DOI 10.1515/cclm-2014-0144 — Clin Chem Lab Med 2014; 52(8): e175–e178

Letter to the Editor

Anna Ruotolo, Maria Donata Di Taranto, Maria Nicoletta D'Agostino, Gennaro Marotta, Marco Gentile, Maria Nunziata, Marta Sodano, Rosa Di Noto, Luigi Del Vecchio, Paolo Rubba and Giuliana Fortunato*

The novel variant p.Ser465Leu in the *PCSK9* gene does not account for the decreased LDLR activity in members of a FH family

Keywords: apolipoprotein B (*APOB*); familial hypercholesterolemia; low-density lipoprotein receptor (LDLR) activity; proprotein convertase subtilisin/kexin type 9 (*PCSK9*).

***Corresponding author: Giuliana Fortunato,** Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, via S. Pansini 5, 80131 Napoli; and CEINGE Biotecnologie Avanzate s.c. a r.l., via Gaetano Salvatore 486, 80145 Napoli, Italy, Phone: +39 0817464200, Fax: +39 0817462404, E-mail: fortunat@unina.it
Anna Ruotolo, Maria Nicoletta D'Agostino, Rosa Di Noto and Luigi Del Vecchio: Dipartimento di Medicina Molecolare e Biotecnologie Mediche, Università degli Studi di Napoli Federico II, Naples, Italy; and CEINGE Biotecnologie Avanzate s.c. a r.l., Naples, Italy
Maria Donata Di Taranto: IRCCS Fondazione SDN, Naples, Italy
Gennaro Marotta, Marco Gentile, Maria Nunziata, Marta Sodano and Paolo Rubba: Dipartimento di Medicina Clinica e Chirurgia, Università degli Studi di Napoli Federico II, Naples, Italy

To the Editor,

Familial hypercholesterolemia (FH) is a genetically heterogeneous lipid disorder with a frequency of 1:300/1:500 for heterozygotes in many populations [1]. The pathogenesis of FH is caused by a dysfunctional lipid metabolism leading to high concentrations of total and low-density lipoprotein (LDL) cholesterol, however, a great phenotypic variability is observed.

Mutations in the LDL receptor gene (*LDLR*) are the main cause of FH, whereas the apolipoprotein B (*APOB*) and proprotein convertase subtilisin/kexin type 9 (*PCSK9*) genes are involved in a lower percentage of cases [2]. *PCSK9* regulates the *LDLR* expression inducing its lysosomal degradation [3]. Some variants in *PCSK9* increase its capacity to promote LDLR degradation [gain of function mutations (GOF)] leading to FH, whereas other mutations cause a decreased PCSK9 activity [loss of function (LOF)] [3]. The molecular diagnosis of FH implies that a new mutation should be deeply studied and accompanied by a functional characterization before one can claim its pathogenicity. Herein, we present the rare case of a family with a suspect of FH in which two members carried a new variant in the *PCSK9* gene and two members carried a known variant in the *APOB* gene.

The proband (I.2; Figure 1) is a 68-year-old woman with hypercholesterolemia, hypertriglyceridemia as well as a clinical history of hypertension, hyperglycemia, carotid and peripheral atherosclerosis referred to the Dipartimento di Medicina Clinica e Chirurgia, Università degli Studi di Napoli Federico II. The study was performed according to the current version of the Helsinki Declaration. Informed consent was obtained for each patient or control. The proband's father died of a heart attack at the age of 64 and her mother has 7.8 mmol/L of total cholesterol. The patient reported that she suspended previous therapies with atorvastatin or rosuvastatin due to the appearance of myalgia. Laboratory analyses in absence of therapy revealed an altered lipid profile as shown in Table 1. The proband (I.2) showed elevated serum levels of total cholesterol, LDL-cholesterol, triglycerides as well as increased apoB levels and LDL score. No sign of corneal arcus or tendinous xanthomata was found, leading to a diagnosis of possible FH according to the Simon Broome criteria [5]. Sequencing and MLPA analysis of the *LDLR* gene performed as previously described [6] did not reveal any mutation or large rearrangements in the proband. Sequencing of the 12 exons with flanking intron sequences of the *PCSK9* gene showed that the proband (I.2) (Figure 1) was heterozygous for the new variant c.1394C>T in exon 9, corresponding to the amino acid substitution p.Ser465Leu.

We verified the absence of the variant in 150 chromosomes from normocholesterolemic individuals. To evaluate the segregation of the new variant with the

Table 1 Serum lipid profile of the patients.

	I.1[a]	Proband (I.2)[a]	II.1[a]	II.2[a]	Proband (I.2)[b]	II.1[c]	II.2[c]
Total cholesterol, mmol/L	4.2	11.6	6.5	6.2	4.8	5.7	5.9
LDL-cholesterol, mmol/L	2.5	8.4	4.3	4.6	2.3	3.7	4.1
HDL-cholesterol, mmol/L	1.1	1.5	1.4	1.4	1.3	1.5	1.5
Triglycerides, mmol/L	1.2	3.7	1.6	0.6	2.6	1.1	0.5
ApoB, g/L	0.81	1.97	not available	not available	0.9	1.00	1.00
LDL score (sdLDL[d]/LDL)	not available	37.1%	not available	not available	not available	4.2%	3.9%

[a]Values in absence of therapy; [b]Values after 3 years of therapy with simvastatin plus ezetimibe; [c]Values during a low fat diet; [d]sdLDL, small dense LDL; percentage of small dense LDL/total LDL were determined as described in Gentile et al. [4].

hypercholesterolemic phenotype, the mutation was searched in the proband's daughters (II.1 and II.2; Figure 1) aged 43 and 36 who showed elevated total cholesterol and LDL-cholesterol levels only partially corrected by a low fat diet (Table 1) without corneal arcus or tendinous xanthomata. The variant p.Ser465Leu was also identified in one of the proband's daughters (II.2).

To predict the effects of the substitutions we used four different in silico algorithms suggesting that the p.Ser465Leu variant could affect the functionality of the protein: Polyphen (http://genetics.bwh.harvard.edu/pph2) returns the result Probably damaging (score 1.00 at Hum Div and 0.045 at HumVar); SIFT (http://sift.jcvi.org): Not tolerated (score 0.02); Mutation taster (http://www.mutationtaster.org): Disease causing (score 3.95); PMut (http://mmb.pcb.ub.es/): Pathological. BLASTP analysis confirmed that the amino acid residue involved in the substitution is conserved in 15/16 species. These algorithms cannot predict the GOF or LOF effect and it has been reported that predictions of GOF mutations should be interpreted with caution [7]. To assess the effects of the variant, two assays were performed to quantify the residual LDLR activity and the LDLR amount on the surface of patient's cells as an indirect evaluation of the *PCSK9* effect. In fact, PCSK9 is secreted into the plasma and binds directly to LDLR on the cell surface; then, following endocytosis, it triggers LDLR degradation [3].

Peripheral blood mononuclear cells were isolated from patients and controls and incubated for 48 h in a medium with lipoprotein deficient serum (in order to upregulate LDLR) supplemented with ionomycin plus PMA to stimulate T-lymphocytes. Residual activity of LDLR was evaluated by measuring the binding and uptake of a fluorescently labeled LDL (1,19-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate-conjugated LDL, Life Technology, Foster City, CA, USA), namely DiI-LDL, incubated with the cells for 3 h at 37 °C as previously described [8]. Amount of LDLR protein on the cell surface was evaluated by measuring the binding of an anti-LDLR antibody (Progen Biotechnik, Heidelberg, Germany) on stimulated T-lymphocytes. Cells were collected and incubated at 4 °C for 1 h with the primary antibody diluted 1:20 followed by an incubation at 4 °C for 45 min with the secondary FITC conjugated anti-rabbit antibody (BD Biosciences San Jose, CA, USA) diluted 1:200. Fluorescence intensities were measured with the FACSCanto (Becton-Dickinson,

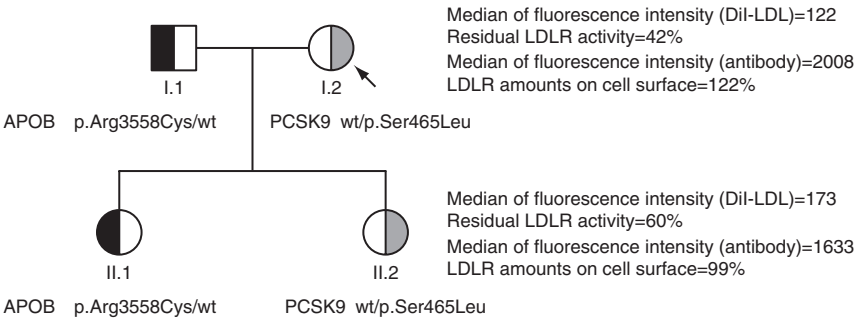

Figure 1 Pedigree of the FH family.
The proband is indicated by an arrow. White and gray symbols indicate heterozygosity for *PCSK9* mutation p.Ser465Leu. Heterozygosity for p.Arg3558Cys mutation in *APOB* is indicated by symbols in black and white. Residual LDLR activities were calculated based on a mean of median fluorescence intensities of the cells from two controls equal to 287; the LDLR amounts were calculated based on a mean of median fluorescence intensities of the cells from two controls obtained with anti-LDLR antibody equal to 1643.

Franklin Lakes, NJ, USA) flow cytometer. LDLR activity or amount of LDLR protein on the cell surface was calculated as the ratio between the median fluorescence intensity of the patients' cells and the mean of median fluorescence intensities of the cells from two controls.

LDLR residual activity in the proband (I.2) and her daughter (II.2) who both bore the c.1394C>T variant, was 42% and 60%, whereas the amounts of LDLR protein on the cell surface were 122% and 99%, respectively (Figure 1).

The decreased LDLR activity observed in both the proband and her daughter (42% and 60%, respectively) together with a small increase or normal LDLR amounts could indicate the dysfunction in LDLR endocytosis as the cause of increased levels of LDL cholesterol in both the mother and the daughter. Our results do not support a pathological role of the new *PCSK9* variant, although it cannot be ruled out because a defect in endocytic pathway could mask its effect, since endocytosis is required for its action as recently described [9]. In addition, other mechanisms of PCSK9 action unrelated to LDR can be responsible for increased cholesterol levels [10].

Furthermore, since the proband's daughter II.1 showed hypercholesterolemia without mutations in *PCSK9*, the genetic screening was extended to the *LDLR* and to all the 29 exons with intron-exon boundaries of *APOB* gene. A mutation in exon 26 of the *APOB* gene (c.10672C>T/p.Arg-3558Cys) was identified in this patient and successively in her father (I.1) who showed a normolipidemic profile.

The p.Arg3558Cys mutation in the *APOB* gene was previously described as pathogenic for FH and associated with a decreased receptor binding affinity of LDL ranging from 30% to 70% and with a high variability of total cholesterol levels ranging from 4.2 mmol/L to 10.9 mmol/L [11]. The above findings are supported by our observations, since p.Arg3558Cys mutation is associated with a normal lipid profile in the patient I.1, but at the same time it is associated with a mild hyperlipidemic profile in the patient II.1.

In conclusion, the new variant p.Ser465Leu in *PCSK9* is not associated to a decreased LDLR amount on the cell membrane and does not explain the reduced LDL activity which is the cause of the FH phenotype. This case report highlights the importance of a deep study of genetic variants before claiming their pathogenicity.

Acknowledgments: We gratefully acknowledge the contribution of the Biobank of CEINGE. Work supported by grants from Regione Campania (Convenzione CEINGE-Regione Campania, DGRC 1901/2009), from IRCCS Fondazione SDN and from POR Campania FSE 2007-2013, Project CREME.

Conflict of interest statement

Authors' conflict of interest disclosure: The authors stated that there are no conflicts of interest regarding the publication of this article. Research support played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.
Research funding: None declared.
Employment or leadership: None declared.
Honorarium: None declared.

Received February 10, 2014; accepted February 12, 2014; previously published online March 6, 2014

References

1. Hopkins PN, Toth PP, Ballantyne CM, Rader DJ. National lipid association expert panel on familial hypercholesterolemia. Familial hypercholesterolemias: prevalence, genetics, diagnosis and screening recommendations from the National lipid association expert panel on familial hypercholesterolemia. J Clin Lipidol 2011;5:S9–17.
2. Bertolini S, Pisciotta L, Rabacchi C, Cefalù AB, Noto D, Fasano T, et al. Spectrum of mutations and phenotypic expression in patients with autosomal dominant hypercholesterolemia identified in Italy. Atherosclerosis 2013;227:342–8.
3. Tibolla G, Norata GD, Artali R, Meneghetti F, Catapano AL. Proprotein convertase subtilisin/kexin type 9 (PCSK9): from structure function relation to therapeutic inhibition. Nutr Metab Cardiovasc Dis 2011;21:835–43.
4. Gentile M, Panico S, Jossa F, Mattiello A, Ubaldi S, Marotta G, et al. Small dense LDL particles and metabolic syndrome in a sample of middle-aged women. Findings from Progetto Clin Chim Acta 2008;388:179–83.
5. Minhas R, Humphries SE, Qureshi N, Neil HA. Controversies in familial hypercholesterolaemia: recommendations of the NICE guideline development group for the identification and management of familial hypercholesterolaemia. Heart 2009;95:584–7.
6. Romano M, Di Taranto MD, D'Agostino MN, Marotta G, Gentile M, Abate G, et al. Identification and functional characterization of LDLR mutations in familial hypercholesterolemia patients from Southern Italy. Atherosclerosis 2010;210:493–6.
7. Flanagan SE, Patch AM, Ellard S. Using SIFT and PolyPhen to predict loss-of-function and gain-of-function mutations. Genet Test Mol Biomarkers 2010;14:533–7.
8. Romano M, Di Taranto MD, Mirabelli P, D'Agostino MN, Iannuzzi A, Marotta G, et al. An improved method on stimulated

T-lymphocytes to functionally characterize novel and known LDLR mutations. J Lipid Res 2011;52:2095–100.
9. Wang Y, Huang Y, Hobbs HH, Cohen JC. Molecular characterization of proprotein convertase subtilisin/kexin type 9-mediated degradation of the LDLR. J Lipid Res 2012;53:1932–43.
10. Sun XM, Eden ER, Tosi I, Neuwirth CK, Wile D, Naoumova RP, et al. Evidence for effect of mutant PCSK9 on apolipoprotein B secretion as the cause of unusually severe dominant hypercholesterolaemia. Hum Mol Genet 2005;14: 1161–9.
11. Pullinger CR, Gaffney D, Gutierrez MM, Malloy MJ, Schumaker VN, Packard CJ, et al. The apolipoprotein B R3531C mutation: characteristics of 24 subjects from 9 kindreds. J Lipid Res 1999;40:318–27.