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RESEARCH REPORT

Clinical, Biochemical, and Molecular Characterization of Novel Mutations in *ABCA1* in Families with Tangier Disease

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Abstract Tangier disease is a rare, autosomal recessive disorder caused by mutations in the *ABCA1* gene and is characterized by near absence of plasma high-density lipoprotein cholesterol, accumulation of cholesterol in multiple tissues, peripheral neuropathy, and accelerated atherosclerosis. Here we report three new kindreds with Tangier disease harboring both known and novel mutations in *ABCA1*. One patient was identified to be homozygous for a nonsense mutation, p.Gln1038*. In a remarkably large Tangier disease pedigree with four affected siblings, we identified compound heterozygosity for previously reported missense variants, p.Arg937Val and p.Thr940Met, and show that both of these mutations result in significantly impaired cholesterol efflux in transfected cells. In a third pedigree, the proband was identified to be compound heterozygous for two novel mutations, a frameshift (p.Ile1200Hisfs*4) and an intronic variant (c.4176-11T>G), that lead to the creation of a cryptic splice site acceptor and premature truncation, p.Ser1392Argfs*6. We demonstrate that this mutation arose de novo, the first demonstration of a pathogenic de novo mutation in *ABCA1* associated with Tangier disease. We also report results of glucose tolerance testing in a Tangier disease kindred for the first time, showing a gene–dose relationship between ABCA1 activity and glucose tolerance and suggesting that Tangier disease patients may have substantially impaired islet function. Our findings provide insight into the diverse phenotypic manifestations of this rare disorder, expand the list of pathogenic mutations in *ABCA1*, and increase our understanding of how specific mutations in this gene lead to abnormal cellular and physiological phenotypes.

Introduction

Plasma levels of high-density lipoprotein cholesterol (HDL-C) are inversely related to risk of atherosclerotic cardiovascular disease. Much of our knowledge of HDL particle function and metabolism has been derived from the study of rare, inherited disorders of HDL. Tangier disease is one such extremely rare, autosomal recessive disorder and is caused by mutations in the ATP-binding cassette transporter, subfamily A, member 1 (*ABCA1*) gene (OMIM entry #205400). Tangier disease is characterized by a near absence of HDL-C in plasma and cholesterol accumulation in the cells of the reticuloendothelial system leading to the classic clinical manifestations of enlarged orange tonsils, peripheral neuropathy, and hypersplenism. Patients with Tangier disease are also reported to be at increased risk for atherosclerosis (Clee et al. 2000; van Dam et al. 2002), although the relationship between *ABCA1* gene mutations, HDL, and atherosclerosis is controversial (Brunham et al. 2008a). Heterozygous mutations in *ABCA1* also cause familial HDL deficiency and are a common cause of low HDL-C in the general population (Cohen et al. 2004; Alrasadi et al. 2006; Tietjen et al. 2012).

The *ABCA1* gene is expressed widely throughout the body, and studies in mice with targeted inactivation of *Abca1* in specific tissues have elucidated discrete phenotypes associated with *Abca1* function in the liver, intestine, macrophages, adipocytes, pancreatic islets, and brain (Brunham et al. 2006a, 2007, 2009; Karasinska et al. 2009; Timmins et al. 2005; Chung et al. 2011). However, the study of human phenotypes associated with complete *ABCA1* deficiency has been more challenging, owing to the rarity of this condition. To date, approximately 180 mutations have been reported in the *ABCA1* gene, and these are associated with a range of clinical, biochemical, and cellular phenotypes (Singaraja et al. 2003; Brunham et al. 2006b; Stenson et al. 2009). Understanding the clinical, biochemical, and cellular impact of pathogenic mutations in *ABCA1* has greatly increased our understanding of the crucial physiological role of this transporter in regulating cellular cholesterol homeostasis throughout the body. Here we investigated three kindreds in which one or more individual presented with extremely low levels of HDL-C. We identified homozygosity or compound heterozygosity for pathogenic mutations in *ABCA1* in 6 individuals, thus confirming the diagnosis of Tangier disease, and assessed the impact of these mutations on clinical and biochemical phenotypes.

Materials and Methods

Genomic DNA Sequencing and Mutation Detection

Genomic DNA was extracted from the blood or saliva using Qiagen DNeasy kits (Qiagen GmbH). The coding exons of the *ABCA1* gene were amplified using PCR primers spanning intron/exon boundaries (primer sequences available on request). Gel- or column-purified PCR products were subject to sequencing using an ABI 3700 instrument in the forward and reverse directions. Sequence trace files were assembled into contigs and aligned to the *ABCA1* consensus sequence using CodonCode aligner, and mutations were detected by manual inspection. All mutations were named according to Human Genome Variation Society guidelines (Uehara et al. 2008) with respect to the *ABCA1* reference sequences NM_005502.3 (in which nucleotide position 1 corresponds to nucleotide A of the ATG start methionine) and protein sequence NP_005493.2. This study was approved by the institutional review board of the University of British Columbia (UBC-C&W CREB: H96-70297) and the University Hospital Gasthuisberg.

cDNA Synthesis and Sequencing

For RNA isolation, whole blood was collected in PAXgene Blood RNA tube (Qiagen GmbH). The tubes were incubated at room temperature for a minimum of 4 h to ensure complete lysis of blood cells. RNA was isolated and purified with the PAXgene Blood RNA Kit according to the manufacturer's instructions (Qiagen GmbH). A total of 0.5–1 μg of isolated RNA was primed with 50 μM oligo $(dT)_{20}$. First-strand cDNA synthesis was performed from RNA using Superscript III according to the manufacturer's instructions (Invitrogen). The *ABCA1* cDNA was PCR amplified using eight sets of primer pairs designed to amplify the *ABCA1* cDNA sequence including the entire 5′UTR and parts of the 3′UTR (sequences available on request). All PCR products were purified using either QIAquick PCR purification or gel extraction according to the manufacturer's instructions (Qiagen GmbH) and sequenced in the forward and reverse directions. Mutations were detected as described above. Cryptic splice sites were predicted using the Automated Splice Site and Exon Definition Analyses (ASSEDA) tool (http://splice.uwo.ca).

Immunoblotting and Immunofluorescence

ABCA1 cDNA constructs containing wild-type ABCA1, ABCA1-Ala937Val, and ABCA1-Thr940Met were obtained from Blue Heron Biotech. HEK293 cells were transiently transfected with pCI-neo (empty vector), pCMVV6AC-hABCA1 (wild type), pCMVV6AC-hABCA1-Ala937Val, or pCMVV6AC-hABCA1-Thr940Met using Fugene6 (Promega) for 48 h. Transiently transfected HEK293 cells were lysed in lysis buffer (10 mM Tris pH 8.0, 1% Triton X-100, complete protease inhibitors [Roche]) for 30 min on ice with vortexing every 10 min. Thirty micrograms of total protein was loaded and separated on 7.5% acrylamide gels. Protein was transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). ABCA1 was immunoblotted using an anti-ABCA1 monoclonal antibody generated to its C-terminus (Wellington et al. 2002a). Anti-GAPDH primary antibody (Millipore), and anti-mouse-HRP-conjugated secondary antibody (Jackson IR Laboratories) were also used in immunoblotting analyses.

For immunofluorescence, human embryonic kidney (HEK293) cells were grown on 20 mm coverslips. After 36 h of transfection with ABCA1-GFP constructs, cells were fixed with ice cold 100% methanol for 6 min followed by three washes with 1X PBS and permeabilized by three washes with 1X PBS containing 0.3% Triton X-100. After blocking with 4% BSA for 45 min, cells were incubated with rabbit anti-GFP antibody (Abcam) at a 1:200 dilution at 4°C overnight, followed by Alexa 555-labeled goat anti-rabbit secondary IgG (Molecular Probes) at a 1:200 dilution for 1 h. The cells were then mounted on slides with mounting medium containing DAPI (H-1200, Vector Labs) and imaged for ABCA1 and DNA using an Olympus FV1000 confocal microscope.

Cholesterol Efflux

HEK293 cells were plated to near confluency in 24-well tissue culture dishes in DMEM containing 4,500 mg/L glucose and supplemented with 10% FBS and 4 mM L-glutamine. Twenty-four hours after cells were transiently transfected with ABCA1 vectors as described above, 1 μCi/mL of [^{3}H] cholesterol (Perkin–Elmer) was added to the cells. Sixteen hours following loading with [^{3}H] cholesterol, cells were equilibrated in DMEM for 1 h, followed by efflux in the presence or absence of 20 μg/mL of apoA-I (Lee Biosolutions) for 4 h. Radioactivity was measured from the collected supernatant and from cells lysed with 0.1 N NaOH. Both the supernatant and cell lysates were diluted and measured in high flash-point scintillation cocktail fluid (Perkin–Elmer). Efflux data represent the mean plus or minus standard error of six independent experiments, each performed in triplicate. Efflux was calculated as the number of counts in supernatant divided by the number of counts in cell lysates plus supernatant and represents the difference of the efflux in the presence and absence of apoA-I, normalized to wild-type ABCA1. Differences between groups were compared using a one-way ANOVA test with a Neuman–Keulls posttest (GraphPad Prism).

Results

Clinical Description

The proband of Family 1 was a 22-year-old female who presented with gastrointestinal complaints. Clinical examination did not disclose evidence of corneal opacities, yellowish tonsils, or peripheral neuropathy. Abdominal imaging revealed evidence of splenomegaly. Upper and lower gastrointestinal endoscopy was performed with biopsies of the stomach, duodenum, and colon. Staining of colon biopsy specimens with Oil Red O revealed dramatic lipid accumulation in the colonic mucosa (Fig. 1a). Electron micrographs of the biopsied tissue revealed lipid-laden macrophages in the lamina propria with cholesterol crystals, as previously described (Meersseman et al. 2010). Biochemical analysis revealed extremely low HDL-C (0.08 mmol/L [3.1 mg/dL]), low total cholesterol (1.3 mmol/L [50 mg/dL]) and LDL-C (0.7 mmol/L [27 mg/dL]), and undetectable apoA-I levels. Triglycerides were 1.22 mmol/L (108 mg/dL). Both parents displayed low HDL-C (7th and 11th percentile for age and gender in the father and mother, respectively), while the proband's sister had normal plasma lipoprotein levels (Fig. 1b).

The proband of Family 2 was a 26-year-old male referred because of low levels of HDL-C and thrombocytopenia. He was clinically well and free of cardiovascular symptomatology. Previously, he had complained of numbness in his fingers. Nerve conduction studies had shown reduction in amplitude in the right median nerve on motor conduction and in the right median, right ulnar, and left median nerves on sensory conduction, interpreted by the patient's neurologist to be consistent with neuropathy of mixed origin. Clinical examination did not reveal evidence of corneal opacities or yellowish tonsils. He took isoretinoic acid for the treatment of acne. Three siblings of the proband were clinically well. There was no family history of premature coronary artery disease in any members of the family. The proband's father had died of cancer.

Laboratory investigations in the proband revealed low levels of total plasma cholesterol (1.68 mmol/L [65 mg/dL]) and LDL-C (0.89 mmol/L [34 mg/dL]). HDL-C was extremely low (0.2 mmol/L [7.7 mg/dL]) and apoA-I was undetectable. Triglycerides were 1.28 mmol/L (114 mg/dL).

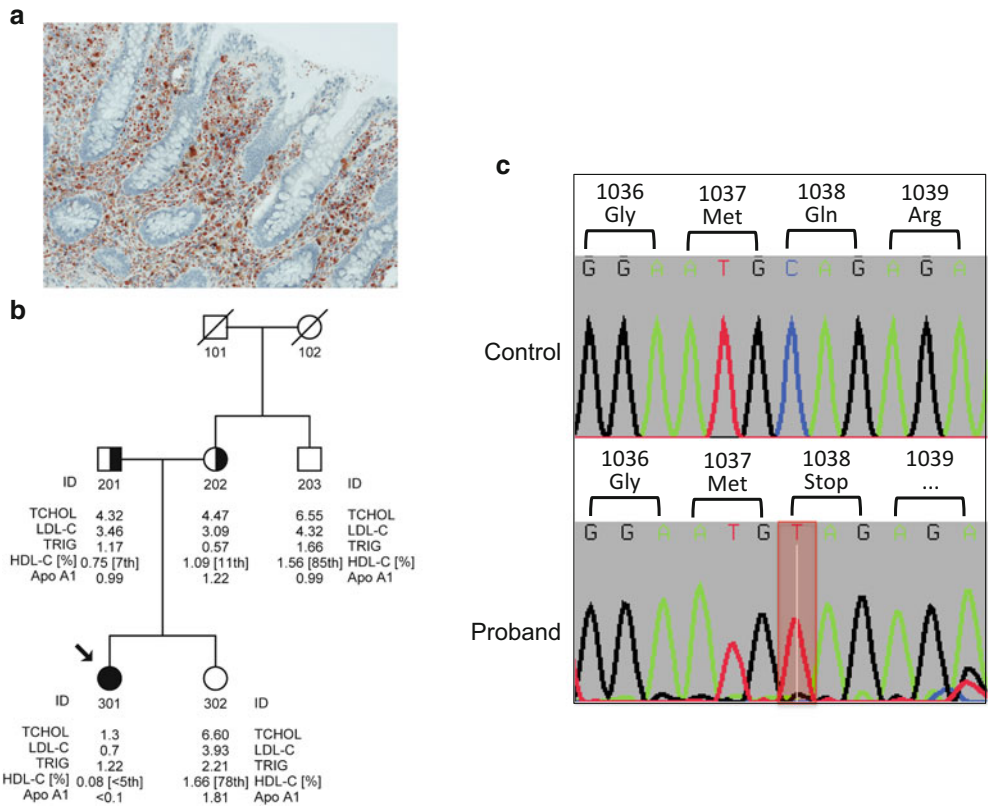

Fig. 1 (**a**) Photomicrograph of a colonic biopsy from the proband of Family 1 with staining of neutral lipids with Oil Red O. Marked accumulation of neutral lipids is observed within the colonic mucosa. (**b**) Pedigree of Family 1. The proband is indicated by an *arrow*. Homozygous affected individuals are shown as *filled symbols*; heterozygous individuals are shown as *half-filled symbols*. Plasma lipid levels are shown below each individual. *Circles*, females; *squares*, males; *diagonal lines*, deceased. Tchol, total cholesterol; Trig, triglycerides. Units are in mmol/L. (**c**) Sequence chromatogram showing *ABCA1* consensus sequence and the sequence of the proband with a homozygous C>T mutation leading to p.Gln1038*. The amino acid translation of the sequence is shown

The proband's father and mother both had moderately low HDL-C levels, 0.86 mmol/L (33 mg/dL) and 0.74 mmol/L (29 mg/dL), representing the 14th and less than the 5th percentile, respectively. All three of the proband's siblings had extremely low levels of HDL-C and apoA-I in keeping with a clinical diagnosis of Tangier disease. Screening of the extended family revealed three additional relatives with low levels of HDL-C (individuals 204, 207, and 318; Fig. 2a).

The proband of Family 3 presented at 4 years of age with abdominal pain without vomiting, steatorrhea, or constipation. She was subsequently found to have splenomegaly, thrombocytopenia (platelet count 143 $\times$ 10^9/L, normal range 200–490 $\times$ 10^9/L), and anemia (hematocrit 0.298, normal range 0.310–0.390). Extended investigations including bone marrow aspirate and biopsy did not reveal an explanation for these findings. On clinical examination she had no corneal opacities and the neurological examination was normal with no evidence of peripheral neuropathy. She was noted to have abnormal yellowish coloration of her tonsils. Other investigations revealed levels of total cholesterol, HDL-C, LDL-C, and apoA-I that were below than the lower limits of detection of the reference laboratory. Plasma triglycerides were elevated at 5.08 mmol/L (452 mg/dL), arguing against the possibility of abetalipoproteinemia. The proband's father had moderately reduced HDL-C (0.64 mmol/L [25 mg/dL], less than the 5th percentile). However, the proband's mother had normal levels of HDL-C (1.36 mmol/L [52 mg/dL], 49th percentile) (Fig. 3a).

Genetic Analysis

In each of these three families, a diagnosis of Tangier disease was suspected on the basis of the characteristic extremely low levels of HDL-C. We applied dideoxy terminator sequencing of the 49 coding exons of the *ABCA1* gene in each of these cases to establish a molecular diagnosis of Tangier disease. In the proband of Family 1, we identified a homozygous c.3112C>T mutation in exon 22 that leads to a stop mutation in the encoded protein, p.Gln1038* (Fig. 1c). Sequencing of the remaining family members indicated that both parents were heterozygous for

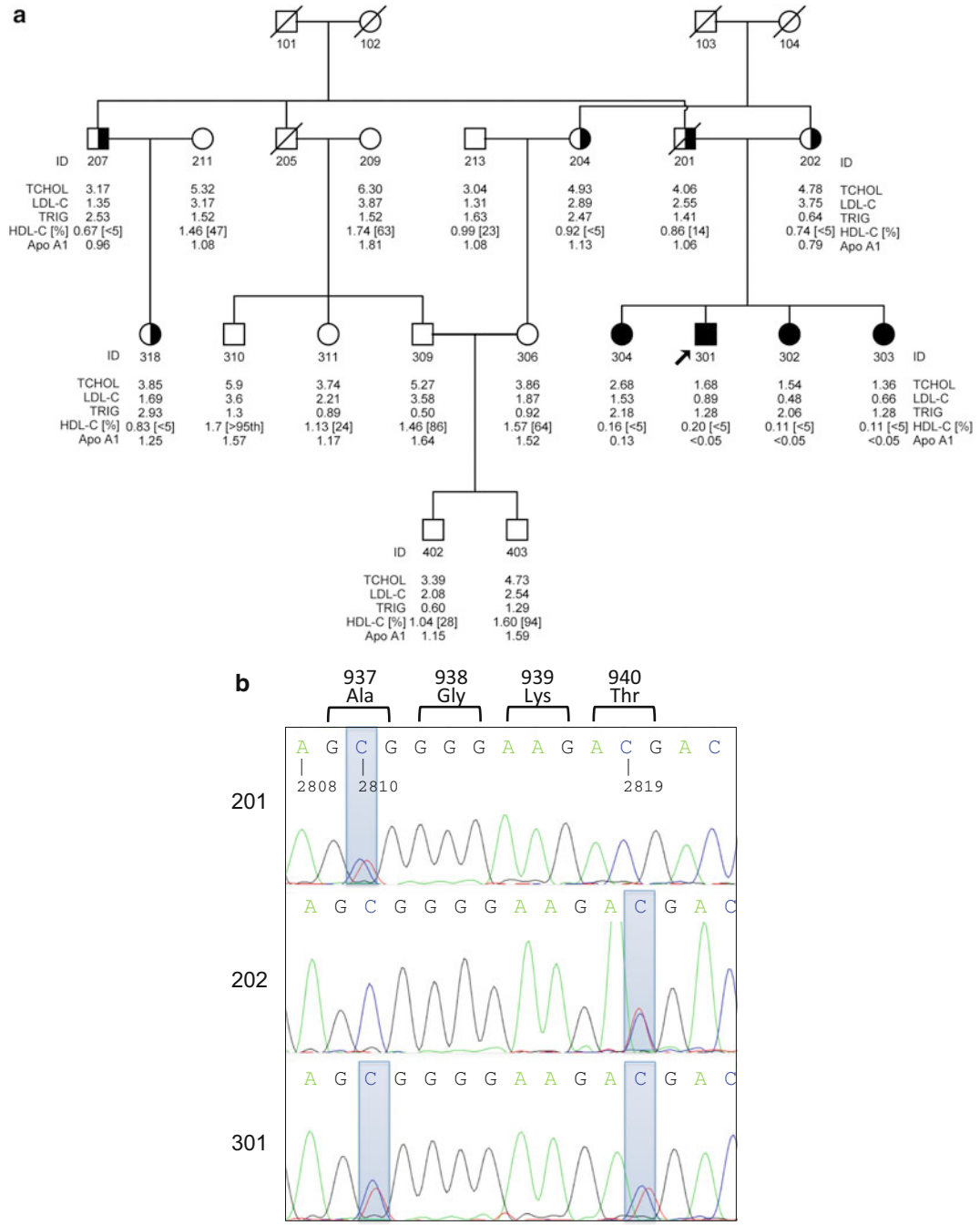

Fig. 2 (**a**) Pedigree of Family 2. The proband is indicated by an *arrow*. Homozygous-affected individuals are shown as *filled symbols*; heterozygous individuals are shown as *half-filled symbols*. Plasma lipid levels are shown below each individual. *Circles*, females; *squares*, males; *diagonal lines*, deceased. Tchol, total cholesterol; Trig, triglycerides. Units are in mmol/L. (**b**) Sequence chromatogram from individuals 201, 202, and 301. The proband (301) is compound heterozygous for c.2810C>T, resulting in a p.Arg937Val substitution, and c.2819C > T, resulting in p.Thr940Met. The proband's father (201) is heterozygous for c.2810C>T while the mother (202) is heterozygous for c.2819C>T, indicating that these two mutations reside on different alleles

this mutation while the proband's sister was homozygous wild type. This mutation was previously reported in a patient with Tangier disease who was compound heterozygous for this mutation and a second missense variant (Candini et al. 2010).

Sequencing of the *ABCA1* gene in the proband of Family 2 disclosed two heterozygous missense mutations, both in exon 19, c.2810C>T, resulting in a p.Arg937Val substitution, and c.2819C>T, resulting in p.Thr940Met (Fig. 2b). We sequenced exon 19 in the remaining members of the

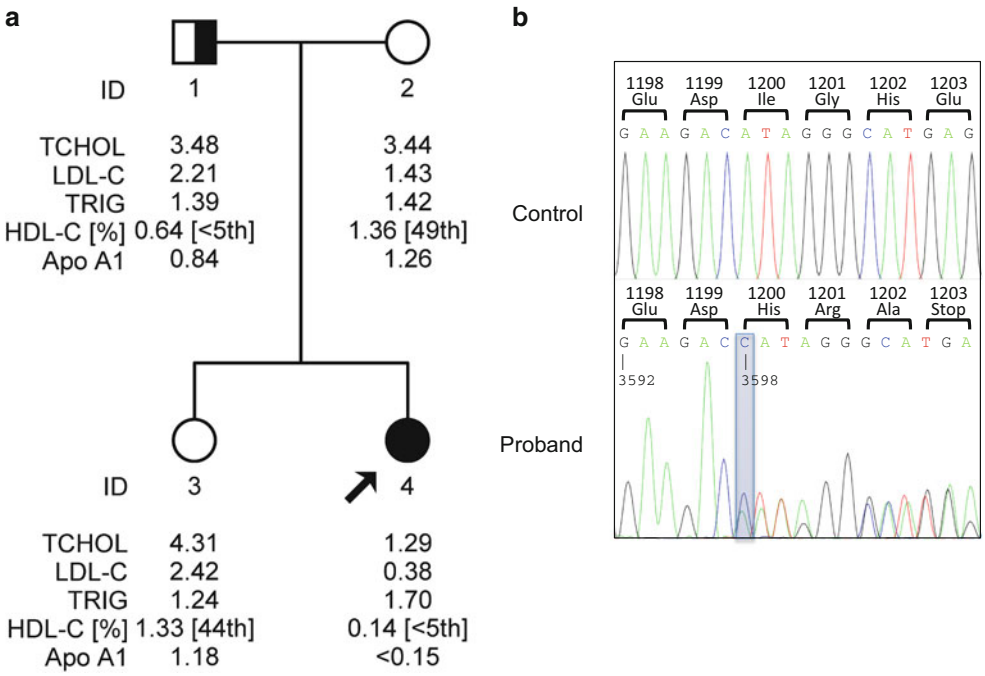

Fig. 3 (**a**) Pedigree of Family 3. The proband is indicated by an *arrow*. Homozygous-affected individuals are shown as *filled symbols*; heterozygous individuals are shown as *half-filled symbols*. Plasma lipid levels are shown below each individual. *Circles*, females; *squares*, males; *diagonal lines*, deceased. Tchol, total cholesterol; Trig, triglycerides. Units are in mmol/L. (**b**) Sequence chromatogram showing *ABCA1* exon 25 consensus sequence (control) and that of the proband. The chromatogram depicts a single nucleotide insertion, c.3597_3598insC, predicted to result in a frameshift and premature truncation at amino acid 1203 (p.Ile1200Hisfs*4)

immediate family and found that the father was heterozygous for p.Arg937Val while the mother was heterozygous for p.Thr940Met, indicating that these two mutations reside on different alleles (Fig. 2b). All three siblings of the proband were compound heterozygotes for these two mutations. Sequencing of the extended family members identified two additional heterozygotes for p.Arg937Val (individuals 207 and 318) and one additional heterozygote for p.Thr940Met (individual 204), with complete co-segregation of these mutations with the low HDL-C phenotype in this pedigree (Fig. 2a). Both p.Arg937Val and p.Thr940Met have been previously reported in patients with Tangier disease (Bodzioch et al. 1999; Uehara et al. 2008).

Sequencing of the *ABCA1* gene in the proband of Family 3 revealed a novel heterozygous insertion mutation in exon 25, c.3597_3598insC, leading to a frameshift and premature protein truncation at amino acid 1203 (p.Ile1200Hisfs*4) (Fig. 3b). We confirmed that the proband's father also carried this mutation, while the mother did not. Subsequent analysis of the remaining coding sequence of the *ABCA1* gene in the proband did not reveal a second mutation. However, based on the proband's characteristic phenotype, we hypothesized that she must harbor a second *ABCA1* mutation. We therefore examined the flanking intronic sequences of the *ABCA1* gene and identified a T>G mutation in intron 29, 11 nucleotides upstream of the start of exon 30 (c.4176-11T>G) (Fig. 4a). This variant is absent in the 1,000 genomes database, indicating that it is extremely rare or private. Intriguingly, this mutation is strongly predicted to create a cryptic splice acceptor site 10 nt upstream of the natural acceptor (T at position −11: −2.9 bits versus G at position −11: 5.8 bits) (Fig. 4b).

To determine the impact of this intronic variant on mRNA splicing, we extracted RNA from the whole blood from the proband and performed RT-PCR, followed by PCR amplification and sequencing of the *ABCA1* cDNA. This identified an insertion mutation at the boundary of exons 29 and 30, corresponding to the insertion of the final 10 nucleotides of intron 29 and thus confirming that c.4176-11T>G creates a cryptic splice acceptor (Fig. 4b). This mutation leads to a frameshift and premature stop mutation in the protein at amino acid position 1397, p. Ser1392Argfs*6. Sequencing of both genomic DNA and reverse-transcribed cDNA and from the proband's mother revealed that she neither carried the intronic mutation nor had abnormal mRNA splicing (data not shown). Sequence analysis of the father's genomic DNA showed that he also did not carry c.4176-11T>G, indicating that this mutation occurred de novo in the proband.

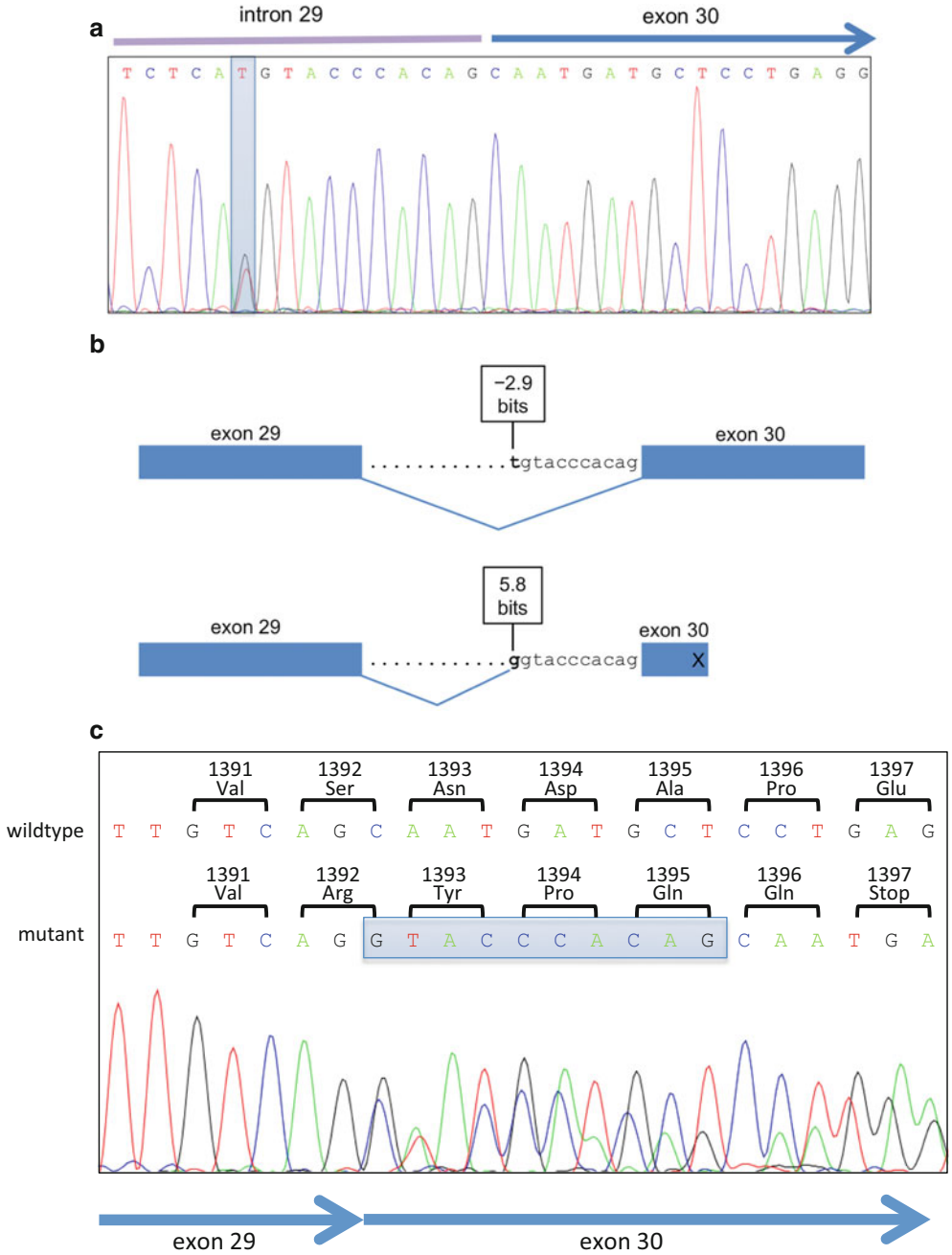

Fig. 4 An intronic mutation in *ABCA1* leads to the creation of a cryptic splice site acceptor and a frameshift in the mRNA. (**a**) Sequence chromatogram from genomic DNA from the proband depicting a heterozygous T>G mutation 11 nucleotides upstream of the start of exon 30. (**b**) This mutation is strongly predicted to increase the strength of a cryptic splice site acceptor that would lead to aberrant mRNA splicing. (**c**) Sequence chromatogram of reverse-transcribed cDNA from the proband depicting a 10 bp insertion mutation (highlighted in the *blue box*) at the beginning of exon 30 corresponding to the last 10 bp of the intron 29 sequence (gtacccacag). The sequence of the wild-type and mutant alleles is shown above the chromatogram with the amino acid translation. The insertion mutation leads to premature truncation of the protein at amino acid 1397 (p.Ser1392Argfs*6). This indicates that the intron 29 mutation in panel A results in creation of a cryptic splice acceptor with abnormal mRNA splicing

Cholesterol Efflux

While premature truncation mutations in *ABCA1* have been shown to be uniformly deleterious (Wellington et al. 2002b), missense mutations in *ABCA1* are associated with a wide range of phenotypes, ranging from no functional impact to complete loss of function (Singaraja et al. 2006). While p.Arg937Val has been shown to be a functionally null mutation (Landry et al. 2006), the functional impact of p.Thr940Met has not been previously assessed. To

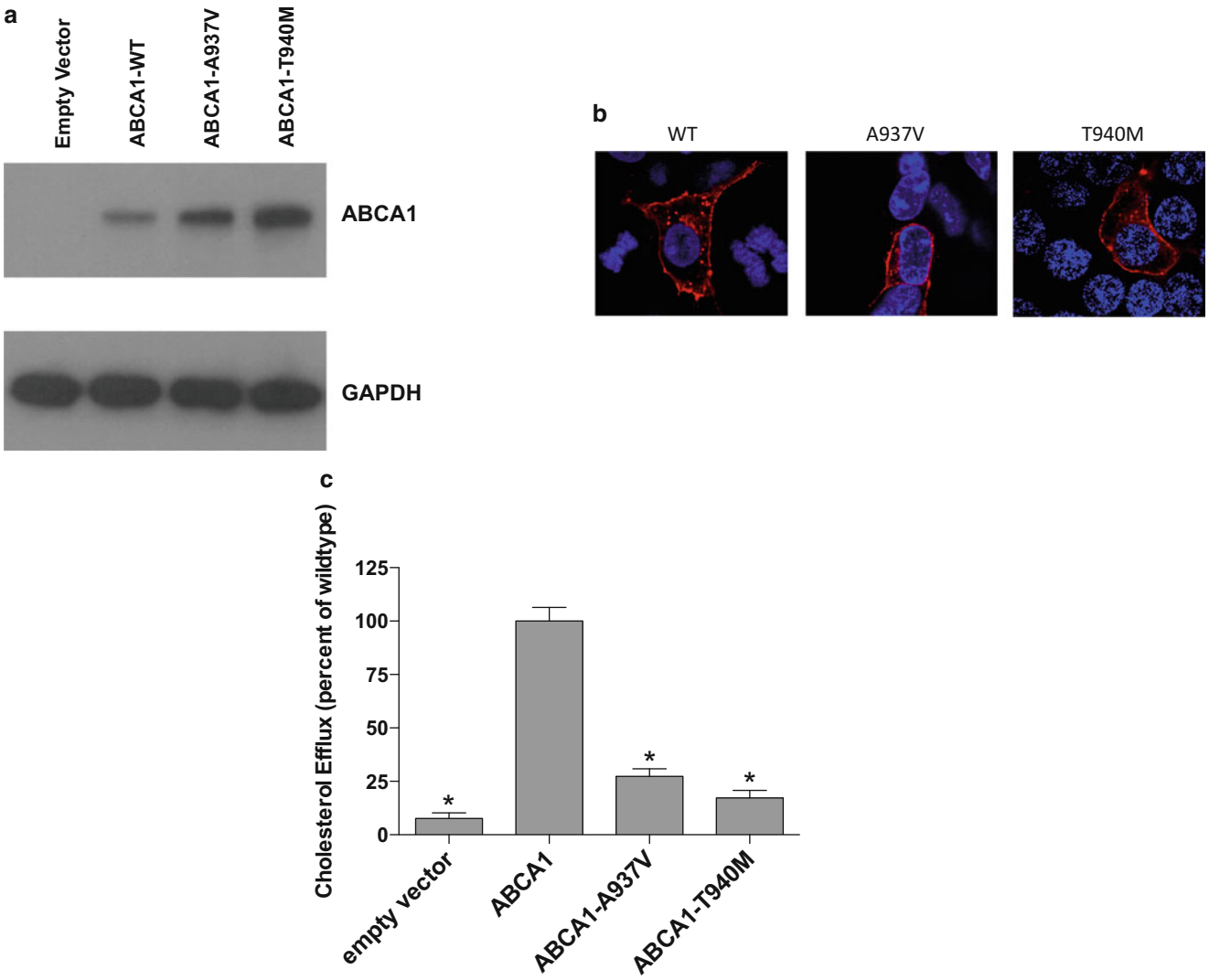

Fig. 5 Functional analysis of *ABCA1* missense mutations. (**a**) cDNA constructs containing wild-type ABCA1, or the p.Arg937Val or p. Thr940Met mutations, were obtained. HEK293 cells were transiently transfected with either empty vector or these ABCA1 alleles and protein expression determined by Western blot. Both mutations were robustly expressed relative to wild-type ABCA1. (**b**) Immunofluorescence revealed a normal pattern of subcellular localization of both mutants. hABCA1 was probed with an anti-GFP primary antibody. *Blue*: DAPI, Red: hABCA1. (**c**) Cholesterol efflux assays demonstrated that both mutations had significantly impaired apoA-I-dependent cholesterol efflux activity relative to wild-type ABCA1. *, $p < 0.01$ compared to wild-type ABCA1

determine the functional impact of this mutation on the ABCA1 protein, we obtained cDNA clones expressing wild-type ABCA1 as well as ABCA1-Arg937Val and ABCA1-Thr940Met. HEK293 cells were transiently transfected with these plasmids and ABCA1 protein expression was determined by Western blot. Both mutations were robustly expressed (Fig. 5a). Subcellular localization was determined by immunofluorescence and revealed a similar pattern of expression of these mutations compared to wild-type ABCA1, with prominent localization to the plasma membrane as well as intracellular compartments (Fig. 5b). We next quantified apoA-I -dependent cholesterol efflux in HEK293 cells transiently transfected with these alleles. Consistent with previous reports, we observed significantly less cholesterol efflux in cells which express p.Arg937Val compared to wild-type ABCA1 (Landry et al. 2006). Cells expressing p.Thr940Met also elicited significantly less efflux compared to wild-type ACBA1 (Fig. 5c). These data indicate that both p.Thr940Met and p.Arg937Val result in near complete loss of ABCA1-mediated cholesterol efflux and are functionally null alleles.

Glucose Metabolism

ABCA1 plays a crucial role in glucose homeostasis, and previous studies have shown that heterozygous *ABCA1*

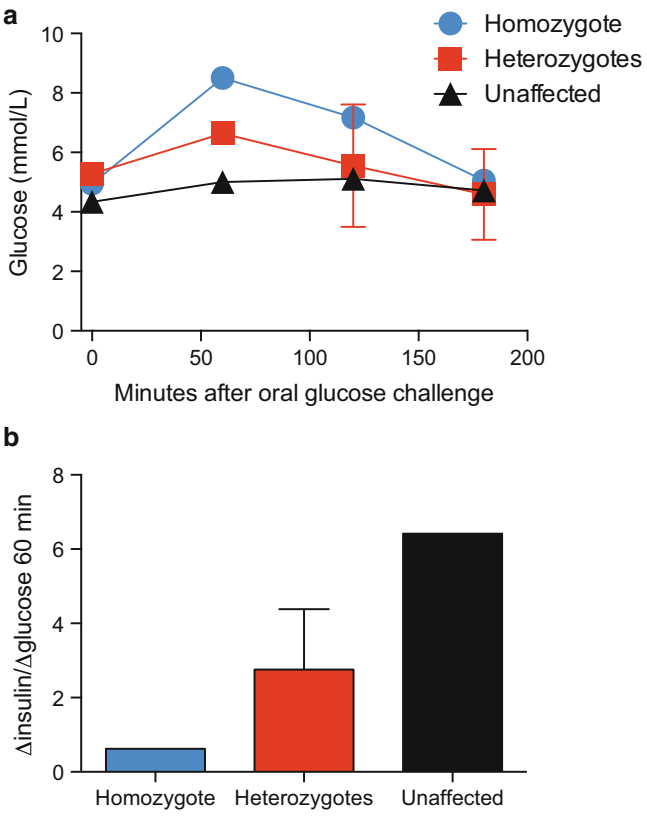

Fig. 6 Glucose metabolism in Family 1. (**a**) Oral glucose tolerance testing results. A standard 75 g oral glucose load was administered to fasting individuals and plasma glucose measured at the indicated time points. (**b**) Acute insulin response to glucose. The change in insulin divided by the change in glucose over the first 60 min of the oral glucose tolerance test is shown. The homozygous-affected individual has markedly blunted acute insulin response to glucose, suggesting impaired islet function

mutation carriers have impaired islet function (Brunham et al. 2007; Vergeer et al. 2010). However, there is a paucity of data regarding glucose homeostasis in Tangier disease subjects. We performed glucose tolerance testing in the proband and the immediate family members of Family 1. The proband did not have a previous history of dysglycemia and was lean (body mass index 22.4) with no evidence of insulin resistance. Despite her normal fasting blood glucose (4.9 mmol/L, normal range 3.6–5.5 mmol/L) and glycosylated hemoglobin level (4.6%, normal range 4.0–6.0%), the proband displayed substantial glucose intolerance relative to her unaffected sister, with the two heterozygous parents displaying an intermediate phenotype (Fig. 6a). The acute insulin response to glucose, a measure of islet function, was markedly reduced in the proband, with a lesser reduction in the heterozygous parents (Fig. 6b), suggesting a gene–dose relationship between ABCA1 activity and glucose intolerance and islet dysfunction in this family.

Discussion

Here we report three Tangier disease kindreds harboring both known and novel mutations in the *ABCA1* gene. To date ~100 patients have been described with molecularly confirmed Tangier disease. Approximately 50 mutations in the *ABCA1* gene have been reported in association with Tangier disease and a further ~130 mutations associated with familial low HDL-C or other phenotypes (Brunham et al. 2006b; Stenson et al. 2009). We report an additional six individuals with Tangier disease, as well as the description of two novel *ABCA1* mutations.

We show that the missense mutations p.Arg937Val and p.Thr940Met both result in near complete loss of apoA-I-dependent cholesterol efflux activity, despite normal subcellular localization to the plasma membrane. Both of these mutations occur near the first nucleotide-binding domain of the ABCA1 protein, a region known to be functionally critical for the transporter (Singaraja et al. 2003), and our results showing normal subcellular localization suggest that these mutations may have impaired ATP binding, thus resulting in defective cholesterol efflux. Very recently, p.Thr940Met was also reported in a targeted re-sequencing study of more than 6,000 individuals in which it was observed in a single individual with low cholesterol (Service et al. 2014). Our in vitro functional characterization of this mutation showing defective cholesterol efflux provides a biological basis and functional validation of that association.

In Family 1, we demonstrated that despite a low BMI, the affected proband had substantial islet dysfunction and that the heterozygous parents had a more mild impairment in glucose tolerance. ABCA1 is highly expressed in the pancreatic beta cell where it is essential for preventing excess cholesterol accumulation. Absence of beta cell *Abca1* in mice leads to islet cholesterol overload and dysfunctional exocytosis of insulin-containing granules (Brunham et al. 2007; Kruit et al. 2012). Heterozygous carriers of *ABCA1* mutations have impaired glucose tolerance and islet function (Vergeer et al. 2010), but the phenotype of homozygous Tangier disease patients as regards glucose homeostasis is less clear. One hypothesis is that the marked reduction in total plasma cholesterol and LDL cholesterol that typically accompanies Tangier disease may limit the degree of islet cholesterol accumulation and glucose intolerance, similar to what has been observed in mice, thereby limiting any impact on islet dysfunction in Tangier disease patients (Brunham et al. 2008b). However, whether the level of residual ABCA1 activity or the plasma cholesterol concentration is more important for determining islet function in humans is unknown. One previous study reported impaired glucose tolerance in four unrelated Tangier disease probands, but that study lacked appropriate

controls and, in addition, all four Tangier disease patients in that study met diagnostic criteria for diabetes based on fasting blood sugar, suggesting ascertainment bias in the results (Koseki et al. 2009). Our results in Family 1 using family member controls with normal fasting blood sugars show a gene–dose relationship with glucose tolerance and suggest that Tangier disease patients may have even greater islet dysfunction than heterozygous *ABCA1* mutation carriers. Our data suggest that Tangier disease patients should be screened for glucose tolerance even in the presence of normal fasting blood glucose or glycosylated hemoglobin. However, it is important to note that our results are from a single, small family and further investigation of glucose homeostasis in larger Tangier disease families using well-matched, related controls will be required to clarify the phenotype of glucose metabolism in Tangier disease.

A remarkable feature of Family 2 is the observation that all four siblings inherited two mutant alleles of *ABCA1* and have clinical and genetically confirmed Tangier disease. The probability of observing this pattern of inheritance based on Mendelian segregation is 1/256. Preferential transmission of the Tangier disease chromosome has not been previously reported, suggesting that this represents a chance occurrence. Nevertheless, the large size of this pedigree with 4 Tangier disease patients, 3 heterozygotes, and multiple unaffected individuals provides an opportunity for future investigation into disease phenotypes associated with Tangier disease in a controlled setting.

The proband of Family 3 initially posed a diagnostic dilemma for two reasons: firstly, this individual's biological mother had normal levels of HDL cholesterol (49th percentile for age and gender), arguing against a carrier status for a functional *ABCA1* mutation, and, secondly, examination of the coding sequence of the *ABCA1* gene in the proband disclosed only one mutation (c.3597_3598insC). This puzzle was solved by the identification of a novel, de novo intronic mutation in the proband (c.4176-11T>G) that resulted in aberrant mRNA splicing and protein truncation, thus both explaining the severe phenotype of the proband (being compound heterozygous for two truncation mutations) and providing a mechanism for why her mother is phenotypically normal. To our knowledge, this represents the first documentation of a de novo mutation in the *ABCA1* gene causing Tangier disease and suggests that even in the absence of phenotypically affected parents, investigation of patients with extremely low HDL cholesterol for Tangier disease may be warranted in the appropriate clinical setting.

The patient we report in Family 3 is among the youngest patients described with Tangier disease. One previous study described Tangier disease in a four-month-old infant with gastrointestinal complaints (Lachaux et al. 1998), although molecular confirmation of the diagnosis was not available at that time. In general, reports of Tangier disease in extremely young children are unusual. However, the determinants of age of onset of the clinical manifestations of Tangier disease are not known. The most common presenting complaints in patients ultimately identified as having Tangier disease are peripheral neuropathy and yellowish tonsils (Assmann et al. 2013). Both the four-year-old proband we describe in Family 3 of this report and the four-month-old patient previously described (Lachaux et al. 1998) presented with abdominal symptoms, suggesting that this may be a more common presentation in very young children with Tangier disease. While the confirmation of Tangier disease in this patient provides a molecular explanation for her plasma lipoprotein abnormalities, given her atypical presentation, it remains possible that not all features of her presentation are attributable to *ABCA1* deficiency.

In summary, we provide molecular evidence of Tangier disease in 6 patients and report two novel mutations in the *ABCA1* gene, as well as document the functional impact of two previously reported missense mutations in *ABCA1*. We also report the first documented de novo mutation in *ABCA1*, a novel intronic mutation that creates a cryptic splice site acceptor leading to a frameshift in the mRNA and premature truncation. Our results expand our understanding of the clinical and biochemical phenotypes associated with specific mutations in the *ABCA1* gene.

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Compliance with Ethics Guidelines

Conflict of Interest

The authors of this manuscript declare that they have no conflict of interest.

Informed Consent

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study. No identifying information about patients is included in this manuscript.

Animal Rights

This article does not contain any studies with animal subjects performed by any of the authors.

Author Contributions

Liam R. Brunham: Designed and conducted experiments, characterized patients, analyzed data, wrote manuscript, guarantor

Martin H. Kang: Conducted experiments

Clara Van Karnebeek: Characterized patients, wrote manuscript

Singh N. Sadananda: Conducted experiments

Jennifer A. Collins: Wrote manuscript

Lin-Hua Zhang: Conducted experiments

Bryan Sayson: Wrote manuscript

Fudan Miao: Conducted experiments

Sylvia Stockler: Characterized patients, wrote manuscript

Jiri Frohlich: Characterized patients

David Cassiman: Characterized patients

Simon W. Rabkin: Characterized patients

Michael R. Hayden: Designed experiments, characterized patients, wrote manuscript

References

Alrasadi K, Ruel IL, Marcil M, Genest J (2006) Functional mutations of the ABCA1 gene in subjects of French-Canadian descent with HDL deficiency. Atherosclerosis 188(2):281–291

Assmann G, von Eckardstein A, Brewer HB (2013) Familial analphalipoproteinemia: Tangier disease. In: Scriver CR, Beaudet AL, Sly WS, Valle D, Vogelstein B (eds) The online metabolic and molecular bases of inherited disease, Chapter 22, McGraw-Hill, New York

Bodzioch M, Orso E, Klucken J et al (1999) The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease. Nat Genet 22(4):347–351

Brunham LR, Kruit JK, Iqbal J et al (2006a) Intestinal ABCA1 directly contributes to HDL biogenesis in vivo. J Clin Invest 116 (4):1052–1062

Brunham LR, Singaraja RR, Hayden MR (2006b) Variations on a gene: rare and common variants in ABCA1 and their impact on HDL cholesterol levels and atherosclerosis. Annu Rev Nutr 26:105–129

Brunham LR, Kruit JK, Pape TD et al (2007) Beta-cell ABCA1 influences insulin secretion, glucose homeostasis and response to thiazolidinedione treatment. Nat Med 13(3):340–347

Brunham LR, Kastelein JJ, Hayden MR (2008a) ABCA1 gene mutations, HDL cholesterol levels, and risk of ischemic heart disease. JAMA 300(17):1997–1998, author reply 1998

Brunham LR, Kruit JK, Verchere CB, Hayden MR (2008b) Cholesterol in islet dysfunction and type 2 diabetes. J Clin Invest 118(2):403–408

Brunham LR, Singaraja RR, Duong M et al (2009) Tissue-specific roles of ABCA1 influence susceptibility to atherosclerosis. Arterioscler Thromb Vasc Biol 29(4):548–554

Candini C, Schimmel AW, Peter J et al (2010) Identification and characterization of novel loss of function mutations in ATP-binding cassette transporter A1 in patients with low plasma high-density lipoprotein cholesterol. Atherosclerosis 213(2):492–498

Chung S, Sawyer JK, Gebre AK, Maeda N, Parks JS (2011) Adipose tissue ATP binding cassette transporter A1 contributes to high-density lipoprotein biogenesis in vivo. Circulation 124 (15):1663–1672

Clee SM, Kastelein JJ, van Dam M et al (2000) Age and residual cholesterol efflux affect HDL cholesterol levels and coronary artery disease in ABCA1 heterozygotes. J Clin Invest 106(10):1263–1270

Cohen JC, Kiss RS, Pertsemlidis A, Marcel YL, McPherson R, Hobbs HH (2004) Multiple rare alleles contribute to low plasma levels of HDL cholesterol. Science 305(5685):869–872

Karasinska JM, Rinninger F, Lutjohann D et al (2009) Specific loss of brain ABCA1 increases brain cholesterol uptake and influences neuronal structure and function. J Neurosci 29(11):3579–3589

Koseki M, Matsuyama A, Nakatani K et al (2009) Impaired insulin secretion in four Tangier disease patients with ABCA1 mutations. J Atheroscler Thromb 16(3):292–296

Kruit JK, Wijesekara N, Westwell-Roper C et al (2012) Loss of both ABCA1 and ABCG1 results in increased disturbances in islet sterol homeostasis, inflammation, and impaired beta-cell function. Diabetes 61(3):659–664

Lachaux A, Sassolas A, Loras-Duclaux I, Le Gall C, Bouvier R (1998) Digestive tract involvement in an infant and a child with Tangier disease. J Pediatr Gastr Nutr 27(2):234–236

Landry YD, Denis M, Nandi S, Bell S, Vaughan AM, Zha X (2006) ATP-binding cassette transporter A1 expression disrupts raft membrane microdomains through its ATPase-related functions. J Biol Chem 281(47):36091–36101

Meersseman W, Vanderschueren S, De Vos R, Cassiman D (2010) Lysosomal lipid vacuoles in macrophages located in the colon. J Inherit Metab Dis 33(3):303–304

Service SK, Teslovich TM, Fuchsberger C et al (2014) Re-sequencing expands our understanding of the phenotypic impact of variants at GWAS loci. PLoS Genet 10(1):e1004147

Singaraja RR, Brunham LR, Visscher H, Kastelein JJ, Hayden MR (2003) Efflux and atherosclerosis: the clinical and biochemical impact of variations in the ABCA1 gene. Arterioscler Thromb Vasc Biol 23(8):1322–1332

Singaraja RR, Visscher H, James ER et al (2006) Specific mutations in ABCA1 have discrete effects on ABCA1 function and lipid phenotypes both in vivo and in vitro. Circ Res 99(4):389–397

Stenson PD, Mort M, Ball EV et al (2009) The Human Gene Mutation Database: 2008 update. Genome Med 1(1):13

Tietjen I, Hovingh GK, Singaraja R et al (2012) Increased risk of coronary artery disease in Caucasians with extremely low HDL cholesterol due to mutations in ABCA1, APOA1, and LCAT. Biochim Biophys Acta 1821(3):416–424

Timmins JM, Lee JY, Boudyguina E et al (2005) Targeted inactivation of hepatic Abca1 causes profound hypoalphalipoproteinemia and kidney hypercatabolism of apoA-I. J Clin Invest 115 (5):1333–1342

Uehara Y, Tsuboi Y, Zhang B et al (2008) POPC/apoA-I discs as a potent lipoprotein modulator in Tangier disease. Atherosclerosis 197(1):283–289

van Dam MJ, de Groot E, Clee SM et al (2002) Association between increased arterial-wall thickness and impairment in ABCA1-driven cholesterol efflux: an observational study. Lancet 359 (9300):37–42

Vergeer M, Brunham LR, Koetsveld J et al (2010) Carriers of loss-of-function mutations in ABCA1 display pancreatic beta-cell dysfunction. Diabetes Care 33(4):869–874

Wellington CL, Walker EK, Suarez A et al (2002a) ABCA1 mRNA and protein distribution patterns predict multiple different roles and levels of regulation. Lab Invest 82(3):273–283

Wellington CL, Yang YZ, Zhou S et al (2002b) Truncation mutations in ABCA1 suppress normal upregulation of full-length ABCA1 by 9-cis-retinoic acid and 22-R-hydroxycholesterol. J Lipid Res 43(11):1939–1949