

Selective Intestinal Cobalamin Malabsorption with Proteinuria (Imerslund-Gräsbeck Syndrome) in Juvenile Beagles

J.C. Fyfe, S.L. Hemker, P.J. Venta, B. Stebbing, and U. Giger

Background: Selective intestinal cobalamin malabsorption with mild proteinuria (Imerslund-Gräsbeck syndrome; I-GS), is an autosomal recessive disorder of dogs caused by mutations in *AMN* or *CUBN* that disrupt cubam function and which can present as a medical emergency.

Objectives: To describe the clinical, metabolic, and genetic bases of I-GS in Beagles.

Animals: Four cobalamin-deficient and 43 clinically normal Beagles and 5 dogs of other breeds.

Methods: Clinical description and candidate gene genetic study. Urinary organic acid and protein excretion were determined by gas-chromatography and SDS-PAGE, respectively. Renal cubilin protein expression was assessed on immunoblots. Mutation discovery was carried out by PCR amplification and DNA sequencing of exons with flanking splice sites and cDNA of *CUBN* and *AMN*. Genotyping was performed by restriction enzyme digestion of PCR amplicons.

Results: Juvenile-affected Beagles exhibited failure to thrive, dyshematopoiesis with neutropenia, serum cobalamin deficiency, methylmalonic aciduria, hyperammonemia, and proteinuria. Affected dogs' kidneys lacked detectable cubilin protein. All affected dogs were homozygous for a single-base deletion in *CUBN* exon 8 (*CUBN* c.786delC), predicting a translational frameshift, and the 2 parents tested were heterozygous.

Conclusions: The *CUBN* mutation in juvenile I-GS Beagles causes a more severe cobalamin malabsorption than in Border Collies with a different *CUBN* defect, but is similar to I-GS caused by *AMN* mutations in Giant Schnauzers and Australian Shepherds. Awareness of the disorder and breed predispositions to I-GS is crucial to precisely diagnose and promptly treat hereditary cobalamin malabsorption and to prevent disease in those dogs at risk in future generations.

Key words: Cubam; Cytopenia; Inborn error of metabolism; Methylmalonic aciduria; Vitamin B₁₂.

Selective intestinal cobalamin (vitamin B₁₂) malabsorption accompanied by mid- to low-molecular weight proteinuria is an autosomal recessive trait (OMIA #000565-9615) in Giant Schnauzers,¹ Australian Shepherds,² and Border Collies.^{3–5} The syndrome is called Imerslund-Gräsbeck syndrome (I-GS; OMIM #261100), after the 2 investigators who simultaneously described it in human families of Norway and Finland.^{6,7} Intestinal malabsorption of the single nutrient coupled with failed renal tubular reabsorption of a subset of proteins filtered by the glomerulus is because of mutations that disrupt cubam function in those tissues. Cubam⁸ is a heteromeric, multi ligand, endocytic receptor composed of 2 distinct protein subunits, cubilin (*CUBN*)⁹ and amnionless (*AMN*).¹⁰ While several *AMN* and *CUBN* mutations occur in humans with I-GS,¹¹ only 2 *AMN* mutations,² and most recently, a single *CUBN* mutation,¹² have been found in 3 different affected dog breeds.

The subunit encoded by *CUBN* is a large (~460 kDa), glycosylated protein of the ileal and

Abbreviations:

| | |
|----------|---|
| AMN | amnionless |
| bp | base pair(s) |
| CUBN | cubilin |
| Cubam | functional receptor complex composed of AMN and CUBN subunits |
| DNA | deoxyribonucleic acid |
| I-GS | Imerslund-Gräsbeck syndrome |
| kb | kilobase(s) |
| kDa | kiloDalton(s) |
| MMA | methylmalonic acid |
| PCR | polymerase chain reaction |
| SDS-PAGE | sodium dodecylsulfate polyacrylamide gel electrophoresis |
| VHUP | Veterinary Hospital of the University of Pennsylvania |
| VSS | Veterinary Specialist Services |

proximal renal tubule brush border that provides binding sites for various specific ligands, including the intrinsic factor-cobalamin complex (IF-Cbl), albumin, vitamin D-binding protein, haptoglobin, apolipoprotein A1, and others.¹³ The *CUBN* coding gene comprises 67 exons on canine chromosome 2, and the mRNA is 11.3 kb. The subunit encoded by *AMN* is an ~50 kDa glycosylated type I transmembrane protein that interacts with *CUBN* to provide membrane anchorage for the complex and endocytic signal motifs that initiate clathrin-coated pit nucleation.¹⁴ The *AMN* coding gene comprises 12 exons on canine chromosome 8, and the mRNA is 1.54 kb. Studies of human and canine tissues harboring mutations in either gene demonstrate that each subunit depends on the near-normal structure of the other for epithelial

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brush border expression and function of the cubam receptor complex.^{2,15}

In canine and human I-GS patients, cobalamin malabsorption can be life-threatening due to the resulting vitamin deficiency and catastrophic metabolic derangements, unless rapidly and specifically treated with parenteral cobalamin. The accompanying proteinuria is benign and persists lifelong despite treatment.^{1–5,12,16,17} Cubam expression is disrupted by distinct *AMN* mutations² in Giant Schnauzers and Australian Shepherds and by a *CUBN* mutation in Border Collies.¹² Early onset cobalamin deficiency also occurs in Beagles in the United States and Australia.^{18,19} Here, we provide clinical, biochemical, and molecular genetic evidence that this severe I-GS is caused by a *CUBN* mutation and can be effectively treated if promptly diagnosed.

Materials and Methods

Animals

Four cobalamin-deficient Beagles were studied. Clinical findings of two were previously reported.^{18,19} Those of the other two were determined from medical records of the referring clinics, the Veterinary Hospital of the University of Pennsylvania (VHUP), and Veterinary Specialist Services (VSS). All routine clinical tests reported here were performed on fresh samples at the time of original clinical investigations and by methods available to each clinic via commercial or in-hospital laboratories. In addition, dogs related to an affected dog from a large family of Beagles, gathered by Veterinary Specialist Services (VSS) in Brisbane, Australia were studied. Whole blood or tissue for DNA isolation was available from all dogs in the study, urine was available from 2 affected dogs as well as normal control Beagles. Blood was stored at 4°C in ethylenediaminetetraacetic acid for up to 2 weeks before DNA isolation. Kidney and liver of 1 affected Beagle were snap frozen during necropsy and stored at –80°C. DNA samples of unrelated clinically normal Beagles and other breed dogs were from the laboratory archives. Urinary organic acids and proteins were assessed and identified as previously described.¹² Urine was stored at –80°C for up to 1 month before study. The studies were approved by the Institutional Animal Care and Use Committee.

Immunoblotting

Protein expression was assessed by *CUBN* immunoblotting of detergent extracted kidney cortex homogenate proteins separated on 4–20% gradient SDS-polyacrylamide gels. *CUBN* was detected on polyvinylidene difluoride (PVDF) membranes incubated sequentially with 1:30,000 dilution of previously characterized rabbit polyclonal anti-dog *CUBN* serum,²⁰ goat antirabbit IgG-horse radish peroxidase conjugate^a (1:20,000), and chemiluminescence reagents.^b Gel loading was assessed by detection of DNA-PK protein using a previously described²¹ antibody (1:1,000; generous gift from Dr Kathryn Meek, Michigan State University). Relative quantification of western blot signals was performed with the Image Lab software of the Bio-Rad Chemi Doc XRS⁺ Molecular Imager.^c

CUBN and *AMN* Sequencing

Genomic DNA was prepared from whole blood or frozen tissue by standard methods.² *CUBN* exons and flanking splice-site

sequences were amplified by PCR from genomic DNA (PCR primer sequences are in Table S1 of reference 12). A portion of *CUBN* cDNA was amplified by RT-PCR from kidney total RNA as previously described² using oligo-dT priming of the RT reaction and PCR primers 5'-GCCCTCTGTGCTCAGTTGATG T-3' (*CUBN* c.482–503, exon 6) and 5'-GTGGCAGCCTCCATT ATTGACT-3' (*CUBN* c.1053–1074, exon 9). Exons and flanking splice-site sequences of *AMN* were amplified similarly from genomic DNA (PCR primer sequences in Table S1). Amplicons were sequenced using the amplifying primers and assembled for comparison to the CanFam 3.1 canine reference genome assembly and *AMN* and *CUBN* cDNA sequences (GenBank accession nos. AY368152.1 and AF137068.1, respectively). New *AMN* genomic sequences generated in this study have been deposited in GenBank (accession no. KF445236) because *AMN* exons 1, 2, and 4–9 are missing from the canine reference sequence due to two large gaps in the assembly and areas of poor sequence quality. *AMN* maps to CanFam 3.1 coordinates Chr8:70,798,913–70,807,835.

CUBN Mutation Genotyping

The *CUBN* exon 8 mutation site was amplified from genomic DNA using the primers 5'-AGGGTTTTCCAGTCACGACGC TTTCTTGCTCATTTCGTGAT-3' and 5'-GCATGCTGGAG ATTGCACAC-3', creating a 170 bp amplicon. The underlined nucleotide in bold font of the second primer introduces a sequence change in the amplicon that creates an *Msl* I restriction enzyme recognition site (CAYNNNNRTG) in the normal allele that is not in the native sequence and which is lost in the mutant allele. The first 20 bases of the forward primer do not match the dog sequence but are included to increase the resolution of the diagnostic bands. Amplification products were digested with *Msl* I, and fragments were separated on 4% agarose gels.

Results

Clinical Presentations

Case 1. An intact female Beagle was presented to a primary care clinic at 4 months of age with a history of inappetence for 1 month and lethargy for a few days. The puppy was thin (body condition score [BCS] 3/9) and was found to have ancylostomiasis, coccidiosis, demodecosis, and dermatophytosis. Despite routine antiparasite treatments over the next month, the puppy continued to have a poor appetite, developed intermittent diarrhea and focal alopecia, and became thinner (BCS 2.5/9). Diagnostic tests at 5 months of age revealed a normocytic-normochromic and regenerative anemia (Hct 27% [normal reference range 35–58]; MCV 63 fL [62–77], MCHC 35 g/dL [30–35]; reticulocytes 85,600/μL [<60,000]) with normal leukogram (WBC 8,100/μL [5,300–19,000]; neutrophils 5,022/μL [3,100–14,400]). There was hypoproteinemia (albumin 2.6 g/dL [2.5–3.7], globulin 1.7 g/dL [2.4–4.0]). Serum electrolyte concentrations and an ACTH-stimulation test were normal. There was nonmeasurable cobalamin in serum, slightly increased folate concentrations (11.5 μg/L [4.8–8.6]), and normal trypsin-like immunoreactivity (17 μg/L [5–35]). A diagnosis of intestinal disease caused by bacterial overgrowth was made, and the puppy was treated with daily oral tylosin and 1 subcutaneous injection of 0.5 mg cyanocobalamin.

At 7 months of age, the dog presented with recent vomiting, continued intermittent diarrhea, lethargy, and inappetence. Because of the continued hypoproteinemia, a protein-losing enteropathy with possible liver disease was considered. She received fluids IV and antiemetic treatment and was discharged the next day after eating and seeming to be clinically improving.

At 9 months of age, she was examined twice within 3 days by the primary care veterinarians for recurrence of vomiting and diarrhea, worsening of her clinical signs (lethargy, inappetence, weakness), and continued poor body condition (4.5 kg). Routine laboratory tests showed anemia (Hct 33%), leukopenia (WBC 520/ μ L; neutrophils 310/ μ L), thrombocytopenia (114,000/ μ L [177,000–398,000]), and hypoproteinemia (albumin 1.7 g/dL, globulin 1.9 g/dL). She received supportive care including fluids administered IV, antiemetics, sucralfate, and antibiotics, and was transferred to VHUP the following day.

On presentation, the dog was lethargic, hardly responsive, dehydrated, hypothermic (97.9°F), and thin. She vomited blood and had hematochezia. Repeat testing confirmed normochromic-normocytic anemia, leukopenia, and thrombocytopenia. Peripheral blood smears revealed nuclear hypersegmentation of neutrophils and some metarubricytes. Additionally, there was hyperglycemia (202 mg/dL [60–110]), hypokalemia (2.4 mmol/L [2.5–5.5]); hypoproteinemia (albumin 1.6 g/dL, globulin 1.5 g/dL), hypocalcemia (4.4 mg/dL [9.8–11.7]), hypomagnesemia (1.2 mg/dL [1.6–2.5]) hypocholesterolemia (88 mg/dL [128–317]), and hyperammonemia (175 μ mol/L [11–35]). Urine methylmalonic acid (MMA) concentration was 5,830 mmol/mmol creatinine (normal <4). Despite intensive care including supplementation of cobalamin, calcium, and magnesium, the dog developed aspiration pneumonia and died of cardiopulmonary arrest within a day. Tissue samples were immediately frozen for subsequent molecular analysis. Necropsy demonstrated hypocellular bone marrow with hemosiderosis, severe necrotizing enteropathy, and moderate regionally extensive acute interstitial bacterial pneumonia.

Case 2. A juvenile male Beagle with signs of cobalamin deficiency was investigated at VHUP. It had similar presentation and clinical course to case 1 but was successfully treated. Clinical details were published previously.¹⁸ Aggressive parenteral cyanocobalamin administration led to complete clinical and laboratory normalization within 5 days and return to normal weight for age within 3 months. With lifelong treatment (~50 μ g cyanocobalamin/kg body weight, SC, q3–4 weeks), the dog lived for 9 years without recurring signs except for persistent proteinuria (1+ to 2+ on a dipstick). Blood and urine were obtained for molecular analysis. Urine protein-creatinine ratio was 0.3 [<0.4], but SDS-PAGE of concentrated urine proteins demonstrated selective excretion of discrete medium- to low-molecular weight proteins identified as albumin, vitamin D-binding protein, haptoglobin, and apolipoprotein A-I, each of which is a cubilin ligand (Fig 1, lane 1). This pattern of protein excretion was

not observed in urine of a normal Beagle (lane 2) or healthy dogs of other breeds.¹²

Cases 3 and 4. A male Beagle was presented at 4.5 months of age for poor appetite and failure to gain weight in the prior 2 months. Diagnostic tests revealed nonregenerative, normochromic-normocytic anemia (Hct 30%; MCV 67 fL; MCHC 34 g/dL; reticulocytes 32,000/ μ L with normal leukogram). There was hypoproteinemia (albumin 2.5 g/dL, globulin 1.7 g/dL). Total T4 (22 nmol/L), pancreatic lipase (138 μ g/L), and serum bile acids before and after a meal (3 and 3 μ mol/L, respectively) were normal. No specific therapy was instituted. One month later, the puppy still had no weight gain and remained anemic, but 5 nucleated RBCs/100 WBC [<2 NRBC is normal] and neutropenia (2,500/ μ L) were noted in the CBC. Prior awareness of cases in Beagles¹⁹ in the local area made cobalamin deficiency the primary diagnostic rule-out. Cyanocobalamin injections (50 μ g/kg body weight, SC, every 2 weeks) corrected all clinical signs and hematologic abnormalities. The dog's appetite returned to normal in a day, and its weight normalized in a month. Case 3 shared close ancestry with a female Beagle that was failing to thrive from 7 months of age (Case 4) and which was identified as case 1 in the case series of congenital cobalamin deficiency in Beagles from Brisbane.¹⁹ Case 4 responded completely to parenteral cobalamin administration and remains in remission with once to twice monthly cyanocobalamin injections (50 μ g/kg body weight, SC) since diagnosis 7 years previously. Blood was collected for genetic analysis from both case 3 and 4 affected dogs, 2 of their parents, and 3 other clinically normal members of the kindred.

CUBN Protein Expression

Selective proteinuria in case 2 comprising cubilin ligands as demonstrated in this study suggested failed

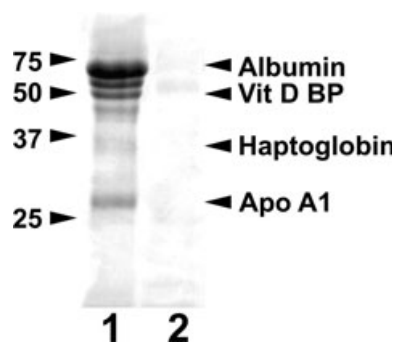


Fig 1. Selective proteinuria in a Beagle with I-GS. Shown are lanes from a silver-stained 15% SDS-PAGE gel loaded with urine proteins of an affected (case 2; lane 1) and a clinically normal Beagle (lane 2). Proteins in each lane were concentrated from urine samples containing 50 μ g of creatinine. The affected dog was in clinical and metabolic remission at the time of urine collection because of previous parenteral cyanocobalamin administration. Migration of molecular weight (kDa) markers is indicated to the left.

cubilin expression in proximal tubular cells. Therefore, on the assumption that the Beagles in each case exhibited the same molecular defect, we generated immunoblots of kidney cortex proteins from case 1 and from 2 normal control dogs (Fig 2). Cubilin expression was undetectable in tissue of the affected dog (lane 1) even though the amount of total protein loaded in that lane was 2.5-fold more than in the control lanes, where cubilin was readily apparent (lanes 2 and 3). Expression of the control protein, DNA-PK was evident in affected and control dog's kidneys in amounts that correlated with the different amounts of protein loaded.

AMN and CUBN Sequences and Mutation Discovery

We amplified the 12 *AMN* exons with flanking splice sites and 2.32 kb of intron sequence from case 2 genomic DNA. There were no exonic or splice sequence variants, but we noted heterozygosity at several intronic sites across the locus (Table S1). The 67 *CUBN* exons with flanking splice sites and 20,488 bp of intronic sequence were amplified from case 1 genomic DNA. There were no sequence variations in splice sites and no heterozygous sites. The only sequence variation likely to affect protein coding was a homozygous deletion of a single cytosine in exon 8 (*CUBN* c.786delC; Fig 3A). The same mutation was found in cDNA of case 1 by sequencing RT-PCR products and in genomic DNA of case 2. The single-base deletion predicts a shift in the translation reading frame and a premature stop codon 45 codons after the deletion (p.C264Sfs*45).

CUBN Mutation Genotyping

The exon 8 deletion was not observed in DNA of 5 normal Beagles by sequencing the mutation site. For larger studies, we designed a convenient allele-specific genotyping assay for the deletion based on PCR amplification of genomic DNA followed by restriction enzyme digestion of the 170 bp amplicon. The reverse primer introduced a change in the amplicon sequence

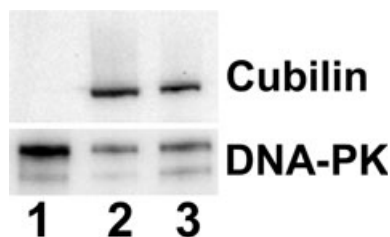


Fig 2. Lack of cubilin protein in kidney of a Beagle with I-GS. Detergent-solubilized protein of kidney cortex homogenates of an affected (case 1; lane 1, 50 μ g protein) and 2 clinically normal control dogs (lanes 2 and 3, 20 μ g protein each) were separated by nonreducing, 4–20% gradient SDS-PAGE and blotted. In the upper panel, the blot was developed by incubation with rabbit antidog cubilin serum. In the lower panel, the blot was developed by incubation with a mouse monoclonal anti-human DNA-PK antibody as a control for loading and protein integrity.

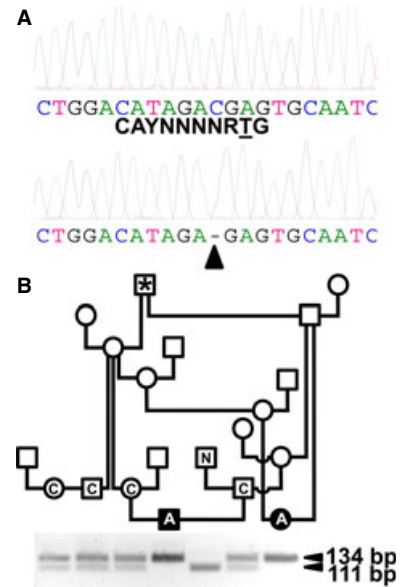


Fig 3. *CUBN* exon 8 cytosine deletion and genotyping assay in Beagles. Panel A shows electrophoretograms of sequence from the Beagle I-GS mutation site of a normal (top) and an affected dog (case 1; bottom). The single cytosine deletion (*CUBN* c.786delC) is indicated by an arrowhead. Panel B shows results of an allele-specific, *MspI* restriction digest of PCR products amplified from genomic DNA. The sequence of the *MspI* recognition site is indicated in black below the normal sequence in panel A. The underlined T of the recognition site is introduced to the amplicon sequence by the reverse PCR primer. The 170 bp PCR product amplified from normal dog genomic DNA has two *MspI* restriction sites, but the Beagle I-GS deletion obliterates one site. Thus, the diagnostic fragment length from the normal allele is 111 bp, while that of the deleted allele is 134 bp. The control cut fragment of 36 bp is not shown. Lanes of a genotyping gel (4% agarose) are aligned beneath symbols for Beagles of the Australian kindred that were tested. Affected dogs are indicated by filled symbols and clinically normal dogs by open symbols; squares are males and circles are females. The affected female at the lower right was case 4 (case 1 in reference 19), and the affected male was case 3 of this report. The asterisk indicates an ancestor that was common to each of the parents of affected offspring available for testing. The letter in each symbol indicates the genotype determined by testing: affected (A), carrier (C), and normal (N).

that created an *MspI* cleavage site when amplified from the normal but not from the mutant allele. Therefore, the normal allele resulted in digestion products of 111 and 23 bp, whereas the deletion allele resulted in a product of 134 bp. Digested DNA amplified from either allele both demonstrated an additional 36 bp product because of a designed “control cut” to indicate adequate function of the restriction enzyme with each sample. To validate the assay, we tested the previously sequenced samples from 2 affected and 5 normal dogs and found no discordance.

The restriction digest assay was applied to samples from cases 3 and 4 and related dogs that originated in Australia (Fig 3B) as well as to unrelated normal dogs. While the two affected dogs of the available kindred

were homozygous for the mutant allele, 2 parents of affected dogs and 3 other clinically normal dogs of the kindred were heterozygous. Three others in the kindred were homozygous for the normal allele. The deleted allele was not observed in any of 40 clinically normal Beagles from North America, nor was the Beagle mutation observed in I-GS affected dogs of 4 other breeds and a mongrel.

Discussion

We describe here the clinicopathologic features, biochemical abnormalities, and the molecular genetic basis of selective cobalamin malabsorption with proteinuria (I-GS) in 4 juvenile Beagles. The identical *CUBN* mutation caused I-GS in each of these cases appearing over a period of 15 years on 2 continents. Clinical signs of cobalamin deficiency in Beagles with inherited cobalamin malabsorption manifest in the juvenile period as fetal stores of the vitamin wane.^{18,19} This is somewhat different from I-GS in Border Collies caused by a different *CUBN* mutation, wherein onset of signs is often delayed into early adulthood and clinical signs in some cases are milder.^{1-5,12} Consistent signs in Beagles, but also in Giant Schnauzers and Australian Shepherds with I-GS caused by *AMN* mutations (Fig 4), include inappetence and failure to thrive often observed from 8 to 12 weeks of age; dyshematopoiesis characterized by anemia, neutropenia with nuclear hypersegmentation, metarubricytosis, and megaloblastic changes in bone marrow; megaloblastic changes of the intestinal epithelium; and metabolic disruptions characterized by methylmalonic acidemia/uria and hyperhomocysteinemia caused by inhibition of cobalamin-dependent enzymes. Variable signs of secondary metabolic disruptions include hyperammonemia, hypoglycemia, ketoacidosis,

and hypoproteinemia. If diagnosis and treatment are delayed, affected dogs exhibit a chronic course of inappetence, lethargy, failure to gain weight and muscle mass, intermittent vomiting and diarrhea, seizures, and/or a lethal metabolic crisis. It is tragic when failure to diagnose the disorder results in morbidity or death because parenteral cyanocobalamin administration (~1 mg/dog/month) initiated early provides rapid and sustained hematopoietic and metabolic remission leading to a normal healthy lifespan.

In case 1, an initial presumptive diagnosis of “bacterial overgrowth” obscured the significance of cobalamin deficiency. By the time of transfer to a tertiary veterinary care facility at 9 months of age, the dog had signs and laboratory findings indicative of severe metabolic decompensation and died despite symptomatic emergency intervention. Case 2 had a clinical course and signs¹⁸ as severe as case 1, but the important difference affecting a positive outcome was the clinical suspicion of an inborn error of metabolism leading to timely diagnosis and specific treatment maintained for life. Case 3 of this report and the seven Beagles of unknown relationship seen in the Brisbane area from 2006 to 2008¹⁹ presented with similar signs and course of disease, but benefited from the clinician’s prior experience that heightened the diagnostic likelihood of cobalamin deficiency. Each dog had a robust clinical and laboratory response to parenteral cyanocobalamin administration. Proteinuria was not documented in those cases, but the selective proteinuria of I-GS in dogs is not quantitatively different from what is observed in many ill and some healthy dogs (1+ to 2+ protein on a dipstick). The specific proteinuria of canine or human I-GS is indicative of failed cubam function in renal tubules, and therefore persists despite cobalamin replenishment.^{1,12,15}

Presumptive cobalamin malabsorption coupled with demonstrated urinary loss of cubilin ligands indicates a functional defect of cubam in intestine and kidney. Cases of early onset cobalamin deficiency in geographically dispersed Beagles (Australia and United States) constitute a breed predisposition that is highly suggestive of an inherited disorder. Homozygosity by descent of a disease-causing mutation is a prerequisite of recessive inheritance, as has been documented for I-GS in the other canine breeds.^{1,2,12} Moreover, because the undiagnosed and untreated disease is so devastating in Beagles, it is unlikely that affected dogs were used in matings that produced the affected offspring. Findings in this study wherein affected dogs are homozygous for the deleted allele and parents of affected dogs are heterozygous are consistent with the hypothesis of autosomal recessive inheritance of Beagle I-GS.

Case 2 was heterozygous at sites across the *AMN* genomic locus (Table S1b), findings incompatible with simple recessive inheritance of I-GS, and exhibited no putative *AMN* mutation. Moreover, prior studies^{1,2,20,22} demonstrated that tissues from I-GS dogs harboring *AMN* mutations continue to express readily detectable cubilin protein on immunoblots despite the fact that it does not reach the epithelial brush border

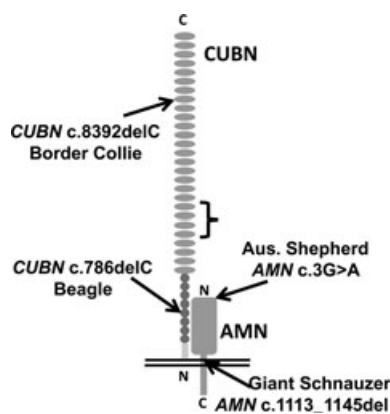


Fig 4. Cubam structure showing sites of I-GS causing mutations in dogs. The cartoon illustrates the protein domain structure of cubam composed of CUBN and AMN subunits. The N and C termini of each protein are indicated. The bracket around CUB domains 5–8 indicates the intrinsic factor–cobalamin binding site. The epithelial cell apical plasma membrane is indicated by double horizontal lines, and the extracellular side is to the top. Each site of breed-specific mutation causing canine I-GS is indicated by an arrow.

and provide cubam function. This understanding coupled with undetectable cubilin on western blots of kidney cortex from case 1, made *CUBN* the most likely candidate gene. Accordingly, we found a single base deletion in exon 8 of *CUBN* that was homozygous in affected Beagles. This frameshift mutation predicts an early translation termination codon in the *CUBN* mRNA that almost assuredly activates the intracellular mechanisms of nonsense-mediated mRNA decay,²³ rather than allowing production of a truncated and/or nonfunctional protein. Border Collies with I-GS also have a single cytosine deletion, but it is in exon 53 of *CUBN* and much further 3' in the mRNA. Ten- to 20-fold reduced steady-state mRNA and a small amount of residual expression of full-length cubilin protein that retains IF-Cbl binding activity were demonstrated in the ileum and kidney of affected Border Collies.¹² Residual cubilin expression, which we do not see in the affected Beagle, might explain why diagnosis in some Border Collies is much delayed compared to Beagles and the other breeds where cubam expression is abrogated entirely.²

Though the deletion allele was not observed in 40 unrelated normal Beagles from the United States, this number is not sufficient to reliably estimate an allele frequency in the breed. This contrasts with the I-GS mutation (c.8392delC) that has an estimated 0.06 allele frequency in Canadian and US Border Collies¹² and nearly the same allele frequency in border collies of the continental EU.²⁴ The increased *CUBN* mutation frequency in Border Collies could be because of the seemingly later age of diagnosis of I-GS in that breed (8–48 months), the more varied and less severe signs, and the possibility that mildly affected dogs might have been used for breeding. For instance, we recently diagnosed I-GS in a nearly 4-year-old Border Collie whose signs were intermittent pyrexia and chronic neutropenia. The earlier median age (4 months) of presentation of severe disease in Beagles is more similar to that of Giant Schnauzers and Australian Shepherds with *AMN* mutations.^{1,2}

5'-adenosyl- and methyl- forms of cobalamin are essential cofactors for 2 essential enzymes of intermediate metabolism, L-methylmalonyl-CoA mutase and 5-methyltetrahydrofolate-homocysteine methyltransferase (aka methionine synthase), respectively. Suppression of substrate flux through catabolic pathways mediated by these enzymes due to cobalamin cofactor deficiency directly causes increased methylmalonic acid (MMA) and homocysteine concentrations in plasma and methylmalonic acid in urine. Inhibition of methionine synthase activity also traps tetrahydrofolate in the 5-methyl form, creating cellular deficiency of 5, 10- methylene tetrahydrofolate required for a one-carbon transfer reaction by thymidylate synthase to support DNA synthesis.²⁵ Thus, cobalamin deficiency slows nuclear maturation in rapidly proliferating hematopoietic and intestinal epithelial cells creating the recognized megaloblastic changes in these tissues. Moreover, increased concentrations of mitochondrial pools of acyl-CoA esters secondarily

inhibit additional metabolic pathways including the urea cycle, gluconeogenesis, and glycine cleavage leading to hyperammonemia, hypoglycemia, and hyperglycinemia.²⁶ Therefore, the signs of chronic cobalamin deficiency are global, because of disruption of several metabolic pathways, and could be confused with hepatoencephalopathy, perhaps due to portocaval shunt, or other disorders.

In the young Beagle with cobalamin deficiency, inappetence, lack of appropriate weight gain, and failure to thrive are predominating client complaints. None of these signs is specific, so a high index of suspicion based on breed predisposition and age-of-onset is needed for efficient diagnosis of hereditary cobalamin malabsorption. Neutropenia with hypersegmentation and metarubricytosis are peripheral signs of megaloblastosis and classic hematologic features of cobalamin deficiency that are observed in these cases. However, cobalamin-deficient dogs do not exhibit the erythrocyte macrocytosis that is common in human cobalamin deficiency, an observation that remains unexplained.¹ Determinations of low serum cobalamin or high urinary MMA concentrations are confirmatory. Acquired cobalamin malabsorption can be present in young dogs accompanying such conditions as gastrointestinal dysbiosis, surgical resection of distal small intestine or exocrine pancreatic insufficiency. Chronic cobalamin deficiency of whatever cause can create secondary generalized gastrointestinal malabsorption because of megaloblastic changes of the intestinal epithelium.²⁷ Therefore, some cases of chronic diarrhea are not effectively treated until body cobalamin stores have been replenished.

I-GS is one of few inborn errors of metabolism that can be treated readily and inexpensively. Parenteral administration of cyanocobalamin has a wide margin of safety, even in megadoses of several mg because any of the water-soluble vitamin B₁₂ that does not bind immediately to the plasma carrier, transcobalamin (TC), is rapidly excreted by glomerular filtration. Therefore, following sample collection for definitive diagnosis, early therapeutic intervention based upon clinical suspicion may save a patient's life. Determination of post-treatment serum cobalamin concentrations can be misleading, however, because cobalamin leaves the plasma space by receptor-mediated endocytosis of the TC-cobalamin complex and subsequently binds as cofactor to the mutase and methionine synthase enzymes, both of which have long half-lives. When cobalamin malabsorption is a lifelong condition, as in I-GS or ileal resection, and after cobalamin deficits have been replenished, a simple regimen of 1 mg cyanocobalamin administered SC every 3–4 weeks is sufficient for normal health, growth, reproduction, and activity, even in a mid- to large-sized dog. This report should raise the clinicians' awareness of I-GS and the clinical presentation. While low serum cobalamin concentration, methylmalonic aciduria, and response to treatment are suggestive, breed-specific *AMN* and *CUBN* mutation tests allow for precise I-GS diagnosis. Genetic tests also allow diagnosis before the develop-

ment of clinical signs and detection of asymptomatic carriers to guide breeding decisions.

Footnotes

^a cat. # 141506, Kirkegaard & Perry Laboratories, Inc, Gaithersburg, MD

^b Western Lightning ECL Plus, PerkinElmer, Inc, Waltham, MA

^c Bio-Rad Laboratories, Inc, Hercules, CA

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References

1. Fyfe JC, Giger U, Hall CA, et al. Inherited selective intestinal cobalamin malabsorption and cobalamin deficiency in dogs. *Pediatr Res* 1991;29:24–31.
2. He Q, Madsen M, Killeney A, et al. Amnionless function is required for CUBN brush-border expression and intrinsic factor-cobalamin (vitamin B₁₂) absorption in vivo. *Blood* 2005;106:1447–1453.
3. Morgan LW, McConnell J. Cobalamin deficiency associated with erythroblastic anemia and methylmalonic aciduria in a Border Collie. *J Am Anim Hosp Assoc* 1999;35:392–395.
4. Battersby IA, Giger U, Hall EJ. Hyperammonaemic encephalopathy secondary to selective cobalamin deficiency in a juvenile Border Collie. *J Small Anim Pract* 2005;46:339–344.
5. Lutz S, Sewell AC, Reusch CE, Kook PH. Clinical and laboratory findings in Border Collies with presumed hereditary juvenile cobalamin deficiency. *J Am Anim Hosp Assoc* 2013;49:197–203.
6. Imerslund O. Idiopathic chronic megaloblastic anemia in children. *Acta Paediatr Scand* 1960;49(Suppl. 119):1–115.
7. Gräsbeck R, Gordin R, Kantero I, Kuhlback B. Selective vitamin B₁₂ malabsorption and proteinuria in young people. *Acta Med Scand* 1960;167:289–296.
8. Fyfe JC, Madsen M, Højrup P, et al. The functional cobalamin (vitamin B₁₂)-intrinsic factor receptor is a novel complex of CUBN and amnionless. *Blood* 2004;103:1573–1579.
9. Aminoff M, Carter JE, Chadwick RB, et al. Mutations in *CUBN*, encoding the intrinsic factor-vitamin B₁₂ receptor, CUBN, cause hereditary megaloblastic anaemia 1. *Nat Genet* 1999;21:309–313.
10. Tanner SM, Aminoff M, Wright FA, et al. Amnionless, essential for mouse gastrulation, is mutated in recessive hereditary megaloblastic anemia. *Nat Genet* 2003;33:426–429.
11. Tanner SM, Sturm AC, Baack EC, et al. Inherited cobalamin malabsorption. Mutations in three genes reveal functional and ethnic patterns. *Orphanet J Rare Dis* 2012;7:56.

12. Fyfe JC, Hemker SL, Venta PJ, et al. An exon 53 frameshift mutation in *CUBN* abrogates cubam function and causes Imerslund-Gräsbeck syndrome in dogs. *Mol Genet Metab* 2013;109:390–396.

13. Nielsen MJ, Rasmussen MR, Andersen CBF, et al. Vitamin B₁₂ transport from food to the body's cells - a sophisticated, multistep pathway. *Nat Rev Gastroenterol Hepatol* 2012;9:345–354.

14. Pedersen GA, Chakraborty S, Steinhauser AL, et al. AMN directs endocytosis of the intrinsic factor-vitamin B₁₂ receptor cubam by engaging ARH or Dab2. *Traffic* 2010;11:706–720.

15. Storm T, Emma F, Verroust PJ, et al. A patient with CUBN deficiency. *N Engl J Med* 2011;364:89–91.

16. Wahlstedt-Fröberg V, Pettersson T, Aminoff M, et al. Proteinuria in CUBN-deficient patients with selective vitamin B₁₂ malabsorption. *Pediatr Nephrol* 2003;18:417–421.

17. Broch H, Imerslund O, Monn E, et al. Imerslund-Gräsbeck anemia: A long-term follow-up study. *Acta Paediatr Scand* 1984;73:248–253.

18. Fordyce HH, Callan MB, Giger U. Persistent cobalamin deficiency causing failure to thrive in a juvenile Beagle. *J Small Anim Pract* 2000;41:407–410.

19. Barron PM, Wouda RM. Congenital cobalamin deficiency in seven Beagle puppies. *Aust Vet Pract* 2008;38:126–132.

20. Xu D, Fyfe JC. Cubilin expression and posttranslational modification in the canine gastrointestinal tract. *Am J Physiol Gastrointest Liver Physiol* 2000;279:G748–G756.

21. Meek K, Kienker L, Dallas C, et al. SCID in Jack Russell Terriers: A new animal model of DNA-PKcs deficiency. *J Immunol* 2001;167:2142–2150.

22. Fyfe JC, Ramanujam KS, Ramaswamy K, et al. Defective brush-border expression of intrinsic factor-cobalamin receptor in canine inherited intestinal cobalamin malabsorption. *J Biol Chem* 1991;266:4489–4494.

23. Kervestin S, Jacobson A. NMD: A multifaceted response to premature translational termination. *Nat Rev Mol Cell Biol* 2012;13:700–712.

24. Owczarek-Lipska M, Jagannathan V, Drögemüller C, et al. A frameshift mutation in the cubilin gene (*CUBN*) in border collies with Imerslund-Gräsbeck syndrome (selective cobalamin malabsorption). *PLoS ONE* 2013;8:e61144.

25. Scott J, Weir D. Folate/vitamin B₁₂ inter-relationships. *Essays Biochem* 1994;28:63–72.

26. Fenton WA, Gravel RA, Rosenblatt DS. Disorders of propionate and methylmalonate metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The Metabolic & Molecular Basis of Inherited Disease*, 8th ed. New York: McGraw-Hill; 2001:2165–2193.

27. Arvanitakis C. Functional and morphological abnormalities of the small intestinal mucosa in pernicious anemia—a prospective study. *Acta Hepatogastroenterol (Stuttg)* 1978;25:313–318.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. (a) PCR primers used to amplify fragments of AMN genomic DNA. (b) Heterozygous sequence variations in case 2 *AMN*.