

## **New rare genetic variants of LMF1 gene identified in severe hypertriglyceridemia**

Rene Valero, Bruno Vergès, Marine Dancer, Mathilde Di Filippo, Oriane Marmontel, Maria del Carmen Piombo Rivarola, Noël Peretti, Cyrielle Caussy, Michel Krempf, Bruno Verges, et al.

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

3  
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36

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38

39 **Short title:** *LMFI* new rare variants in hyperchylomicronemia

40

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43

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46

47

48 **Abstract**

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

53 Objective: The aim of this study was to assess the frequency of *LMFI* variants in a cohort of  
54 385 hyperchylomicronemic patients without homozygous or compound heterozygous  
55 deleterious mutations identified in *LPL*, *APOA5*, *APOC2*, *GPIHBP1* genes, and to determine  
56 their functionality.

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

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

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

73

74

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

78

79

## 80 **1. Introduction**

81

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

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

104

## 105 **2. Material and Methods**

106

### 107 **A. Patients and populations studied**

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

120

### 121 **B. Genomic DNA analysis**

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

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

133 The rs identification number of each variant identified was checked in the dbSNP  
134 database (<https://www.ncbi.nlm.nih.gov/gate2.inist.fr/snp/>) and Alamut® Visual 2.9 software  
135 (*Refseq* accession numbers for *LMFI*, NM\_022773.2, NP\_073610.2). The allele frequency of  
136 *LMFI* variants was compared to two European general populations, 1000 Genomes Europe  
137 (<http://www.internationalgenome.org/1000-genomes-browsers>) and ExAC European non-  
138 Finnish (<http://exac.broadinstitute.org/>). For nonsynonymous missense coding variants, *in*  
139 *silico* analyses were performed using the PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>)  
140 (12) and SIFT ([http://sift.dna.org/www/Extended\\_SIFT\\_chr\\_coords\\_submit.html](http://sift.dna.org/www/Extended_SIFT_chr_coords_submit.html)) (13)  
141 software to predict the possible impact of the observed amino acid substitutions on the  
142 structure and function of the human protein.

143

### 144 **C. Human post-heparin LPL activity**

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

148

### 149 **D. Functional analysis of *LMFI* variants**

#### 150 *1. Human LMFI and LPL cDNA constructs*

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



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

161

## 162 *2. Cell transfections*

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

177

## 178 *3. Real time quantitative PCR analysis*

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

190

#### 191 4. *LPL* activity assay in cell culture media

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

196 The optimal volume of medium sample (40  $\mu\text{l}$ ) was tested for sensitivity and linearity of the  
197 *LPL* activity assay (supplemental figure 1 and 2). A strong correlation between the *LPL*  
198 activity and the volume was found in the same way as the initial plasma assay ( $R=0.99$ ,  
199  $p<0.01$ ) (14) (supplemental figure 1). The *LPL* activity assay was linear between 2 and 6  
200 hours after heparin addition (supplemental figure 2). The longer incubation time tested (6  
201 hours) was conserved to have enough power to discriminate the effect of *LMFI* variants.  
202 Inter-assay reproducibility was verified using an internal control; the variation coefficient was  
203 8 % (mean 22.3  $\mu\text{mol/L/min}$ ).

204

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

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

219

## 220 **E. Statistical analysis**

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

## 225 **3. Results**

226

### 227 **A. *LMFI* coding variants identification**

228           The 385 hyperchylomicronemic patients explored (free of homozygous or compound  
229 heterozygous mutations in *LPL*, *APOC2*, *APOA5* or *GPIIIBP1* genes) were middle-aged  
230 (mean +/- standard deviation (SD): 43.2 +/-16.2 years old). They were mainly men (70.5 %).  
231 35 subjects (9.1 %) had diagnosis of hyperchylomicronemia before they were 20 years old.

232           In this cohort, 34 different *LMFI* coding variants were identified: 15 synonymous and  
233 19 nonsynonymous (18 missense variants and one nonsense variant p.Trp464Ter) (Table 1).  
234 The complete list of identified variants, including variants in adjacent intronic regions covered  
235 by the genetic analyses, is available in Supplemental Table 3.

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

247

## 248 **B. Functional analysis of *LMFI* variants**

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

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

263

## 264 **C. Case reports**

### 265 *1. p.Trp464Ter heterozygote and family explorations*

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

273 permanent HTG and acute pancreatitis (5) and was accordingly shown to totally abolish *in*  
274 *vitro* LPL activity in our *in vitro* study. The proband (II-2) inherited the heterozygous  
275 p.Trp464Ter from her mother (I-2). The heterozygous p.Arg451Trp *LMFI* rare variant,  
276 inherited from her normotriglyceridemic father (I-1), did not alter LPL activity in our model  
277 (Figure 1). Her normolipidemic sister (II-1) had the same *LMFI* genotype. The proband (II-2)  
278 also inherited from her mother a new heterozygous rare variant of *GPIHBP1* gene  
279 (NM\_178172.5 (*GPIHBP1*): c.424C>G)) not previously described in literature neither in  
280 dbSNP nor in ExAC databases, which is predicted to be “tolerated” in SIFT and “possibly  
281 damaging” in PolyPhen-2 prediction software. The signal peptide p.Cys14Phe common  
282 variant of *GPIHBP1* (c.41G>T, rs11538389) was also identified at heterozygous state in the  
283 siblings (II-1, II-3) inherited from the mother (I-2). The proband (II-2), her mother (I-2) and  
284 her brother (II-3) exhibited the  $\epsilon3\epsilon4$  genotype of *APOE* gene (Figure 2). No additional  
285 variants were identified on *LPL*, *APOA5* or *APOC2* genes in the proband.

286

## 287 2. p.Gly172Arg homozygote

288

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

299 7.22 mmol/L and HbA1C 8.4 % under insulin treatment. His brother and sister were also  
300 known to have intermittent severe HTG (TG > 10 mmol/L), however they refused to be  
301 explored.

302

## 303 **4. Discussion**

304 This study identified 19 nonsynonymous *LMFI* coding variants in a large cohort of  
305 hyperchylomicronemic patients (without homozygous or compound heterozygous deleterious  
306 mutations identified in *LPL*, *APOA5*, *APOC2*, *GPIHBP1* genes): 10 of them were identified  
307 for the first time in hyperchylomicronemia. This finding confirms the rarity of *LMFI* variants  
308 since 16.9 % of patients harbored missense or nonsense *LMFI* variants, and only 4.7 % were  
309 carriers of variants suspected to be deleterious based on their *in vitro* effect on LPL activity.  
310 In patients with TG > 10 mmol/L, a close frequency of missense variants of 17.6 % was  
311 reported in a smaller population (n=85) (7). In populations including both moderate and  
312 severe HTG, the frequency of missense *LMFI* variants was variable, lower (5.1 %) in the  
313 cohort reported by De Castro-Oros et al. (118 subjects with TG > 5.6 mmol/L) (9) and higher  
314 (28 %) in a larger cohort reported by Johansen et al. (413 subjects with TG > 3.37 mmol/L)  
315 (8).

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

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

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



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

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

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

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

394 The study has some limitations. For instance, due to their paucity it was not possible to  
395 provide any association studies of these rare *LMF1* variants with plasma TG in the general  
396 population. Systematic post-heparin LPL activity and LPL mass assessment in variant carriers

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

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

409

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495

496 **Legends for Figures and Tables**

497

498 **Figure 1. Functional analysis of *LMFI* variants**

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

502 \* $p < 0.05$  compared to wild type (WT). £, LPL activity of p.Trp464Ter and p.Tyr439Ter, was  
503  $< 2\%$  of WT.

504

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

507 Arrow: proband; Filled symbols: homozygotes for *LMFI* variants; Half-filled symbols:  
508 heterozygotes for *LMFI* variants.

509 <sup>a</sup> maximum TG documented. <sup>b</sup> pathological lipid value for age

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

512

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

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

518

519 **Tables**

520 **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 <sup>a,d,f</sup>	2.9	29/2	11.5	9.0	21.0	T	B	(7,8)
2	c.413C>G	p.Ser138Cys	rs200382562	2/0 <sup>a</sup>	0.3	0/0	0	0.1	0.2	T	PRD	
2	c.428C>T	p.Thr143Met	rs375529211	1/0 <sup>b</sup>	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 <sup>c</sup>	1.2	6/0	2.1	2.5	3.0	D	B	(7,8)
8	c.1091G>A	p.Arg364Gln	rs35168378	15/0 <sup>c,d</sup>	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 <sup>e</sup>	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 <sup>e</sup>	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 <sup>b,f</sup>	1.4	1/0	0.3	0.8	0.9	D	POD	(7,8)

<sup>a</sup> One subject with p.Ser138Cys is also heterozygote for p.Gly36Asp

<sup>b</sup> p.Thr143Met heterozygous variant is associated with p.Pro562Arg heterozygous variant

<sup>c</sup> p.Arg354Trp heterozygous variant is systematically associated with p.Arg364Glu heterozygous variant

<sup>d</sup> One heterozygous subject with p.Arg364Gln is also heterozygote for p.Gly36Asp

<sup>e</sup> p.Trp464Ter variant is associated with p.Arg451Trp heterozygous variant

<sup>f</sup> Two subjects with p.Pro562Arg are also heterozygotes for p.Gly36A

