lipids. A: Immunoblot of $C3^{WT}$ or $C3^{QK}$ (top) along with endogenous mouse apoE (bottom). B: Total TAG in pooled plasma samples at 0, 1, and 2 h after P407 injection are shown. C: Total CE in pooled plasma samples at 0, 1, and 2 h after P407 injection are shown. D–F: Pooled plasma at time zero (D), at 1 h (E), and at 2 h (F) were fractionated by cumulative rate flotation ultracentrifugation, mass of TAG (left), CE (middle), and PC (right) in each fraction was quantified and plotted. The lipid-rich $VLDL_1$ fractions collected at 2 h after P407 injection are shown in F, left (inset).

LXRa, CD-36, and 3-hydroxy-3-methylglutaryl-CoA reductase mRNAs was significantly higher in $C3^{QK}$ mice as compared with $C3^{WT}$ or vector control mice (**Fig. 6A**). We also determined hepatic DNL in these mice using [^{3}H]acetic acid (injected via tail vein) labeling for 3 h. Data presented in Fig. 6B indicate that ^{3}H-radioactivity was incorporated significantly more into FA, DAG, TAG, PC, Chol, and CE in the livers of $C3^{QK}$ mice than in $C3^{WT}$ or vector control mice. These data suggest that $C3^{QK}$ expression in *apoc3*-null mice exerts a profound impact on hepatic lipogenesis.

Expression of $C3^{QK}$ variant in *apoc3*-null mice causes hyperlipidemia

Mass measurement of plasma lipids showed that under chow diet conditions, there was a significant increase in fasting plasma concentrations of FA (by 30%) (**Fig. 7A**), TAG (by 40%) (Fig. 7B), Chol (by 24%) (Fig. 7C), and CE (by 18%) (Fig. 7D) in $C3^{QK}$ mice when compared with $C3^{WT}$.

Analysis of Gln38Lys mutation in human APOC3 gene

To determine the prevalence of *APOC3* Gln38Lys (p.Q58K) in humans, we performed next-generation sequencing of 1,557 DNA samples from patients exhibiting dyslipidemia phenotypes, including hypertriglyceridemia, hypercholesterolemia, and combined hyperlipidemia, from Canadian of predominantly European origin, with some of Chinese, African, and South-Asian origin (18, 19). *APOC3* p.Q58K was not found in these 1,557 dyslipidemia samples, nor was there any occurrence of this variant in the ExAC databases containing exome or genome sequence information on 60,706 individuals. Together, these data confirm the rarity of the *APOC3* p.Q58K variant in humans.

DISCUSSION

The present study of the naturally occurring apoC-III Q38K variant provides new evidence for an intracellular role that human apoC-III plays in the assembly and secretion of hepatic TAG-rich $VLDL_1$. Although apoC-III is a rather small protein of 79 amino acids, there are at least seven coding sequence variations reported to date. Three loss-of-function variants, namely, Lys58Glu (11), Ala23Thr (10), and Arg19X (21), are associated with a hypotriglyceridemia phenotype and may confer cardioprotective effect. Two missense mutations, Thr74Ala (22, 23) and Asp45Asn (24), have not been associated with lipid or lipoprotein abnormalities. Another newly identified missense mutation Asp25Val variant has been associated with renal insufficiency and hypotriglyceridemia (25). The Gln38Lys variant (14), on the other hand, was associated with hypertriglyceridemia, and the current study suggests Gln38Lys being a gain-of-function mutation in promoting $VLDL_1$ production. However, genomic analysis of DNA samples of dyslipidemia patients, from various ethnic groups, as well as samples from normal controls (total of >65,000 individuals), did not detect the Gln38Lys variant. Thus, the Gln38Lys is a rare variant in the human population.

The present data, together with experimental evidence obtained from our previous studies of $C3^{AT}$ (12) and $C3^{KE}$ variants (13), have suggested three roles that apoC-III may play intrahepatocellularly. First, apoC-III is required for the formation of MALD, a putative lipid substrate of $VLDL_1$ (13). Second, apoC-III promotes incorporation of the lipid substrates into VLDL during $VLDL_1$ assembly/secretion (9). Third, expression of apoC-III (i.e., $C3^{QK}$) enhances hepatic DNL (Figs. 2, 6), which is associated with hyperlipidemia,

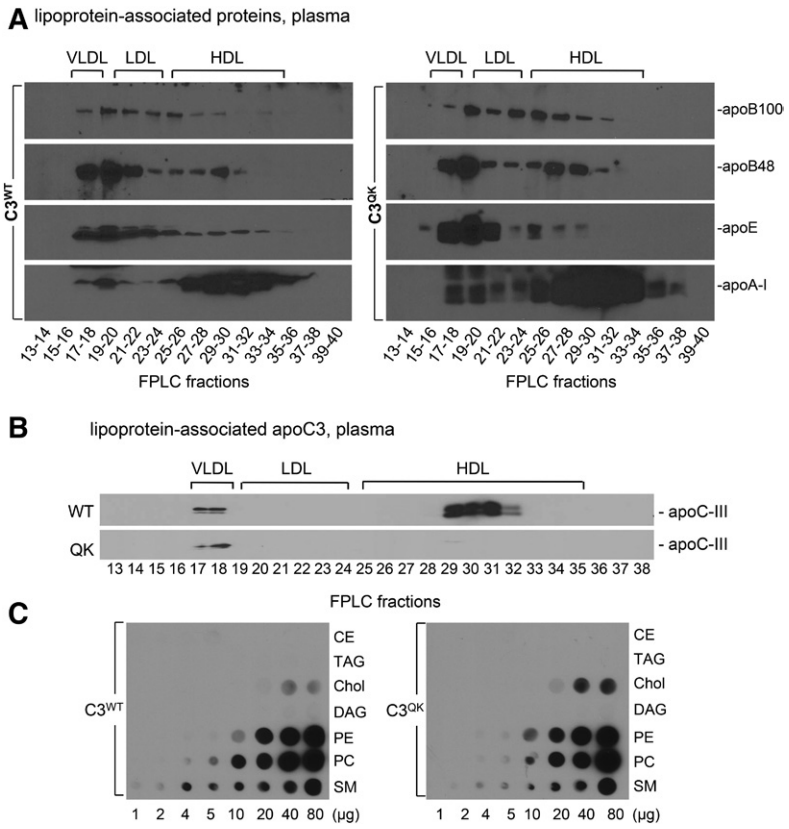

Fig. 4. Expression of $C3^{QK}$ mutant in *apoc3*-null mice promotes the association of apoB100, apoE, and apoC3 to plasma VLDL. Plasma collected from mice at 2 h after P407 injection were pooled and fractionated by size exclusion chromatography (FPLC). A: Western blot images of ApoB-100, apoB-48, apoE, and apoA-I (left, $C3^{WT}$; right, $C3^{QK}$). B: Western blots of apoC-III in FPLC fractionated plasma of $C3^{WT}$ and $C3^{QK}$ mice. C: Comparison of lipid-binding properties of apoC-III protein from $C3^{WT}$ and $C3^{QK}$.

even under chow-diet conditions, upon $C3^{QK}$ expression (Fig. 7). Nearly all of these functions are lost in the loss-of-function variants $C3^{KE}$ (13); in contrast, the gain-of-function variant $C3^{QK}$ (this study) enhances these functions.

The mechanism by which apoC-III can play such a profound role in hepatic lipid metabolism is unclear. Regardless, the amphipathic nature of apoC-III α-helices appears to be critical to its function. The Gln^{38} residue resides in the putative water-oil interface of the amphipathic helix 3 (Fig. 1A). Substituting Gln^{38} with Lys introduces a positive charge at the interface, rendering helix 3 a perfect type A amphipathic helix (26). Previous studies have shown that changes in the net positive or negative charges of individual helices of apoC-III may affect its lipid binding properties (10, 13, 27). It has also been suggested that changes in charge distribution of apoC-III may exert an effect on the LPL-catalyzed lipolysis or receptor-mediated reuptake of lipoproteins (1, 10). Although under the present in vitro assay conditions, $C3^{QK}$ did not show gross changes in binding to lipid species, subtle differences in binding to Chol was noticed between $C3^{WT}$ and $C3^{QK}$ (Fig. 4C). Thus, charge distribution and the related amphipathicity of α-helices of apoC-III are important structural elements underlying its functions.

Results of the present study highlight the role that the Gln38Lys variant may play in lipogenesis. Overexpression of WT human apoC-III in mice resulting in certain degree of hepatosteatosis has been reported previously (28). Under our experimental conditions, hepatosteatosis was not consistently observed in *apoc3*-null mice transfected with adenovirus-encoded human apoC-III (i.e., $C3^{WT}$). However, expression of the Gln38Lys variant (i.e., $C3^{QK}$) in *apoc3*-null mice using the adenovirus vector invariably resulted in moderate levels of steatosis (Fig. 5B, C), indicating that this gain-of-function mutant could exert a potent lipogenic effect in the liver.

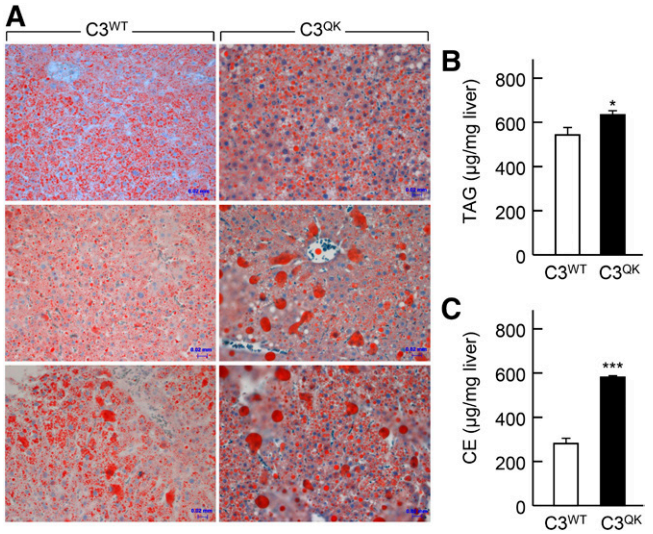

Fig. 5. Expression of $C3^{QK}$ mutant under high-fat diet condition in *apoc3*-null mice promotes hepatic steatosis. A: Oil Red O staining of liver sections of *apoc3*-null mice fed with a high-fat diet for 1 week and then injected with adenovirus constructs encoding $C3^{WT}$ or $C3^{QK}$. Liver samples collected from the mice at 48 h after injection were frozen fresh and sectioned by using cryostat and then stained with Oil Red O and visualized under light microscope. Images of intrahepatic lipid droplets from three different mice liver sections per group are shown. Scale bar, 20 μm. B, C: Lipid mass measurements of liver TAG (B) and CE (C) from $C3^{QK}$ and $C3^{WT}$ mice are shown. Data are expressed as mean ± SD. * $P < 0.05$; *** $P < 0.001$ (n = 3–6 mice per group).

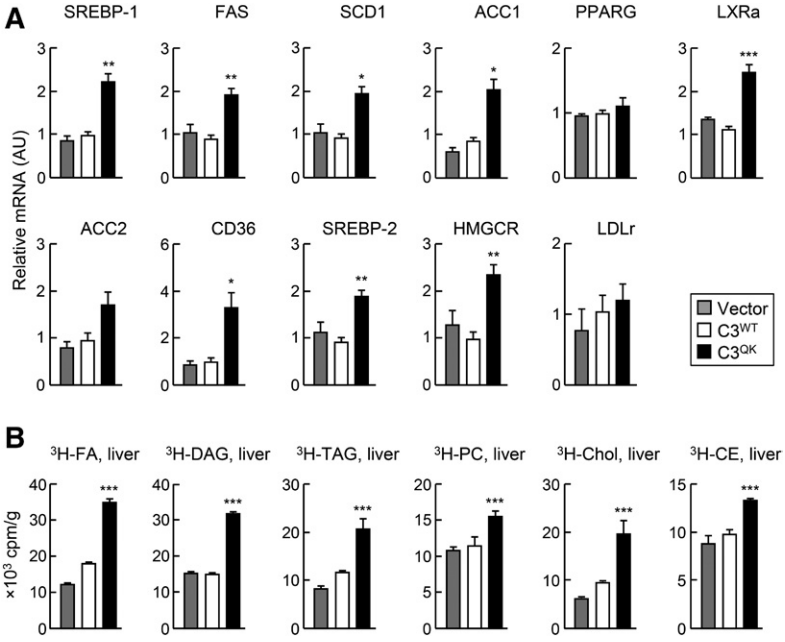

Fig. 6. Expression of $C3^{QK}$ in mice increases the expression of lipogenesis genes and hepatic DNL. A: Relative mRNA expression profile of genes involved in lipogenesis in the livers of mice injected with adenovirus constructs of vector control or $C3^{WT}$ or $C3^{QK}$. Data are expressed as mean ± SD. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ (n = 3–5 mice per treatment). B: In vivo hepatic DNL in mice. Bar graph shows the radioactivity associated with the indicated lipids extracted from the livers of mice 3 h after tail vein injection of [^{3}H] acetic acid. Data are expressed as mean ± SD. *** $P < 0.001$ (n = 3–5 mice per treatment).

Overexpression of apoC-III in mice has also been shown to increase diet-induced obesity, which could be reversed by expression of physiological levels of CE transfer protein (29), and these mice were found predisposed to develop diet-induced hepatic steatosis and hepatic insulin resistance (28). On the other hand, mice deficient in apoC-III failed to develop hypertriglyceridemia, even after inducing diabetes with streptozotocin under chow-diet condition (30). However, apoC3 deficiency appears to promote high-fat diet-induced obesity in mice, presumably owing to enhanced hydrolysis of plasma TAG catalyzed by LPL, and the resulting FAs are subsequently taken up by adipose tissues and further aggravate insulin resistance due to decreased whole-body insulin sensitivity and unbridled endogenous glucose production (31). D-glucose and farnesoid X receptor (FXR) ligands additively regulate the expression of FXR and its target genes, small heterodimer partner and *apoC3* in the liver of Zuker rats (32). Studies with mice lacking a liver-specific insulin receptor show that glucose induces *apoC3* mRNA transcription via a mechanism involving the transcription factors carbohydrate response element binding protein and hepatocyte nuclear factor-4α (33). These studies together suggest that apoC-III may serve as a critical component in the complex interplay between lipid and carbohydrate metabolism.

In summary, the present study provides new experimental evidence for an intracellular function of apoC-III in regulating hepatic lipid metabolism by enhancing the hepatic DNL that provides the required lipid substrates (TAG, CE, Chol, PC, etc.) to be incorporated into the VLDL assembly process, resulting in the secretion of lipid-rich buoyant $VLDL_1$ particles from the cells and mice expressing the gain-of function variant $C3^{QK}$.

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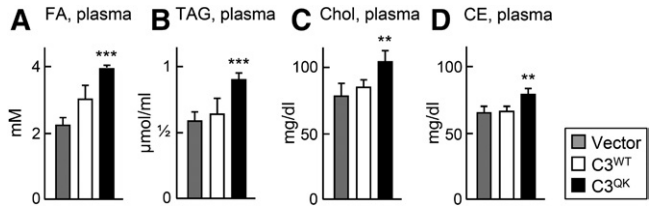

Fig. 7. Expression of $C3^{QK}$ in mice causes hyperlipidemia. Twelve week old apoC3-null mice maintained on normal chow diet were injected with adenovirus constructs encoding $C3^{WT}$ or $C3^{QK}$ or empty vector. At 48 h after injection, mice were fasted for 8 h, blood was collected, and then levels of FA (A), TAG (B), Chol (C), and CE (D) in the plasma were quantified. Data are expressed as mean ± SD. ** $P < 0.01$; *** $P < 0.001$ (n = 5 mice per treatment).

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