C7 VERTEBRA HOMEOTIC TRANSFORMATION IN DOMESTIC DOGS- ARE PUG DOGS BREAKING MAMMALIAN EVOLUTIONARY CONSTRAINTS?

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Abstract

The number of cervical vertebrae in mammals is almost constant at seven, regardless of their neck length, implying that there is selection against variation in this number. Homebox (*Hox)* genes are involved in this evolutionary mammalian conservation and homeotic transformation of cervical into thoracic vertebrae (cervical ribs) is a common phenotypic abnormality when *Hox* gene expression is altered. This relatively benign phenotypic change can be associated with fatal traits in humans. Mutations in genes upstream of *Hox*, inbreeding and stressors during organogenesis can also cause cervical ribs. The aim of this study was to describe the prevalence of cervical ribs in a large group of domestic dogs of different breeds and explore a possible relation with other congenital vertebral malformations (CVM) in the breed with the highest prevalence of cervical ribs. By phenotyping we hoped to give clues as to the underlying genetic causes. Twenty computed tomography studies from at least two breeds belonging to each of the nine groups recognised by the Federation Cynologique Internationale, including all the brachycephalic "screw-tailed" breeds that are known to be overrepresented for CVM's were reviewed. The Pug dog was more affected by cervical ribs than any other breed (46%) ($p<0.001$) and was selected for further analysis. No association was found between the presence of cervical ribs and vertebral body formation defect, bifid spinous process, caudal articular process hypoplasia/aplasia and an abnormal sacrum, which may infer they have a different etiopathogenesis. However, Pug dogs with cervical ribs were more likely to have a transitional thoraco-lumbar vertebra ($p= 0.041$) and a pre-sacral vertebral count of 26 ($p<0.001$). Higher C7/T1 dorsal spinous processes ratios were associated with the presence of cervical ribs ($P<0.001$) supporting this is a true homeotic transformation. Relaxation of the stabilizing selection has likely occurred and the Pug dog appears to be a good naturally occurring model to further investigate the aetiology of cervical ribs, other congenital vertebral anomalies and numerical alterations.

Introduction

The number of cervical vertebrae in mammals is almost constant at seven, regardless of their neck length (Schulz, 1961; Leboucq, 1895; Starck, 1978; Badlangana et al. 2009). Interestingly, the vertebral count in other regions does vary among mammals, implying there is a selection against variation in the number of cervical vertebrae (Schulz, 1961; Leboucq, 1895).

Manatees (Trichechus) and sloths (Bradypus and Choloepus) are exceptions to this evolutionary rule (Buchholtz & Stepien, 2009; Varela-Lasheras et al. 2011; Buchholtz et al. 2007). This relaxation of stabilizing selection leading to persistence of features, against which there is normally a strong selection, can also be found in domesticated mammals through artificial selection. Due to the relaxation of stabilizing selection through human care, dogs with polydactyly can breed and reproduce despite the strong evolutionary constraint on polydactyly in amniotes (Galis et al. 2001; Galis et al. 2010).

Homebox *(Hox)* genes are involved with this evolutionary mammalian conservation and have proven to be critical regulators in the establishment of axial skeleton morphology during embryogenesis in all vertebrae classes (Krumlauf, 1994; Wellik 2007). It is generally assumed that shifts of the boundary of vertebral regions are associated with shifts of *Hox* expression patterns and therefore, homeotic transformations of cervical into thoracic vertebrae (C7 transitional vertebra commonly known in humans as cervical ribs) is a common phenotypic abnormality. Abnormal expression of *Hox* genes does not just lead to vertebral column anomalies, but also neural problems, increased susceptibility to early childhood cancer, other congenital anomalies and stillbirths (Galis, 1999; Hostikka et al. 2009; Furtado et al. 2011; ten Broek et al 2012; Schumacher, 1992; Gladstone et al. 1932). Upstream organizers of *Hox* genes are equally crucial in the head-to-tail patterning of the vertebral column

An extreme selection in humans against homeotic transformations of cervical vertebrae occurs (ten Broek et al 2012) and the total incidence of cervical ribs in people is low (0.04-6.2%) (Henderson, 1913; Spadlinski et al. 2016). Two previous studies in dogs have described congenital vertebral malformations of the whole vertebral column in 145 and 228 cases submitted to necropsy and reported a cervical ribs prevalence that varied from 2.1% to 11.4% (Morgan, 1968; Breit and Kunzel 1998). These studies date from 1968 and 1998 and the dog population has changed in size, genetic diversity, breed prevalence and phenotypic characteristics over time (The Kennel Club 2016, Vila et al. 1999)

Normal dogs have seven cervical, 13 thoracic, seven lumbar, three sacral and a variable number of caudal vertebrae. The first and second vertebrae present easily distinct recognisable anatomical features. The rest of cervical vertebrae differ slightly from each other, except for the expanded sagittal plate-like transverse processes of C6. Thoracic vertebrae have shorter bodies compared to cervical and lumbar vertebrae and articulate with a rib. The spinous process of the first thoracic vertebra is the longest of all vertebrae, is its most conspicuous feature and its length gradually decreases with successive vertebrae (Evans and de Lahunta, 2013) (**Figure 1**).

Congenital vertebral malformations (CVM) are relatively common in brachycephalic “screw-tailed” breed dogs, such as the French bulldogs, the English bulldogs, Boston terriers and Pug dogs (Gutierrez-Quintana et al. 2014; Steffen et al. 2004; Wrzosek et al. 2014). Unlike CVM’s in other less frequently seen regions, thoracic CVM’s have been described and compared in some brachycephalic dogs. Overall, Pug dogs had significantly fewer vertebral body formation defects and more thoracolumbar transitional vertebrae and bifid spinous process compared to the other two breeds. Despite having fewer vertebral body formation defects, when present, these had greater clinical relevance. Furthermore, all French and English bulldogs in the study had a normal thoracic vertebral count, while 25% of the Pug dogs had 12 instead of 13 thoracic vertebrae (Ryan et al. 2017). Therefore, the Pug dog appeared to be a good naturally occurring canine model to phenotype and further investigate the genetic background of CVM's.

The aim of this study was to describe the frequency of cervical ribs in a large group of domestic dogs of different breeds. We were particularly interested in breeds reported to be commonly affected by CVM's and we wanted to explore a possible relation between them in a naturally occurring model, providing clues as to their genetic causes. Given the high number of thoraco-lumbar transitional vertebrae previously identified in Pug dogs (Ryan et al. 2017), we hypothesised that a high number of cervical ribs compared to other dog breeds and species would be identified. We also hypothesised that having cervical ribs would be associated with a higher incidence of other CVM's.

Material and methods

Ethical approval from the University of Glasgow, School of Veterinary Medicine was obtained for this study.

To quantify the frequency of cervical ribs in different breeds of domestic dogs, the medical records of the Royal Veterinary College, University of Glasgow Small Animal Hospital, Vet Extra Neurology and Fitzpatrick Referrals were retrospectively reviewed to identify computed tomographic studies (CTs) that included the C7 vertebra regardless of their clinical indication. A total of 19 breeds with at least two breeds belonging to each of the nine groups recognised by the Federation Cynologique Internationale (FCI) (FCI 2017) were examined. These included Basset hounds, Beagle, Border collie, Chow-chow, Cocker spaniel, German shepherd dog, German pointer, Greyhound, Hungarian Vizsla, Husky, Labrador retriever, Dachshund, Staffordshire bull terrier, Whippet, West Highland white terrier and all brachycephalic "screw-tailed" breeds (French bulldog, the English bulldog, Boston terrier and Pug dog) known to be overrepresented for CVM's were selected for analysis (Gutierrez-Quintana et al. 2014; Ryan et al. 2017). A maximum of 20 randomly selected CTs were reviewed for each breed. Dogs underwent imaging investigations under sedation or general anaesthesia for a variety of clinical indications (related or unrelated to spinal disease). The breed having the highest frequency of cervical ribs was then selected for further analysis. All the available CTs that included the entire vertebral column (including or excluding coccygeal vertebrae) or C7 and the thoracic region were then reviewed and the signalment (age, breed, sex and colour) was recorded when available. We also retrieved and reviewed orthogonal radiographic views of dogs with thoracic CVM's causing neurological deficits from the breed that was selected for further analysis.

Computed tomographic studies and radiographs were reviewed by a board-certified neurologist (RG) and a third-year neurology resident (JB). Images were displayed using an open-source Workstation DICOM viewer (Osirix Imaging Sofware, v 3.9.2, Pixmeo, Geneva, Switzerland). Congenital vertebral malformations were identified and classified into vertebral body formation defects (commonly known as hemivertebra) (Gutierrez-Quintana et al. 2014), transitional vertebrae and dorsal process formation defects (e.g. bifid spinous process or fusion of the dorsal spinal process). Ambiguous vertebrae located at the border between two segments of the vertebral column and possessing characteristics of both segments were considered to be transitional vertebra (Simoens, 1983). The total number of pre-sacral vertebrae was recorded and the sacrum was classified as being normal or abnormal. The caudal articular processes from T9-L2 of whole body CTs were classified as normal or hypoplastic/aplastic. All dogs were considered to have seven cervical vertebrae, even if two large ribs were identified at C7. When identified on CT, cervical ribs were classified as being either short (less than 1.5cm) or long (more than 1.5cm), and with or without articulation. One of the key morphological differences between cervical and thoracic vertebrae is the comparatively long dorsal spinous process in the latter. To test whether other aspects of the C7 vertebrae took on thoracic vertebrae characteristics, the heights of the dorsal spinous processes of C7 and T1 vertebrae were measured and the C7/T1 dorsal spinous process height ratio was calculated. When identified on radiographs, the presence or absence of cervical ribs was recorded with no further sub classification. Thoraco-lumbar vertebrae were considered thoracic if they were transitional (including transverse process asymmetry) and lumbar if they had bilateral and normal size symmetric transverse processes. Dogs in which L7 was considered to have fused with the sacrum (absent intervertebral disc) were considered to have six lumbar vertebrae.

Statistical Analysis

All analyses were done using SAS 9.4 (SAS Inst. Inc., Cary, NC). Data was analysed using non-parametric methods (Wilcoxon-Mann-Whitney, Kruskal-Wallis and Chi-square/Fisher tests, and Spearman correlation) except for the analysis of C7/T1 dorsal spinous process ratio depending on the presence of transitional vertebrae, cervical ribs and joints. The later was analysed using t-test. Alpha level for determination of significance was 0.05 and multiple comparison were analysed using Bonferroni correction. Given that CT provides better bone definition compared to radiography, data from orthogonal radiographic views of dogs with thoracic CVM's causing neurological deficits was analysed separately and only to study the relation between CVM's.

Results

Frequency of cervical ribs in domestic pure breed dogs

Twenty CTs were avaiable for review from each of the breeds mentioned above except for the Husky (n=15), the Basset hound (n=15), the German pointer (n=13), the Miniature Snauchzer (n=6) and the Chow-chow (n=4). The percentage of dogs with cervical ribs from each of the reviewed breeds is illustrated in **Figure 2**. Cervical ribs were more common in the Pug dog compared to any other breed and was therefore selected for further anaylsis (45/97-46%) (P<0.001). The overall prevalence of cervical ribs was 14% (61\430). Excluding the Pug dog the percentage decreased to 4.8% (16/333).

Further analysis in the breed wih highest frequency of cervical ribs: the Pug dog

A total of 51 CTs of the whole vertebral column and 46 cervico-thoracic CTs from Pug dogs with no clinical signs caused by CVM's (scanned for respiratory signs (n=81), mestastic check (n=6), orthopaedic problems (n=6) and gastrointestinal signs (4)) and 33 thoracic radiographs from Pug dogs with neurological deficits caused by CVM's (radiographed for T3-L3 myelopathy) Pug dogs were available and reviewed (n=130 Pug dogs).

A total of 57/130 Pug dogs had cervical ribs, 50/130 had vertebral body formation defects (**Figure 3**), 48/130 had bifid spinous process (without myelomeningocele) (**Figure 4**), 52/130 had a thoraco-lumbar transitional vertebra (**Figure 5**), 12/51 had a lumbo-sacral transitional vertebra (**Figure 6**) and 47/51 had caudal articular process hypoplasia/aplasia (**Figure 7**). T13 was absent in 31/130 and L7 was absent in 17/51. Out of 51 whole vertebral column CTs, twenty-eight (55%) had 26 pre-sacral vertebrae, 23 (45%) had 27 pre-sacral vertebrae and eleven had no transitional vertebrae (**Table 1**).

Mean and median age were 38 and 31 months respectively. Sixty-one Pug dogs were female and 69 were male. Females (30/45-65%) were affected more frequently by cervical ribs than males (15/45-29%) (P=0.012). Coat colour was only available for Pug dogs with a whole-body CT. No association between coat colour and the presence of any transitional vertebrae, vertebral body formation defects or a bifid spinous process was identified. In most cases, cervical ribs were bilateral (32/45-71%). Among cases with unilateral changes (13/45-29%), the left (10/13-77%) side was more frequently affected compared to the right (3/13-23%) (P=0.04; **Table 2**). Cervical ribs were classified as short with no joint in 35% (27/77) of the cases, long with no joint in 2.5% (2/77), short with a joint in 34% (26/77) and long with a joint in 28.5% (22/77) of the cases (**Figure 8**).

No association was found between the presence of vertebral body formation defects, transitional vertebra, bifid spinous process, caudal articular process hypoplasia/aplasia and an abnormal sacrum in the CTs of Pug dogs with no clinical signs associated to CVM's or in the radiographs of Pug dogs with CVM's causing neurological deficits. Pug dogs in which cervical ribs were identified on CTs were more likely to have a thoraco-lumbar transitional vertebra (P= 0.041) than those without cervical ribs, though this was not replicated in our analysis of radiographs. Pug dogs with cervical ribs (P=0.025) or a lumbo-sacral transitional vertebra ($P<0.001$) were also more likely to have a pre-sacral vertebral count of 26 instead of 27. The pre-sacral vertebral count was not associated with the presence of hemivertebrae, bifid spinous process or an abnormal sacrum. Longer C7 dorsal spinal processes and higher C7/T1 ratios were associated with the presence of cervical ribs ($P<0.001$) and were correlated (r=0.44 and r = 0.55 respectively) with the added length of left and right cervical ribs (**Figure 9**). Higher C7/T1 ratios were also associated with having fewer vertebrae affected by caudal articular process hypoplasia/aplasia (r = -0.36; P = 0.010).

Discussion

The previously reported prevalence of cervical ribs in domestic dogs varied from 2.1% to 11.9% (Morgan, 1968; Breit and Kunzel 1998). The present study investigated a larger number of dogs equally distributed between breeds and found an overall similarly low prevalence, but identified the Pug dog as being significantly more affected than any other breed. The German Shepherd (15%), the Hungarian Vizsla (15%) and the Boxer (10%) also exhibited unusually high percentages of cervical ribs.

It has been proposed that cervical ribs may be largely composed of ossification sites that disappear after birth (Chernoff and Rogers, 2004). One of the key morphological differences between cervical and thoracic vertebrae is the comparatively long dorsal spinous process in the latter. Longer C7 dorsal spinous processes and high C7/T1 dorsal spinous process height ratios were associated with the presence of cervical ribs in this study, suggesting this is a true homeotic transformation of a cervical vertebra into a thoracic one and not an unmasking of vestigial cervical ribs left behind in evolution. The Spearmen correlation between C7/T1 ratio and the added length of left and right cervical ribs was moderate, suggesting that longer cervical ribs are not always associated with longer C7 dorsal spinous process. In combination with the length of the cervical ribs, this ratio may be used as an objective measure of the degree of C7 homeotic transformation.

In humans, bilateral cervical ribs are the most common; and when unilateral, they are traditionally considered to be more frequently left-sided. Although inconsistent results have been reported, females seem to be more frequently affected (Furtado et al. 2011; Galis et al 2006; Bots et al. 2011, Schumacher et al. 1992). The results of this study were in accordance with these findings. Deficient signalling in mice leads to delayed somite formation on the right side, causing a diminished coordination between the development of the left and right somites, and leading to a differential expression of the *Hox* genes (Vermot et al. 2005; Griesinger et al. 2005).

Mammals under harsh conditions and stressors like hyperthermia or exposure to boric acid during organogenesis (Chernoff and Rogers, 2004; Steigenga et al. 2006; Wery et al. 2003, Li and Shiota, 1999) and mutations that influence *Hox* gene expression can also cause cervical ribs. T-gene (also known as brachyury) knockdown in vivo experiments cause T1 transformation of cervical vertebrae in 30% of mice (Yamaguchi, 1999; Pennimpede et al. 2012) and a spontaneous mutation in this same gene, has been associated with a cervical vertebral homeotic transformation in the *Bos Taurus* with vertebral and spinal dysplasia (VSD) (Kromic et al. 2015). Data suggests that a distinct amino acid position (p.66) in the *T-gene* is relevant for a coordinated Wnt–brachyury–HOX signaling cascade, which is important for cervical vertebral and spinal cord development (Galis, 1999; Yamaguchi, 1999; Pennimpede et al. 2012). To the authors' knowledge, no other spontaneous mutation in mammalian genes causes a homeotic transformation of cervical vertebrae similar to VSD. Some familial cases of cervical ribs with a probable autosomal-dominant mode of inheritance have been reported in human literature (Weston, 1956; Schapera 1987). A high incidence of cervical ribs has been observed in mammals with a high rate of inbreeding (e.g 33% in mammoths before extinction (Reumer et al. 2014), 11% in minipigs (KD, 1998) or 7.5% in humans (Palma and Carine, 1990)). Pedigrees were not available for analysis in any of the dogs included in this study; and therefore, the mode of inheritance or the degree of inbreeding could not be studied. Familial cases could potentially help determine the genetic basis of cervical ribs.

Children with neuroblastoma, brain tumours, leukaemia, soft tissue sarcoma, Wilms tumour, and Ewing sarcoma also have a high incidence of cervical ribs. Pug dogs are not known to be overrepresented for any of the above (Gough and Thomas, 2010). Cervical ribs have also been associated with human stillbirths (Furtado et al. 2011; ten Broek et al 2012). Among the 100 most popular breeds in a Norwegian dog population, the Pug dog ranked eighth highest for stillbirths and fourth highest for total perinatal mortality risk at the individual puppy. Mean litter size was like other similar size dog breeds (Parker et al. 2017; Tonnessen et al. 2012). Our study was based on the United Kingdom Pug dog population and the prevalence of cervical ribs in the Norway Pug dog population is currently unknown, making comparisons difficult. Significant associations between congenital anomalies and cervical ribs in stillborn human foetuses have been found (ten Broek et al 2012). One study did not identify this association; however, an association with aneuploidy was identified, implying that congenital anomalies would have been detected if they had survived to be born (Furtado et al. 2011). We only evaluated the available images for the presence of other CVM's and not for other major anomalies (renal agenesis, ventricular septum defects, anal atresia, etc.).

Most human adults with cervical ribs are asymptomatic, but some develop thoracic outlet syndrome (TOS) (Chang et al. 2013). Thoracic outlet syndrome represents a group of distinct disorders that result from the compression of one or more neurovascular elements as they traverse the thoracic outlet. Unlike humans in which the brachial plexus and the subclavian artery pass through the interscalene triangle, which is formed by first rib and the ventral and middle components of the scalene muscles (Ferrante and Ferrante, 2017); in domestic mammals, the ventral scalene muscle is not separated from the middle component and the interscalene triangle is not formed (Evans and de Lahunta, 2013). The scalene muscles can play an important role in the pathophysiology of this syndrome (Ferrante and Ferrante, 2017a), of which some forms typically occur in athletes after vigorous activity (Ferrante and Ferrante, 2017b). None of the Pug dogs in our study presented with clinical signs suggestive of TOS. To the authors' knowledge, TOS has not been reported in dogs, and the anatomical difference between species and the fact that that Pug dogs are not too athletic may be some of the reasons why; however, given the high prevalence of cervical ribs within the breed, clinicians should bear in mind this differential diagnosis in Pug dogs with cervical ribs and compatible clinical signs.

Even though they do not usually cause clinical signs in humans, cervical ribs and transitional lumbo-sacral vertebra are associated with dermatomal variations and alterations in function (Makhoul and Machleder, 1992; Roos, 1996; Seyfert 1997; Kim et al. 2008). Lumbo-sacral transitional vertebrae can predispose dogs to cauda equina syndrome (Fluckiger et al. 2006), asymmetrical hip joint development and more severe hip dysplasia (Komsta et al. 2015; Fluckiger et al. 2017). The prevalence of lumbo-sacral transitional vertebrae in some dog breeds has been reported as high as 22%; however, the overall prevalence is low (3.5%) (Damur-Djuric et al. 2006). Despite high prevalence of lumbo-sacral transitional vertebrae (12/51-24%), none of the Pugs dogs in our study presented with clinical signs suggestive of hip dysplasia and/or cauda equina syndrome. Pug dogs are predisposed to hip dysplasia, with an approximate prevalence of 70% (OFA 2016). To the author's knowledge, the Pug dog is not predisposed to cauda equina syndrome (Meij and Bergknut 2010). If spinal stenosis occurs in the absence of clinical signs, and how clinically significant hip dysplasia is in Pug dogs, remains to be explored.

The prominent and sharp ventral process of C1, the large transverse processes of C6, the first and last rib, the first lumbar transverse process and the last lumbar vertebra are all useful anatomical landmarks for localisation during the surgical approach to vertebral column in dogs (Sharp and Wheeler 2005). Two out of 51 (4%), 8/51 (16%) and 23/51 (45%) of Pug dogs with a whole body CTs were considered to have a C5, a C6 and a C7 abnormal transverse process morphology, respectively, and 52/130 (40%) were considered to have a thoraco-lumbar transitional vertebra. Given the frequency of altered anatomical surgical landmarks, special consideration should be taken when using them to localise during surgery in pug dogs.

No association was found between the presence of vertebral body formation defects, transitional vertebrae, bifid spinous process or an abnormal sacrum, and this may reflect a different etiopathogenesis. However, an association between cervical ribs and thoracolumbar transitional vertebrae was identified using CT. A possible reason why this was not significant when analysing radiographs is that CT provides better bone definition compared to radiography, and the presence of cervical ribs could had been underestimated. In sloths, cervical disruptions occur coincident with abnormalities at the thoraco-lumbar, lumbo-sacral, and sacro-caudal boundaries. In people, significant associations between cervical ribs and sacralisation (Erken et al. 2002) and thoraco-lumbar transitional vertebrae have been found (Bots et al. 2011). Thoraco-lumbar transitional vertebrae have also been associated with a higher incidence of concomitant lumbo-sacral transitional vertebrae (Carrino et al. 2011) and vice versa (Nakajima et al. 2014).

A total of 49 bifid spinous processes were identified in 47/130 (36%) Pug dogs. This prevalence is in accordance with a previous study in which bifid spinous process was identified exclusively at T1 in Pug dogs (Ryan et al. 2017). One Pug dog in our study had bifid spinous process at C5 and C6 and another Pug dog had bifid spinous process at T5. It is currently unclear why bifid spinous process occurs more frequently at T1 and what the clinical relevance of this finding may be. A higher than normal incidence of cervical ribs has been previously identified in foetuses and infants with open spina bifida (myelomeningocele) (5/112-4.5%) and spina bifida (4/10-40%), suggesting a possible relation between both malformations (Galis et al. 2006; Naik et al. 1978). Our study identified no association between bifid spinous process and cervical ribs.

Twenty-eight out of 51 (55%) Pug dogs had 26 pre-sacral vertebrae instead of the fixed 27 pre-sacral vertebrae common to all the canine species (de Lahunta et al. 2015). Having 26 pre-sacral vertebrae was associated with the presence of cervical ribs or a lumbo-sacral transitional vertebra. In humans, the presence of lumbo-sacral transitional vertebra is associated with an anomalous total number of vertebrae, but the presence of thoraco-lumbar transitional vertebra is not (Carrino et al. 2011). The mechanisms of regulation of the number of segments in the vertebral column and its associations with homeotic transformations are still not well understood (Gomez and Pourquie 2009).

Hypoplasia or aplasia of the T9-L2 caudal articular processes are thought to sometimes lead to the formation of fibrous tissue and cause a constrictive myelopathy along these spinal cord segments in Pug dogs (Fisher et al. 2013, Breit, 2002). The association between cervical ribs and thoraco-lumbar transitional vertebra identified in our study supports the previously demonstrated fact that cervical ribs appear to be accompanied by homeotic changes of several adjacent cervical and thoracic vertebrae, and sometimes all thoracic vertebrae (ten Broek et al 2012; Oostra et al 2005). As a result, the normal vertebral anatomy, including the caudal articular processes of T9-T13, could be altered. However, we found no association between the presence of caudal articular process hypoplasia/aplasia, the presence of vertebral body formation defects, transitional vertebra at any location or bifid spinous process. Furthermore, higher C7/T1 dorsal spinous process ratios were associated with having fewer vertebrae affected by caudal articular process hypoplasia/aplasia, but the correlation coefficient was low. The reasoning behind this finding remains unknown.

Mutations in *Hox* genes are almost absent in humans, other than a few mutations in caudal *Hox* genes (e.g. HoxA11, Hox A13, HoxD10, HoxD13) (Kosaki et al. 2002, Dobbs et al. 2006) and supposedly because most mutations are lethal at a very young stage. As supported by this study, cervical ribs are rarely the only homeotic change along the vertebral column and are often associated with shifts of the thoraco-lumbar and lumbo-sacral boundary (Varela-Lasheras et al. 2011). This combination of homeotic transformations can be well explained by mutations of genes upstream of *Hox* (Alkema et al 1995, Ikeya and Takada 2001, van den Akker et al 2002). Alternatively, it can be caused by mutations in several *Hox* genes at the same time, which is unlikely, given that such mutations are already not found in a single *Hox* gene. Therefore, it is more likely that mutations in genes upstream of *Hox* genes are involved in the induction of cervical ribs in dogs and other mammals.

To the authors' knowledge, no previous CT based study in veterinary literature including a large number of dogs has described CVM's of the whole vertebral column (excluding coccygeal vertebrae) in any specific breed. Due to the high percentage of altered pre-sacral vertebral counts and the high number and variety of CVM's identified in the Pug dog, it appears to be a good naturally occurring model to further investigate the aetiology of CVM's and numerical alterations.

Conclusion

Despite the high prevalence of C7 homeotic transformation, affected Pug dogs survive to adulthood, and reproduce; whereas most conceived individuals with a deviation number of cervical vertebrae die prenatally, or as infant (ten Broek et al 2012). We hypothesise that stabilizing selection relaxed due to human care and the pleiotropic constraints broke to allow the evolution of novelties in body plans. Sloths, manatees, *Bos taurus* with VDS and Pug dogs are unique examples of mammals with a naturally occurring exceptional number of cervical vertebrae. Further research is required to answer questions regarding the aetiology and frequent natural occurrence of the different CVM's and numerical anomalies of the axial skeleton in Pug dogs.

Author contributions

J.B.: concept/design, acquisition of data, data analysis/interpretation, drafting of the manuscript, assembled figures, critical revision of the manuscript and approval of the article. S.D.D.: acquisition of data, critical revision of the manuscript and approval of the article. R.J.L: acquisition of data, critical revision of the manuscript and approval of the article. J.P: acquisition of data, critical revision of the manuscript and approval of the article. E.M: data analysis/interpretation, critical revision of the manuscript and approval of the article. C.S: critical revision of the manuscript and approval of the article. S.B.: acquisition of data, critical revision of the manuscript and approval of the article. J.J.S.: critical revision of the manuscript and approval of the article. C.R.: acquisition of data, critical revision of the manuscript and approval of the article. N.F.: critical revision of the manuscript and approval of the article. R.G: concept/design, data analysis/interpretation, drafting of the manuscript, critical revision of the manuscript and approval of the article.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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Table 1. Number (N) and percentages (%) of Pug dogs with different congenital vertebral malformations and number of pre-sacral vertebrae identified on whole body computed tomography studies (WBCT), cervico-thoracic computed studies (C-TCT) and radiographs.

	WBCT % (affected/total)	C-TCT % (affected/total)	Radiographs % (affected/total)	Total N and %
Homeotic free	22 (11/51)	33 (15/46)	45 (15/33)	32 (41/130)
C7 cervical ribs	45 (23/51)	48 (22/46)	36 (12/33)	44 (57/130)
Transitional TL	53 (27/51)	33 (15/46)	30 (10/33)	40 (52/130)
Transitional LS	24 (12/51)	-	-	24 (12/51)
Bifid spinous process	27 (14/51)	46 (21/46)	39 (13/33)	37 (48/130)
Hemivertebra	16 (8/51)	21 (9/46)	100 (33/33)	38 (50/130)
Articular process hypoplasia/aplasia	92 (47/51)	-	-	92 (47/51)
Absent T13 vertebra	22 (11/51)	24 (11/46)	27 (9/33)	24 (31/130)
Absent L7 vertebra	33 (17/51)	-	-	33 (17/51)
26 pre-sacral vertebrae	55 (28/51)	-	-	55 (28/51)
27 pre-sacral vertebrae	45 (23/51)	-	-	45 (23/51)

Table 2. Distribution of C7 vertebra cervical ribs in Pug dogs according to gender and colour coat.

	Bilateral	Unilateral right	Unilateral left	% (affected/total number)
C7 cervical ribs	32	3	10	46 (45/97)
Gender:				
• **Female**	22	2	6	65 (30/46)
• **Male**	10	1	4	29 (15/51)
Colour coat:				
• **Fawn**	10	0	3	50 (13/26)
• **Black**	8	1	1	40 (10/25)

Figure 1. Lateral (A), ventral (B) and dorsal (C) 3D-computed tomography reconstructed images of the axial skeleton (excluding some caudal vertebrae), ribs, sternum and pelvis of a Pug dog with no congenital vertebral malformations.

Figure 2. Bar chart illustrating the number of computed tomography studies reviewed and the number and percentage of dogs with C7 homeotic transformation from the breeds included in the study.

Figure 3. Sagittal reconstructed computed tomography image of the thoracic vertebral column of a Pug dog with a vertebral body formation defect (arrow) and ventral spondylosis.

Figure 4. Transverse computed tomography image (A) and 3D-computed tomography reconstruction image (caudo-cranial oblique view-B) of the T1vertebra of a Pug dog with a bifid spinous process.

Figure 5. Transverse computed tomography image of T13 (A) and 3D-computed tomography reconstruction image (dorsal view-B) of T11, T12 and T13 vertebrae of a Pug dog. T13 vertebra is a transitional thoraco-lumbar vertebra and has two long thick transverse processes (no joints).

Figure 6. Transverse computed tomography image of L7 (A) and 3D-computed tomography reconstruction image (ventral view-B) of L6, L7 and the sacrum of a Pug dog. L7 is a lumbo-sacral transitional vertebra and has asymmetric transverse processes.

Figure 7. Two 3D-computed tomography reconstructed images (dorsal views) of T11, T12, T13, L1 and L2 vertebrae of a Pug dog. Image A illustrates normal caudal articular processes. Image B has bilateral hypoplastic/aplastic caudal articular processes at T11-T12, T12-T13 (small arrow), unilateral hypoplastic/aplastic caudal articular processes at T13-L1 (medium sized arrow), and normal caudal articular processes at L1-L2 (large arrow).

Figure 8. Transverse computed tomography images (left) and 3D-computed tomography reconstructions (right) of the C7 vertebra of multiple Pug dogs: (A) Normal vertebra (B) Vertebra with bilateral short ribs and no joint (C) Vertebra with a left long rib and no joint, and a right long rib with a joint not visible in the transverse image (D) Vertebra with bilateral short ribs and joints (E) Vertebrae with bilateral long ribs and joints.

Figure 9. Spearman correlation for the added length (cm) of left and right cervical ribs and the dorsal spinous process C7/T1 ratio, r = 0.55.