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New rare genetic variants of *LMF1* gene identified in hyperchylomicronemic patients

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Abstract

Context: *LMF1* (lipase maturation factor 1) gene encodes for a protein involved in lipoprotein lipase and hepatic lipase maturation. Homozygous mutations in *LMF1* leading to hyperchylomicronemia are rare in the literature and a few additional rare *LMF1* variants have been described with poor functional studies.

Objective: The aim of this study was to assess the frequency of *LMF1* variants in a cohort of 385 hyperchylomicronemic patients without homozygous or compound heterozygous deleterious mutations identified in *LPL, APOA5, APOC2, GPIHBP1* genes, and to determine their functionality.

Methods: *LMF1* coding variants were screened using dHPLC followed by direct sequencing. *In silico* studies were performed with SIFT and PolyPhen-2 softwares, followed by *in vitro* functional studies using HEK-293T cells co-transfected with vectors encoding human *LPL* and *LMF1 c*DNA. LPL activity was measured in cell culture medium after heparin addition using human VLDL-TG as substrate.

Results: 19 nonsynonymous coding *LMF1* variants were identified in 65 patients. 10 variants were newly described in hyperchylomicronemia. *In vitro*, p.Gly172Arg, p.Arg354Trp, p.Arg364Gln, p.Arg537Trp *LMF1* variants decreased LPL activity and p.Trp464Ter variant completely abolished LPL activity. We identified a young girl heterozygote for p.Trp464Ter variant and a homozygote carrier of p.Gly172Arg variant with a near 50 % decreased LPL activity *in vitro* and *in vivo.*

Conclusion: The study confirms the rarity of *LMF1* variants in a large cohort of hyperchylomicronemic patients. *LMF1* variants are likely to be involved in multifactorial hyperchylomicronemia. Partial *LMF1* defects could be associated with intermittent hyperchylomicronemia as described for p.Gly172Arg homozygous and p.Trp464Ter heterozygous carriers.

Précis: In a cohort of 385 unexplained hyperchylomicronemic patients, we identified 19 nonsynonymous coding variants, including 8 new variants. Five variants had functional impact on *in vitro* LPL activity.

1. Introduction

Severe hypertriglyceridemia (HTG), defined by plasma triglycerides (TG) > 10 mmol/L, is more likely to have genetic causes than moderate HTG. Monogenic familial hyperchylomicronemia syndrome (FCS), a rare autosomal recessive disease, is due to homozygous or compound heterozygous loss of function mutations in genes that regulate TG rich-lipoprotein lipolysis such as lipoprotein lipase (*LPL),* apolipoprotein C2 (*APOC2),* apolipoprotein *A5 (APOA5),* glycosylphosphatidylinositol anchored high density lipoprotein binding protein 1 *(GPIHBP1)* genes. Most frequently, hyperchylomicronemic patients exhibit multigenic sporadic severe HTG (multifactorial chylomicronemia, MCM) with a large interplay of life style factors or comorbidities such as metabolic syndrome, obesity, type 2 diabetes and a combination of common small-effect variants and/or rare heterozygous large effect variants in genes involved in the regulation TG metabolism, with incomplete penetrance (1,2). *LMF1* (lipase maturation factor 1) gene encodes for a transmembrane protein located in the endoplasmic reticulum, critical for LPL, hepatic lipase (HL) and endothelial lipase (EL) maturation, by acting as a lipase chaperone (3). Common or rare *LMF1* variants are infrequently reported in the literature. Only 3 nonsense homozygous mutations of *LMF1* gene leading to hyperchylomicronemia have been identified (4-6). A few other rare variants have been published in moderate to severe HTG with no clear evidence for their functionality (7-9).

The aim of the present study was to assess the frequency of *LMF1* variants by systematic sequencing among a large cohort of 385 hyperchylomicronemic patients without homozygous or compound heterozygous deleterious mutations previously identified in *LPL, APOA5, APOC2, GPIHBP1* genes and to determine their functionality.

2. Material and Methods

A. Patients and populations studied

The *LMF1* gene was systematically sequenced in a cohort of 385 unrelated patients with documented episodes of hyperchylomicronemia without homozygous or compound heterozygous mutations previously identified in *LPL, APOC2*, *APOA5* or *GPIHBP1* genes. Hyperchylomicronemia was defined by fasting plasma TG concentration over 15 mmol/L with a TG to total cholesterol ratio (g/L) above 2.5 (10) or plasma fasting TG over 10 mmol/L with a familial history of HTG. The family of the index cases harboring a nonsense *LMF1* rare variant identified in the cohort was also explored. Another cohort of 144 dyslipidemic patients without HTG were also screened for *LMF1* variants and served as control population. Clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki. Written informed consent was obtained from all subjects included in the study and the children's parents, before DNA collection, blood sampling and heparin injection for post-heparin LPL activity analyses.

B. Genomic DNA analysis

Genomic DNA was extracted from EDTA peripheral blood using the FlexiGene® DNA kit (Qiagen, Milan, Italy). The *LMF1* DNA sequences (50 ng) were amplified by PCR with 11 primers pairs detailed in Supplemental Table 1 (Invitrogen Life Technologies, Carlsbad, CA, USA). PCR products were analyzed for sequence variation by denaturing high-performance liquid chromatography (dHPLC) (Transgenomic, Glasgow, United Kingdom) in a 1:1 mixture with control (wild type, WT) amplicons. Variant sequences were confirmed by direct sequencing (3500 Dx Genetic Analyzer, Thermo Fisher Scientific, Waltham, MA, USA). The *GPIHBP1* gene direct sequencing for family A was performed as previously

described (11). The exon 4 of *APOE* gene was PCR amplified and directly sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The rs identification number of each variant identified was checked in the dbSNP database (https://www-ncbi-nlm-nih-gov.gate2.inist.fr/snp/) and Alamut® Visual 2.9 software (*Refseq* accession numbers for *LMF1,* NM_022773.2, NP_073610.2). The allele frequency of *LMF1* variants was compared to two European general populations, 1000 Genomes Europe (http://www.internationalgenome.org/1000-genomes-browsers) and ExAC European non-Finnish (http://exac.broadinstitute.org/). For nonsynonymous missense coding variants, *in silico* analyses were performed using the PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/) (12) and SIFT (http://siftdna.org/www/Extended_SIFT_chr_coords_submit.html) (13) software to predict the possible impact of the observed amino acid substitutions on the structure and function of the human protein.

C. Human post-heparin LPL activity

Post-heparin LPL activity in human plasma was determined as previously described (14), apart from non-esterified fatty acids (NEFA) concentration which was measured on an Architect C16000 analyzer (Abbott Laboratories, Chicago, IL, USA).

D. Functional analysis of *LMF1* variants

1. Human LMF1 and LPL cDNA constructs

Human *LMF1* and *LPL* cDNA WT sequences were inserted into two different pCMV6-XL4 vectors which were used for transfection (Origene, Rockville, MD, USA), creating a pCMV6-XL4-LMF1-WT vector and a pCMV6-XL4-LPL vector. The *LMF1* missense variants predicted to be probably damaging or deleterious *in silico* (p.Ser138Cys,

p.Thr143Met, p.Gly172Arg, p.Gly228Val, p.Arg354Trp, p.Arg364Gln, p.Gly410Arg, p.Arg451Trp, p.Arg461His, p.Trp464Ter, p.Ala469Thr, p.Arg537Trp, p.Pro562Arg), and the two nonsense mutations previously described (p.Tyr439Ter and p.Trp464Ter) were introduced into the pCMV6-XL4-LMF1-WT vector using QuikChange II XL Site-Directed Mutagenesis Kit (Agilent technologies Santa Clara, CA, USA) according to the manufacturer's protocol. All constructs were verified by sequencing.

2. Cell transfections

Human embryonic kidney 293T (HEK-293T) cells (ATCC CRL-11268) were maintained at 37°C in a 5 % CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing glucose 25 mM, glutamine 2mM, penicillin 100 units/ml, streptomycin sulfate 100µg/ml and supplemented with 10% decompleted fetal calf serum. HEK-293T cells were plated into 6-well plates ($6.5x10^5$cells/well) 24 hours before transfection and were maintained in 10% decompleted fetal calf serum 1 hour before transfection. Transient transfections were performed with jetPRIME® transfection reagent (Polyplus transfection™, Illkirch, France) using 2µL of jetPRIME® for 1 µg of DNA according to the manufacturer's protocol. HEK-293T cells were co-transfected with 1.6 µg of pCMV6-XL4-LPL and 5 ng of a pCMV6-XL4-LMF1 (WT or variants). Heparin (10 U/mL) was added 24 hours after transfection, 6 hours before removing the supernatant and cell lysis. At the end of the experiment, media samples were collected; cells were washed with PBS and lysed in TRI Reagent (Roche, Meylan, France). Media samples and lysed cells were stored at -80°C until use. All transfection experiments were performed in triplicate and repeated three times.

3. Real time quantitative PCR analysis

LPL and *LMF1* mRNAs concentrations in the cell lysates were assessed by reverse transcription (RT) followed by real-time quantitative PCR (RT-qPCR). Total RNA was extracted from cell lines with TRI Reagent (Roche, Meylan, France), according to the manufacturer's protocol. RNA concentration was measured with Nanodrop-ND1000 (Labtech, Uckfield, United Kingdom). One µg of RNA was used for RT reaction using the Primescript RT kit (Takara, Dalian, Japan) according to the manufacturer's instructions. Quantitative PCR were performed in duplicate on a Rotor-Gene 6000 (Qiagen, Milan, Italy) using the Absolute qPCR SYBR Green Mix (ABgene, Illkirch, France). Quantitative data were defined by cycle threshold (Ct) normalized on an internal standard hypoxanthine phosphoribosyltransferase 1 (*HPRT)* gene. Details for primers and RT-qPCR conditions for each mRNA are available upon request to the corresponding author.

4. LPL activity assay in cell culture media

LPL activity assay in previously collected and frozen cell culture media, was adapted from the plasma post-heparin LPL activity method described by Di Filippo et al. (14), using human VLDL-TG prepared by ultracentrifugation of heat-inactivated normolipidemic human serums diluted as substrate.

The optimal volume of medium sample (40 µl) was tested for sensitivity and linearity of the LPL activity assay (supplemental figure 1 and 2). A strong correlation between the LPL activity and the volume was found in the same way as the initial plasma assay ($R=0.99$, $p<0.01$) (14) (supplemental figure 1). The LPL activity assay was linear between 2 and 6 hours after heparin addition (supplemental figure 2). The longer incubation time tested (6 hours) was conserved to have enough power to discriminate the effect of *LMF1* variants. Inter-assay reproducibility was verified using an internal control; the variation coefficient was 8 % (mean 22.3 µmol/L/min).

Briefly, 40 µL of sample medium were added to 180 µL of VLDL-TG substrate (final TG concentration 7 +/- 0.3 mmol/L) in 510 µL of buffer A. Upon incubation at 37°C, NEFA concentration was assayed hourly during 4 hours. LPL activity was expressed as µmol/L/min of released NEFA. The LPL activity detectable in culture medium of non-transfected HEK cells was very low, closed to the minimal detectable LPL activity previously established for this assay (1.68 µmol/L/min) (ref plos one), consistent with a very low expression of *LPL* and *LMF1* gene measured in RT-qPCR (supplemental table 2).

Hepatic lipase activity was undetectable in cell media samples. Two *LMF1* mutants p.Tyr439Ter and p.Trp464Ter previously known to affect LMF1 function were used as controls (4,5). Basal LPL activity detected in culture media of cells transfected with human *LPL* and WT *LMF1* (pCMV6-XL4-LPL and LMF1-WT) was used as internal control in each assay. Data were adjusted on the internal control and on transfection efficacy using *LPL* and *LMF1* mRNA quantified by RT-qPCR. Results are presented normalized with respect to WT *LMF1* samples.

E. Statistical analysis

Statistical analyses were performed using SPSS 13.0 software. Data are presented as mean +/- standard error of the mean (SEM). A parametric Student's paired t-test was used for comparison of means following a normal distribution. A two-tailed p value < 0.05 was considered as significant.

3. Results

A. *LMF1* coding variants identification

The 385 hyperchymomicronemic patients explored (free of homozygous or compound heterozygous mutations in *LPL*, *APOC2*, *APOA5* or *GPIHBP1* genes) were middle-aged (mean +/- standard deviation (SD): 43.2 +/-16.2 years old). They were mainly men (70.5 %). 35 subjects (9.1 %) had diagnosis of hyperchylomicronemia before they were 20 years old.

In this cohort, 34 different *LMF1* coding variants were identified: 15 synonymous and 19 nonsynonymous (18 missense variants and one nonsense variant p.Trp464Ter) (Table 1). The complete list of identified variants, including variants in adjacent intronic regions covered by the genetic analyses, is available in Supplemental Table 3.

A total of 65 (16.9 %) patients harbored at least one *LMF1* nonsynonymous coding variants. Fifteen patients were carriers of two *LMF1* coding variants. The p.Arg354Trp variant was systematically associated with the p.Arg364Glu variant supporting a complete linkage disequilibrium between these 2 variants. All nonsynonymous coding variants were found in the cohort with a minor allele frequency (MAF) < 5 %, similar to that found in the general population, except for p.Gly36Asp which appears to be a common polymorphism in the general population. Among the 19 nonsynonymous coding variants, 10 are reported for the first time in hyperchylomicronemic patients and 7 of them had not been previously reported neither in European general populations and were not identified in the control dyslipidemic cohort, except for p.Phe279Leu variant. Only 2 variants, p.Gly36Asp and p.Gly172Arg, were identified at homozygous state in two distinct patients (Table 1).

B. Functional analysis of *LMF1* variants

The 10/18 missense variants, predicted to be "probably damaging" by PolyPhen-2 or "deleterious" by SIFT by *in silico* studies, were selected for functional analyses. The p.Thr143Met variant, predicted to be "possibly damaging", was added since it was associated with p.Pro562Arg in a patient, in order to investigate the role of each variant (Table 1). The two previously described nonsense variant (p.Trp464Ter identified in our cohort, and p.Tyr439Ter) were used as positive controls in the model as both were already shown to severely affect LMF1 function (4,5).

The functionality of *LMF1* variants was assessed using an *in vitro* assay measuring LPL activity in the culture media of HEK-293T cells co-transfected with *LPL* and *LMF1* expression vector (WT or variants). Four newly identified variants (p.Gly172Arg, p.Arg354Trp, p.Arg364Gln, p.Arg537Trp) significantly reduced LPL activity in the culture media by 38 to 79 %, but to a lesser extent than the two previously characterized nonsense mutations (p.Tyr439Ter and p.Trp464Ter (4,5) which abolished LPL activity in our model. Unexpectedly, one variant (p.Thr143Met) substantially increased LPL activity (Figure 1).

C. Case reports

1. p.Trp464Ter heterozygote and family explorations

The p.Trp464Ter mutation heterozygous carrier was found in an 8 months old girl who exhibited severe HTG (21 mmol/L) upon an episode of severe acute gastro-enteritis leading to hospitalization, without acute pancreatitis. Her plasma TG rapidly decreased and then normalized under diet therapy after the gastro-enteritis resolution. Her parents and siblings had normal plasma TG, except her young brother who had a mild non-fasting mixed dyslipidemia at 6 months while he was being breast fed (Figure 2). This p.Trp464Ter mutation has been previously described to be deleterious in a homozygous patient with severe

permanent HTG and acute pancreatitis (5) and was accordingly shown to totally abolish *in vitro* LPL activity in our *in vitro* study. The proband (II-2) inherited the heterozygous p.Trp464Ter from her mother (I-2). The heterozygous p.Arg451Trp *LMF1* rare variant, inherited from her normotriglyceridemic father (I-1), did not alter LPL activity in our model (Figure 1). Her normolipidemic sister (II-1) had the same *LMF1* genotype. The proband (II-2) also inherited from her mother a new heterozygous rare variant of *GPIHBP1* gene (NM_178172.5 (GPIHBP1): c.424C>G)) not previously described in literature neither in dbSNP nor in ExAC databases, which is predicted to be "tolerated" in SIFT and "possibly damaging" in PolyPhen-2 prediction software. The signal peptide p.Cys14Phe common variant of *GPIHBP1* (c.41G>T, rs11538389) was also identified at heterozygous state in the siblings (II-1, II-3) inherited from the mother (I-2). The proband (II-2), her mother (I-2) and her brother (II-3) exhibited the ε3ε4 genotype of *APOE* gene (Figure 2). No additional variants were identified on *LPL, APOA5* or *APOC2* genes in the proband.

*2. p.*Gly172Arg *homozygote*

The homozygous *p.*Gly172Arg *LMF1* variant was found in a 65-years-old man who had several episodes of hyperchylomicronemia, since he was 30 years old. His maximum documented TG was 50 mmol/L, under a hypolipidic diet. He had one episode of acute pancreatitis when he was 35 years old, leading to a partial pancreatectomy after the discovery of a congenital malformation of the bile ducts. He subsequently developed a secondary exocrine pancreatic insufficiency treated by oral exogenous pancreatic lipases. He suffered from type 2 diabetes discovered when he was 45 in a context of obesity (BMI 30 kg/m^2), and was subsequently treated with insulin. He presented multiple cardiovascular complications (coronaropathy, severe peripheral arterial disease). His post-heparin LPL activity was low but not abolished: 13.5 µmol/L/min (Normal range: 22.0-47.6 µmol/L/min) with concomitant TG

7.22 mmol/L and HbA1C 8.4 % under insulin treatment. His brother and sister were also known to have intermittent severe HTG (TG > 10 mmol/L), however they refused to be explored.

4. Discussion

This study identified 19 nonsynonymous *LMF1* coding variants in a large cohort of hyperchylomicronemic patients (without homozygous or compound heterozygous deleterious mutations identified in *LPL, APOA5, APOC2, GPIHBP1* genes): 10 of them were identified for the first time in hyperchylomicronemia. This finding confirms the rarity of *LMF1* variants since 16.9 % of patients harbored missense or nonsense *LMF1* variants, and only 4.7 % were carriers of variants suspected to be deleterious based on their *in vitro* effect on LPL activity. In patients with TG > 10 mmol/L, a close frequency of missense variants of 17.6 % was reported in a smaller population (n=85) (7). In populations including both moderate and severe HTG, the frequency of missense *LMF1* variants was variable, lower (5.1 %) in the cohort reported by De Castro-Oros et al. (118 subjects with TG > 5.6 mmol/L) (9) and higher (28 %) in a larger cohort reported by Johansen et al. (413 subjects with TG > 3.37 mmol/L) (8).

So far, only 3 nonsense *LMF1* mutations were previously found in homozygous patients and considered to be causally involved in hyperchylomicronemia (p.Tyr439Ter, p.Tyr460Ter, p.Trp464Ter) (4-6). Several *LMF1* missenses variants have been identified in moderate or severe hypertriglyceridemic cohorts (7-9) but only one study provided functional data for 7 missenses variants. None of these missenses variant had any significant impact on *in vitro* LPL activity (7).

In the present study, functional data are provided for 12 missense variants. Three of them were already studied by Surendran et al. (p.Arg354Trp, p.Arg364Gln and p.Pro562Arg) (7). Among the 10 new variants, 2 were found deleterious *in vitro* (p.Gly172Arg and p.Arg537Trp). The two variants p.Arg354Trp and p.Arg364Gln, without deleterious effect in Surendran's study (7), were shown to have a detrimental impact on the LPL activity in our study. Moreover, we found a similar defect in LPL activity for both pTyr439Ter and p.Trp464Ter nonsense mutation whereas Cefalu et al. (5) and Surendran et al. (7) reported a milder effect of p.Trp464Ter. As both nonsense *LMF1* mutations are responsible for a protein truncation at 77 and 81 %, a similar defect on LPL activity could be expected. These discrepancies may be explained by the different *in vitro* models used. Surendran et al. tested *LMF1* variants functionality in cld-mutant hepatocytes (*LMF1* deficient mouse cell model) co-expressing *LPL* and *LMF1*. LPL activity was measured using a labeled triolein emulsion as substrate (7), whereas we used HEK-293T human cells and a human substrate (a pool of human VLDL-TG). In addition, the quantity of DNA used for transfection and the incubation time, which were not specified by Surendran et al. (7), may also be different and were shown to have a significant impact on the results (15). The very low LPL activity detectable in culture medium of non-transfected HEK cells is not able to account for these discrepancies since it was at the limit of assay sensitivity and our results were adjusted on the WT LPL activity. Moreover, in agreement with the result of our *in vitro* functional test, the post-heparin LPL activity in the homozygous carrier of the p.Gly172Arg was consistently found reduced by 50%.

Thus, the newly identified homozygous p.Gly172Arg, is likely to explain the phenotype of the carrier since no additional variant was detected in the other main genes involved in hyperchylomicronemia. The causal relationship between the other heterozygous missense variants and the hyperchylomicronemic phenotype could be more questionable. In

another study, we assessed the release of LPL activity in human plasma, 60 minutes after heparin injection (post heparin lipase activity, PHLA60), in addition to the most commonly used 10 minutes time point after heparin injection (PHLA10). In the 3 patients harboring the heterozygous p.Arg364Gln (and for two of them also the p.Arg354Trp), no significant difference was found in PHLA10 but a significant 50% decrease of PHLA60 was observed, compared to controls (14.9 +/- 4.8 vs 33.7 +/- 7.2 µmol/L/min, $p<0.005$) (16). Thereby, these data suggest that this p.Arg364Gln (and p.Arg354Trp) variant could also affect plasma lipolysis *in vivo* through a milder defect in LPL availability. These missense variants (p.Gly172Arg, p.Arg354Trp, p.Arg364Gln) are located in the two cytoplasmic loops B and D, involved in LPL binding and maturation. It is likely that *LMF1* heterozygous missenses variants, although they might affect simultaneously the maturation of 3 lipases (LPL, HL, and EL) are not pivotal for the severity of the hyperchylomicronemic phenotype. However, they could contribute to modulate the phenotype, in combination with additional effects of rare variants already described in MCM, leading to a polygenic predisposition to severe HTG unmasked in presence of deleterious nutritional or pathological conditions (1,2).

Regarding non-sense *LMF1* mutations, our p.Trp464Ter patient is the first *LMF1* heterozygous patient described to be hyperchylomicronemic, although the transient phenotype corresponded to MCM. Interestingly this mutation was previously identified in an adult homozygous carrier (TG 8-27 mmol/L) who had several episodes of acute pancreatitis but who almost normalized his TG under treatment (5). His heterozygous 2 year-old son was normotriglyceridemic (5). Heterozygous carriers of no other non-sense *LMF1* variants have been described to be hyperchylomicronemic in the literature and only 2/7 exhibited border line HTG below 2 mmol/L (6). The three *LMF1* truncations and the most deleterious missense variant herein (p.Arg537Trp) alter the C terminal domain, which is free in the luminal side of the endoplasmic reticulum, and considered as essential for LPL maturation (21).

Consistently, the segregation study in the p.Trp464Ter proband family revealed that her normotriglyceridemic sister and mother shared the same heterozygous mutation, in addition to the p.Arg451Trp variant, found to be non-deleterious in our functional study. These findings raise the question of the real involvement of the p.Trp464Ter mutation in the hyperchylomicronemic phenotype of the patient. A new heterozygous mutation of *GPIHBP1* with uncertain pathogenesis and no clear segregation with the phenotype was also found. To date no missense heterozygous mutation of *GPIHBP1* gene has been reported to be responsible for hyperchylomicronemic phenotype (17). Even if the contribution of another deleterious mutation in an unknown gene cannot be excluded, this family supports the paradigm that the association of several heterozygous variants in crucial genes involved in intravascular lipolysis (including *LMF1)* may alter LPL activity in critical conditions, such as severe infection in this patient, and may be responsible for intermittent impairment of LPL activity. It is noteworthy that, moderate HTG is a common feature in severe infectious state. Endotoxins and inflammatory cytokines such as TNF, IL-1, IL-2 or IL-6, have been involved in increased VLDL hepatic production, decreased VLDL clearance and moreover altered LPL expression in adipocytes and myocytes *in vitro*, with decreased post-heparin LPL activity in humans (18-20).

Curiously, the p.Thr143Met variant significantly increases *in vitro* LPL activity, suggesting a gain of function variant. We and others previously identified increased *in vivo* LPL activity in patients with MCM (14,22). Collective considerations of these clinical findings strongly suggest that additional mechanisms apart from defects in intravascular lipolysis are involved in MCM.

The study has some limitations. For instance, due to their paucity it was not possible to provide any association studies of these rare *LMF*1 variants with plasma TG in the general population. Systematic post-heparin LPL activity and LPL mass assessment in variant carriers

could have been valuable in order to document *in vivo* the functionality of the new rare variants.

To conclude, the identification of several new functional *LMF1* variants in a large cohort of patients with a history of unexplained hyperchylomicronemia supports the concept that genetic variations of *LMF1* are involved in MCM, in combination with other heterozygous gene variants regulating TG lipolysis. Moreover, our data strongly support the new concept that partial *LMF1* defects, such as the homozygous p.Gly172Arg and the heterozygous p.Trp464Ter mutations, could be involved in intermittent hyperchylomicronemic phenotypes mostly found in MCM. Although *LMF1* variants and mutations are rare, *LMF1* gene study remains important to decipher the complex genetic patterns involved in both monogenic, a challenge now simplified by the access to Next Generation Sequencing.

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Legends for Figures and Tables

Figure 1. Functional analysis of *LMF1* variants

LMF1 variants functionality was assessed by measuring the LPL activity released in culture media of HEK-293T cells co-transfected with human *LPL* and *LMF1* (WT or variants) cDNA. Each histogram represents the mean of three individual transfections ± SEM.

*p<0.05 compared to wild type (WT). £, LPL activity of p.Trp464Ter and p.Tyr439Ter, was < 2 % of WT.

Figure 2. Pedigree, lipid parameters, and genotypes of the family of *LMF1* p.Trp464Ter heterozygous carrier.

Arrow: proband; Filled symbols: homozygotes for *LMF1* variants; Half-filled symbols: heterozygotes for LMF1variants.

[a] maximum TG documented. [b] pathological lipid value for age

HTZ: heterozygote; HDLc: HDL cholesterol; LDLc: LDL cholesterol; TC: total cholesterol; TG: triglycerides; WT: wild-type haplotype.

Table 1: *LMF1* nonsynonymous coding variants identified in the hyperchylomicronemic cohort (n=385)

B, benign; D, damaging; HCM, hyperchylomicronemic; HMZ, homozygote; HTZ, heterozygote; MAF, minor allele frequency; NA, not available; POD, possibly damaging; PRD, probably damaging; Ref, references; T, tolerated.

Tables

Table 1

Exon	Position	Variant	Rs number	HCM cohort n = 385		Control dyslipidemic cohort n = 144		MAF in 1000 Genomes EUR (%)	MAF in EXAC Eur nonFinn (%)	SIFT prediction	PolyPhen-2 prediction	Ref*
				HTZ/ HMZ	MAF (%)	HTZ/ HMZ	MAF (%)					
1	c.95C>T	p.Ala32Val	rs199831082	1/0	0.1	0/0	0	0.0	0.0	T	B	
1	c.107G>A	p.Gly36Asp	rs111980103	20/1[a,d,f]	2.9	29/2	11.5	9.0	21.0	T	B	(7,8)
2	c.413C>G	p.Ser138Cys	rs200382562	2/0[a]	0.3	0/0	0	0.1	0.2	T	PRD	
2	c.428C>T	p.Thr143Met	rs375529211	1/0[b]	0.1	0/0	0	0.0	0.0	T	POD	
3	c.514G>A	p.Gly172Arg	rs201406396	0/1	0.3	0/0	0	0.0	0.0	T	PRD	
5	c.683G>T	p.Gly228Val	NA	1/0	0.1	0/0	0	NA	NA	T	PRD	(8)
6	c.837C>A	p.Phe279Leu	rs61745065	1/0	0.1	1/0	0.3	0.0	0.0	T	B	
7	c.1001C>G	p.Ser334Cys	rs765992133	1/0	0.1	0/0	0	NA	0.0	T	POD	
7	c.1052G>A	p.Arg351Gly	rs192520307	7/0	0.9	2/0	0.7	0.2	0.2	T	B	(7,8)
7	c.1060C>T	p.Arg354Trp	rs143076454	9/0[c]	1.2	6/0	2.1	2.5	3.0	D	B	(7,8)
8	c.1091G>A	p.Arg364Gln	rs35168378	15/0[c,d]	2.0	7/0	2.4	3.0	3.6	T	PRD	(7,8)
8	c.1228G>A	p.Gly410Arg	rs199713950	1/0	0.1	0/0	0	0.1	0.0	T	PRD	
9	c.1292C>A	p.Ala431Asp	rs115416993	1/0	0.1	0/0	0	0.0	0.1	T	B	
9	c.1351C>T	p.Arg451Trp	rs138205062	5/0[e]	0.7	0/0	0	0.4	0.5	D	PRD	(8,9)
9	c.1382G>A	p.Arg461His	rs557053661	1/0	0.1	0/0	0	0.0	0.0	D	PRD	
9	c.1391G>A	p.Trp464Ter	rs587777626	1/0[e]	0.1	0/0	0	NA	NA			(4)

9	c.1405G>A	p.Ala469Thr	rs181731943	1/0	0.1	2/0	0.7	0.2	0.1	D	PRD	(8)
11	c.1609C>T	p.Arg537Trp	rs555435528	1/0	0.1	0/0	0	NA	0.0	D	PRD	
11	c.1685C>G	p.Pro562Arg	rs4984948	11/0 [b,f]	1.4	1/0	0.3	0.8	0.9	D	POD	(7,8)

[a] One subject with p.Ser138Cys is also heterozygote for p.Gly36Asp

[b] p.Thr143Met heterozygous variant is associated with p.Pro562Arg heterozygous variant

[c] p.Arg354Trp heterozygous variant is systematically associated with p.Arg364Glu heterozygous variant

[d.] One heterozygous subject with p.Arg364Gln is also heterozygote for p.Gly36Asp

[e] p.Trp464Ter variant is associated with p.Arg451Trp heterozygous variant

[f.] Two subjects with p.Pro562Arg are also heterozygotes for p.Gly36A