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

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29   **Abstract**

30   The number of cervical vertebrae in mammals is almost constant at seven, regardless of their

31   neck length, implying that there is selection against variation in this number. Homebox (*Hox*)

32   genes are involved in this evolutionary mammalian conservation and homeotic

33   transformation of cervical into thoracic vertebrae (cervical ribs) is a common phenotypic

34   abnormality when *Hox* gene expression is altered. This relatively benign phenotypic change

35   can be associated with fatal traits in humans. Mutations in genes upstream of *Hox*, inbreeding

36   and stressors during organogenesis can also cause cervical ribs. The aim of this study was to

37   describe the prevalence of cervical ribs in a large group of domestic dogs of different breeds

38   and explore a possible relation with other congenital vertebral malformations (CVM) in the

39   breed with the highest prevalence of cervical ribs. By phenotyping we hoped to give clues as

40   to the underlying genetic causes. Twenty computed tomography studies from at least two

41   breeds belonging to each of the nine groups recognised by the Federation Cynologique

42   Internationale, including all the brachycephalic "screw-tailed" breeds that are known to be

43   overrepresented for CVM's were reviewed. The Pug dog was more affected by cervical ribs

44   than any other breed (46%) (p<0.001) and was selected for further analysis. No association

45   was found between the presence of cervical ribs and vertebral body formation defect, bifid

46   spinous process, caudal articular process hypoplasia/aplasia and an abnormal sacrum, which

47   may infer they have a different etiopathogenesis. However, Pug dogs with cervical ribs were

48   more likely to have a transitional thoraco-lumbar vertebra (p= 0.041) and a pre-sacral

49   vertebral count of 26 (p<0.001). Higher C7/T1 dorsal spinous processes ratios were

50   associated with the presence of cervical ribs (P<0.001) supporting this is a true homeotic

51   transformation. Relaxation of the stabilizing selection has likely occurred and the Pug dog

52   appears to be a good naturally occurring model to further investigate the aetiology of cervical

53   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

77 organizers of *Hox* genes are equally crucial in the head-to-tail patterning of the vertebral

78 column

79 An extreme selection in humans against homeotic transformations of cervical vertebrae

80 occurs (ten Broek et al 2012) and the total incidence of cervical ribs in people is low (0.04-

81 6.2%) (Henderson, 1913; Spadlinski et al. 2016). Two previous studies in dogs have

82 described congenital vertebral malformations of the whole vertebral column in 145 and 228

83 cases submitted to necropsy and reported a cervical ribs prevalence that varied from 2.1% to

84 11.4% (Morgan, 1968; Breit and Kunzel 1998). These studies date from 1968 and 1998 and

85 the dog population has changed in size, genetic diversity, breed prevalence and phenotypic

86 characteristics over time (The Kennel Club 2016, Vila et al. 1999)

87 Normal dogs have seven cervical, 13 thoracic, seven lumbar, three sacral and a variable

88 number of caudal vertebrae. The first and second vertebrae present easily distinct

89 recognisable anatomical features. The rest of cervical vertebrae differ slightly from each

90 other, except for the expanded sagittal plate-like transverse processes of C6. Thoracic

91 vertebrae have shorter bodies compared to cervical and lumbar vertebrae and articulate with a

92 rib. The spinous process of the first thoracic vertebra is the longest of all vertebrae, is its most

93 conspicuous feature and its length gradually decreases with successive vertebrae (Evans and

94 de Lahunta, 2013) (**Figure 1**).

95 Congenital vertebral malformations (CVM) are relatively common in brachycephalic "screw-

96 tailed" breed dogs, such as the French bulldogs, the English bulldogs, Boston terriers and Pug

97 dogs (Gutierrez-Quintana et al. 2014; Steffen et al. 2004; Wrzosek et al. 2014). Unlike

98 CVM's in other less frequently seen regions, thoracic CVM's have been described and

99 compared in some brachycephalic dogs. Overall, Pug dogs had significantly fewer vertebral

100 body formation defects and more thoracolumbar transitional vertebrae and bifid spinous

101 process compared to the other two breeds. Despite having fewer vertebral body formation

102 defects, when present, these had greater clinical relevance. Furthermore, all French and

103 English bulldogs in the study had a normal thoracic vertebral count, while 25% of the Pug

104 dogs had 12 instead of 13 thoracic vertebrae (Ryan et al. 2017). Therefore, the Pug dog

105 appeared to be a good naturally occurring canine model to phenotype and further investigate

106 the genetic background of CVM's.

107 The aim of this study was to describe the frequency of cervical ribs in a large group of

108 domestic dogs of different breeds. We were particularly interested in breeds reported to be

109 commonly affected by CVM's and we wanted to explore a possible relation between them in

110 a naturally occurring model, providing clues as to their genetic causes. Given the high

111 number of thoraco-lumbar transitional vertebrae previously identified in Pug dogs (Ryan et

112 al. 2017), we hypothesised that a high number of cervical ribs compared to other dog breeds

113 and species would be identified. We also hypothesised that having cervical ribs would be

114 associated with a higher incidence of other CVM's.

115 **Material and methods**

116 Ethical approval from the University of Glasgow, School of Veterinary Medicine was obtained

117 for this study.

118 To quantify the frequency of cervical ribs in different breeds of domestic dogs, the medical

119 records of the Royal Veterinary College, University of Glasgow Small Animal Hospital, Vet

120 Extra Neurology and Fitzpatrick Referrals were retrospectively reviewed to identify

121 computed tomographic studies (CTs) that included the C7 vertebra regardless of their clinical

122 indication. A total of 19 breeds with at least two breeds belonging to each of the nine groups

123 recognised by the Federation Cynologique Internationale (FCI) (FCI 2017) were examined.

124 These included Basset hounds, Beagle, Border collie, Chow-chow, Cocker spaniel, German

125   shepherd dog, German pointer, Greyhound, Hungarian Vizsla, Husky, Labrador retriever,

126   Dachshund, Staffordshire bull terrier, Whippet, West Highland white terrier and all

127   brachycephalic "screw-tailed" breeds (French bulldog, the English bulldog, Boston terrier

128   and Pug dog) known to be overrepresented for CVM's were selected for analysis (Gutierrez-

129   Quintana et al. 2014; Ryan et al. 2017). A maximum of 20 randomly selected CTs were

130   reviewed for each breed. Dogs underwent imaging investigations under sedation or general

131   anaesthesia for a variety of clinical indications (related or unrelated to spinal disease). The

132   breed having the highest frequency of cervical ribs was then selected for further analysis. All

133   the available CTs that included the entire vertebral column (including or excluding coccygeal

134   vertebrae) or C7 and the thoracic region were then reviewed and the signalment (age, breed,

135   sex and colour) was recorded when available. We also retrieved and reviewed orthogonal

136   radiographic views of dogs with thoracic CVM's causing neurological deficits from the breed

137   that was selected for further analysis.

138   Computed tomographic studies and radiographs were reviewed by a board-certified

139   neurologist (RG) and a third-year neurology resident (JB). Images were displayed using an

140   open-source Workstation DICOM viewer (Osirix Imaging Sofware, v 3.9.2, Pixmeo, Geneva,

141   Switzerland). Congenital vertebral malformations were identified and classified into vertebral

142   body formation defects (commonly known as hemivertebra) (Gutierrez-Quintana et al. 2014),

143   transitional vertebrae and dorsal process formation defects (e.g. bifid spinous process or

144   fusion of the dorsal spinal process). Ambiguous vertebrae located at the border between two

145   segments of the vertebral column and possessing characteristics of both segments were

146   considered to be transitional vertebra (Simoens, 1983). The total number of pre-sacral

147   vertebrae was recorded and the sacrum was classified as being normal or abnormal. The

148   caudal articular processes from T9-L2 of whole body CTs were classified as normal or

149   hypoplastic/aplastic. All dogs were considered to have seven cervical vertebrae, even if two

150   large ribs were identified at C7. When identified on CT, cervical ribs were classified as being

151   either short (less than 1.5cm) or long (more than 1.5cm), and with or without articulation.

152   One of the key morphological differences between cervical and thoracic vertebrae is the

153   comparatively long dorsal spinous process in the latter. To test whether other aspects of the

154   C7 vertebrae took on thoracic vertebrae characteristics, the heights of the dorsal spinous

155   processes of C7 and T1 vertebrae were measured and the C7/T1 dorsal spinous process

156   height ratio was calculated. When identified on radiographs, the presence or absence of

157   cervical ribs was recorded with no further sub classification. Thoraco-lumbar vertebrae were

158   considered thoracic if they were transitional (including transverse process asymmetry) and

159   lumbar if they had bilateral and normal size symmetric transverse processes. Dogs in which

160   L7 was considered to have fused with the sacrum (absent intervertebral disc) were considered

161   to have six lumbar vertebrae.

162   **Statistical Analysis**

163   All analyses were done using SAS 9.4 (SAS Inst. Inc., Cary, NC). Data was analysed using

164   non-parametric methods (Wilcoxon-Mann-Whitney, Kruskal-Wallis and Chi-square/Fisher

165   tests, and Spearman correlation) except for the analysis of C7/T1 dorsal spinous process ratio

166   depending on the presence of transitional vertebrae, cervical ribs and joints. The later was

167   analysed using t-test. Alpha level for determination of significance was 0.05 and multiple

168   comparison were analysed using Bonferroni correction. Given that CT provides better bone

169   definition compared to radiography, data from orthogonal radiographic views of dogs with

170   thoracic CVM's causing neurological deficits was analysed separately and only to study the

171   relation between CVM's.

172   **Results**

173   *Frequency of cervical ribs in domestic pure breed dogs*

174   Twenty CTs were avaiable for review from each of the breeds mentioned above except for

175   the Husky (n=15), the Basset hound (n=15), the German pointer (n=13), the Miniature

176   Snauchzer (n=6) and the Chow-chow (n=4). The percentage of dogs with cervical ribs from

177   each of the reviewed breeds is illustrated in **Figure 2**. Cervical ribs were more common in the

178   Pug dog compared to any other breed and was therefore selected for further anaylsis (45/97-

179   46%) (P<0.001). The overall prevalence of cervical ribs was 14% (61\430). Excluding the

180   Pug dog the percentage decreased to 4.8% (16/333).

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

182   A total of 51 CTs of the whole vertebral column and 46 cervico-thoracic CTs from Pug dogs

183   with no clinical signs caused by CVM's (scanned for respiratory signs (n=81), mestastic

184   check (n=6), orthopaedic problems (n=6) and gastrointestinal signs (4)) and 33 thoracic

185   radiographs from Pug dogs with neurological deficits caused by CVM's (radiographed for

186   T3-L3 myelopathy) Pug dogs were available and reviewed (n=130 Pug dogs).

187   A total of 57/130 Pug dogs had cervical ribs, 50/130 had vertebral body formation defects

188   (**Figure 3**), 48/130 had bifid spinous process (without myelomeningocele) (**Figure 4**), 52/130

189   had a thoraco-lumbar transitional vertebra (**Figure 5**), 12/51 had a lumbo-sacral transitional

190   vertebra (**Figure 6**) and 47/51 had caudal articular process hypoplasia/aplasia (**Figure 7**).

191   T13 was absent in 31/130 and L7 was absent in 17/51. Out of 51 whole vertebral column

192   CTs, twenty-eight (55%) had 26 pre-sacral vertebrae, 23 (45%) had 27 pre-sacral vertebrae

193   and eleven had no transitional vertebrae (**Table 1**).

194   Mean and median age were 38 and 31 months respectively. Sixty-one Pug dogs were female

195   and 69 were male. Females (30/45-65%) were affected more frequently by cervical ribs than

196   males (15/45-29%) (P=0.012). Coat colour was only available for Pug dogs with a whole-

197   body CT. No association between coat colour and the presence of any transitional vertebrae,

198   vertebral body formation defects or a bifid spinous process was identified. In most cases,

199   cervical ribs were bilateral (32/45-71%). Among cases with unilateral changes (13/45-29%),

200   the left (10/13-77%) side was more frequently affected compared to the right (3/13-23%)

201   (P=0.04; **Table 2**). Cervical ribs were classified as short with no joint in 35% (27/77) of the

202   cases, long with no joint in 2.5% (2/77), short with a joint in 34% (26/77) and long with a

203   joint in 28.5% (22/77) of the cases (**Figure 8**).

204   No association was found between the presence of vertebral body formation defects,

205   transitional vertebra, bifid spinous process, caudal articular process hypoplasia/aplasia and an

206   abnormal sacrum in the CTs of Pug dogs with no clinical signs associated to CVM's or in the

207   radiographs of Pug dogs with CVM's causing neurological deficits. Pug dogs in which

208   cervical ribs were identified on CTs were more likely to have a thoraco-lumbar transitional

209   vertebra (P= 0.041) than those without cervical ribs, though this was not replicated in our

210   analysis of radiographs. Pug dogs with cervical ribs (P=0.025) or a lumbo-sacral transitional

211   vertebra (P<0.001) were also more likely to have a pre-sacral vertebral count of 26 instead of

212   27. The pre-sacral vertebral count was not associated with the presence of hemivertebrae,

213   bifid spinous process or an abnormal sacrum. Longer C7 dorsal spinal processes and higher

214   C7/T1 ratios were associated with the presence of cervical ribs (P<0.001) and were correlated

215   (r=0.44 and r = 0.55 respectively) with the added length of left and right cervical ribs (**Figure**

216   **9**). Higher C7/T1 ratios were also associated with having fewer vertebrae affected by caudal

217   articular process hypoplasia/aplasia (r = -0.36; P = 0.010).

218   **Discussion**

219 The previously reported prevalence of cervical ribs in domestic dogs varied from 2.1% to

220 11.9% (Morgan, 1968; Breit and Kunzel 1998). The present study investigated a larger

221 number of dogs equally distributed between breeds and found an overall similarly low

222 prevalence, but identified the Pug dog as being significantly more affected than any other

223 breed. The German Shepherd (15%), the Hungarian Vizsla (15%) and the Boxer (10%) also

224 exhibited unusually high percentages of cervical ribs.

225

226 It has been proposed that cervical ribs may be largely composed of ossification sites that

227 disappear after birth (Chernoff and Rogers, 2004). One of the key morphological differences

228 between cervical and thoracic vertebrae is the comparatively long dorsal spinous process in

229 the latter. Longer C7 dorsal spinous processes and high C7/T1 dorsal spinous process height

230 ratios were associated with the presence of cervical ribs in this study, suggesting this is a true

231 homeotic transformation of a cervical vertebra into a thoracic one and not an unmasking of

232 vestigial cervical ribs left behind in evolution. The Spearmen correlation between C7/T1 ratio

233 and the added length of left and right cervical ribs was moderate, suggesting that longer

234 cervical ribs are not always associated with longer C7 dorsal spinous process. In combination

235 with the length of the cervical ribs, this ratio may be used as an objective measure of the

236 degree of C7 homeotic transformation.

237 In humans, bilateral cervical ribs are the most common; and when unilateral, they are

238 traditionally considered to be more frequently left-sided. Although inconsistent results have

239 been reported, females seem to be more frequently affected (Furtado et al. 2011; Galis et al

240 2006; Bots et al. 2011, Schumacher et al. 1992). The results of this study were in accordance

241 with these findings. Deficient signalling in mice leads to delayed somite formation on the

242 right side, causing a diminished coordination between the development of the left and right

243   somites, and leading to a differential expression of the *Hox* genes (Vermot et al. 2005;

244   Griesinger et al. 2005).

245   Mammals under harsh conditions and stressors like hyperthermia or exposure to boric acid

246   during organogenesis (Chernoff and Rogers, 2004; Steigenga et al. 2006; Wery et al. 2003, Li

247   and Shiota, 1999) and mutations that influence *Hox* gene expression can also cause cervical

248   ribs. T-gene (also known as brachyury) knockdown in vivo experiments cause T1

249   transformation of cervical vertebrae in 30% of mice (Yamaguchi, 1999; Pennimpede et al.

250   2012) and a spontaneous mutation in this same gene, has been associated with a cervical

251   vertebral homeotic transformation in the *Bos Taurus* with vertebral and spinal dysplasia

252   (VSD) (Kromic et al. 2015). Data suggests that a distinct amino acid position (p.66) in the *T-*

253   *gene* is relevant for a coordinated Wnt–brachyury–HOX signaling cascade, which is

254   important for cervical vertebral and spinal cord development (Galis, 1999; Yamaguchi, 1999;

255   Pennimpede et al. 2012). To the authors' knowledge, no other spontaneous mutation in

256   mammalian genes causes a homeotic transformation of cervical vertebrae similar to VSD.

257   Some familial cases of cervical ribs with a probable autosomal-dominant mode of inheritance

258   have been reported in human literature (Weston, 1956; Schapera 1987). A high incidence of

259   cervical ribs has been observed in mammals with a high rate of inbreeding (e.g 33% in

260   mammoths before extinction (Reumer et al. 2014), 11% in minipigs (KD, 1998) or 7.5% in

261   humans (Palma and Carine, 1990)). Pedigrees were not available for analysis in any of the

262   dogs included in this study; and therefore, the mode of inheritance or the degree of

263   inbreeding could not be studied. Familial cases could potentially help determine the genetic

264   basis of cervical ribs.

265   Children with neuroblastoma, brain tumours, leukaemia, soft tissue sarcoma, Wilms tumour,

266   and Ewing sarcoma also have a high incidence of cervical ribs. Pug dogs are not known to be

267 overrepresented for any of the above (Gough and Thomas, 2010). Cervical ribs have also

268 been associated with human stillbirths (Furtado et al. 2011; ten Broek et al 2012). Among the

269 100 most popular breeds in a Norwegian dog population, the Pug dog ranked eighth highest

270 for stillbirths and fourth highest for total perinatal mortality risk at the individual puppy.

271 Mean litter size was like other similar size dog breeds (Parker et al. 2017; Tonnessen et al.

272 2012). Our study was based on the United Kingdom Pug dog population and the prevalence

273 of cervical ribs in the Norway Pug dog population is currently unknown, making

274 comparisons difficult. Significant associations between congenital anomalies and cervical

275 ribs in stillborn human foetuses have been found (ten Broek et al 2012). One study did not

276 identify this association; however, an association with aneuploidy was identified, implying

277 that congenital anomalies would have been detected if they had survived to be born (Furtado

278 et al. 2011). We only evaluated the available images for the presence of other CVM's and not

279 for other major anomalies (renal agenesis, ventricular septum defects, anal atresia, etc.).

280

281 Most human adults with cervical ribs are asymptomatic, but some develop thoracic outlet

282 syndrome (TOS) (Chang et al. 2013). Thoracic outlet syndrome represents a group of distinct

283 disorders that result from the compression of one or more neurovascular elements as they

284 traverse the thoracic outlet. Unlike humans in which the brachial plexus and the subclavian

285 artery pass through the interscalene triangle, which is formed by first rib and the ventral and

286 middle components of the scalene muscles (Ferrante and Ferrante, 2017); in domestic

287 mammals, the ventral scalene muscle is not separated from the middle component and the

288 interscalene triangle is not formed (Evans and de Lahunta, 2013). The scalene muscles can

289 play an important role in the pathophysiology of this syndrome (Ferrante and Ferrante,

290 2017a), of which some forms typically occur in athletes after vigorous activity (Ferrante and

291 Ferrante, 2017b). None of the Pug dogs in our study presented with clinical signs suggestive

292   of TOS. To the authors' knowledge, TOS has not been reported in dogs, and the anatomical

293   difference between species and the fact that that Pug dogs are not too athletic may be some of

294   the reasons why; however, given the high prevalence of cervical ribs within the breed,

295   clinicians should bear in mind this differential diagnosis in Pug dogs with cervical ribs and

296   compatible clinical signs.

297   Even though they do not usually cause clinical signs in humans, cervical ribs and transitional

298   lumbo-sacral vertebra are associated with dermatomal variations and alterations in function

299   (Makhoul and Machleder, 1992; Roos, 1996; Seyfert 1997; Kim et al. 2008). Lumbo-sacral

300   transitional vertebrae can predispose dogs to cauda equina syndrome (Fluckiger et al. 2006),

301   asymmetrical hip joint development and more severe hip dysplasia (Komsta et al. 2015;

302   Fluckiger et al. 2017). The prevalence of lumbo-sacral transitional vertebrae in some dog

303   breeds has been reported as high as 22%; however, the overall prevalence is low (3.5%)

304   (Damur-Djuric et al. 2006). Despite high prevalence of lumbo-sacral transitional vertebrae

305   (12/51-24%), none of the Pugs dogs in our study presented with clinical signs suggestive of

306   hip dysplasia and/or cauda equina syndrome. Pug dogs are predisposed to hip dysplasia, with

307   an approximate prevalence of 70% (OFA 2016). To the author's knowledge, the Pug dog is

308   not predisposed to cauda equina syndrome (Meij and Bergknut 2010). If spinal stenosis

309   occurs in the absence of clinical signs, and how clinically significant hip dysplasia is in Pug

310   dogs, remains to be explored.

311   The prominent and sharp ventral process of C1, the large transverse processes of C6, the first

312   and last rib, the first lumbar transverse process and the last lumbar vertebra are all useful

313   anatomical landmarks for localisation during the surgical approach to vertebral column in

314   dogs (Sharp and Wheeler 2005). Two out of 51 (4%), 8/51 (16%) and 23/51 (45%) of Pug

315   dogs with a whole body CTs were considered to have a C5, a C6 and a C7 abnormal

316   transverse process morphology, respectively, and 52/130 (40%) were considered to have a

317   thoraco-lumbar transitional vertebra. Given the frequency of altered anatomical surgical

318   landmarks, special consideration should be taken when using them to localise during surgery

319   in pug dogs.

320   No association was found between the presence of vertebral body formation defects,

321   transitional vertebrae, bifid spinous process or an abnormal sacrum, and this may reflect a

322   different etiopathogenesis. However, an association between cervical ribs and thoracolumbar

323   transitional vertebrae was identified using CT. A possible reason why this was not significant

324   when analysing radiographs is that CT provides better bone definition compared to

325   radiography, and the presence of cervical ribs could had been underestimated. In sloths,

326   cervical disruptions occur coincident with abnormalities at the thoraco-lumbar, lumbo-sacral,

327   and sacro-caudal boundaries. In people, significant associations between cervical ribs and

328   sacralisation (Erken et al. 2002) and thoraco-lumbar transitional vertebrae have been found

329   (Bots et al. 2011). Thoraco-lumbar transitional vertebrae have also been associated with a

330   higher incidence of concomitant lumbo-sacral transitional vertebrae (Carrino et al. 2011) and

331   vice versa (Nakajima et al. 2014).

332   A total of 49 bifid spinous processes were identified in 47/130 (36%) Pug dogs. This

333   prevalence is in accordance with a previous study in which bifid spinous process was

334   identified exclusively at T1 in Pug dogs (Ryan et al. 2017). One Pug dog in our study had

335   bifid spinous process at C5 and C6 and another Pug dog had bifid spinous process at T5. It is

336   currently unclear why bifid spinous process occurs more frequently at T1 and what the

337   clinical relevance of this finding may be. A higher than normal incidence of cervical ribs has

338   been previously identified in foetuses and infants with open spina bifida (myelomeningocele)

339   (5/112-4.5%) and spina bifida (4/10-40%), suggesting a possible relation between both

340   malformations (Galis et al. 2006; Naik et al. 1978). Our study identified no association

341   between bifid spinous process and cervical ribs.

342   Twenty-eight out of 51 (55%) Pug dogs had 26 pre-sacral vertebrae instead of the fixed 27

343   pre-sacral vertebrae common to all the canine species (de Lahunta et al. 2015). Having 26

344   pre-sacral vertebrae was associated with the presence of cervical ribs or a lumbo-sacral

345   transitional vertebra. In humans, the presence of lumbo-sacral transitional vertebra is

346   associated with an anomalous total number of vertebrae, but the presence of thoraco-lumbar

347   transitional vertebra is not (Carrino et al. 2011). The mechanisms of regulation of the number

348   of segments in the vertebral column and its associations with homeotic transformations are

349   still not well understood (Gomez and Pourquie 2009).

350   Hypoplasia or aplasia of the T9-L2 caudal articular processes are thought to sometimes lead

351   to the formation of fibrous tissue and cause a constrictive myelopathy along these spinal cord

352   segments in Pug dogs (Fisher et al. 2013, Breit, 2002). The association between cervical ribs

353   and thoraco-lumbar transitional vertebra identified in our study supports the previously

354   demonstrated fact that cervical ribs appear to be accompanied by homeotic changes of several

355   adjacent cervical and thoracic vertebrae, and sometimes all thoracic vertebrae (ten Broek et al

356   2012; Oostra et al 2005). As a result, the normal vertebral anatomy, including the caudal

357   articular processes of T9-T13, could be altered. However, we found no association between

358   the presence of caudal articular process hypoplasia/aplasia, the presence of vertebral body

359   formation defects, transitional vertebra at any location or bifid spinous process. Furthermore,

360   higher C7/T1 dorsal spinous process ratios were associated with having fewer vertebrae

361   affected by caudal articular process hypoplasia/aplasia, but the correlation coefficient was

362   low. The reasoning behind this finding remains unknown.

363   Mutations in *Hox* genes are almost absent in humans, other than a few mutations in caudal

364   *Hox* genes (e.g. HoxA11, Hox A13, HoxD10, HoxD13) (Kosaki et al. 2002, Dobbs et al.

365   2006) and supposedly because most mutations are lethal at a very young stage. As supported

366   by this study, cervical ribs are rarely the only homeotic change along the vertebral column

367   and are often associated with shifts of the thoraco-lumbar and lumbo-sacral boundary

368   (Varela-Lasheras et al. 2011). This combination of homeotic transformations can be well

369   explained by mutations of genes upstream of *Hox* (Alkema et al 1995, Ikeya and Takada

370   2001, van den Akker et al 2002). Alternatively, it can be caused by mutations in several *Hox*

371   genes at the same time, which is unlikely, given that such mutations are already not found in

372   a single *Hox* gene. Therefore, it is more likely that mutations in genes upstream of *Hox* genes

373   are involved in the induction of cervical ribs in dogs and other mammals.

374

375   To the authors' knowledge, no previous CT based study in veterinary literature including a

376   large number of dogs has described CVM's of the whole vertebral column (excluding

377   coccygeal vertebrae) in any specific breed. Due to the high percentage of altered pre-sacral

378   vertebral counts and the high number and variety of CVM's identified in the Pug dog, it

379   appears to be a good naturally occurring model to further investigate the aetiology of CVM's

380   and numerical alterations.

381

382   **Conclusion**

383   Despite the high prevalence of C7 homeotic transformation, affected Pug dogs survive to

384   adulthood, and reproduce; whereas most conceived individuals with a deviation number of

385   cervical vertebrae die prenatally, or as infant (ten Broek et al 2012). We hypothesise that

386   stabilizing selection relaxed due to human care and the pleiotropic constraints broke to allow

387   the evolution of novelties in body plans. Sloths, manatees, *Bos taurus* with VDS and Pug

388 dogs are unique examples of mammals with a naturally occurring exceptional number of

389 cervical vertebrae. Further research is required to answer questions regarding the aetiology

390 and frequent natural occurrence of the different CVM's and numerical anomalies of the axial

391 skeleton in Pug dogs.

392 **Author contributions**

393 J.B.: concept/design, acquisition of data, data analysis/interpretation, drafting of the

394 manuscript, assembled figures, critical revision of the manuscript and approval of the article.

395 S.D.D.: acquisition of data, critical revision of the manuscript and approval of the article.

396 R.J.L: acquisition of data, critical revision of the manuscript and approval of the article. J.P:

397 acquisition of data, critical revision of the manuscript and approval of the article. E.M: data

398 analysis/interpretation, critical revision of the manuscript and approval of the article. C.S:

399 critical revision of the manuscript and approval of the article. S.B.: acquisition of data,

400 critical revision of the manuscript and approval of the article. J.J.S.: critical revision of the

401 manuscript and approval of the article. C.R.: acquisition of data, critical revision of the

402 manuscript and approval of the article. N.F.: critical revision of the manuscript and approval

403 of the article. R.G: concept/design, data analysis/interpretation, drafting of the manuscript,

404 critical revision of the manuscript and approval of the article.

405

406 **Conflict of interest statement**

407 None of the authors has any financial or personal relationships that could inappropriately

408 influence or bias the content of the paper.

409

410 An abstract with some of the results of this study was submitted to the European College of

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666 **Table 1**. Number (N) and percentages (%) of Pug dogs with different congenital vertebral

667 malformations and number of pre-sacral vertebrae identified on whole body computed

668 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)

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

678   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)

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694 **Figure 1.** Lateral (A), ventral (B) and dorsal (C) 3D-computed tomography reconstructed

695 images of the axial skeleton (excluding some caudal vertebrae), ribs, sternum and pelvis of a

696 Pug dog with no congenital vertebral malformations.
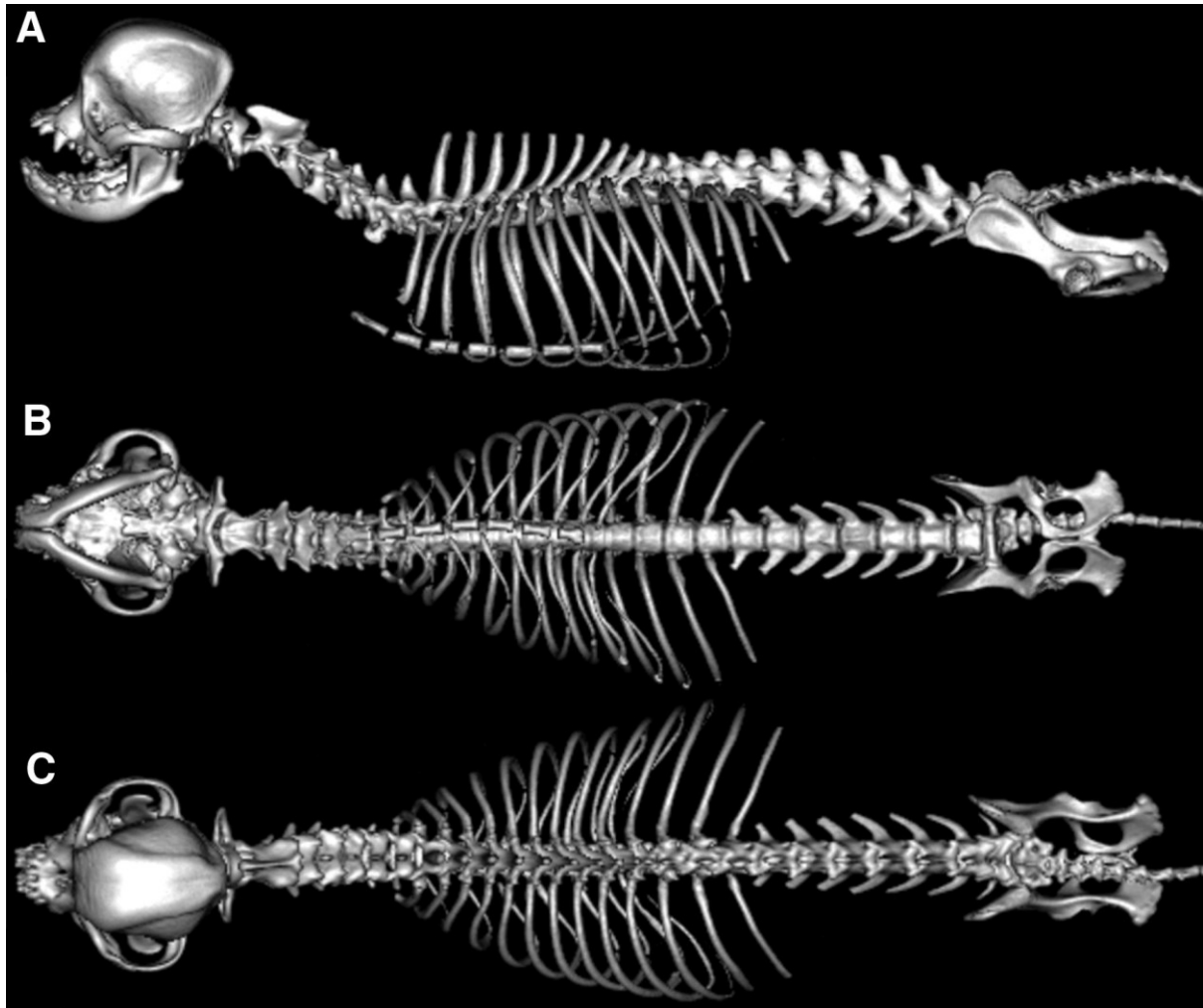
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700   **Figure 2.** Bar chart illustrating the number of computed tomography studies reviewed and

701   the number and percentage of dogs with C7 homeotic transformation from the breeds

702   included in the study.

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705   **Figure 3.** Sagittal reconstructed computed tomography image of the thoracic vertebral

706   column of a Pug dog with a vertebral body formation defect (arrow) and ventral spondylosis.
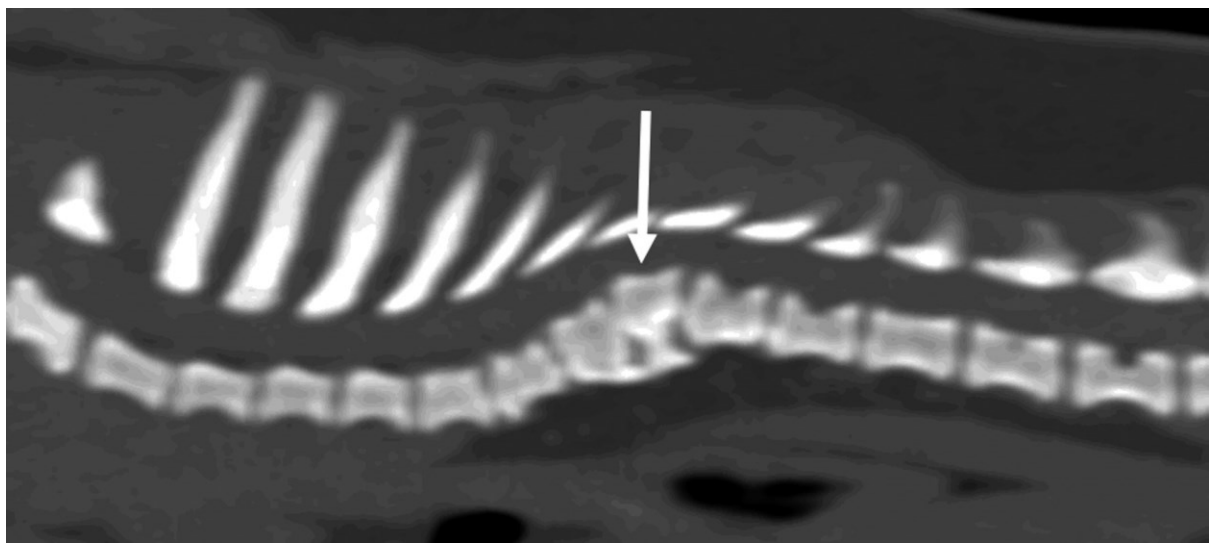
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709   **Figure 4.** Transverse computed tomography image (A) and 3D-computed tomography

710   reconstruction image (caudo-cranial oblique view-B) of the T1vertebra of a Pug dog with a

711   bifid spinous process.
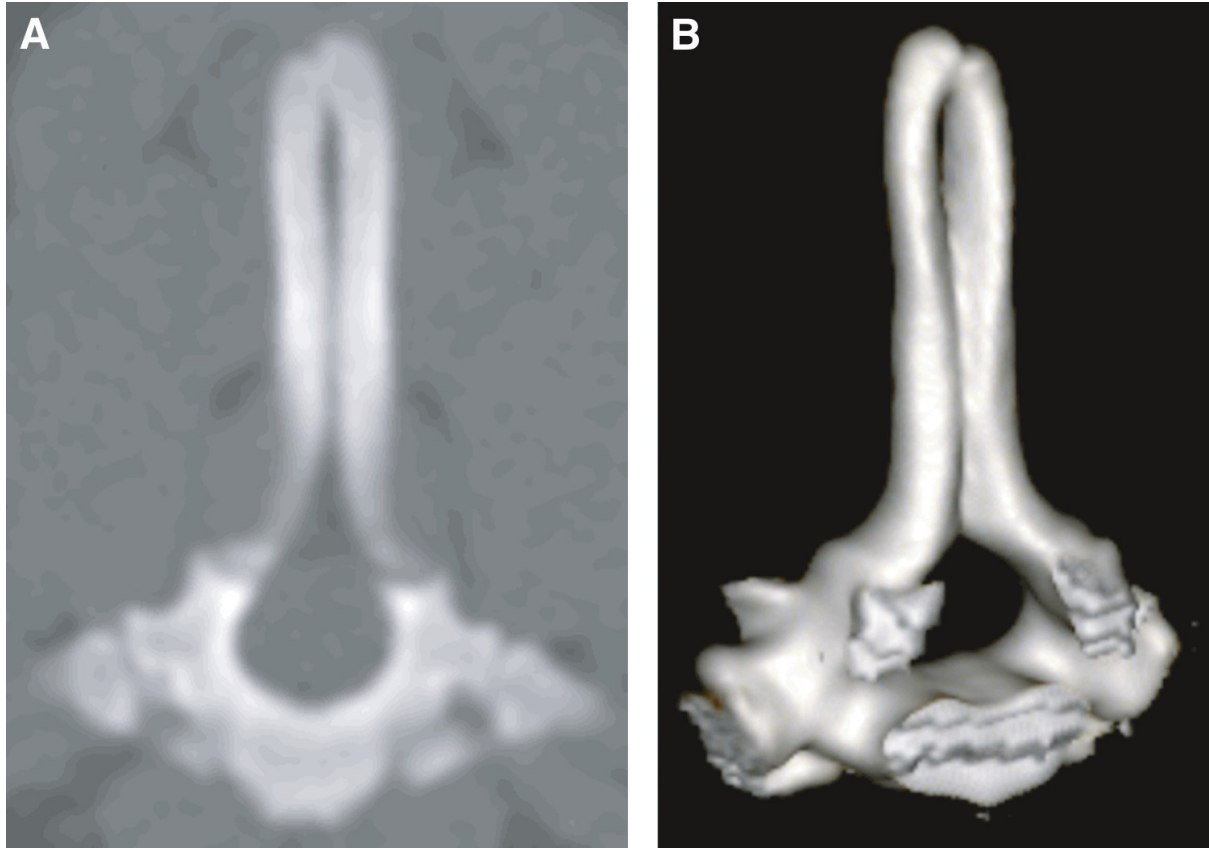
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715 **Figure 5.** Transverse computed tomography image of T13 (A) and 3D-computed tomography

716 reconstruction image (dorsal view-B) of T11, T12 and T13 vertebrae of a Pug dog. T13

717 vertebra is a transitional thoraco-lumbar vertebra and has two long thick transverse processes

718 (no joints).
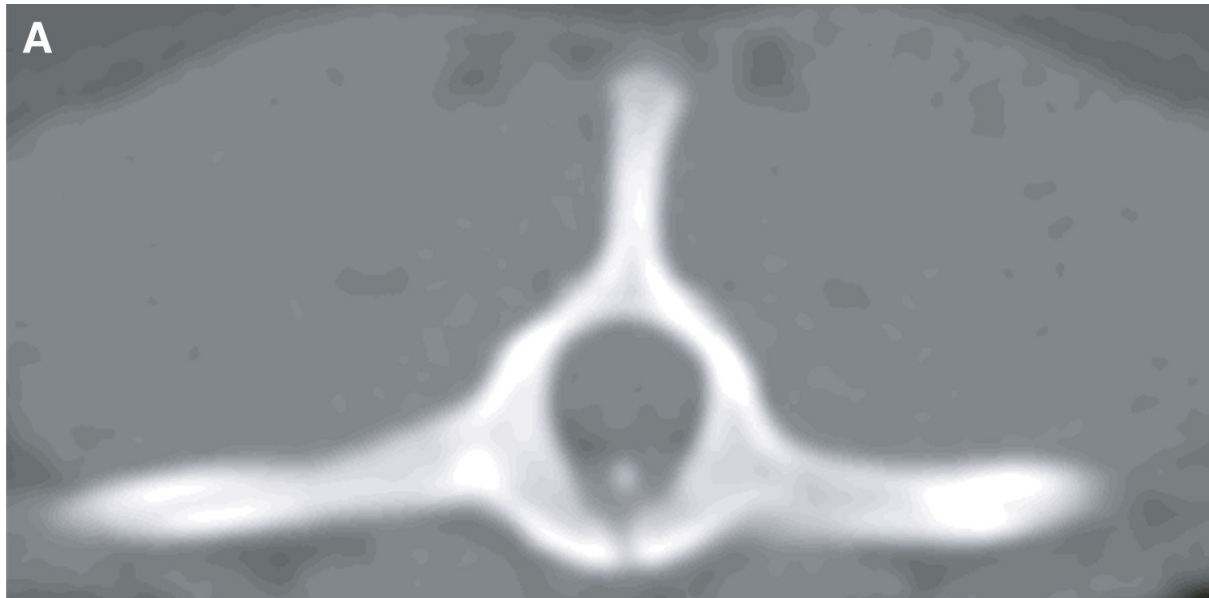
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722   **Figure 6**. Transverse computed tomography image of L7 (A) and 3D-computed tomography

723   reconstruction image (ventral view-B) of L6, L7 and the sacrum of a Pug dog. L7 is a lumbo-

724   sacral transitional vertebra and has asymmetric transverse processes.
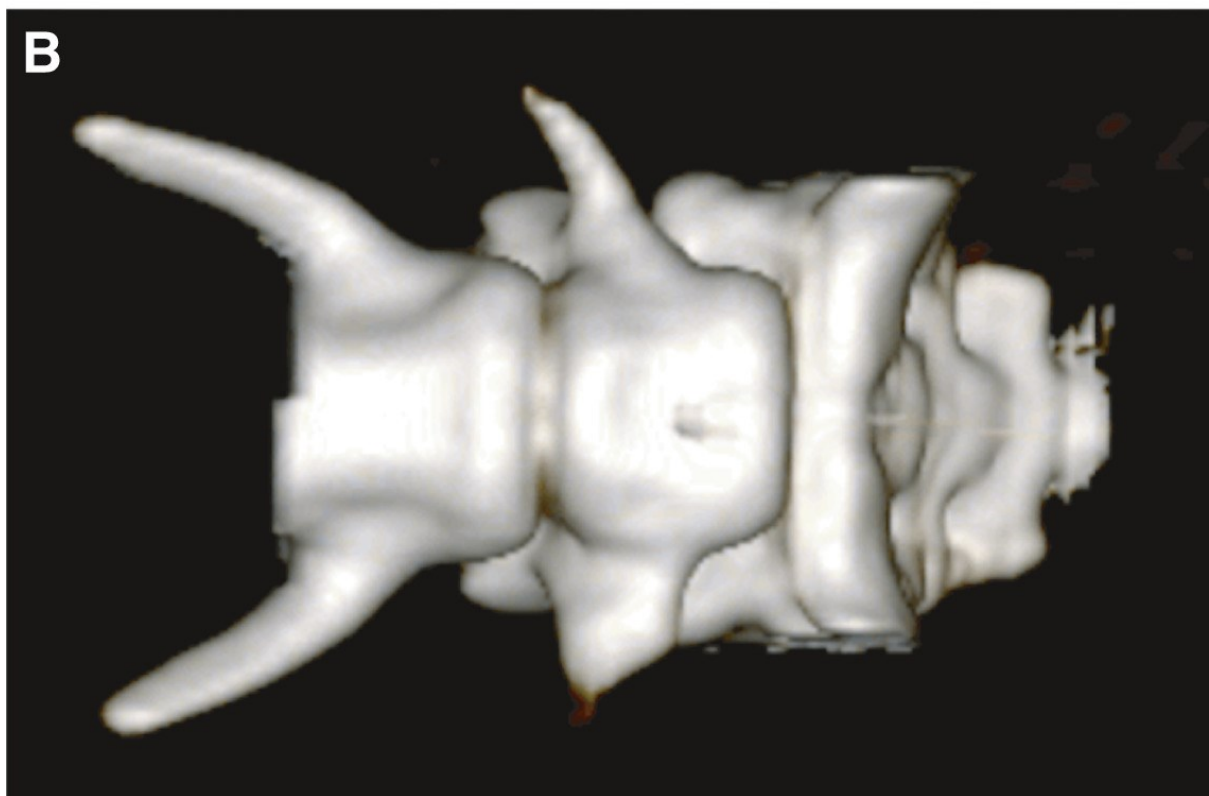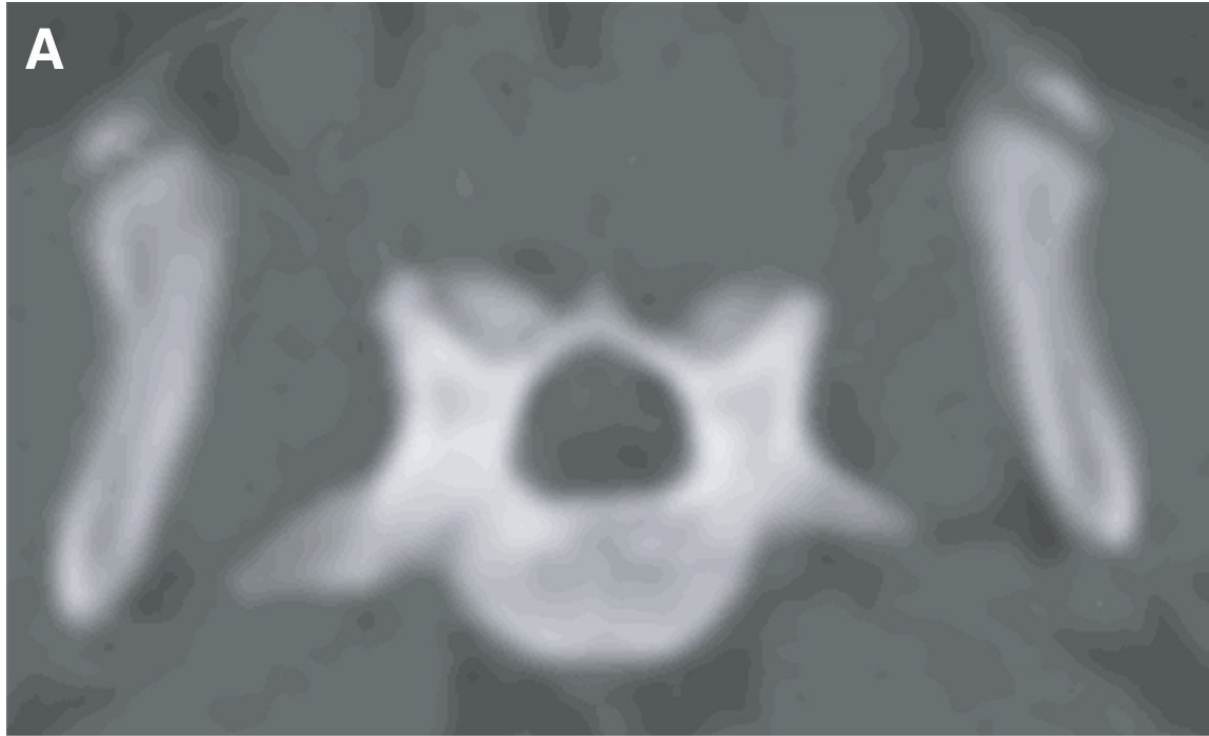
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727 **Figure 7**. Two 3D-computed tomography reconstructed images (dorsal views) of T11, T12,

728 T13, L1 and L2 vertebrae of a Pug dog. Image A illustrates normal caudal articular processes.

729 Image B has bilateral hypoplastic/aplastic caudal articular processes at T11-T12, T12-T13

730 (small arrow), unilateral hypoplastic/aplastic caudal articular processes at T13-L1 (medium

731 sized arrow), and normal caudal articular processes at L1-L2 (large arrow).
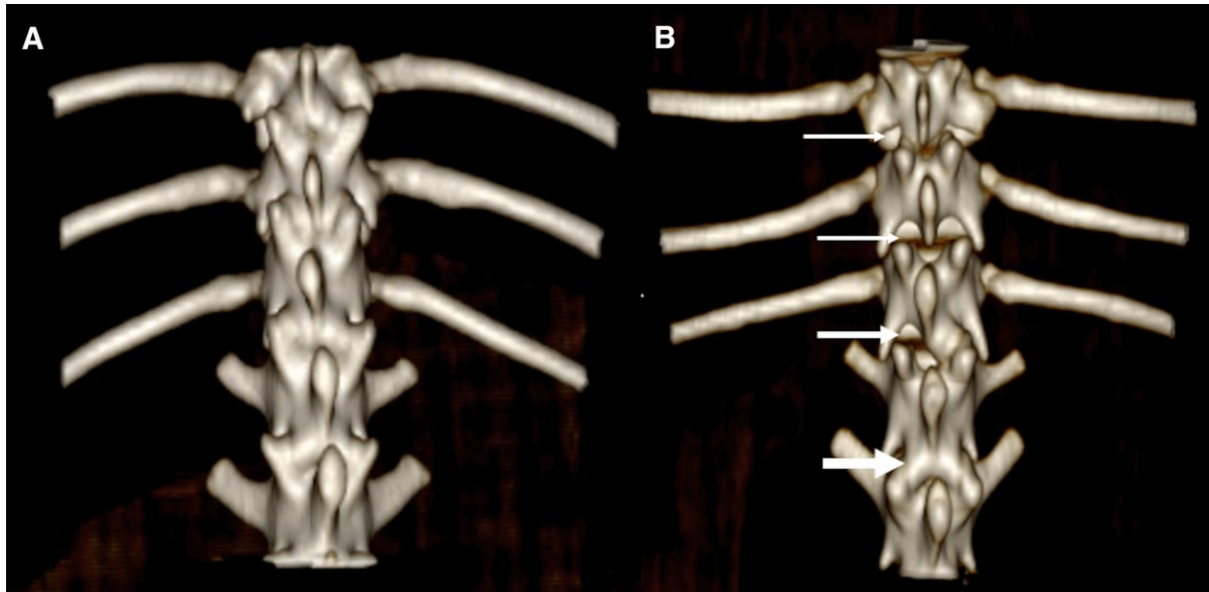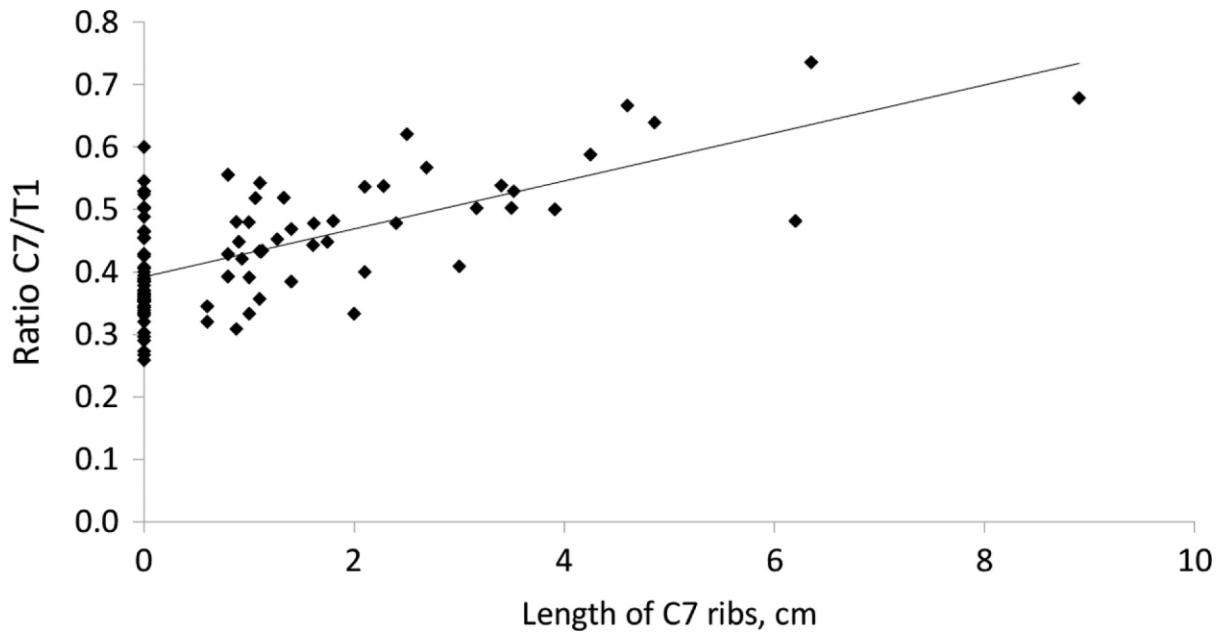

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735　**Figure 8.** Transverse computed tomography images (left) and 3D-computed tomography

736　reconstructions (right) of the C7 vertebra of multiple Pug dogs: (A) Normal vertebra (B)

737　Vertebra with bilateral short ribs and no joint (C) Vertebra with a left long rib and no joint,

738　and a right long rib with a joint not visible in the transverse image (D) Vertebra with bilateral

739　short ribs and joints (E) Vertebrae with bilateral long ribs and joints.

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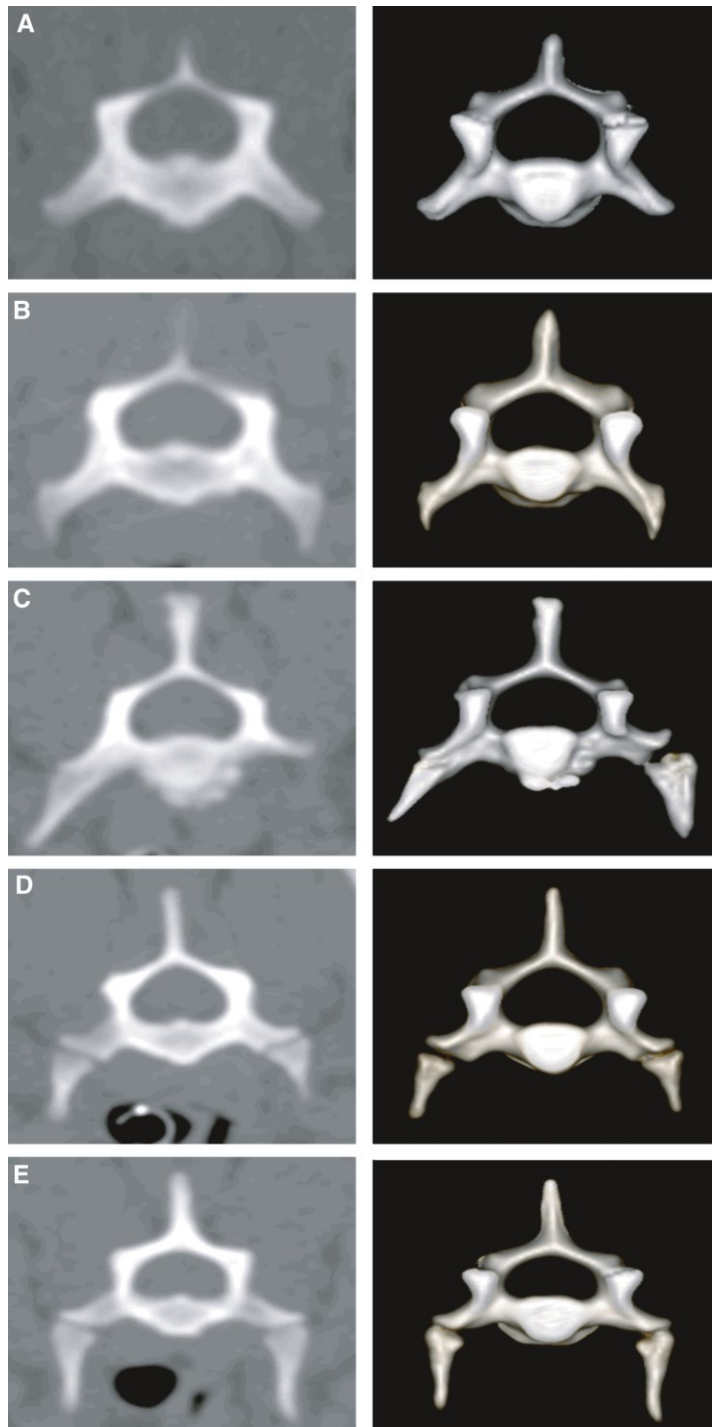
741   **Figure 9.** Spearman correlation for the added length (cm) of left and right cervical ribs and

742   the dorsal spinous process C7/T1 ratio, r = 0.55.

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