Identification of ZNF471 as a modulator of DNA methylation and plasma lipid levels in cardiovascular diseases

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Running title

Impact of ZNF471 on DNA methylation and plasma lipid levels

Abstract.

Context. Plasma lipid concentrations are closely linked to the development of cardiovascular diseases (CVD). Plasma levels of Low-Density Lipoprotein cholesterol are positively correlated to the incidence of CVD whereas those of High-Density Lipoprotein Cholesterol (HDL-C) are considered as protective. Recent studies revealed that epigenetic mechanisms, such as DNA methylation, might contribute to the control of blood lipid concentrations and might affect cardiovascular risk. However, underlying molecular mechanisms are unknown.

Objective. The goal of this study was to identify novel pathways controlling DNA methylation and the determination of plasma lipid levels.

Methods and Results. Epidemiological studies in the MARTHA cohort led us to identify the association of a non-synonymous single nucleotide polymorphism (nSNP), rs11667052, located in a gene coding for a novel transcription factor, *ZNF471*, with both DNA methylation at *CETP* CpG sites and circulating cholesterol levels. Moreover, the *ZNF471* nSNP was associated to DNA methylation levels at *CETP* CpG sites, plasma CETP activity and to CVD in the ECTIM (Etude Cas-Témoin de l'Infarctus du Myocarde) study. Expression of human ZNF471 (hZNF471) in the liver of ApoB/CETP transgenic mice led to an increased DNA methylation at a *CETP* CpG site (+17%, $p<0.05$) and a reduced hepatic *CETP* mRNA levels (-78%, $p<0.05$) as compared to control animals. Plasma CETP activity and total cholesterol levels were decreased (-19% and -10%, respectively, $p<0.05$) upon hZNF471 expression, whereas an elevation of atheroprotective HDL-C (+57%, $p<0.05$) was observed. Plasma from hZNF471 mice showed a higher capacity to promote macrophage cholesterol efflux than those from control mice (+35%, $p<0.05$). In addition, liver expression of additional DNA methylation-sensitive key genes controlling lipid metabolism, *Lpl* (Lipoprotein lipase), *Pltp* (Phospholipid Transfer Protein) and *Abcg1* (ATP-Binding Cassette G1), was equally reduced in hZNF471 mice. In the Cardiogenics cohort, methylation levels of CpG sites in *CETP*, *ABCG1*, *LPL* and *PLTP* genes were associated with *ZNF471* expression levels in circulating monocytes and derived macrophages; such an association being influenced by *ZNF471* rs11667052.

Conclusions Our findings indicate that epigenetic modulation of lipid metabolism genes by ZNF471 might impact plasma lipid and lipoprotein levels and the incidence of CVD.

Introduction.

Plasma lipids and lipoproteins levels, such as low-density, high-density lipoproteins cholesterol (LDL-C, HDL-C, respectively) and post-prandial triglycerides(TG)-rich lipoproteins (TRL) are well-established risk factors for cardiometabolic disorders (CMD) including coronary artery disease (CAD), obesity and type 2 diabetes (T2D) that represent the most prevalent cause of morbidity and mortality in Western countries. These CMD risk factors are modifiable and demonstrate strong inter-individual variability whose deep characterization can help identifying novel therapeutic targets for cholesterol management and CMD prophylaxis.

Thus, because plasma LDL-C levels are positively correlated to the incidence of CAD[1], targeting the reduction of LDL-C has been widely validated as being an efficacious therapeutic goal in atherosclerosis and CAD. Pharmacological drugs such as statins (cholesterol synthesis inhibitors) have successfully achieved this goal during the last decades[2]. New promising molecules, including PCSK9 inhibitors, are currently tested in clinical trials and should help to strengthen the therapeutic arsenal available for CAD patients, although those molecules are intended to a very restricted population of patients. Indeed, PCSK9 inhibitors are proposed to patients for whom first-line treatment like statins cannot lower efficiently their cholesterol due to serious genetic defects as it is case in familial hypercholesterolemic patients carrying homozygous mutations in *APOB*, *LDLR* and *PCSK9* genes.

However, whatever the therapeutic option, a residual CAD risk still persists in treated patients[2] and new therapies are therefore needed in order to reduce this risk. Novel therapeutic strategies currently explored include the pharmacological increase of HDL-C[3], as circulating HDL-C levels are inversely associated to CAD risk[1,4]. Such association results from the atheroprotective properties of HDL particles notably the one related to reverse cholesterol transport (RCT). Indeed, HDL particles promote cholesterol efflux from the arterial wall, mostly from intimal lipid-laden macrophages, and its transport back to the liver for elimination. Through the RCT pathway, HDLs are proposed to reduce lipid accumulation in the arterial wall, atherosclerosis development and CAD related outcomes[5] and mortality[6]. More importantly, recent studies reported that cholesterol efflux from macrophage to HDL, is strongly inversely associated with both carotid intima-media thickness and the likelihood of angiographic coronary artery disease[7].

Recent studies pointed out that circulating lipid levels variability may be controlled by epigenetic mechanisms including DNA methylation. Thus, DNA methylation regulation of several genes implicated in lipid metabolism was reported to be associated with blood lipid levels, however molecular mechanisms underlying those effects are largely unknown[8]. We presently identified ZNF471, a transcription factor of

the zinc finger family, as a modulator of the expression of genes involved in the lipoprotein metabolism likely through an epigenetic mechanism which requires DNA methylation at CpG sites.

Materials & Methods.

In vivo expression of human ZNF471 in mice

Twelve-week aged male Tg human Apolipoprotein B (ApoB) / CETP (ApoB/CETP) mice (generated by breeding human CETP transgenic mice (line 5203) with mice transgenic for the human apoB gene as previously descrived[9]) fed on a standard chow diet were retro-orbitally injected with 40µg of either a hZNF471 expression vector (pCMV6-hZNF471-GFP, Origene#RG215696) or a control pmax-GFP vector (Lonza) using an *in vivo* transfection reagent (PepJet DNA In Vivo Transfection Reagent, SignaGen Laboratories) (injected volume = 200µl) under isoflurane anesthesia. Seven days following injection, blood samples were collected in Microvette tubes (Sarstedt) by retro-orbital bleeding under isoflurane anesthesia. Mice were weighted, euthanized and livers were isolated for RNA extraction and DNA methylation analysis by bisulfite conversion and pyrosequencing. Plasma samples were analyzed with an Autoanalyzer (Konelab 20) using reagent kits from Roche (total cholesterol) and ThermoElectron (HDL-cholesterol). A liver piece was excised, minced and digested in Hank's balanced salt solution (HBSS, Gibco, Invitrogen, Cergy Pontoise, France) with 2.5 mg/mL collagenase D (Roche, Boulogne Billancourt, France) for 30 min at 37°C under shaking and dissociated through a 200 µm pored cell strainer (Franklin lakes, NJ, USA). Hepatocytes were isolated following a brief centrifugation for 1 min at 1,000 rpm and stored at -80°C until use.

RNA extraction, reverse-transcription and quantitative-PCR

Total RNA from liver or isolated hepatocytes were extracted using the NucleoSpin RNA II kit (Macherey-Nagel) according to the manufacturer's instructions. Reverse transcription and real-time qPCR using a LightCycler LC480 (Roche) were performed as previously described[10]. Expression of mRNA levels was normalized to mouse hypoxanthine phosphoribosyltransferase 1 (*Hprt1*), mouse non-POU domain containing, octamer-binding housekeeping gene (*Nono*), mouse heat shock protein 90kDa alpha (cytosolic), class B member 1 (*Hsp90ab1*) and mouse cyclophilin A (*CycA*). Data were expressed as a fold change in mRNA expression relative to control values.

DNA methylation analyses in the MARTHA study

We measured genome-wide DNA methylation in whole blood samples of 350 individuals of the MARTHA (MARseille Thrombosis Association) study using the dedicated Illumina HumanMethylation450 array. A detailed description of the quality controls and the normalization procedures applied to the methylation

array data has previously been published[11]. Briefly, bisulfite conversion was performed on 1 µg genomic DNA for each sample using the Qiagen EpiTect 96 Bisulfite Kit and 200 ng of bisulfite-converted DNA at 50 ng/µl was independently amplified, labeled and hybridized to Infinium HumanMethylation450 BeadChip microarrays. For each sample, the intensities of the methylated and unmethylated signals were measured at 485,577 CpG sites using the Illumina iScan (with default settings). Three CpG sites at the *CETP* locus were investigated in the present work: cg09889350, cg12564453 and cg1660091. DNA methylation levels were expressed as a β-value, a continuous variable over the [0–1] interval, representing the percentage of methylation of a given CpG site.

Association of *ZNF471* genotypes with *CETP* CpG levels was tested using a linear regression analysis adjusted for age and sex. Correlation analyses between *CETP* CpG and lipids levels were investigated while adjusting for age, sex and cell type composition determined by specific biological counts of lymphocytes, monocytes, neutrophils, eosinophils and basophils.

DNA methylation analysis of CETP CpG sites in the ECTIM study

Sodium bisulfite conversion was performed from 300 ng genomic DNA using EZ-96 DNA Methylation kit (ZymoResearch) from patients included in the ECTIM study (Cases, n=361, Controls, n=461, Study for Myocardial Infarction). From the resulting bisulfite DNA, a relative quantitative PCR was performed using 2 TaqMan MGB probes (Life Technologies) specific to methylated or unmethylated studied CpG and specific labelled with FAM or VIC reporter. The primers and probes design was performed with Primer Express software from the bisulfite converted DNA sequence. The primers and probes were chosen with no CpG island in there sequence, except the one to study. The sequences of the probes and primers were:

For cg09889350:

probe C $^{5'}$ AGTTTTATGTTTCGTG$^{3'}$(FAM) – probe T $^{5'}$ AGTTTTATGTTTTGTG$^{3'}$ (VIC)

primer F $^{5'}$ TAAAAATGGTGTAGATGGTGG$^{3'}$ – primer R $^{5'}$ CATAATTATCAAACAATAATATATAAATAACC$^{3'}$.

For cg125644538:

probe C $^{5'}$ AGTTTGGAGTTCGT$^{3'}$(FAM) – probe T $^{5'}$ TTAGTTTGGAGTTTGT$^{3'}$ (VIC)

primer F $^{5'}$ GTGGGTGTTTATGAAAAGATTT$^{3'}$ – primer R $^{5'}$ CTAAAACCAAAAAAACCCTACTAC$^{3'}$.

For cg16660091:

probe C $^{5'}$ TTAGGTTGAACGGT$^{3'}$(FAM) – probe T $^{5'}$ TAGGTTGAATGGTT$^{3'}$ (VIC)

primer F $^{5'}$ GGTGTAGATGGTGGAGGG$^{3'}$ – primer R $^{5'}$ AACATAATTATCAAACAATAATATATAAATAAC$^{3'}$.

Relative quantitative PCR was performed on a ABIPRISM7000 instrument (Life Technology) with Rox Passive Reference from 1.5µl of bisulfite converted DNA in total volume of 14µl. The reaction mix contained 200 nM of each MGB probe (Life technologies), 900 nM of each primer (Sigma – HPLC purified), 0.1 mM dNTP, 5 mM MgCl2, 0.1X Rox (Euromedex), 0.4U Immolase Taq and 1X ImmoBuffer (Bioline). The amplification program was: 95°C 10 min following by 45 cycles of 95°C 15sec - 58°C 1min. For each sample, the Ct for the two probes were determined with the same threshold, this threshold being defined with control DNA for which methylated value was known. For each sample, the formula "% methylation = $100/[1+2^{(Ctmeth-Ctunmeth)}]$" was applied to calculate the methylated percentage of the studied CpG. Association of *ZNF471* genotypes with CETP CpG levels was tested using a linear regression analysis adjusted for age

Analysis of DNA methylation by bisulfite conversion and pyrosequencing in mouse liver

Liver DNA from ApoB/CETP mice was extracted using the DNeasy Blood and Tissue kit (Qiagen) and converted through bisulfite treatment for methylation analysis using the Premium Bisulfite kit (Diagenode) according to the manufacturer's instructions. Amplification of a 366 bp fragment of the human CETP promoter gene encompassing 11 CpG sites and including the 3 incriminated CpG sites (cg09889350, cg12564453 and cg16660091) was carried out by polymerase chain reaction using the following primers (forward : 5'-AAGACTCGGCAGCATCTCCATATTGATATTTATATATTAGGAGGGTAG-3' and reverse : 5'-GCGATCGTCACTGTTCTCCAAAAACAAATAAAAATTAAAATACTCTTATT-3'). Quality of amplicons was validated onto a 2% agarose gel and PCR products were pyrosequenced on a PyroMark Q96 MD system using the PyroMark Gold Q96 CDT reagent kit and Pyro Q-CpG software (Qiagen).

Cellular free cholesterol efflux assays

Human THP-1 macrophages were labeled with 1µCi/ml [3H]cholesterol for 24 hours in the presence of 50µg/ml acetylated LDL (acLDL) in a RPMI medium containing 2mM Glutamine, 50mM Glucose and 0.2% BSA (RGGB). Cells were then equilibrated in RGGB medium for an additional period of 16 hours. Cellular cholesterol efflux to 0.5% mouse serum was assayed in RGGB medium for a 4-hour chase period. Finally, culture media were harvested and cleared of cellular debris by brief centrifugation. Cell associated radioactivity was determined by extraction in hexan-isopropanol (3:2), evaporation of the

solvent and liquid scintillation counting (Wallac Trilux 1450 Microbeta). The percentage of cholesterol efflux was calculated as 100 x (medium cpm) / (medium cpm + cell cpm).

Cardiogenics study population

Individuals included in this analysis have been collected as part of the Cardiogenics Consortium[12]. They were composed of 479 individuals of white European origin with (n=241) or without (n=238) a history of coronary artery disease and recruited in five centres (Cambridge and Leicester, UK; Lübeck and Regensburg, Germany; Paris, France). Fasting venous blood samples were collected from all the participants into EDTA. All patients have been typed for genome-wide genotypes from whole blood DNA using the Human Quad Custom 670 array (Illumina). mRNA expression from isolated CD14+ monocytes and derived macrophages were quantified using the HumanRef-8 v3 Beadchip array (Illumina)[13]. Large-scale analysis of methylation in whole blood DNA was previously conducted using the Infinium Human Methylation450K array (Illumina)[11].

Expression quantitative trait loci (eQTL) and DNA methylation analysis

The effect of *ZNF471* expression on DNA methylation was analyzed by selecting all CpG sites for *CETP*, *LPL*, *ABCG1* and *PLTP* after adjustment for age, sex, centre, blood cell composition as fixed effects with batch and chip as random effects. Association of *ZNF471* rs11667052 on gene expressions was tested using regression linear models under the assumption of additive allele effects with the same adjustments as above. Note that this polymorphism was available on the DNA array and had not had to be imputed.

Statistical analysis

Data are shown as mean ± SEM. Experiments were performed in triplicate and values correspond to the mean from at least three independent experiments. Comparisons of 2 groups were performed by a 2-tailed Student's t test and comparisons of 3 or more groups were performed by ANOVA with Newman–Keuls post-test. All statistical analyses were performed using Prism software from GraphPad (San Diego, CA, USA).

Results.

Association of a nonsynonymous polymorphism of *ZNF471* with DNA methylation at the CETP locus.. In a sample of 350 patients with venous thrombosis from the MARTHA study[14], whose whole blood DNA was epityped with the Illumina H450K DNA methylation array, we observed an association between the nonsynonymous rs62123030 (G 0,8 > C 0,2) polymorphism of *ZNF471* and whole blood DNA methylation levels at five CpG sites located in the *CETP* gene promoter (cg09889350, cg01706698, cg12564453, cg16660091 and cg26624021). As an illustration, the common *ZNF471* rs62123030 G was associated with increased DNA methylation at CpG cg09889350 (Figure 1A). We replicated this association in an independent sample of 822 individuals part of the ECTIM study[15], a case-control study for arterial thrombosis. In the ECTIM sample composed of 361 patients with myocardial infarction and 461 healthy controls, all males, whole blood DNA methylation at *CETP* CpG sites were measured using a bisulfite PCR assay. As shown in Figure 1B, the common rs62123030 G allele was significantly ($p = 0.0025$) associated with increased CpG cg09889350 levels, consistently to what observed in the MARTHA cohort epityped for the H450K array. Similar patterns were obtained for the four other *CETP* CpG sites (data not shown). Of note, this association was slightly enhanced in the sample of 361 cases (additive effect of the G allele $\beta = +0.27$, $p = 0.02$) than in the sample of 461 controls ($\beta = +0.13$, $p = 0.10$). Also of interest were the observations of lower *CETP* CpG cg09889350 levels in cases than in controls ($p=5.83\ 10^{-4}$) and in smokers compared to non-smoker individuals ($p= 1.25\ 10^{-4}$) of the ECTIM study (Figure 2). Despite differences in study sampling scheme, DNA methylation measurements and data processing between the hypothesis-generating MARTHA study and the replication ECTIM study, those data provide strong support for *ZNF471* genetic polymorphisms (the rs62123030 or any other variation in very strong linkage disequilibrium with it i.e $r^2 >0.8$) associated with whole blood DNA methylation levels at the *CETP* locus.

Interestingly, methylation levels at the *CETP* CpG sites were correlated to plasma HDL, LDL and total cholesterol in both MARTHA and ECTIM, and these correlations were modulated by the rs62123030 genotype. For example, in the ECTIM population, the correlation between CpG cg09889350 and HDL-C was $\rho = 0.11$ ($p = 0.02$) in the 459 carriers of the GG genotype and $\rho = 0.01$ ($p =0.84$) in the 330 carriers of the C allele. As CETP activity had been previously measured in ECTIM participants[16], we investigated where this genotype also influences the correlation between CpG cg09889350 levels and CETP activity. In GG carriers, CpG levels and CETP activity were not correlated ($\rho =0.05$; $p = 0.30$) while they were negatively correlated in C carriers ($\rho = -0.15$; $p = 4.72\ 10^{-3}$).

Taken together, those data indicate that methylation of *CETP* CpG sites and plasma lipid levels are affected by a polymorphism located in the *ZNF471* gene, suggesting that *ZNF471* genetically influences the epigenetic regulation of *CETP* that further contributes to circulating lipid levels variability and cardiovascular diseases.

***In vitro* expression of human ZNF471 represses CETP mRNA levels in human hepatocytes.** In order to test if ZNF471 modulates the expression of CETP, we overexpressed ZNF471 using an expression vector coding for a human ZNF471 protein tagged with GFP. Analysis of the cellular localization of ZNF471-GFP in both Hep3B and CHO cells confirmed the nuclear localization of ZNF471 as previously reported (Ref. Cao Oncogene 2018) (Figure 3A). Interestingly, the transient overexpression of human ZNF471 (hZNF471) in HepG2 cells (Figure 3B) led to a significant decrease of *CETP* mRNA levels (Figure 3C; -22%, $p<0.05$) which is coherent with a repressive mechanism involving an increased DNA methylation of *CETP* CpG sites as observed in our epidemiological studies.

***In vivo* expression of human ZNF471 affects plasma lipid levels and macrophage cholesterol efflux.** In order to validate our findings *in vivo*, we evaluated the effect of the expression of hZNF471 in the Tg human Apolipoprotein B (ApoB) / CETP (ApoB/CETP) (as CETP is lacking in mice) mouse model which displays a “humanized” lipid metabolism more prone to reproduce effects expected on human lipid metabolism. Indeed, plasma cholesterol in mice is mostly carried by HDL (ApoA-I-containing lipoproteins) and not by LDL or VLDL (ApoB-containing lipoproteins) as it occurs in humans. Introduction of the human ApoB transgene in CETP Tg mice allows a plasma phenotype similar to that observed in humans, with a predominance of plasma cholesterol carried by ApoB-containing particles.

To achieve this goal, hZNF471 was expressed in ApoB/CETP mice following the retro-orbital injection (PepJet DNA In Vivo Transfection Reagent) of a hZNF471 expression vector (pCMV6-hZNF471-GFP). Retro-orbital injection of this cationic peptide (PepJet DNA In Vivo Transfection Reagent) was described to allow a high in vivo delivery efficiency to the liver which is the key organ controlling lipid metabolism.

Injection of the hZNF471 expression vector in ApoB/CETP mice led to a significant decrease of plasma total cholesterol levels as compared to animals injected with a control plasmid (pmaxGFP) after 7 days (Figure 4A). More importantly, an increase of plasma HDL-C levels was observed following injection of hZNF471 whereas those of animals injected with the control vector were not significantly affected. In agreement with this marked increase of plasma HDL-C levels, capacity of plasmas from mice

injected with the hZNF471 expression vector to promote cholesterol efflux from human THP-1 macrophages was enhanced by 35% ($p<0.05$) (Figure 4C) as compared to control animals. This result is of particular interest as plasma cholesterol efflux capacity from macrophages, a metric of HDL function, is strongly inversely associated with both carotid intima-media thickness and the likelihood of angiographic coronary artery disease[7].

In vivo experiments in a "humanized" lipid metabolism mouse model indicate that the expression of human *ZNF471* is able to reduce the atherogenicity of plasma lipid phenotype.

Modulation of liver expression of key lipid metabolism genes following in vivo expression of human ZNF471. Seven days following the injection of the hZNF471 expression vector, a robust expression of hZNF471 mRNA levels was detected in hepatocytes isolated from livers of ApoB/CETP mice (Figure 5A). In addition, liver expression of hZNF471 led to an increased methylation of a promoter *CETP* CpG site, a reduction of *CETP* mRNA levels together with a decrease of plasma CETP activity as compared to control animals (Figure 5B-C). Moreover, liver gene expression analysis revealed that the increased expression of hZNF471 represses not only *CETP*, but also three additional murine genes involved in lipid metabolism, *Pltp, Lpl* and *Abcg1* coding respectively for Phospholipid Transfer Protein, Lipoprotein Lipase and ATP-Binding Cassette G1 (Figure 6 and supplemental Figure 1). Taken together these results indicate that ZNF471 not only modulates the expression of CETP but of a set of genes controlling lipoprotein metabolism.

Association of CpG sites DNA methylation levels into genes identified in epidemiological and in vivo studies with ZNF471 rs11667052 and ZNF471 expression in human monocytes and derived macrophages. To determine if these genes might be targets of ZNF471 in humans, eQTL analysis was performed in a subset of the Cardiogenics cohort. Analysis of *ZNF471* expression in human monocytes and derived macrophages from 479 individuals of the Cardiogenics Consortium indicated that the expression of *ZNF471* in human monocytes was significantly correlated to whole blood methylation levels at *CETP* CpG sites (cg26624021, cg01706698, cg03232842, cg09889350 and cg16660091) but also to those at CpG sites located in candidates genes (*PLTP*, cg20105087, cg26321644 and cg01236081; *ABCG1*, cg00222799 and cg26767954 and *LPL*, cg22108175 and cg04649769) (Table 1); the most significant effect being observed at the *PLTP* CpG site cg20105087 ($p=0.002$). Highly significant correlations were observed between *ZNF471* expression in human macrophages and methylation levels

at *CETP* CpG sites (cg09889350, cg26624021, cg01706698, cg16660091, cg03232842 and cg12564453) and to a lesser degree at the *LPL* CpG site cg07263235 (Table 1). However, no correlations were detected at CpG sites in the *PLTP* and *ABCG1* genes in macrophages.

In this subset of individuals of the Cardiogenics cohort, the non-synonymous ZNF471 rs11667052 variant (C/A: 0,76/0,24, Met→Ile), which is in complete linkage disequilibrium (LD) with rs62123030 (r^2=1) was associated with whole blood methylation at *CETP* (cg01706698, cg03232842, cg09889350, cg16660091, cg26624021, cg12564453 and cg03186069), *LPL* (cg20435463) and *ABCG1* (cg05639842) CpG sites (Table 2). Further analysis revealed an interaction between rs11667052 and *ZNF471* expression on whole blood DNA methylation levels at *CETP*, *ABCG1*, *LPL* and *PLTP* CpG sites (Table 3). As a whole, those findings indicate that both the *ZNF471* rs11667052 (or any other variant in perfect LD with it) and *ZNF471* monocyte/macrophage expression are associated with whole blood DNA methylation at *CETP* CpG sites but also at CpG sites in *LPL*, *ABCG1* and *PLTP* genes then supporting the idea that ZNF471 might modulate the expression of these genes through DNA methylation.

Discussion.

Altogether, the present study points out to a novel transcription factor, ZNF471, as a new player in modulating plasma cholesterol levels through epigenetic modulation of key genes controlling lipid metabolism. Those data indicate that *ZNF471* SNPs may be valid indicators of the action of ZNF471 on DNA methylation and potentially the expression of several genes involved in the lipid metabolism in humans. In this context, it can be envisaged that exploration of the *ZNF471* genotype could be a useful tool in precise medicine for modulation of cholesterol metabolism.

Indeed, the present transversal exploration of the role of ZNF471 by epidemiological, *in vitro* and *in vivo* studies led us to propose that this transcription factor, for which only few information are available in the literature, represses the expression of *CETP* and others genes involved in cholesterol metabolism i.e. *LPL*, *PLTP* and *ABCG1* very likely through the methylation of CpG sites within these genes. In agreement with our findings, DNA methylation of *CETP*, *PLTP, LPL* and *ABCG1* genes was recently reported to contribute independently to plasma lipid levels in hypercholesterolemic individuals[17]. Moreover, DNA methylation at *ABCG1* CpG sites was reported to be associated with plasma HDL-C levels in several studies and to predict incident coronary heart disease events [18–21]. A role of ZNF471 on CVD is supported by our observation in ECTIM that the modulation of methylation at *CETP* CpG sites by the *ZNF471* SNP was associated with myocardial infarction.

Our study does not however provide direct evidences that ZNF471 modulates the DNA methylation of the target genes. Our knowledge on molecular mechanisms through which ZNF transcription factors control the gene expression allows us to draw a mechanism for the mode of action of ZNF471. Family of ZNF contains more than 400 different proteins, expressed in various tissues in human[22]. ZNF471 possesses a classical zinc finger structure allowing the three-dimensional stabilization of α helix and β sheet: two cysteine (C) and two histidine (H) (C_2H_2 type) residues are linked through this ion zinc. This three-dimensional arrangement allows attachment of the ZNF protein on DNA sequence. ZNF contains a KRAB (Krüppel Associated Box) domain, which has a strong repressor activity. KRAB domain permits the recruitment and binding of KAP1 (KRAB-Associated Protein 1) protein, a corepressor implicated in the recruitment of others partners. Indeed, this ZNF-KRAB-KAP1 complex is able to modulate epigenetic through histones modifications (acetylation and/or methylation) and/or DNA methylation[23,24]. To achieve this role, the complex is able to recruit HP1 (Heterochromatin Protein 1) and to read methylation levels on the lysine 9 of histone 3, HDAC (Histone Deacetylase), HAT (Histone Acetylase) or DNMT (DNA Methyltransferase) proteins[25,26]. For instance, ZNF202, a C_2H_2 ZNF type

transcription factor such as ZNF471, has been showed to be a modulator of apolipoproteins *Apo A-IV*, *Apo C-III* and *Apo E*[27] and *ABCA1* (ATP-Binding Cassette A1) and *ABCG1* gene expression[28], all of them being implicated in lipid metabolism. Thus, the reduced expression of *ABCA1* and *ABCG1* by ZNF202 transfection diminishes phospholipid and cholesterol efflux to Apo A-I and HDL from Raw 264.7 murine macrophages[28].

We were not able to detect any modification in the expression of *KAP1*, *KDM1A* (Lysine Demethylase 1A), *DNMT1* or *DNMT3* following ZNF471 expression (data not shown). Taken together, we therefore propose that ZNF471 represses the expression of CETP, LPL, PLTP and ABCG1 through its binding to their DNA sequence and the recruitment of KAP1 and DNMT3A or DNMT3B; these latter being able to perform the *de novo* DNAmethylation at CpG sites. Unfortunately, the use of anti-GFP or anti-ZNF471 antibodies commercially available did not allow to immunoprecipitate ZNF471 and associated proteins in order to validate this mechanism. Ongoing transcriptomic (RNA-sequencing) and DNA methylation (Reduced Representation Bisulfite Sequencing) analysis in liver samples from hZNF471 and control mice will provide important information about the role of ZNF471 on gene expression. However, we need to keep in mind that ZNF471 is a transcription factor that very likely modulates the expression of numerous genes implicated in cellular pathways other than lipid metabolism. Indeed, ZNF471 expression was previously characterized in human tumors; *ZNF471* gene being hypermethylated in patients with squamous cell carcinoma of the tongue[29] or gastric cancer[30]. As a consequence, the reduced ZNF471 expression was associated with a reduced survival of patients[30].

Although *ABCG1* expression was not reported to determine plasma HDL-C concentrations[31–33], a large body of evidence indicates that a reduced expression of *PLTP*, *LPL* and *CETP* led to an increase of circulating levels of HDL-C [34,35]. More especially, CETP inhibitors have been developed for a therapeutic use as elevated plasma HDL-C levels are largely described to be inversely associated with in ASCVD[36]. In this context, the inhibition of *CETP*, *LPL*, *PLTP* expression and in some extend that of *ABCG1*, observed in the present study following the expression of *ZNF471* is coherent with an elevation of plasma HDL-C levels and a subsequent increased capacity of plasma to promote macrophage cholesterol efflux. Therefore, our findings suggest that the pharmacological increase of *ZNF471* expression might have a benefical action on ASCVD through the raising of HDL-C. Interestingly, the expression of human *ZNF471* in ApoB/CETP mice was equally accompanied by a reduction of total cholesterol levels; such a decreased of non-HDL cholesterol being detected in patients treated with CETP inhibitors[36].

Beyond of the use of pharmacological tools for increasing ZNF471 which remain to be identified, our study rather led us to propose that the *ZNF471* genotype could serve as a biological marker for stratifying patients and to propose a personalized medicine strategy for treating cardiovascular diseases through lipid-lowering therapy. More especially, the *ZNF471* rs11667052 (or any other variant in perfect LD with it) might be used to predict the response of patients to CETP inhibitors which mostly failed to reduce CVD in clinical trials[37–39]. Indeed, among the four CETP inhibitors developed by pharmaceutical companies, only Anacetrapib was reported to decrease the risk of CV events[40]. Then, it can be anticipated that individuals homozygous for the rare allele of *ZNF471* rs11667052 (AA, low *ZNF471* expression) which are expected to exhibit both a high CETP expression and activity would benefit from the CETP inhibition. By contrast, the CC carriers (high *ZNF471* expression) would be poor responders of CETP inhibition because of the low CETP expression and activity in those individuals. Such a pharmacogenomic approach was recently used in order to predict the response to the CETP inhibitor Dalcetrapib on the incidence of CVD[41]. Thus, Tardif et al. identified a polymorphism of the Adenylate Cyclase 9 (*ADCY9*) gene able to identify patients who will benefit from the treatment with Dalcetrapib. However, the use of this genetic biomarker failed to predict the response to Evacetrapib, another CETP inhibitor, indicating that *ADCY9* SNPs was a biomarker to the Dalcetrapib drug rather than the CETP inhibition[42]. In this context, the *ZNF471* rs11667052 might be a useful pharmacogenomic tool to identify patients for which CETP inhibitors would be efficient for reducing CVD.Although additional investigations are needed to elucidate the molecular mechanisms through which ZNF471 exerts its epigenetic control of gene expression, the present study provides important information in the understanding of the relation between DNA methylation and determination of plasma lipid levels. Finally, our findings identify ZNF471 as a potential therapeutic target and a novel pharmacogenomic tool for personalized medicine.

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Tables.

Cell type	Methylation probe	Gene	β	SE	*P* Value
Monocyte	cg20105087	*PLTP*	0.026	0.008	0.002
	cg26624021	*CETP*	0.050	0.018	0.005
	cg01706698	*CETP*	0.020	0.007	0.006
	cg03232842	*CETP*	0.026	0.010	0.011
	cg00222799	*ABCG1*	0.024	0.010	0.019
	cg26767954	*ABCG1*	-0.004	0.002	0.020
	cg22108175	*LPL*	-0.029	0.013	0.022
	cg04649769	*LPL*	0.017	0.007	0.022
	cg26321644	*PLTP*	-0.007	0.003	0.035
	cg09889350	*CETP*	0.027	0.013	0.039
	cg16660091	*CETP*	0.031	0.015	0.042
	cg01236081	*PLTP*	0.014	0.007	0.045
Macrophage	cg09889350	*CETP*	0.041	0.011	1.18E-04
	cg26624021	*CETP*	0.054	0.015	2.20E-04
	cg01706698	*CETP*	0.021	0.006	4.78E-04
	cg16660091	*CETP*	0.041	0.012	9.85E-04
	cg03232842	*CETP*	0.028	0.009	1.29E-03
	cg12564453	*CETP*	0.023	0.009	0.011
	cg07263235	*LPL*	0.022	0.011	0.044

Table 1. Association between methylation levels at selected *CETP*, *ABCG1*, *LPL* and *PLTP* CpG sites and *ZNF471* expression in human monocytes and derived macrophages. Estimates are for a unit increase in log2 *ZNF471* expression (ILMN_2191720).

Methylation probe	Gene	β	SE	*P* Value
cg01706698	*CETP*	-0.0234	0.0024	3.08E-23
cg03232842	*CETP*	-0.0288	0.0034	1.56E-17
cg09889350	*CETP*	-0.0306	0.0044	2.46E-12
cg16660091	*CETP*	-0.0347	0.0051	9.68E-12
cg26624021	*CETP*	-0.0373	0.0060	7.24E-10
cg12564453	*CETP*	-0.0202	0.0037	7.04E-08
cg03186069	*CETP*	-0.0031	0.0013	0.0169
cg20435463	*LPL*	-0.0079	0.0035	0.0266
cg05639842	*ABCG1*	-0.0015	0.0008	0.0437

Table 2. Association between methylation levels at selected *CETP*, *ABCG1* and *LPL* CpG sites and the non-synonymous *ZNF471 SNP* rs11667052. Values are for the rs11667052 A allele.

Cell type	Methylation probe	Gene	*P* Value
Monocyte	cg01176028	*ABCG1*	0.008
	cg07072366	*LPL*	0.018
	cg23611945	*PLTP*	0.027
	cg03232842	*CETP*	0.038
	cg14982472	*ABCG1*	0.043
	cg08918749	*LPL*	0.046
	cg22108175	*LPL*	0.046
	cg01706698	*CETP*	0.047
	cg11662315	*ABCG1*	0.049
Macrophage	cg00222799	*ABCG1*	0.002
	cg09863247	*PLTP*	0.002
	cg06500161	*ABCG1*	0.007
	cg05046272	*ABCG1*	0.013
	cg20214535	*ABCG1*	0.013
	cg26768067	*ABCG1*	0.016
	cg02370100	*ABCG1*	0.017
	cg08918749	*LPL*	0.019
	cg07397296	*ABCG1*	0.020
	cg07072366	*LPL*	0.024
	cg23611945	*PLTP*	0.039
	cg16125291	*LPL*	0.042
	cg27243685	*ABCG1*	0.044

Table 3. *P* values of the interaction test between rs11667052 polymorphism and *ZNF471* expression on whole blood DNA methylation levels at selected *CETP, ABCG1, LPL* and *PLTP* CpG sites.

Figures.

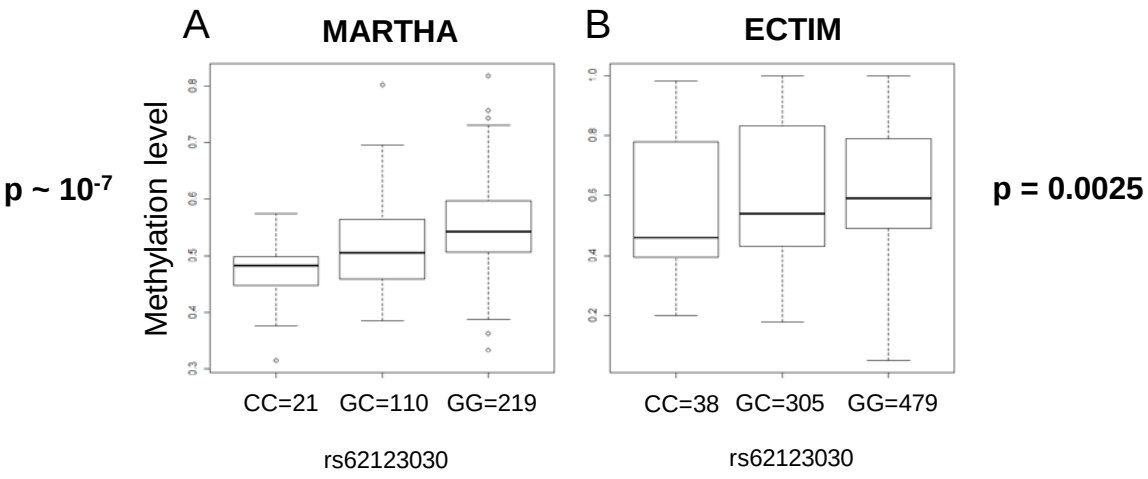


Figure 1. Association of a ZNF471 nSNP (rs62123030) with whole blood DNA methylation levels at CETP CpG#1 (cg09889350) site in the MARTHA (A) and ECTIM (B) studies. In ECTIM, association was performed on normalized values adjusting for age and case-control status. Only males were studied.

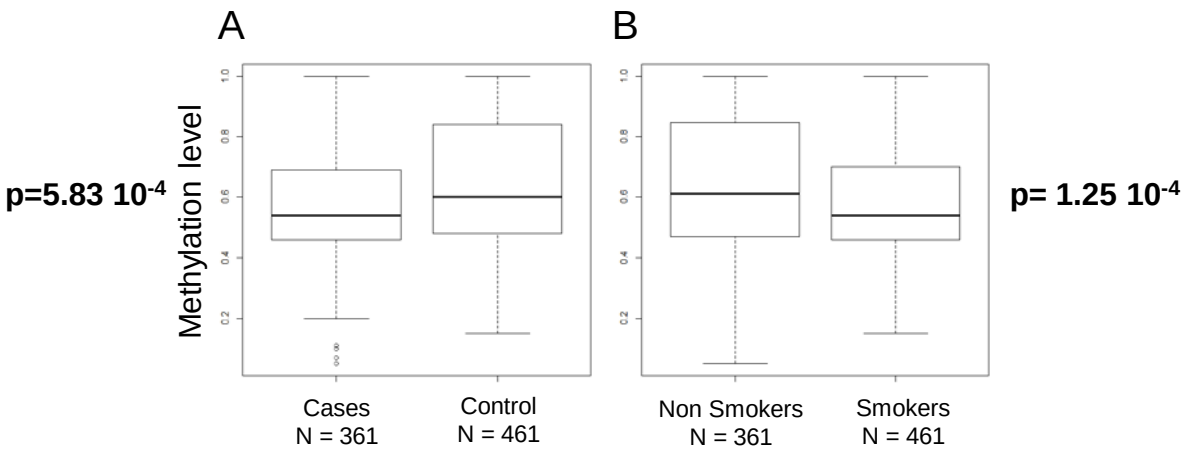


Figure 2 . Association of CETP CpG#1 (cg09889350) levels with arterial thrombosis (A) and smoking (B) in the ECTIM study.

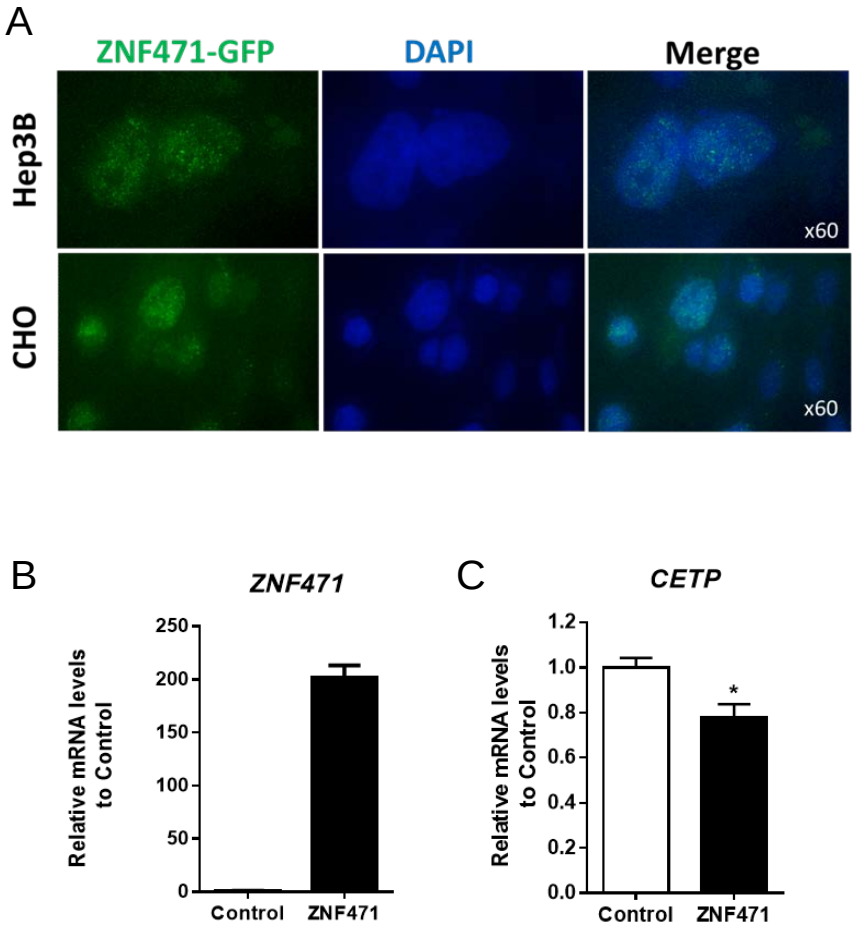


Figure 3 . Expression of hZNF471 *in vitro* reduces CETP expression. A. Nuclear localization of ZNF471-GFP in Hep3B and CHO cells following the transient transfection with an expression vector coding for a human ZNF471-GFP fusion protein. ZNF471-GFP in green and DAPI in blue. Analysis of ZNF471 (B) and CETP (C) mRNA levels in HepG2 cells transfected with an expression vector coding for human ZNF471or a control vector. *p<0.05 versus control.

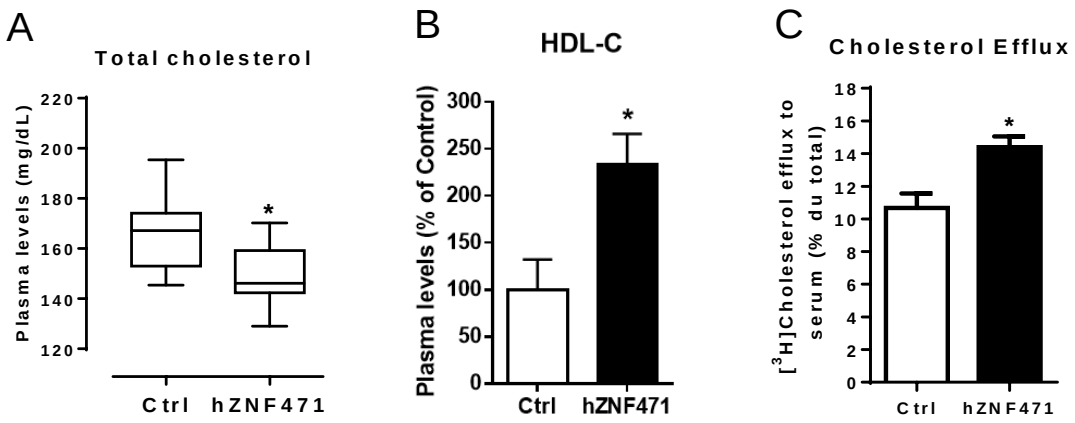


Figure 4. Expression of hZNF471 *in vivo* decreases circulating cholesterol levels. ApoB/CETP mice were retro-orbitally injected with an expression vector coding for human ZNF471 (hZNF471) or a control vector (Ctrl). Quantification of circulating total cholesterol (A), HDL-C levels (B) and capacity of serum to promote macrophage cholesterol efflux (C) 7 days following the injection. *p<0.05, **p<0.05. n=7-8 animals per group.

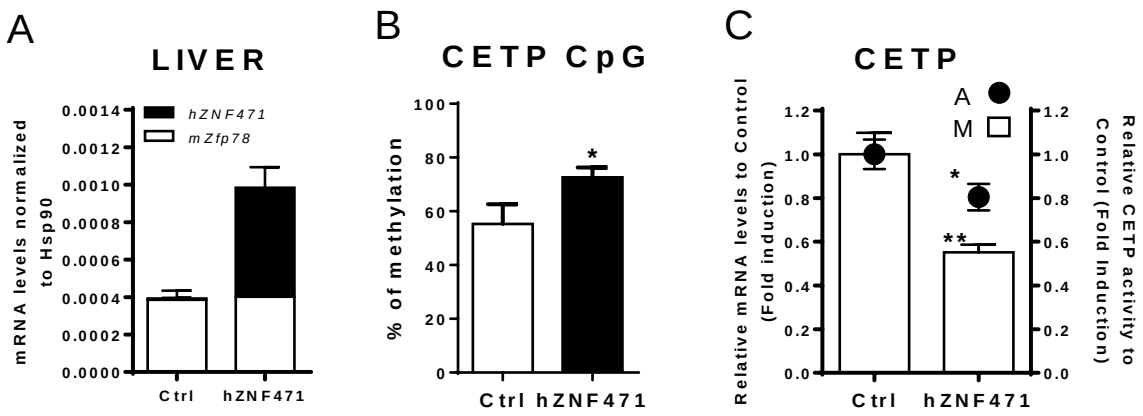


Figure 5. Expression of hZNF471 *in vivo* reduces liver CETP expression . ApoB/CETP mice were retro-orbitally injected with an expression vector coding for human ZNF471 (hZNF471) or a control vector (Ctrl). Quantification of human *ZNF471* and the mouse *Zfp78* orthologue mRNA levels *(A).* Analysis of a CETP promoter CpG site methylation by bisulfite conversion (B). Quantification of hepatic *CETP* (C, ☐) mRNA levels, and measurement of plasma CETP activity (C, ●) 7 days following the injection. *p<0.05, **p<0.05. n=7-8 animals per group.

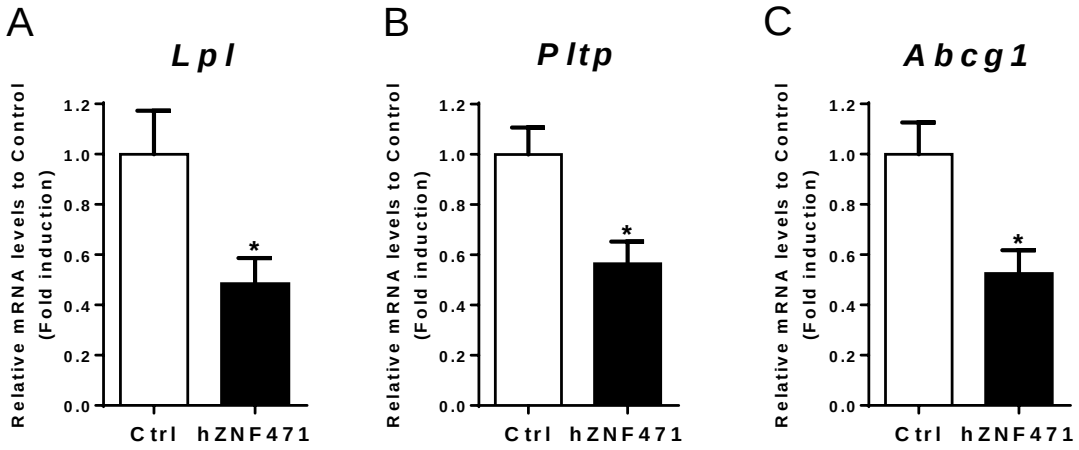
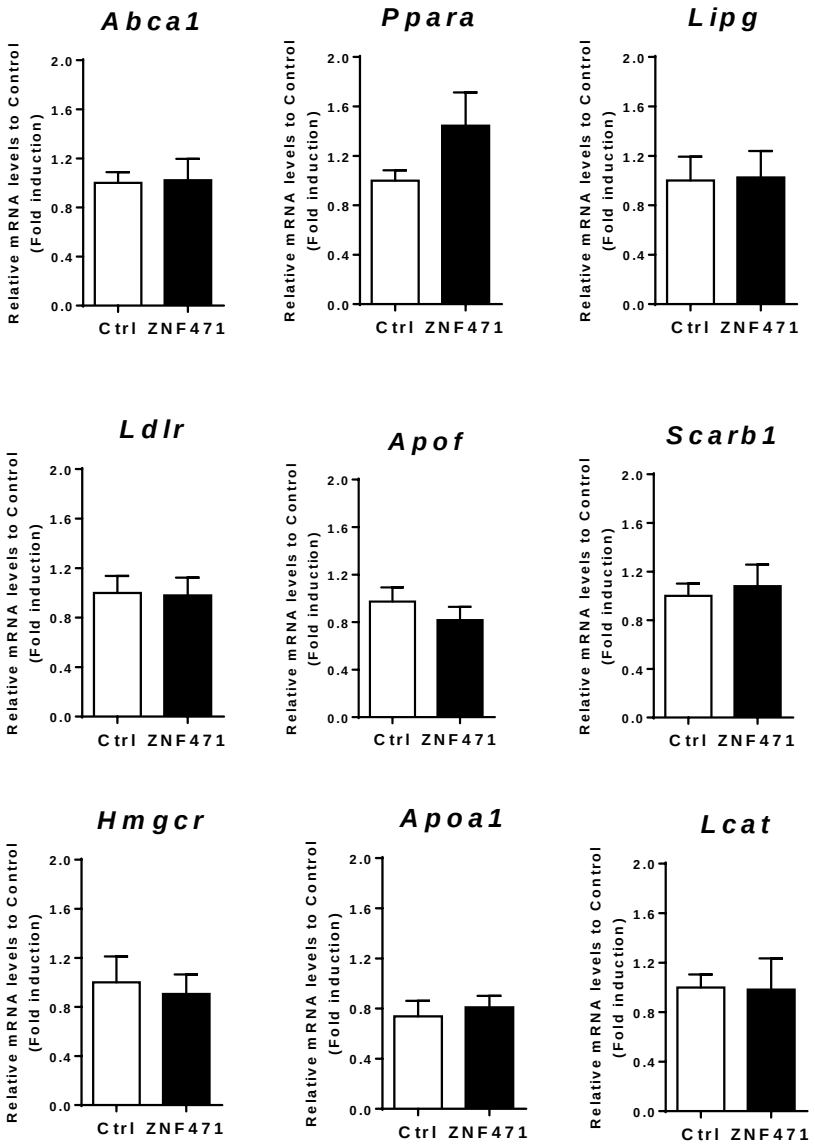


Figure 6. Expression of hZNF471 *in vivo* reduces liver expression of key lipid metabolism genes. ApoB/CETP mice were retro-orbitally injected with an expression vector coding for human ZNF471 (hZNF471) or a control vector (Ctrl). Quantification of hepatic *Lpl* (A), Pltp (B) and Abcg1 (C) mRNA levels 7 days following the injection. *p<0.05, n=7-8 animals per group.

Supplemental figure.



Supplemental Figure. Impact of hZNF471 *on* liver expression of others lipid metabolism genes. ApoB/CETP mice were retro-orbitally injected with an expression vector coding for human ZNF471 (hZNF471) or a control vector (Ctrl). Quantification of mRNA levels 7 days following the injection. n=7-8 animals per group.