L-2 hydroxyglutaric aciduria in a South African Staffordshire Bull Terrier

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L-2 hydroxyglutaric aciduria is an autosomal recessive error of metabolism that manifests as an encephalopathy. The most common presenting signs are seizures, tremors, ataxia and/or dementia. Some affected dogs show only subtle behavioural changes. Amongst canines, the condition has been best described in Staffordshire Bull Terriers. Although this is the first reported case in South Africa, at least three other affected dogs have been indentified by polmerase chain reaction (PCR) in this country. Affected dogs have normal haematology, serum biochemistry and routine urine analysis. This report discusses the advantages and limitations of the three main diagnostic modalities, namely: magnetic resonance imaging, urine gas chromatography-mass spectrometry and genetic testing. The aim of this report is to increase awareness of the condition, assist diagnosis in encephalopathic dogs and improve detection of carriers amongst breeding stock.

Introduction

L-2 hydroxyglutaric aciduria (L2-HGAU) was first reported in humans in 1980 (Duran *et al.* 1980). To date, case reports on well over 100 patients have been published (Kranendijk *et al.* 2012; Steenweg *et al.* 2010). In 2003, Abramson and others described six-affected Staffordshire Bull Terriers (SBT) in the United Kingdom (Abramson *et al.* 2003). Since then, L2-HGAU has also been documented in three Yorkshire Terriers (YRT) (Farias *et al.* 2012; Sanchez-Masian *et al.* 2012) and one West Highland White Terrier (Garosi *et al.* 2005). The causative mutations in YRT differ from those in SBT (Farias *et al.* 2012; Penderis *et al.* 2007; Sanchez-Masian *et al.* 2012). Whilst all tested SBT have had the same mutation in the L2 hydroxyglutarate dehydrogenase gene (*L2-HGDH*) (Penderis *et al.* 2007), over 80 different mutations of *L2-HGDH* have been demonstrated in affected humans (Kranendijk *et al.* 2012; Steenweg *et al.* 2010).

Case history

A 5-year-old, 18.3 kg, female, neutered SBT was referred to the first author for further investigation of an episode of bizarre behaviour or dementia.

In retrospect, her owners had observed mild ataxia for 11 months prior to presentation; she appeared somewhat unsteady for a few seconds when she woke up, occasionally banged into walls on cornering and at times had difficulty climbing steps. Signs would wax and wane and were too subtle to prompt consultation with a veterinarian.

The first episode of bizarre behaviour occurred nine months prior to presentation, lasted for a day and resolved without treatment. The dog appeared disorientated and aggressive, was pacing and restless, panting and hyperactive. She did not respond to commands where she was normally biddable.

The episode that prompted the investigation lasted for a week. Initially, she became unusually aggressive toward the neighbour's dog and attacked the garbage bins. Signs gradually worsened and two days later she had stopped responding to commands, spent all night pacing and appeared disorientated with impaired spatial perception and balance. She urinated indoors and showed no awareness of the fact that this had happened. On the third symptomatic day she was presented to her usual veterinarian. She was treated with alprazolam (1.75 mg bid) (Adco-Alzem, Adcock Ingram Pharmaceuticals, Bryanston, South Africa), which caused transient sedation, after which the above behaviour resumed. The next day procaine benzylpenicillin with dihydrostreptomycin (dose not recorded) (Depomycin, Intervet-Schering Plough, Isando, South Africa), alprazolam, dexamethazone (dose not recorded) (Dexa 0.2 Phenix, Virbac, Halfway House, South Africa) and phenobarbitone (30 mg bid) (Lethyl, Aspen Pharmacare, Gallo Manor, South Africa) were administered. Her owners reported that she had appeared calmer and had slept for the first time in two days following these treatments. She was referred to a specialist the following day, which was Day 5 of this episode.

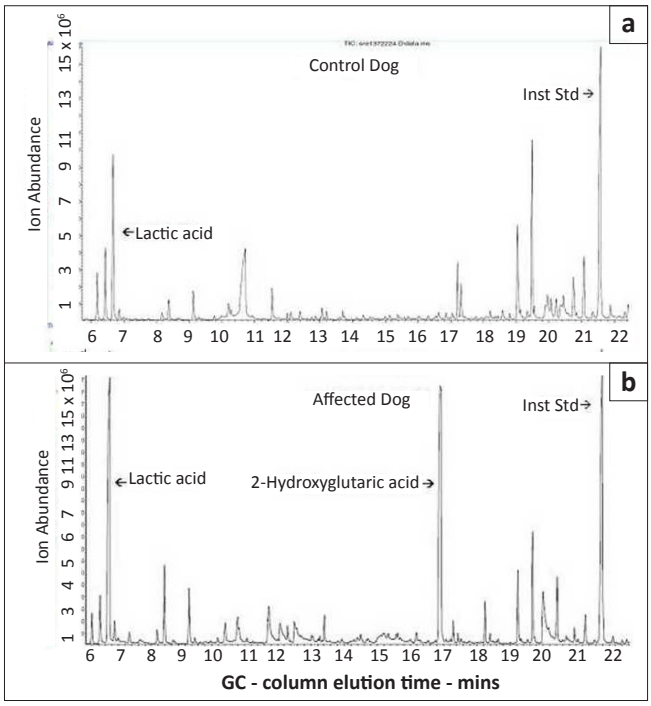
On clinical examination the dog appeared disorientated. She was staring into space, becoming fixated on and staring at objects (e.g. a lead), pacing, circling, and walking into a wall. A complete clinical and neurological examination revealed no further abnormalities.

Results of haematology, serum biochemistry, electrolytes, random cortisol, basal ammonia and a bile acid stimulation test were all within normal reference ranges. Urine analysis revealed only hyposthenuria (SG 1.005).

Anaesthesia was induced with intravenous propofol at 3.33 mg/kg (Propofol 1.0% Fresenius, Fresenius Kabi, Halfway House, South Africa) and was maintained with a constant rate infusion of propofol 0.20 mg/kg/min in saline (5 mL/kg/min) (Sabax Sodium Chloride 0.9%, Adcock Ingram, Johannesburg, South Africa). The following MRI sequences were acquired using an eight-channel head array coil in a GE 1.5T Sigma Excite HD MRI scanner: transverse T2, transverse T2 FLAIR, sagittal T1, sagittal T2 and coronal T2 FLAIR and axial T1. Further sagittal and transverse T1 sequences were acquired after intravenous administration of 1.8 mmol gadolinium (Dotarem, Gd-DOTA 27.93% m/v, 0.5 mmol/mL, Guerbet, Villepinte, France). The MRI was interpreted by one of the co-authors (SB). A subtle bilateral symmetrical T2 hyperintensity was evident, involving the regions of the basal ganglia, central and posterior tectum. No contrast enhancement was evident and no other abnormalities were reported. MRI changes were consistent with a metabolic or toxic encephalopathy.

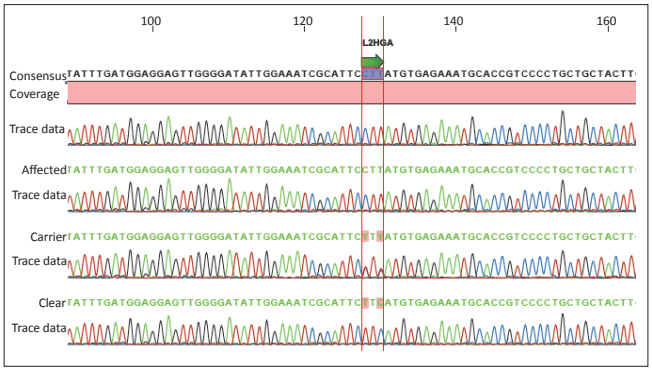
A single free flow urine sample was collected from the patient and submitted to the UCT/NHLS laboratory (Red Cross Children's Hospital, Cape Town) for organic acid analysis by gas chromatography–mass spectrometry (GC-MS) as previously described (Van der Watt *et al.* 2010). Urine from a clinically normal five-year-old female entire Doberman bitch was used as a control. The organic acid spectrum from the patient revealed significant lactic aciduria and a large 2-hydroxyglutaric acid component (quantified at 360 mmol/mol creatinine). 2-Hydroxyglutaric acid was not detected in the control canine urine (Figure 1). The patient's urine organic acid profile was highly suggestive of L2-HGAU, as previously reported in SBTs (Abramson *et al.* 2003).

EDTA blood was submitted to Inqaba Biotec (Hatfield, Pretoria, South Africa) to determine whether the causative mutation was present. DNA was extracted and followed with a polymerase chain reaction (PCR) to amplify the gene of interest. The causative mutation is known to be in exon 10 of chromosome 8 in SBT and consists of two single-nucleotide substitutions separated by a single T nucleotide (c[1297TRC; 1299cRt]). This results in the substitution of two adjacent amino acids: proline replaces leucine at position 433 and tyrosine replaces histidine at position 434 (Penderis *et al.* 2007). An ABI PRISM™ 3130 Genetic Analyzer was used to determine the nucleotide sequences in the amplicons. The dog was shown to be homozygous for the dicodon mutation in L2-*HGDH* (Figure 2).



X-axis, gas chromatography column compound elution times; Y-axis, Fragment ion abundancee in arbitrary units
Note: The scales on the Y-axis differ.

FIGURE 1: Aligned GC-MS spectra of urine organic acids from (a) a control dog and (b) the Staffordshire Bull Terrier investigated for 2-hydroxyglutaric aciduria.



Note: This chromatogram shows the nucleotide sequence of the *L2-HGDH* gene, the gene which when mutated results in clinical signs of L2-HGAU. The consensus sequence (top row) is the nucleotide sequence of the sample. The trace data show the actual nucleotides that were detected in this sample. Below the trace data line, three reference samples were sequenced, namely: a dog that was diagnosed as affected by L2-HGA, a heterozygote and a clear or normal dog. The two vertical red lines indicate the mutations. A carrier has two nucleotides at both mutation positions indicated with a 'Y' at both positions. This figure shows that the gene sequence of the sample tested corresponded to the sequence of the reference sample from an affected dog.

FIGURE 2: Chromatogram of the nucleotide sequences.

The patient was maintained on low doses of phenobarbitone (30 mg in the morning and 45 mg in the evening; serum phenobarbitone 49.8 µmol/L – therapeutic range 43 µmol/L –172 µmol/L) until she was relocated with her owners. On the three occasions when the first author observed the patient in the three months after her initial diagnosis she still appeared mildly dazed. The dose was reduced to 15 mg in the morning and 30 mg in the evening at some stage, at the recommendation of another veterinarian. Supplementation with 100 mg riboflavin daily started immediately after diagnosis. Her owners considered her back to normal and no further severe episodes were observed for the remainder of her life. She was treated with both medications until she

ruptured bilateral cruciate ligaments, prompting euthanasia 16 months after diagnosis.

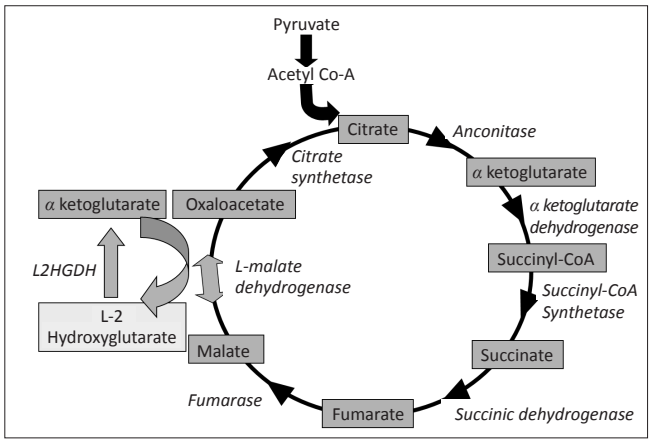
Discussion

This is the first report of L2-HGAU in SBT in South Africa. Samples from 200 South African SBT were submitted to Inqaba Biotec, a commercial laboratory, for determination of the dogs' *L2-HGDH* status between 2009 and 2012. The laboratory detected 46 heterozygotes (23%) and four (2%) affected dogs (H. van der Zwan [Inqaba Biotec] pers. comm., 04 Dec. 2012). This confirms that the mutation is present in the South African SBT population and dogs showing clinical signs of L2-HGAU are likely to be presented periodically to veterinarians.

A study of 130 normal UK SBT and 131 normal Finnish SBT selected from two DNA archives detected *L2-HGDH* heterozygotes in 11% of both populations (Short *et al.* 2010). The same authors searched for the SBT *L2-HGDH* mutations in residual blood samples submitted for routine phenobarbitone monitoring from over 1000 epileptic dogs of other breeds and found no carrier dogs (Short *et al.* 2010). Thus, the mutations described by Penderis *et al.* (2007) are either specific to the SBT breed or occur at a very low frequency in epileptic dogs of other breeds. The higher proportion of carriers and affected dogs recorded by Inqaba Biotec is expected because their data merely reflect the incidence of positive samples submitted to a commercial laboratory and do not represent a random sampling of the South African SBT population. Staffordshire Bull Terrier breeders who had become aware of L2-HGAU and were screening their breeding stock submitted most samples. It is not known what proportion of the sampled SBTs was symptomatic or had known, affected relatives. Carrier and affected dogs identified by the laboratory originated from different breeding lines and lived in kennels dispersed throughout South Africa.

L2-HGAU has been called a deficiency of 'metabolic repair'. L2-HGA is produced because the mitochondrial enzyme L-malate dehydrogenase is not specific for its primary substrate, oxaloacetate, but is also able to reduce α-ketoglutarate to L2-hydroxyglutaric acid (L2-HGA). L2-HGA has no known metabolic function. It is converted back to α-ketoglutarate by L2-hydroxyglutaric acid dehydrogenase (L2-HGDH). Thus, L2-HGDH ensures that the functionless L2-HGA does not accumulate in the cells of normal animals, in other words, it ensures metabolic repair (Rzem *et al.* 2004) (Figure 3).

When a mutated *L2-HGDH* results in a non-functional enzyme, L2-HGA accumulates. Markedly increased L2-HGA levels can be measured in CSF, plasma and urine in affected individuals (Rzem *et al.* 2004; Steenweg *et al.* 2010) but the authors are not aware of any publication proving accumulation of this substance in astrocytes or any other neurons. *L2-HGDH* is most active in the brain (Sanchez-Masian *et al.* 2012); this may explain why neurological signs predominate in affected animals. L2-HGA has been shown to



Note: The citric acid cycle (Krebs cycle, tricarboxylic acid cycle) is part of aerobic respiration. Acetyl-CoA enters the mitochondrion and is converted to water and carbon dioxide, whilst NAD^+ is reduced to NADH. NADH enters the oxidative phosphorylation pathway to produce ATP and thereby energy that can be used in other metabolic processes. L-malate dehydrogenase converts malate to oxaloacetate. The reaction is reversible. In fact, in isolation, it favours malate production (malate can leave the mitochondrion, be converted back to oxaloacetate in the cytoplasm, which can then enter gluconeogenesis). L-malate dehydrogenase is not specific to malate and oxaloacetate: it can also convert α- ketoglutarate to L-2 hydroxyglutaric acid (L2-HGA). L2-hydroxyglutarate dehydrogenase (L2-HGDH) is bound to mitochondrial membranes (Rzem *et al.* 2004) and converts this molecule that has no known biological function back to α - ketoglutarate. In patients with a genetic mutation that causes a malfunctioning L2-HGDH, L2-HGA accumulates in cells, which eventually disrupts metabolic pathways and causes clinical signs.

FIGURE 3: Citric acid cycle and L2-hydroxyglutraic aciduria.

cause oxidative stress – it inhibits mitochondrial creatinine kinase in the cerebellum and may also inhibit glutamate-dependent pathways, as it is a structural analogue of this common neurotransmitter substance (Rzem *et al.* 2004). It is this oxidative stress that was thought to be responsible for the astrocyte vacuolation observed on histopathological sections of an affected dog's brain (Scurrell *et al.* 2008).

Affected humans usually show clinical signs before they are seven years old (Kranendijk *et al.* 2012) but, as signs may be subtle and/or non-specific, diagnosis may be delayed as in this case (Abramson *et al.* 2003; Patay *et al.* 2012; Weimar *et al.* 2012). Clinical signs shown by both species include ataxia, dementia and seizures. In dogs, signs of dementia may include head pressing, getting trapped in corners, hyperactivity, aggression, lethargy or attention seeking (Farias *et al.* 2012). Dogs show behavioural changes and/or loss of learnt behaviour, whilst behavioural problems are reported in 32% and psychomotor delay in 93% of humans. Disorientation, hypermetria and tremors are reported in dogs, whilst 82% of humans specifically show cerebellar ataxia with intention tremors (Steenweg *et al.* 2010). Thirty-eight percent of people also show extrapyramidal signs, such as involuntary movements, inability to initiate movement, tremor and muscle spasm. Owners have observed muscle stiffness and fatigue with exercise in affected dogs, whilst 40% of human patients show hypotonia progressing to spasticity (Abramson *et al.* 2003). Some dogs show periodic exacerbation of clinical signs that may last for minutes to days. In contrast, clinical signs are slowly progressive in humans and periodic exacerbations are not commonly reported. Many dogs will show only one or two of the above signs and some may be so subtle that the owner considers the dog to be normal (Abramson *et al.* 2003). In both species

the extent of the neurological compromise does not appear to be related directly to the severity of the aciduria (Abramson *et al.* 2003). In humans, it has not been possible to associate different mutations with different clinical manifestations (Steenweg *et al.* 2010).

On MRI, both species show T2 hyperintensity in peripheral subcortical white matter as well as in grey matter of the thalamus, basal ganglia, globus pallidus, caudate and dentate nuclei (Penderis *et al.* 2007; Steenweg *et al.* 2010). Diffuse oedema, manifesting as a subtle T1 hypointensity without contrast enhancement, is sometimes reported (Abramson *et al.* 2003). In addition, humans may show cerebellar atrophy, macrocephaly and may also have an increased risk of brain tumours (Aghili, Zahedi & Rafiee 2009; Kranendijk *et al.* 2012). To the authors' knowledge, none of these have been reported in dogs. Because the extensive grey matter changes in dogs are usually symmetrical, inexperienced viewers may overlook them. In addition, other breed-associated polioencephalopathies may have similar MRI lesions (Abramson *et al.* 2003). Referral to a veterinary diagnostic imaging specialist is thus advised.

A full *post mortem* study of an affected SBT found no macroscopic changes. Histopathological changes were restricted to the brain. There were marked spongiform changes in the astrocytes of the cerebral cortex, thalamus, cerebellum and brainstem. Grey matter was most severely affected (Scurrell *et al.* 2008). In humans, spongiform changes are most severe in the subcortical white matter (Penderis *et al.* 2007). Thus, histopathological changes correspond to those found on MRI. It is not clear why the distribution of the most severe lesions differs between humans and dogs (Abramson *et al.* 2003).

Routine haematology, serum biochemistry and in-house urine analysis are typically normal (Scurrell *et al.* 2008) and the hyposthenuria in this patient was probably a side effect of phenobarbitone treatment.

Routine GC-MS detects the presence of 2-hydroxyglutaric acid in the urine, but does not determine chirality. L2-HGAU must be differentiated from D-2 hydroxyglutaric aciduria (D2-HGAU), isolated cases of which have been reported in dogs (Abramson *et al.* 2003). In humans, two types of congenital D2-HGAU, as well as D,L-2 hydroxyglutaric aciduria, have been described (Kranendijk *et al.* 2012). To complicate matters, in humans, D2-HGAU has been associated with other errors of metabolism, for example, a skeletal dysplasia resulting in metaphyseal chondromatosis and multiple Acyl-CoA dehydrogenase deficiency. Lastly, it has been observed as an acquired mutation in patients with glioma and acute myeloid leukaemia (Kranendijk *et al.* 2012). There is no reason why these errors of metabolism would not occur in dogs, thus all of these should be considered as a differential diagnosis for a dog with high 2-hydroxyglutaric acid levels in the urine. In humans, clinical signs and MRI changes may often distinguish these conditions, but confirmation of chirality and genetic testing are necessary for a complete work-up (Kranendijk *et al.* 2012). Urine GC-MS would be an appropriate screening tool for L2-HGAU in encephalopathic dogs of breeds other than SBT. Dogs with a 2-hydroxyglutaric acid spike in their urine may not have L2-HGAU, but the disease can be excluded in dogs without one.

Elevated lysine in the CSF, plasma and urine has been noted in some humans and dogs with L2-HGAU, whilst some had elevated urine cysteine and/or arginine (Abramson *et al.* 2003). As high urine lysine decreases absorption of cysteine, ornithine and arginine in the proximal convoluted tubules, increased loss of these four amino acids may be interrelated (Abramson *et al.* 2003). Urine or plasma amino acid levels were not quantified in this case, but are of value in detecting other metabolic encephalopathies, for example, multiple Acyl-CoA dehydrogenase deficiency and maple syrup urine disease (MSUD). Elevated urine lactate has been reported in one person with L2-HGAU (Barth *et al.* 1998).

More than 80 different mutations of *L2-HGDH* that can result in a non-functional enzyme and clinical signs of L2-HGAU have been documented in humans (Kranendijk *et al.* 2012). Such genetic heterogeneity should be anticipated amongst dogs. Indeed, a different *L2-HGDH* mutation has already been described in YRT (Farias *et al.* 2012). Genetic screening is by its nature specific to a known mutation and cannot be used to exclude the disease. It is, however, the only way in which carriers can be detected. Genetic screening and GC-MS thus have complimentary roles in the diagnosis of L2-HGAU.

L2-HGAU is incompletely understood and there is no specific treatment for dogs or humans at present (Penderis *et al.* 2007). Humans with better-characterised organic acidurias are treated with dietary protein restriction and appropriate amino acid, mineral and vitamin supplementation (Kolker *et al.* 2012; Penderis *et al.* 2007; Van der Watt *et al.* 2010). The efficacy of such supplementation of precursors is likely to vary depending on the nature of the underlying mutation as well as whether any normal enzyme is present in the particular patient. Supplementation with 100 mg riboflavin once daily improved neurological function and markedly decreased L2-HGA secretion in the urine of two of three children with L2-HGAU in whom its use is reported (Kranendijk *et al.* 2012; Yilmaz 2009). Riboflavin (Vitamin B2) is bound to an ADP molecule to form flavin adenine dinucleotide (FAD). Flavin adenine dinucleotide is a co-factor for L2-HGDH (Yilmaz 2009). A diet supplemented with FAD and levocarnitine chloride improved clinical signs in another human (Samuraki *et al.* 2008). A lysine-restricted diet was offered to one dog with concurrently elevated plasma lysine levels, but the patient refused to eat it (Farias *et al.* 2012). Such a diet was not tested in this SBT, as her lysine status was not known. In dogs with seizures, phenobarbitone at 3 mg/kg appears effective at controlling clinical signs (Abramson *et al.* 2003; Sanchez-Masian *et al.* 2012). In the SBT described here, it is believed that the phenobarbitone acted as a sedative during the period of acute exacerbation that prompted

investigation. The dose was reduced to 15 mg in the morning and 30 mg in the evening, but was not discontinued in the hope that it might suppress another period of dementia.

The therapeutic effect of either of the interventions used in this case is difficult to assess in this dog, as her severe manifestations were episodic and uncommon, she only lived for 16 months post diagnosis and she was never off medication again. This case does confirm previous observations that some mildly affected individuals may remain acceptable pets despite the limited scope of current treatment options.

Conclusion

To the authors' knowledge, this is the first report of L2-HGAU in a South African SBT. The aim of this report is to raise awareness of this condition in the SBT breed in South Africa for two reasons. The first is so that colleagues can encourage breeders to screen breeding stock prior to mating. At least 46 carrier dogs have already been identified in South Africa and 11% of two overseas convenience samples of clinically normal SBT were carriers. Heterozygotes are asymptomatic but unwittingly mating two carriers is likely to result in clinically affected pups. Some affected dogs have clinical signs that are vague and may even be considered a cute quirk or 'just a Staffie thing' by some owners. Nevertheless, even mildly affected individuals should not be bred because all offspring will at least be carriers and subsequent homozygous offspring could be a great deal more compromised. The second reason is so that colleagues consider genetic screening on SBT showing consistent neurological signs, as this potentially avoids having to do more invasive or expensive diagnostic testing. There may be further mutations causing L2-HGAU in dogs, just as there are over 80 described in humans (Kranendijk *et al*. 2012). For this reason, urine organic acids should be determined in dogs showing consistent clinical signs or MRI changes but whose genetic test suggests they are unaffected.

Acknowledgement

Competing interests

The authors declare that they have no financial or personal relationship(s) which may have inappropriately influenced them in writing this article.

Authors' contributions

M.B. (King Edward Veterinary Referral Hospital) was the primary clinician responsible for looking after the patient, coordinating work-up, writing the main body of the article, performing the literature search and coordinating everyone else. H.H. (UCT/NHLS Department of Chemical Pathology) performed GC-MS on urine, wrote the section on the 2-hydroxyglutaric acid analysis of the urine for the article, performed an extensive review of the article and added further references pertinent to the GC-MS. H.v.d.Z. (Inqaba biotechnical industries [Pty] Ltd.) performed the PCR, supplied data on other Staffordshire Bull Terriers tested, wrote the section on the PCR analysis for the article and reviewed the remainder of the article. S.B. (Drs Visser, Erasmus, Vawda & Partners) reviewed the MRI, checked that the imaging findings were correctly reported in the article and reviewed the remainder of the article.

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