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## **Molecular and functional analysis of two new *MTTP* gene mutations in an atypical case of abetalipoproteinemia.**

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**Running head:** Functional analysis of two new *MTTP* mutations

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**List abbreviations in the order cited**

ABL abetalipoproteinemia

*MTTP* microsomal triglyceride transfer protein large subunit gene

MTP microsomal triglyceride transfer protein large subunit

PDI protein disulfide isomerase

P4HB prolyl 4-hydroxylase, beta polypeptide

ApoB apolipoprotein B

MCT medium chain triglyceride

PCSK9 proprotein convertase subtilisin/kexin type 9

ANGPTL3 angiopoietin-like 3

## **Abstract**

Abetalipoproteinemia (ABL) is an inherited disease characterized by the defective assembly and secretion of apolipoprotein B-containing lipoproteins caused by mutations in the microsomal triglyceride transfer protein large subunit (MTP) gene (*MTTP*). We report here a female patient with an unusual clinical and biochemical ABL phenotype. She presented with severe liver injury, low levels of LDL-cholesterol, subnormal levels of vitamin E, but only mild fat malabsorption and no retinitis pigmentosa or acanthocytosis. Our objective was to search for *MTTP* mutations and to determine the relationship between the genotype and this particular phenotype.

The subject exhibited compound heterozygosity for two novel *MTTP* mutations: one missense mutation (p.Leu435His) and an intronic deletion (c.619-5\_619-2del). COS-1 cells expressing the missense mutant protein exhibited negligible levels of MTP activity. In contrast, the minigene splicing reporter assay showed an incomplete splicing defect of the intronic deletion with 26% of the normal splicing being maintained in the transfected HeLa cells.

The small amount of MTP activity resulting from the residual normal splicing in the patient explains the atypical phenotype observed. Our investigation provides an example of a functional analysis of unclassified variations, which is an absolute necessity for the molecular diagnosis of atypical ABL cases.

**Supplementary key words:** dyslipidemias, familial hypocholesterolemia, chylomicrons, lipoprotein/assembly, hepatosteatosis, genetics, gene expression, genotype-phenotype correlation, functional analysis, splicing

Abetalipoproteinemia (ABL; OMIM#200100) is an autosomal recessive hypocholesterolemia usually detected during infancy due to failure to thrive, severe diarrhoea and a lipid malabsorption syndrome. Sixty years ago, Bassen and Kornzweig described this disease for the first time (1) and forty years later, the role of MTP (microsomal triglyceride transfer protein large subunit) was discovered (2-5). MTP forms a heterodimer with protein disulfide isomerase (PDI) also named prolyl 4-hydroxylase beta polypeptide (P4HB) which is responsible of the assembly of apolipoprotein B (ApoB) containing lipoproteins in the liver and the intestine. Following the discovery of the first mutations, many mutations have been identified in the microsomal triglyceride transfer protein large subunit gene (*MTTP*) in patients (3-6). The identification of mutations in the *MTTP* gene in DNA from patients is important for establishing the diagnosis of ABL in the context of two other hereditary hypocholesterolemias (homozygous familial hypobetalipoproteinemia OMIM#107730 and Anderson's disease/Chylomicron retention disease, OMIM#246700) which are due to mutations in the *APOB* and *SAR1B* genes, respectively.

To date, mutations in the *MTTP* gene have been established in about 50 cases of ABL (7-12). In some instances, the prediction of altered MTP function is readily made on the basis of premature stop codons, mutations in canonical splice sites or frameshift mutations. In other cases, the functional consequences have been difficult to predict (13-15), even if the mutations co-segregate with clinical phenotype. The measurement of MTP activity (2, 3, 6, 11, 13, 16, 17) and the consequence of intronic mutations (9, 11) have been studied in only a few cases due to the necessity of additional intestinal or hepatic biopsies.

ABL patients are usually diagnosed during infancy and they exhibit marked lipid malabsorption, very low levels of cholesterol and triglycerides, the absence of

ApoB in the plasma, the absence of chylomicrons after fat loading, and essential fatty acid and fat-soluble vitamin deficiencies (particularly vitamin E). Hepatic steatosis has not been reported uniformly (although it is frequent), and hepatic fibrosis or cirrhosis has been reported in a few cases (18, 19). We report here an atypical case of ABL presenting in childhood with severe liver injury, hypocholesterolemia associated with a low (but not absent) levels of plasma ApoB and a subnormal level of plasma vitamin E. Furthermore, the patient exhibited normal development into adulthood. ABL was suspected because of the presence, at the ultrastructural level, of large amounts of free lipid droplets accumulated in the cytoplasm of enterocytes and hepatocytes in intestinal and liver biopsies.

The patient was found to be compound heterozygous for two novel mutations in the *MTTP* gene, one intronic (c.619-5\_619-2 del, from her mother) and the other a missense mutation (p.Leu435His, from her father). The possible functional impact of these two mutations was not readily apparent. Since no additional biopsy material was available from the patient, two different assays were established. COS-1 cells were transfected with wild type and several mutant *MTTP* cDNAs in order to evaluate the impact of the missense mutation on MTP activity and HeLa cells were transfected with wild-type and mutant minigenes to evaluate possible defects in splicing.

## **Material and methods**

### ***Patient (diagnosis and follow-up)***

A 4 year-old girl was referred for an anicteric chronic hepatitis fortuitously discovered 10 months previously. The level of blood transaminases were consistently elevated and ranged from 5 to 7 times the normal values associated with bilirubinemia in the absence of inflammation or hepatic insufficiency. Upon clinical

examination, the liver was moderately enlarged and firm, but there was no other sign of chronic liver disease. The patient's growth was normal. The patient's parents altered her diet to avoid fatty foods following episodes of diarrhoea in the first months of life. The levels of total cholesterol and ApoB in the plasma were found to be low, whereas the level of triglyceride was normal (Table1). The parents were not related and they had normal levels of blood lipids.

A perendoscopic jejunal biopsy, performed as previously described (20), showed a white mucosa. Electron microscopic examination of the biopsy showed enterocytes with many small or very large free lipid droplets scattered throughout the cytoplasm (Figure 1a, 1b). Examination by ultrasound showed that the liver was moderately enlarged and slightly hyper-echogenic. A percutaneous liver biopsy showed extensive fibrosis (scored F3 according to the METAVIR scoring system (21)) which was associated with a massive micro- and macro-vesicular steatosis. Large amounts of free lipid droplets were observed in the cytoplasm by electron microscopy (Figure 1c). Abetalipoproteinemia was suspected although retinitis pigmentosa and acanthocytosis were absent and the blood vitamin E level was only slightly decreased. The fecal fat content, measured over a 3 day period, was also normal (mean 2.8 g/24h, normal < 3g/24h). However, when the child consumed a normal fat diet (33% fat), diarrhoea occurred and steatorrhoea was 24 g/day. After an oral fat load (Table1), the level of serum triglycerides increased slightly and a small amount of chylomicrons appeared in the plasma, suggesting that some intestinal lipid absorption occurs accompanied by low levels of intestinal lipoprotein secretion.

A diet limited in saturated fatty acids (22% of total energy) and enriched with medium chain triglycerides (MCT) was begun and resulted in the elimination of diarrhoea, progressive improvement in liver function (transaminases 2 to 4 X normal

at the age of 7 years, 2 to 3 X normal at 14 years, normal values at 18 years of age), the normalization of the levels of essential fatty acids and fat soluble vitamins, and normal growth and puberty. At the age of 14 years, the patient no longer adhered to the MCT diet and 36% of total energy intake was composed of fat. Hepatitis recurred (transaminases increased to 150 to 200 X normal) but resolved when the MCT diet was re-instituted.

The patient is now 22 years of age and is in good health. Fat soluble vitamins are in the normal range without any supplementation. The levels of transaminases are < 1.5 X normal and hepatic steatosis persists (16% of hepatic parenchyma as determined by magnetic resonance imaging). There are no neurological, ophthalmological or retinal abnormalities.

Blood samples, intestinal and liver biopsies were obtained from the patient using the procedures and the experimental methods approved by INSERM (RBM 0256) and by a bioethics committee (Comité Consultatif de Protection des Personnes dans la Recherche Biomédicale de Paris Bichat-Claude Bernard, Paris, France, CCPPRB Bichat-C. Bernard-2003/05). Informed, written consent was obtained from the patient's parents.

### ***Mutation analysis***

Following the extraction of genomic DNA from the blood (Nucleon Bac3, GE Healthcare®, Chalfont St. Giles, UK, <http://www.gehealthcare.com>), each of the coding exons, and the flanking intronic junctions, of the *MTTP*, *APOB*, proprotein convertase subtilisin/kexin type 9 (*PCSK9*) and angiopoietin-like 3 (*ANGPTL3*) genes was amplified by PCR. The amplicons were sequenced directly with the BigDye®

Terminator v3.1 Cycle Sequencing Kit on an ABI PRISM 3130 or 3730 DNA sequencer (Applied Biosystems, Foster City, USA, [www.appliedbiosystems.com](http://www.appliedbiosystems.com)).

### ***In Silico Analysis***

Analysis of the mutations was performed with Alamut v2.0 (Interactive Software), Polyphen (<http://genetics.bwh.harvard.edu/pph/>) and SIFT ([http://sift.jcvi.org/www/SIFT\\_aligned\\_seqs\\_submit.html](http://sift.jcvi.org/www/SIFT_aligned_seqs_submit.html)). Several algorithms were used for computational scoring of 3' splice site based on different concepts using default parameter settings (Neural Network Splice Prediction (NNSplice) (22), MaxEntScan (23), Splice site Finder Like, GeneSplicer (24), Human Splicing Finder (HSF) (25)).

### ***Protein expression***

To evaluate the p.Leu435His protein expression, two assays were performed. First, to measure wild type and mutant MTP activities, we transfected COS-1 cells with the *MTTP* and *P4HB* cDNA. Second, to compare the production of wild type and p.Leu435His, we transfected COS-1 cells with wild type and mutated *MTTP* cDNA C-terminally tagged with GFP.

### **MTP activity**

A 2699 bp fragment containing the entire *MTTP* coding sequence, extending from c.-5 to c.\*9 (NM\_000253.2) and a 1582 bp fragment containing the entire *P4HB* coding sequence (from c.-14 to c.1527, NM\_000918.3) were obtained by RT-PCR from 1 µg of human liver total RNA (Cat.No.636531, Clontech, Mountain View, U.S.A, [www.clontech.com](http://www.clontech.com)) with the Transcriptor High Fidelity cDNA Synthesis Sample Kit

(Cat.No.05081963001 Roche Applied Science, Indianapolis, USA, [www.roche-applied-science.com](http://www.roche-applied-science.com)).

The *MTTP* cDNA was inserted, with the In-Fusion Advantage PCR Cloning Kit (Cat.No.639616, Clontech, Mountain View, U.S.A, [www.clontech.com](http://www.clontech.com)), into the *Kpn* I site of the pBudCE4.1 expression vector (Cat.No.V532-20 Invitrogen, Carlsbad, U.S.A, [www.invitrogen.com](http://www.invitrogen.com)) downstream of the human elongation factor 1 $\alpha$ -subunit promoter. The *P4HB* cDNA was inserted into the *Hind* III site of the same vector, downstream of the human cytomegalovirus immediate-early promoter, allowing the production of the two proteins from the same plasmid.

The p.Leu435His (c.1304T>A), p.Leu435Glu (c.1303\_1305delinsGAA), p.Leu435Val (c.1303C>G) and p.Cys194Stop (c.582C>A) mutants were constructed from the wild type sequence with the QuickChange II XL Site-Directed Mutagenesis Kit (Cat.No.200521 Stratagene, Agilent Technologies, Cedar Creek, USA, [www.agilent.com](http://www.agilent.com)) according to the manufacturer's instructions.

Transient expression of MTP and P4HB in COS-1 cells (Cat. No. CRL-1650 ATCC) was carried out by transfecting 6  $\mu$ g of plasmid per T25 flask in the presence of 9  $\mu$ L of FuGENE® HD (Cat.No.4709691001 Roche Applied Science, Indianapolis, USA, [www.roche-applied-science.com](http://www.roche-applied-science.com)), according to the manufacturer's instructions.

COS-1 cells were harvested by trypsinization 48 hours post-transfection and disrupted by sonication. Triglyceride transfer from donor to acceptor vesicles was measured by a fluorescent-labelled method using a commercial kit (R100 MTP activity, Chylos Inc, USA, [www.chylos.com](http://www.chylos.com)) according to the manufacturer's instructions and previously described fluorescent methods (26-28). The results are expressed as % transfer/mg total proteins/h. The method was evaluated with

intestinal and hepatic biopsies and the MTP activities were in agreement with previous published results (data not shown).

#### Visualisation of MTP in transfected cells

A fragment containing the *MTTP* cDNA, extending from c.1 to c.2682 (NM\_000253.2) of each sequence (wild type, p.Cys194Stop, p.Leu435His) was amplified from the previously obtained pBudCE4.1 vector. The *MTTP* cDNA was inserted, with the In-Fusion Advantage PCR Cloning Kit into the *KpnI* site of pAcGFP1-N1 expression vector (Cat.No.632469 Clontech, Mountain View, U.S.A, www.clontech.com) between the immediate early promoter of the CMV (PCMV IE) and the AcGFP1 cDNA allowing the fusion of the MTP and the GFP proteins. COS-1 cells were electroporated with 0.5 µg of MTP-AcGFP1 plasmid per 80 000 cells (MicroPorator, DigitalBio Technology and Neon Transfection System 10µL Kit, Cat.No. MPK1096, Invitrogen Carlsbad, U.S.A, www.invitrogen.com) according to the manufacturer's instructions (1050V, 30ms, 2 pulses). Intracellular fluorescence was observed with a Nikon Eclipse TE 2000-U microscope 48h after transfection.

#### ***Minigene Splicing Reporter Assay***

A 371 bp *MTTP* fragment (the last 147 bases of intron 5, 140 bp of exon 6, and the first 84 bases of intron 6) was amplified from the patient's DNA and inserted (In-Fusion Advantage PCR Cloning Kit) into the *Nde I* restriction site of the pTB minigene vector (29). The transfection of normal and mutant minigenes into HeLa cells and RT-PCR procedures were as previously described (30).

The fluorescence of the ethidium bromide bands obtained following gel electrophoresis of RT-PCR products was integrated under unsaturated conditions

(Quantity One<sup>®</sup> 1-D Analysis Software Cat. No170-9600, BIO-RAD, www.bio-rad.com) to derive band intensities.

### ***Metabolic labelling of intestinal biopsies***

Intestinal biopsies from normal individuals and from the patient were placed into organ culture and metabolically labelled with [<sup>35</sup>S] methionine as described previously (31). After homogenization and solubilization, the labelled intestinal biopsy extracts and the corresponding media were immunoprecipitated with polyclonal antibodies against Apo B (Rabbit polyclonal antibodies to Apo B were the gifts of Dr A. Mazur of Institut National de la Recherche Agronomique, Champanelle, France) and MTP (31). For semi-quantitative analysis, densitometric analysis was performed using the PC version of NIH Image software (Scion Image) after photography with a computer-assisted camera (GS-800 Calibrated Densitometer, BIO-RAD). The values were normalized with respect to the amount of TCA precipitable incorporated material in the biopsies.

## **Results**

### ***Identification of mutations***

Sequencing was performed on the genomic DNA of the patient and her parents. Two novel variants of the *MTTP* gene were identified in the patient. The first, inherited from her father, is a change from T to A at position c.1304 in exon 10 (Supplemental Data, Figure 1A) (Nucleotide numbering starts at A of the ATG initiating codon and exon 1 is the first coding exon) which changes the amino acid Leu 435 to a His. The second, inherited from her mother, is a 4 bp deletion in intron

5: c.619-5\_619-2 del (Supplemental Data, Figure 1B). This deletion of a repeated motif (TTTA) is upstream the acceptor site. These mutations were found neither in our panel of 100 normal alleles from unrelated subjects nor in the 1000 genomes database (32).

The patient is also heterozygous for a well described polymorphism p.Gln95His inherited from her father. The frequency of this allele is between 5.4 and 6% in a healthy adult Caucasian population (England) and in a sample of 270 unrelated French Canadian men (6, 33) and 6.2% in a cohort of abetalipoproteinemia (6).

The patient had no mutation in the *APOB*, *PCSK9* or *ANGTL3* genes.

#### ***Analysis of c.1304 T>A, p.Leu435His:***

The missense mutation (p.Leu435His) is located in exon 10 and affects a highly conserved residue in the middle  $\alpha$ -helical domain of MTP. The substitution changes a hydrophobic to a hydrophilic, basic residue (Grantham distance: 99 [0-215]). This missense mutation is predicted by Polyphen to be “probably damaging” with a score of 0.99 and by SIFT to “affect protein function”.

To determine whether the p.Leu435His missense mutation produces a protein that is functional or not, we expressed, in COS-1 cells, the wild type MTP as well as the p.Leu435His and several other mutants (p.Leu435Glu, p.Leu435Val and p.Cys194Stop). These different mutations were tested in COS-1 cells to evaluate the sensitivity and specificity of our MTP activity assay: p.Leu435Glu is expected to be as severe as p.Leu435His as it changes the hydrophobic Leu to a hydrophilic acidic Glu, whereas p.Leu435Val is expected to be a mild change (Leu and Val are both hydrophobic and closely related amino acids). The introduction of a premature stop

codon at the position p.Cys194 is expected to lead to the complete abolition of MTP activity since the ApoB and PDI binding sites are absent from the mutant. Further, the homozygous p.Cys194Stop mutation has been reported in an ABL patient (8).

As shown in Figure 2, cells expressing p.Leu435His (the patient's mutation), p.Cys194Stop, or p.Leu435Glu have negligible levels of MTP activity (0.30 to 1.9% TG transfer/30µg protein/h) as compared to cells expressing p.Leu435Val or the wild type protein (24.15 to 27.25 % TG transfer/30µg protein/h respectively). The activity of the p.Leu435His MTP is not significantly different from the p.Cys194Stop truncated protein and, thus, must be considered as having a negligible MTP activity as compared to the wild-type protein. RT-PCR of the *MTTP* and *P4HB* transcripts in transfected COS-1 cells shows that the level of expression from each vector is comparable for the wild type and the different mutant transfected cDNA (Supplemental Data, Figure 2A and 2B). Fluorescent tagged MTP showed an identical cellular distribution (Supplemental Data, Figure 2C). The p.Leu435His mutant, therefore, is functionally defective.

### ***Analysis of c.619-5\_619-2del:***

#### Splicing predictions

All the algorithms predicted an effect on splicing with a decrease in the score for the acceptor site of intron 5 ranging from -10% for SSF-like to a complete abolition for GeneSplicer, (-44% for MaxEnt Scan, -38.9% for NNSplice, -79% for HSF). Given that all the algorithms predicted an effect on splicing, experimental analysis of splicing was performed. HeLa cells transfected with wild type or mutant minigenes were analyzed (size and sequence) by RT-PCR to determine the presence of abnormal transcript processing of the mutant.

## Minigene splicing reporter assay

Transfection of the wild type minigene into HeLa cells produced, as expected, a 386 bp RT-PCR fragment (Figure 3, lanes 1, 2 3). In contrast, transfection of the mutant c.619-5\_619-2del minigene produced 2 different RT-PCR fragments: a small amount of a fragment of normal size (386bp) and a large amount of a 248 bp product (lanes 4, 5, 6). The sequence of the 386 bp PCR product includes exon 6 of *MTTP* whereas exon 6 is skipped in the 248 bp product (Supplemental Data, Figure 3). This result shows that c.619-5\_619-2del mutation produces two differently spliced transcripts: one containing exon 6 and the other without exon 6. Exon 6 skipping would result in a protein containing the first 206 MTP amino acids but followed by 26 aberrant amino acids and a premature stop codon. *In vivo*, this mutant mRNA might be targeted for nonsense mediated decay and degraded. By densitometry of the agarose gel, the amount of normal splicing for the mutant minigene is estimated to be 26% of that of the wild type (Figure 3). These data indicate that the c.619-5\_619-2del is a splice site mutation which can lead to the skipping of exon 6. However, the effect of this mutation on splicing is not complete and about 26% of the transcript is correctly spliced.

## ***Biosynthesis of ApoB and MTP in intestinal organ culture***

Immunoprecipitation with polyclonal antibodies to ApoB of the medium and the total homogenate of the organ culture of intestinal biopsies from the patient showed the synthesis of an ApoB48 identical in size to that of normal control subjects (Figure 4). Analysis by densitometry with correction for the amount of incorporated TCA

precipitable incorporated radioactivity showed that there is an intracellular retention of ApoB48 (1.6 fold more) and a decreased ApoB48 secretion (1.3 fold less).

Immunoprecipitation with polyclonal antibodies to MTP of the total homogenate of the organ culture of intestinal biopsies from the patient showed the synthesis of a large subunit of identical in size to that of normal control subjects. However, by densitometric analysis, there was 2.49 fold less MTP synthesized in the patient's biopsy as compared to that of the normal control subject after correction. A protein band corresponding to the molecular mass of PDI co-immunoprecipitated with the MTP in the patient as was observed for the normal control subject (identical in amount as compared to that of the normal subject after correction).

## **Discussion**

In this paper, we describe two new mutations in the *MTTP* gene: p.Leu435His and a 4 bp deletion in intron 5: c.619-5\_619-2 del. Since the impact of these mutations was unclear and the phenotype was atypical, cellular functional tests were used to further understand this new case of ABL.

The diagnosis of ABL is made readily if the following criteria are present during the first months of life: severe diarrhoea, failure to thrive, undetectable vitamin E, severe hypocholesterolemia and hypotriglyceridemia with undetectable amounts of plasma ApoB, and the absence, post-prandially, of chylomicrons in the serum. Our patient presented with a mild phenotype and lacked the major criteria for the diagnosis of ABL. Surprisingly, the patient was referred only for anicteric chronic hepatitis with major liver steatosis at 4 years of age. The other causes of hypobetalipoproteinemias were ruled out because of the patient's lipids profile and normal lipid values of the parents (34-40). Furthermore, no mutation was found in the

*PCSK9*, *ANGPTL3*, *APOB* genes. The diagnosis of ABL was finally suspected because of hypocholesterolemia and the typical aspect of intestinal and liver biopsies suggesting a defect in the assembly of ApoB-containing lipoproteins.

Seven atypical cases of ABL have been reported (13-15, 19, 41, 42). For five of these patients (Table 2) a molecular diagnosis of the mutation in *MTTP* was established. However, a functional assay of the mutation was performed in only one case (13). Two of the cases (cases 1 and 2 in Table 2) are very surprising. Both the patients have deleterious mutations, however, the first patient exhibits a mild phenotype (42) and the other is almost asymptomatic (13). Two other patients (cases 4 and 5 in Table 2) are homozygous for the same p.Ser590Ile mutation (14, 15). Unfortunately the functional impact of this mutation has not been established. Only the third case (19) (Table 2) resembles our case, clinically and biologically. This patient exhibits compound heterozygosity for a splice-site and a missense mutation, along with liver dysfunction and slightly decreased level of vitamin E.

The p.Leu435His mutation affects an amino-acid in helix 8 of the predicted  $\alpha$ -helical domain of MTP (43). Although this helix is not included in the major binding site for PDI and ApoB (43-45), the MTP activity of the mutant protein is very low and similar to that of “severe” mutations. The p.Leu435His mutation leads to a non-functional protein probably by affecting the MTP folding. Misfolded MTP may be targeted for degradation in the intestine or the liver; however, this recognition of misfolded proteins cannot be observed in heterologous expression systems (transfected COS-1 cells).

The c.619-5\_619-2del is probably responsible for the mild phenotype as it produces two transcripts. One is normal-sized and contains exon 6 of the *MTTP*. The other transcript is aberrantly spliced and lacks exon 6. The phenotype may result

from the correctly spliced RNA which is able to produce an active MTP. Although there may be some variability in the amounts of correctly spliced RNA between HeLa cells and the liver or the intestine, the marked effect of the intronic deletion observed in HeLa cells most likely reflects what might be expected in other cell types. A possible molecular mechanism for this splicing anomaly, predicted by the *in silico* analysis, is the shortening of the polypyrimidine tract by the deletion of one TTTA repeat. Short polypyrimidine tracts have been showed to be associated with variable levels of correctly spliced transcripts in the CFTR gene (46) thus providing evidence for incomplete penetrance of some splicing mutations in disease.

In our patient, the lipid transfer activity of the MTP produced from the normally spliced transcript may be sufficient to allow the assembly and secretion of a relatively limited amount of ApoB-containing lipoproteins by liver and intestine and could explain the presence of the lipid absorption (as shown by metabolic labelling biosynthesis data) observed in the intestine of the patient. Both the intracellular accumulation and the decreased secretion of ApoB48 from the intestine are consistent with a partial defect in the assembly of ApoB which leads to a partial defect in secretion of ApoB48 (unusual in ABL). In addition, the data also suggest that a low amount of intestinal MTP was present with a normal molecular weight and with an intact PDI binding domain. Finally, the presence of a limited amount of fat absorption and lipoprotein secretion could explain the normal level of vitamin E and the normal development with only a low fat diet as treatment. However, the residual MTP activity is not sufficient to prevent the accumulation of lipids in the enterocytes and the hepatocytes.

In conclusion, our study of this atypical case of abetalipoproteinemia shows that the combination of molecular diagnosis and functional analysis resulted in a

definitive diagnosis of MTP deficiency in a patient when insufficient biopsy material was available for the analysis of the MTP protein or RNA. Second, the functional studies of the substitution of Leu435 to a charged amino acid (acid Glu or basic His) highlight the requirement for this hydrophobic un-charged residue for MTP activity. Third, in cases of unusual phenotype, the functional characterization of the *MTTP* mutants allows a better understanding of the milder ABL phenotype.

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**Figure 1: Ultrastructure of intestinal and liver biopsies after a 12-hour fast.**

**Figure 2: Triglyceride transfer activity of normal and mutant MTP in COS-1 cells over a period of 48h.**

**Figure 3: Size separation of RT-PCR products as determined by 2% agarose gel electrophoresis:**

**Figure 4: Intestinal biosynthesis of ApoB48 and MTP in organ culture**

Table 1: Biological data for the patient and the parents.

	TC mmol/L	TG mmol/L	HDLC mmol/L	LDLC mmol/L	ApoB g/L	ApoA1 g/L	VitE µmol/L	VitA µmol/L	VitD nmol/L	ALT UI/L	AST UI/L	ALB g/L	BMI kg/m <sup>2</sup>
<i>Reference interval*</i>	2.58-4.39	0.34-1.25	>1.03	1.81-3.10	0.5-1.2	1.1-2.0	11-35	1.4-3.0	34-130	<30	<30	35-50	
Patient (at diagnosis, 4yrs of age)	1.68	0.58	0.85	0.57	< 0.18	ND	9.9	1.7	159	287	272	32	14
Oral fat load (5yrs) T0	2.27	1.15	2.17		0.20	0.93							
Oral fat load T 90 min	2.06	1.50	1.86		0.20	0.99							
Patient (22yrs old) (reduced fat diet)	2.24	0.64	1.70	0.26	ND	ND	18.9	3	96	49	41	45	17.8
Father	5.42	0.97	1.42	3.57									
Mother	4.64	1.84	1.52	2.77	1.01	1.19				18	14		

TC total cholesterol, TG triglyceride, HDLC HDL cholesterol, LDLC LDL cholesterol, Vit vitamin, ALT Alanine aminotransferase, AST Aspartate aminotransferase, ALB albumin, BMI body mass index, ND : not determined

\* normal values for 4 a year old child.

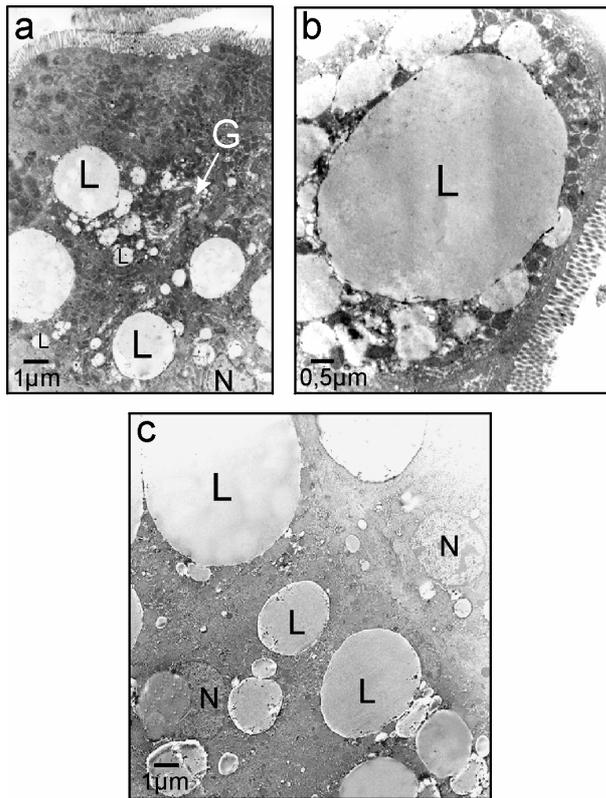
Oral fat load with 15g of fat.

Table 2: Atypical mild cases of ABL reported in the literature

	case 1	case 2	case 3	case 4	case 5
ref.	(42)	(13)	(19)	(15)	(14)
mutation	[c. 61+2T>C]+ [c.419-420insA]	homozygous p.Asn780Tyr	[c. 61+1G>C]+ [p.Ile564Thr]	homozygous p.Ser590Ile	homozygous p.Ser590Ile
age at diagnosis (year)	13	27	1.25	52	24
age at present (year)	32	27	5	52	44
context of diagnosis	NA	during a routine medical examination	hepatomegaly + liver dysfunction	during a routine medical examination	NA
malabsorption	+	0	NA	+	+
liver impairments	ALT, AST, GGT increased	mild fatty liver	hepatomegaly, steatosis, fibrosis	NA	NA
neurological impairments	reduced reflexes	0	0	0	0
ophthalmological impairments	minor xerophthalmia, abnormal dryness of membrane of the eyes	0	0	atypical retinitis pigmentosa	retinopathy
Vit E (µmol/L)	14 (RI: 12-36) on replacement therapy	<2.4	10 (RI: 18-34)	23 (RI: 12-46)	NA
TC (mmol/L)	0.90	0.87	1.25 to 2.36	0.85	NA
TG (mmol/L)	0.50	0.03	0.11 to 1.14	0.06	NA
HDLC (mmol/L)	0.50	0.59	NA	0.68	0.56
LDLC (mmol/L)	0.07	NA	NA	0.16	NA
ApoB (g/L)	0.06	0.006	< 0.007	NA	NA
MTTP activity	NA	negligible	NA	NA	NA

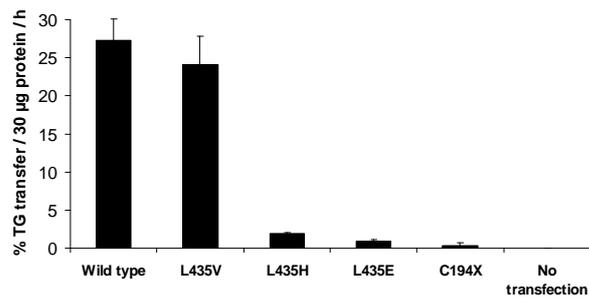
TC total cholesterol, TG triglyceride, HDLC HDL cholesterol, LDLC LDL cholesterol, Vit + present, 0 absent, NA not available, RI reference interval

**Figure 1**



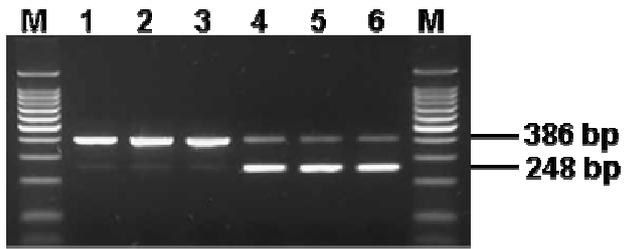
The enterocytes (a,b) and hepatocytes (c) were engorged with numerous small or very large lipid droplets (L) free in the cytoplasm. The Golgi apparatus (G) is empty (b). Hepatic intercellular spaces were sometimes enlarged with fibrosis (c). The cell nucleus is labelled N.

**Figure 2**



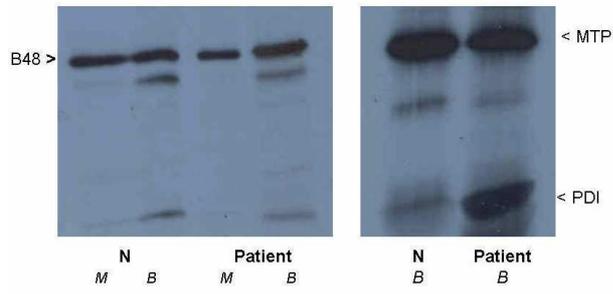
Cell homogenates were used to measure triglyceride transfer from donor to acceptor vesicles using fluorescent-labelled method membranes. Columns and bars represents means  $\pm$  SD (n=2).

**Figure 3**



M: molecular weight marker, lanes 1, 2, 3: normal minigene, lane 4 5 6: mutant (c.619-5\_619-2del) minigene.

**Figure 4**



ApoB48 was synthesized by the duodenal biopsy (B) and secreted in the medium (M) with the same molecular weight as normal control (N). MTP from the patient had the same molecular weight as normal control (N). PDI was coimmunoprecipitated: the binding of MTP with PDI was intact in the intestinal biopsy.

## Supplemental Data 1 : Sequencing of genomic DNA

Figure 1A : Sequencing of the exon 10 of the *MTTP* gene

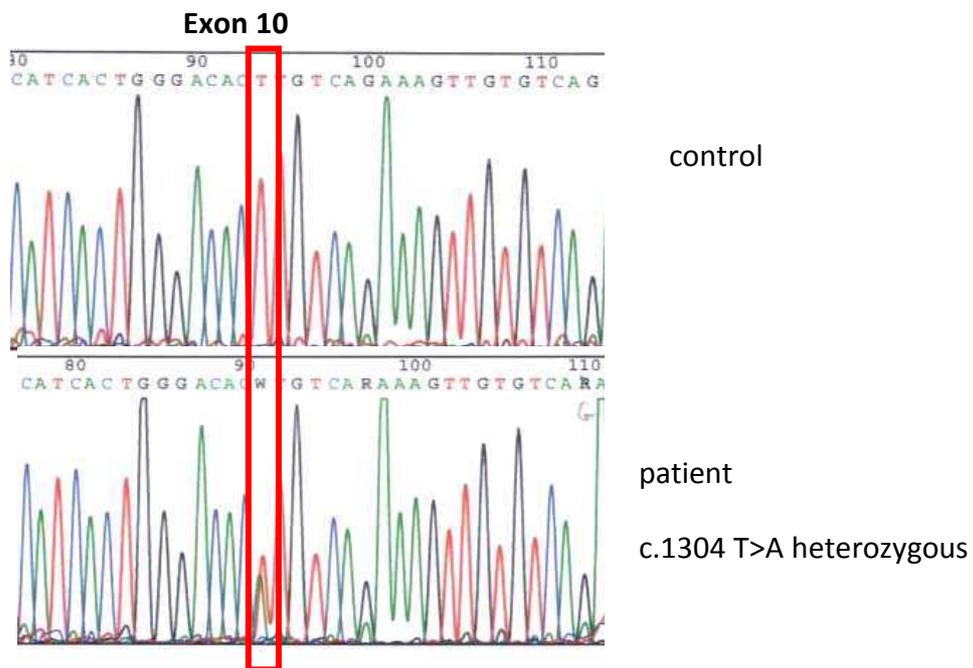
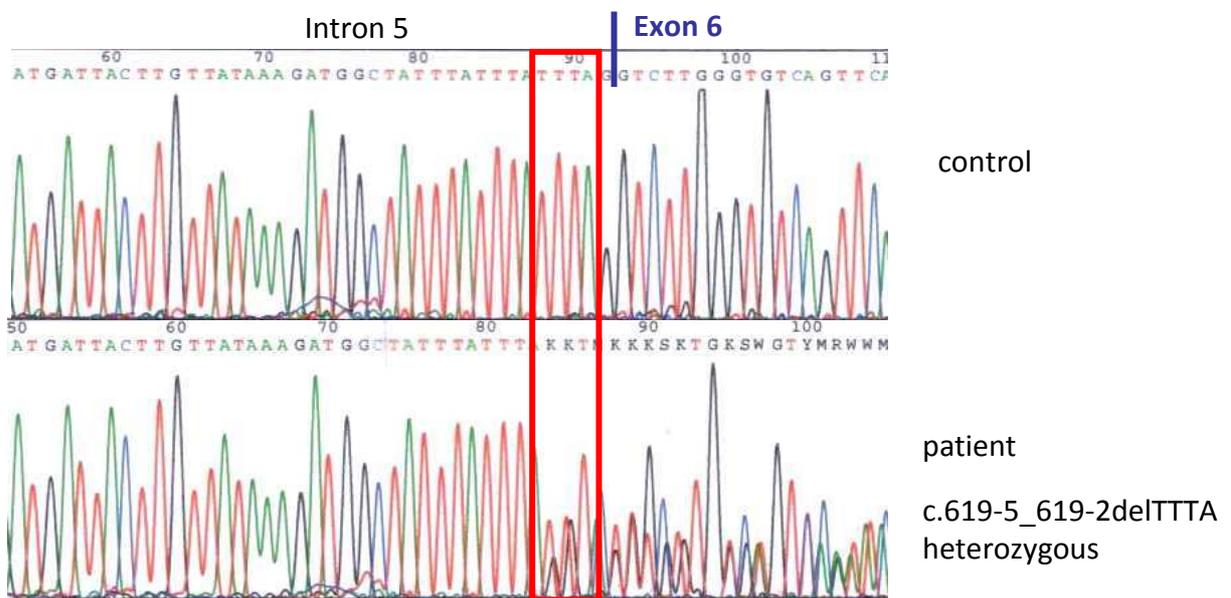
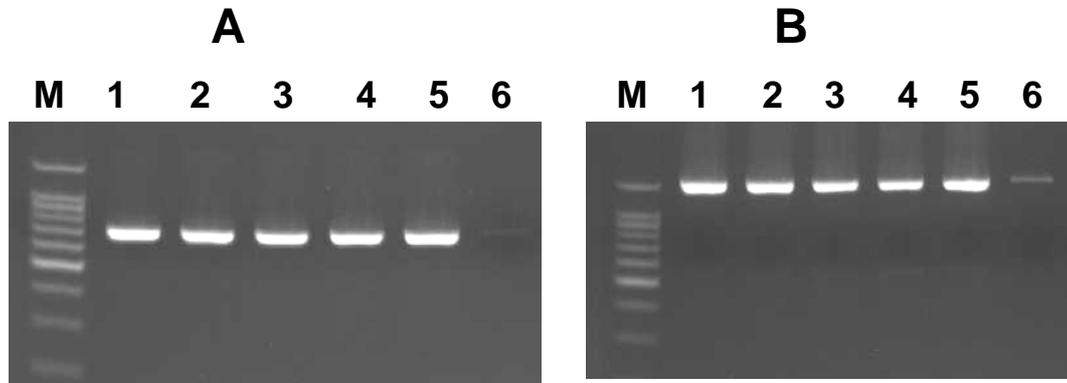


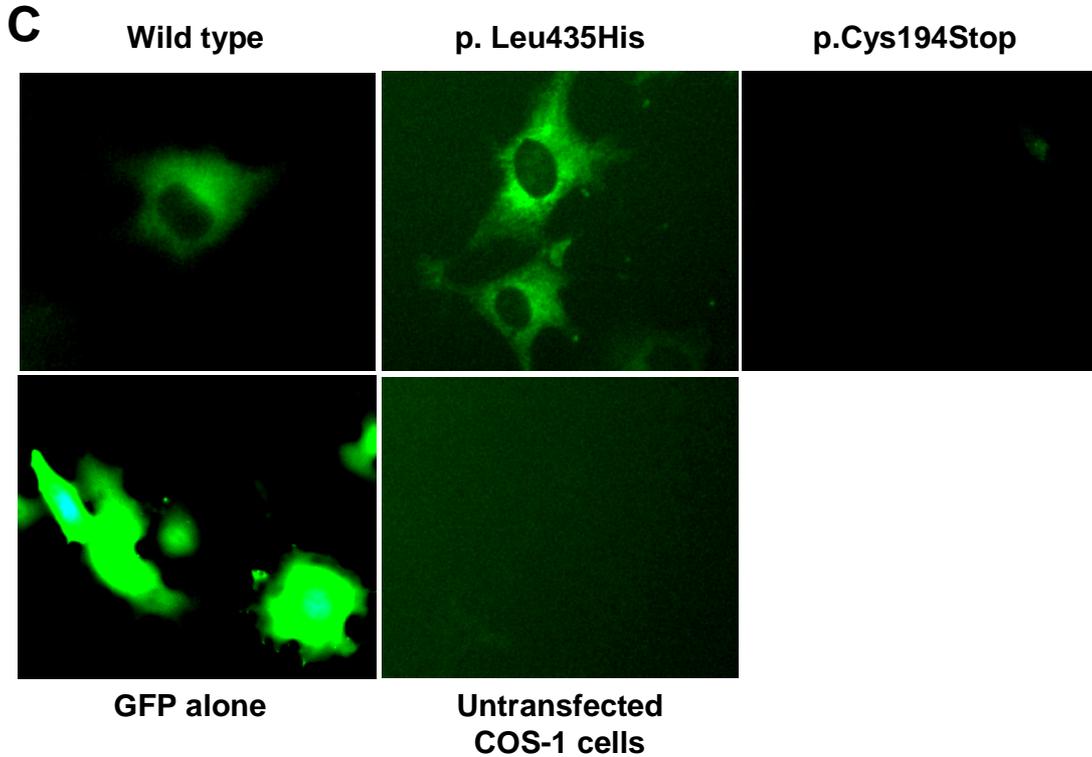
Figure 1B : Sequencing of the intron 5 and exon 6 of the *MTTP* gene



## Supplemental Data Figure 2



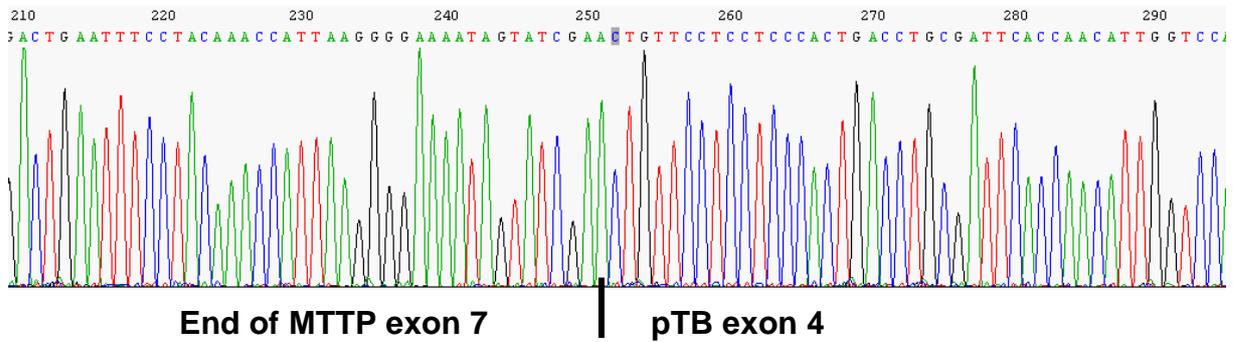
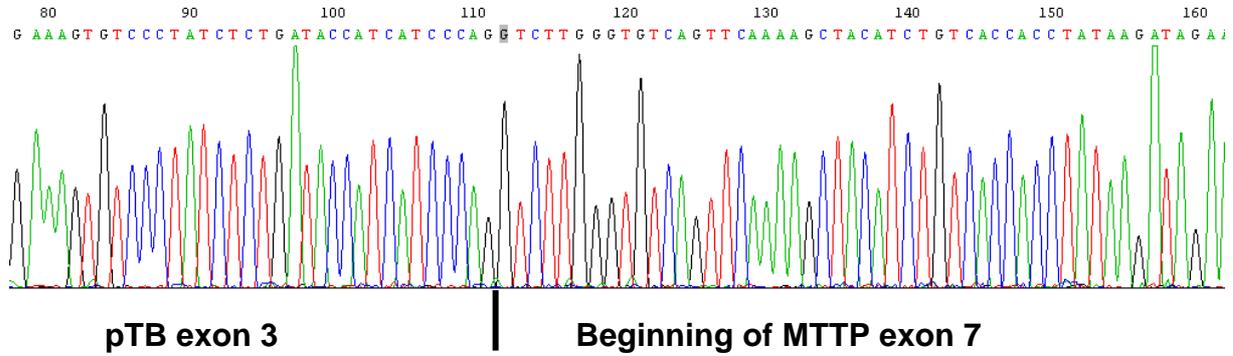
5  $\mu$ L of RT-PCR products were loaded on a 2% agarose gel. **A**: Total RNA was extracted from COS-1 cells transfected with the wild type MTP c.DNA (1) or the p.Leu435His c.DNA (2) or the p.Leu435Val c.DNA (3) or the p.Leu435Glu c.DNA (4) or the p.Cys194Stop c.DNA (5) or untransfected (6). After reverse transcription, amplification of a 706 bp fragment was performed with primers cMTTP-946F and cMTTP-1098rev. **B**: same as **A** but a 1582 bp PDI c.DNA fragment was amplified with primers PDI-Hind3-1 and PDI-Hind3-2.



COS-1 cells were transfected with MTP-AcGFP1 fusion vector by microporation. Fluorescence was observed using Nikon Eclipse TE 2000-U microscope (objective 20) 48 hours after transfection.

### Supplemental Data Figure 3

Sequencing of the 386 bp RT- PCR product from minigene transfection :



Sequencing of the 248 bp RT- PCR product from minigene transfection :

