# Transient erythropoietic protoporphyria associated with chronic hepatitis and cirrhosis in a cohort of German shepherd dogs

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**Over the course of one year, slight jaundice and ascites suggestive of chronic liver disease occurred in 17 German shepherd dogs from one breeding colony. Blood analyses, performed twice with a six-month interval, revealed elevated serum activities of liver enzymes in 13 dogs. In addition, four young adult German shepherd dogs that showed severe ascites, slight jaundice and increased serum liver enzyme activities were referred for further evaluation. Because of their poor prognosis these four dogs were euthanased. There were no signs of photosensitivity. Postmortem examinations revealed macronodular darkened livers, which were characterised histopathologically by cirrhosis associated with aggregates of brown pigments showing a striking orange birefringence in polarised light. Ultrastructurally, the crystalline pigments were typical of protoporphyrins. High-performance liquid chromatographic analysis of liver samples revealed very high levels of protoporphyrins (mean 9550 nmol/g wet liver, reference value 0·41 nmol/g wet liver) and low activities of ferrochelatase (mean 0·274 mmol/mg protein/hour, reference value 0·684 nmol/mg protein/hour). Twenty-six months after the onset of the hepatopathies, the clinical condition of the 13 surviving dogs had improved and their serum liver enzyme activities were normal. The clinical histories and pedigree analyses were not in concordance with an inherited form of protoporphyria. There was no known history of exposure to toxic substances or drugs. The findings are in accordance with a transient erythropoietic protoporphyria associated with hepatic complications, presumably caused by exposure to a porphyrinogenic, ferrochelatase-inhibitory substance of unknown origin.**

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PORPHYRIAS are diseases caused by marked deficiencies of enzymes of the haem biosynthetic pathway; they may be either inherited or acquired. Haem is an essential component of haemoglobin, myoglobin and the cytochromes of the mitochondrial respiratory chain. Eight enzymes are involved in the precisely regulated pathway of haem synthesis, in which haem represses, via negative feedback regulation, the regulatory first level enzyme δ-aminolevulinic acid synthase (ALAS) (Moore and others 1987, Sassa and Kappas 2000, Anderson and others 2001). Deficiencies in one or more of the eight enzymes of the haem synthesis pathway contribute to the various forms of porphyria and the accumulations of toxic haem precursors in tissues (Sassa and Kappas 2000). The mitochondrial enzyme ferrochelatase (FeC, protoheme ferrolyase) catalyses the final and rate-dependent step in the pathway by the insertion of ferrous iron ($Fe^{2+}$) into the protoporphyrin-IX molecule to form haem. In erythropoietic protoporphyria, a deficiency of FeC causes accumulations of toxic protoporphyrin-IX in various organs, especially the liver (Sassa and Kappas 2000, Anderson and others 2001).

In vitro and in vivo models show that heavy metals such as lead, cadmium, mercury and several other xenobiotics like heptachlor inhibit FeC directly (Dailey 1987, Jacobs and others 1998, Taira and San Martin de Viale 1998, Bhasin and others 2002). Indirect porphyrinogenic substances including dihydrophyridines, dihydroquinolines, sydnones and the widely used fungicide griseofulvin are dependent on the inactivation of hepatic cytochrome-P450 (c-P450) isozymes. In this reaction, the haem moieties of c-P450 isozymes undergo N-alkylation, leading to the formation of potent FeC-inhibitory N-alkyl-protoporphyrin-IX, such as methyl-, ethyl-, and vinylprotoporphyrin (Stejskal and others 1975, De Matteis and others 1980). Because FeC is the rate-controlling enzyme in the synthesis of haem, accumulations of protoporphyrin-IX can result from c-P450 isozyme-inducing drugs, such as dexamethasone, phenobarbital (Kimmet and others 1996) and sulphonamides (De Matteis 1974), as a result of overstimulation of haem synthesis. Secondary inhibition of FeC occurs in cases of iron depletion after treatment or intoxication with $Fe^{2+}$ chelating agents (Smith and others 1997).

Different traits of inheritance in human porphyrias have been well established (Sassa and Kappas 2000). In human beings, erythropoietic protoporphyria (EPP) is characterised by an autosomal dominant coinheritance of a FeC gene mutation combined with a low expression of the normal FeC allele (Gouya and others 2004). Various genetic mutations are known to be expressed as reductions in FeC activity (Rufenacht and others 1998). In Limousin cattle, an autosomal recessive inherited form of EPP occurs that is believed to be due to a point gene mutation that encodes a minor change in the structure of FeC (Bloomer and others 1982, 1987, Jenkins and others 1998, Pence and Liggett 2002).

The hepatotoxic capacities of protoporphyrin-IX are attributed to its cytotoxicity to hepatocytes and biliary epithelium, initiating cholestatic liver disease and cirrhosis (Meerman and others 1999). Comparable hepatobiliary lesions result from either inherited or induced forms of EPP. The end-stage protoporphyric liver is characterised by dark brown to black discoloration and cirrhosis; the protoporphyrin-IX pigments show a bright orange birefringence, often displayed in a typical 'Maltese cross' when examined under polarised light (Klatskin and Bloomer 1974, Troyer and others 1991). Ultrastructural investigations of hepatic tissue samples in EPP reveal liver cell damage and crystalline deposits typical of porphyrins (Komatsu and others 2000), and the biochemical abnormalities include increased liver and faecal concentrations of protoporphyrin-IX and low blood activities of FeC (Hindmarsh and others 1999). Porphyrins can be excited by light and generate free radicals and singlet oxygen

**TABLE 1: Mean (sd) serum liver enzymes, total bilirubin and bile acids in German shepherd dogs with erythropoietic protoporphyria at the onset of the clinical signs, six months later and 26 months later**

| Measurements | Onset of liver disease Dogs 1 to 4 | Onset of liver disease Dogs 5 to 16 | Six months (dogs 6 to 17) | 26 months (dogs 5 to 17) |
|---|---|---|---|---|
| Alkaline phosphatase (U/l) | 324 (199) (<190)* | 199·6 (106·0) (<190)* | 437 (337) (<190)* | 19·2 (6·9) (15-69)† |
| Alanine aminotransferase (U/l) | 267 (117) (<50)* | 237 (193) (<50)* | 367 (148) (<50)* | 59·1 (24·5) (23-90)† |
| Glutamate dehydrogenase (U/l) | 7·3 (3·5) (<7)* | 5·6 (3·8) (<7)* | 13·0 (11·5) (<7)* | |
| Aspartate aminotransferase (U/l) | | | | 28·3 (8·5) (10-29)† |
| Total bilirubin (µmol/l) | 5·1 (1·7) (0-10·3)* | 5·1 (1·7) (0-10·3)* | 6·8 (3·4) (0-10·3)* | |
| Bile acids (µmol/l) | | | | 3·1 (1·6) (0-8)† |

* Reference ranges: Clinic for Companion Animals, School of Veterinary Medicine Hannover, Germany
† Reference ranges: Department of Clinical Science of Companion Animals, Utrecht University, The Netherlands

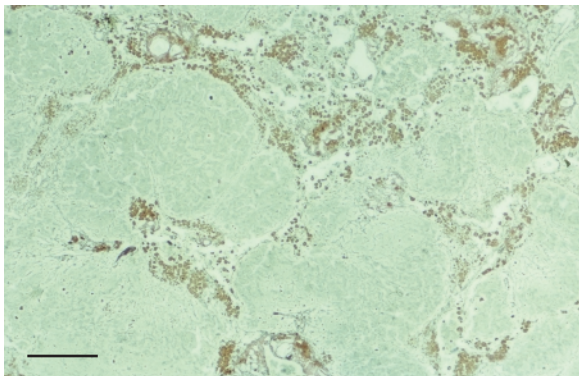

**FIG 1: Liver from a dog with erythropoietic protoporphyria, showing abnormal hepatic architecture consisting of regenerative hyperplastic parenchymal nodules surrounded by portoportal and portocentral fibrotic septa containing large amounts of dark brown pigment-laden macrophages. Reticulin stain. Bar=500 µm**

molecules, which may incite cytotoxic lipid peroxidation and cross-linking of membrane proteins. These changes account for the photodynamic lesions of unpigmented skin that are frequently observed in porphyria (Goldstein and Harber 1972).

EPP must be distinguished from the congenital form of the disease, which is caused by an inherited defect of uroporphyrinogen-III-cosynthase. It has been described in cattle (Rimington 1936, Opsomer and de Kruif 1991) and rarely in pigs and domestic cats (Giddens and others 1975, Roels and others 1995). Dermal photosensitisation, which is practically exclusively observed in cattle, and brownish discolorations of bone, teeth and urine associated with toxic accumulations of uroporphyrinogen-I and coproporphyrinogen-I exemplify the congenital form of the disease.

This paper describes a transient spontaneous form of EPP in a cohort of German shepherd dogs that developed hepatobiliary lesions and typical morphological and biochemical changes.

## MATERIALS AND METHODS

The 17 dogs were housed individually in kennels and allowed separate access to a fenced outdoor area with ample shaded areas for no more than 30 minutes a day. They were fed a commercial complete dog food with fresh water ad libitum. No toxic substances had been recorded in the dogs' accommodation and the dogs were not known to have received any pharmaceutical treatments. All the dogs were affected in the course of a year and four of them (1 to 4) were euthanased because of their poor prognosis. Blood samples from these four were analysed for liver enzymes, total bilirubin and bile acids, and tissue samples were taken for histopathology and electron microscopy. In the less severely affected dogs, blood samples were taken twice, at the onset of the clinical signs (from dogs 5 to 16) and six months later (from dogs 6 to 17): follow-up blood samples were taken 26 months after the onset of the liver disease from all 13 surviving dogs.

Dogs 1 to 4 were examined postmortem macroscopically and histopathologically. Liver samples were fixed in 10 per cent neutral buffered formalin and embedded in paraffin; 4 µm thick sections were cut and stained with haematoxylin and eosin, van Giesson's stain, Gordon-Sweet silver staining for reticulin, Perl's Prussian blue for iron and rubeanic acid for copper.

Fresh liver biopsies from dogs 1 and 2 were frozen in liquid nitrogen and analysed for free protoporphyrin-IX and the activity of FeC by high-performance liquid chromatography (HPLC) with fluorescence detection, described respectively by Bailey and Needman (1986) and Li and others (1987). Reference values for these components were determined by the same methods in liver biopsies taken from eight young adult healthy beagle dogs (four males and four females) with no relationship to the 17 dogs from the cohort.

For ultrastructural examination, 10 µm thick sections were cut from the formalin-fixed, paraffin-embedded liver samples and mounted on glass slides. After routine processing for electron microscopy, a Beem-embedding capsule size 3 (EMS) was fixed on the glass-mounted tissue samples with Durcupan-epoxy resin (Sigma-Aldrich), which was polymerised and hardened overnight. The glass slides were then detached from the Beem capsule-embedded tissue by submerging them in liquid nitrogen. The tissue samples were trimmed with a razor blade to approximately 0·5 x 0·5 mm, and ultra-thin sections (50 mm) were cut with a diamond knife by using a type 20S ultramicrotome (Leica). The sections were examined in a Philips CM-10 transmission electron microscope (FEI) with an acceleration voltage of 80 kV.

## RESULTS

The results of the blood analyses performed at the onset of clinical signs and repeated six months later are shown in Table 1. The mean age of the dogs at onset was 1·5 years (range six months to 3·5 years). The serum activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT) and glutamate dehydrogenase (GLDH) were high, and even higher after six months. However, the bilirubin levels were within the reference range. The serum levels of total protein, creatinine and urea were normal, indicating that the dogs' renal function was not impaired (data not shown). In a follow-up study 26 months after the onset of the clinical signs, there were no signs of hepatic disease or any other abnormalities (Table 1).

At the onset of the clinical disease, two male dogs (1 and 3) and two females (2 and 4), aged 18 months to three years, showed signs of severe chronic liver failure. Their general condition was poor and they had slight yellowish discoloration of the mucous membranes and severe ascites. Blood analyses revealed high serum activity of ALP (839, 987, 234 and 1180 U/l), ALT (140, 99, 252 and 632 U/l) and GLDH (13·3, 7·1, 22·5 and 86·2 U/l) (Table 1). Their prothrombin time was abnormally long (14·2 to 15·0 s, reference range 6·0 to 9·5 s), indicating disturbed blood coagulation; their activated partial thromboplastin time (15·4 to 20·4 s, reference range 14·5 to

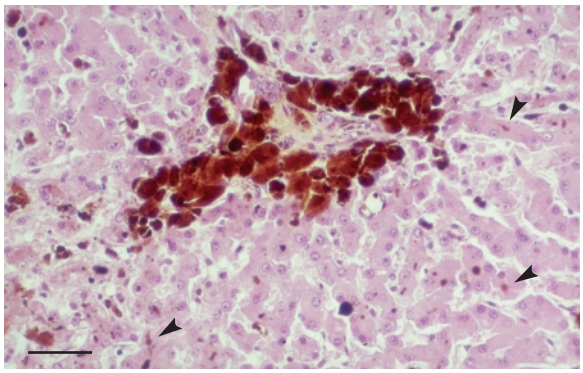

**FIG 2: Accumulations protoporphyric pigment in the liver of one of the dogs, showing conglomerates of dark brown-pigmented macrophages in a perivenous pattern and numerous bile plugs (some indicated by arrowheads) with the same dark brown discoloration. Haematoxylin and eosin. Bar=50 µm**

19·0 s) was within the normal range. Ultrasonography and explorative laparotomy revealed that the livers of these four dogs had an irregular multinodular appearance.

Postmortem examinations of the four dogs revealed that their livers were dark brown to black, with a multinodular surface, consistent with macronodular cirrhosis. On cut surface, the parenchyma had a marked increase in consistency combined with a pale, irregular arboreal branching pattern. The extrahepatic bile ducts and other organs, including the integument, appeared to be grossly normal.

The microscopic lesions were similar in all four dogs and displayed, to a greater or lesser extent, a distortion of the hepatic lobular architecture (Fig 1). The portal tracts showed extensive fibrous expansion with portoportal and portocentral bridging, exhibiting moderate bile ductule proliferation and moderate amounts of mixed inflammatory infiltrates. These fibrous bands replaced collapsed hepatic parenchyma and surrounded variably sized hyperplastic nodules of broad, bilayered hepatocytic cords. In many places there was moderately sized areas of confluent hepatocytic necrosis and randomly disseminated solitary necrotic hepatocytes. Most characteristically, in all four cases, there was excessive amounts of granular dark brown pigment in conglomerates of macrophages in the portal fibrous septa and in Kupffer cells (Fig 2). Widespread canalicular bile plugs and pigment granules were observed in the hepatocytic cytoplasm, which had the same brown colour. The pigments in both the macrophages and the bile plugs displayed a striking bright orange birefringence when examined in polarised light (Fig 3), often with a central dark Maltese cross typical of porphyrins (Fig 4). The dark brown pigments were consistently negative for iron and copper.

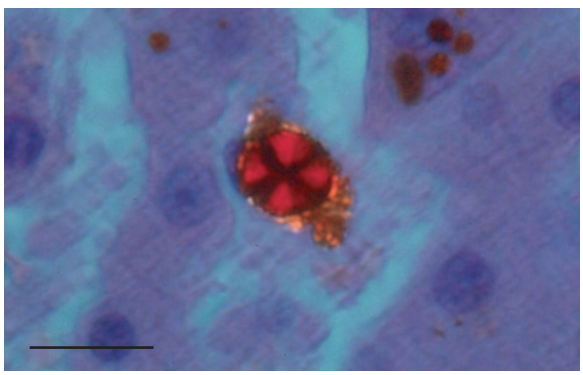

**FIG 4: Protoporphyric pigment observed in polarised light displaying a bright orange-red birefringence with a central dark Maltese cross typical of porphyrin crystals. Haematoxylin and eosin. Bar=20 µm**

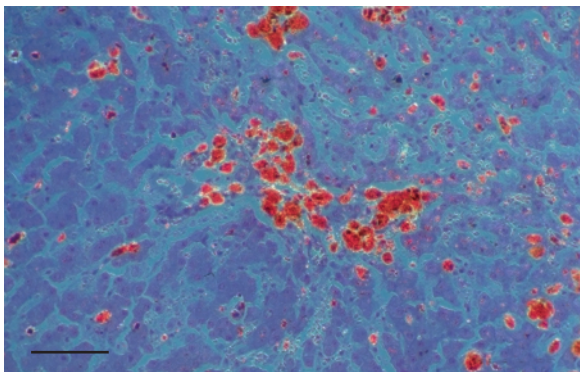

**FIG 3: Accumulations of protoporphyric pigment in the liver of one of the dogs viewed in polarised light, showing a striking bright orange birefringence of the pigment-laden macrophages and bile plugs. Haematoxylin and eosin. Bar=100 µm**

At the ultrastructural level there were extensive deposits of pigment scattered diffusely in swollen macrophages and Kupffer cells, and less marked deposits in hepatocytes. Most of the deposits were composed of numerous electron-dense, randomly distributed, slender, needle-like crystals (Fig 5), but there were also some slightly curved slender crystals in a rosette-like pattern with a radial arrangement (Fig 6). In the hepatocytes and macrophages the crystals were mostly free in the cytoplasm, whereas in Kupffer cells, parts of a surrounding single membrane could occasionally be observed. The crystals varied in size, ranging from 45 to 650 nm in length and 7 to 20 nm in width, with electron-dense margins and more lucent centres (Fig 5). No association between the crystalline accumulations and particular cell organelles could be established.

HPLC analyses of the liver samples from dogs 1 and 2 showed very high levels of protoporphyrin-IX (9854·5 and 9325·5 nmol/g wet liver, reference value 0·41 nmol/g wet liver) and low activities of FeC (0·306 and 0·241 nmol/mg protein/hour, reference value 0·684 nmol/mg protein/hour) (Table 2). Urinalyses of dogs 1, 2, 3 and 4 were negative for porphyrins.

## DISCUSSION

Although a rare disorder, EPP associated with hepatic lesions is a well documented condition both in human beings and in several animal species.

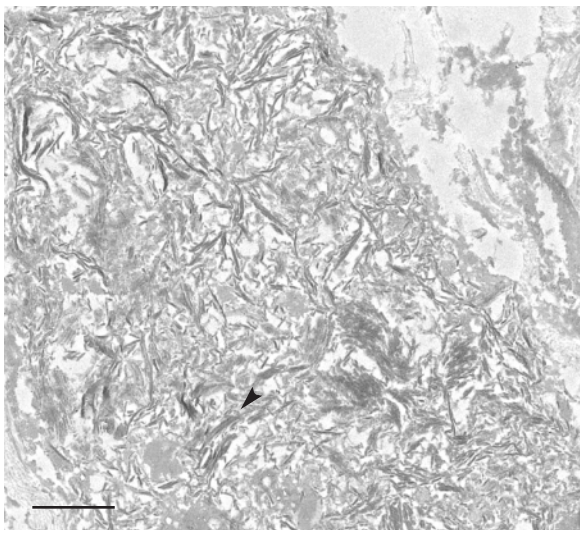

**FIG 5: Electron micrograph of the liver from one of the dogs with erythropoietic protoporphyria, showing intracellular straight needle-like and slightly curved crystals typical of protoporphyrins, some of the crystals have an electron-lucent centre and electron-dense margins (arrowhead). Bar=1 µm**

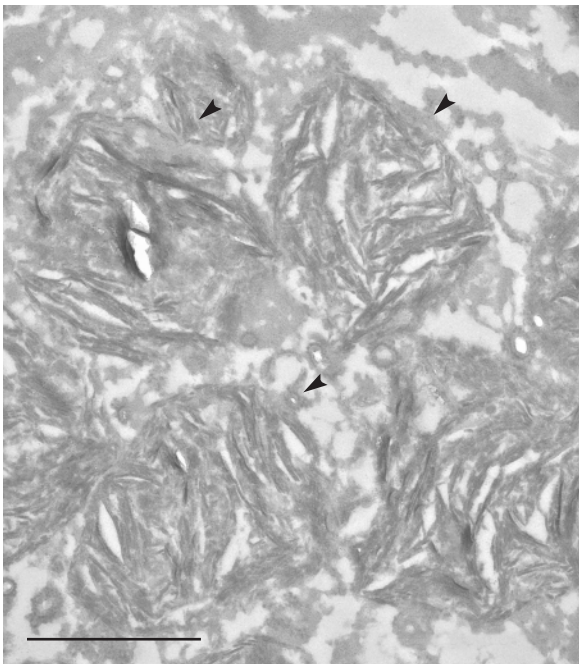

**FIG 6: Electron micrograph showing a hepatic macrophage containing protoporphyric crystals in a radially arranged multiple rosette pattern (arrowheads). Bar=1 µm**

**TABLE 2: Concentrations of free protoporphyrin-IX and the activities of ferrochelatase in the livers of two severely affected dogs compared with the mean values in eight healthy control dogs**

| Measurement | Dog 1 | Dog 2 | Normal dog |
|---|---|---|---|
| Free protoporphyrin-IX (nmol/g wet weight) | 9854·5 | 9325·5 | 0·41 |
| Ferrochelatase (nmol/mg protein/hour) | 0·306 | 0·241 | 0·684 |

The macroscopic changes and the histological lesions in the livers of the four dogs were characteristic of the hepatic lesions associated with accumulations of protoporphyrin-IX described by Greijdanus-van der Putten and others (2000) and Libbricht and others (2003). Since hepatic fibrosis is regarded as a non-specific reaction in areas of hepatic damage, the portoportal and portocentral bridging fibrosis observed in these cases, together with the presence of large quantities of porphyrin pigments in macrophages, is suggestive of the cytotoxicity of protoporphyrin-IX.

The ultrastructural investigations revealed extensive deposits of crystals in Kupffer cells, macrophages, bile ducts and hepatocytes, the appearance and dimensions of which were similar to those described by Komatsu and others (2000) and characteristic of protoporphyrins. Although typical intracellular crystalline rosette-like patterns were observed, the crystalline starburst formations in bile plugs described by Komatsu and others (2000) and Matilla and Molland (1974) were not observed. It was not possible to make an accurate evaluation of the ultrastructural liver cell damage because the electron microscopy was performed on formalin-fixed, paraffin-embedded tissue samples. However, the crystals preserved their characteristic properties.

The key factor in the pathogenesis of EPP is a reduction in the activity of FeC, which allows the toxic immediate haem precursor protoporphyrin-IX to accumulate in the liver (Sassa 2000); in contrast with preceding haem precursors, it is highly hydrophobic and excreted solely via bile in the faeces. As a result it is not cleared by the kidneys and there is no discoloration of urine (Hindmarsch and others 1999, Meerman 2000). Urine samples from the severely affected dogs tested negative for porphyrins. The diagnostic biochemical markers of EPP are decreased activities of FeC in the liver and increased concentrations of protoporphyrin-IX in the blood, liver and faeces (Hindmarsch and others 1999). Although no analyses of protoporphyrin-IX in blood and faeces were done, the other specific diagnostic markers showed the characteristic changes. Compared with eight healthy dogs, the concentration of protoporphyrin-IX in the liver was greatly increased and the activity of FeC was reduced by approximately 60 per cent (Table 2).

The high serum concentrations of liver enzymes at the onset of the clinical signs and the increases observed six months later, together with the histopathological lesions, are compatible with a chronic liver disease. However, in the follow-up study 26 months later, the dogs' liver enzymes were within the reference ranges (Table 1) and the dogs were apparently healthy. All the dogs had apparently been affected by liver failure to different degrees; 13 of the 17 dogs recovered completely, suggesting that the cause had been transient.

In human beings there are several mutations in the FeC gene that reduce the activity of the enzyme (Rufenacht and others 1998). In Limousin cattle, a similar point mutation occurs (Jenkins and others 1998). The inherited forms of EPP are progressive and there is no spontaneous clinical recovery. No inherited form of porphyria has been described in dogs. It is unlikely that the 17 dogs had a genetic defect in the FeC gene because there was no age predilection, and a pedigree analysis revealed no familial correlations to support an inherited form of the disease. Moreover, the disease was not progressive because 13 of the dogs made a full clinical recovery and had normal serum liver enzyme activities approximately two years after the onset of the disease.

Greijdanus-van der Putten and others (2000) reported that EPP associated with both cholestasis and chronic hepatitis could be induced experimentally in dogs, and EPP associated with cirrhosis can be readily induced in mice with griseofulvin (Meerman and others 1999, Plosch and others 2002). Combinations of direct and indirect protoporphyrinogenic substances produce marked deposits of protoporphyrin-IX (Kimmet and others 1996, Smith and others 1997) and are associated with severe liver damage, as in the cases reported here. It is therefore possible that one or more unidentified porphyrinogenic substances may have induced the disease in these dogs.

The porphyrias are frequently associated with dermal lesions, for example, in cattle (Rimington 1936, Schelcher and others 1991, Armstrong and others 2002) and in one dog (Lapras and others 1972). However, in the present cases there were no dermal abnormalities, possibly because the dogs' thick haircoat and the short periods for which they were allowed outdoors (approximately 30 minutes per day), protected them; in addition, the outdoor kennel bordered a wooded area with ample shade.

This cohort of dogs apparently suffered a transient form of EPP of variable severity, presumably induced by a FeC-inhibiting agent or by a combination of porphyrinogenic substances of unknown origin. To the best of the authors' knowledge, it is the first report of a spontaneous transient form of the disease in dogs.

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