Case:

Cobalamin deficiency in a collie cross bedlington terrier

(A case of Marmite deficiency?)

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Case presentation with additional findings:

Sapphie, a 1 year, 2 month old female entire grey collie cross bedlington terrier, presented to the referring veterinary practice with a history of intermittent lethargy, variable appetite and poor weight gain. Prior to presentation Sapphie had been off food for 24 hours. Significant findings on haematology included moderate neutropaenia (1.7 $x10^9$/L, reference interval; 2.5-12.5$x10^9$/L) and mild lymphopaenia (0.4$x10^9$/L, reference interval; 0.5-4.8$x10^9$/L). A two-week course of oral amoxicillin/clavulanic acid was prescribed. Appetite and demeanour improved briefly, but nine days later, clinical signs of listlessness, depression and wheezing were apparent. Injectable dexamethasone and amoxicillin/clavulanic acid were administered. Thoracic and upper airway radiographs, performed to rule out a tracheal or bronchial foreign body, were unremarkable. Due to concern over Sapphie's ongoing depression and poor body condition, referral was recommended.

On Day 1, at presentation to the Queen Mother Hospital for Animals (Royal Veterinary College), Sapphie was noted to be quiet but alert and underweight (9.9kg), with a body condition score of 3/9. Further significant clinical findings included pale mucous membranes, bradycardia (60bpm) and a grade II/VI systolic murmur over the left heart base. Differential diagnoses considered at this point included a portosystemic shunt (PSS) and hypoadrenocorticism.

Haematology and biochemistry results are shown in Tables 1 and 2. On Day 1 Sapphie had mild, normocytic, normochromic, non-regenerative anaemia, and a marked lymphopaenia. Significant blood smear findings included low numbers of hypersegmented neutrophils and rare to occasional nucleated red blood cells (Images 1 and 2). Serum biochemistry findings included a mild hyperbilirubinaemia, moderate hypocalcaemia, mild hypoglobulinaemia and mild hypokalaemia.

On Day 4, a moderate neutropaenia, moderate lymphopaenia, continued non-regenerative anaemia, mild hyperbilirubinaemia and a moderate increase in amylase and lipase activity was noted. Serum electrolyte concentrations, an ACTH stimulation test (to rule out hypoadrenocorticism) and bile acid stimulation test (to screen for a PSS) results were within reference intervals (WRI). Serum cobalamin concentration was below the limit of detection and serum folate concentration was mildly increased. Urinalysis results (Table 3) revealed a pH of 5, +2 heme protein, +1 proteinuria and a specific gravity

of 1.047. Urine protein:creatinine ratio was WRI. A urine sample was submitted to the Metabolic Genetic Screening Laboratory, University of Pennsylvania for a methylmalonic acid (MMA) spot test.

Daily injections of amoxicillin/clavulanic acid were commenced due to neutropaenia.

On Day 6, haematology results revealed a continued moderate neutropaenia and moderate lymphopaenia, with worsening non-regenerative anaemia (absolute reticulocyte concentration; 0/µL). Biochemistry demonstrated moderately increased plasma ammonium concentration and an increased trypsin-like immunoreactivity (TLI). Cobalamin measurement was repeated to confirm the initial result and concentrations were found again to be below the limits of detection.

Echocardiography revealed adequate systolic function and a mild increase in left aortic outflow velocity, which explained the murmur. Systolic blood pressure was WRI. On abdominal ultrasonography the liver was unremarkable, further ruling out a PSS, and a focus of inflammation, to explain the neutropaenia, was not found.

On Day 7, 0.4mg of cobalamin was administered subcutaneously (Vitbee 250, 0.025%w/v, Dechra Veterinary products) due to the repeated low cobalamin concentrations and a urine sample was submitted to the Metabolic Genetic Screening Laboratory, University of Pennsylvania for a methylmalonic acid (MMA) spot test.

Sapphie was discharged with the most likely differential diagnoses being cobalamin deficiency and/or cyclic haematopoiesis and an acute episode of pancreatitis. Amoxicillin/clavulanic acid was continued due to ongoing neutropaenia.

On Day 11, Sapphie was re-examined to assess the response to parenteral cobalamin. An improvement was noticed as Sapphie was now bright and alert and weighed 10.1kg. However, her mucous membranes were still pale. Neutrophil and monocyte concentrations were WRI, but a moderate lymphopaenia remained. HCT appeared to be improving with marked regeneration present (absolute reticulocyte concentration; 510,000/µL). Based upon her clinical improvement a course of four weekly injections of 50µg/kg cobalamin was prescribed.

On Day 15 results of the MMA spot test, taken on Day 4, revealed a severe methylmalonic aciduria which was attributed to a cobalamin malabsorption defect.

On Day 18, Sapphie appeared very bright. Haematological results revealed a resolution of the anaemia, but with a very mild neutropaenia and a mild lymphopaenia. Cobalamin concentration was now WRI.

On Day 21, RBC and neutrophil concentrations were WRI. There was a mild lymphopaenia. Due to the cyclical nature of fluctuations in neutrophil and monocyte concentrations 1ml of blood in an EDTA tube was submitted to Pinmoore Animal Laboratory, Tarpoley, Cheshire to detect a defect in the ELA-2 gene, which has been associated with canine cyclic haematopoiesis.

On Day 32, the CBC was unremarkable. Plasma ammonium concentration was found to be increased. It was discovered later that this was an unfasted sample. cPLI concentration, to assess the pancreatitis, was WRI.

Results of the cyclic neutropaenia screening test found Sapphie to be homozygous negative for a defect in ELA-2.

On Day 123, haematology parameters were WRI. Cobalamin concentrations were once again below detectable limits.

Table 1. Haematology results

Parameter	Day 1	Day 4	Day 6	Day 11	Day 18	Day 21	Day 32	Day 123	Reference Interval	Unit
RBC	5.16	5.10	4.17	4.73	5.87	6.25	6.25	6.53	5.50-8.50	$x10^{12}/L$
HGB	11.7	11.7	9.5	11.2	14.4	15.0	15.1	15.3	12.0-18.0	g/dL
HCT	0.35	0.34	0.28	0.33	0.42	0.47	0.44	0.44	0.37-0.55	
MCV	68.2	67.3	67.5	69.4	71.8	74.5	70.4	67.5	60.0-77.0	f/L
MCH	22.7	23.0	22.7	23.7	24.6	24.1	24.2	23.4	19.5-24.5	p/g
MCHC	33.2	34.1	33.6	34.1	34.2	32.3	34.3	34.7	31.0-37.0	g/dL
Platelets	276	302	163	>120 #	326	279	322	389	150-900	$x10^9/L$
WBC	4.7	2.4	3.4	6.9	3.9	4.3	8.4	11.2	6.0-17.1	$x10^9/L$
Neutrophils	4.6	1.3	2.0	5.9	2.7	3.1	6.6	5.9	3.0-11.5	$x10^9/L$
Lymphocytes	0.1	0.5	0.6	0.7	0.8	0.8	1.1	4.3	1.0-4.8	$x10^9/L$
Monocytes	0.10	0.70	0.80	0.30	0.30	0.20	0.70	0.70	0.15-1.0	$x10^9/L$
Eosinophils	0.0	0.0	0.0	0.0	3.0	0.2	0.1	0.3	0.0-1.3	$x10^9/L$
Reticulocytes	-	-	0	510,000	-	-	-	-	<60,000	/µL
Polychromasia	rare	neg	neg	+++	+	rare	rare	-		

Parenteral cobalamin administered on Day 7
(#) Moderate platelet clumping present

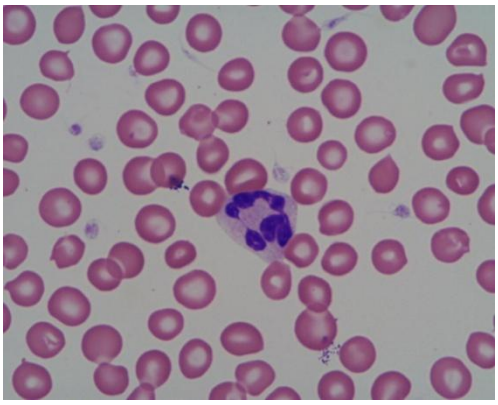

Image 1. Day 1 blood smear: hypersegmented neutrophil. 100x oil; Modified Wright's stain.

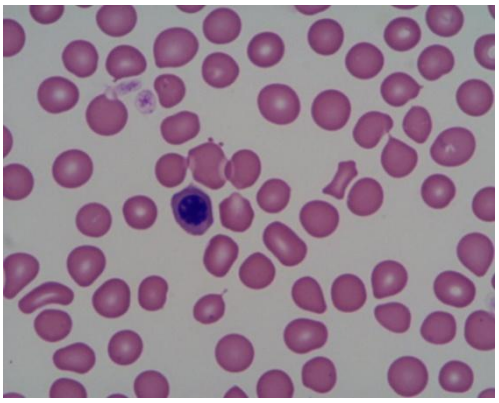

Image 2. Day 1 blood smear: polychromatophilic rubricyte. 100x oil; Modified Wright's stain.

Table 2. Biochemistry results								
Parameter	Day 1	Day 4	Day 6	Day 18	Day 32	Day 123	Reference interval	Unit
Total protein	55.7	63.1	-	-	-	61.2	49.0-71.0	g/L
Albumin	36.4	39.8	-	-	-	29.2	28.0-39.0	g/L
Globulin	19.3	23.3	-	-	-	32.0	21.0-41.0	g/L
Sodium	141	153	-	-	-	146	140-153	mmol/L
Potassium	3.6	4.4	-	-	-	4.4	4.1-5.3	mmol/L
Chloride	110	109	-	-	-	111	107-115	mmol/L
Calcium	1.57	2.60	-	-	-	2.62	2.13-2.70	mmol/L
Free calcium	0.94	-	-	-	-	-	1.13-1.33	mmol/L
Inorg. phosphorus	1.6	1.5	-	-	-	1.5	0.8-2.0	mmol/L
Urea	7.8	9.2	-	-	-	8.3	3.0-9.1	mmol/L
Creatinine	87	99	-	-	-	80	20-150	µmol/L
Cholesterol	4.5	5.5	-	-	-	6.6	3.3-8.9	mmol/L
Total Bilirubin	5.3	5.6	-	-	-	1.5	0.0-2.4	µmol/L
Amylase	1137	2826	-	-	-	-	176-1245	U/L
Lipase	902	1650	-	-	-	-	72-1115	U/L
ALT	17	23	-	-	-	23	13-88	U/L
CK	294	141	-	-	-	128	61-394	U/L
ALP	66	64	-	-	-	41	19-285	U/L
Bile acids pre pran	-	2.8	-	-	-	-	0.1-5.0	µmol/L
Bile acids post pran	-	3.3	-	-	-	-	0.1-5.0	µmol/L
ACTH pre	-	<27	-	-	-	-	<250	nmol/L
ACTH post	-	123	-	-	-	-	<500	nmol/L
TLI	-	-	>55	-	-	-	0-35	µg/L
cPLI	-	-	-	-	69	-	0-200	µg/L
Cobalamin	-	<150	<150	413	-	<150	>200	ng/L
Folate	-	15.0	14.0	13.3	-	8.8	7.1-14.4	µg/L
Ammonium	-	-	91	-	120	-	0-70	µmol/L
Urinary MMA	-	POS	-	-	-	-		
UPC	-	0.17	-	-	-	-	0.00-0.50	

Table 3. Urinalysis results (Bayer Labstix) Day 4	
Specific gravity	1.047
pH	5
nitrate	Negative
protein	1+
glucose	Negative
ketones	Negative
bilirubin	Negative
heme protein	2+

Discussion:

This report describes a 1 year, 2 month old collie cross bedlington terrier who presented for vague signs of lethargy, poor weight gain, variable appetite, non-regenerative anaemia and leukopaenia. The initial differential diagnoses considered were PSS, hypoadrenocorticism, cobalamin deficiency and/or cyclic haematopoiesis, and an acute episode of pancreatitis. Based on the findings of low serum cobalamin, increased plasma ammonium concentration, methylmalonic aciduria, certain haematological findings, age, breed association, and clinical response to parenteral cobalamin, a presumptive diagnosis of hereditary cobalamin deficiency was reached.

Cobalamin is a water-soluble vitamin synthesised by microorganisms and is essential for DNA, heme, and fatty acid synthesis [1-3]. It is involved in a number of metabolic processes such as cell growth, and peripheral and central nervous system function[4]. For carnivores and omnivores the nutritional requirement is met through ingestion of animal protein. Once ingested, cobalamin is released from food in the stomach and is then bound to a nonspecific cobalamin binding protein of salivary and gastric origin, termed haptocorrin. In the duodenum haptocorrin is degraded by pancreatic proteases and cobalamin is transferred from haptocorrin to a glycoprotein, termed intrinsic factor (IF), produced by the stomach and pancreas in dogs. The process is facilitated by the high affinity of cobalamin for IF at a neutral pH[5-6]. In the microvillus pits of the apical brush border of the ileal enterocytes the cobalamin-IF complex is internalised into the cell via endocytosis[7]. This is mediated by a specific multiligand apical membrane protein receptor on the cell surface termed the cubam receptor[8-9], comprised of the protein cubilin and amnionless. Once in the cell IF is degraded in enterocyte lysosomes and the cubam receptor is recycled back to the cell surface. Cobalamin is released into the cytosol of the cell and is then either escorted to its destination enzymes in the mitochondria and cytosol, or it is exported through the basolateral membrane where it binds to transcobalamin in the circulation[10].

Cobalamin acts as a cofactor for two main enzymes. The first enzyme is methionine synthase, involved in the conversion of homocysteine to methionine. Abnormalities in Sapphie's haematology may be related to decreased activity of this enzyme resulting in decreased methionine levels and increased levels of its precursor, homocysteine (not measured). This decreased activity results in a metabolic trap for folate, preventing the conversion of 5-methyl tetrahydrofolate to tetrahydrofolate, which would otherwise make folate accessible. The build up of 5-methyl tetrahydrofolate decreases the amount of essential folate co-factors, thus decreasing nucleic acid synthesis[3]. Dividing cells are arrested in the S phase of the cell cycle. Rapidly dividing cells of myeloid and erythroid lineage experience the most pronounced effects. Bone marrow samples were not obtained but usual findings include hypersegmented neutrophils, large band forms, giant metamyelocytes and reduced erythroid precursors[11]. Neutropaenia and hypersegmented neutrophils (Image 1) noted on Sapphie's blood smear examinations were attributed to cobalamin deficiency which has been known to cause dysmyelopoiesis[11]. Rare nucleated red blood cells were also noted in the absence of any polychromasia (Image 2). Rubricytes have been documented in cobalamin deficient dogs and have been interpreted as evidence of ineffective erythropoiesis. However, in this case, numbers were very low and may not have been of clinical significance. Cyclic haematopoiesis was considered a possible differential due to the cyclical fluctuations noted in neutrophil and monocyte concentrations, the non-regenerative anaemia and the breed of dog. A defect in neutrophil elastase (ELA-2) is one of the mutations suspected in dogs with cyclic haematopoiesis. ELA-2 encodes the neutrophil granule serine protease, neutrophil elastase, which has been implicated in the regulation of granulopoiesis. Sapphie was found to be negative for ELA-2 mutation (homozygous N/N) and responded well to cobalamin

therapy, hence cobalamin deficiency was considered the most likely cause of her haematological abnormalities. It is not entirely understood why the neutrophil concentrations increased on Day 11 but were low again on Day 18.

The second enzyme which cobalamin acts as a cofactor for is methylmalonyl-CoA mutase, involved in the conversion of methylmalonyl-CoA to succinyl CoA[12]. The increase in Sapphie's plasma ammonium concentration on Day 6 may be attributed to a decreased activity of methylmalonyl-CoA, resulting in increased precursor accumulation and methylmalonic acid production (MMA). This increase in MMA concentration was detected in Sapphie's urine. Increased MMA may indirectly impair the urea cycle by decreasing carbamoyl phosphate synthetase I (CPS I). CPS I is the rate limiting enzyme in the urea cycle catalysing the formation of carbamoyl phosphate[13]. This reaction requires magnesium adenine triphosphate and free Mg^{2+}, with N-acetylglutamate (NAG). Increased concentrations of MMA may inhibit NAG, thus indirectly inhibiting CSP I and thereby decreasing the amount of ammonium which can be processed in the urea cycle. Plasma ammonium concentrations were measured on 2 separate occasions with the second result being discounted as the dog had just eaten. Due to the vast improvement in clinical signs the owners declined further blood sampling, so it is unclear if the plasma ammonium concentrations returned to WRI. Urea cycle dysfunction due to cobalamin deficiency was considered the most likely reason for this biochemical abnormality.

Trypsin-like immunoreactivity (TLI) was initially measured to rule out exocrine pancreatic insufficiency. EPI may cause cobalamin deficiency due to reduced production of intrinsic factor (IF) from the pancreas, an essential step in its metabolism. However, TLI was greater than the upper reference interval, which may have been consistent with acute or chronic pancreatitis. Diagnostic sensitivity for pancreatitis has been reported to be about 33-50% for TLI[14]. Mildly elevated serum amylase and lipase concentrations were also noted. A canine specific pancreatic lipase (cPLI) measurement on Day 32 was WRI. Unfortunately cPLI was not measured earlier to confirm the suspicion of pancreatitis.

It is unclear why there was a moderate hypocalcaemia (both total and free) on Day 1. Sapphie did not receive any calcium supplementation, yet on Day 4, concentrations were WRI. Pancreatitis was considered a potential cause for hypocalcaemia, although the lack of direct association between changes in pancreatic markers and serum calcium concentration raised doubt for this differential. The pathogenesis by which pancreatitis causes hypocalcaemia is unknown. It may involve abnormal hormonal regulation, passage of calcium into damaged cells, binding of calcium to plasma fatty acids or to fatty acids in the peritoneum which have been liberated by the actions of escaped pancreatic lipase[15]. Another electrolyte change noted was a mild hypokalaemia which may have reflected decreased intake.

Bilirubin concentration was mildly increased in initial serum samples (Day 1 and 4). Cholestasis was considered an unlikely differential for this finding, based upon the lack of ultrasonographically detectable bile duct abnormalities, along with WRI ALP activity and serum bile acid concentration. Cobalamin deficient humans have been documented with increased serum bilirubin concentrations. A link between hyperhomocysteinaemia, haemolysis and subsequent hyperbilirubinaemia has been speculated. Homocystein may act as a haemotoxin facilitating the accumulation of reactive oxygen species. Alternativly it may cause a vasculitis, leading to microangiopathic damage to erythrocytes. Cobalamin deficiency and intramedullary haemolysis is a well reported phenomenon[16]. As hallmarks of oxidative or microangiopathic damage to erythrocytes were not encountered in blood smears from this patient, and without measurements of haptoglobin or homocystein concentrations a link between hyperhomocysteinaemia and hyperbilirubinaemia could not be documented.

Urinalysis revealed a mild proteinuria, without an increase in UPC, which likely reflected the presence of heme protein. Proteinuria has been reported in previous cases of cobalamin deficiency[17-19]. It is believed that absence of the cubam receptor complex in proximal renal tubular cells results in reduced reabsorption of albumin and other cubam ligands[9]. In the human literature it is reported that proteinuria often persists, even after treatment with cobalamin is initiated[20]. We have not been able to obtain subsequent urinalysis results to assess for persistent proteinuria.

Cobalamin deficiency may manifest as a result of gastrointestinal disease, small intestinal bacterial overgrowth or malabsortion due to intestinal inflammation or exocrine pancreatic insufficiency (EPI). Cobalamin deficiency may also be a primary inherited defect transmitted by an autosomal recessive mode in dogs and humans[21]. It has been described previously in an eight month old male neutered border collie[17], a 14 month-old border collie[20], giant schnauzers[19,21], and beagles[18]. A recent study on Chinese Shar Pei's with cobalamin deficiency demonstrated an association with microsatellite markers DTR13.6 and REN13N11, located on chromosome 13[4]. Although Sapphie did not demonstrate clinical signs of gastrointestinal disease or EPI, without intestinal biopsy an acquired cobalamin deficiency cannot be completely ruled out. However, given the dramatic improvement in body condition, demeanour and weight gain after therapy, an inborn error in the uptake or intracellular processing of cobalamin was considered more likely. It is unknown if other members of the litter have been affected.

Treatment for cobalamin deficiency is life-long parenteral cobalamin supplementation. With initiation of cobalamin supplementation (Day 7) an erythroid regenerative response developed (three days into therapy) and neutropaenia eventually resolved. Weight gain and improved body condition were noted early into therapy. Although on Day 123 Sapphie had a very low Cobalamin concentration, this is not an unusual finding. Cobalamin is taken up by tissues from the plasma and bound to enzymes with a long half-life, allowing it to remain active even when, as in Sapphie's case, cobalamin concentrations are below the limits of detection[18]. Similar findings of subnormal cobalamin concentrations have been reported previously in cobalamin deficient dogs on long term supplementation[17]. The prognosis for inherited cobalamin deficiency is very good and Sapphie continues to do well on weekly injections of cobalamin.

In conclusion the common features that Sapphie shared with previously reported dogs suffering from inherited cobalamin deficiency included:

- presenting clinical signs of juvenile age of onset, poor weight gain, lethargy
- haematological and biochemical changes being neutropaenia, non-regenerative anaemia, hyperammonaemia, cobalamin concentrations below detectable limits, methylmalonic aciduria
- rapid response to treatment with parenteral cobalamin.

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