Post-Heparin LPL Activity Measurement Using VLDL As a Substrate: A New Robust Method for Routine Assessment of Plasma Triglyceride Lipolysis Defects

Mathilde Di Filippo1,2,*, Christophe Marçais2,3, Sybil Charrière2,4, Oriane Marmontel1, Martine Broyer1, Mireille Delay3, Micheline Merlin3, Axel Nollace1, René Valéro5, Michel Lagarde2, Valérie Pruneta-Deloche2, Philippe Moulin2,4, Agnès Sassolas1,2

1 UF Dyslipidémies Cardiobiologie, Département de Biochimie et Biologie Moléculaire, Centre de Biologie et de Pathologie Est, Laboratoire de Biologie Médicale Multi Sites, Hospices Civils de Lyon, Bron, France, 2 INSERM U1060, INSA de Lyon, INRA U1235, Univ Lyon-1, Université de Lyon, Villeurbanne, Oullins, France, 3 Laboratoire de Biochimie spécialisée, Centre de Biologie Sud, Centre Hospitalier Lyon-Sud, Laboratoire de Biologie Médicale Multi Sites, Hospices Civils de Lyon, Pierre-Bénite, France, 4 Fédération d’endocrinologie, maladies métaboliques, diabète et nutrition, Hôpital Louis Pradel, Hospices Civils de Lyon, Bron, France, 5 Département de Nutrition, Maladies Métaboliques, Endocrinologie, APHM, Hôpital de la Timone, Aix-Marseille Université, UMR_S 1062, UMR_A1260, Marseille, France

**Abstract**

**Background:** Determination of lipoprotein lipase (LPL) activity is important for hyperchylomicronemia diagnosis, but remains both unreliable and cumbersome with current methods. Consequently by using human VLDL as substrate we developed a new LPL assay which does not require sonication, radioactive or fluorescent particles.

**Methods:** Post-heparin plasma was added to the VLDL substrate prepared by ultracentrifugation of heat inactivated normolipidemic human serums, diluted in buffer, pH 8.15. Following incubation at 37°C, the NEFA (non esterified fatty acids) produced were assayed hourly for 4 hours. LPL activity was expressed as µmol/l/min after subtraction of hepatic lipase (HL) activity, obtained following LPL inhibition with NaCl 1.5 mmol/l. Molecular analysis of LPL, GPIHBP1, APOA5, APOC2, APOE genes was available for 62 patients.

**Results:** Our method was reproducible (coefficient of variation (CV): intra-assay 5.6%, inter-assay 7.1%), and tightly correlated with the conventional radiolabelled triolein emulsion method \( n = 26, r = 0.88 \). Normal values were established at \( 34.8 \pm 12.8 \) µmol/l/min (mean ± SD) from 20 control subjects. LPL activities obtained from 71 patients with documented history of major hypertriglyceridemia showed a trimodal distribution. Among the 11 patients with a very low LPL activity (< 10 µmol/l/min), 5 were homozygous or compound heterozygous for LPL deleterious mutations, 3 were compound heterozygous for APOA5 deleterious mutations and the p.S19W APOA5 susceptibility variant, and 2 were free of any mutations in the usual candidate genes. No homozygous gene alteration in LPL, GPIHBP1 and APOC2 genes was found in any of the patients with LPL activity >10 µmol/l/min.

**Conclusion:** This new reproducible method is a valuable tool for routine diagnosis and reliably identifies LPL activity defects.

**Introduction**

Lipoprotein lipase (LPL) (glycerol ester hydrolase, EC 3.1.1.34) plays a crucial role in the metabolism of very low density lipoproteins (VLDL) and chylomicrons [1,2]. A large pool of this enzyme bound to heparin sulfate proteoglycans and glycosylphosphatidylinositol-anchored high density lipoproteins binding protein 1 (GPIHBP1) through ionic linkage [3,4], is releasable into plasma following the intravenous injection of heparin. Familial LPL deficiency is a rare autosomal recessive disorder which is characterized by primary hypercholesterolemia due to homozygous or compound heterozygous mutations of LPL gene [5] as well as to homozygous mutations of APOC2, GPIHBP1, APOA5 or LMF1 genes [6–8]. Additionally, sporadic forms of severe hypertriglyceridemia commonly result from complex interactions between environmental and genetic factors leading to various degrees of LPL deficiency [6]. Consequently, LPL activity measurement remains primordial in order to decipher the mechanisms underlying determinants of these heterogeneous phenotypes and bring irreplaceable information in case of negative or unavailable molecular diagnosis.

However, the LPL activity assay remains difficult. Methods using commercial triglyceride (TG) emulsions as a substrate are hampered by a high NEFA concentration at the basal state and a lack of specificity due to the presence of mono and diglycerides [9,10]. The use of trioleyl substrates with the incorporation of

---

*E-mail: mathilde.di-filippo@chu-lyon.fr

**Citation:** Di Filippo M, Marçais C, Charrière S, Marmontel O, Broyer M, et al. (2014) Post-Heparin LPL Activity Measurement Using VLDL As a Substrate: A New Robust Method for Routine Assessment of Plasma Triglyceride Lipolysis Defects. PLoS ONE 9(5): e96482. doi:10.1371/journal.pone.0096482

**Editor:** Paulo Lee Ho, Instituto Butantan, Brazil

**Received** November 8, 2013; **Accepted** April 7, 2014; **Published** May 2, 2014

**Copyright:** © 2014 Di Filippo et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** The authors have no support or funding to report.

**Competing Interests:** The authors have declared that no competing interests exist.
radioactive tracers (3H) by sonication under strictly controlled conditions by Nilsson-Ehle et al published in 1972 [11], was a major improvement. However, this time-consuming assay needs to be performed in triplicate in order to minimize the intra-assay error [12–15]. Despite several improvements, most of the current methods using radiolabelled (3H or 14C) substrates cannot be run on automats and require cumbersome strategies to inhibit hepatic lipase activity. Some chromophoric [16–20] or fluorescent [21] substrates have been used; however the preparation of these substrates is problematic, the derivatives were unstable and the method not sensitive enough [21]. In a recent method, Basu D et al [15] used a commercially fluorescent substrate (EnzChek), esterified by BODIPY-C12 at the sn-1 position of glycerol, which was stable and solubilised with a specific detergent (Zwittergent). Although highly appealing, this method has not been tested in human post-heparin plasmas.

Therefore, we decided to set up a new reliable method sensitive enough to allow human post-heparin LPL activity measurement in routine conditions without requiring sonication, nor use of a fluorescent or radioactive substrate. Seeking reliability, we preferred to choose a natural substrate composed of pooled human VLDL, thereby also providing the optimal amount of human apoC-II.

**Materials and Methods**

**Ethics Statement**

Clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki. Informed written consent was obtained from the control subjects and the patients before DNA sampling and heparin injection. The study protocol was approved by our institutional ethical committee (Comité d’Ethique du CHU de Lyon).

**Patients**

Twenty adults control subjects (9 men, 11 women, mean age 52 ± 17 years) were recruited in order to determine normal post-heparin LPL activity (without history of hypertriglyceridemia or diabetes). 71 hypertriglyceridemic patients with documented history of type V dyslipidemia (TVHTG) were assessed with the new method (Plasma TG concentration (TG) > 15 mmol/l or familial history of hypertriglyceridemia with TG > 10 mmol/l). Patients characteristics are summarized in Table 1.

Fasting venous blood samples were drawn into EDTA tubes on ice before (T0) and 10 min (T10) after a 50 U/kg intravenous heparin injection. The tubes were immediately centrifuged at 4°C and plasma was frozen at −80°C.

**VLDL substrate preparation**

VLDL substrate was used for the LPL assay: 20 ml of human serum were pooled from set of 10 human serum samples (TG 0.9–1.75 mmol/l, HDL-cholesterol > 0.9 mmol/l, total cholesterol 3.2–5.5 mmol/l). VLDL from the pool were isolated in 1.2 ml polycarbonate tubes (Beckman) by preparative ultracentrifugation (d = 1.006 kg/l) using a Sorval Discovery M150 SE ultracentrifuge (Sorval S140 AT 017 Rotor: 140000 RPM (1042000 max) for 70 minutes). Floating VLDL were collected, stored at +4°C and used within 48 h; 360 μl of VLDL substrate at a final TG concentration of 7.3 mmol/l was used for both total post heparin and hepatic lipase activity assay. Before use, the VLDL substrate was heated at 56°C for one hour in order to inactivate any residual endogenous lipase activity.

| Table 1. Subjects features. | n | Age year | Sex % of men | BMI kg/m² | Smoker % of smoker | Diabetes % |
|-----------------------------|---|----------|--------------|------------|---------------------|------------|
| 0 Controls                  | 20| 51.7 (22–76) | 22 | 55 NA | 0 |
| 1 HTG without mutation group no mutation in PL, GPIHBP1, AGAS, APOC2 | 15| 45.3 (13–61) | 3 | 75.0 (21.9–34.7) | 20 |
| 2 HTG minor polymorphism group: APOA5 p.S19W or SNP1 heterozygote, APOAS p.S19W | 20| 48.4 (23–80) | 2 | 75.0 (19.8–34.0) | 50 |
| 3 HTG minor mutation group: homozygous mutation of APOAS, heterozygous mutation of APOAS, heterozygous mutation of APOAS, heterozygous mutation of APOAS (p.0.00) | 7| 42.3 (36–50) | 1 | 71.0 (20.3–32.3) | 60 |
| 4 HTG minor mutation group: homozygous mutation of APOAS, heterozygous mutation of APOAS, heterozygous mutation of APOAS, heterozygous mutation of APOAS | 8| 48.4 (32–64) | 8 | 50.0 (21.7–40.0) | 38 |
| 5 HTG major mutation group: homozygous mutation of APOAS and polymorphism | 5| 23.3 (15–40) | 6 | 60.0 (21.7–21.0) | 33 |
| 6 Patients with anti-APO antibodies and low LPL activity | 3| 54.0 (55–70) | 3 | 67.0 (21.8–22.3) | NA |
Michaelis constant
The Michaelis constant (K_m) was determined as the inverse of the Lineweaver-Burk line intercept using 6 different VLDL substrates with final TG concentration ranging from 0.80 to 6.80 mmol/l.

LPL activity assay
The inactivated VLDL substrate and the mixture for the enzymatic reaction were kept on ice until the start of the reaction at 37°C.
Total post-heparin lipase activity (PHLA) (A): 180 µl of VLDL substrate was mixed with 540 µl of buffer A (Tris 50 mmol/l, MgCl2 3 mmol/l, CaCl2 1.5 mmol/l, dodecylbetain laurylbetain Ramidus AB, Idenion, SE-223 70 Lund, Sweden) detergent 0.03%, pH 8.15) leading to a 1.8 mmol/l final TG concentration; 10 µl of post-heparin plasma sample (T10) were added and mixed; 120 µl of substrate was mixed with 540 µl of buffer A (Tris 50 mmol/l, NaCl 2 mmol/l, dodecylbetain detergent 0.03%, pH 8.15) leading to a 1.8 mmol/l final TG concentration; 10 µl of post-heparin plasma sample (T10) were added and mixed; 120 µl of substrate was mixed with 540 µl of buffer A (Tris 50 mmol/l, NaCl 2 mmol/l, dodecylbetain detergent 0.03%, pH 8.15) leading to a 1.8 mmol/l final TG concentration; 10 µl of post-heparin plasma sample (T10) were added and mixed; 120 µl of substrate was mixed with 540 µl of buffer A (Tris 50 mmol/l, NaCl 2 mmol/l, dodecylbetain detergent 0.03%, pH 8.15) leading to a 1.8 mmol/l final TG concentration. Calculation of the enzymatic activity was made in the linear part of the curve (see results) between 60 and 180 minutes. The PHLA activity was expressed in mmol/l/min of released NEFA.
Hepatic Lipase (HL) activity (B): the assay was conducted with the same procedure, but with 20 µl of post-heparin plasma sample and buffer A was substituted by buffer B (Tris 50 mmol/l, NaCl 2 mmol/l, dodecylbetain detergent 0.03%, pH 8.15) in order to inhibit the LPL activity; the final NaCl concentration in the mix was 1.5 mmol/l.
LPL activity was obtained by the subtraction of hepatic lipase activity, measured with buffer B from total activity obtained with buffer A and expressed as mmol/l/min of produced NEFA. The enzymatic reaction was controlled using, as the external standard, a sample of a frozen pool of PHLA plasmas obtained from 8 control subjects.

LPL assay comparison with the radiolabelled method
A radiolabelled 14C-triolein emulsion was used as previously described [22]. The assays were performed in triplicate.

Molecular diagnosis
Following the extraction of genomic DNA from blood (Nucleon BAC3, GE Healthcare, Chalfont St. Giles, UK), the encoding regions, and the flanking intronic junctions of LPL, APOA5, APOC2, GPIHBP1 and APOE genes were PCR amplified as previously reported [23-26]. The amplicons were directly sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3730 DNA sequencer (Applied Biosystems, Foster City, USA).
In silico analyses of the mutations were performed with Alamut v2.0 (Interactive Software, Polyphen (http://www.genetics.bwh. harvard.edu/pph/) and SIFT (http://sift.jcvi.org/www/SIFT _aligned_seq_submit.html).

Statistical analysis
Statistical analyses were performed using SPSS 17.0 software. T-test or non-parametric Mann Whitney tests were performed in order to compare mean/median of LPL activities between groups. Shapiro-Wilk test was used to assess the normality of the distribution of the post heparin LPL activities. p-values (two sided) less than 5% were considered significant. Linear regressions were performed to determine the correlation between LPL activity and age or the conventional radiolabelled method.

Results
Optimization of substrate
(see Supplemental data S1 and Figure S1)
First, we verified that similar lipoprotein profiles were obtained from different VLDL substrate pools (n = 40, the coefficient of variation (CV) was <5% for cholesterol and TG concentrations and <10% for apoB, C-II and C-III) (Table S1). Second, the mean K_m of the reaction was lower than 1.5 mmol/l (mean 1.42 mmol/l). In order to determine the optimal TG concentration chosen for the mixture, we tested 2 patient samples (PHLA activity 17.4 and 49.2 mmol/l/min) with 7 different TG concentrations in the mixture (ranging from 0.45 to 3.45 mmol/l); the activity was decreased (−44, −67%) with low TG levels (<0.90 mmol/l) and slightly increased (+17, +24%) with high TG concentration (>2.7 mmol/l) in the mixture (see Figure S2). Consequently, we established the optimal final concentration of TG in the assay mixture at 1.8 mmol/l.

Limit of detection
The minimal level of detectable LPL activity was determined through two methods: first, we performed the assay using 6 pre-heparin control plasmas and second, with 5 inactivated (56°C one hour) control post-heparin plasmas. We discovered that the minimal detectable LPL activity (mean±3SD) was 2.7 µmol/l/min for the pre-heparin plasmas (from VLDL-bound LPL, or other circulating lipases) and 1.68 µmol/l/min for the heat inactivated post-heparin plasmas.

Imprecision
The coefficient of variation (CV) of the intra-assay reproducibility was 5.6% for an LPL activity of 31.5 µmol/l/min (SD: 1.8 µmol/l/min).

The inter-assay reproducibility was studied by including a single sample from a PHLA frozen control pool in 16 consecutive series
of independent LPL determinations; the CV was 7.1% (mean: 29.1±2.1 μmol/l/min). This sample allows an internal quality control of each assay.

Comparison with conventional radiolabelled method

26 post-heparin plasma LPL-HL activities obtained from hypertriglyceridemic patients with history of documented major hyperchylomicronemia were assayed for LPL activity by both methods (our new method versus the conventional 14C triolein method). LPL activities showed a strong positive correlation (r = 0.88, p<0.001) (Figure 2A).

LPL activity in controls and TVHTG patients

Normal values of LPL activity were established at 34.8±12.8 μmol/l/min (mean±SD; extreme values 10.6 to 62.2) in 20 control subjects (Figure 2B); the LPL activity was unchanged according to age (no correlation between LPL activity and age, r = 0.063) and gender: mean 30.9±11.7 μmol/l/min and 39.6±13.0 μmol/l/min in 11 females and 9 males respectively (p = 0.261).

Interestingly, LPL activities from the 71 TVHTG patients were widely distributed (activity range 0 to 103 μmol/l/min; mean: 30.5±18.2 μmol/l/min) with evidence for a non normal distribution (p<0.01) (Figure 2B). 11 patients had a low LPL activity (<

---

**Figure 1. Kinetics and linearity.** Figure 1A. PHLA and HL kinetics in 3 patients. LPL+HL (line); HL (dotted line); Patient 1 (triangle); Patient 2 (circle); Patient 3 (square); Patient 4 (vertical line). Figure 1B. Linearity test. Patient 1 (black diamond); Patient 2 (white square); Patient 3 (grey triangle); Patient 4 (black square); Patient 5 (grey square); Patient 6 (grey circle).

doi:10.1371/journal.pone.0096482.g001

**Figure 2. Correlation between LPL activity assays in TVHTG and controls patients.** Figure 2A. Correlation with conventional method (n = 26, r = 0.88, p<0.001). y = 8.93 x + 1.21; R² = 0.77. Figure 2B. Distribution of LPL activity in TVHTG patients and controls subjects. TVHTG patients (grey square); controls (black square).

doi:10.1371/journal.pone.0096482.g002
10 μmol/l/min (mean + SD: 3.6+2.4 μmol/l/min) and the LPL activities in the other patients with LPL between 10 and 55 μmol/l/ min were normally distributed around the median of this group (p = 0.97, median: 31.6 μmol/l/min, mean: 32.1±8.6, n = 51). A group of 6 TVHTG patients had high LPL activity despite hypertriglyceridemia.

LPL activity and molecular diagnosis in hypertriglyceridemic patients with TVHTG

Among the 71 patients with history of major hypertriglyceridemia included, molecular assessment was available for 62 patients; all of which were examined for LPL gene mutations. 58 patients had a molecular diagnosis for additional candidate genes involved in the regulation of LPL activity (APOA5, GPIHBP1, APOC2, APOE). 5 new mutations were found: p.V227G in LPL gene, p.Y110LfsX158, p.Q295X and p.R343C in APOA5 gene and p.Q246R in APOE gene (See Table S2).

Mutations in LPL, GPIHBP1, APOA5 or APOC2 genes were not found in 15/58 (26%) TVHTG patients (Figure 3, lane 1). In this group, most patients (13/15) presented normal LPL activity whereas 2 patients had both a very low LPL activity (1.6 and 3.7 μmol/l/min) and a clear familial history of hyperchylomicronemia. 1 of these 2 patients was found to be a carrier of p.Q246R a new missense heterozygous APOE gene mutation.

LPL activity was found in the reference interval in the 20/58 patients who harboured only APOA5 or APOC2 susceptibility polymorphisms (APOA5 p.S19W, APOA5 haplotype 2 or APOC2 p.K41T) (lane2). LPL activity was also found in the reference interval in the 7/58 patients who had either heterozygous LPL or APOA5 deleterious mutation as well as in a single patient with a homozygous deleterious APOA5 missense mutation (lane 3).

Conversely all the 5 homozygous or compound heterozygous patients with deleterious LPL or GPIHBP1 mutations had a drastically reduced LPL activity (lane 5 and see Table S2).

We considered a subgroup of TVHTG patients (8/58) defined as compound heterozygotes for a deleterious APOA5 mutation and, on the second allele, a susceptibility polymorphism (either APOA5-haplotype 2 or APOA5-haplotype 3 (p.S19W)) (lane 4). 3 of these patients presented a low LPL activity < 10 μmol/l/min while 5 had a normal LPL activity.

3 patients had LPL auto-antibodies, only 1/3 displayed a low LPL activity (lane 6) at the time of blood drawing (3.1 μmol/l/min); this patient was also heterozygous for the LPL p.P200LfsX7 frame shift mutation and was previously reported with low LPL activity using the triolein emulsion conventional assay [27]. Overall, drastically reduced LPL activity (<10 μmol/l/min) was discovered in 11 TVHTG patients (TG mean ±SD: 20.5±27.0 mmol/l): 5 of which were identified with obvious causal genotypes in either LPL or GPIHBP1 genes and 4 of which had genotypes most likely to contribute to their hyperchylomicronemia. Additionally, 2 pediatric patients with family history of dominant hypertriglyceridemia had a clear lipolysis defect although there was no causal genotype yet identified in candidate genes.

Compared to patient with low LPL activity (<10 μmol/l/min), the 47 genotyped (TVHTG) patients with LPL activity over 10 μmol/l/min had milder hypertriglyceridemia (8.9 ±8.0 mmol/l p<0.01). In this group, no correlation between LPL activity and

![Figure 3. LPL activity and molecular diagnosis.](image-url)
plasma TG concentration was found ($r = 0.14$). None of these patients had either homozygous or combined heterozygous deleterious mutations in $LPL$, $APOC2$, or $GPIHBP1$.

**Discussion**

We propose a robust, very reproducible and convenient method to determine LPL activity in human post-heparin plasma. The absence of radiolabelled emulsion prevents the requirement of unreliable sonication under stringent conditions and avoid the use of costly and problematic reagent. Human VLDL constitute the natural substrate of LPL with an optimal composition in apoC-II and apoC-III similar to the concentrations found in normal plasma. The use of a pool of 10 plasmas obtained from control subjects provides a reproducible composition and lipolysis ability. Since fresh VLDL substrate for each LPL activity assay was needed, care was taken to control each assay with a pool of PHLA control plasmas. The dilution of the VLDL substrate was performed in a TRIS buffer in order to provide the necessary ions, optimal pH and suitable detergent conditions for an optimal reaction. A pH of 8.15 was considered as the optimal pH for the LPL activity $[9,17,28–30]$. The low HL activity found with our new method could be due to the fact that VLDL is not the optimal substrate for HL $[31]$. Since the kinetics of PHLA with a human VLDL substrate was not yet documented, we ascertained the linearity and the Michaelis constant ($K_m^*$) of the enzymatic reaction. The $K_m^*$ found using human VLDL was in the range of several $K_m^*$ reported using artificial TG emulsions (0.1 to 2.5 mmol/l) $[9,16,32]$. We chose to work with a substrate concentration of 1.8 mmol/l; within these conditions, the upper linearity of the LPL activity is sufficient (70 mmol/l/min); however the highest LPL activity level needs a decrease of sample volume while the lowest LPL activity may need an increase of volume. The use of a substrate TG concentration set at 1.8 mmol/l offers many advantages; firstly, the substrate can be prepared from normal sera; secondly, the required amount of TG VLDL is low.

The robustness of the method is optimal. The inter-assay reproducibility (CV 7.1%) is lower than those of most conventional triolein methods (mean CV 11.6%, range 5–25%) reported from 11 different methods by Henriksen $[12–15,17,29,33,34]$. The correlation with a radiolabelled triolein method was tight ($r = 0.88$), similar to that obtained by Imamura (dioleoyl substrate versus labelled triolein, $r = 0.79$) $[p = 0.21]$ $[35]$. Surprisingly, the other published methods with non-labelled soluble substrates did not provide any correlation with the conventional radio-labelled emulsion method $[15–19]$.

Post-heparin LPL activity level in our normal controls (34.84–12.8 μmol/l/min) was in the low range of those reported by the conventional method $[24,36]$. However, a huge heterogeneity is found amongst the published methods. The differences in normal LPL activity levels could be due to the use of different substrates, various doses of injected heparin (from 10 to 100 UI/kg body weight), apoC-II concentrations, pH of the reaction mixture, concentration of albumin, and method of inhibition of LPL and HL. All of these discrepancies were well described by Henriksen et al., who reported 11 published LPL activity assays, the reference values ranging from 25 to 362 mU/ml $[34]$.

Our method provides a convenient tool to identify patients with a major defect in LPL activity. All patients with homozygous or compound heterozygous mutations of $LPL$ and $GPIHBP1$ were below the cut off point of 10 μmol/l/min, which is in agreement with previous reports for $LPL$ and $GPIHBP1$ genes mutations $[14,37]$. Interestingly, except these 11 patients, all the other TVHTG patients had LPL activities similar to control subjects, with several high LPL values, as previously reported by Coca-Prieto $[14]$. As expected, our 4 patients with heterozygous LPL mutations were identified in the group of TVHTG patients with normal LPL activity except for one patient who additionally had neutralising anti LPL antibodies $[27]$. Surprisingly, LPL activity has been poorly documented in heterozygous LPL mutation carriers with a history of type V hyperlipidemia. According with our findings, Surendram $[8]$ recently described 2 $LPL$ and 1 $GPIHBP1$ new heterozygous missense mutations with normal LPL activity. All these findings are consistent with the knowledge that under dietetic conditions, patients with heterozygous LPL mutations have normal or mildly increased TG levels due to sufficient residual LPL activity $[30]$. Interestingly, LPL activity levels in our patients with $APOA5$ mutations were highly heterogeneous. Normal activity was found in one patient with a homozygous missense $APOA5$ mutation (p.R343C) predicted to alter apoaV function. A very low LPL activity was found in 3 diabetic patients compound heterozygous for $APOA5$ p.Q139X mutation and $APOC2$ s19W polymorphism. These patients were previously reported as having a very low activity using LPL activity emulsion method $[24]$. Finally, LPL activity was normal for 5 patients with other heterozygous deleterious $APOA5$ mutations combined with the heterozygous p.S19W polymorphism. It remains possible that these variants are present on the same haplotype, leaving a functional $APOA5$ allele in some of these patients. The discrepancy of LPL activity in $APOA5$ deficient patients underlines the complexity of the phenotype and the necessity to have access to LPL activity in order to understand the high phenotypic variability of these patients. Occurrence of transient severe LPL deficiency might correspond to complex and not yet fully elucidated gene-gene interactions or gene-environment interactions.

In TVHTG patients, over half did not harbour any genetic mutation in $LPL$, $APOA5$, $APOC2$, $GPIHBP1$, $APOE$ 34% had only minor $APOA5$ or $GPIHBP1$ defects and 26% were not found to harbour any variants in candidate genes. This distribution is in complete accordance with Surendram $[8]$ who had 47% HTG patients without mutations in candidate genes (26% with only polymorphism and 21% with no variants) and reports from other teams $[7]$. However LPL activity was not systematically reported in these series. Our new method identified 2 TVHTG patients with an apparent autosomal dominant transmission, a very low LPL activity, no deleterious mutation in any usual candidate genes (including $LMPI$) and no evidence for autoimmunity (Figure 3, lane 1).

These findings illustrate the importance of accurate routine LPL activity assessment in order to decipher the phenotype of TVHTG patients. The discrepancy between hyperchylomicronemia and normal LPL activities raises the question of the role of additional regulators of heparin releasable LPL and/or molecular mechanisms leading to transient LPL deficiencies. Although less decisive than molecular genetic testing for etiologic diagnosis, the finding of low LPL activity in patients without mutation or conversely very high activity in patients supposed to have major genetic defect, raise some major questions about the regulation of TG metabolism and bring valuable information for diagnosis. Due to its robustness and its excellent reproducibility, this new method using human VLDL as natural substrate provides a convenient tool in order to explore the underlying mechanisms of misunderstood hyperchylomicronemia.
Figure S1 Optimization of the assay: triglycerides concentration in the mixture. Triglycerides concentration 25% (line); 50% (dotted line) Pool 23 (diamond); Pool 24 (circle). PHLA kinetics of 1 control plasma activity with 2 different VLDL substrates used at 2 TG concentrations (25 and 50%, i.e. TG 1.8 and 3.6 mmol/l).

(DOC)

Figure S2 PHLA measured with different triglycerides concentration substrate in the reaction. Patient 1 PHLA = 17.4 μmol/l/min (black line); Patient 2 PHLA = 49.2 μmol/l/min (grey line) 100% of activity was fixed with triglycerides in the mixture at 1.8 mmol/l.

(DOC)

References

1. Erkel RH (1989) Lipoprotein lipase. A multifunctional enzyme relevant to common metabolic diseases. N Engl J Med 320: 1060–1069.
2. Olivecrona G, Olivecrona T (1995) Triglyceride lipases and atherosclerosis. Curr Opin Lipidol 6: 291–305.
3. Dallinga-Thouw GM, Franssen R, Mooy HL, Visser ME, Hassing HC, et al. (2010) The metabolism of triglyceride-rich lipoproteins revisited: new players, new insight. Atherosclerosis 211: 1–8.
4. Davies BS, Beigneux AP, Fong LG, Young SG (2012) New wrinkles in triglyceride lipoprotein biology. Curr Opin Lipidol 23: 35–42.
5. Hayden MR, Ma Y (1992) Molecular genetics of human lipoprotein lipase deficiency. Mol Cell Biochem 113: 171–176.
6. Johansen CT, Kathiresan S, Hégéle RA (2011) Genetic determinants of plasma triglycerides. J Lipid Res 52: 189–206.
7. Gotoda T, Shirai K, Ohba T, Kobayashi J, Yokoyama S, et al. (2012) Diagnosis and management of type I and type V hyperlipoproteinemia. J Atheroscler Thromb 19: 1–12.
8. Surendran RP, Visser ME, Heemelaar S, Wang J, Peter J, et al. (2012) Mutations in LPL, APOC2, APOA5, GPHNIP1 and LMF1 in patients with severe hypertriglyceridemia. J Intern Med 272: 185–196.
9. Boberg J, Carlson LA (1964) Determination of Heparin-Induced Lipoprotein Lipase Activity in Human Plasma. Clin Chim Acta 10: 420–427.
10. Biale Y, Shafrir E (1969) Lipolytic activity toward tri- and monoglycerides in human postheparin plasma. Clin Chim Acta 23: 413–419.
11. Nilsson-Ehle P, Torquato H, Belfrage P (1972) Rapid determination of lipoprotein lipase activity in human adipose tissue. Clin Chim Acta 166: 359–367.
12. Henderson AD, Richardson W, Ellefsen RS (1993) Hepatic and lipoprotein lipases selectively assayed in postheparin plasma. Clin Chem 39: 218–223.
13. Eriksson JW, Buren J, Svensson M, Olivecrona T, Olivecrona G (2003) Postprandial regulation of blood lipids and adipose tissue lipoprotein lipase in type 2 diabetes patients and healthy control subjects. Atherosclerosis 166: 339–345.
14. Coca-Prieto I, Valldestil P, Olivecrona G, Ariza MJ, Rioja J, et al. (2009) Lipoprotein lipase activity and mass, apolipoprotein C-II mass and polymorphisms of apolipoproteins E and A5 in subjects with prior acute hypertriglyceridemic pancreatitis. BMC Gastroenterol 9: 46.
15. Basu D, Manjuri J, Jin W (2011) Determination of lipoprotein lipase activity using a novel fluorescent lipase assay. J Lipid Res 52: 826–832.
16. McFarland JT, Rojas C (1986) 1,2-Dipalmitoyl-3-O-(m-2-furylacryloyl)glycerol: a chromophoric substrate for lipoprotein lipase. Biochem Biophys Acta 876: 438–449.
17. Dousset N, Negre A, Salvayre R, Regolle P, Dang QQ, et al. (1988) Use of a fluorescent radiolabeled triacylglycerol as a substrate for lipoprotein lipase and hepatic triglyceride lipase. Lipids 23: 605–608.
18. Rawyler A, Siegenthaler PA (1989) A single and continuous spectrophotometric assay for various lipolytic enzymes, using natural, non-labelled lipid substrates. Biochim Biophys Acta 1004: 337–344.
19. Wircher I, Sandel W, Boeckin A, Kostner GM, Zechner R, et al. (1996) Quantification of lipoprotein lipase (LPL) by dissociation-enhanced lanthanide fluorescence immunoassay. Comparison of immuno-reactivity of LPL mass and enzyme activity of LPL. Immunol Methods 192: 1–11.
20. Panteghni M, Bonora R, Pagano F (2001) Measurement of pancreatic lipase activity in serum by a kinetic colorimetric assay using a new chromogenic substrate. Ann Clin Biochem 38: 363–370.

Table S1 Lipids and apoproteins of the VLDL substrates (n = 40 different pools of VLDL).

(DOC)

Table S2 Genetic variants identified in candidate genes in TVHTG patients.

(DOC)

Acknowledgments

We would like to thank Pr Gilles Rionfol, Pr Gérard Finet, Dr François Sassolas, and Pierre-Valentin Lalitte for their contribution in control recruitment, and Dr Bénédicte Mousson de Camaret and Marjorie Dumoux for technical assistance.

Author Contributions

Conceived and designed the experiments: MDF CM SC ML PM AS. Performed the experiments: OM MB MD MM AN VPD. Analyzed the data: MDF CM SC OM MB MD MM AN VPD. Contributed reagents/materials/analysis tools: OM MB MD MM AN VPD. Wrote the paper: MDF CM SC ML PM AS RV.

New Post-Heparin Lipoprotein Lipase Activity Assay