Diet1, bile acid diarrhea, and FGF15/19: mouse model and human genetic variants[S]

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Abstract Diet1 modulates intestinal production of the hormone, fibroblast growth factor (FGF)15, which signals in liver to regulate bile acid synthesis. C57BL/6ByJ mice with a spontaneous *Diet1*-null mutation are resistant to hypercholesterolemia compared with wild-type C57BL/6J mice through enhanced cholesterol conversion to bile acids. To further characterize the role of Diet1 in metabolism, we generated *Diet1*$^{-/-}$ mice on the C57BL/6J genetic background. C57BL/6J *Diet1*$^{-/-}$ mice had elevated bile acid levels, reduced *Fgf15* expression, and increased gastrointestinal motility and intestinal luminal water content, which are symptoms of bile acid diarrhea (BAD) in humans. Natural genetic variation in *Diet1* mRNA expression levels across 76 inbred mouse strains correlated positively with *Ffg15* mRNA and negatively with serum bile acid levels. This led us to investigate the role of *DIET1* genetic variation in primary BAD patients. We identified a *DIET1* coding variant (*rs12256835*) that had skewed prevalence between BAD cases and controls. This variant causes an H1721Q amino acid substitution that increases the levels of FGF19 protein secreted from cultured cells. We propose that genetic variation in *DIET1* may be a determinant of FGF19 secretion levels, and may affect bile acid metabolism in both physiological and pathological conditions.—Lee, J. M., J. R. Ong, L. Vergnes, T. Q. de Aguiar Vallim, J. Nolan, R. M. Cantor, J. R. F. Walters, and K. Reue. **Diet1, bile acid diarrhea, and FGF15/19: mouse model and human genetic variants. *J. Lipid Res.* 2018.** 59: **429–438.**

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Cholesterol is eliminated from the body through the catabolism of cholesterol to bile acids, and subsequent loss of bile acids in the feces (1–3). The elimination of bile acids through the intestine is the basis for the reduction of serum cholesterol levels through the use of bile acid binding resins, which sequester bile acids in the intestine to form an insoluble complex (4–6). More recently, we identified a mutant mouse model that exhibits constitutive conversion of cholesterol to bile acids and elimination through feces and urine, leading to resistance to dietary cholesterol (7, 8). The underlying mutation occurs in the *Diet1/Malrd1* gene, which encodes a 236 kDa protein that is expressed exclusively in epithelial cells lining the small intestinal villi and kidney proximal tubules (7). Because *Diet1* is the original gene name and all studies published thus far use *Diet1*, we will continue to use this designation here. Diet1 influences enterohepatic signaling between the small intestine and liver to modulate hepatic bile acid synthesis. Specifically, Diet1 deficiency leads to reduced production and/or secretion by enterocytes of the hormone, fibroblast growth factor (FGF)15 (FGF19 in humans) (7). FGF15/19 is secreted by the ileum into the enterohepatic circulation, and downregulates bile acid synthesis by signaling through hepatic coreceptors (FGF receptor 4 and β klotho) (2, 9–11).

The augmentation of flux through the bile acid synthetic pathway has been successfully used therapeutically to control plasma cholesterol levels (2, 12, 13). However, the amount of bile acids lost through excretion must be carefully regulated, as increased bile acid levels in the intestine can trigger the flow of water into the colon, resulting in bile acid malabsorption, also known as bile acid diarrhea (BAD) (2, 14). One of the mechanisms by which elevated intestinal bile acids may influence the severity of BAD is by

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Abbreviations: BAD, bile acid diarrhea; FGF, fibroblast growth factor; HMDP, Hybrid Mouse Diversity Panel; MAF, minor allele frequency; MAM, meprin/A5-protein/PTPmu; SeHCAT, selenium homocholic acid taurine; SNV, single nucleotide variant.

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increasing colonic motility (15, 16). BAD may be classified into three types based on distinct etiologies (15). Type 1 BAD was first recognized in association with surgical resection or disease of the terminal ileum, which leads to impaired reabsorption of bile acids from the intestinal lumen (17). This results in elevated fecal bile acid levels and is treated with bile acid sequestrants (18). Type 2 BAD (referred to herein as "primary BAD") was recognized in 1976 as a similar syndrome without obvious ileal disease (19). Primary BAD appears to be heterogeneous in etiology (20–22). Type 3 BAD encompasses chronic diarrheas caused by other conditions that can affect bile acid absorption, such as cholecystectomy or bacterial overgrowth in the small intestine (23).

Primary BAD is the most common of the bile acid malabsorption syndromes, estimated to affect about 1% of the European population, and accounting for about 30% of cases with chronic diarrhea and diarrhea-predominant irritable bowel syndrome (24). The definitive test for primary BAD is the measurement of fecal bile acid levels, but this is demanding and not usually performed clinically. Alternatively, BAD may be defined by administering a radiolabeled bile acid [selenium homocholic acid taurine (SeHCAT)] and determining the amount retained in the body after 7 days (25, 26). Patients with primary BAD eliminate the radiolabel more rapidly than controls, as determined by scanning with a γ camera (25). This test is available in Europe, but not in the United States. An additional screening method for BAD is the quantification of the bile acid synthetic intermediate, C4 (7α-hydroxycholest-4-ene-3-one), which is inversely correlated with SeHCAT retention (27).

A relationship between BAD and FGF19 levels has been identified by Walters and colleagues (28). BAD patients were diagnosed by reduced SeHCAT retention in the absence of bile acid sequestrants. In BAD patients, FGF19 levels were lower than normal and there was a positive correlation between FGF19 and percent SeHCAT retention (28). There was also an inverse relationship between FGF19 and C4 levels (16, 29).

Animal studies provide independent support for a key role of FGF15/19 in bile acid homeostasis. In monkeys, administration of anti-FGF19 antibodies produced severe bile acid loss and diarrhea (30). Furthermore, mice carrying a knockout mutation for FGF15 exhibited excess fecal bile acid secretion, as observed in humans (31). As described earlier, Diet1-deficient mice exhibit very low levels of FGF15 in the small intestine and constitutive upregulation of hepatic bile acid synthesis (7). Providing FGF15 to Diet1-deficient mice led to normalization of bile acid synthetic gene expression, demonstrating that Diet1 functions upstream of FGF15 to modulate bile acid levels (7).

In the current study, we characterize the effects of mouse *Diet1*-null mutation on an isogenic C57BL/6J background and find that these mice experience traits reminiscent of BAD in humans. We further assess whether natural genetic variation in *Diet1* expression across more than six dozen inbred mouse strains is correlated with *Fgf15* expression and bile acid traits. We expand our investigation to evaluate the presence of functional *DIET1* variants in humans, and characterize a nonsynonymous coding variant that has a significantly different prevalence in control and BAD subjects, and which influences FGF19 levels secreted from cultured cells. These studies expand our knowledge of the role of variation in Diet1 levels and the sequence in traits associated with bile acid metabolism.

MATERIALS AND METHODS

Diet1$^{-/-}$ congenic mice

C57BL/6J and C57BL/6ByJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). *Diet1*$^{-/-}$ congenic mice were generated by backcrossing the *Diet1* allele from C57BL/6ByJ mice onto the C57BL/6J background for eight generations. The *Diet1* allele was genotyped using PCR primers specific for the mutant and wild-type alleles, as described previously (7). Heterozygous mice from the N8 backcross generation were intercrossed to produce wild-type (*Diet1*$^{+/+}$) and *Diet1*$^{-/-}$ mice. Mice were placed on an atherogenic diet (TD88051; Harlan Laboratories, Madison, WI), as previously described (7), for 3 or 6 weeks before tissues were collected. All mouse studies were conducted in accordance with and approved by the Institutional Animal Research Committee of the University of California, Los Angeles.

Mouse plasma lipid and bile acid measurements

Mice were fasted for 16 h and blood was collected from the retro-orbital sinus under isoflurane anesthesia. Enzymatic assays for total cholesterol and HDL cholesterol were performed as described (32). Total bile acid levels were assessed using Diazyme total bile acids assay kit (DZ042A-K; Diazyme Laboratories, Poway, CA).

Hybrid Mouse Diversity Panel analysis

The Hybrid Mouse Diversity Panel (HMDP) strains have been described previously (33). Briefly, 76 inbred and recombinant inbred mouse strains (three to five mice per strain) were assessed at 8 weeks for gene expression in the ileum for *Diet1* and *Fgf15*. Biliary bile acid and cholesterol levels were determined by calorimetric assays from Diazyme and Thermo Fisher (A12216; Thermo Fisher Scientific, Grand Island, NY), respectively. Correlation analyses were performed in Prism (v7, GraphPad Software, La Jolla, CA).

Gene expression and quantitative PCR

Liver and small intestine were isolated from mice following a 16 h fast and RNA was prepared using Trizol (Invitrogen). Primer sequences for quantitative PCR of *Diet1*, *Fgf15*, *Asbt*, *Cyp7a1*, Cyp8b1, *Cyp27*, *Fgfr4*, and *B2m* (as a normalization gene) were reported previously (7). *Rpl4* primer sequences were 5′gatgagctgtatggcacttgg3′ for forward primer and 5′cttgtgcatgggcaggtt3′ for reverse primer.

Determination of gastrointestinal transit time

Mice were fed an atherogenic diet for 2 weeks. After an overnight fast with ad libitum water, mice were gavaged with a semiliquid solution (0.2 ml) of 5% Evans blue and 1.5% methylcellulose (without additional food), as previously described (34). Mice were continuously monitored and the time to produce the initial blue fecal pellet was determined as a measure of gastrointestinal transit time.

Intestinal water content

Mice were fed an atherogenic diet for 2 weeks, then fasted for 18 h with ad libitum water, and refed for 3 h with atherogenic diet. The small intestine and colon were excised precisely and the contents collected by gentle extrusion. Contents were weighed, desiccated for 3 days, and then weighed again to determine the fluid and solid weights, as described previously (35–37). Weights of the water and dry intestinal contents were expressed as absolute amounts, and after normalization to intestinal lengths.

Study subjects

Genomic DNA was studied in an initial cohort of 44 subjects that has been described before (38) (**Table 1**). Twenty-two of the subjects had chronic diarrhea due to primary BAD, with SeHCAT 7 day retention between 1% and 7%. The control group of 22 subjects had normal bowel habits and no evidence of malabsorption. Both groups were of predominantly Caucasian origin with no subjects of African descent. The DNA samples were obtained under the approval of the local Research Ethics Committees (Institutional Review Boards) of Hammersmith, Queen Charlotte's and Chelsea, Acton, and Wrightington, Wigan, and Leigh Hospitals. Informed consent was obtained from all subjects. The DNA was sequenced under a protocol approved by the University of California, Los Angeles Institutional Review Board.

Genomic DNA was also studied in an additional cohort of patients, who have also been previously described (28) (Table 1). This cohort consisted of a prospectively recruited set of 78 patients with chronic diarrhea who underwent SeHCAT testing and FGF19 measurements. This cohort was also predominantly Caucasian in origin.

DIET1 sequencing

The exons and flanking intron sequences of *DIET1* were amplified by PCR using the oligonucleotide primer sequences listed in supplemental Table S1. PCR products were treated with recombinant exonuclease I and shrimp alkaline phosphatase (Agilent Technologies, Santa Clara, CA), and sequenced on a Biosystems 3730 capillary DNA analyzer using BigDye terminator cycle sequencing reagents. Variants were confirmed by amplification of an independent PCR product and sequencing both strands.

The *rs12256835* genotyping

Genomic DNA was extracted from patient blood using QiAmp DNA mini kit (Qiagen, Manchester, UK) according to the manufacturer's protocols. Samples were genotyped for the *rs12256835* variant using a TaqMan allelic discrimination assay (Life Sciences, Paisley, UK). PCR was performed on 96-well reaction plates and analyzed using a StepOnePlus RT-PCR machine (Life Sciences) to obtain an allelic discrimination plot, including a negative water control. Reaction volume was 15 μl per well, including 7.5 μl TaqMan genotyping Master Mix (2×), 0.375 μl SNP assay mix (40×), 6.125 nuclease-free water, and 1 μl genomic DNA. Standard PCR conditions were: 95°C for 10 min, followed by 40 cycles of amplification at 95°C for 15 s, and 60°C for 1 min, followed by 60°C for 30 s.

Serum FGF19 measurements

Serum FGF19 levels were measured by commercial ELISA assay (FGF19 Quantikine ELISA; R&D Systems, Minneapolis, MN), as previously described. Aliquots (100 μl) of serum were assayed in duplicate according to the manufacturer's protocols.

Expression constructs and site-directed mutagenesis

Expression plasmids for human Diet1-V5 and FGF19-Myc were generated previously (7). Site-directed mutagenesis was performed on the plasmid expressing Diet1-1721H to generate Diet1-1721Q using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies) with primers gaagctcactgtgcacagtatacaagcacaacag (hDIET1_H1721Q-F) and ctgttgtgcttgtatactgtgcacagtgagcttc (hDIET1_H1721Q-R). Site-directed mutagenesis was performed on the plasmid expressing Diet1-1712D to generate Diet1-1721G using primers cataagccagattgctctggtaggtctgatgaagc (hDIET1_H1712G-F) and gcttcatcagacctaccagagcaatctggcttatag (hDIET1_H1712G-R).

FGF19 secretion assay

The effect of Diet1 on FGF19 secretion was assessed as previously described (7). Briefly, HEK293T cells (American Type Culture Collection, Manassas, VA) were transfected (in triplicate or quadruplicate) with pcDNA6 expression plasmids for FGF19-Myc in combination with a V5-tagged control gene construct (Kdm5c), Diet1-1721H, Diet1-1721Q, Diet1-1712D, or Diet1-1712G (BioT transfection reagent; Bioland, Paramount, CA). After 24 h, an equal amount of fresh medium was added to each well. The following day, culture medium was collected to quantify the levels of FGF19 secreted in 24 h, and cells were collected to quantify cellular FGF19 and Diet1 protein levels by Western blot. Diet1 was detected with rabbit anti-V5 (1:5,000; Bethyl Laboratories, Montgomery, TX); FGF19 was detected with rabbit anti-Myc (1:10,000; Bethyl Laboratories); and β-actin was detected with a mouse monoclonal antibody (1:10,000; Sigma, St. Louis, MO). After secondary antibody application, protein bands were detected with ECL2 chemiluminescent reagent (ThermoScientific, Rockford, IL) and the Bio-Rad ChemiDoc XRS+ system with a CCD camera and ImageLab software (Bio-Rad, Hercules, CA). The protein band intensity from the resulting image was quantified using the ImageLab software. The amount of FGF19 was normalized to Diet1 protein levels. Western blot images shown in Fig. 6A were adjusted and cropped in Adobe Photoshop.

Diet1/FGF19 coimmunoprecipitation

Protein coimmunoprecipitation was performed as previously described (7). HEK293T cells were transfected as described for the FGF19 secretion assay above. After 48 h, cells were lysed in PBS containing 1% NP-40 (Sigma), 1× Complete Mini protease inhibitor cocktail (Roche, Indianapolis, IN), and 0.1% phosphatase inhibitors 2 and 3 (Sigma). Cell lysates were incubated overnight at 4°C with 1 μg of mouse anti-V5 antibody (Life Technologies, Grand Island, NY), 4 μg of mouse anti-Myc antibody (Millipore, Billerica, MA), or 4 μg nonspecific mouse IgG (Jackson ImmunoResearch, West Grove, PA). Protein A/G

TABLE 1. Clinical characteristics of BAD case/control cohorts

	Group 1	Group 2	All Subjects
Number	44	78	122
Age range (years)	27–80	17–90	17–90
Male:female	13:31	34:43	47:74
SeHCAT retention range (%)	0.5–7.3[a]	0.4–90	0.4–90

[a]Non-BAD subjects were not assessed for percent SeHCAT retention.

PLUS agarose beads (Santa Cruz Biotechnologies, Paso Robles, CA) were added for 3 h at 4°C. After three washes with 1× PBS + 0.1% NP-40, protein was eluted from the beads with 1× loading buffer and 1% β-mercaptoethanol, boiled for 5 min, and analyzed by Western blot, as described in the preceding section.

Statistical analysis

Fisher's exact tests and one-way ANOVA were performed using VasserStats (http://www.vassarstats.net/). Two-way ANOVA was performed using NCSS or GraphPad Prism. Linear regressions were performed with WinStat for Excel. Pair-wise comparisons were made using Student's *t*-test.

RESULTS

Generation of *Diet1*$^{-/-}$ C57BL/6J mice

We previously identified the *Diet1* gene in the C57BL/6ByJ mouse strain, which carries a naturally occurring mutation leading to a null allele for *Diet1* (7). Because the C57BL/6ByJ mouse strain is >99% genetically identical to C57BL/6J mice, the latter strain was previously used as the wild-type control in studies of Diet1 (7, 8, 39, 40). However, to ensure that all effects of Diet1 deficiency could be attributed to the *Diet1*-null mutation (and not influenced by C57BL/6ByJ and C57BL/6J variation at loci other than *Diet1*), we generated a congenic strain by breeding the *Diet1*-null allele onto the C57BL/6J background for eight generations. As expected, the resulting C57BL/6J *Diet1*$^{-/-}$ mice had undetectable *Diet1* mRNA expression in the ileum (**Fig. 1A**).

We assessed the C57BL/6J *Diet1*$^{-/-}$ mice for traits originally observed in the C57BL/6ByJ mice (7) and found that the effects on bile acid metabolism were recapitulated. Data shown are for mice fed a short-term (3 week) atherogenic diet, which enhanced the phenotypic differences between wild-type and Diet1-deficient mice (8). As observed previously for C57BL/6ByJ mice (7, 8, 39), C57BL/6J *Diet1*$^{-/-}$ mice exhibited approximately 2-fold lower plasma cholesterol and lipoprotein levels and >4-fold higher plasma bile acid levels than *Diet1*$^{+/+}$ mice (Fig. 1B, C). Intestinal gene expression was altered in the congenic strain similarly to C57BL/6ByJ mice, with dramatically reduced *Fgf15* mRNA and increased *Asbt* mRNA (Fig. 1D). Diet1 deficiency in C57BL/6ByJ mice did not influence hepatic *Fgfr4* gene expression and increased the expression of cholesterol synthetic genes (*Cyp27*, *Cyp7a1*, and *Cyp8b1*) (8). C57BL/6J *Diet1*$^{-/-}$ mice similarly exhibited wild-type *Fgfr4* and elevated *Cyp27* levels, but effects on *Cyp7a1* and *Cyp8b1* exhibited biological variability (supplemental Fig. S1A). Thus, the plasma and intestinal phenotypes originally observed in C57BL/6ByJ mice were present in Diet1-deficient C57BL/6J mice.

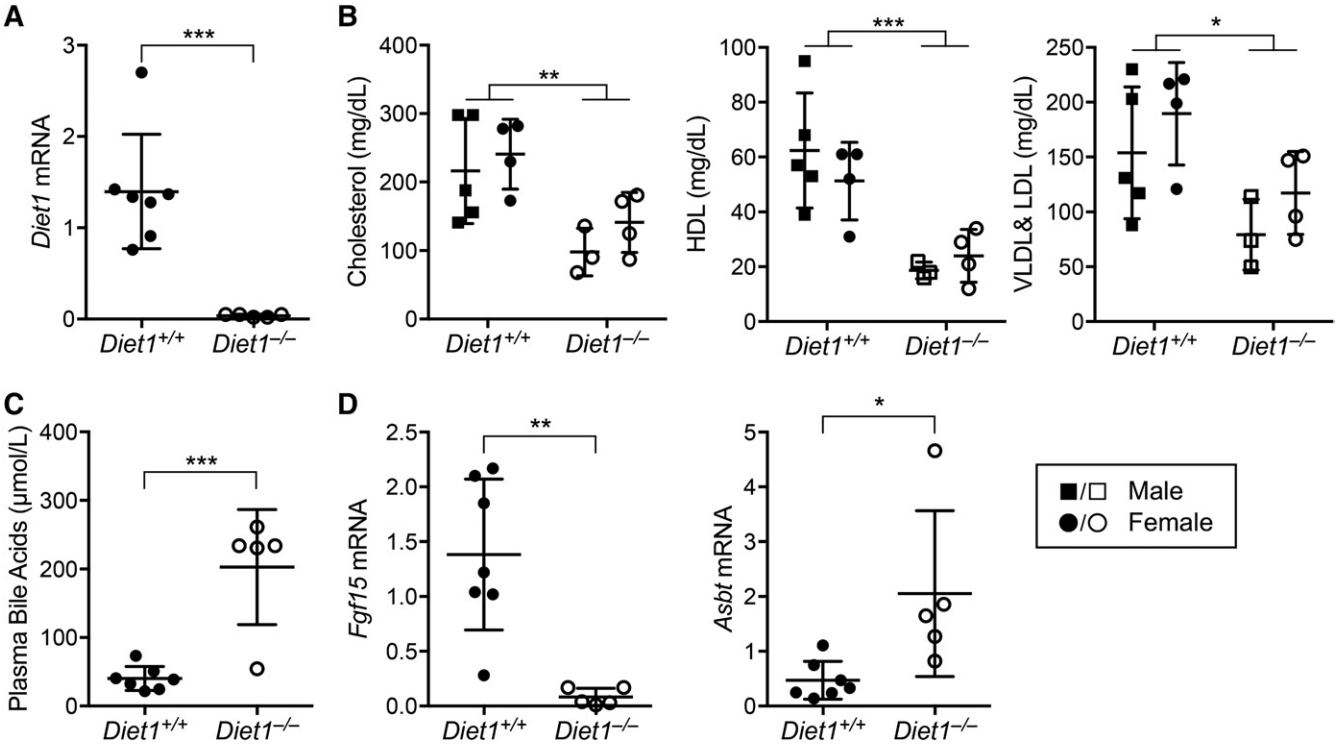

Fig. 1. Altered bile acid, cholesterol, and ileal gene expression levels in C57BL/6J *Diet1*$^{-/-}$ mice. A: Confirmation of Diet1 deficiency in C57BL/6J *Diet1*$^{-/-}$ mouse ileum. B: Reduced plasma total, HDL, and VLDL cholesterol levels in C57BL/6J *Diet1*$^{-/-}$ mice compared with isogenic *Diet1*$^{+/+}$ mice fed an atherogenic diet for 3 weeks (**$P < 0.01$ and ***$P < 0.001$ on two-way ANOVA). C: Elevated plasma bile acid levels in C57BL/6J *Diet1*$^{-/-}$ mice fed an atherogenic diet for 3 weeks. D: Reduced ileal *Fgf15* and elevated *Asbt* expression in C57BL/6J *Diet1*$^{-/-}$ mice fed an atherogenic diet 3 weeks. For all panels, n = 5–7 mice per genotype, as indicated. For panels A, C, and D, statistical analyses via Student's *t*-test; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Diet1 deficiency causes intestinal diarrhea in the mouse

We hypothesized that Diet1 is required for normal gastrointestinal function, and assessed intestinal motility and the presence of diarrhea (**Fig. 2A**). *Diet1*$^{-/-}$ mice exhibited normal food and water intake and body weight while eating an atherogenic diet (Fig. 2B; supplemental Fig. S1B). However, *Diet1*$^{-/-}$ mice had reduced transit time of an intragastric bolus through the intestinal tract (Fig. 2C), indicating increased intestinal motility (16, 21, 30). The increase in intestinal motility in conjunction with increased bile acid levels in feces (8) raised the possibility that these animals may experience BAD. We assessed the intestinal content of water and solids after acute feeding of the atherogenic diet for 3 h (see the scheme in Fig. 2A). Small and large intestine were collected and intestinal contents extruded and weighed. Following evaporation, the weight difference in intestinal contents was used to calculate the content of water and solids. *Diet1*$^{-/-}$ mice had increased water content of both small and large intestine per unit of intestinal length, indicating diarrhea (Fig. 2D, E). Increased water content was also observed in the small intestine of *Diet1*$^{-/-}$ mice when determined in fasted mice (supplemental Fig. S1C). By contrast, the intestinal solids content was not affected by Diet1 (Fig. 2D, E), suggesting a secretory diarrhea in *Diet1*$^{-/-}$ mice. The elevated bile acid levels, increased gastrointestinal motility, and increased

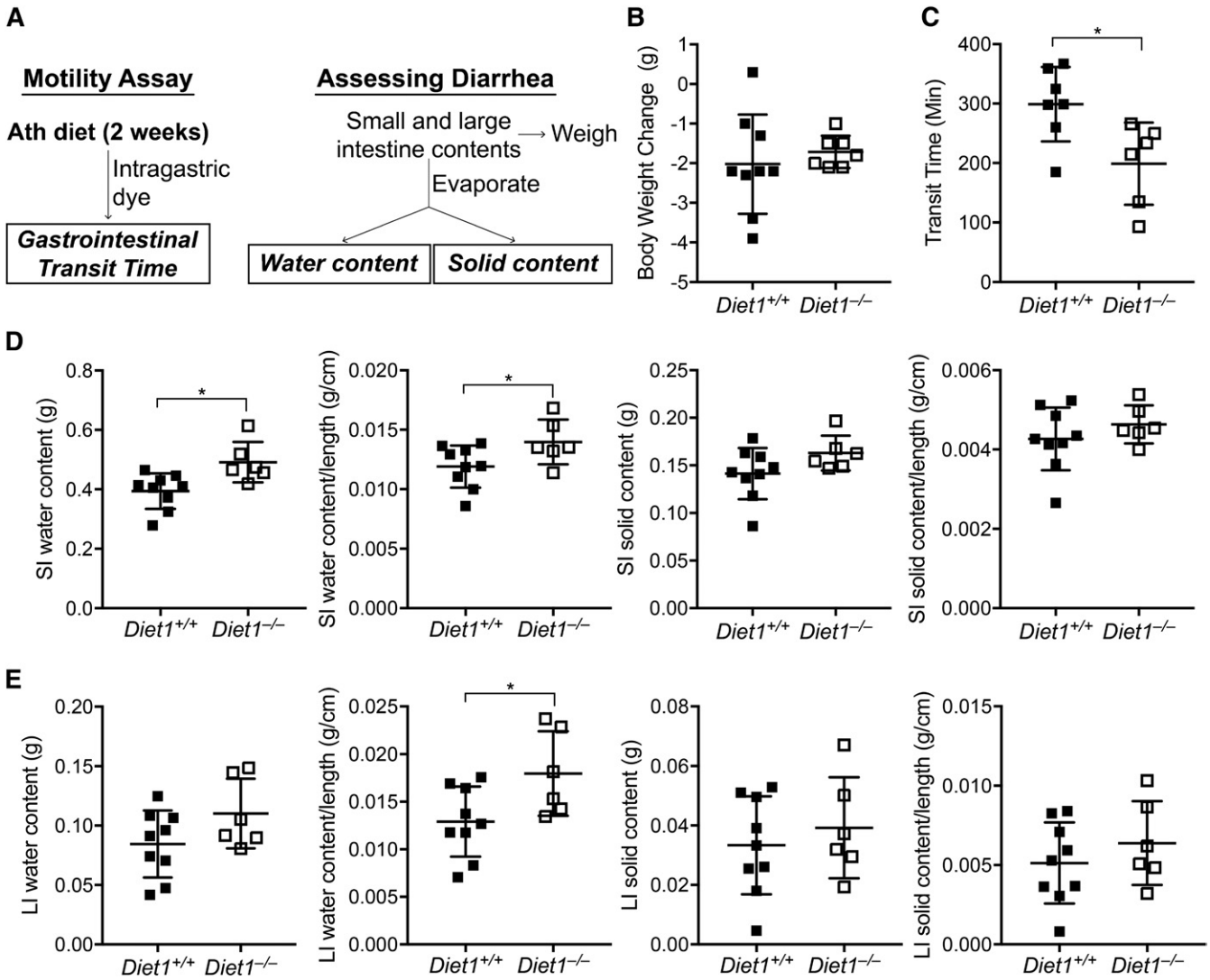

Fig. 2. *Diet1*$^{-/-}$ mice exhibit reduced gastrointestinal transit time and diarrhea. A: Study design to measure gastrointestinal transit time and diarrhea. Mice were fed an atherogenic diet for 2 weeks and gastrointestinal transit time was measured after gastric administration of a bolus containing dye. The time to first fecal pellet containing dye was determined. Diarrhea was assessed in fed animals by quantifying small and large intestine water and solid contents (described further in the Materials and Methods). B: *Diet1*$^{-/-}$ and *Diet1*$^{+/+}$ mice have similar body weight after 2 weeks on atherogenic diet. C: *Diet1*$^{-/-}$ mice exhibit reduced total gastrointestinal transit time, indicating increased intestinal motility. D: Small intestinal (SI) water content is increased in *Diet1*$^{-/-}$ mice when expressed as absolute amount and when normalized to intestinal length. There were no differences in SI solids content between genotypes. E: Large intestine (LI) water content normalized to length is increased in *Diet1*$^{-/-}$ mice. There were no differences in LI solids content between genotypes. Statistical analyses via Student's *t*-test; $^{*}P < 0.05$; n = 6–9.

intestinal water content in the absence of alterations in dry content suggested parallels between *Diet1*$^{-/-}$ mice and patients with BAD.

Genetic variation in mouse *Diet1* expression positively correlates with *Fgf15* mRNA levels and negatively correlates with biliary bile acid and cholesterol levels

Our data, thus far, indicated that complete deficiency of Diet1 influences *Fgf15* mRNA and plasma bile acid and cholesterol levels (Fig. 1) (7, 8). We wondered whether more modest naturally occurring variations in *Diet1* expression levels across different genetic backgrounds would also correlate with these traits. To assess this, we analyzed *Diet1* and *Fgf15* mRNA levels in ileum, biliary cholesterol levels, and bile acid levels across 76 inbred mouse strains, which comprise the HDMP (33). We observed a positive correlation between *Diet1* and *Fgf15* expression levels and a negative correlation between *Diet1* mRNA and both bile acid and cholesterol levels in gallbladder (**Fig. 3**). These results strengthen the relationship between *Diet1* expression levels and these parameters, and indicate that small variations in *Diet1* expression levels are associated with differences in *Fgf15* mRNA, bile acid, and cholesterol levels.

Sequence variation in *DIET1*

As a recently annotated gene, *DIET1* probes were not available in existing exome sequencing platforms when we began our study, and *DIET1* has not been resequenced in any disease cohort. To evaluate the potential role of rare or common *DIET1* variants in primary BAD, we sequenced the complete *DIET1* coding region and exon/intron junctions in a case-control cohort (22 cases, 22 controls). The basic characteristics of the cohort are summarized in Table 1. Primary BAD cases were defined as individuals with very low SeHCAT retention levels (<7.3%), and controls were randomly selected from patients with normal bowel habits (38).

We observed a total of 34 variants distributed along the length of *DIET1* (**Fig. 4**). Of these, 25 produce nonsynonymous amino acid substitutions, including two novel single nucleotide variants (SNVs) observed in single individuals from the Case group, one novel SNV observed in a single individual from the Control group, and a frame-shift mutation at position 1221 detected in a single control subject (supplemental Fig. S2; supplemental Tables S2, S3). Because these variants occurred at low frequency, there was no statistical evidence of an enrichment of rare variants in the primary BAD cases, and we did not pursue these variants further.

A common *DIET1* variant with skewed frequency distribution between primary BAD case and control subjects

Among the nonsynonymous variants identified in *DIET1*, one previously recorded common variant [*rs12256835*, 5163 T>G; minor allele frequency (MAF) 0.125] was present at statistically different levels in BAD cases and controls (Fisher's exact test: nominal $P = 0.0005$; adjusted for multiple testing, $P = 0.0175$) (supplemental Table S3). The *rs12256835* major allele encodes a histidine residue at position 1721 of the Diet1 protein (Fig. 4), and the minor allele encodes a glutamine residue (H1721Q). The minor (Q) allele was detected exclusively in control samples (11 of 44 alleles). This polymorphism has a frequency of 0.3181 in dbSNP (build 150), suggesting that it is a common source of structural variation in the Diet1 protein in the general population. Based on existing database information from dbSNP, the allele frequency for *rs12256835* is similar in European and East Asian populations (MAF 0.17–0.25, respectively), but deviates substantially in African populations (MAF 0.63). However, individuals of African descent were not present in our study sample, and cannot account for the difference in *rs12256835* allele frequency that we observed between BAD cases and controls.

We investigated the association of the *rs12256835* genotype with BAD in an additional 78 individuals, for an extended sample of 122 individuals (see Table 1 for the characteristics of the initial group, the extended group, and the total cohort). The individuals with primary BAD (defined as SeHCAT ≤10%) were more likely to have the T allele than the G allele (Fisher's exact test: $P = 0.035$; **Table 2**). The reduced frequency of the minor allele (G) in those with primary BAD in the extended

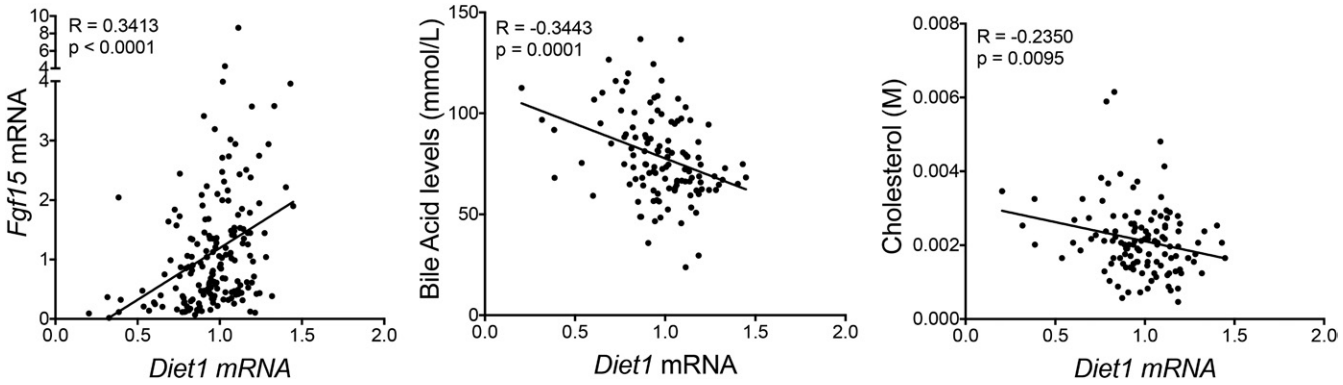

Fig. 3. *Diet1* mRNA levels correlate positively with *Fgf15* mRNA, and negatively with biliary bile acid and cholesterol levels across 76 mouse strains. *Diet1* mRNA levels in ileum vary across 76 inbred mouse strains, and correlate positively with *Fgf15* mRNA levels. *Diet1* mRNA levels correlate negatively with bile acid and cholesterol levels in bile collected from the gallbladder. Each point on the graphs represents a single mouse strain (three to five mice per strain) from the HDMP (see the Materials and Methods). The mice were fed a standard chow diet.

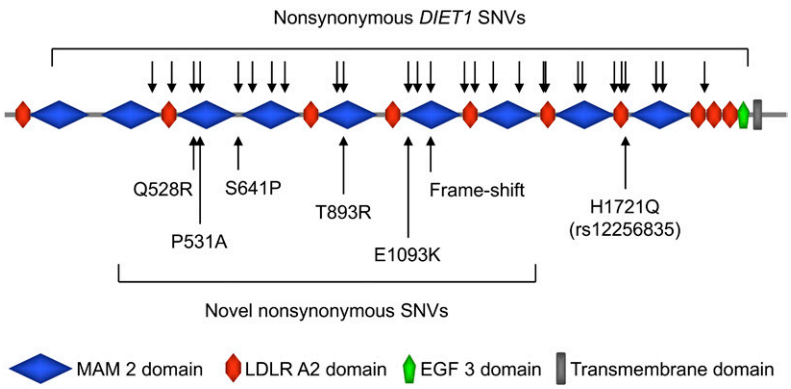

Fig. 4. Location of nonsynonymous variants in human Diet1 protein. Schematic of Diet1 protein domain structure. Arrows above the diagram indicate the 25 nonsynonymous variants and one single-nucleotide deletion detected in cases and controls. Arrows below the diagram indicate the novel nonsynonymous variants, the frame-shift mutation, and the position of *rs12256835*.

sample group was consistent with our observations in the initial cohort.

Walters and colleagues previously established a positive relationship between percent SeHCAT retention and FGF19 levels (28). To extend our analysis of the *rs12256835* genotype association with BAD, we determined fasting FGF19 levels in the group of 78 patients described above. The cohort was separated into BAD cases (SeHCAT ≤10%) and controls (SeHCAT >10%) and classified according to the genotype at *rs12256835* (TT vs. TG and G/G). FGF19 values were significantly different between BAD cases and controls ($P = 0.0088$; two-way ANOVA), and there was a significant interaction between *rs12256835* genotype and BAD case/control status ($P = 0.016$; **Table 3**). Post hoc Student's *t*-tests showed that, in BAD cases, FGF19 levels were significantly lower in those with the T/T genotype compared with those with one or more G alleles ($P = 0.0488$, Table 3).

The Diet1 H1721Q variant influences FGF19 production

The data described above suggested that the Diet1 protein variants encoded by the two alleles at *rs12256835* may have functional differences. The Diet1 protein has a predicted modular structure, with repeating interspersed LDL receptor and meprin/A5-protein/PTPmu (MAM) domains (7). The H1721Q amino acid change occurs at the transition between the seventh LDL receptor domain and the adjacent MAM domain (Fig. 4). We previously demonstrated that Diet1 influences bile acid levels by modulating FGF15 or FGF19 production in mouse intestine and in a human intestinal cell line, respectively (7). We also observed partial colocalization of Diet1 and FGF19 in cultured enterocytes, and demonstrated that the proteins coimmunoprecipitate (7). We therefore assessed whether the Diet1 H and Q variants differ in the ability to bind FGF19 and/or to promote FGF19 secretion.

To assess whether the H-to-Q substitution alters Diet1/FGF19 protein interaction, we coexpressed epitope-tagged Diet1 and FGF19 in cultured cells, immunoprecipitated the protein complex, and quantified the amount of each Diet1 isoform pulled down by FGF19. Expression of the 1721H and 1721Q Diet1 variants at similar levels (see the "Lysate" lanes in **Fig. 5**) led to the pull-down of a similar proportion of Diet1 by immunoprecipitation of FGF19 (black boxes in Fig. 5). Thus, the two Diet1 isoforms interacted with FGF19 similarly.

To assess the ability of the two Diet1 protein variants to promote FGF19 protein secretion, we coexpressed V5-tagged Diet1-1721H or -1721Q with Myc-tagged FGF19 in HEK293T cells (**Fig. 6A**, B). Cells were collected 48 h after transfection to quantify intracellular FGF19, and medium was collected during the final 24 h to quantify secreted FGF19. FGF19 was quantified by Western blot analysis and normalized to the amount of Diet1 protein expressed (Fig. 6B), as well as to actin levels (supplemental Fig. S3). As a control for specificity of the Diet1 effect, we analyzed the levels of FGF19 secretion in response to expression of an irrelevant protein with a similar molecular weight to Diet1 (Kdm5c-V5). A representative experiment (from a total of nine individual experiments) is shown in Fig. 6A. The presence of Diet1-1721H or -1721Q protein increased FGF19 production compared with the control protein. The amount of intracellular FGF19 was similar for the 1721H or 1721Q Diet1 variants, but the amount of FGF19 secreted into the culture medium in 24 h was greater for Diet1-1721Q than Diet1-1721H (Fig. 6A, B; supplemental Fig. S3). This relationship was confirmed in the meta-analysis of nine independent experiments (each performed in triplicate or quadruplicate). The Diet1-1721Q variant enhanced FGF19 secretion by approximately 70% compared with Diet1-1721H (Fig. 6B; $P < 0.01$). By contrast, another Diet1 variant identified

TABLE 2. Allele frequencies for *rs12256835* in all subjects

	T Allele	G Allele	*P*	Odds Ratio (95% CI)
SeHCAT ≤10%	83	13	0.035	0.470 (0.235, 0.940)
SeHCAT >10%	111	37		

The number of T and G alleles genotyped in 122 subjects are shown, segregated as BAD cases (SeHCAT ≤10%) and controls (SeHCAT >10%). The third column lists the *P*-value of association as calculated by Fisher's exact test, and the odds ratio (with 95% CIs indicated) is given in the final column.

TABLE 3. The *rs12256835* genotype and FGF19 levels

		FGF19 (pg/ml)	
		T/T	T/G and G/G
SeHCAT ≤10%	Mean ± SEM	119 ± 19[a]	253 ± 56
	Number	14	12
SeHCAT >10%	Mean ± SEM	246 ± 24	215 ± 28
	Number	30	22

Fasting FGF19 levels for a cohort of 78 patients are shown, segregated as BAD cases (SeHCAT ≤10%) and controls (SeHCAT >10%) and classified according to the genotype at *rs12256835*. FGF19 values were log-normalized before statistical analysis. FGF19 values were significantly different between BAD cases and controls [$P = 0.0088$ (two-way ANOVA)], and there was a significant interaction between *rs12256835* genotype and BAD case/control status ($P = 0.016$).

[a]Post hoc Student's *t*-tests confirmed lower FGF19 levels in those with primary BAD carrying the T/T genotype ($P < 0.05$).

in our study and located in the same protein domain (D1712G) did not affect FGF19 secretion (Fig. 6C, D). Thus, it appears that the differential effect of the 1721H and 1721Q Diet1 proteins on FGF19 secretion is related to the specific amino acid substitution.

DISCUSSION

The current study expands our understanding of Diet1 deficiency on intestinal function and assesses the effects of *Diet1/DIET1* genetic variation on traits related to FGF15/19 and bile acid metabolism. We evaluated a new *Diet1*$^{-/-}$ mouse strain on a C57BL/6J background in comparison to isogenic C57BL/6J wild-type mice (in contrast to the previous studies comparing C57BL/6ByJ mice, which carry the natural *Diet1*-null allele, to C57BL/6J mice). The *Diet1*$^{-/-}$ C57BL/6J mice recapitulated previous phenotypes in intestinal gene expression, as well as plasma lipid and bile acid levels (7, 8). We also found that Diet1 deficiency increases gastrointestinal transit time and intestinal water content, which, together with elevated bile acid levels and reduced FGF15 levels, parallel primary BAD in humans. The increased intestinal water content was not apparent when *Diet1*$^{-/-}$ mice were fed a standard chow diet (unpublished observations), suggesting that elevated circulating bile acid levels that are stimulated by short-term atherogenic diet contributed to the diarrheal phenotype, as is seen in patients with BAD.

In addition to effects of the extreme genetic condition of Diet1 deficiency, we assessed whether modest naturally occurring variations in Diet1 influence traits that are affected in *Diet1*$^{-/-}$ mice. We found that genetic variation in *Diet1* intestinal mRNA expression across a panel of 76 inbred mouse strains correlated positively with *Fgf15* mRNA levels, and negatively with bile acid and cholesterol levels. These results underscore that small changes in *Diet1* mRNA levels (such as might occur among individuals in the human population) influence *Fgf15* expression levels, as well as bile acid and cholesterol levels. In the future, it will be interesting to determine whether the genetic variations in *Diet1* expression levels are associated with *cis*-acting regulatory sequence variants that differ across mouse strains.

The phenotype of *Diet1*$^{-/-}$ mice led us to investigate whether *DIET1* genetic variation influences the severity of BAD in humans. We identified a prevalent (MAF of approximately 30% across populations) nonsynonymous variant in the *DIET1* coding region that is associated with SeHCAT retention levels in BAD patients and which alters levels of FGF19 secretion in cultured cells. Previous genetic studies of primary BAD have focused on a few candidate genes with established roles in bile acid metabolism. A rare mutation in *SLC10A2/ASBT* was identified in one family with congenital BAD, but variations in *SLC10A2* appear not to be associated with adult-onset primary BAD (41, 42). Because *DIET1* was not annotated until recently (7), this gene is not represented on existing expression microarrays or exome sequencing platforms, and little gene expression or sequence data are available for this gene in human samples. Our *DIET1* resequencing identified several rare nonsynonymous amino acid substitutions and a frame-shift

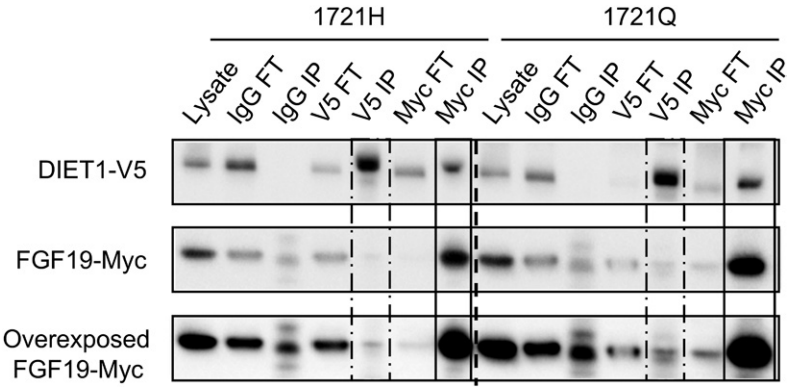

Fig. 5. Diet1-1721H and -1721Q isoforms do not differentially affect Diet1-FGF19 interaction. Coimmunoprecipitation of Diet1-1721H and Diet1-1721Q with FGF19. FGF19-Myc was coexpressed in HEK293T cells with Diet1-1721H-V5 or Diet1-1721Q-V5. Input lanes show similar levels of expression of the two Diet1 isoforms. Bands corresponding to precipitation of FGF19 with Diet1-1721H are on the left of the dashed line, and bands corresponding to precipitation of FGF19 with Diet1-1721Q are on the right of the dashed line. Dashed boxes indicate immunoprecipitation of Diet1 (V5 IP); solid boxes indicate immunoprecipitation of FGF19 (Myc IP). Both Diet1 isoforms interact with FGF19 with similar efficiency. A representative blot from eight experiments is shown.

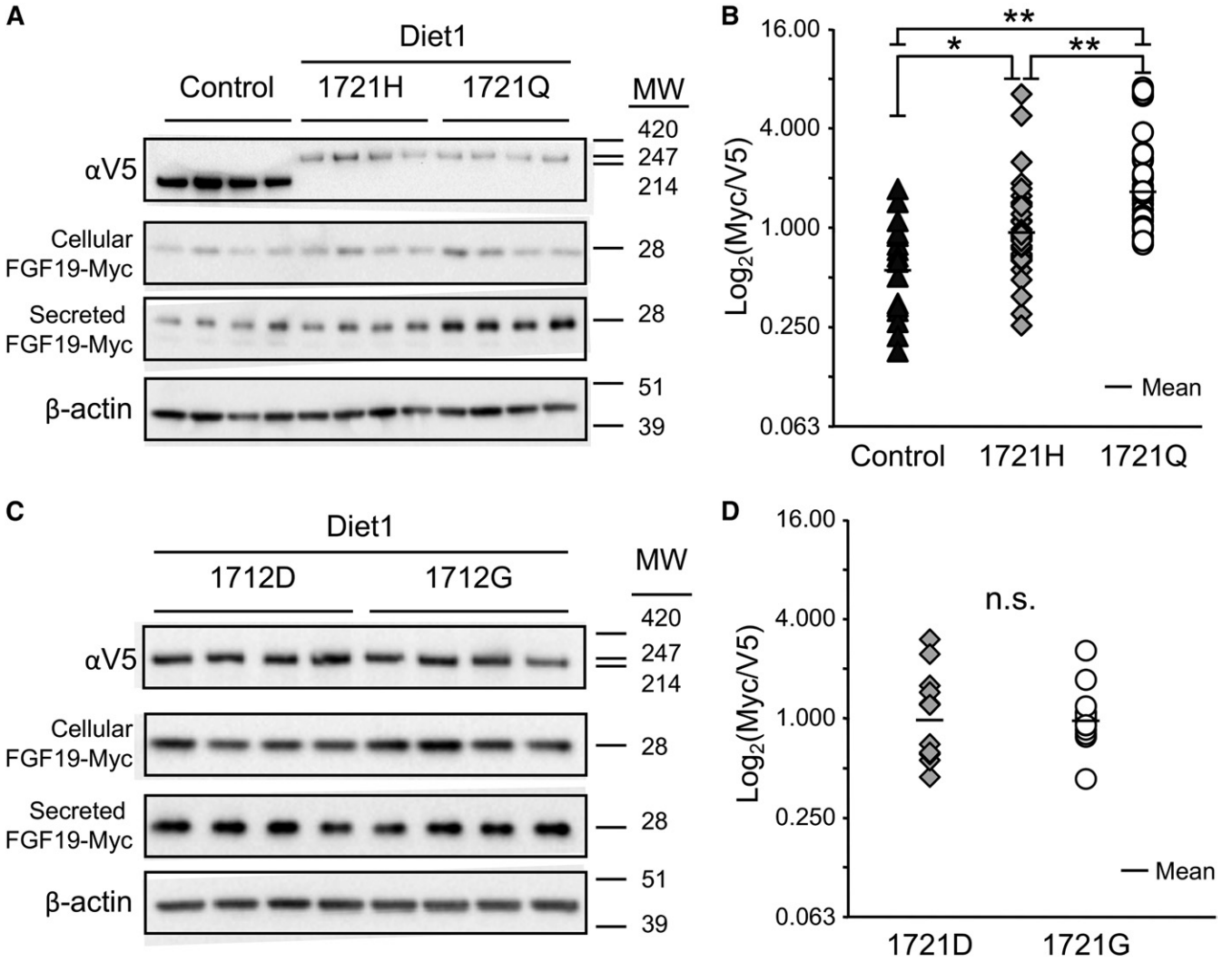

Fig. 6. *DIET1* variant *rs12256835* influences secretion of FGF19 in a cell-based assay. A: Representative results of an FGF19 secretion assay comparing Diet1-1721H and -1721Q. FGF19-Myc was expressed in HEK293T cells in combination with either Diet1-1721H-V5, Diet1-1721Q-V5, or an irrelevant control protein (Kdm5c-V5). Cellular Diet1 protein levels and secreted FGF19 levels were visualized by Western blot. MW, molecular mass in kD. B: Quantification of FGF19 secretion from nine experiments of the type shown in A. Each point is one independent transfection (done in triplicate or quadruplicate), representing the amount of FGF19 secreted per unit of cellular V5 expression for control protein (Kdm5c), Diet1-1721H, or Diet1-1721Q. The overall *P*-value for all three conditions is $P = 3.70 \times 10^{-7}$ (one-way ANOVA). Individual comparisons were made using Tukey's HSD post hoc test on $\log_2$-normalized values. Small horizontal lines represent geometric mean of samples for each protein isoform (*$P < 0.05$, **$P < 0.01$). C: Representative results of an FGF19 secretion assay comparing Diet1-1721D and -1721G. Details are as in A. D: Quantification of FGF19 secretion from five experiments of the type shown in C. No statistically significant difference (n.s.) in the amount of extracellular FGF19 was detected between Diet1-1712D and -1712G ($P = 0.945$; one-way ANOVA).

mutation. One common *DIET1* variant (*rs12256835*), which alters amino acid composition (H1721Q), was significantly associated with BAD status.

We found that Diet1-1721Q protein promotes increased FGF19 levels in the medium in a cultured cell assay system compared with Diet1-1721H. This effect is likely due to the specific position and amino acid alteration, as a D1712G nonconservative substitution that is located only nine amino acids away had no effect on FGF19 secretion. A full understanding of the role of residue 1721 in Diet1 protein function will require additional protein structure-function studies. An attractive hypothesis is that H1721Q amino acid change affects Diet1 protein conformation and/or interactions with other cellular proteins, potentially affecting FGF19 trafficking and secretion.

Our findings raise the possibility that the Diet1 H1721Q variant could contribute to the 7-fold variation in plasma FGF19 levels that have been observed in healthy individuals (11, 43). Resequencing *DIET1* in additional subjects will be required to reveal whether *DIET1* rare mutations or additional common variants contribute to variations in bile acid metabolism.

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