Cubilin Maintains Blood Levels of HDL and Albumin

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ABSTRACT

Cubilin is an endocytic receptor highly expressed in renal proximal tubules, where it mediates uptake of albumin and filtered forms of apoA-I/HDL. Cubilin deficiency leads to urinary loss of albumin and apoA-I; however, the consequences of cubilin loss on the homeostasis of blood albumin and apoA-I/HDL have not been studied. Using mice heterozygous for cubilin gene deletion (cubilin HT mice), we show that cubilin haploinsufficiency leads to reduced renal proximal tubular uptake of albumin and apoA-I and significantly increased urinary loss of albumin and apoA-I. Moreover, cubilin HT mice displayed significantly decreased blood levels of albumin, apoA-I, and HDL. The levels of albumin and apoA-I protein or mRNA expressed in the liver, kidney, or intestine of cubilin HT mice did not change significantly. The clearance rate of small HDL3 particles (density > 1.13 g/ml) from the blood increased significantly in cubilin HT mice. In contrast, the rate of clearance of larger HDL2 particles from the blood did not change significantly, indicating a decreased half-life for HDL particles capable of filtering through the glomerulus. On the basis of these findings, we conclude that cubilin deficiency reduces renal salvage and delivery back to the blood of albumin and apoA-I, which decreases blood levels of albumin and apoA-I/HDL. These findings raise the possibility that therapeutic increase of renal cubilin expression might reduce proteinuria and increase blood levels of albumin and HDL.


HDL and albumin are major blood components with links to cardiovascular disease.1–3 As such, understanding the metabolism and homeostatic regulatory mechanisms of these constituents may hold keys to new therapeutic approaches.

Cubilin is a multiligand receptor capable of mediating the endocytosis of albumin and HDL, as well as its major apolipoprotein component, apoA-I.4–6 However, the significance of cubilin to the homeostasis of HDL and albumin in the blood has not been studied. Cubilin and its coreceptor LDL-related protein-2 (megalin) are expressed by absorptive cells, including proximal tubule cells (PTCs).7 At present, cubilin- and megalin-mediated endocytic uptake represents the only established process by which PTCs reabsorb proteins from the glomerular filtrate.5,6,8,9 Genetic alterations that affect renal cubilin expression in humans, dogs, and mice result in urinary wastage of an array of macromolecules, including albumin and apoA-I.10,11 Although several studies demonstrate the role of cubilin in mediating renal uptake of apoA-I and albumin,5,10,11 the precise fate of these proteins following PTC uptake has been an open question. Although a general view is that ligand endocytosis by cubilin and megalin leads to lysosomal degradation of ligands, several lines of evidence indicate that cubilin-megalin-mediated uptake may also participate in a process by which certain ligands are targeted for transcytosis and delivery back to the blood. For example, in cultured renal PTCs, megalin mediates transcytosis of transcobalamin-B12 complex12 and retinol-binding protein in...
complex with retinol/vitamin A. Megalin also mediates transcytosis of thyroglobulin, Shh, and the megalin-cubilin binding protein, receptor-associated protein. Indirect evidence for cubilin playing a similar role in ligand transcytosis comes from findings showing that albumin is transcytosed back to circulation via the proximal tubule epithelium, as well as the fact that cubilin-mediated uptake of intrinsic factor–B12 complex in the gut leads to its release into blood in the form of transcobalamin–B12.

Whether or not renal cubilin-mediated uptake of apoA-I and albumin from the glomerular filtrate might be part of a salvage process that affects levels of these proteins in the blood is not known. Here, we sought to define the relationships between genetic cubilin deficiency and renal uptake/urinary loss of apoA-I and albumin, along with the extent to which cubilin deficiency influences blood levels of each constituent, as well as HDL.

RESULTS

Cubilin Heterozygous Mice

Cubilin gene (Cubn) deletion mice were previously generated by Cubn exon 1–6 deletion and an EGFP cassette insertion (Cubn+/del exon 1–6;EGFP). As previously shown, cubilin-null mice are embryonic lethal. However, Cubn+/del exon 1–6;EGFP mice (Cubn heterozygous mice) develop without any obvious abnormalities and are physically similar to wild-type (WT) littermates. BUN, urinary creatinine excretion, and total urine output levels did not significantly differ between Cubn heterozygous and WT mice (data not shown). As shown in Figure 1, Cubn heterozygous mice had significantly reduced cubilin protein levels in the extracts of ileum and kidney cortex compared with WT mice. Cubilin coreceptor (megalin) levels in Cubn heterozygous mice kidneys were not significantly different compared with WT (Figure 1A).

Effect of Cubilin Deficiency on Blood HDL Levels

To determine the effect of cubilin deficiency on the blood levels of HDL, multiple approaches were applied to analyze plasma or serum from fasted mice. First, serum lipoprotein levels were analyzed by nuclear magnetic resonance. As shown in Figure 2, HDL-associated cholesterol (HDL-C) was significantly reduced by approximately 27% in serum from Cubn heterozygous mice compared with WT. Similarly, HDL particle concentration was also significantly reduced by about 29% in serum from Cubn heterozygous mice compared with WT. By contrast, VLDL and LDL particle concentrations in serum did not significantly differ between Cubn heterozygous mice and WT mice. Next, pooled plasma samples were separated by fast protein liquid chromatography (FPLC) and the lipid content of each fraction analyzed. The fractions corresponding to the HDL peak (fractions 35–42) contained cholesterol, cholesterol ester, and phospholipids, which were significantly reduced by approximately 25% in the serum from Cubn heterozygous mice compared with WT mice (Figure 3). In contrast, these lipids did not significantly differ in the non-HDL fractions in serum from Cubn heterozygous mice compared with WT mice. Likewise, triglycerides, which are mostly associated with non-HDL lipoproteins, were not significantly different in serum from Cubn heterozygous mice compared with WT mice. Finally, plasma apoA-I concentration was analyzed by a two-antibody sandwich ELISA using purified apoA-I as standard. Congruent with the above approaches, apoA-I concentration in plasma from Cubn heterozygous mice decreased by about 30% compared with WT mice (Figure 4A).

Effect of Cubilin Deficiency on Urinary ApoA-I Loss

To determine the effect of cubilin deficiency on urine levels of apoA-I, urine samples were analyzed by the two-antibody sandwich ELISA using purified apoA-I as standard. As shown in Figure 4B, apoA-I levels were significantly increased by approximately 4.5-fold in the urine of Cubn heterozygous mice compared with WT mice. This urinary loss amounted to an average of 45.44 ng of apoA-I per day in the urine of Cubn heterozygous mice compared with 9.04 ng/d in the WT mice (average daily excretion, 0.5 mg of creatinine in 1 ml of urine). To determine whether or not there was a correlation between
To determine the effect of cubilin deficiency on urinary apoA-I loss and decreased plasma apoA-I levels, urinary apoA-I concentrations were plotted against plasma apoA-I levels (Figure 4C). A significant inverse correlation was observed between urinary apoA-I and plasma apoA-I concentration in both Cubn heterozygous and WT groups (WT, Spearman $r = -0.78$, $P=0.003$; Cubn heterozygous (HT), Spearman $r = -0.7$, $P=0.02$).

**Effect of Cubilin Deficiency on Blood Albumin Levels**

To determine the effect of cubilin deficiency on blood levels of albumin, plasma samples from fasted mice were analyzed using a two-antibody sandwich ELISA. Plasma albumin was significantly reduced by approximately 17% in Cubn heterozygous mice compared with WT (Figure 5A). Because albumin constitutes a large portion of blood protein content, total plasma protein was also quantified using a bicinchoninic acid protein assay. As shown in Figure 5B, total plasma protein was significantly reduced by about $12\%$ in Cubn heterozygous mice compared with WT mice.

**Effect of Cubilin Deficiency on Urinary Albumin Loss**

To determine the effect of cubilin deficiency on urine levels of albumin, spot urine and 16-hour urine samples were analyzed using the two-antibody sandwich ELISA. Albumin levels were significantly increased by approximately 2.4-fold in the urine of Cubn heterozygous mice compared with WT mice (Figure 5C). This urinary loss amounted to a daily average of 232.83 $\mu$g of albumin in the urine of Cubn heterozygous mice compared with 96.84 $\mu$g/d in the WT. Urine volumes and creatinine levels at 16 and 24 hours were not significantly different between Cubn heterozygous and WT mice (data not shown).

**Cubilin Deficiency Does Not Alter Intestinal or Renal ApoA-I/HDL and Albumin Biosynthesis**

To determine whether cubilin deficiency affected apoA-I/HDL biosynthesis, quantitative PCR was used to measure tissue mRNA levels of apoA-I and Abca1, which catalyzes the rate-limiting step in the lipidation of apoA-I into nascent HDL particles. As shown in Figure 6, A and F, cubilin mRNA levels were significantly decreased by approximately 50% in the intestine and kidney of Cubn heterozygous mice compared with WT mice. However, there was no significant difference in apoA-I and Abca1 mRNA levels in the intestine, liver, or kidney of Cubn heterozygous mice compared with WT mice (Figure 6). Similarly, no significant difference was observed in albumin mRNA levels in the intestine or liver of Cubn heterozygous mice compared with WT mice (data not shown). ApoA-I and Abca1 immunoblot analysis of extracts of these tissues was also performed. Consistent with findings from the mRNA analyses, no significant differences were observed in apoA-I and Abca1 protein levels in the extracts of intestine or liver from Cubn heterozygous mice compared with WT mice (Supplemental Figure 1).

**Effect of Cubilin Deficiency on ApoA-I/HDL Fractional Clearance**

The clearance from blood of intravenously injected human HDL fractions was measured in Cubn heterozygous and WT mice. At 48 and 72 hours after injection, levels of human HDL$_2$ were significantly reduced (by about 50%) in Cubn heterozygous mice compared with WT mice (Figure 7A). The area under the curve for apoA-I/HDL plasma clearance in Cubn heterozygous mice was significantly reduced (by approximately 29%) compared with WT mice (Figure 7A). Similarly, the average half-life of these HDL particles was also reduced by about 28% in Cubn heterozygous mice compared with WT mice (half-life in WT mice, 15–18 hours; half-life in HT mice, 9–15 hours). These observations indicate that apoA-I/HDL fractional clearance is significantly increased in Cubn heterozygous mice compared with WT mice. As a control, the fractional clearance of the larger HDL$_2$ fraction was also determined. Most of HDL$_2$ should not be filtered through the glomerulus because of its larger size. As shown in Figure 7B, the plasma concentration of human HDL$_2$ in WT mice was not significantly different compared with Cubn heterozygous mice at all time points. Furthermore, the area under the curve for HDL$_2$ plasma clearance in WT mice was also not significantly different compared with Cubn heterozygous mice (Figure 7B).

**Effect of Cubilin Deficiency on ApoA-I Localization in Kidney Cortex**

To determine the effect of cubilin deficiency on apoA-I uptake by proximal tubules, apoA-I localization was analyzed in the...
kidney cortex by immunofluorescence microscopy. Previously, we showed that cubilin expression is monoallelic. Thus, proximal tubules in the Cubn heterozygous mice stochastically express either the targeted Cubn allele (containing the EGFP cassette) or the WT cubilin allele. The enhanced green fluorescent protein (EGFP)–expressing proximal tubule cells are cubilin deficient, and their brush borders had significantly less bound albumin than proximal tubules with WT cubilin expression. Similarly, we show here that apoA-I localization in the brush border of EGFP-expressing cubilin-deficient proximal tubules in the Cubn heterozygous mouse kidney is greatly reduced (Figure 8). The level of apoA-I localization to proximal tubule brush borders was quantified by counting the number of proximal tubules with detectable apoA-I immunofluorescence in 109 images from four WT and five HT mice. The number of apoA-I–positive proximal tubules was significantly reduced, by 36%, in Cubn heterozygous mice compared with WT mice (Figure 8C).

**DISCUSSION**

Here we show that cubilin haploinsufficiency in mice leads to decreased PTC uptake of both albumin and apoA-I and significantly increased loss of albumin and apoA-I in the urine. Importantly, our studies demonstrate that decreased PTC uptake of albumin and apoA-I causes significant decreases in plasma levels of albumin, apoA-I, and HDL particle concentration. This was supported by an inverse correlation between urinary loss of apoA-I and plasma apoA-I levels and analyses of HDL half-life and clearance rate from blood. Indeed, cubilin HT mice had significantly reduced blood half-life and increased blood clearance rate of small HDL particles. In contrast, there was not a significant difference in the clearance rate from blood of HDL2, larger particles that are not filtered through the glomerulus. On the basis of these findings, we inferred that the increased urinary loss of apoA-I/HDL would result in a continuous decline in blood HDL levels until it is counterbalanced by an increase in synthesis or decrease in degradation of apoA-I/HDL, resulting in a new homeostatic level of apoA-I/HDL that is lower than WT levels. We did not find a significant difference in apoA-I biosynthesis at the mRNA or protein level in cubilin HT mouse tissues compared with WT. Thus, a decrease in apoA-I/HDL degradation is likely to provide the counterbalance measure to allow blood apoA-I/HDL levels in cubilin HT to reach steady state.

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**Figure 3.** FPLC gel filtration analysis of serum shows reduced serum HDL levels in Cubn+/del exon 1–6;EGFP mice. (A) Cholesterol. (B) Cholesterol ester. (C) Phospholipids. (D) Triglyceride levels in fractions of pooled serum samples (6 WT and 6 HT) subjected to gel filtration using Superose 6 column chromatography. Serum samples were obtained from age-matched adult male Cubn+/del exon 1–6;EGFP (HT) and WT mice after a 15-hour fast.

**Figure 4.** Cubn+/del exon 1–6;EGFP mice have reduced plasma apoA-I and increased urinary apoA-I levels, which are inversely correlated. (A and B) Levels of apoA-I in plasma (A) and urine (B) samples obtained from age-matched 1-year-old male Cubn+/del exon 1–6;EGFP (HT) and WT mice. Samples were analyzed by a two-antibody sandwich ELISA using purified apoA-I as standard. Horizontal lines in A and B indicate median values for each data group. P value calculations in A and B were based on Mann–Whitney U tests. (C) The inverse correlation of plasma apoA-I and urinary apoA-I levels from Cubn+/del exon 1–6;EGFP (HT) (Spearman r = −0.70, P = 0.02; linear regression R² = 0.47, P = 0.04) and WT mice (Spearman r = −0.78, P = 0.003; linear regression R² = 0.37, P = 0.05).
Our conclusion that cubilin-mediated salvage of albumin is tied to blood albumin homeostasis is consistent with findings from other studies. In a study of doxorubicin-induced rat kidney injury, RNA interference–mediated silencing of renal cubilin expression not only led to increased albuminuria, but also resulted in reduced blood albumin levels. In studies of protein overload in mice, BSA overload caused urinary wastage of cubilin ligands and resulted in reduced serum albumin levels without any changes in glomerular function or ultrastructure.

The data here, together with findings from other studies, point to a mechanism involving PTC cubilin-mediated uptake of apoA-I and albumin from the glomerular filtrate, leading to transcytosis of these ligands across the renal proximal tubule cells and delivery back into circulation. Support for such a mechanism comes from live renal imaging studies showing transcytosis of albumin in rat PTCs and other studies demonstrating the in vitro transcytosis of megalin ligands. The underlying mechanisms by which cubilin-mediated uptake would lead to ligand transcytosis remains to be established. In a recent study, a significant reduction in plasma albumin levels and decreased half-life of albumin in the blood were observed in mice transplanted with kidneys deficient in neonatal Fc receptor (FcRn). FcRn is expressed by renal podocytes and PTCs and binds albumin under acidic conditions. However, FcRn-deficient PTCs retained the capacity to bind albumin at their apical brush borders. This is consistent with other studies showing that FcRn is expressed within the cytoplasm and not at the plasma membrane. Together, these studies suggest that after cubilin-mediated endocytosis, albumin might then bind to FcRn in acidic vesicles and become routed for transcytosis. Therefore, at least in case of albumin, the transcytosis pathway might involve cooperation of cubilin and FcRn.

A role for renal cubilin-mediated salvage in maintaining HDL and albumin homeostasis could have implications for cardiovascular diseases (CVD). Clinical evidence indicates that HDL-C blood levels and serum albumin levels are inversely correlated with risk of coronary heart disease. Emerging evidence indicates that this salvage pathway might be affected in human disease, with consequential effects on HDL and albumin homeostasis. For example, elevated urinary albumin excretion was associated with a doubling of risk of CVD and all-cause mortality in a study of elderly persons. This study also showed an inverse correlation between urinary levels of albumin and plasma levels of HDL-C. Similarly, patients with chronic renal failure have decreased plasma HDL concentration. Furthermore, a missense cubilin variant is associated with albuminuria in both the general population and individuals with diabetes.

Decreased renal cubilin-mediated salvage may affect HDL homeostasis in diabetes. In mouse models of diabetes, levels of renal cubilin are decreased. Although renal cubilin levels have
not been directly analyzed in diabetic patients, one study of microalbuminuric patients with type 1 diabetes reported aberrant shedding of cubilin in urine along with cubilin ligands, albumin, and apoA-I. Other studies demonstrate that diabetic patients have lower blood HDL levels. Furthermore, a study of normoalbuminuric patients with type 1 diabetes and normal glomerular function reported a correlation between lower levels of total HDL-C and smaller HDL₃-C with albuminuria, but not larger HDL₂-C. Similarly, HDL size distribution has shifted toward large particles in patients with proteinuria. However, further work is required to analyze cubilin expression in these patient populations and determine whether a causal link exists between any changes in cubilin expression and altered HDL levels. Demonstrating such a link would emphasize the need to upregulate the renal cubilin-mediated salvage pathway to prevent CVD in these patient populations.

CONCISE METHODS

Animals

All studies involved the use of 2- to 12-month-old male mice: either cubilin heterozygous with an EGFP cassette insertion in a site in which cubilin exons 1–6 were deleted (Cubn+/del exon 1–6;EGFP) or age- and sex-matched WT littermates on a mixed 129Sv/C57BL/6 genetic background. Mice were housed in a Medical University of South Carolina (MUSC) animal care facility, and mouse experiments were conducted with approval from the MUSC Institutional Animal Care and Use Committee with adherence to the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals. The mean body weight was 32.84 g for WT mice and 33.07 g for cubilin heterozygous mice. Both WT and Cubn heterozygous mice were fed Harlan Teklad Global 2918 rodent chow during this study. Genotypes of progeny were determined by PCR performed with tail-clip genomic DNA using a three-primer set: cubilin sense strand primer, 5′-AGCCACGCTTATTACTTACATGGTG-3′ (residues 1041331-10413332 in NT_039202.7); cubilin antisense strand primer, 5′-TGACCCCTCACAAGTTGAACAG-3′ (residues 10413656-10413635 in NT_039202.7); and EGFP antisense strand primer, 5′-GGTCTTGTAGTTGCCGTCGT-3′ (residues 1281-1262 in GU045599.1). Cycling parameters for PCR amplification were 98°C for 5 minutes and then 40 cycles of 98°C for 0.5 minute, 55°C for 1 minute and 72°C for 1 minute. For WT animals, the expected size of the amplicon is about 350, whereas amplicons of about 350 and 520 bp are produced from Cubn heterozygous mice.

Immunoblot Analysis

Segments of small intestine, liver, and kidney cortex were homogenized in 1% Triton X-100, 0.5% Tween 20, 0.5 M NaCl, and 50 mM Hepes, pH 7.5, containing a protease inhibitor cocktail (Complete-mini, EDTA-free; Roche, Germany) using a Polytron-aggregate (Brinkmann Instruments, Switzerland). Extracts were clarified by centrifugation at 14,000g for 30 minutes at 4°C. Protein concentration in extracts was quantified using Pierce BCA Protein Assay Kit (Rockford, IL). Equal amounts of protein were loaded and analyzed by SDS-PAGE. 

Figure 7. Cubn+/del exon 1–6;EGFP mice have increased fractional clearance of small HDL particles. (A) Fractional clearance of human HDL₃ (d>1.13 g/ml) supplemented with 6% purified apoA-I infused into Cubn+/del exon 1–6;EGFP (HT) (n=6) and WT (n=3) mice. Areas under the curves in A were statistically different (P=0.05). (B) Fractional clearance of human HDL₂ (d=1.11–1.13 g/ml) infused into WT and HT mice (n=3 and 5, respectively). Analysis showed no statistically significant difference between the two data sets in B (i.e., area under the curves, P=0.11).

Figure 8. Cubn+/del exon 1–6;EGFP mouse renal proximal tubules show reduced apical levels of apoA-I. (A) Image of a WT mouse kidney section immunolabeled with anti-apoA-I (red) and anti-EGFP (green). Nuclei were stained with Draq5. (B) Cubn+/del exon 1–6;EGFP kidney section labeled similarly to A. Note that very little or no apoA-I is present on the apical aspects of tubules expressing EGFP. (C) Graph showing the percentage of anti-apoA-I immunolabeling positive proximal tubules counted from 109 low-magnification fields from four WT and five HT mice. Horizontal line indicates median values for each data group. The P value was calculated using a Mann–Whitney U test.
amounts of protein from the extracts were loaded onto NuPAGE 4%–
12% polyacrylamide gradient, Bis-Tris gels in the presence of SDS (no
reducing agent). After electrophoresis, the proteins were transferred
onto polyvinylidene fluoride membranes, which were blocked with
5% nonfat milk in Tris-buffered saline and probed with antibodies di-
luted in Tris-buffered saline containing 0.5% Tween-20, 5% nonfat
milk. Antibodies used in immunoblotting included goat anti-cubilin
A20 (Santa Cruz Biotechnology), rabbit anti–smooth muscle actin (Ab-
cam, Cambridge, MA), goat anti–apoA-I (ab7614; Abcam), and rabbit
anti–ABCA1 (NB400–105; Novus Biologic, Littleton, CO). Rabbit anti-
porcine megalin (rb6286) was described previously.46 Chemilumines-
cent detection of bound antibodies was achieved using the Pierce ECL
Western blotting substrate.

Immunohistochemistry

Mouse kidneys were fixed initially by perfusion fixation followed by
immersion of kidney segments in 4% paraformaldehyde PBS for 12
hours. Kidney segments were then embedded in paraffin and sectioned
at 6-μm thickness. Tissue sections were incubated with rabbit polyclonal
anti-EGFP IgG (Abcam) and goat anti–apoA-I (ab7614; Abcam). Tissue
sections were incubated with donkey anti-goat and donkey anti-rabbit
Alexa Fluor (488 or 568) conjugates (Invitrogen, Carlsbad, CA). Nuclei
were stained using Draq5 (Cell Signaling Technology, Danvers, MA).
Immunolabeled sections were analyzed using a Leica SP5 confocal mi-
roscope (Leica Microsystems, Inc., Exton, PA).

Urine Analysis

Urine was collected from male mice over a 5-day period. Mice were
individually placed in diuresis cages from 5 PM to 8 AM with 10% su-
crose, but no food. From 8 AM to 5 PM, the mice were returned to
regular cages and given food and water. This cycle was repeated for 5
days. Spot urine samples were collected from mice on regular chow
diet and pooled from 5 consecutive days. Urinary creatinine levels were
determined by a two-antibody sandwich ELISA (Abcam). Albumin levels in
urine (diluted 1:500 in PBS) were measured by ELISA using the Mouse Albumin
ELISA Quantitation Set (Bethyl Laboratories, Inc., Montgomery, TX). ApoA-I levels in urine
(diluted 1:20 in PBS) were measured by a two-antibody sandwich
ELISA using goat anti-mouse apoA-I (ab7614) and rabbit anti-mouse
apoA-I (ab20453; Abcam) and purified apoA-I as a standard
(Calbiochem, Darmstadt, Germany).

Blood ApoA-I and Albumin Analysis

Blood from male mice fasted for 6 hours was drawn by retro-orbital
bleeding using heparinized micro-pipettes (Drummond Scientific
Co., Broomall, PA). Drawn blood was kept on ice for approximately 1
hour, then subjected to centrifugation (2000g) and the plasma super-
natant collected. ApoA-I levels in plasma (diluted 1:20,000 in PBS)
were determined by a two-antibody sandwich ELISA (Abcam). Al-
bumin levels in plasma (diluted 1:400,000 in PBS) were determined by
a two-antibody sandwich ELISA (Bethyl Laboratories).

FPLC

FPLC separation of serum lipoproteins from pooled plasma samples
(6 WT and 6 heterozygous mice) was achieved by gel filtration using
two Superose 6HR 10/30 columns (Amersham Pharmacia Biotech,
Piscataway, NJ) in series as previously described.47 Cholesterol, cho-
esterol ester, phospholipid, and triglyceride levels in each fraction
were measured using enzymatic or immunoturbidimetric kits from
Sigma-Aldrich (St. Louis, MO) and Wako Chemicals (Richmond,
VA).

Analysis of Blood Lipoprotein Levels by Nuclear
Magnetic Resonance

Quantitative analysis of serum lipoprotein levels from WT mice and
heterozygotes (from fasted males) was performed by LipoScience, Inc.
(Raleigh, NC) using nuclear magnetic resonance spectroscopy as
described.22

Quantitative PCR

RNA from ileum, liver, and kidney cortex was isolated using RNA-Stat
60 (Tel-test, Inc., Friendswood, TX), and quality was assessed on an
Agilent Bioanalyzer. cDNA was prepared using the iScript cDNA
Synthesis Kit (Bio-Rad, Hercules, CA) according to manufacturer
instructions. Quantitative PCR (qPCR) was performed using iQ SYBR
Green Supermix (Quanta Biosciences, Inc., Gaithersburg, MD)
reagents and a C1000 Thermal Cycler (Bio-Rad). The following
qPCR primers were used: Cubilin sense, 5’-ATTTTCTCTGGGGTTTGT-
TAG-3’ and Cubilin antisense, 5’-TAAGTTTCCCTCCTGCTCTAG-3’
(NM_001081084.2); ApoA-I sense, 5’-AACGCAACCTGCGCTGAGG-
3’ and ApoA-I antisense, 5’-AAAGCCTATGGGGGTTGG-3’; Aba1
sense, 5’-CGTCTCCGGGAAGTGTCTA-3’ and Aba1 antisense,
5’-CTAGAGATGACAAAGGAGTGGAGA-3’; and Albumin sense, 5’-
AGCCCCAGCTACGGCGG-3’ (NM_013454.3)48 and Albumin
antisense, 5’-GGGTAGCTGAGAAGGTTGTTG-3’ (NM_009654.2).
Genes used for qPCR standardization included: β-actin sense, 5’-
CGGGACCTGACAGACTACCCT-3’ and β-actin antisense,
5’-AAGCGCTCTTGGCAATA-3’ (NM_007393.3); Hprt1 sense, 5’-
ATCAATTATGCCGAGATTGTTA-3’ and Hprt1 antisense,
5’-CACACAGAGGGCGACAATGT-3’ (NM_013556); and Rn18s
sense, 5’-CGCCGGCTAGGTTAAATCT-3’ and Rn18s antisense
5’-CGAACCTCCTGACTTCTTCT-3’ (NR_003278.2).

Preparation of Human Plasma HDL Subfractions

Human plasma HDL2 (d=1.11–1.13 g/ml) and HDL3 (d=1.13–1.25
g/ml) were isolated by density gradient ultracentrifugation as previ-
ously described.49 Total protein and apoA-I concentration was de-
termined using Pierce BCA Protein Assay Kit and apoA-I ELISA as
described above.

Human HDL Fractional Clearance in Mice

The clearance of HDL subfractions was measured in male mice
following tail vein injection with HDL2 (approximately 15 μg/g body
weight) or HDL3 supplemented with 6% purified apoA-I (Calbiochem)
(approximately 40 μg/g).50 After the tail vein injection, blood samples
were collected at 10 minutes by retro-orbital and subsequently by tail
vein bleeding at 1, 3, 6, 12, 24, 48, and 72 hours. Human HDL subfrac-
tion concentrations in mouse plasma were determined by a two-antibi-
body ELISA using rabbit anti–apoA-I (NB110–55465; Novus Biologic)
and sheep anti–apoA-I (NB600–1538). The antibodies used in this
ELISA are specific to human apoA-I and display no cross-reactivity to mouse apoA-I. Standard curves for these assays were generated using purified apoA-I (Calbiochem) diluted in 5% mouse plasma. The concentration of human HDL measured for each time point was normalized to the concentration measured at 10 minutes for each mouse and average values plotted on a log scale.

**Statistical Analyses**

Comparisons between WT and heterozygous mice were performed with GraphPad Prism (GraphPad Software, La Jolla, CA) using the Mann–Whitney U test. Horizontal lines depict median values.

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**DISCLOSURES**

None.

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