Novel missense *MTTP* gene mutations causing abetalipoproteinemia

Sharon A. Miller,^a John R. Burnett,^{b,c} Mike A. Leonis,^d C. James McKnight,^e Frank M. van Bockxmeer,^{c,f} and Amanda J. Hooper^{a,b,c,*}

Schools of ^aPathology & Laboratory Medicine and ^bMedicine & Pharmacology, University of Western Australia, Perth, Australia; ^cDepartment of Clinical Biochemistry, PathWest Laboratory Medicine WA, Royal Perth Hospital, Perth, Australia; ^dDivision of Gastroenterology, Hepatology & Nutrition, Cincinnati Children's Hospital Medical Center and Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH, USA; ^eDepartment of Physiology & Biophysics, Boston University School of Medicine, Boston, MA, USA; ^fSchool of Surgery, University of Western Australia, Perth, Australia

Dr. Sharon Miller, E-mail: sharon.miller@health.wa.gov.au

Clin/Prof. John Burnett, E-mail: john.burnett@health.wa.gov.au

A/Prof. Mike Leonis, E-mail: mike.leonis@cchmc.org

A/Prof. C. James McKnight, E-mail: cjmck@bu.edu

Prof. Frank M. van Bockxmeer, E-mail: frank.vanbockxmeer@health.wa.gov.au

*To whom correspondence should be addressed:

Dr. Amanda Hooper, Department of Clinical Biochemistry, PathWest Laboratory Medicine WA, Royal Perth Hospital, GPO Box X2213, Perth, WA 6847, Australia

Tel: +61 8 9224-2982 Fax: +61 8 9224-1789

E-mail: amanda.hooper@health.wa.gov.au

Abstract

Objective: The microsomal triglyceride transfer protein (MTTP) plays a critical role in the formation of hepatic very low density lipoprotein. Abetalipoproteinemia (ABL) is a rare, naturally-occurring extreme form of MTTP inhibition, which is characterized by the virtual absence of apolipoprotein (apo)B-containing lipoproteins in blood. The goal of this study was to examine the effect that four novel *MTTP* missense mutations had on protein interactions, expression and lipid-transfer activity, and to determine which mutations were responsible for the ABL phenotype observed in two patients.

Approach and Results: In two patients with ABL, we identified in *MTTP* a novel frameshift mutation, (p.K35Ffs*37), and four novel missense mutations, namely G264R, Y528H, R540C and N649S. When transiently expressed in COS-7 cells all missense *MTTP* mutations interacted with apoB17, apoB48 and protein disulfide isomerase. Mutations Y528H and R540C, however, displayed negligible levels of MTTP activity and N649S displayed a partial reduction relative to the wild-type MTTP. In contrast, G264R retained full lipid-transfer activity.

Conclusions: These studies indicate that missense mutations Y528H, R540C and N649S appear to cause ABL by reducing MTTP activity rather than by reducing binding of MTTP with protein disulfide isomerase or apoB. The region of MTTP containing amino acids 528 and 540 constitutes a critical domain for its lipid-transfer activity.

Keywords: abetalipoproteinemia, microsomal triglyceride transfer protein, lipid-transfer activity, missense mutations

1. Introduction

Abetalipoproteinemia (ABL; OMIM 200100) is a rare, autosomal recessive disorder of lipoprotein metabolism, which is characterized by a virtual absence of plasma apolipoprotein (apo) B-containing lipoproteins, such as chylomicrons, very low density lipoprotein (VLDL) and low density lipoprotein (LDL) [1, 2]. ABL is caused by mutations in the microsomal triglyceride transfer protein (*MTTP*) gene [1-3] and affected individuals often present in childhood with failure to thrive, fat malabsorption, acanthocytosis, and vitamin E deficiency [4, 5]. Patients with ABL cannot be distinguished clinically from compound heterozygous and homozygous familial hypobetalipoproteinemia (OMIM 101130), which is caused by mutations in the *APOB* gene [5-7].

MTTP is an endoplasmic reticulum protein that catalyses the transfer of neutral lipids between membrane vesicles and is essential for the assembly of apoB-containing lipoproteins by the liver and small intestine [8]. MTTP is a heterodimer composed of a P subunit (~55 kDa), which is a multi-functional, ubiquitously expressed protein, protein disulfide isomerase (PDI) and a larger M subunit (~97 kDa, 894 amino acids), which is primarily expressed in hepatocytes and enterocytes. Based on the sequence homology of MTTP with lipovitellin, the M subunit is proposed to have three major structural domains: N-terminal β -barrel (amino acid residues 22-297), which mediates the interaction with apoB, the central α -helical domain (amino acid residues 298-603), which associates with both PDI and apoB, and the C-terminal lipid-binding domain (amino acid residues 604-894), which is involved in lipid-binding and lipid-transfer activity [8-12].

To date more than 30 *MTTP* mutations causing ABL have been described. The majority of these are frameshift, nonsense and splice-site mutations which are located throughout the entire *MTTP* gene and are predicted to encode truncated forms of MTTP resulting in a complete loss of function [1, 2, 13-17]. Five missense *MTTP* mutations; L435H, R540H, S590I, G746E, and N780Y have been found to cause ABL and *in vitro* studies of these mutations has provided a valuable insight into the structure and function of MTTP [15, 17-22].

R540H, the first *MTTP* missense mutation described to cause ABL, prohibits the association of the M subunit with the PDI subunit, causing the mutant MTTP to remain as an insoluble aggregate and it does not support apoB secretion. R540H is thought to disrupt the R540-H531-E570 internal salt bridge, thereby affecting binding with PDI and subsequently the loss of both lipid-transfer activities [10, 15, 20].

The four other mutations described were found to all lack lipid-transfer activity, but still maintain the ability to bind to the PDI subunit. Mutations N780Y and G764E are thought to affect the tertiary structure of the carboxyl-terminal lipid-binding domain, thus disrupting its lipid-binding capabilities [18, 20]. L435H is located in the predicted α -helical domain and is thought to affect MTTP folding [22]. S590I is also located in the α -helical domain and it is thought to potentially augment a hydrophobic patch, consisting of residues S590, F585, M587 and A589, by increasing the hydrophobicity of the pocket [20].

In the present study we report two patients with ABL. The first was found to be a compound heterozygote for two novel mutations in the *MTTP* gene, one deletion (c.103-127del25) and the other a missense mutation (Y528H). The second was found to carry three novel missense mutations: G264R, R540C and N649S. To determine whether any of these missense

mutations are responsible for the ABL phenotype, a series of *in vitro* experiments were performed to assess the effect that each of these missense mutations had on protein interactions, expression levels and lipid-transfer activity.

2. Materials and Methods

2.1. Clinical data

Patient 1: A 4-month old girl was referred for evaluation of failure to thrive. Her plasma biochemistry results revealed a strikingly-low total cholesterol at 1.0 mmol/L, with extremely low levels of LDL-cholesterol and apoB (**Table 1**). The child's parents were in good health and had normal plasma lipid profiles, consistent with a recessive mode of inheritance i.e. ABL. She has been supplemented with high dose vitamin E (100 mg/kg/day) up through her most recent followup visit at age 7, and has remained healthy with normal development and function.

Patient 2: A 37-year-old man was referred for investigation after hypocholesterolemia was found on routine biochemical testing (**Table 1**). His plasma total cholesterol was 1.3 mmol/L, with detectable, but extremely low LDL-cholesterol and apoB. Family members were unavailable for study.

2.2. *MTTP* gene sequencing and mutation analysis

Patient 2 and the family of Patient 1 gave informed consent to genetic testing. Briefly, genomic DNA was extracted from peripheral blood using a standard Triton X-100 salting out procedure. All 18 exons and flanking intronic junctions of the *MTTP* gene were amplified by PCR using AmpliTaq Gold (Invitrogen) and sequenced in forward and reverse directions using Big Dye Terminator chemistry (Applied Biosystems). Chromatograms were aligned to reference sequence NM_000253. Mutations were confirmed by sequencing of a second PCR product.

In order to exclude compound heterozygous and homozygous familial hypobetalipoproteinemia in Patient 2, plasma was screened for truncated apoB variants by immunoblotting using the apoB monoclonal antibody 1D1, kindly provided by Prof. Ross Milne (University of Ottawa Heart Institute, Ottawa, Ontario, Canada), using chemiluminescent detection (Amersham Biosciences). In addition, exons 1 to 25 of the *APOB* gene were PCR-amplified and sequenced.

2.3. *In silico* analysis

Mutations were analyzed using PolyPhen-2 [23] (<http://genetics.bwh.harvard.edu/pph2/>) and SIFT [24] (<http://sift.jcvi.org/>).

2.4. Modeling

The MTTP model was built using the human MTTP sequence and the high resolution structure of lamprey lipovitellin [25] as inputs to the program Modeller 9.12 [26]. The Modeller program's alignment differs from that of Read *et al.* [11] primarily after the two regions of lipovitellin sequence that are missing from the electron density in the crystal structure in the C-sheet region. Modeller does not align any MTTP sequence to these regions whereas the Read *et al.* [11] model does. This offset allows Modeller to fit the entire sequence of MTTP to the lipovitellin structure with reasonable sequence correspondence.

2.5. Construction of mutant MTTP cDNA

The plasmid vector pMTTP, containing the entire coding sequence of the large subunit of MTTP subcloned into plasmid pRC/neo (Invitrogen), was used as a template to prepare mutant pMTTP plasmids containing Y528H, G264R, R540C and N649S. Site-directed mutagenesis was performed using the Quikchange® II Site-Directed Mutagenesis Kit

(Stratagene) according to the manufacturer's instructions. Plasmid DNA was harvested using the QIAGEN Maxiprep Kit and sequenced to confirm the presence of all mutations. Plasmid pMTTP was kindly provided by Prof. Zemin Yao, University of Ottawa Heart Institute, Canada.

2.6. Transient transfection and detection of MTTP

COS-7 cells, an immortalized African green monkey fibroblast-like kidney cell line, were cultured in 6-well plates to an 80% confluency and transfected with 3 µg of wild-type or mutant pMTTP using the fuGENE® 6 transfection reagent (Promega). After 48 h, cells were washed three times with ice-cold phosphate buffered saline (PBS) and harvested in PBS. Cells were lysed using EBC lysis buffer (50 mM TRIS-HCl [pH 8], 120 mM NaCl, 0.5% NP-40, 100 mM NaF, 100 µM leupeptin hemisulphate, 1 mM phenylmethylsulfonyl fluoride) on ice for 15 min and insoluble material was removed by centrifugation (12000 x g, 10 min). Protein concentration was determined by Bradford Assay (Sigma). Protein samples were subjected to SDS-PAGE (8%) under reducing conditions and blotted onto a supported nitrocellulose membrane (Bio-Rad). MTTP was detected using a primary anti-MTTP Ab, SC-33116 and a secondary donkey anti-goat IgG-HRP Ab, SC-2020 (Santa Cruz Biotechnology). MTTP was visualized using Luminata Classico Western HRP substrate (Millipore) and exposed on x-ray film. Protein sizes were estimated using a broad range protein ladder (Bio-Rad).

2.7. Co-immunoprecipitation

As described above, COS-7 cells were transfected with pMTTP either alone or in conjunction with pB17 or pB48. After lysis, soluble protein (450 µg) in a final volume of 300 µL EBC buffer was pre-cleared by incubation with protein A-sepharose™ CL-4B beads (GE Healthcare) for 1 h at 4°C prior to incubation with capture Ab (anti-PDI [RL90, Sapphire Bioscience], 1D1 anti-apoB) coupled with protein A-sepharose™ CL-4B beads (GE Healthcare) for 4 h at 4°C. Beads were washed four times with NETN-100 mM NaCl (20 mM TRIS-HCl [pH 8], 1 mM EDTA, 100 mM NaCl, 0.5% NP-40) and once with NETN-900 mM NaCl (20 mM TRIS-HCl [pH 8], 1 mM EDTA, 900 mM NaCl, 0.5% NP-40). All centrifugation steps were carried out at 500 x g, 1 min. Beads were resuspended in 2 x Laemmli sample buffer, boiled, pelleted and the supernatants were subjected to SDS-PAGE and Western blot analysis. Plasmids pB17 [27] and pB48 [28] were kindly provided by Prof. Zemin Yao, University of Ottawa Heart Institute, Canada.

2.8. MTTP activity assay

MTTP activity in cells was measured using a fluorescently labeled triglyceride kit (Chylos) following the manufacturer's instructions. In brief, cells transfected with MTTP were washed extensively with ice-cold PBS and homogenized in 300 µL buffer K (1 mM TRIS-HCl, pH 7.6, 1 mM MgCl₂ and 1 mM EGTA) with 100 µM leupeptin hemisulphate, 1 mM phenylmethylsulfonyl fluoride. Cells lysates were homogenized through a fine tip (19G) needle twenty times and protein concentration was determined by Bradford Assay (Sigma). Homogenates were centrifuged in a Beckman airfuge at 100,000 x g for 1 h. Supernatants were used for MTTP lipid-transfer assays in triplicate. To the wells of a black microtitre plate, 10 µL of supernatants, 5 µL of substrate vesicles and 85 µL of distilled H₂O were added and incubated for 3 h at room temperature prior to fluorescence reading using 460 nm excitation and 520 nm emission wavelengths in a fluorescent plate reader (FLUOstar Omega, BMG LABTECH, Software version 1.30). Total fluorescence was calculated by disrupting vesicles with isopropanol. To determine the percentage lipid-transfer, background fluorescence (fluorescence values containing no MTTP) was subtracted from each sample containing MTTP and this value was further divided by the total fluorescence of vesicles.

3. Results

3.1. Identification of mutations

DNA sequencing of *MTTP* revealed that Patient 1 was a compound heterozygote for two novel mutations. The first, inherited from her mother is a 25 bp deletion at position c.103-127del25 in exon 2 that is predicted to lead to a frameshift after Tyr³⁴ and the production of 36 new amino acids followed by a stop codon (p.K35Ffs*37). The second mutation inherited from her father, is a single base change, 1582T>C in exon 12 that leads to a missense mutation, Y528H. This results in the conversion of a tyrosine to histidine at amino acid position 528 and the subsequent loss of a hydrophobic side chain. This mutation affects a highly conserved residue in the central α -helical domain of MTTP and is located in helix 13 with contacts to the loop between the C-terminus of helix 14 and the N-terminus of helix 15 (Fig 1). *In silico* analysis of Y528H using PolyPhen-2 and SIFT predicts it to be probably damaging (score of 1) and damaging (score of 0.04), respectively.

Patient 2 did not have a truncated apoB species in plasma, nor were any mutations detected in exons 1 to 25 of *APOB*. However, sequencing of *MTTP* revealed three novel missense mutations: c.790G>C in exon 7 (G264R), c.1618C>T in exon 12 (R540C) and c.1946A>G in exon 13 (N649S). As family members were unavailable for study, it is unknown on which allele/chromosome these mutations are located. Mutation G264R results in the conversion of a glycine to arginine at amino acid position 264, which occurs in the N-terminal β -barrel of MTTP. Mutation R540C results in the conversion of an arginine to cysteine at amino acid position 540 and the subsequent gain of a hydrophobic side chain. This mutation is located at the N-terminal end of helix 14 in the central α -helical domain of MTTP (Fig 1). A different mutation at this position (R540H) has previously been associated with ABL [15]. Mutation N649S results in the conversion of an asparagine to serine at amino acid position 649, which is located on the 3d strand of the C-terminal lipid-binding domain of MTTP facing the hydrophobic pocket (Fig 1). Of the three missense mutations, R540C was predicted to be the most damaging by PolyPhen-2 and SIFT with results of probably damaging (score of 1) and damaging (score of 0), respectively.

3.2. Functional characterization of mutant MTTP

To determine whether any of the four missense mutations affect the expression of MTTP in COS-7 cells, the plasmids encoding wild-type MTTP and mutant MTTP (G264R, Y528H, R540C and N649S) were transiently transfected into COS-7 cells, which do not endogenously express MTTP [29]. MTTP expression was evaluated by Western blot and all constructs produced immunoreactive proteins of the predicted size (~97 kDa) and with comparable expression levels to that of the wild-type MTTP (Fig 2) i.e. none of the mutations significantly affected translation efficiency. The constitutively expressed GAPDH was used as a loading control.

Co-immunoprecipitation studies were performed to assess the affect the mutations may have on the interaction of MTTP with PDI. PDI was immunoprecipitated with an anti-PDI mAb, subjected to Western blotting, then probed for both MTTP and PDI. MTTP and PDI were detected in all immunoprecipitates, indicating that all mutants interact strongly with PDI (Fig 3A).

Co-immunoprecipitation studies were also performed to analyze the effect of the four missense mutations on the interaction between MTTP and apoB. Wild-type and mutant MTTP and apoB17 or apoB48 were co-transfected into COS-7 cells and apoB immunoprecipitated with 1D1, subjected to Western blotting, then probed for both MTTP and apoB. MTTP was detected in association with both apoB17 and apoB48 indicating that the four missense mutations are unlikely to have a deleterious effect on the MTTP interaction with apoB (Fig 3B,C). To assess any non-specific binding of MTTP to either protein A-sepharose beads or the capture antibody (anti-PDI mAb or 1D1 anti-apoB mAb), bead-only controls and isotype controls were included in preliminary co-immunoprecipitation experiments. MTTP was not detected in either control (results not shown).

The lipid-transfer activity of the four missense mutations was determined. COS-7 cells transfected with wild-type MTTP and mutant MTTP were lysed to release the contents of the microsomes and soluble proteins were separated from insoluble proteins by high speed centrifugation. Supernatants (containing only soluble proteins) were analyzed for MTTP lipid-transfer activity and immunoblotted for the MTTP 97 kDa subunit. Only G264R retained its lipid-transfer activity to levels comparable to that of the wild-type, whereas the lipid-transfer activities of Y528H, R540C and N649S were significantly reduced by 90%, 88% and 43%, respectively (Fig 4A). The reductions in lipid-transfer activity were proportional to the amount of soluble MTTP recovered (Fig 4B). The MTTP 97 kDa subunit for the Y528H and R540C mutants was virtually absent from the supernatants and greatly reduced for N649S. Analysis of the centrifuged pellets of Y528H and R540C located the majority of the MTTP 97 kDa subunit, indicating that aggregation had occurred rendering it insoluble at 100,000 x g. As expected, lipid-transfer activity was not detected in the pellets of R528H and R540C (results not shown).

To ensure the observed loss of lipid-transfer activity was due to the identified mutations, site-directed mutagenesis was performed to revert back the three plasmids containing the Y528H, R540C and N649S mutations to wild-type. Lipid-transfer activity was restored in all revertants to levels similar to that of the wild-type, indicating that the loss of activity was a biological consequence of the mutation (results not shown). To confirm the sensitivity and specificity of the lipid-transfer activity assay, several other mutations were evaluated (Fig 5). As expected, the conversion of Tyr⁵²⁸ to Lys (Y528K) and Arg⁵⁴⁰ to Ala (R540A) (similar in hydrophobicity to the mutants) reduced lipid-transfer activity, with activity being only 9% and 21% of the wild-type, respectively. However, conversion of Tyr⁵²⁸ to Phe (Y528F) and Arg⁵⁴⁰ to Lys (R540K) (similar in hydrophobicity to the wild-type) did not reduce lipid-transfer activity. The R540H mutation was also evaluated as it had previously been identified as causing ABL by rendering MTTP catalytically inactive [15]. The R540H mutation reduced lipid-transfer activity to 9% of the wild-type, consistent with previous studies [15].

4. Discussion

In this study, we identified five novel mutations in *MTTP* in two patients with ABL, namely, p.K35Ffs*37, G264R, Y528H, R540C and N649S. We then performed a series of *in vitro* experiments to assess the effect that each of these missense mutations had on protein interactions, expression levels and lipid-transfer activity.

Sequencing *MTTP* of the first patient revealed two novel mutations, one frameshift, c.103-127del25 and one missense Y528H. The frameshift mutation, inherited maternally, is predicted to produce a severely truncated protein and is therefore likely to be functionally defective. The paternal missense mutation Y528H occurs in a highly conserved region of *MTTP* and Tyr⁵²⁸ is also conserved across species. In addition, mutational analysis using SIFT and PolyPhen-2 predicted the mutation to be damaging, indicating that this mutation is likely to have a critical role in MTTP function. Expression experiments performed on Y528H showed that although binding to apoB17, apoB48 and PDI was not inhibited, lipid-transfer activity was significantly reduced. This indicates that mutation Y528H is likely a functionally defective missense mutation and together with the p.K35Ffs*37 frameshift mutation accounts for the ABL phenotype.

The Y528H mutation is located in the central α -helical domain of MTTP and affects an amino acid at the carboxyl terminus of helix 13. Tyr⁵²⁸ is predicted to be surface exposed (Fig 1) and is not thought to participate in PDI binding directly [10]. The conversion of Tyr⁵²⁸ to histidine results in a loss of a hydrophobic side chain, which may lead to protein misfolding and aggregation, and consequently the loss of function observed with this mutation. Furthermore, converting Tyr⁵²⁸ to the hydrophilic lysine reduced lipid-transfer activity to a similar level as histidine (<10% of wild-type), whereas converting Tyr⁵²⁸ to the hydrophobic phenylalanine did not impede lipid-transfer activity, supporting the requirement for a hydrophobic amino acid at this site.

Sequencing *MTTP* of the second patient revealed three novel heterozygous missense mutations: G264R, R540C and N649S. Mutational analysis of these three variants using SIFT and PolyPhen-2 predicted R540C to be the most damaging. Expression experiments performed on these three missense mutations showed no reduction in binding to apoB17, apoB48 or PDI, however, lipid-transfer activity of R540C and N649S was reduced by 88% and 43%, respectively. Although the R540C mutation is likely to be functionally defective, mutant N649S still retained more than 50% of its lipid-transfer activity as compared to the wild-type and this may be sufficient for the assembly and secretion of a limited amount of apoB-containing lipoproteins in the liver or intestine. These two mutations located on different alleles may account for the mild clinical ABL phenotype observed in this patient, however, as the phase of the three mutations could not be determined, no definite conclusion can be made.

The R540C mutation affects an amino acid at the N-terminal end of helix 14 (537-550) and is located in the central α -helical domain of MTTP (Fig 1), which is believed to form part of a buried salt bridge (R540-N531-E570) uniting the carboxyl terminus of helix 13, the amino terminus of helix 14 and the loop preceding helix 16 [10]. It has previously been shown that disruption of the internal salt bridge can have a major effect on MTTP structure [20]. The R540C mutation may be mislinked during folding resulting in aberrant disulfide bond formation, which could therefore affect the local structure and stability of MTTP. The Arg⁵⁴⁰ site has previously been implicated in ABL. R540H was the first missense mutation in the 97

kDa subunit of MTTP to be reported [15] and it was found to render MTTP catalytically inactive and reduced the association of the M subunit with PDI [15, 20]. It was suggested that a positively charged residue is required at amino acid position 540 for a successful interaction with PDI and the formation of an active complex [15]. The R540C mutation, which converts the positively charged, hydrophilic arginine to an uncharged, hydrophobic cysteine, renders MTTP catalytically inactive, supporting the requirement for a positively charged amino acid at this site. Similarly, conversion of Arg⁵⁴⁰ to lysine, a positively charged amino acid, showed normal MTTP activity [10].

To determine whether other mutations at this residue would affect MTTP activity, Arg⁵⁴⁰ was converted to alanine, an amino acid with similar properties to cysteine. This mutation reduced lipid-transfer activity by 79%, further supporting the requirement for a positive charge at amino acid position 540. An additional mutation converting Arg⁵⁴⁰ to the uncharged, hydrophilic asparagine also reduced lipid-transfer activity, but by a more modest 58% (results not shown), indicating that the loss of a positive charge alone is not enough to completely abolish MTTP function. It is likely that the complete loss of function of MTTP, due to mutations R540H and R540C, is a result of both the loss of a positive charge and a change in hydrophobicity.

The N649S mutation is located in the C-terminal lipid-binding domain and forms part of the predicted C-sheet domain (amino acids 603-712) comprising six anti-parallel β -strands and a short helix [11]. Located deep inside the hydrophobic pocket, Asn⁶⁴⁹ is likely to be shielded from other proteins and in contact with lipids (Fig 1). The N649S mutation converts the hydrophilic asparagine to the smaller, but still polar serine, and was found to only partially disrupt the lipid-transfer activity of MTTP, which may account for this patient's milder or atypical phenotype. The structure of the lipid-binding cavity also comprises an A-sheet (amino acids 725-829), which contains four β -strands and two α -helices, designated A (amino acids 725-736) and B (amino acids 781-786). The two helices, located at the entrance to the MTTP cavity, are predicted to mediate lipid acquisition and binding [11]. Mutations N780Y and G746E located within the A-sheet have been shown to cause ABL by hindering lipid-transfer activity [11, 18, 20]. It is possible that the N649S mutation may affect the stability or conformation of the lipid-binding cavity, thereby reducing MTTP activity. Two additional missense mutations located in the C-sheet of the lipid-binding cavity have also been reported, G661A [19] and G661S [30], but neither has been characterized.

The G264R mutation, located in the N-terminal β -barrel (Fig 1), has no observable impact on PDI, apoB17 or apoB48 binding or lipid-transfer activity. The mutation was predicted to be tolerated by SIFT and only possibly damaging by PolyPhen-2. It is likely that this mutation has no critical effect on MTTP function and that the ABL phenotype observed in Patient 2 is a consequence of mutations R540C and N649S. It is possible that mutation G264R could lose all or part of its functionality when combined with one of the other two mutations. This would have little effect on the allele containing mutation R540C as this mutation alone is functionally defective, however, combined with N649S on a different allele to R540C it may further inhibit MTTP activity.

Our studies show that all four mutants interact with PDI, despite the negligible lipid-transfer activities of Y528H and R540C. Both Y528H and R540C formed insoluble aggregates and pelleted at 100,000 x g. It is believed that a stable interaction between MTTP and PDI results in a fully soluble and active protein [31, 32]. The results from this study, however, show that Y528H and R540C both co-immunoprecipitate with PDI despite being catalytically inactive.

This is consistent with Khatun *et al.* [20] and Mann *et al.* [10], who found that R540H (insoluble and catalytically inactive) also co-immunoprecipitated with PDI albeit to a lesser extent than the wild-type. It has also been suggested that the interaction between R540H and PDI is weaker than that of the wild-type [20]. A weaker interaction between Y528H, R540C and PDI may similarly account for the insolubility observed during the lipid-transfer activity assay, however, the results in this study did not indicate any significant reduction in binding.

The two patients discussed in this paper presented with contrasting ABL phenotypes. Patient 1 was a 4-month old girl who presented with failure to thrive, low levels of total cholesterol, LDL-cholesterol and apoB, and undetectable levels of α -tocopherol. This patient received dietary fat restriction with fat-soluble vitamin supplements to promote health and normal development [33]. Patient 2, however, was not diagnosed until the age of 37. This patient had measureable LDL-cholesterol, apoB and α -tocopherol levels, which may account for his lack of neurological symptoms. Mutations were identified in the *MTTP* gene of both patients. Patient 1 had a deletion in one chromosome and a missense mutation in the other, both of which are predicted to result in a functionally defective MTTP protein. These results provide a biochemical explanation for the severe ABL phenotype of patient 1. Patient 2, however, had three missense mutations, only one of which was completely functionally defective, R540C. Mutation N649S only partially disrupted MTTP activity and mutation G264R appeared to have no effect on MTTP structure or function. The N649S mutation, if located on a different chromosome to R540C, may offer a biological explanation for the mild ABL phenotype observed in this patient.

5. Conclusions

Our study describes five novel *MTTP* mutations in two patients with ABL, one frameshift mutation, predicted to result in a severely truncated functionally defective protein, and four missense mutations. Functional analysis of the missense mutations has provided a biochemical explanation for mutations Y528H, R540C and N649S. Our studies highlight the requirement for a positively charged, hydrophilic residue at Arg⁵⁴⁰ and a hydrophobic residue at Tyr⁵²⁸ for MTTP activity. This study is also the first to characterize a mutation in the C-sheet domain of the C-terminal lipid-binding cavity and demonstrates that mutations in this domain also have the ability to inhibit lipid-transfer activity.

6. Acknowledgments

Sources of funding

This work was supported by National Health and Medical Research Council Project Grant 1010133 (to AJH, JRB and FvB). JRB is a recipient of Practitioner Fellowship from the Royal Perth Hospital Medical Research Foundation.

7. References

- [1] Sharp D, Blinderman L, Combs KA, et al. Cloning and gene defects in microsomal triglyceride transfer protein associated with abetalipoproteinaemia. *Nature* 1993; 365:65-69.
- [2] Shoulders CC, Brett DJ, Bayliss JD, et al. Abetalipoproteinemia is caused by defects of the gene encoding the 97 kDa subunit of a microsomal triglyceride transfer protein. *Hum Mol Genet* 1993; 2:2109-2116.
- [3] Burnett JR, Bell DA, Hooper AJ, Hegele RA. Clinical utility gene card for: Abetalipoproteinaemia. *Eur J Hum Genet* 2012; 20(8):doi:10.1038/ejhg.2012.1030.
- [4] Berriot-Varoqueaux N, Aggerbeck LP, Samson-Bouma M, Wetterau JR. The role of the microsomal triglyceride transfer protein in abetalipoproteinemia. *Annu Rev Nutr* 2000; 20:663-697.
- [5] Hooper AJ, van Bockxmeer FM, Burnett JR. Monogenic hypocholesterolaemic lipid disorders and apolipoprotein B metabolism. *Crit Rev Clin Lab Sci* 2005; 42:515-545.
- [6] Burnett JR, Bell DA, Hooper AJ, Hegele RA. Clinical utility gene card for: Familial Hypobetalipoproteinaemia (APOB). *Eur J Hum Genet* 2012; 20(8):doi:10.1038/ejhg.2012.1085.
- [7] Hooper AJ, Burnett JR. Molecular genetics of hypobetalipoproteinaemia and abetalipoproteinaemia. *eLS* 2013:doi:10.1002/9780470015902.a9780470025000.
- [8] Hussain MM, Shi J, Dreizen P. Microsomal triglyceride transfer protein and its role in apoB-lipoprotein assembly. *J Lipid Res* 2003; 44:22-32.
- [9] Bradbury P, Mann CJ, Kochl S, et al. A common binding site on the microsomal triglyceride transfer protein for apolipoprotein B and protein disulfide isomerase. *J Biol Chem* 1999; 274:3159-3164.

- [10] Mann CJ, Anderson TA, Read J, et al. The structure of vitellogenin provides a molecular model for the assembly and secretion of atherogenic lipoproteins. *J Mol Biol* 1999; 285:391-408.
- [11] Read J, Anderson TA, Ritchie PJ, et al. A mechanism of membrane neutral lipid acquisition by the microsomal triglyceride transfer protein. *J Biol Chem* 2000; 275:30372-30377.
- [12] Hussain MM, Iqbal J, Anwar K, Rava P, Dai K. Microsomal triglyceride transfer protein: a multifunctional protein. *Front Biosci* 2003; 8:s500-506.
- [13] Magnolo L, Najah M, Fancello T, et al. Novel mutations in SAR1B and MTTP genes in Tunisian children with chylomicron retention disease and abetalipoproteinemia. *Gene* 2013; 512:28-34.
- [14] Narcisi TM, Shoulders CC, Chester SA, et al. Mutations of the microsomal triglyceride-transfer-protein gene in abetalipoproteinemia. *Am J Hum Genet* 1995; 57:1298-1310.
- [15] Rehberg EF, Samson-Bouma ME, Kienzle B, et al. A novel abetalipoproteinemia genotype. Identification of a missense mutation in the 97-kDa subunit of the microsomal triglyceride transfer protein that prevents complex formation with protein disulfide isomerase. *J Biol Chem* 1996; 271:29945-29952.
- [16] Berthier MT, Couture P, Houde A, et al. The c.419-420insA in the MTP gene is associated with abetalipoproteinemia among French-Canadians. *Mol Genet Metab* 2004; 81:140-143.
- [17] Wang J, Hegele RA. Microsomal triglyceride transfer protein (MTP) gene mutations in Canadian subjects with abetalipoproteinemia. *Hum Mutat* 2000; 15:294-295.
- [18] Ohashi K, Ishibashi S, Osuga J, et al. Novel mutations in the microsomal triglyceride transfer protein gene causing abetalipoproteinemia. *J Lipid Res* 2000; 41:1199-1204.

- [19] Di Leo E, Lancellotti S, Penacchioni JY, et al. Mutations in MTP gene in abeta- and hypobeta-lipoproteinemia. *Atherosclerosis* 2005; 180:311-318.
- [20] Khatun I, Walsh MT, Hussain MM. Loss of both phospholipid and triglyceride transfer activities of microsomal triglyceride transfer protein in abetalipoproteinemia. *J Lipid Res* 2013; 54:1541-1549.
- [21] Al-Shali K, Wang J, Rosen F, Hegele RA. Ileal adenocarcinoma in a mild phenotype of abetalipoproteinemia. *Clin Genet* 2003; 63:135-138.
- [22] Di Filippo M, Crehalet H, Samson-Bouma ME, et al. Molecular and functional analysis of two new MTTP gene mutations in an atypical case of abetalipoproteinemia. *J Lipid Res* 2012; 53:548-555.
- [23] Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. *Nat Methods* 2010; 7:248-249.
- [24] Ng PC, Henikoff S. Predicting deleterious amino acid substitutions. *Genome Res* 2001; 11:863-874.
- [25] Thompson JR, Banaszak LJ. Lipid-protein interactions in lipovitellin. *Biochemistry* 2002; 41:9398-9409.
- [26] Eswar N, Webb B, Marti-Renom MA, et al. Comparative protein structure modeling using Modeller. *Curr Protoc Bioinformatics* 2006; Chapter 5:Unit 5 6.
- [27] Vukmirica J, Nishimaki-Mogami T, Tran K, et al. The N-linked oligosaccharides at the amino terminus of human apoB are important for the assembly and secretion of VLDL. *J Lipid Res* 2002; 43:1496-1507.
- [28] Vukmirica J, Tran K, Liang X, et al. Assembly and secretion of very low density lipoproteins containing apolipoprotein B48 in transfected McA-RH7777 cells. Lack of evidence that palmitoylation of apolipoprotein B48 is required for lipoprotein secretion. *J Biol Chem* 2003; 278:14153-14161.

- [29] Leiper JM, Bayliss JD, Pease RJ, Brett DJ, Scott J, Shoulders CC. Microsomal triglyceride transfer protein, the abetalipoproteinemia gene product, mediates the secretion of apolipoprotein B-containing lipoproteins from heterologous cells. *J Biol Chem* 1994; 269:21951-21954.
- [30] Vongsuvanh R, Hooper AJ, Coakley JC, et al. Novel mutations in abetalipoproteinaemia and homozygous familial hypobetalipoproteinaemia. *J Inherit Metab Dis* 2007; 30:990.
- [31] Lamberg A, Jauhiainen M, Metso J, et al. The role of protein disulphide isomerase in the microsomal triacylglycerol transfer protein does not reside in its isomerase activity. *Biochem J* 1996; 315 (Pt 2):533-536.
- [32] Wetterau JR, Combs KA, McLean LR, Spinner SN, Aggerbeck LP. Protein disulfide isomerase appears necessary to maintain the catalytically active structure of the microsomal triglyceride transfer protein. *Biochemistry* 1991; 30:9728-9735.
- [33] Lee J, Hegele RA. Abetalipoproteinemia and homozygous hypobetalipoproteinemia: a framework for diagnosis and management. *J Inherit Metab Dis* 2014; 37:333-339.

Figure 1. Structure of the 97 kDa subunit of the MTTP complex (M subunit) based on lamprey lipovitellin. Ribbon diagram colored to show the different domains. β -barrel domain, blue-cyan; helical domain, green; C-sheet, green-yellow and A-sheet, yellow-red. Mutations are shown in the structure as space filling representations colored by atom.

Figure 2. Transient expression of wild-type and mutant MTTP in COS-7 cells. Expression constructs containing either wild-type or mutant MTTP were transfected into COS-7 cells. Forty eight hours after transfection, cells were collected and lysed. MTTP and GAPDH were detected with anti-MTTP pAb and anti-GAPDH pAb, respectively.

Figure 3. Co-immunoprecipitation of MTTP with PDI, B17 and B48 in COS-7 cells. Expression constructs containing either wild-type or mutant MTTP were transfected into COS-7 cells alone or in combination with an expression construct containing either apoB17 or apoB48. Forty eight hours after transfection, cells were collected and lysed. Co-immunoprecipitation was performed with either anti-PDI RL90 mAb or anti-apoB 1D1 mAb. Immunoreactive products were detected with anti-PDI RL90 mAb or anti-apoB 1D1 mAb, and anti-MTTP pAb. *A* Wild-type and mutant MTTP interact with protein disulfide isomerase in COS-7 cells. *B* Wild-type and mutant MTTP interact with apoB17 in COS-7 cells. *C* Wild-type and mutant MTTP interact with apoB48 in COS-7 cells. Repetition of the experiments yielded similar results.

Figure 4. A Triglyceride transfer activity of wild-type and mutant MTTP in transfected COS-7 cells. Transfer activities are expressed as the percentage of the total triglyceride transferred from donor to acceptor membranes. The results are the mean of triplicate assay $\pm$ standard deviation from a representative experiment. *B* Equal aliquots of soluble MTTP (centrifuged at 100,000 $\times$ g to remove membrane associated or insoluble proteins) and total COS-7 cell lysates were analyzed by SDS-PAGE and immunoblotting. Asterisk denotes statistical significance (<0.05) relative to control, determined by two-tailed Student t-test.

Figure 5. Triglyceride transfer activity of wild-type and mutant MTTP in transfected COS-7 cells. Transfer activities are expressed as the percentage of the total triglyceride transferred from donor to acceptor membranes. The results are the mean of triplicate assay $\pm$ standard deviation from a representative experiment. Asterisk denotes statistical significance (<0.05) relative to control, determined by two-tailed Student t-test.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5.

Table 1. Biochemistry of patients with abetalipoproteinemia

Analyte	Patient 1	Patient 2	Reference Limit/Interval
Total cholesterol (mmol/L)	0.96	1.30	<5.5
HDL-cholesterol	0.78	0.81	>1.0
LDL-cholesterol (mmol/L)	Undetectable	0.13	<3.0
ApoB (g/L)	Undetectable	0.14	<1.00
ALT (U/L)	109	52	<35
AST (U/L)	137	49	<35
α -Tocopherol (μ mol/L)	Undetectable	10	18-46

ALT, alanine aminotransferase; AST, aspartate aminotransferase; HDL, high-density lipoprotein.