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REPORT

An *APOA5* 3′ UTR Variant Associated with Plasma Triglycerides Triggers *APOA5* Downregulation by Creating a Functional miR-485-5p Binding Site

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APOA5 c.*158C>T (rs2266788), located in the 3′ UTR, belongs to *APOA5* haplotype 2 (APOA5*2), which is strongly associated with plasma triglyceride levels and modulates the occurrence of both moderate and severe hypertriglyceridemia. Individuals with APOA5*2 display reduced *APOA5* expression at the posttranscriptional level. However, the functionality of this haplotype remains unclear. We hypothesized that the hypertriglyceridemic effects of APOA5*2 could involve miRNA regulation in the *APOA5* 3′ UTR. Bioinformatic studies have identified the creation of a potential miRNA binding site for liver-expressed miR-485-5p (MIRN485-5p) in the mutant *APOA5* 3′ UTR with the c.*158C allele. In human embryonic kidney 293T (HEK293T) cells cotransfected with an *APOA5* 3′ UTR luciferase reporter vector and a miR485-5p precursor, c.*158C allele expression was significantly decreased. Moreover, in HuH-7 cells endogenously expressing miR-485-5p, we observed that luciferase activity was significantly lower in the presence of the c.*158C allele than in the presence of the c.*158T allele, which was completely reversed by a miR-485-5p inhibitor. We demonstrated that the rare c.*158C *APOA5* allele creates a functional target site for liver-expressed miR-485-5p. Therefore, we propose that the well-documented hypertriglyceridemic effect of APOA5*2 involves an *APOA5* posttranscriptional downregulation mediated by miR-485-5p.

Hypertriglyceridemia (HTG [MIM 145750 and 144600]) is a common metabolic disease resulting from complex interactions between genetic and environmental factors.[1,2] Lipoprotein lipase (*LPL* [MIM 609708]) and its regulators, such as apolipoprotein CII (*APOC2* [MIM 608083]), apolipoprotein CIII (*APOC3* [MIM 107720]), and apolipoprotein A-V (*APOA5* [MIM 606368]), play a major role in triglyceride (TG) metabolism. Deleterious mutations in these genes cause hyperchylomicronemia (MIM 144650 and 238600), and various SNPs have been associated with both mild and severe HTG.[2]

ApoAV, encoded by *APOA5*, is a liver-expressed 366 amino acid apolipoprotein that binds to very-low-density lipoprotein, high-density lipoprotein, and chylomicrons in plasma.[3] Its involvement in TG metabolism was first demonstrated in mouse models: *Apoa5*-knockout mice showed a 4-fold increase in plasma TG concentrations, whereas *Apoa5* overexpression in mice significantly reduced TG levels.[3,4] In mice, apoAV lowers plasma TG levels by increasing lipoprotein lipase (LPL) activity, as confirmed by in vitro and in vivo studies.[5–7] However, its mechanism of TG regulation is still not completely understood.

In humans, *APOA5* plays a critical role in HTG physiopathology. Deleterious *APOA5* mutations were found to be involved in familial hyperchylomicronemia by inducing a LPL activity defect.[8–10] Moreover, two common *APOA5* variant haplotypes modulate triglyceridemia and the expression of mild and severe HTG.[3,10–15] *APOA5* haplotype 3 (APOA5*3) carries the c.56C>G (p.Ser19Trp) polymorphism (rs3135506; RefSeq accession number NM_052968.4), which alters apoAV signal peptide and causes reduced levels of mature peptide in vitro.[16] A second *APOA5* haplotype (APOA5*2) includes the C rare allele of the c.*158C>T SNP (rs2266788; RefSeq NM_052968.4), which is located in the *APOA5* 3′ UTR (previously described as *APOA5* SNP1, c.1891T>C, or c.1259T>C) and is in strong linkage disequilibrium with three additional SNPs: g.4430C>T (rs662799; RefSeq NG_015894.1; previously described as *APOA5* SNP3 or g.−1131T>C), c.−3A>G (rs651821; RefSeq NM_052968.4), and c.162−43A>G (rs2072560; RefSeq NM_052968.4; previously described as *APOA5* SNP2).[3,11] The frequency of APOA5*2 is approximately 7% in populations of European descent and is strongly associated with both mild and severe HTG.[10,14,15] This strong association with plasma TG concentrations was confirmed in genome-wide association studies (GWASs).[12,13,17–19] Clinical data have shown that subjects with the c.*158C minor allele have 20%–30% higher plasma TG levels than do those without this allele.[3] Some studies have also shown a decreased plasma apoAV concentration in subjects with APOA5*2.[20–24] Several arguments have suggested that APOA5*2 might modulate *APOA5* expression at the posttranscriptional

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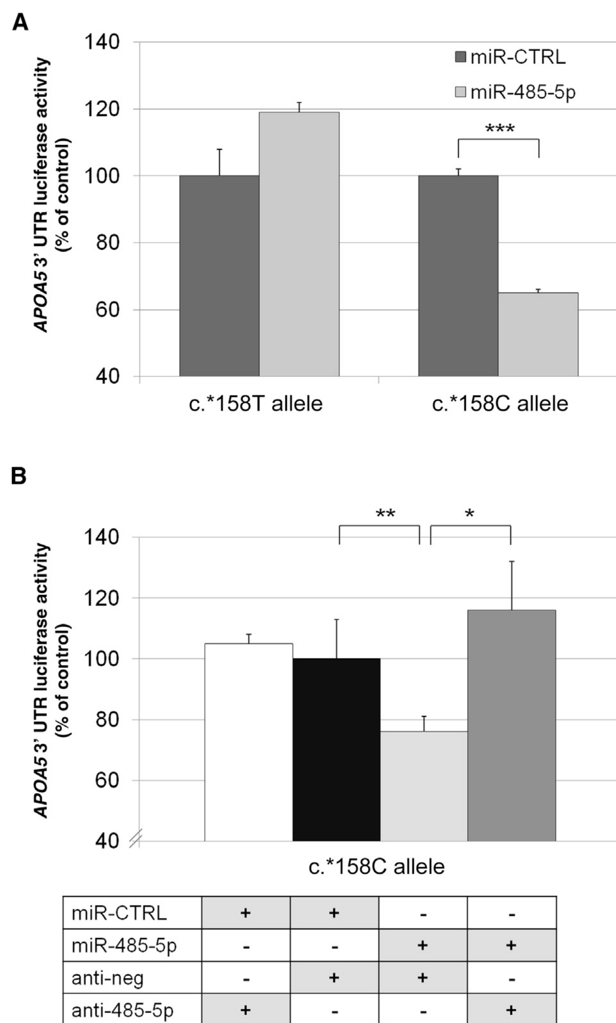

Figure 1. The *APOA5* 3′ UTR with the c.*158C Allele Creates a miR-485-5p Seed Site

(A) Luciferase activity of the pEZX-C or pEZX-T construct with miR-485-5p or miR control (miR-CTRL).

(B) Luciferase activity of the pEZX-C construct with miR-485-5p or miR-CTRL in the presence of either a miR-485-5p inhibitor (anti-485-5p) or a negative control inhibitor (anti-neg).

The *APOA5* 3′ UTR full-length sequence (c.*158T allele) was inserted into pEZX-MT01 (GeneCopoeia, Labomics SA) downstream of a firefly luciferase reporter gene, creating a pEZX-MT01-*APOA5*-c.*158T vector (pEZX-T). Site-directed mutagenesis was performed with the QuickChange II Site-Directed Mutagenesis Kit (Agilent Technology) with mutated primers (forward 5′-GGGTGC TGTCTCCTGCACATCCAGCCTCCTGCG-3′ and reverse 5′-CGCA GGAGGCTGGATGTGCAGGAGACAGCAGCC-3′) for introduction of the c.*158C allele, creating a pEZX-MT01-*APOA5*-c.*158C vector (pEZX-C). All constructs were verified by sequencing. HEK293T cells (3×10^5 cells/well) were plated onto 12-well plates 48 hr before transfection and were maintained in a 5% decompleted fetal calf serum 1 hr before transfection. Cells were cotransfected with 800 ng of the pEZX-T or pEZX-C vector and 200 ng of miR-485-5p precursor or scramble nontargeting control (miR-CTRL) (pEZX-MR04 vectors, GeneCopoeia and Labomics). For anti-miR experiments (B), 24 hr before transfections with pEZX-C, HEK293T cells were pretransfected with anti-485-5p or anti-neg (mirVana, Life Technologies; final concentration 30 nM). All transient transfections were performed according to the manufacturer's protocol with the use of ExGen500 transfection reagent (EUROMEDEX) and 5 μl of ExGen500 for 1 μg of DNA. Luciferase activity was measured 48 hr after transfection on cell lysate with the Dual-Glo Luciferase Reporter Assay System (Promega) with a GLOMAX20/20 luminometer (Promega). *Renilla* luciferase activity was used as an internal control for normalizing to the corresponding firefly luciferase activity. All transfection experiments were performed in triplicate and repeated three times. Results are expressed as luciferase activity relative to that of control samples (pEZX-C vector cotransfected with miR-CTRL and anti-neg for miR inhibitor experiments in B). Data represent the mean ± SEM. p values were determined by a Student's paired t test (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). NS, nonsignificant.

level. Similar *APOA5* mRNA levels were found in mice overexpressing human *APOA5*, either wild-type or with APOA5*2.[25] Moreover, *APOA5* mRNA levels were similar in human hepatic biopsies in subjects with either wild-type *APOA5* or APOA5*2.[20] However, the underlying mechanisms involved in APOA5*2 dysfunction remain unknown, e.g., Palmen et al. did not confirm in vitro the hypothesis that within APOA5*2, the c.−3A>G Kozak sequence polymorphism could alter *APOA5* mRNA translation.[24] Consequently, we investigated the potential implication of miRNAs in the posttranscriptional regulation of mutant *APOA5*.

miRNAs are evolutionally conserved 19–22 nucleotides of noncoding RNA that posttranscriptionally downregulate gene expression by binding target mRNAs. This process leads to mRNA degradation or translation repression. Recent data have suggested that miRNAs predominantly decrease mRNA stability through base pairing with the 3′ UTR of target mRNAs. The recognition of target mRNA by miRNA involves a small complementary sequence from 2 to 7 nucleotides long.[26,27] Sequence alteration by SNPs can either generate or destroy miRNA binding sites in mRNAs.[28] Such a finding was reported for obesity-associated c.*2270A>G (rs8887; RefSeq NM_001080400), which creates an illegitimate miR-522 binding site in the 3′ UTR of perilipin 4 (*PLIN4* [MIM 613247]) and promotes its downregulation in adipose tissue.[29]

Within APOA5*2, c.*158C>T is the only SNP located in the 3′ UTR. We hypothesized that the *APOA5* 3′ UTR c.*158C>T rare variant might create an illegitimate binding site for miRNAs and thus result in *APOA5* posttranscriptional inhibition. This could lead to downregulation of lipolysis and a subsequent increase in plasma TG levels.

Using five distinct software tools for miRNA target prediction, we performed bioinformatic studies to determine whether c.*158C>T affects the binding of specific miRNAs (Table S1, available online). First, we used PITA[30] and RegRNA[31] to investigate the potential effect of the c.*158C allele on miRNA target sites and how it compares to that of the c.*158T allele. The miRNA-mRNA binding scores were stronger in the presence of the C allele than in the presence of the T allele for three miRNAs: miR-485-5p (MIRN485-5p) and miR-1255a (MIRN1255a) were predicted by the two programs, whereas miR-3188 (MIRN3188) was only predicted by RegRNA (Table S2). Second, we utilized three additional programs (microRNA.org,[32] TargetScan,[33] and Diana-microT[34]) to confirm the potential binding of

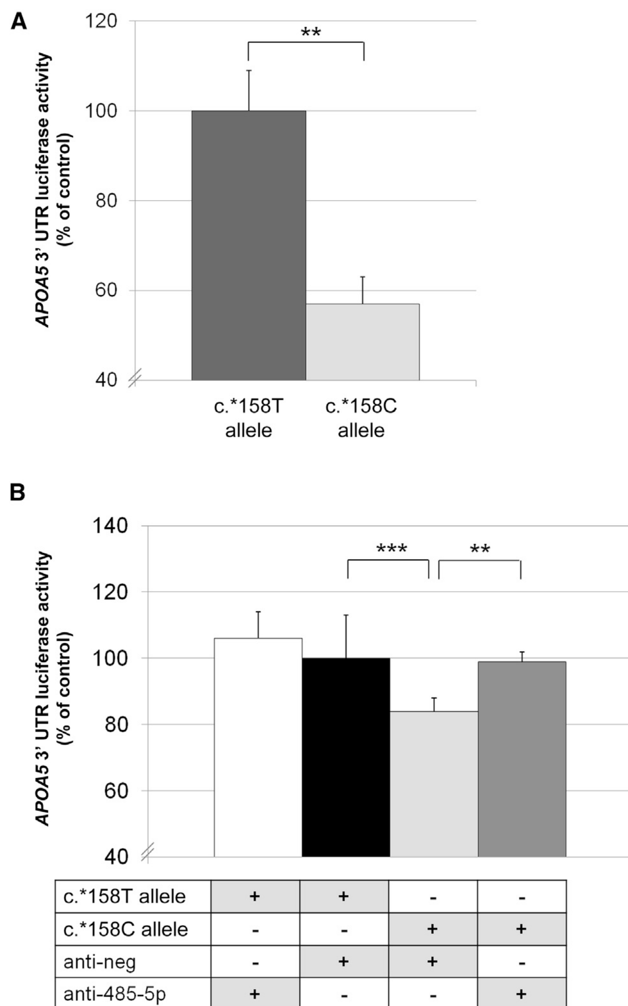

Figure 2. Endogenous miR-485-5p Downregulates Luciferase Activity of the *APOA5* 3′ UTR with the c.*158C Allele in HuH-7 Cells

(A) Luciferase activity of c.*158C (pEZX-C) is expressed as relative to that of c.*158T (pEZX-T).

(B) Luciferase activity of c.*158C (pEZX-C) is expressed as relative to that of c.*158T (pEZX-T) in the presence of either anti-485-5p or anti-neg.

HuH-7 cells (5×10^4 cells/well) were plated onto 12-well plates and transfected with 1 μg of pEZX-T or pEZX-C vector as described for HEK293T cells (Figure 1). Anti-miR experiments were performed 24 hr after incubation with anti-485-5p or anti-neg as previously described (Figure 1). Experiments were performed in triplicate, and data are expressed as luciferase activity relative to that of control samples (pEZX-C vector cotransfected with miR-CTRL and anti-neg). Data represent the mean ± SEM. p values were determined by a Student's paired t test (**$p < 0.01$, ***$p < 0.001$). NS, nonsignificant.

these three miRNAs on the c.*158C allele: miR485-5p binding was confirmed by the three programs, miR3188 binding was confirmed only by microRNA.org, and miR1255a binding was not confirmed (Table S2). Consequently, these results indicate that the c.*158C allele might create a potential illegitimate target site at least for miR-485-5p. Given the discrepancies between the programs, we decided to also consider miR-1255a and miR-3188 for in vitro binding validation.

Luciferase expression vectors containing the C or T allele (pEZX-C or pEZX-T, respectively) were constructed for functional assessment of the binding of the three candidate miRNAs on the mutant *APOA5* 3′ UTR (Figure 1A). Human embryonic kidney 293T (HEK293T) cells (ATCC CRL-11268) were cotransfected with pEZX-C or pEZX-T and with either a miRNA precursor (miR-485-5p, miR-1255a, or miR-3188) or a control mimic (miR-CTRL). Compared to control miRNA, the *APOA5* 3′ UTR containing the c.*158C allele showed a significant, 35% (±6%) decrease in luciferase activity in the presence of miR-485-5p ($p < 0.001$). Meanwhile, the 3′ UTR containing the c.*158T allele showed a nonsignificant increase in the level of luciferase activity in the presence of miR-485-5p in comparison to control miRNA (Figure 1A). However, when the c.*158T or c.*158C allele was expressed in the presence of miR-1255a (Figure S1A) or miR-3188 (Figure S1B), there was no significant difference in comparison to expression with the miR-CTRL. The specific effect of miR-485-5p on the luciferase activity of the *APOA5* 3′ UTR c.*158C allele was assessed with a mir-485-5p inhibitor (anti-485-5p) or a negative control inhibitor (anti-neg). The anti-485-5p fully reversed the decrease in luciferase activity of the *APOA5* 3′ UTR c.*158C allele, whereas in the same conditions, the anti-neg had no significant effect (Figure 1B).

These results confirm that in vitro miR-485-5p is capable of targeting the *APOA5* 3′ UTR c.*158C sequence and of partially repressing its luciferase activity.

Because *APOA5* is exclusively expressed in the liver, only liver-expressed miRNAs would potentially regulate *APOA5* expression. We sought to determine whether miR-485-5p was expressed in the liver by using quantitative RT-PCR on RNA extracted from human hepatic cells (HepG2 [ATCC HB-8065] and HuH-7 [human hepatoma cell line, JCRB-0403]) and mouse and human liver tissue (generous gift from the pathology laboratory of Centre Hospitalier Lyon Sud). miR-485-5p was expressed in both human and mouse hepatic tissue and in all hepatic cell lines tested and was most highly expressed in human hepatic tissue (Figure S2).

To confirm our results obtained with HEK293T cells, we investigated endogenous miR-485-5p functionality in human hepatic cell line HuH-7, expressing miR-485-5p. HuH-7 cells were transfected with the luciferase expression vectors pEZX-C or pEZX-T. We confirmed the specific effect of endogenous miR-485-5p by using anti-485-5p or anti-neg cotransfected with pEZX-C or pEZX-T. We observed that the *APOA5* 3′ UTR c.*158C allele showed significantly less luciferase activity than did the c.*158T allele (−43 ± 16%, $p < 0.01$) (Figure 2A). This decrease was completely reversed in the presence of anti-485-5p, but not in the presence of anti-neg (Figure 2B). These results demonstrate that endogenous hepatic miR-485-5p is able to target the mutant *APOA5* 3′ UTR with the c.*158C allele and decrease *APOA5* 3′ UTR luciferase expression.

Our in silico studies primarily identified the *APOA5* 3′ UTR with the c.*158C rare allele as a potential

miR-485-5p target site. Our in vitro studies subsequently established that the *APOA5* c.*158C minor allele creates an illegitimate and functional miR-485-5p binding site. In the human liver, miR-485-5p might therefore downregulate mutant *APOA5* at the posttranscriptional level, which could explain the strong GWAS-confirmed association between hypertriglyceridemia and APOA5*2, which bears c.*158C>T.[3,11–15,17–19]

Clinical studies have suggested a decreased apoAV concentration in subjects with APOA5*2.[20–24] An association between c.*158C>T genotypes and plasma apoAV levels has not been specifically reported. Nevertheless, because this SNP is in complete linkage disequilibrium with g.4430C>T (rs662799, g.−1131T>C) on APOA5*2,[3,35] data can be extrapolated from g.4430C>T studies. Subjects with the g.4430C rare allele (T/C + C/C genotypes) were found to have a substantially significant 23%–27% decrease in plasma apoAV concentration in comparison to subjects with the g.4430 T/T genotype.[20,25] In a recent large study, Kim et al. provided support for the relevance of plasma apoAV levels in plasma TG concentrations. This study clearly showed a close inverse association between apoAV and TGs in a cohort of 754 hypertriglyceridemic individuals (TGs > 150 mg/dl). Compared to the individuals with the g.4430 T/T major genotype, individuals with T/C and C/C genotypes displayed a 11% and 19% apoAV decrease, respectively, associated with a 9% and 18% TG increase, respectively.[36]

Several studies have suggested that APOA5*2 might modulate *APOA5* expression at the posttranscriptional level.[20,24] Nevertheless, functional analysis of APOA5*2 polymorphisms have hitherto failed to identify the mechanisms involved in the regulation of *APOA5* expression.[16,24,25] Our results support a miRNA posttranscriptional regulation of APOA5*2. Consistent with our study, Palmen et al. performed a functional analysis of APOA5*2 SNPs by using a luciferase reporter construct including part of APOA5*2: the g.4430C>T, c.−3A>G, and c.*158C>T rare variants. Palmen et al. reported approximately the same decrease in luciferase expression in HuH-7 cells as in our study. Although miRNA regulation was not suspected at that time, their findings are consistent with a targeting of APOA5*2 by endogenous miR-485-5p expressed in this cell type. Additionally, Palmen et al. showed an equally reduced luciferase expression with the construct including only one *APOA5* variant: the c.*158C allele.[24] As in our results, these previous findings demonstrate that the c.*158C>T rare variant alone provides luciferase modulation in this model.

Additionally, the data presented in this study are in line with several previous studies that demonstrated that SNPs located either in miRNA genes or in mRNAs can affect miRNA-mRNA recognition and either abrogate or create miRNA binding sites.[28] This could account for diseases or phenotypic traits in various cellular pathways.[29,37] In plasma TG metabolism, the miR-410 seed site disruption induced by *LPL* 3′ UTR SNP c.*1671T>C (rs13702; RefSeq NM_000237.2) was recently associated with a significant decrease in plasma TG concentration.[38]

Because *APOA5* is exclusively expressed in the liver, we crucially provided evidence of miR-485-5p expression in human liver tissue. This is in accordance with the discovery of miR-485-5p in human fetal liver[39] and its recently documented hepatic expression.[40] It is encoded in a miRNA cluster within an intergenic region located in chromosome 14 and is involved in neurologic pathways or diseases such as synaptic plasticity, Alzheimer disease,[41] and ependymomas.[42] It has also been shown to be downregulated in ovarian epithelial tumors.[43] miR-485-5p was not reported to affect lipid metabolism before our study. However, because *APOA5* c.*158C>T regulation would appear through the creation of an illegitimate target site, it is not obvious that miR-485-5p might physiologically regulate TG metabolism.

Therefore, our work provides a comprehensive mechanism for the APOA5*2 hypertriglyceridemic effect. We propose that, in humans, the miR-binding site created by the c.*158C rare allele in the *APOA5* 3′ UTR causes liver posttranscriptional downregulation of *APOA5* by miR-485-5p, a miRNA expressed in the human liver. This downregulation might at least partially account for the reported expression of APOA5*2, its effect of rising plasma TG concentrations in humans, and the strong association between APOA5*2 and both mild and severe hypertriglyceridemia. It remains to be clarified whether additional interactions with other APOA5*2 SNPs are also required.

Supplemental Data

Supplemental Data include two figures and two tables and can be found with this article online at http://www.cell.com/AJHG.

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Web Resources

The URLs for data presented herein are as follows:

DIANA microT v.4, http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microtv4/index
microRNA.org, http://www.microrna.org
Online Mendelian Inheritance in Man (OMIM), http://omim.org
PITA, http://genie.weizmann.ac.il/pubs/mir07/mir07_prediction.html

RegRNA, http://regrna.mbc.nctu.edu.tw/
TargetScan 6.2, http://www.targetscan.org/

References

1. Marçais, C., Bernard, S., Merlin, M., Ulhmann, M., Mestre, B., Rochet-Mingret, L., Revol, A., Berthezene, F., and Moulin, P. (2000). Severe hypertriglyceridaemia in Type II diabetes: involvement of apoC-III Sst-I polymorphism, LPL mutations and apo E3 deficiency. Diabetologia *43*, 1346–1352.
2. Johansen, C.T., Kathiresan, S., and Hegele, R.A. (2011). Genetic determinants of plasma triglycerides. J. Lipid Res. *52*, 189–206.
3. Pennacchio, L.A., Olivier, M., Hubacek, J.A., Cohen, J.C., Cox, D.R., Fruchart, J.C., Krauss, R.M., and Rubin, E.M. (2001). An apolipoprotein influencing triglycerides in humans and mice revealed by comparative sequencing. Science *294*, 169–173.
4. van der Vliet, H.N., Schaap, F.G., Levels, J.H., Ottenhoff, R., Looije, N., Wesseling, J.G., Groen, A.K., and Chamuleau, R.A. (2002). Adenoviral overexpression of apolipoprotein A-V reduces serum levels of triglycerides and cholesterol in mice. Biochem. Biophys. Res. Commun. *295*, 1156–1159.
5. Fruchart-Najib, J., Baugé, E., Niculescu, L.S., Pham, T., Thomas, B., Rommens, C., Majd, Z., Brewer, B., Pennacchio, L.A., and Fruchart, J.C. (2004). Mechanism of triglyceride lowering in mice expressing human apolipoprotein A5. Biochem. Biophys. Res. Commun. *319*, 397–404.
6. Merkel, M., Loeffler, B., Kluger, M., Fabig, N., Geppert, G., Pennacchio, L.A., Laatsch, A., and Heeren, J. (2005). Apolipoprotein AV accelerates plasma hydrolysis of triglyceride-rich lipoproteins by interaction with proteoglycan-bound lipoprotein lipase. J. Biol. Chem. *280*, 21553–21560.
7. Grosskopf, I., Baroukh, N., Lee, S.J., Kamari, Y., Harats, D., Rubin, E.M., Pennacchio, L.A., and Cooper, A.D. (2005). Apolipoprotein A-V deficiency results in marked hypertriglyceridemia attributable to decreased lipolysis of triglyceride-rich lipoproteins and removal of their remnants. Arterioscler. Thromb. Vasc. Biol. *25*, 2573–2579.
8. Priore Oliva, C., Pisciotta, L., Li Volti, G., Sambataro, M.P., Cantafora, A., Bellocchio, A., Catapano, A., Tarugi, P., Bertolini, S., and Calandra, S. (2005). Inherited apolipoprotein A-V deficiency in severe hypertriglyceridemia. Arterioscler. Thromb. Vasc. Biol. *25*, 411–417.
9. Marçais, C., Verges, B., Charrière, S., Pruneta, V., Merlin, M., Billon, S., Perrot, L., Drai, J., Sassolas, A., Pennacchio, L.A., et al. (2005). Apoa5 Q139X truncation predisposes to late-onset hyperchylomicronemia due to lipoprotein lipase impairment. J. Clin. Invest. *115*, 2862–2869.
10. Charrière, S., Cugnet, C., Guitard, M., Bernard, S., Groisne, L., Charcosset, M., Pruneta-Deloche, V., Merlin, M., Billon, S., Delay, M., et al. (2009). Modulation of phenotypic expression of APOA5 Q97X and L242P mutations. Atherosclerosis *207*, 150–156.
11. Pennacchio, L.A., Olivier, M., Hubacek, J.A., Krauss, R.M., Rubin, E.M., and Cohen, J.C. (2002). Two independent apolipoprotein A5 haplotypes influence human plasma triglyceride levels. Hum. Mol. Genet. *11*, 3031–3038.
12. Kraja, A.T., Vaidya, D., Pankow, J.S., Goodarzi, M.O., Assimes, T.L., Kullo, I.J., Sovio, U., Mathias, R.A., Sun, Y.V., Franceschini, N., et al. (2011). A bivariate genome-wide approach to metabolic syndrome: STAMPEED consortium. Diabetes *60*, 1329–1339.
13. Tan, A., Sun, J., Xia, N., Qin, X., Hu, Y., Zhang, S., Tao, S., Gao, Y., Yang, X., Zhang, H., et al. (2012). A genome-wide association and gene-environment interaction study for serum triglycerides levels in a healthy Chinese male population. Hum. Mol. Genet. *21*, 1658–1664.
14. Wang, J., Ban, M.R., Kennedy, B.A., Anand, S., Yusuf, S., Huff, M.W., Pollex, R.L., and Hegele, R.A. (2008). APOA5 genetic variants are markers for classic hyperlipoproteinemia phenotypes and hypertriglyceridemia. Nat. Clin. Pract. Cardiovasc. Med. *5*, 730–737.
15. Charriere, S., Bernard, S., Aqallal, M., Merlin, M., Billon, S., Perrot, L., Le Coquil, E., Sassolas, A., Moulin, P., and Marcais, C. (2008). Association of APOA5 -1131T>C and S19W gene polymorphisms with both mild hypertriglyceridemia and hyperchylomicronemia in type 2 diabetic patients. Clin. Chim. Acta *394*, 99–103.
16. Talmud, P.J., Palmen, J., Putt, W., Lins, L., and Humphries, S.E. (2005). Determination of the functionality of common APOA5 polymorphisms. J. Biol. Chem. *280*, 28215–28220.
17. Comuzzie, A.G., Cole, S.A., Laston, S.L., Voruganti, V.S., Haack, K., Gibbs, R.A., and Butte, N.F. (2012). Novel genetic loci identified for the pathophysiology of childhood obesity in the Hispanic population. PLoS ONE *7*, e51954.
18. Kettunen, J., Tukiainen, T., Sarin, A.P., Ortega-Alonso, A., Tikkanen, E., Lyytikäinen, L.P., Kangas, A.J., Soininen, P., Würtz, P., Silander, K., et al. (2012). Genome-wide association study identifies multiple loci influencing human serum metabolite levels. Nat. Genet. *44*, 269–276.
19. Willer, C.J., Sanna, S., Jackson, A.U., Scuteri, A., Bonnycastle, L.L., Clarke, R., Heath, S.C., Timpson, N.J., Najjar, S.S., Stringham, H.M., et al. (2008). Newly identified loci that influence lipid concentrations and risk of coronary artery disease. Nat. Genet. *40*, 161–169.
20. Hahne, P., Krempler, F., Schaap, F.G., Soyal, S.M., Höffinger, H., Miller, K., Oberkofler, H., Strobl, W., and Patsch, W. (2008). Determinants of plasma apolipoprotein A-V and APOA5 gene transcripts in humans. J. Intern. Med. *264*, 452–462.
21. Ishihara, M., Kujiraoka, T., Iwasaki, T., Nagano, M., Takano, M., Ishii, J., Tsuji, M., Ide, H., Miller, I.P., Miller, N.E., and Hattori, H. (2005). A sandwich enzyme-linked immunosorbent assay for human plasma apolipoprotein A-V concentration. J. Lipid Res. *46*, 2015–2022.
22. Dallinga-Thie, G.M., van Tol, A., Hattori, H., van Vark-van der Zee, L.C., Jansen, H., and Sijbrands, E.J.; DALI study group (2006). Plasma apolipoprotein A5 and triglycerides in type 2 diabetes. Diabetologia *49*, 1505–1511.
23. Vaessen, S.F., Schaap, F.G., Kuivenhoven, J.A., Groen, A.K., Hutten, B.A., Boekholdt, S.M., Hattori, H., Sandhu, M.S., Bingham, S.A., Luben, R., et al. (2006). Apolipoprotein A-V, triglycerides and risk of coronary artery disease: the prospective Epic-Norfolk Population Study. J. Lipid Res. *47*, 2064–2070.
24. Palmen, J., Smith, A.J., Dorfmeister, B., Putt, W., Humphries, S.E., and Talmud, P.J. (2008). The functional interaction on in vitro gene expression of APOA5 SNPs, defining haplotype APOA52, and their paradoxical association with plasma triglyceride but not plasma apoAV levels. Biochim. Biophys. Acta *1782*, 447–452.
25. Ahituv, N., Akiyama, J., Chapman-Helleboid, A., Fruchart, J., and Pennacchio, L.A. (2007). In vivo characterization of human APOA5 haplotypes. Genomics *90*, 674–679.

26. Fabian, M.R., and Sonenberg, N. (2012). The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. Nat. Struct. Mol. Biol. *19*, 586–593.
27. Bartel, D.P. (2009). MicroRNAs: target recognition and regulatory functions. Cell *136*, 215–233.
28. Gong, J., Tong, Y., Zhang, H.M., Wang, K., Hu, T., Shan, G., Sun, J., and Guo, A.Y. (2012). Genome-wide identification of SNPs in microRNA genes and the SNP effects on microRNA target binding and biogenesis. Hum. Mutat. *33*, 254–263.
29. Richardson, K., Louie-Gao, Q., Arnett, D.K., Parnell, L.D., Lai, C.Q., Davalos, A., Fox, C.S., Demissie, S., Cupples, L.A., Fernandez-Hernando, C., and Ordovas, J.M. (2011). The PLIN4 variant rs8887 modulates obesity related phenotypes in humans through creation of a novel miR-522 seed site. PLoS ONE *6*, e17944.
30. Kertesz, M., Iovino, N., Unnerstall, U., Gaul, U., and Segal, E. (2007). The role of site accessibility in microRNA target recognition. Nat. Genet. *39*, 1278–1284.
31. Huang, H.Y., Chien, C.H., Jen, K.H., and Huang, H.D. (2006). RegRNA: an integrated web server for identifying regulatory RNA motifs and elements. Nucleic Acids Res. *34* (Web Server issue), W429–W434.
32. Betel, D., Wilson, M., Gabow, A., Marks, D.S., and Sander, C. (2008). The microRNA.org resource: targets and expression. Nucleic Acids Res. *36* (Database issue), D149–D153.
33. Grimson, A., Farh, K.K., Johnston, W.K., Garrett-Engele, P., Lim, L.P., and Bartel, D.P. (2007). MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol. Cell *27*, 91–105.
34. Maragkakis, M., Vergoulis, T., Alexiou, P., Reczko, M., Plomaritou, K., Gousis, M., Kourtis, K., Koziris, N., Dalamagas, T., and Hatzigeorgiou, A.G. (2011). DIANA-microT Web server upgrade supports Fly and Worm miRNA target prediction and bibliographic miRNA to disease association. Nucleic Acids Res. *39* (Web Server issue), W145–W148.
35. Olivier, M., Wang, X., Cole, R., Gau, B., Kim, J., Rubin, E.M., and Pennacchio, L.A. (2004). Haplotype analysis of the apolipoprotein gene cluster on human chromosome 11. Genomics *83*, 912–923.
36. Kim, J.Y., Kim, O.Y., Paik, J.K., Lee, S.H., and Lee, J.H. (2013). Association of apolipoprotein A-V concentration with apolipoprotein A5 gene -1131T>C polymorphism and fasting triglyceride levels. J. Clin. Lipidol. *7*, 94–101.
37. Wynendaele, J., Böhnke, A., Leucci, E., Nielsen, S.J., Lambertz, I., Hammer, S., Sbrzesny, N., Kubitza, D., Wolf, A., Gradhand, E., et al. (2010). An illegitimate microRNA target site within the 3′ UTR of MDM4 affects ovarian cancer progression and chemosensitivity. Cancer Res. *70*, 9641–9649.
38. Richardson, K., Nettleton, J.A., Rotllan, N., Tanaka, T., Smith, C.E., Lai, C.Q., Parnell, L.D., Lee, Y.C., Lahti, J., Lemaitre, R.N., et al. (2013). Gain-of-function lipoprotein lipase variant rs13702 modulates lipid traits through disruption of a microRNA-410 seed site. Am. J. Hum. Genet. *92*, 5–14.
39. Fu, H., Tie, Y., Xu, C., Zhang, Z., Zhu, J., Shi, Y., Jiang, H., Sun, Z., and Zheng, X. (2005). Identification of human fetal liver miRNAs by a novel method. FEBS Lett. *579*, 3849–3854.
40. Liang, Y., Ridzon, D., Wong, L., and Chen, C. (2007). Characterization of microRNA expression profiles in normal human tissues. BMC Genomics *8*, 166.
41. Cohen, J.E., Lee, P.R., Chen, S., Li, W., and Fields, R.D. (2011). MicroRNA regulation of homeostatic synaptic plasticity. Proc. Natl. Acad. Sci. USA *108*, 11650–11655.
42. Costa, F.F., Bischof, J.M., Vanin, E.F., Lulla, R.R., Wang, M., Sredni, S.T., Rajaram, V., Bonaldo, Mde.F., Wang, D., Goldman, S., et al. (2011). Identification of microRNAs as potential prognostic markers in ependymoma. PLoS ONE *6*, e25114.
43. Kim, T.H., Kim, Y.K., Kwon, Y., Heo, J.H., Kang, H., Kim, G., and An, H.J. (2010). Deregulation of miR-519a, 153, and 485-5p and its clinicopathological relevance in ovarian epithelial tumours. Histopathology *57*, 734–743.