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Normal serum ApoB48 and red cells vitamin E concentrations after supplementation in a novel compound heterozygous case of abetalipoproteinemia

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HIGHLIGHTS

- Clinically typical abetalipoproteinemia (ABL) proband with two *MTTP* mutant alleles.
- ABL with normal apoB48 concentration but deficient serum fat soluble vitamins.
- c.1868G > T undergoes abnormal splicing and infrequently p.R623L functional variant.
- Requirement of methods detecting heterozygous copy number variation on *MTTP*.
- Normal vitamin E in red cells in case of normal apoB48 secretion.

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ABSTRACT

Background and aims: Abetalipoproteinemia (ABL) is a rare recessive monogenic disease due to *MTTP* (microsomal triglyceride transfer protein) mutations leading to the absence of plasma apoB-containing lipoproteins. Here we characterize a new ABL case with usual clinical phenotype, hypocholesterolemia, hypotriglyceridemia but normal serum apolipoprotein B48 (apoB48) and red blood cell vitamin E concentrations.
Methods: Histology and MTP activity measurements were performed on intestinal biopsies. Mutations in *MTTP* were identified by Sanger sequencing, quantitative digital droplet and long-range PCR. Functional consequences of the variants were studied *in vitro* using a minigene splicing assay, measurement of MTP activity and apoB48

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secretion.
Results: Intestinal steatosis and the absence of measurable lipid transfer activity in intestinal protein extract supported the diagnosis of ABL. A novel *MTTP* c.1868G > T variant inherited from the patient's father was identified. This variant gives rise to three mRNA transcripts: one normally spliced, found at a low frequency in intestinal biopsy, carrying the p.(Arg623Leu) missense variant, producing *in vitro* 65% of normal MTP activity and apoB48 secretion, and two abnormally spliced transcripts resulting in a non-functional MTP protein. Digital droplet PCR and long-range sequencing revealed a previously described c.1067 + 1217_1141del allele inherited from the mother, removing exon 10. Thus, the patient is compound heterozygous for two dysfunctional *MTTP* alleles. The p.(Arg623Leu) variant may maintain residual secretion of apoB48.
Conclusions: Complex cases of primary dyslipidemia require the use of a cascade of different methodologies to establish the diagnosis in patients with non-classical biological phenotypes and provide better knowledge on the regulation of lipid metabolism.

1. Introduction

Abetalipoproteinemia (ABL; OMIM 200100) is a rare autosomal recessive disorder characterized by extremely low levels of plasma total cholesterol (TC) and triglycerides (TG) due to an almost complete absence of apolipoprotein B (apoB)-containing lipoprotein (apoB-LP) secretion [1]. This defect affects both apoB48-containing chylomicrons (CM) and apoB100-containing very-low density lipoproteins (VLDL) leading to an absence of low density lipoproteins (LDL) [2,3]. ABL is caused by a deficiency of the microsomal triglyceride transfer protein (MTP) large subunit encoded by *MTTP*, the microsomal triglyceride transfer protein gene [2–4]. The underlying mechanism involves the absence of MTP-protein disulfide isomerase (PDI) heterodimer that acts as a chaperone for the production of apoB-LP. This complex is required for the transfer of neutral lipids to nascent primordial apoB-LP and may facilitate their fusion with luminal lipid droplets [5,6]. In case of MTP deficiency, apoB cannot be lipidated properly and is degraded by the proteasome [7,8].

ABL subjects are unable to secrete CM and VLDL from the intestine and liver, respectively. The absence of CM leads to lipid malabsorption, symptoms related to lipid-soluble vitamin and essential fatty acid deficiencies [9–11]. The absence of VLDL secretion induces liver steatosis leading to fibrosis and cirrhosis in many cases [11]. To date, at least 59 deleterious *MTTP* variants have been reported in ABL patients [11–17]. The molecular diagnosis is considered as the “gold-standard” diagnostic test, allowing the diagnosis of atypical ABL patients [11,15,18] and establishing the differential diagnosis *versus* co-dominant familial compound heterozygous or homozygous hypobetalipoproteinemia (Ho-FHBL; OMIM 107730) due to deleterious variants on the *APOB* gene, encoding apoB [11, 19]. Parents of ABL cases usually exhibit normal lipid profile whereas parents of Ho-FHBL cases exhibit mild hypobetalipoproteinemia [19,20].

We report herein a new case of ABL occurring in childhood with typical clinical and lipid profile. However, the diagnosis was delayed by her normal plasma apoB48 concentration, mild hypolipoproteinemia in both parents, and the requirement of additional methods to Sanger sequencing allowing the subsequent identification of heterozygous copy number variations (CNV).

2. Materials and methods

2.1. Patient

The patient is the first child of a non-consanguineous French family. She was delivered full-term after a normal pregnancy. Her birth measurements were normal (weight: 3.5 kg, height: 50 cm).

2.2. Lipid profile, fat load, apoB electrophoresis, and vitamin E determination

Lipid profile, CM, apoB48 and vitamin E concentration and apoB48 characterization can be found in Supplementary Material.

The proband received an oral fat load as described in Supplementary Material to assess the plasma TG concentration response.

2.3. MTP activity on intestinal biopsy

Intestinal biopsies of the proband and 11 control subjects without ABL were processed to obtain protein extracts and determine MTP activity as previously described [18,21,22] and detailed in Supplementary Material. The results are expressed as UI per gram of total protein per hour.

2.4. Variant and in silico analysis

Following the extraction of genomic DNA (gDNA) from the blood, each of the coding exons, as well as 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 and amplicons were sequenced as previously described [11]. Analysis of the mutations was performed using Alamut v2.11 as described in Supplementary Material.

2.5. Protein activity and subcellular localization of the missense variant

Three assays were performed as previously described [13,18,23] to evaluate the c.1868G > T p.(Arg623Leu) expression in COS cells: measurement of MTP activity, apoB48 secretion after cotransfection with the *MTTP* and *P4HB* (encoding PDI) cDNA, and subcellular localization after transfection with wild type (WT) or mutated *MTTP* cDNA C-terminally tagged with GFP (Supplementary Material).

2.6. Minigene splicing reporter assay and analysis of spliced products in HeLa cells after minigene transfection

As NM_000253.3(MTTP):c.1868G > T variant affects the first base of exon 15 and may affect splicing, minigene splicing reporter assay was performed as previously described [18] and as detailed in Supplementary Material.

2.7. RNA extraction from intestinal biopsy sample, PCR amplification and sequencing

Total RNA was isolated, reverse transcribed into first strand cDNA; cDNA was then amplified and the PCR product was cloned into a TOPO vector and sequenced (Supplementary Material).

2.8. Quantitative PCR and large rearrangement characterization

Digital droplet quantitative PCR was performed from gDNA of the proband and her parents to detect CNV as detailed in Supplementary

Material. Sequencing of the genomic deletion breakpoints was performed after long range PCR as previously described [11].

3. Results

3.1. Patient (diagnosis and follow-up)

Diarrhea appeared after the first week of life. She was referred to the hospital at 1 month of age for an acute pyelonephritis and at 4 months of age for growth retardation (weight: 3.8 kg, < -2SD, height: 58 cm, -2SD, cranial perimeter: 37 cm, -2SD), with abdominal distension, diarrhea, and vomiting. Hepatomegaly was observed, blood transaminases were moderately elevated (Table 1) and hepatic steatosis was suspected because of the hyper-refringent liver observed at ultrasound examination. Her prothrombin ratio was decreased (18%) due to vitamin K deficiency (normal factor V). A low-fat infant formula with a high medium-chain TG content (Monogen®, Nutricia, Dublin, Ireland) was started. Liposoluble vitamin replacement was initiated with vitamin E (Vedrop®, Orphan Europe Laboratories, Puteaux – France, 600 U/j), vitamin K (10 mg/week), vitamin A-313 (A313®, Laboratoire Pharma Développement, Corbigny, France, 50 000 U/week), vitamin D (Sterogyl®, Desma Pharma, Chiasso, Switzerland, 1600 U/day) in addition to omega 3 supplementation (Omacor®, Laboratoire Pierre Fabre Médicament, Ussel, France, 1 capsule containing EPA 460 mg and DHA 380 mg, capsules were open and drank twice a week). The treatment stopped the diarrhea and improved growth.

At 14 months of age, her body weight was 9.1 kg (-1SD) and her height was 72 cm (-1SD). She had no hepatomegaly, no diarrhea and no steatorrhea over a 3-day period strict low-fat diet (2–3 g/24 h of lipids). The liver elastometry was normal (4.4 kPa at 1 year of age) but the levels of blood transaminases remained moderately increased (Table 1). At 18 months of age, upper-endoscopy and duodenal biopsies showed vacuolated enterocytes (Fig. 1).

The patient is now 7 years of age and is in good health, without diarrhea under a strict low-fat diet. She presents no neurological, ophthalmological or cardiac abnormalities; a slight hepatic steatosis persisted upon ultrasound examination with normal liver elastometry (3.3 kPa).

3.2. Lipid profile, fat load, and apoB electrophoresis

At diagnosis, the concentrations of TC, TG, apoB, and vitamin E were found to be dramatically low in the patient (Table 1) as were the concentrations of vitamin A 0.25 µmol/L (normal range, NR: 0.5–2.40), vitamin K 44 ng/L (NR: 100–1000) and beta-carotene < 0.10 µmol/L (NR: 0.30–0.89). After supplementation, serum vitamin E concentrations increased (Table 1) but remained under the NR whereas the red cells concentrations were found to be normal (5.4 µmol/L; NR: 2.0–7.8 µmol/L) [24] as were the plasma and serum concentrations of vitamin A (0.77 µmol/L) and K (143 ng/L and 424 ng/L at 3 years of age). When the patient was diagnosed, the parents had serum LDL-C below normal concentration, which was more compatible with Ho-FHBL (dominant) than ABL (recessive); the serum LDL-C concentration of the parents was later found within normal range (Table 1).

When she was 1.5 years old, after an oral fat load (Table 1), the level of serum TG slightly increased from 0.05 to 0.10 mmol/L. However, CM remained undetectable. Plasma ApoB electrophoresis revealed the presence of both apoB100 and apoB48 in the fasting state, and an increase of apoB48 and apoB100 after oral fat load (Fig. 2A and B), suggesting some intestinal lipid absorption and low amounts of intestinal and hepatic lipoprotein secretion.

ApoB48 concentration in fasting plasma at 3 different ages (2, 3.5, and 4 years) was found to range from 8.40 to 11.06 mg/L in the normal IR (mean ± 2SD for controls: 5.81 ± 6.18 mg/L, n = 11). These values were higher than the limit of quantification (0.25 mg/L), and higher than apoB48 levels found in other ABL, in chylomicron retention disease (CMRD), and in Ho-FHBL patients (Fig. 2C).

3.3. MTP activity on intestinal biopsy

TG transfer activity in the intestinal biopsy at 18 months from the patient was not detectable (< 5% of the mean of controls subjects: mean ± SD: 569 ± 101 UI/mg/h, range: 393 to 761 UI/mg/h, n = 11) whereas alkaline phosphatase activity (124 UI/g of protein) was normal (mean ± SD for controls: 114 ± 41 UI/g, range: 56 to 173 UI/g of protein, n = 7). These findings, showing a major MTP deficiency, were in agreement with ABL and not Ho-FHBL.

Table 1
Biological data for the patient and the parents.

	Age	TC	TG	HDL-C	LDL-C	ApoA1	ApoB	PL	vitamin E	ALAT	ASAT
		mmol/L	mmol/L	mmol/L	mmol/L	g/L	g/L	mmol/L	µmol/L	Multiple of ULN	
Patient	4 m	**1.40**	**0.09**	1.42	**ND**	1.07	**< 0.26**	NA	**0.6**	**1.8**	**3.1**
	4.5 m	**1.61**	**0.05**	1.60	**ND**	0.97	**< 0.03**	NA	**8.6**[a]	NA	NA
	1.5 y	**1.26**	**0.05**	1.33	**ND**	NA	**< 0.04**	1.08	**6.4**[b]	NA	NA
	1.5 y T3h[e]	**1.31**	**0.10**	1.27	**ND**	NA	**< 0.04**	1.09	NA	NA	NA
	1.5 y T5h[e]	**1.22**	**0.07**	1.28	**ND**	NA	**< 0.04**	1.09	NA	NA	NA
	3 y 3 m	NA	NA	NA	NA	NA	NA	NA	**7.7**[c]	**3.0**	**2.4**
	3 y 9 m	**1.23**	**< 0.04**	NA	NA	NA	NA	NA	**8.0**[c]	**2.3**	**2.0**
	4 y 3 m	**1.32**	**< 0.04**	1.35	**ND**	NA	**< 0.04**	NA	**9.4**[d]	NA	NA
	5 y	**1.22**	**0.05**	1.21	**ND**	NA	**< 0.04**	NA	**9.2**[d]	NA	NA
	7 y	**1.24**	**< 0.04**	NA	NA	0.94	**< 0.03**	NA	**8.8**[d]	**1.5**	**1.9**
Sister	3 m[f]	3.63	2.42	1.41	1.12	NA	0.63	NA	NA	NA	NA
Father	24 y 9 m	**2.30**	1.33	**0.65**	**1.04**	1.35	**0.50**	NA	NA	NA	NA
	24 y 11 m	3.97	0.91	1.45	2.11	NA	0.58	NA	NA	NA	NA
Mother	26 y	**3.23**	0.76	1.45	**1.42**	1.35	0.55	NA	NA	NA	NA
	27 y	**3.10**	0.86	1.33	**1.38**	1.19	**0.48**	NA	NA	NA	NA
	30 y	3.71	0.57	1.44	2.01	NA	NA	NA	NA	0.3	0.6

[a.] to [d.]: under supplementation: [a].Vedrop® 100UI/kg/j, [b].Vedrop® 120 UI/kg/j, [c].Cambridge® 50UI/kg, [d] Cambridge® 100 mg/kg, [e]numbers of hours after fat load, [f] after meal.

TC: total cholesterol; TG: triglycerides; HDL-C: High Density Lipoprotein cholesterol; LDL-C: low density lipoprotein cholesterol; Apo: apolipoprotein; PL: phospholipids; ALAT: alanine aminotransferase; ASAT: aspartate aminotransferase; ULN: upper limit of normal; m: month; y: year; NA: not available; ND: not detectable. Bold type: under normal range of the laboratory. Vitamin E normal range: 16 to 30 µmol/L

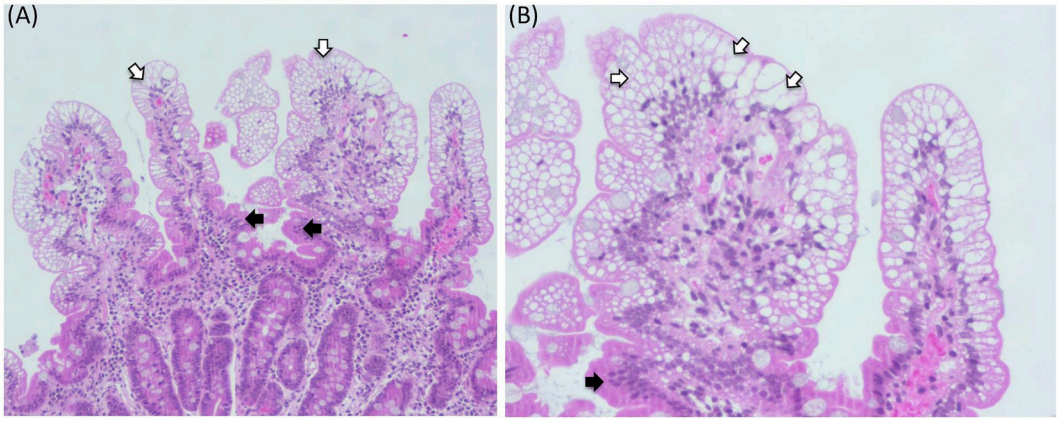

Fig. 1. Histological features in intestinal biopsy.
Duodenal biopsy obtained at 18 months and processed for Hematoxylin- Eosin- Saffron-staining. (A) ×100: intestinal villi mainly composed of vacuolated enterocytes and a few normal enterocytes as well as goblet cells. (B) x 200: focus on lipid droplets of variable sizes within the enterocytes. Black arrow: normal enterocytes; white arrows: vacuolated enterocytes.

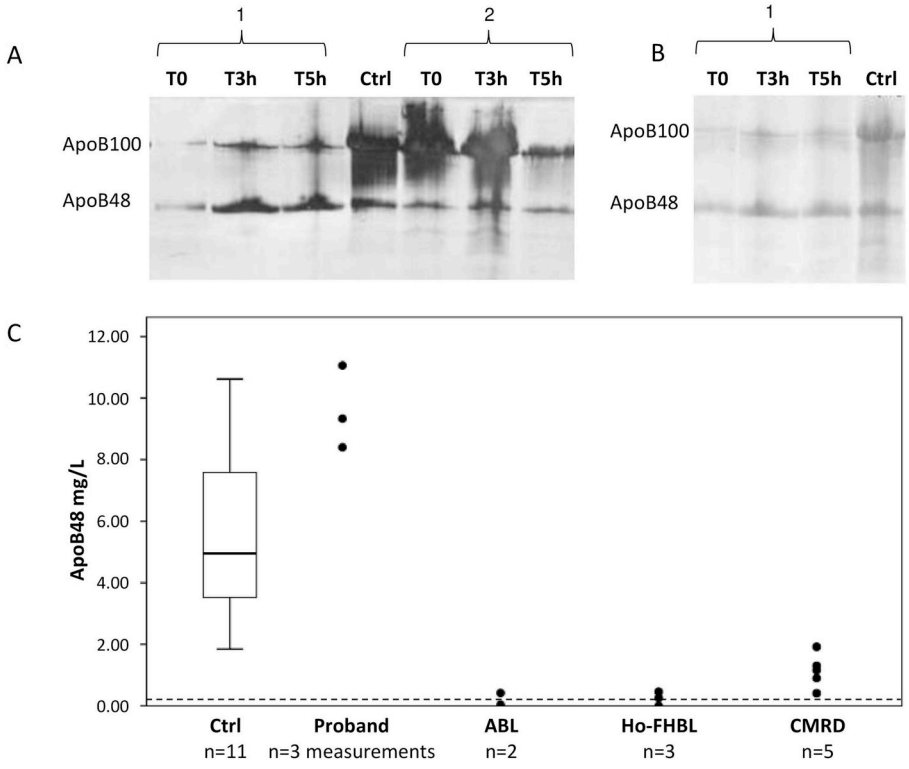

Fig. 2. ApoB electrophoresis and ApoB48 concentration.
(A and B) ApoB SDS-PAGE revealed by silver staining (A) or immunoblotting (B) before (T0) and after fat load for the proband (1), a normal subject (2); compared to a control (Ctrl: VLDL and chylomicrons). (C) Plasma apoB48 concentration in primary hypolipidemias and controls. Ctrl: controls; Proband n = 3 measurements (2, 3.5, and 4 years); ABL: abetalipoproteinemia; Ho-FHBL: homozygous or compound heterozygous hypobetalipoproteinemia; CMRD: chylomicron retention disease; dotted line: limit of quantification (0.25 mg/L).

3.4. Identification of MTTP variants

Two heterozygous, linked, nucleotide substitutions of the *MTTP* gene, both inherited from her father, were identified in the patient using Sanger sequencing. The first, NM_000253.3(MTTP): c.1868G > T leads to p.(Arg623Leu) missense variant and affects the first base on exon 15. This variant is not described in the gnomAD database or in ClinVar. The second, NM_000253.3(MTTP): c.1981G > A, is a well described p.(Gly661Ser) missense variant. The frequency of this allele (rs113337987) is 2.6% in the gnomAD database. The patient had no deleterious variants in the other genes involved in familial hypocholesterolemia (*APOB, PCSK9*, and *ANGTL3*). Her sister was not carrier of these single nucleotide variants on *MTTP*.

3.5. Analysis of functional impacts of c.1868G > T, p.(Arg623Leu)

3.5.1. Impact on MTP activity and ApoB48 secretion

The p.(Arg623Leu) variant affects a likely conserved residue. The substitution changes a basic hydrophilic to a hydrophobic residue. This missense variant is predicted by Polyphen to be “probably damaging” and by SIFT to be “tolerated”.

COS-7 cells expressing *MTTP* c.582C > A (p.(Cys194*)) showed no measurable MTTP activity, but COS-7 cells expressing *MTTP* c.1868G > T (p.(Arg623Leu)) (the patient's variant) showed 60–65% MTTP activity *versus* wild *MTTP* (Fig. 3A). The subcellular localization of the p.Leu623 missense mutant was then compared to the p.Arg623 (WT): both displayed a similar pattern, showing a reticular localization throughout the cytosol (Supplementary Fig. 1).

COS-7 cells expressing *MTTP* c.582C > A (p.(Cys194*)) variant

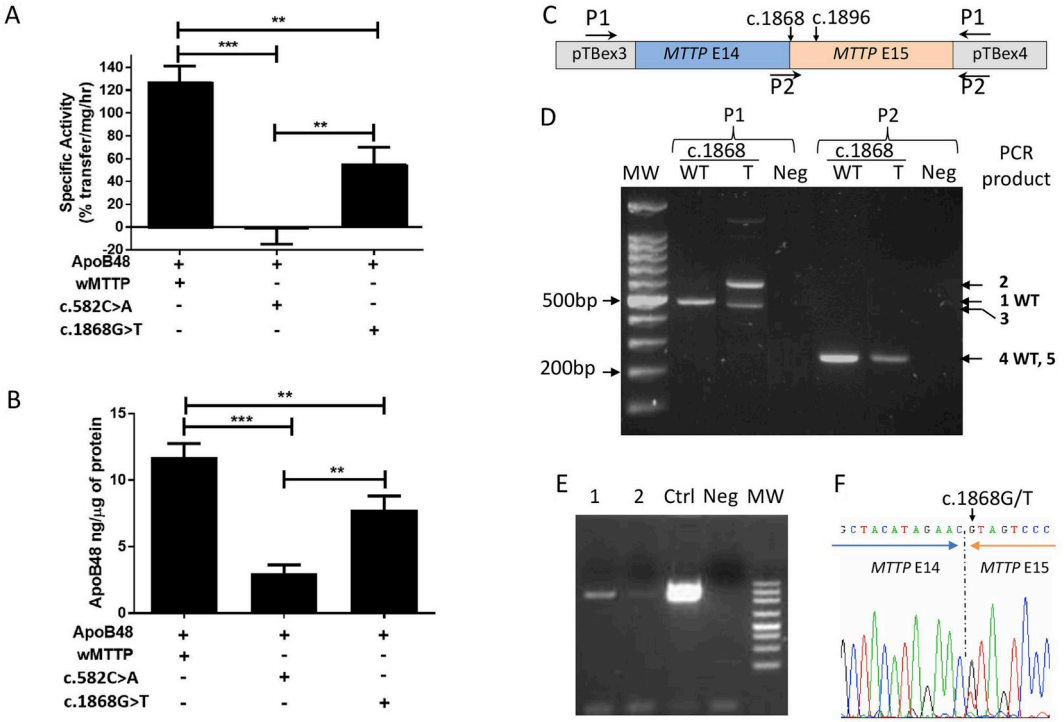

Fig. 3. *In vitro* and *in vivo* characterization of the c.1868G > T, p.(Arg623Leu).
(A) Activity of normal (wMTTP) and mutant *MTTP* in COS-7 cells 72 h after transient transfection; $^{**}p < 0.01$; $^{***}p < 0.001$. (B) ApoB48 secretion in the medium of COS-7 cells 72 h after transient cotransfection in triplicate with plasmid expressing human apoB48 and different indicated *MTTP* expression plasmids. (C) Localization of the two pairs of primers, P1, complementary to the vector, and P2, a forward primer complementary to the exon 14-exon 15 junction (MTP_E14-15chimNL MTP_E14-15chimML) and a reverse primer complementary to the vector from the RT product. (D) *Ex vivo* analysis performed in HeLa cells transfected either with the WT (c.1868G) or mutant (c.1868T) minigene using two pairs of primers. Gel electrophoresis of RT-PCR products obtained with either P1: primer pair 1; P2: primer pair 2; PCR products 1 to 3 were obtained using P1 primer pair: 1: wild type (466bp); 2: 560 bp product retaining complete intron 14; 3: shorter fragment (438bp) including an abnormal exon 15 lacking the 28 first bases; PCR products 4 and 5 were obtained using P2 primer pair. 4: wild type (240bp) with G at position c.1868; 5: 240bp fragment with T at position c.1868. The sequence of these 5 PCR products were shown on Supplementary Figs. 3 and 4. (E) and (F) Sequence analysis of *MTTP* transcription product from intestinal biopsy. After total RNA extraction and reverse transcription from a control biopsy and patient intestinal biopsy, PCR was performed to sequence cDNA from nucleotides 1285 to 2051. PCR products from two different RT for the patient (lanes 1 and 2), and one for the control biopsy (Ctrl) were analyzed by migration on agarose gel (E) and sequenced (F).
Neg: negative control; MW: molecular weight marker.

showed least apoB48 concentration in media, whereas COS-7 cells expressing *MTTP* c.1868G > T (p.(Arg623Leu)) (the patient's variant) showed 35–40% reduced apoB concentration in media compared to wild type (Fig. 3B). There was no significant difference in the level of protein expression of different *MTTP* mutants (Supplementary Fig. 2). These studies suggest that *MTTP* c.582C > A (p.(Cys194*)) variant does not transfer triglyceride and does not support apoB48 secretion. In contrast, *MTTP* c.1868G > T (p.(Arg623Leu)) (the patient's variant) is able to support triglyceride transfer and apoB48 secretion, albeit to a lower extent compared to WT *MTTP*.

3.5.2. *Impact of the c.1868G > T on splicing*

3.5.2.1. Splicing predictions. As this nucleotide change affects the first base of exon 15, all the tested algorithms predicted an effect on splicing with a decrease in the score for the acceptor site of intron 15 ranging from −7.4% for SSF-like to a complete abolition for GeneSplicer, and NNSPLICE.

3.5.2.2. Minigene splicing reporter assay. To study the effect of this variant on splicing, RT-PCR products were analyzed using two pairs of primers (Fig. 3C).

A 466 bp expected RT-PCR fragment was produced after transfection of the WT minigene in HeLa cells (Fig. 3D, PCR product 1 and Supplementary Fig. 3, lanes 1, 2, and 3). In contrast, transfection of the mutant c.1868G > T minigene mainly produced 2 different RT-PCR fragments: a 560 bp product retaining complete intron 14, and a shorter fragment (438bp) including an abnormal exon 15 lacking the 28 first bases (Fig. 3D, PCR products 2 and 3 respectively, Supplementary Fig. 3, lanes 4, 5, 6, and Supplementary Fig. 4). Both transcripts would result in an aberrant protein with a premature stop codon. Minigene experiments indicated that the c.1868G > T leads mainly either to an activation of an exonic cryptic acceptor splice site at c.1896 in exon 15 or to a complete retention of intron 14. A very faint 466 bp fragment, almost undetectable by gel electrophoresis, was also present in products from the mutant minigene.

To better characterize normally spliced products from the mutant minigene, cDNA was amplified with primer pair 2 (Supplementary Fig. 5). A PCR product of the expected size (240 bp) was obtained from RT PCR of both minigenes, WT and mutant, and Sanger sequencing confirmed the presence of the correct junction of exons 14 and 15 for both minigenes (with the presence of G and T at c.1868; Fig. 3D, PCR products 4 and 5, Supplementary Fig. 5). This result confirmed that a very small amount of a normally spliced transcript was also produced from the mutant minigene (Supplementary Fig. 6).

3.5.2.3. Amplification and sequence analysis of cDNA from intestinal biopsies. To study the *in vivo* effect of the c.1868G > T variant, RT-PCR of RNA prepared from the patient's biopsies was carried out spanning nucleotides 1285 to 2051 of *MTTP* cDNA. One PCR product exhibiting the expected size was found (Fig. 3E); this confirmed that the two misspliced mRNA transcripts from the variant containing the premature termination codons were targeted for nonsense-mediated

decay and degraded. As expected, the sequence analysis of this transcript showed the presence of a normal sequence with a G/T mosaicism at positions c.1868 (Fig. 3F) and a G/A at c.1981 (from the paternal p.Gly661Ser variant).

3.6. Detection and characterization of a copy number variation (CNV)

As the patient exhibited a phenotype in agreement with ABL and only one heterozygous variant was found by Sanger sequencing, CNV were studied using digital droplet PCR. No quantitative difference was observed for exons 3, 5, 6, 11, 13 17, 18, and 19 compared to the reference, while exon 10 of the proband and her mother exhibited approximately 50% of amplification products compared to the reference suggesting a heterozygous deletion (Fig. 4A).

After long-range PCR (Fig. 4B) and sequencing, the deletion breakpoints were identified (Fig. 4C and D): the patient, her mother and her sister presented a 2198bp deletion, which begins in the intron 9 and ends in exon 10, the NM_000253.3(MTTP):c.1067+1217_1141del variant, which was previously described as c.1068–2127_1138del. Deletion of the first 71 bases of exon 10 would induce a premature stop codon (p.(Pro357Phefs*4).

4. Discussion

We report the result of in-depth *MTTP* gene analysis in a proband with very low serum TG and TC but normal apoB48 concentration. The phenotype let to suggest either an ABL or Ho-FHBL diagnosis.

The phenotypic differential diagnosis between ABL and Ho-FHBL is often difficult as they can share similar clinical and biological manifestations [11]. The consideration of the lipid profile of the proband's parents is valuable: *MTTP* obligate heterozygotes usually have normal lipid profile while *APOB* heterozygotes are expected to have LDL-C and apoB levels below the 5th percentile according to age and sex [11,19,25]. Accordingly, mild hypocholesterolemia in both proband's parents was suggestive of Ho-FHBL in the proband. However, the parents follow-up showed a fluctuant hypobetaliproteinemic lipid profile, and in the literature, 14% of the heterozygous carriers of deleterious *MTTP* variants were previously found to have low plasma LDL-C and apoB [17]. In these cases, genetic analysis is mandatory to distinguish ABL from Ho-FHBL, and to consistently establish the diagnosis.

In the patient presented herein, intestinal biopsies were available, and the absence of detectable MTP activity suggested the diagnosis to ABL. However, Sanger sequencing detected only a new heterozygous variant of unknown significance, p.(Arg623Leu), on the *MTTP* paternal allele. The p.(Arg623Leu) is predicted to be surface exposed (Supplemental Fig. 7) in C-terminal ß-sheets domain of MTP mediating the lipid-binding and transfer catalytic activity of MTP [20]. Other missense variants in this domain and have been implicated in defective lipid transfer activity [11,26–28]. Since the impact of this missense variant was unclear and furthermore fasting concentration of apoB48 was normal and increasing after fat load, cellular functional tests were undertaken to decipher the functionality of this new variant. Its residual activity, correct subcellular localization and the remaining apoB48 secretion in the medium found *in vitro* strongly suggest that, unlike arginine at position 540 supposed to form a salt bridge with PDI [29], this residue at position 623 is less critical for MTP function. Additionally, *in vitro*, the c.1868T variant produced two major abnormally spliced transcripts and a very low level of a normally spliced transcript carrying the missense variant p.623Leu. Interestingly, *ex vivo*, in intestinal biopsy, only the normally spliced transcript carrying the missense variant was found, but at a very low level. This variant was consequently classified as pathogenic according to the American College of Medical Genetics and Association of Medical Pathologists (ACMG) guidelines [30].

Subsequent quantitative PCR allowed the identification of the maternal deleterious variant. This case underlines the necessity to routinely detect CNV in *MTTP*. Until now, CNV were reported in only 5 ABL-patients, for which the molecular mechanism (uniparental disomy, contiguous large gene deletion, or a homozygous deletion of an exon [11,31–34]) allowed detection by Sanger sequencing, whereas

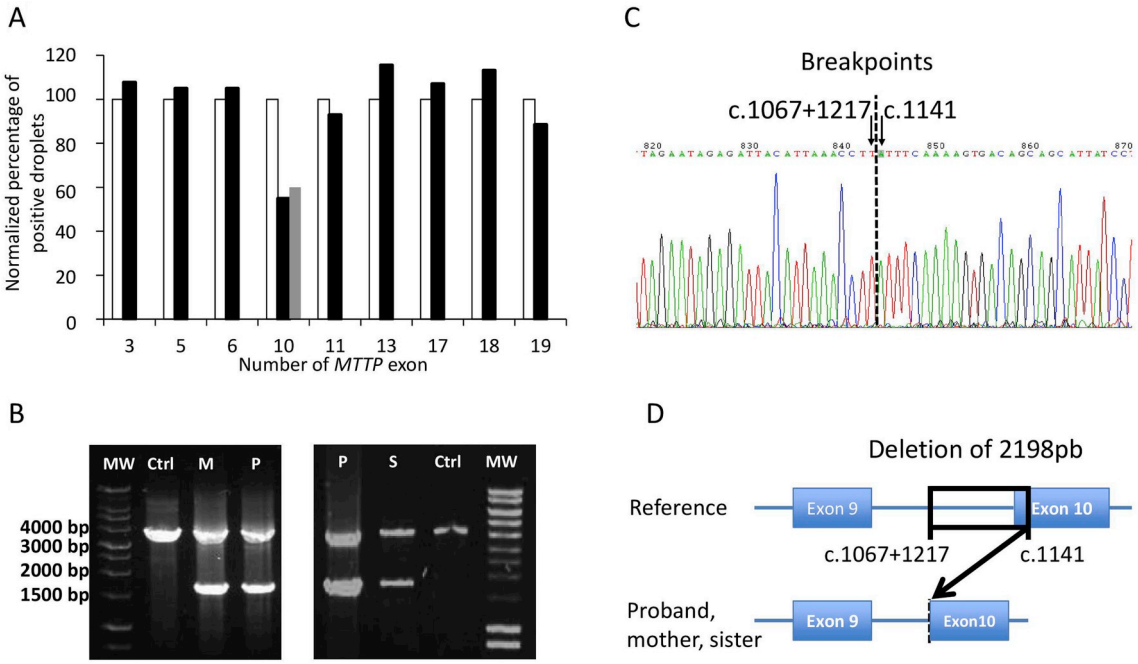

Fig. 4. Characterization of the c.1068–2127_c.1138del large genomic rearrangement.
(A) Digital droplet PCR: normalized percentage of positive droplets was represented for each tested exon. Deletion was suspected in case of percentage lower than 50%. White bars: father, used as control; Black bars: proband; grey bars: mother. (B) Long range amplification from gDNA expected size for control 3876 bp; MW: molecular weight; Ctrl: control; M: mother; P: proband; S: sister. (C) Sequence of the deleted PCR product; the black line shows breakpoints. (D) Schematic representation of the deletion.

heterozygous CNV are undetectable by Sanger sequencing. Consequently, it is of utmost importance to develop methods that allow the detection of CNV such as quantitative PCR or next-generation sequencing (NGS) for routine diagnosis [35–37], and therefore to allow the simultaneous detection of both single nucleotide variations and CNV, as recently demonstrated in other dyslipidemias [38,39].

The same partial deletion of exon 10 has previously been described in another apparently unrelated homozygous patient [11]. The translation product of this variant is predicted to create a premature termination codon eliminating the lipid-binding cavity and the central region involved in the interaction with PDI [5,40,41]. The exon 10 deletion, caused by other molecular defects, leads to the production of a smaller MTTP protein, detectable but unable to interact with PDI, devoid of activity and shown to prevent the apoB secretion [4,29,42].

To the best of our knowledge this is the first report of normal plasma apoB48 levels associated with an increase of apoB after fat load in a clinically typical ABL case. ApoB is usually not detected in serum even after fat load in clinically typical ABL patients [11,43,44]. In the patient described herein, the normal apoB48 secretion could be explained by the residual activity of the heterozygous new missense *MTTP* variant. Although this residual activity was not measurable in the tested biopsy, the limited amount of tissue might have hampered the sensitivity of this *ex vivo* test.

The case presented is of particular interest because it has been previously established that sufficient apoB lipidation was conditional to avoid rapid apoB presecretory degradation [45]. In this patient, normal apoB48 concentration but very low apoB100 amount, undetectable using classical immunoturbidimetric measurement, was found as well as a raise in apoB plasma concentration after fat load whereas serum TG only increased to a maximum of 0.10 mmol/L and CM were not detectable. This observation raises the hypothesis of differential pathways in intestinal and hepatic cells regarding apoB proteasomal degradation and secretion of apoB. In accordance with the patient reported herein, in liver tissue-specific knockout $Mttp^{\Delta/\Delta}$ mice and in cultured murine primary hepatocytes treated with a MTP inhibitor, a partial inactivation of *MTTP* lowered apoB100 levels in plasma by 90 to > 95% whereas the secretion of apoB48 was only slightly decreased, indicating that secretion of apoB100 is much more sensitive to MTTP activity than apoB48 [46,47]. Despite the presence of detectable apoB48, this proband exhibited a complex profile of fat-soluble vitamin deficiency.

Vitamin E is thought to be incorporated into CM and transported by lipoproteins in serum. Despite the normal concentration of apoB48 in the patient presented herein, supporting secretion of apoB48-LP from the intestine, her vitamin E plasma concentrations were as low as those reported in other ABL patients in agreement with the very low level of its serum transporter, the LDL [11,13,20,29,42,48–54]. However, the normal concentration of vitamin E in red blood cells despite the absence of CM detected after fat load, supports additional mechanisms allowing the saving of vitamin E absorption in the context of vitamin E supplementation under a strict low-fat diet and poorly-lipidated apoB48 secretion. This might involve cooperation between regulation of vitamin E transport, HDL production and residual apoB48 secretion by enterocytes [55]. Additional studies will be needed to carefully characterize size and composition of the lipoproteins from the proband, when she will be adult due to the amount of plasma needed in abetalipoproteinemic patient.

In conclusion, we have shown that the main functional impairment of the c.1868G > T *MTTP* variant is a splicing defect that produces a small amount of normally spliced transcript enabling some residual MTP activity and apoB48 secretion. ABL patients with incomplete splicing defects may present with different clinical phenotype according to the molecular defect of the other allele. This case provides further insights into the genetic basis of the phenotypic diversity of ABL, regarding functional domains of MTTP, residual MTP activity, and their impact on apoB48 secretion and vitamin E absorption. It shows that methods allowing CNV detection are required to diagnose complex primary hypolipidemia, for which NGS may be of great interest.

Conflicts of interest

Philippe Moulin has received honoraria paid to his university for participating in clinical trials and/or advisory boards and/or symposia organized by: AKCEA, AMT, Chiesi, and Uniqure. The other authors have nothing to disclose.

Author contributions

Clinical assessments: AR NP.

Experiments: conception and design: MDF SCF AJ CC DB MH; performed the experiments: CD SN SD SR.

Analyzed the data: MDF SCF CD SN OM AJ DB CC MH PM.

Wrote the paper, approve the version to be published, agree to be accountable for all aspects of the work: all authors.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.atherosclerosis.2019.02.016.

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