

Neural tube defects in four Shetland sheepdog puppies: clinical characterisation and computed tomography investigation

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Abstract

Case report Here we report on the occurrence of neural tube defects in four related Shetland sheepdog puppies. Neural tube defects present as a range of congenital malformations affecting the spine, skull and associated structures. Despite the severity of these malformations and their relatively high prevalence in humans, the aetiology is not well understood. It is even less well characterised in veterinary medicine. Affected puppies were investigated using computed tomography and then post-mortem examination. Computed tomography identified a range of brain and spine abnormalities in the affected animals, including caudal anencephaly, encephalocele, spina bifida and malformed vertebrae. Other observed abnormalities in these puppies, including cranioschisis, atresia ani and hydrocephalus, may be secondary to, or associated with, the primary neural tube defects identified.

Conclusion This case report describes multiple related cases of neural tube defects in an Australian cohort of dogs. This study also highlights the potential of advanced imaging techniques in identifying congenital anomalies in stillborn and neonatal puppies. Further research is required to investigate the aetiology of neural tube defects in this group of affected Shetland sheepdogs.

Key words

anencephaly, canine, cranioschisis, encephalocele, neural tube defect (NTD), spina bifida.

Abbreviations list

CNS, central nervous system; CT, computed tomography; OMIA, Online Mendelian inheritance in animals; OMIM, Online Mendelian inheritance in man; NTD(s), neural tube defect(s).

Introduction

Neural tube defects (NTDs) encompass a wide range of congenital malformations of the brain, spinal cord, and vertebrae as well as the soft tissue surrounding these structures. NTDs arise when the process forming the neural tube (neurulation) fails.¹ In humans, NTDs (OMIM: #182940)² are one of the most common congenital anomalies occurring in approximately one in 1000 births.^{3,4} Many types of NTDs are recognised and include anencephaly (absence of a major part of the brain and skull), cranioschisis (failure of the calvarium to close) and spina bifida (incomplete closure of vertebral arches dorsally).¹ Spina bifida may be further described as open (where the spinal cord or meninges are exposed) or closed (where the defect is covered by skin).¹

The aetiology of NTDs has been proposed to be multifactorial with genetics, maternal factors (e.g. folic acid supplementation)^{4,5} and the environment all being potential risk factors for NTDs.⁶ Several lines of evidence support a significant contribution of genetic components to NTDs. Studies have shown up to a 50-fold increased risk of NTDs in siblings of people affected when compared to the general public.⁷ Based on patterns of inheritance in some families, where the affected members of the family were second or third degree relatives, a oligogenic pattern of inheritance has been suggested.⁷

Only a small number of studies have described NTDs in dogs. Of these, there have been reports of cases of anencephaly,⁸ dermoid sinus,⁹ myelomeningocele¹⁰ and spinal dysraphism¹¹ (OMIA: 000044, 000272-9615, 000938-9615, respectively)¹². The occurrence of NTDs in Australian dogs goes largely unreported, with only one report on scattered NTD cases in several breeds identified.¹³ Anecdotally, breeds such as the British bulldog are thought to have a higher occurrence of NTDs;^[cited in 11] however, prevalence data on the rates of NTDs in the general population of any breed are not available.

Considering the prevalence in humans, and the likelihood of undiagnosed cases amongst stillborn and neonatal puppies, it is possible that NTDs in dogs are more common than the literature suggests. The aims of the current study were to describe the gross abnormalities in affected neonates from an extended family of Shetland sheepdogs and then further characterise these cases utilising advanced imaging techniques.

Materials and methods

Study population

Cadavers were collected from severely affected Shetland sheepdogs that were either stillborn, or neonates that required euthanasia. Samples were collected (with consent) under two University of Sydney animal ethics approved projects (2015/902 and 2016/1075).

Pedigree analysis

To determine the relatedness of the litters, pedigree data was collected for each affected puppy, aiming to include a depth of at least six generations. Pedigree analysis was performed using Pedigraph software (version 2.4).¹⁴

Computed tomography (CT)

Post-mortem advanced imaging was used to define the extent of abnormalities in affected animals. As congenital anomalies of the skull and spine were expected, CT was chosen as the best modality and was conducted at the University Veterinary Teaching Hospital Sydney (University of Sydney). Whole-body CT was performed for the four affected cadavers. The CT parameters were adjusted to the relatively small body size of the stillborn and neonatal affected animals (Table 1).

Post-mortem examinations

Cadavers were initially assessed for gross congenital anomalies by a veterinary embryologist (HM). A veterinary pathologist and/or veterinary anatomical pathologist then performed routine macroscopic and microscopic post-mortem examinations.

Results

Study population

Three litters of privately owned Shetland sheepdogs with affected puppies displaying a range of suspected neural tube-related gross abnormalities were included in this study. The following clinical observations were reported by the breeders of affected animals, with tissue samples collected from four affected puppies (one stillborn, three neonates). The rest of the animals were not available for further examination or sampling.

The first litter consisted of one normal male and two affected puppies. The affected puppies were a stillborn male with an open skull defect with protruding tissue within a fluid-filled sac observed at birth (puppy 1 [SHLT0001]; Figure 1a) and a female that was euthanised at three weeks of age due to an abnormal pelvis (puppy 2 [SHLT0003]; Figure 2a). The cadavers from the two affected pups were collected.

The second litter comprised six puppies. Of these, two were grossly normal, two were stillborn (and underweight), and two were grossly abnormal. The first grossly abnormal puppy was underweight, had difficulty suckling and died within 24 hours. Another grossly abnormal male puppy (puppy 3 [SHLT0004]; Figure 3) with initial diagnosis of atresia ani and an absent tail (anury) was euthanised due to welfare concerns at two days old.

A third litter of three puppies included one grossly normal puppy; one puppy that was euthanised shortly after birth due to cleft palate and a distal limb abnormality; and a male puppy (puppy 4 [SHLT0006; Figure 4a] with a dome-shaped head, ataxia and hindlimb paresis. This last puppy was euthanised at three weeks of age due to welfare concerns.

Pedigree analysis

The pedigree compiled included at least six generations for each affected litter, totalling 392 dogs. Several common ancestors (two to six generations back from the affected puppies) were partially shared between the affected litters, but not between all three litters. One common ancestor of interest was identified. This dog (#39) was a common ancestor present in both the maternal and paternal lines of litter one and two, but only on the maternal line of litter three (Supplementary 1).

Computed tomography

The four affected animals investigated displayed a range of abnormalities that involved structures including the skull, brain, spine and pelvis (Table 2). The main abnormalities identified were: caudal anencephaly, maxillary hypoplasia and cleft palate (puppy 1, Figure 1b; puppy 3), hemivertebrae with poor development of the sacrum and abnormally displaced right pelvis and lumbosacral spina bifida (puppy 2, Figure 2b) and hydrocephaly (puppy 4, Figure 4b).

Puppy 1 (Figure 1b) displayed multiple congenital anomalies of the skull including short maxillary (maxillary hypoplasia), nasal and frontal bones. The soft tissues at the level of the rostral maxillary hard palate were absent bilaterally, consistent with severe bilateral cleft palate. A severe flattening of the caudal portion of the cranium and the occipital bones was noted, suggesting this animal had a severe cranioschisis. A suspected anencephaly of the caudal brain was also noted. The skeleton was immature, with incomplete ossification of vertebral arches and open physes, likely consistent with the age of the animal.

In puppy 2 (Figure 2b), the lumbar spine and sacrum were characterised by multiple hemivertebrae associated with the fifth, sixth and seventh lumbar vertebrae. The sacral wings were poorly developed and the right side of the pelvis appeared to be displaced cranially without clear visualisation of sacroiliac joint. A transitional vertebra was present with 12 ribs on the left and 11 on the right (Figure 2b).

The skeleton of puppy 3 displayed characteristics consistent with osteochondrodysplasia, as there was a lack of mineralisation of the centres of ossification of the epiphyses. The palatine bone and the soft tissue planes of the hard palate were not present. These findings are consistent with the presence of a cleft palate. The skull also had severe flattening of the caudal portion of the cranium and occipital bones, with the caudal brain not observed.

The brain of puppy 4 had a marked enlargement of the lateral ventricles and normal third and fourth ventricles, consistent with non-communicating hydrocephalus (Figure 4b).

Gross post-mortem examinations

Gross post-mortem inspection by a veterinary embryologist of the four affected animals and post-mortem examination by veterinary pathologists identified a range of developmental anomalies associated with the head and spine. Unfortunately, the cadavers of puppy 1 and puppy 2 were too autolysed for a full post-mortem examination (the majority of internal viscera were liquefied and no longer intact), but both were examined for evidence of external abnormalities. Puppy 3 and 4 underwent full post-mortem examinations.

Puppy 1 had gross abnormalities of the head including incomplete closure of the calvarium on the midline (cranioschisis; Figure 1), open caudal fontanelle and severe secondary cleft palate. The cleft palate had a wide gap between the two lateral palatine processes, where both the hard and soft palate should have been formed. A patent urachus was also noted in this puppy.

Puppy 2 (full sibling to puppy 1) was found to have abnormalities of the pelvis and caudal spine. The pelvis was found to be tilted dorsocranially to the right. This puppy also had a short, kinked tail. The tail was positioned with the base on the right side of the midline, the main part of the tail kinked to the left side, and the tail tip was located in the midline next to the anal orifice (Figure 2a).

Puppy 3 was found to have atresia ani, anury (absent tail; Figure 3) and premature termination of the rectum 5 mm cranial to the expected location of the anal orifice with associated megacolon. Both testes were in the mid-abdominal cavity, instead of in the inguinal canal, which is considered abnormal for an animal this age. The skull had a mildly open fontanelle and an abnormal head shape (flattened caudally) in comparison to other puppies of the same breed. The brain was also abnormal. Examination of the brain post-fixation revealed a hindbrain (including discernible cerebellum tissue) that was subjectively small when compared to the cerebral cortex/midbrain.

Puppy 4 was found to have a markedly domed dorsal cranium (Figure 4a) with an unusual presentation of open anterior fontanel characterised by bilaterally open frontal-parietal fissures with exposure of underlying dura (Figure 4c). The flat bones on the dorsal cranium were also stretched and thinned (less than 1 mm). The muzzle of puppy 4 was short (Figure 4a). Narrow dental arches were abnormally located closer to the median axis of the maxilla (instead of in close proximity to the lateral/buccal walls of the oral cavity). The tongue was flat and wider than expected. Evaluation of the brain after fixation revealed generalised, mild to moderate loss of distinction between the cerebral sulci/gyri and cerebellar fissures/folia. Similar to puppy 3, the right testis was located mid-abdominal, while the left testis was within the inguinal canal.

Histopathology

Histopathology was not performed on puppy 1 or puppy 2 due to post-mortem degradation of the samples.

Histopathology of the central nervous system (CNS) tissue in puppy 3 was deemed to be of limited value due to the moderate to marked tissue autolysis and freeze-thaw artefacts. Microscopic evaluation of grossly evident distal colon abnormalities was performed and revealed increased diameter of the colonic lumen (supportive of a diagnosis of megacolon). Due to technical slide preparation limitations and marked intestinal autolysis, a definitive site of colonic/rectal termination could not be ascertained histologically. No other significant histological findings were made.

Histopathology of puppy 4 tissues was attempted, particularly of the brain; however, despite fixation, severe autolysis and freeze-thaw artefacts confounded further microscopic assessment and measurements of the ventricles and other relevant structures. No other significant histological findings were made.

Macroscopic and microscopic normal tissues of puppy 3 and puppy 4 are listed (Supplementary 2).

Discussion

Here we describe the occurrence of NTDs in four related Shetland sheepdog puppies. The congenital anomalies observed in these animals demonstrate the range of phenotypes that are associated with failure of neural tube development, as well as related secondary phenotypes. Advanced imaging and post-mortem examination of these puppies further diagnosed and characterised the developmental anomalies reported here. All affected animals were of the same breed and were related, possibly suggesting an inherited component in this cohort of dogs.

The range of phenotypes observed in NTDs reflects the complex nature of neural tube closure. The folding of the neural tube in the primary phase is initiated at several fusion points along the dorsal-caudal axis of the embryo (three rostral fusion points in mice and two to three in humans).^{15, 16} The points of fusion in dogs have not been determined. To create the neural tube, closure starts at each fusion point and proceeds cranially and/or caudally (with the edges coming together like a zipper). The secondary phase of neurulation occurs in the most caudal part of the embryo (the tail bud) and creates the caudal spinal cord including the sacrum and coccygeal region.¹

Fusion sites are of interest as they relate to the range of NTDs observed, as failure to fuse at each point may result in different phenotypes. Failure of the primary phase of neurulation is associated with open NTDs such as encephalocele.⁶ In mice, this is observed when the rostral part of the neural tube fails to fuse, leading to the most severe form of NTD, craniorachischisis, where the brain and spinal cord are exposed from midbrain to the lower spine.¹⁷ During the secondary phase of neurulation, failure of fusion at the caudal neuropore, which forms the most caudal parts of the spine, is associated with closed NTDs such as spina bifida occulta, tethered cord syndrome and dermoid sinuses.^{6, 17, 18}

The presence of different NTDs in the siblings, puppy 1 and puppy 2, demonstrates this variability of phenotype. While several studies in human patients have shown increased risk of NTDs in siblings,⁷ recurrence in families affected by NTDs do not always show consistent phenotypes. It has been estimated that in 30 to 40 % of recurrent cases (e.g. subsequent cases in affected families) the phenotype was different to the original case.⁷ A female gender bias has been reported for anencephaly in humans,⁸ however, the low number of canine reports make it difficult to determine if any gender bias is present in dogs.

While neurulation and neural tube closure occurs during very early pregnancy,¹⁷ it is believed that failure of the calvarium to fuse later in the pregnancy exposes the neural/brain tissues leading to encephalocele (fluid-filled sac with neural/brain tissue) or exencephaly (exposed brain tissue). The exposed tissue then degenerates, leading to partial or full anencephaly.^{1, 19}

The fluid-filled sac with suspected brain/neural tissue observed protruding from the skull in puppy 1 was not available for further diagnostics; however, it was likely to be a case of encephalocele. In puppy 1, this was also accompanied by a cranioschisis, a defect which is likely to be secondary to the failure of normal fusion of the rostral neuropore.¹⁹

Anencephaly is one of the most common concurrent malformations reported with cranioschisis. The partial presence of brain tissue outside the skull is often of telencephalon

origin,¹⁹ the region of the embryonic brain that develops into forebrain structures. In puppy 1, the cranioschisis, likely caused the brain tissue to move anteriorly, leading to exposure and destruction of brain tissue, resulting with a caudal anencephaly.

On CT, puppy 3 was suspected to have a similar malformation, however, post-mortem examination subsequently found that the brain was smaller than expected, with the hindbrain (including cerebellum) anteriorly displaced. This was accompanied by a mildly open fontanelle, a potentially normal presentation for an animal this age. The abnormalities observed in puppy 3, including a flattened skull and smaller brain, may be similar to the report by Huisinga et al. (2010) on a German shepherd with anencephaly, hypoplastic calvarium and flattened base of the skull.⁸

Puppy 1 and puppy 3 were also reported to have incomplete ossification of the vertebral arches on CT with suspected sacral malformations and atresia ani in puppy 3. As ossification of the vertebral arches is not complete at birth,²⁰ the significance of this morphological finding is unclear in puppies of this age (stillborn and two days old, respectively). Therefore, assessment for spina bifida occulta cannot be made. The severe caudal spine and pelvis deformities in puppy 2 are most likely spina bifida occulta, but due to the presence of necrotic tissue at the time of necropsy, the exact type could not be confirmed.

In addition to the described NTDs, several associated congenital anomalies were found in this cohort of puppies. Atresia ani, anury and premature termination of the distal colon with associated dilation were present in puppy 3. While not classified as a NTD, atresia ani is known to often co-occur with NTDs in people, especially with tethered cord syndrome.^{21,22} Co-occurrence of NTDs and atresia ani has also been documented in rat models with short tails, atresia ani and neural tube defects.²³ Interestingly, the rate of perinatal death in an Australian Shetland sheepdog cohort (247 puppies) was increased with 3.6% of puppies reported as having gross congenital defects at birth compared to average rate of 2.8% across multiple breeds.¹³ One case each of spinal dysraphism, atresia ani and anury were also reported in that cohort,¹³ potentially indicating sacral spinal and tail deformities associated with NTDs.

Cleft palate was documented in two of the puppies included in this study (puppy 1 and puppy 3) as well as reported in littermates that were not available for inclusion in this study. Orofacial clefts are common in dogs and may result from maldevelopment of the palate, lip or nose due to inherited and/or environmental causes.²⁴ Cleft palate is not classified as a NTD, though it is often observed to co-occur with NTDs. It is suggested that the development of the orofacial and the neural tube structures share common gene pathways.²⁵⁻²⁷

Many environmental and maternal risk factors have been identified in humans.⁷ Maternal nutrition has been investigated widely in humans. In women, increasing the intake of folic acid has led to significant decreases in the prevalence of NTDs.^{4,5} A substantial proportion of human cases (estimated at around 50%), however, are not sensitive to this measure.⁵ In dogs, one study suggested that folic acid supplementation reduced the occurrence of cleft palate in a population of Boston terriers.²⁸

Given the dense coat of Shetland sheepdogs and the relatively hot climate in Australia, maternal hyperthermia can also be considered as a possible risk factor associated with NTDs.^{7, 29} Of the four affected puppies, however, only one was born in the summer and two others were born during the peak of the winter. While the authors are not aware of any exposure of the dams to teratogenic drugs and/or toxins during the pregnancy, such environmental risks cannot be completely excluded here. For example, the drug griseofulvin has been reported to induce exencephaly in cats.³⁰

Finally, puppy 4 was diagnosed with hydrocephalus on CT and had post-mortem changes consistent with this diagnosis. While not classified as a primary NTD, hydrocephalus often occurs secondary to neural defects due to obstruction of the outflow of cerebrospinal fluid (CSF).³¹ While an underlying NTD was not identified in puppy 4, it was included in this report as it was related to the other affected animals and another puppy in the litter (not available for examination) was reported by the owner to have cleft palate and a distal limb abnormality.

Despite extensive investigation into the mechanisms of NTDs in humans and mice,³² prevalence and genetic data are difficult to find for other species such as dogs. Investigations into NTDs in two dog breeds, the Rhodesian ridgeback (dermoid sinus) and the Weimaraner (spinal dysraphism), have identified associated genetic mutations which are unique to each of these dog breeds: a 133-kb duplication involving three fibroblast growth factor (FGF) genes in Rhodesian (and Thai) ridgebacks and a mutation in the *NKX2-8* homeobox gene in Weimaraners.^{33, 34}

Pedigree analysis identified several common ancestors, potentially supporting a genetic component. One common ancestor of interest was identified but while this dog was an ancestor from both maternal and paternal lines of puppies 1, 2 and 3, it could not be linked to the paternal side of puppy 4.

Live grandsires exclude a sex-linked trait, and the severity of the disease together with the rare reports suggest that dominant inheritance is unlikely. Considering the limited generations studied here, it is still possible that a common ancestor in higher-order generations is responsible for an autosomal recessive mutation (or a mutation with incomplete penetrance). It is also possible, however, that the common ancestors identified may reflect a popular animal or characteristic at the time that has contributed to the establishment of the breed in Australia. Analysis of a wider pedigree and/or genetic investigations is required to exclude or include an inherited mutation.

Cryptorchidism was observed in both puppy 3 (bilateral) and puppy 4 (unilateral). One human study reported spina bifida occulta as a risk factor for testicular cancer, although this study found that the risk was independent of cryptorchidism.³⁵ As cryptorchidism is a well-established risk factor for canine testicular cancer, including in this breed,³⁶ it may be worthwhile to investigate if NTDs are associated with risk of cryptorchidism/testicular cancer in dogs.

Of major concern to the veterinary profession is the welfare impact of NTDs. Animals may be severely affected, leading to euthanasia, while more mildly affected animals may have

ongoing disability that affects their quality of life. Puppies affected by severe NTDs are often stillborn, or due to severe clinical presentation, do not survive or are euthanised in the neonatal period. Often, they are not presented to a veterinarian, making it difficult to confirm the presumed diagnosis and collect prevalence data. The current study has shown that further investigation using non-invasive advanced imaging techniques and detailed post-mortem may aid in diagnosis of NTDs and/or other congenital anomalies in stillborn puppies or in cases of perinatal mortality. The current study only included CT imaging performed on stillborn or euthanised puppies. This modality (and/or other advanced imaging techniques) may also aid in the diagnosis of congenital anomalies in live neonates with indicative clinical presentation(s), or a failure to thrive with unknown underlying cause.

The difficulty in identifying cases makes the recruitment of affected animals for research one of the main challenges in this area. Small sample sizes, an inability to assess all litter mates (or dam/sire), inability to perform histology from autolysed CNS tissues, and a lack of multi-generational data have all limited this research. In future, larger sample sizes, including cases across multiple generations, will allow more insight into this under-researched disorder.

Conclusion

A range of NTD phenotypes in four affected Shetland sheepdogs, including gross features, CT and post-mortem findings, are described here, highlighting the variability in presentation of NTDs.

The use of advanced imaging techniques for investigation of stillborn and/or neonatal loss in dogs is uncommon and post-mortem examinations of such puppies are rarely done. This study suggests that such methods should be utilised more frequently in neonates for diagnosis of congenital anomalies, such as NTDs. Further research on the occurrence and pathogenesis of NTDs in dogs, including genetic, nutritional, or other causes, may reduce the occurrence of these severely debilitating conditions.

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Conflicts of interest and funding

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Tables

Table 1. CT parameters used for imaging of affected animals

Parameter	Value
Thickness	1.5 mm
Increment	-0.75 mm
kV	90
mAs/Slice	100
Resolution	High
Collimation	16 x 0.75
Pitch	0.813
Rotation time	0.5 s
Field of view (FOV)	180 mm
Bone reconstruction filter	1 mm

Table 2. CT abnormalities detected in affected Shetland sheepdog pups

Puppy (M/F) ^a age	Cranial abnormalities	Vertebral abnormalities	Other abnormalities
Puppy 1 (M) stillborn	Maxillary hypoplasia Severe bilateral cleft palate Severe cranioschisis Caudal anencephaly	Incomplete ossification of vertebral arches	
Puppy 2 (F) 3 weeks		Transitional vertebrae Multiple thoracic & lumbar hemivertebrae Lumbosacral spina bifida Short and kinked tail	Maldevelopment of the pelvis
Puppy 3 (M) 2 days	Maxillary hypoplasia Cleft palate Severe flattening of the caudal skull Caudal anencephaly	Incomplete ossification of vertebral arches Anury	Atresia ani
Puppy 4 (M) 3 weeks	Thinning of cranium Hydrocephalus		

^aM=male; F=female

Figure legends

Figure 1. Gross image and CT 3D reconstruction from puppy 1. A) Gross image (left lateral view) showing cranioschisis (closure failure of the calvarium) and encephalocele (fluid-filled sac with protruding neural/brain tissue content). B) 3D reconstruction of full skeleton (left lateral view) showing maxillary hypoplasia (arrow) and cranioschisis (arrow head).

Figure 2. Gross and CT images of puppy 2. A) Gross image (caudal view), with kinked tail and deformed pelvis. B) 3D reconstruction of caudal spine and pelvis (dorsal view). Note the transitional vertebra (12 ribs on the left side (arrow) and 11 on the right side), caudal spine abnormalities including hemivertebrae and incomplete fusion of vertebral arches (spina bifida; arrowhead).

Figure 3. Gross image of puppy 3 (caudal view). Gross image showing atresia ani and anury.

Figure 4. Gross and CT images of puppy 4. A) Gross image with dome-shaped skull and shortened muzzle. B) Sagittal CT image of the head showing hydrocephalus and a dome-shaped skull. C) Post-mortem image of the exposed rostral and caudal aspect of the calvarium. An unusual presentation of open anterior fontanel characterised by bilaterally open frontal-parietal fissures viewed in the rostral part of the skull (bottom of the image).

Supplementary data

Supplementary 1. Pedigree of affected litters

Partial pedigree of affected litters showing relation to each other, and to common ancestor dog #39. Affected puppies are coloured grey. Males are denoted by rectangles, females are denoted by ovals and diamonds denote unknown gender. Siblings of affected animals are denoted as 'sib' followed by their identifying number.

Supplementary 2. Macroscopic and microscopic normal tissues of puppy 3 and puppy 4.

No significant gross abnormalities were detected in the following tissues of both puppy 3 and puppy 4 (skeletal muscle, femur bone, oesophagus, pancreas, adrenal glands), or further tissues that were not autolysed in puppy 4 (coxofemoral joints, heart and great cardiac vessels, tongue, pharynx, eyes and ears).

The following tissues of puppy 3 and puppy 4 had no significant histologic lesions: thymus, heart (right/left ventricle and interventricular septum), tongue, oesophagus, duodenum, jejunum, liver, adrenal gland, urinary bladder, testicle, epididymis, skeletal muscle. Some other tissues were microscopically normal, but only examined in puppy 3 (stomach, ileum, mesenteric lymph node, spleen, and kidney) or puppy 4 (trachea, thyroid gland, caecum and colon (both with prominent GALT), pancreas, umbilicus, urachus (non-patent), and peripheral nerve).

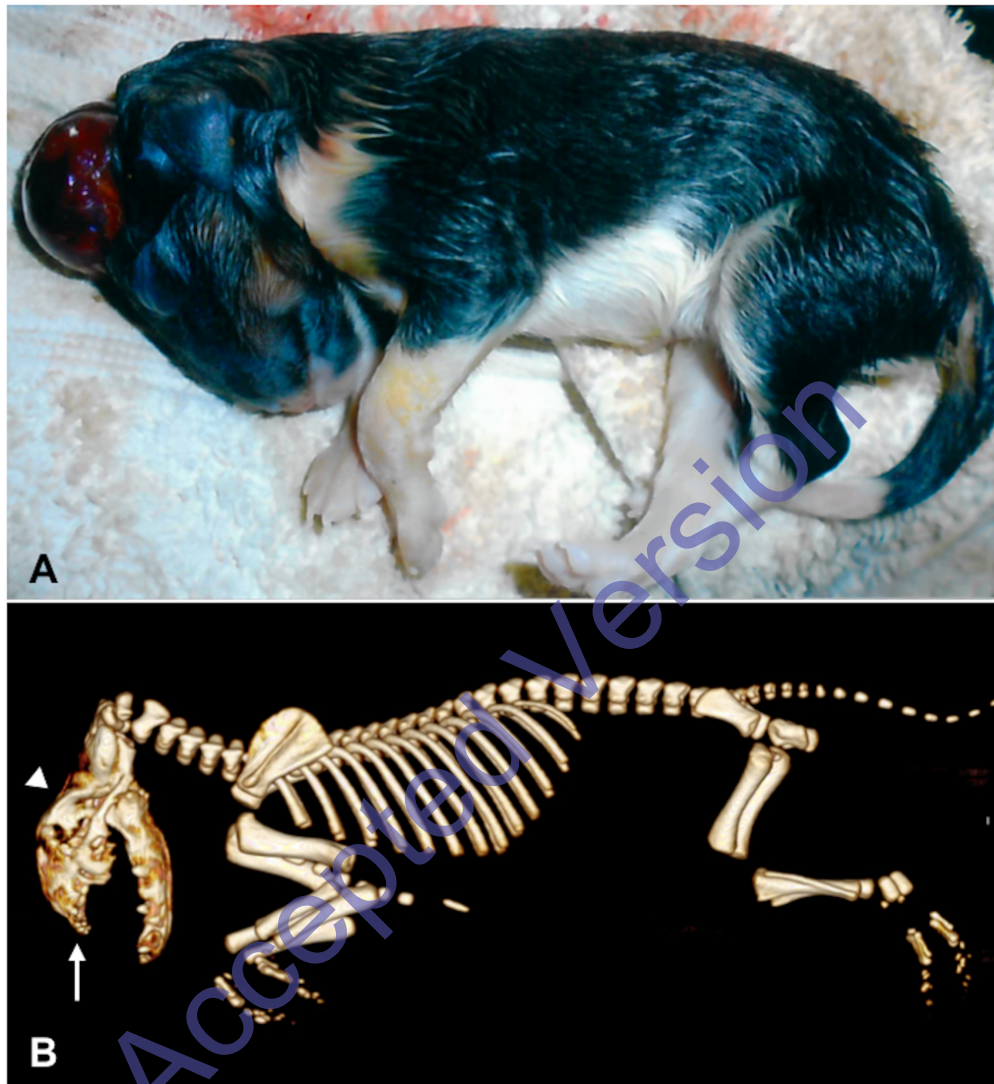


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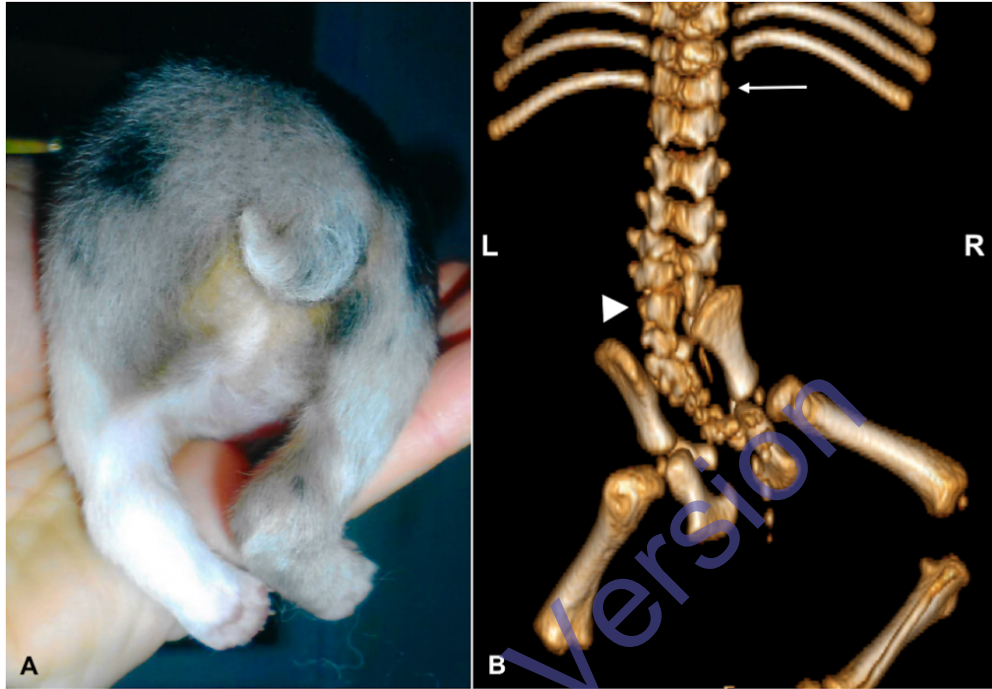


Figure 2. Gross and CT images of pup 2. A) Gross image (caudal view), with kinked tail and deformed pelvis. B) 3D reconstruction of caudal spine and pelvis (dorsal view). Note the transitional vertebra (12 ribs on the left side (arrow) and 11 on the right side), caudal spine abnormalities including hemivertebrae and incomplete fusion of vertebral arches (spina bifida; arrowhead).

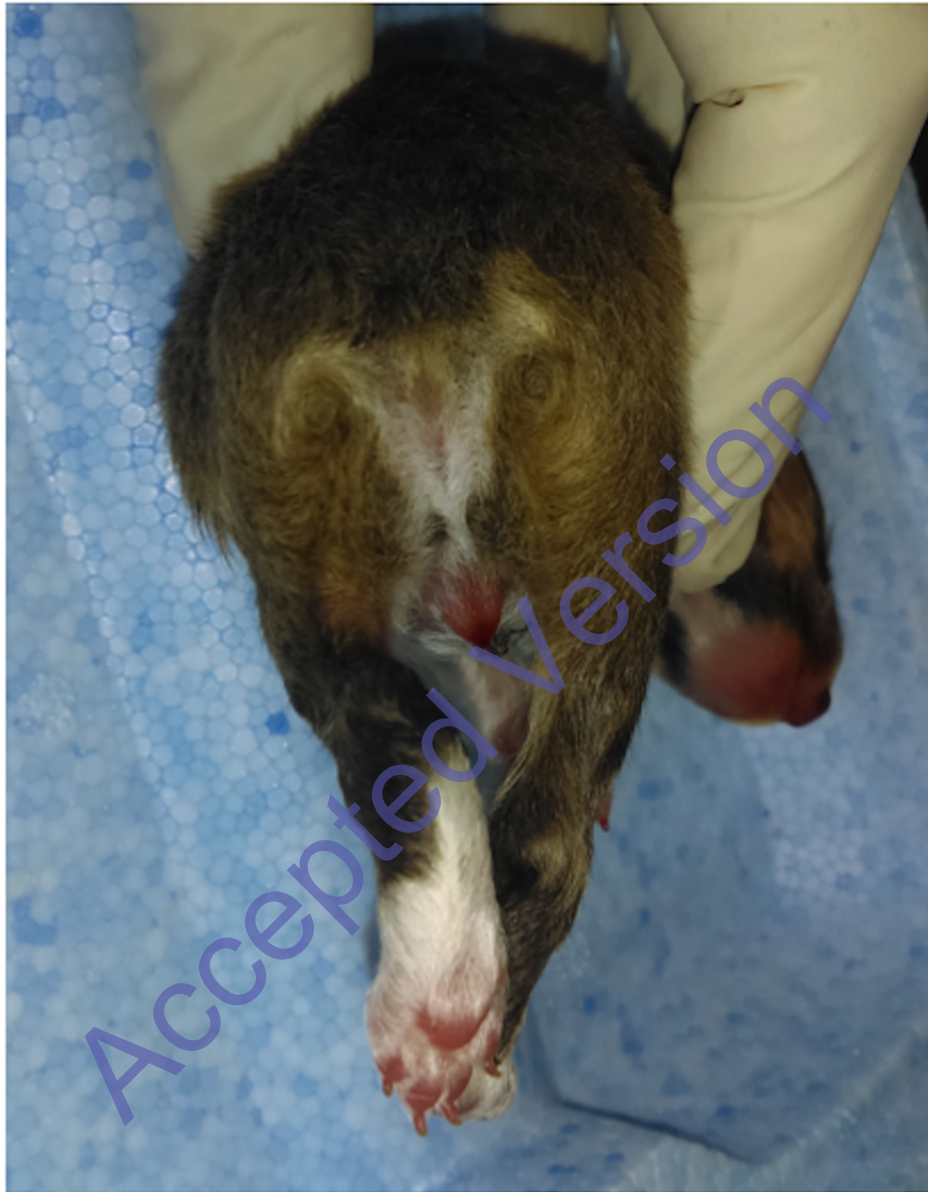


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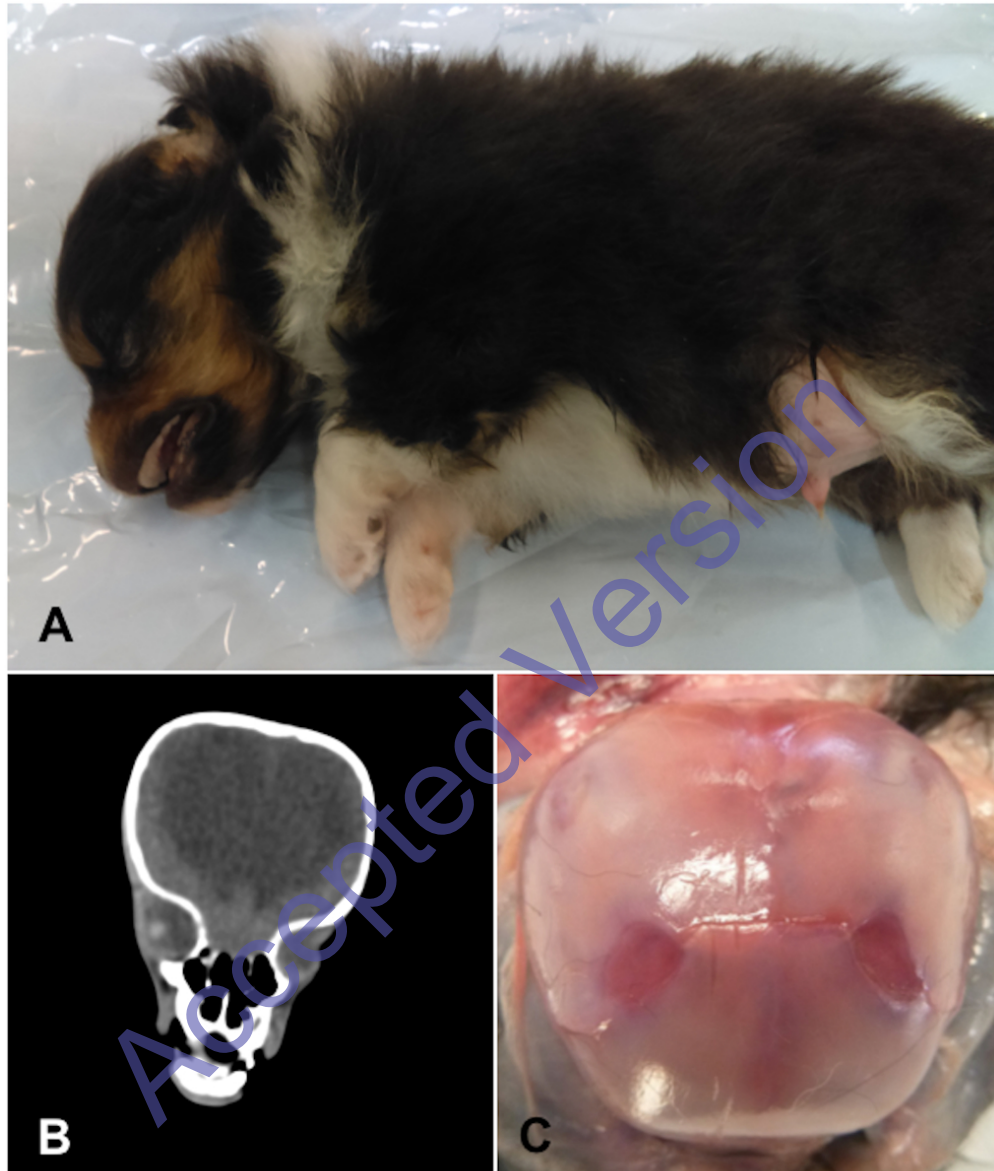
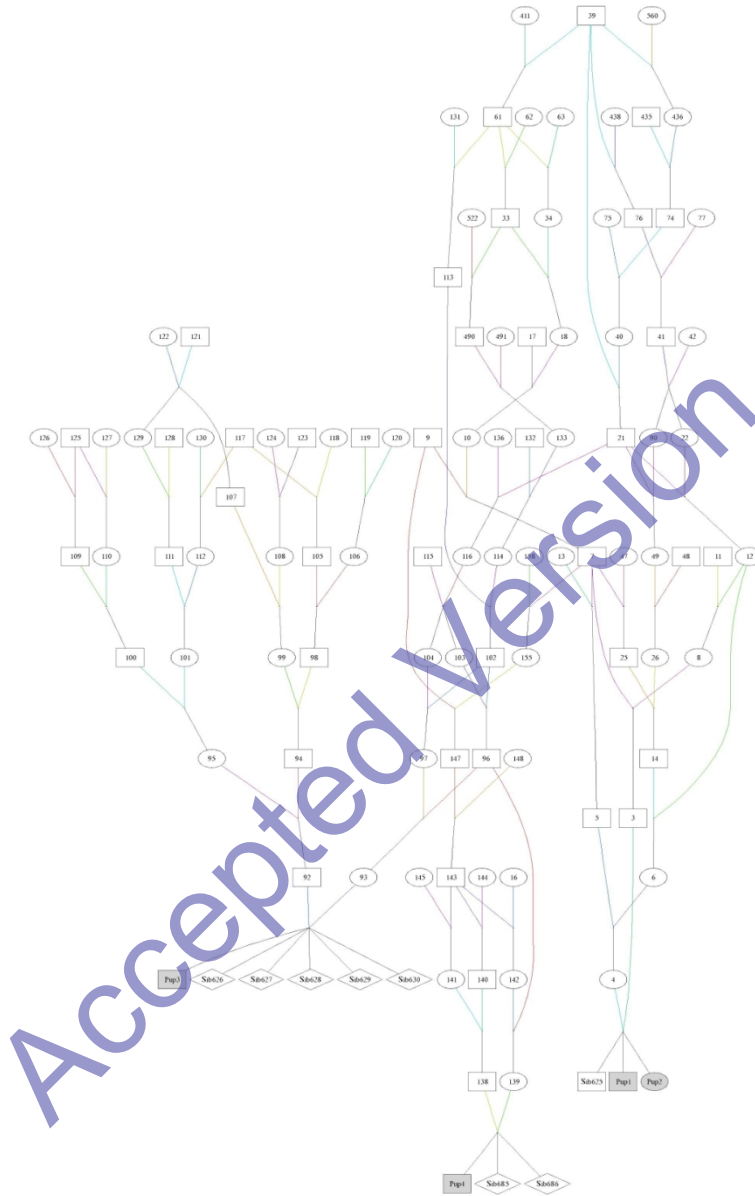


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(Figure legend below, Figure uploaded as file 6)

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Accepted Version