Non-Syndromic Cleft Palate and Transverse Limb Deficiency Segregating in a Family of Dogs

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Abstract

Oro-facial clefts are one of the most common birth defects in humans, most are non-syndromic, and few have established molecular diagnoses. Here we report the morphology and genetic transmission of isolated cleft palate in a naturally occurring dog model. Palate morphology was evaluated grossly, by microcomputed tomography, and by histologic examination of serial coronal sections. In repeated matings of a clinically normal sire/dam pair, 18% (12/68) of live-born pups had full-length cleft of the secondary palate with no other abnormalities. At the gestational stage of normal palate fusion, palate shelves of affected fetuses were above the tongue but did not meet at midline. Mandibles were normal, and oral epithelium and periderm were intact. Genetic transmission was determined in experimental backcross matings of surgically repaired affected dogs with a normal parent, which produced 20 cleft, 11 male and 9 female, and 24 normal-palate pups. Furthermore, all offspring of matings between affected dogs had cleft palate. These data were as expected under the hypothesis of autosomal recessive transmission of the cleft palate trait ([1 df, N = 44] $\chi^2 = 0.36$, $p = 0.55$). About half of cleft offspring produced in backcross matings of which the dam had cleft palate, also exhibited various transverse limb deficiencies. No limb deficiencies occurred in backcross offspring of a dam with normal palate, suggesting a possible maternal effect. This dog family constitutes a large animal model of non-syndromic isolated cleft palate coincident with developmental limb deficiency.

Introduction

Orofacial clefts (OFC) are a group of craniofacial developmental defects that are common in humans and cause significant medical, psychosocial, and financial burdens (Wehby and Cassell 2010). OFC are subdivided into cleft lip only (CLO), cleft lip with or without cleft palate (CL/P) and cleft palate only (CPO). OFC occur either alone or as a feature of various syndromes involving other organ systems. Nonsyndromic CL/P occurs in 1 in ~ 500 to 1 in ~ 2,500 live births, depending on the geographical, racial, and ethnic distribution of the study population (Dixon et al. 2011; Leslie and Marazita 2013). Defects leading to CL/P originate in embryonic development of the upper lip primordia and primary palate (Jiang et al. 2006). Nonsyndromic CPO occurs in 1 in ~ 1,500 live births, and defects leading to CPO originate in failed emergence or fusion of palate shelves to form the secondary palate. There is some overlap of risk factors for each, but CL/P and CPO seldom occur in the same family lines (Dixon et al. 2011; Beaty et al. 2016; Carlson et al. 2019; Huang et al. 2019; Ray et al. 2021). About 40 genetic loci have been associated with nonsyndromic CL/P, and about 10 have been associated with nonsyndromic CPO, but these explain less than a quarter of the estimated total risk of OFC heritability (Carlson et al. 2019; Ludwig et al. 2017; He et al. 2020).

Limb reduction defects (LRDs) are congenital anomalies exhibiting absent or severely hypoplastic structures of one or more limbs. LRD as a group occur in 1 in ~ 1,250-2,200 infants and are often associated with malformations in other organ systems (Froster-Iskenius and Baird 1989; McGuirk et al. 2001; Stoll et al. 2010; Bergman et al. 2020; Elsner et al. 2021; Holmes 2021). Causes of LRD include Mendelian disorders, chromosomal anomalies, teratogen exposure, and presumed vascular disruption

events, but the majority of LRD are unexplained in most surveys. Like the OFC, LRDs are subdivided into categories of anatomical deformity, corresponding in some degree to the developmental stage or process gone awry (Gold et al. 2011). Terminal transverse limb deficiency, or just transverse limb deficiency (TLD), indicates interruption of development along the proximal to distal axis of the limb, such that from the point of amputation all distal portions of the limb are hypoplastic or missing entirely while the more proximal portions are intact (Holmes 2021). Most occur in the pectoral limbs, and points of amputation are typically at the glenohumeral, brachial-antebrachial, antebrachial-carpal, metacarpal-phalangeal, and interphalangeal joints (Gold et al. 2011). TLD is a defining characteristic of Adams-Oliver syndrome, a rare inherited disorder (Lehman and Patel 2016), but evidence suggests a vascular disruption etiology in some patients exhibiting isolated TLD (Graziano et al. 2009; Sadler and Rasmussen 2010; Ordal et al. 2016; Holmes et al. 2018).

OFC and LRD can be co-morbidities in humans and animals, particularly when caused by teratogen exposure (Kochhar et al. 1984; Hurst et al. 1995; Wyszynski and Beaty 1996; Hackshaw et al. 2011). Environmental risk factors common to OFC and the TLD subgroup include maternal factors of suboptimal periconceptional folate consumption (Wyszynski and Beaty 1996; Botto et al. 2004) and teratogen exposures leading to fetal tissue hypoxia, such as maternal tobacco smoking or treatment for hypertension in the first trimester (Wyszynski and Beaty 1996; Scott, Jr 1983; Czeizel et al. 1994; Webster and Abela 2007). We have been investigating nonsyndromic CPO in an outbred colony of research dogs. Here we present characterization of the pathology and results of experimental matings consistent with simple autosomal recessive inheritance of CPO in this family. During breeding experiments some offspring from cleft-affected dams born with CPO also exhibited TLD. While unexpected, the type of limb deficiency and pattern of occurrence suggest various models of causation of both phenotypes.

Methods

Dogs were maintained by personnel of Campus Animal Resources of Michigan State University in a breeding colony for genetic disease research. The average vivarium census over the course of this study included ~ 30 breeding females, of which 6 were in the CPO line. The CPO line were mixed-breed dogs with genetic contributions from curly coated retrievers, giant schnauzers, beagles, and mongrels. Protocols for animal use were approved by the MSU Institutional Animal Care and Use Committee. Dog matings were either brother/sister intercrosses or backcrosses (affected dog X normal parent) performed either by natural or artificial insemination. Palate fusion of newborn pups was determined by physical examination of the oral cavity. Affected pups and littermate controls euthanized on the first postnatal day were processed variously for histologic analysis or X-ray computed microtomography (μ CT) of the head. Processing for light microscopic and immunohistochemical evaluation was as previously described (Freiberger et al. 2021). In some matings, day 0 of gestation was determined by serial measurements of pre-ovulatory serum progesterone concentration, as previously described (Freiberger et al. 2021), and fetal pups were removed by cesarean section on day 39 (d39). Immediately upon removal from the uterus, fetal pups were placed in ice-cold sterile saline, examined under a dissection microscope, and

decapitated. Liver was snap frozen for DNA isolation and PCR-based sex determination, as described previously (Meyers-Wallen et al. 2017).

For histology, d39 fetal heads from 3 cleft-affected and 3 normal littermate pups were fixed in formalin and processed for paraffin embedding. Serial coronal sections (7 μm) were collected from the rostral tip of the *planum nasale* through the back of the eyes. Hematoxylin and eosin (H&E) and immunostaining were performed as described previously (Freiberger et al. 2021). For μCT , heads from 3 cleft-affected and 3 normal newborn littermates were fixed overnight in formalin, skinned, and transferred to 50% ethanol. Micro-CT was performed on a Perkin Elmer QuantumGX system (PerkinElmer, Inc, Waltham, MA, USA) using the following image acquisition parameters: high resolution scan mode, 57-min gantry rotation time, 90kVp/88uA power, 72 mm field of view, 144 μm voxel size, 512 slices at 144 μm slice thickness, and 144 μm^3 voxel resolution. We standardized all images for HU intensity from 0-5000 HU. Image rendering and analysis was performed using Analyze 14.0 (Biomedical Imaging Resource, Mayo Clinic, Rochester, MN, USA). To allow an unobstructed 3D image view of the ventral aspect of the palate, some 3D images were rendered after removing the mandible using the visualization software. We used 3D images to measure the length and width of mandibles, compiling measurements for the right and left mandible as separate areas. To obtain the total length of the mandible, we added measurements from i) the most caudal point of the condylar process to a point ventral to the 4th premolar on the ventral surface of the mandibular body, and ii) from that point to the most rostral extent of alveolar bone, excluding protruding teeth. Additionally, we developed a “curved” measurement function by free drawing several point-to-point measurements along the curved ventral surface of the mandible. Curved lengths were $\sim 4\%$ longer than the simple 3-point measurements. The width of each mandible was measured on a ventral-dorsal view perpendicular to the long axis at the level of the suture between the palatine processes of the maxillae and palatine bones.

A male and 3 female affected pups were raised to six weeks of age for surgical closure of the palate cleft. Each received 2 mL of the dam’s serum subcutaneously on day 1, for passive transfer of immunity, and intermittent feedings of increasing amounts of bitch milk replacer (Esbilac®, PetAg, Hampshire, IL, USA) by stomach tube 8–9 times/day. Hydration was assessed continuously by monitoring skin turgor and fecal consistency and maintained as needed by subcutaneous or enteral administration of warm lactated Ringer’s solution. Growth was monitored by daily weighing. At 3 weeks of age, feedings were supplemented by digital gavage with moistened kibble. Growth was normal throughout the first 6 weeks (exponential, according to the equation $y = 246.34e^{0.0545x}$ with $R^2 = 0.998$) (Alves 2020). At 6 weeks of age and ≥ 2 kg body weight, the affected pups underwent general anesthesia, computed tomography of the head, and 2-layer closure of the cleft, restoring both nasal and oral mucosae of the soft and hard palate. Recovery was uneventful; routine feedings of soft food began the day after surgery, evolving to regular kibble and return to group housing over the next 3 weeks.

For statistical analyses of breeding experiments, we used i) the Chi-Square goodness-of-fit test to compare observed versus expected Mendelian ratios of affected/normal pups; ii) Fishers exact test when experimental values were less than 5; and iii) Odds ratio to assess association of phenotypes with

potential maternal effects. Micro-CT measurements of mandibles of cleft and normal littermates ($n = 3$ in each group) were compared by two-tailed Student's t-test both separately for right and left mandibles and with the sides combined.

Results

Cleft phenotype:

Isolated cleft palate occurred infrequently ($\sim 5\%$) in a breeding colony of mix-bred dogs maintained since 1991, until we began intercrosses between littermates of a pup born with cleft palate. In repeated matings (dog 170 X dog 173, Fig. 1), 12 affected pups (18%), 5 male and 7 females, were born among 68 total offspring. The cleft phenotype was remarkably consistent; at birth there was a 1.5-2 mm wide, full-length midline cleft of the secondary palate, extending caudally from the incisive papilla through the hard and soft palates (Fig. 2b). The sides of the cleft were essentially parallel. There were no clefts of lip or primary palate. No other external morphological abnormalities were apparent in pups born to this dam.

Micro-CT of normal newborn pups showed an ~ 0.6 mm longitudinal midline gap between mineralized bones of the hard palate (Fig. 3a-c), but the gap was filled entirely by soft tissue fused at the midline (Fig. 3c). In cleft littermates, μ CT demonstrated an ~ 3.2 mm wide longitudinal midline gap between bony edges of the bilateral palatine processes of the maxillae and horizontal laminae of palatine bones (Fig. 3d-f). Soft tissue covering the bony tissues reduced the gap to 1.7-2.0 mm (Fig. 3d). Deciduous dentition was complete, and the vomer bone was intact. In 3 normal and 3 cleft littermates, there was no significant difference of the mandibular length or width (curved length: normal [$n = 6$] 36.58 ± 1.77 mm vs. cleft [$n = 6$] 37.70 ± 1.70 mm [mean $\pm$ SD], $p > 0.32$; width: normal [$n = 6$] 4.45 ± 0.52 mm vs. cleft [$n = 6$] 4.05 ± 0.53 mm, $p > 0.25$) nor between the right and left sides by either measurement method. Clinical CT of normal and affected littermates at 6 weeks of age showed the hard palate was continuously ossified across the roof of the oral cavity in normal pups, whereas the cleft palate pups had the same longitudinal gap between bones of the hard palate and tissue of the soft palate (not shown). These scans revealed no other abnormalities in the cleft palate pups.

With intensive nursing care we rescued 1 male and 3 females born with cleft palate and raised them to 6-weeks of age, at which time they each underwent surgical reconstruction of the palate. After recovery from surgery they each went back into the general population and grew normally into healthy adults. At appropriate ages, they each developed full adult dentition and gained reproductive competency. The male routinely exhibited good libido and 500–800 million sperm/ejaculate when collected for artificial insemination. The sperm were $\sim 95\%$ progressively motile with few morphologic abnormalities. The females each had their first estrous cycle between 12 and 14 months of age, and each became pregnant when bred in their second cycles. None have had issues of poor health and are 4.5-7 years of age, at present.

To explore potential pathophysiological mechanisms, we performed histologic analyses on serial coronal sections of heads from fetuses with and without cleft palate. By cesarean section we interrupted backcross matings of each of the 3 surgically repaired affected females with their common sire on estimated d39, the previously defined day of normal palate fusion (Freiberger et al. 2021). Cleft palate was readily identified in 9 of 19 fetuses, 5 males and 4 females (Fig. 1). As expected, palate shelves in normal littermates had begun to fuse along the length of the secondary palate (Fig. 4a-c), as evidenced by dissolution of the epithelia in the medial edge seam. In affected fetuses, however, although the palate shelves were elevated above the tongue, a gap remained between them along the entire length of the palate shelves (Fig. 4d-f). We also tracked palate fusion by immuno-staining with antibodies directed against K17, a cytosolic protein marker for periderm, and p63, a nuclear protein marker for basal epithelial cells. Again, as expected in normal fetuses at this timepoint, the signals for both K17 (red) and p63 (green) were mostly gone along the medial edge seam, except at the epithelial triangles located at extreme nasal and oral ends of the medial edge seam (Fig. 5d, h). For cleft-affected littermates, the K17 and p63 signals on the oral and nasal sides of the palate shelves did not appear different from normal littermates. However, unlike the palate shelves from normal fetuses, the K17 and p63 signals remained robust and continuous along the medial ends of each unfused palate shelf and the vomer (Fig. 5l, p).

Clinical genetics:

Twelve pups, 5 male and 7 female, among 68 total offspring (18%) were born with cleft palate, occurring in 7 matings of clinically normal littermate parents (Fig. 1). While this result was not statistically different from 25% expected for an autosomal recessive trait ($[1 \text{ df}, N = 68] \chi^2 = 1.96, p = 0.16$), the percentage of affected pups accumulating with each new litter had hovered at 18% since the third litter (5/30), suggesting that the deficit was real. Breeding surgically repaired affected dogs provided opportunity for prospective experiments. In backcross experiments, three matings of the affected male (248) to his dam (173) produced 3 cleft-affected pups, 2 male and 1 female, among 11 total offspring (27%). Although again not statistically different from the expected 50% for an autosomal recessive trait in backcross matings ($p = 0.39$), these results and those of the 170 X 173 matings, indicated above, suggested that the dam (173) might be transmitting a factor that suppressed penetrance of the cleft trait. To test that hypothesis, we performed backcross matings of the surgically repaired affected females. Collectively, matings of affected females 286, 329, and 335 (Fig. 1) to their common sire (170) produced 17 cleft-affected pups, 9 male and 8 female, among 33 total offspring, exactly as expected for a simple autosomal recessive trait ($[1 \text{ df}, N = 33] \chi^2 = 0.30, p = 0.86$). Furthermore, 2 matings of the affected male (248) with his affected full sib (286) produced 8 offspring, 3 male and 5 female, all of which had cleft palate, again as expected for a simple recessive trait. Thus, it appeared that penetrance of the cleft trait was reduced only when 173 was the dam.

As often occurs with inbreeding, we exposed a second phenotype among some offspring of matings that did not include dam 173. TLD appeared in offspring of backcrosses to the family sire (170) and intercrosses between cleft-affected dogs (Fig. 1). These matings produced 41 offspring, of which 16 had

normal palate and 25 had cleft palate. Two male normal-palate offspring and 14 of the cleft-palate pups, 4 male and 10 female, had various TLD. An invariable abnormality was absent toenails of digits 3 and 4 on both hindlimbs (Fig. 6d). More severe abnormalities were only in the forelimbs, including various degrees of unilateral or bilateral TLD that were often asymmetric (Fig. 6c, e). The most severe deficiency was bilateral absence, amelia, of forelimbs below the scapulohumeral joint in 6 pups. Abnormalities in the two normal-palate pups were the absent rear toenails (Fig. 6h, i) and unilateral fusion of forepaw digits 4 and 5 with absence of the digit 5 toenail (right forepaw of one and left forepaw of the other) (Fig. 6g). As noted, the limb defects were highly associated with cleft palate (OR = 10.2, 95% CI: 1.9–54). Moreover, we produced 14 pups with limb defects among 25 CPO offspring from matings when the dams had cleft palate, but no limb defects among the 3 CPO offspring from backcross matings when the dam had normal palate ($p = 0.002$).

Discussion

Sporadic occurrence of cleft palate has been reported in many mammalian species (Łobodzińska, 2014), and animal models have been major contributors to understanding normal and abnormal palatogenesis. Mouse models have been particularly useful for dissecting the molecular genetics and mechanisms of craniofacial development, but they are rarely useful as clinical models for post-natal or *in utero* cleft palate repair because mice are small, and the gestational window for post-operative healing without scarring is short (Lorenz and Longaker 2003). Dogs have been used as a large animal model for cleft palate (Martinez-Alvarez et al. 2013; Peralta et al. 2017). However, while there are several reports of dog families with CL/P or CPO as part of a syndrome of midline and/or limb anomalies (Natsume et al. 1994; Villagómez and Alonso, 1998; Kemp et al. 2009; Moura et al. 2012; Wolf et al. 2014; Wolf et al. 2015), there are few reports of familial CPO occurring as an isolated defect (Richtsmeier et al. 1994; Peralta et al. 2017). The use of dogs as a model is also supported by the observation that the incidence of CL/P in dogs can be reduced by dietary folate supplementation (Domosławska et al. 2013), as seen in some human populations (Wyszynski and Beaty 1996; Hackshaw et al. 2011). Overall, sporadic CPO is more common than CL/P or CLO in dogs, but the incidence of OFC subcategories varies significantly across breeds (Peralta et al. 2017; Roman et al. 2019). To date, however, genetic risk variants have been determined for only two OFC in dogs (Wolf et al. 2014; Wolf et al. 2015).

The molecular genetic etiology of CPO in the dog family reported here is not yet known. However, the morphological and histological analyses may provide clues. The immuno-staining for K17 and p63 appeared normal, showing that periderm and basal epithelial formation and function were not grossly affected. At gestational d39, the palate shelves are fusing in normal dog fetuses (Freiberger et al. 2021), but while the palate shelves of affected pups had elevated over the tongue, they had failed to fuse. This could be failure of the palate shelves to grow and elongate horizontally so that the epithelia did not meet at midline. Alternatively, it could be failure of the opposing shelves to fuse at the midline when the leading-edge epithelia met, with subsequent growth of the head causing a relative retraction of the shelves that left the gap. Analyses of mutant mouse models indicate that developmental regulation of mesenchymal cell proliferation, palate shelf elongation, and fusion at the medial epithelial seam involves

multiple signaling pathways, including sonic hedgehog (SHH), wingless-related integration site (WNT), fibroblast growth factor (FGF), bone morphogenic protein (BMP), and transforming growth factor β (TGF β), and that there is extensive cross-regulation between pathways (Gritli-Linde 2007; Lan and Jiang 2009; Iwata et al. 2011; Lane and Kaartinen 2014; Okello et al. 2017; Jia et al. 2017; Reynolds et al. 2019; Sweat et al. 2020). We note that some of the same signaling pathways that affect palate development are involved in limb development (e.g. SHH and FGF), and perturbations can lead to limb deformities like those observed in this CPO family (Mariani et al. 2008; Guerrini et al. 2011; Tickle and Towers 2017; Reynolds et al. 2020). Even with a shared-etiology hypothesis, the large number of genes involved in palate and limb development and the pleiotropic effects of variants in those genes, argue against a candidate gene approach to determining the etiology of CPO in this family. Rather, we propose that a whole genome approach is warranted.

Results of our breeding experiments are not consistent with a simple inheritance model that accounts for both phenotypes. The pedigree suggests that CPO is inherited as an autosomal recessive trait, but possibly with the added complexity of a suppressor allele. If there was an allele suppressing CPO, it appeared to be restricted to dog 173 because the frequency of CPO was as expected of simple recessive inheritance, except when 173 was the dam. One possible model is that a suppressor allele was inherited from 173 as a dominant allele with incomplete penetrance, and the same allele suppressing CPO also suppressed limb defects. However, this model fails to explain the complete absence of limb defects in CPO offspring from crosses with 173. The attractive hypothesis that limb defects were caused by the same recessive allele as caused CPO also requires modification because 11 of 25 offspring with CPO when 173 was not the dam did not exhibit limb defects. An alternative hypothesis is that the family sire (170) harbored a dominant TLD-causing allele that was only fully expressed in CPO offspring. And yet another possibility compatible with the data is that TLD is caused by a recessive allele that segregates independent of the CPO allele but, again, is fully expressed only in CPO offspring.

An explanation of our observations could also be that while CPO is indeed an autosomal recessive trait in this family, the limb defects were not an inherited trait, but instead resulted from insults to developing fetuses created *in utero* by the maternal genome. This model is fully consistent with the pedigree and is also suggested by the type of limb defects observed, notably variably expressed TLD. TLD are unique among LRD in that many are attributed to vascular disruption from placental or fetal blood clots during limb development, such as may occur in cases of maternal thrombophilia (Sadler and Rasmussen 2010; Ordal et al. 2016; Hunter 2000). This model lends itself to the observed maternal effect.

In summary, we describe a dog model for non-syndromic CPO. Backcross matings led to limb defects as a second phenotype in this family. While the two phenotypes may be related genetically, the current pedigree suggests that breeding strategies can be designed to minimize the occurrence of limb defects in these dogs. Thus, this line of dogs represents a well-characterized and highly reproducible large animal model for non-syndromic CPO. Finally, the pedigree suggests models of inheritance for the CPO and TLD phenotypes that provide a genetic framework to analyze future whole genome data.

Declarations

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Conflicts of Interest The authors declare no conflict of interest.

Availability of data and material Not applicable

Code availability Not applicable

Author contributions Study conception and design was by John Fyfe and Brian Schutte. Material preparation, data collection and analysis were performed by John Fyfe, Abdullah Mahmood, Jeremy Hix, Bryden Stanley and Brian Schutte. The first draft of the manuscript was written by John Fyfe and Brian Schutte, and all authors commented on it and subsequent versions of the manuscript. All authors read and approved the final manuscript.

Ethics approval Animal use protocols were approved by the Michigan State University Institutional Animal Use and Care Committee.

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References

1. Alves I (2020) A model of puppy growth during the first three weeks. *Vet Med Sci* 6:946–957. <https://doi.org/10.1002/vms3.322>
2. Beaty TH, Marazita JL, Leslie EJ (2016) Genetic factors influencing risk to orofacial clefts: today's challenges and tomorrow's opportunities. *F1000Res* 5:2800. <https://doi.org/10.12688/f1000research.9503.1>
3. Bergman JEH, Löhner K, van der Sluis CK, Rump P, de Walle HEK (2020) Etiological diagnosis in limb reduction defects and the number of affected limbs: A population-based study in the Northern Netherlands. *Am J Med Genet Part A* 182:2909–2918. <https://doi.org/10.1002/ajmg.a.61875>

4. Botto LD, Olney RS, Erickson JD (2004) Vitamin supplements and the risk for congenital anomalies other than neural tube defects. *Am J Med Genet C Semin Med Genet* 125C:12–21. <https://doi.org/10.1002/ajmg.c.30004>
5. Carlson JC, Anand D, Butali A et al (2019) A systematic genetic analysis and visualization of phenotypic heterogeneity among orofacial cleft GWAS signals. *Genet Epidemiol* 43:704–716. <https://doi.org/10.1002/gepi.22214>
6. Czeizel AE, Kodaj I, Lenz W (1994) Smoking during pregnancy and congenital limb deficiency. *BMJ* 308:1473–1476. <https://doi.org/10.1136/bmj.308.6942.1473>
7. Dixon MJ, Marazita ML, Beaty TH, Murray JC (2011) Cleft lip and palate: understanding genetic and environmental influences. *Nat Rev Genet* 12:167–178. <https://doi.org/10.1038/nrg2933>
8. Domosławska A, Jurczak A, Janowski T (2013) Oral folic acid supplementation decreases palate and/or lip cleft occurrence in pug and Chihuahua puppies and elevates folic acid blood levels in pregnant bitches. *Pol J Vet Sci* 16:33–37. <https://doi.org/10.2478/pjvs-2013-0005>
9. Elsner J, Mensah MA, Holtgrewe M et al (2021) Genome sequencing in families with congenital limb malformations. *Hum Genet* 140:1229–1239. <https://doi.org/10.1007/s00439-021-02295-y>
10. Freiburger K, Hemker S, McAnally R et al (2021) Secondary palate development in the dog *Canis lupus familiaris*. *Cleft Palate Craniofac J* 58:230–236. <https://doi.org/10.1177/1055665620943771>
11. Froster-Iskenius UG, Baird PA (1989) Limb reduction defects in over one million consecutive livebirths. *Teratology* 39:127–135. <https://doi.org/10.1002/tera.1420390205>
12. Gold NB, Westgate M-N, Holmes LB (2011) Anatomic and etiological classification of congenital limb deficiencies. *Am J Med Genet Part A* 155A:1225–1235. <https://doi.org/10.1002/ajmg.a.33999>
13. Graziano C, Carone S, Panza E et al (2009) Association of hereditary thrombocythemia and distal limb defects with a thrombopoietin gene mutation. *Blood* 114:1655–1657. <https://doi.org/10.1182/blood-2009-04-217851>
14. Gritli-Linde A (2007) Molecular control of secondary palate development. *Dev Biol* 301:309–326. <https://doi.org/10.1016/j.ydbio.2006.07.042>
15. Guerrini L, Costanzo A, Merlo GR (2011) A symphony of regulations centered on p63 to control development of ectoderm-derived structures. *J Biomed Biotechnol* 2011:864904. <https://doi.org/10.1155/2011/864904>
16. Hackshaw A, Rodeck C, Boniface S (2011) Maternal smoking in pregnancy and birth defects: a systematic review based on 173,687 malformed cases and 11.7 million controls. *Hum Reprod Update* 17:589–604. <https://doi.org/10.1093/humupd/dmr022>
17. He M, Zuo X, Liu H et al (2020) Genome-wide analyses identify a novel risk locus for nonsyndromic cleft palate. *J Dent Res* 10:1–8. <https://doi.org/10.1177/0022034520943867>
18. Holmes LB (2021) Terminal transverse limb defects. July 14, 2021 Online ahead of print <https://doi.org/10.1002/bdr2.1933>

19. Holmes LB, Westgate M-N, Nasri H, Toufaily MH (2018) Malformations attributed to the process of vascular disruption. *Birth Defects Res* 110:98–107. <https://doi.org/10.1002/bdr2.1160>
20. Huang L, Jia Z, Shi Y et al (2019) Genetic factors define CPO and CLO subtypes of nonsyndromic orofacial cleft. *PLoS Genet* 15:e1008357. <https://doi.org/10.1371/journal.pgen.1008357>
21. Hunter AG (2000) A pilot study of the possible role of familial defects in anticoagulation as a cause for terminal limb reduction malformations. *Clin Genet* 57:197–204. <https://doi.org/10.1034/j.1399-0004.2000.570305.x>
22. Hurst JA, Houlston RS, Roberts A, Gould JS, Tingey W (1995) Transverse limb deficiency, facial clefting and hypoxic renal damage: an association with treatment of maternal hypertension? *Clin Dysmorphol* 4:359–563. <https://doi.org/10.1097/00019605-199510000-00013>
23. Iwata J, Parada C, Chai Y (2011) The mechanism of TGF- β signaling during palate development. *Oral Dis* 17:733–744. <https://doi.org/10.1111/j.1601-0825.2011.01806.x>
24. Jia S, Zhou J, Fanelli C et al (2017) Small-molecule Wnt agonists correct cleft palates in *Pax9* mutant mice *in utero*. *Development* 144:3819–3828. <https://doi.org/10.1242/dev.157750>
25. Jiang R, Bush JO, Lidral AC (2006) Development of the upper lip: morphogenetic and molecular mechanisms. *Dev Dyn* 235:1152–1166. <https://doi.org/10.1002/dvdy.20646>
26. Kemp C, Thiele H, Dankof A et al (2009) Cleft lip and/or palate with monogenic autosomal recessive transmission in Pyrenees shepherd dogs. *Cleft Palate Craniofac J* 46:81–88. <https://doi.org/10.1597/06-229.1>
27. Kochhar DM, Penner JD, Tellone CI (1984) Comparative teratogenic activities of two retinoids: effects on palate and limb development. *Teratog Carcinog Mutagen* 4:377–387. <https://doi.org/10.1002/tcm.1770040407>
28. Lan Y, Jiang R (2009) Sonic hedgehog signaling regulates reciprocal epithelial-mesenchymal interactions controlling palatal outgrowth. *Development* 136:1387–1396. <https://doi.org/10.1242/dev.028167>
29. Lane J, Kaartinen V (2014) Signaling networks in palate development. *Wiley Interdiscip Rev Syst Biol Med* 6:271–278. <https://doi.org/10.1002/wsbm.1265>
30. Lehman A, Wuyts W, Patel MS (2016) Adams-Oliver Syndrome. In: Adam MP, Ardinger HH, Pagon RA et al (eds) *GeneReviews®* [Internet]. University of Washington, Seattle, pp 1993–2021. <https://www.ncbi.nlm.nih.gov/books/NBK355754/>
31. Leslie EJ, Marazita JL (2013) Genetics of cleft lip and cleft palate. *Am J Med Genet C Semin Med Genet* 163C:246–258. <https://doi.org/10.1002/ajmg.c.31381>
32. Łobodzińska A, Gruszczyńska J, Max A et al (2014) Cleft palate in the domestic dog *Canis lupus familiaris* – Etiology, pathophysiology, diagnosis, prevention, and treatment. *Acta Sci Pol Zootechnica* 13:5–28
33. Lorenz HP, Longaker MT (2003) In utero surgery for cleft lip/palate: minimizing the "Ripple Effect" of scarring. *J Craniofac Surg* 14:504–511. <https://doi.org/10.1097/00001665-200307000-00021>

34. Ludwig KU, Böhmer AC, Bowes J et al (2017) Imputation of orofacial clefting data identifies novel risk loci and sheds light on the genetic background of cleft lip ± cleft palate and cleft palate only. *Hum Mol Genet* 26:829–842. <https://doi.org/10.1093/hmg/ddx012>
35. Mariani FV, Ahn CP, Martin GR (2008) Genetic evidence that FGFs have an instructive role in limb proximal–distal patterning. *Nature* 453:401–405. <https://doi.org/10.1038/nature06876>
36. Martínez-Álvarez C, González-Meli B, Berenguer-Froehner B et al (2013) Injection and adhesion palatoplasty: a preliminary study in a canine model. *J Surg Res* 183:654–662. <https://doi.org/10.1016/j.jss.2013.03.009>
37. McGuirk CK, Westgate MN, Holmes LB (2001) Limb deficiencies in newborn infants. *Pediatrics* 108:E64. <https://doi.org/10.1542/peds.108.4.e64>
38. Meyers-Wallen VN, Boyko AR, Danko CG et al (2017) XX Disorder of Sex Development is associated with an insertion on chromosome 9 and downregulation of *RSPO1* in dogs (*Canis lupus familiaris*). *PLoS One* 12:e0186331. <https://doi.org/10.1371/journal.pone.0186331>
39. Moura E, Cirio SM, Pimpão CT (2012) Nonsyndromic cleft lip and palate in boxer dogs: evidence of monogenic autosomal recessive inheritance. *Cleft Palate Craniofac J* 49:759–760. <https://doi.org/10.1597/11-110>
40. Natsume N, Miyajima K, Kinoshita H, Kawai T (1994) Incidence of cleft lip and palate in beagles. *Plast Reconstr Surg* 93:439. <https://doi.org/10.1097/00006534-199402000-00042>
41. Okello DO, Iyyanar PPR, Kulyk WM et al (2017) *Six2* plays an intrinsic role in regulating proliferation of mesenchymal cells in the developing palate. *Front Physiol* 8:955. <https://doi.org/10.3389/fphys.2017.00955>
42. Ordal L, Keunen J, Martin N et al (2016) Congenital limb deficiencies with vascular etiology: Possible association with maternal thrombophilia. *Am J Med Genet A* 170:3083–3089. <https://doi.org/10.1002/ajmg.a.37890>
43. Peralta S, Fiani N, Kan-Rohrer KH, Verstraete FJM (2017) Morphological evaluation of clefts of the lip, palate, or both in dogs. *Am J Vet Res* 78:926–933. <https://doi.org/10.2460/ajvr.78.8.926>
44. Ray D, Venkataraghavan S, Zhang W et al (2021) Pleiotropy method reveals genetic overlap between orofacial clefts at multiple novel loci from GWAS of multi-ethnic trios. *PLoS Genet* 17:e1009584. <https://doi.org/10.1371/journal.pgen.1009584>
45. Reynolds K, Kumari P, Rincon LS et al (2019) Wnt signaling in orofacial clefts: crosstalk, pathogenesis and models. *Dis Models Mech* 12:dmm037051. <https://doi.org/10.1242/dmm.037051>
46. Reynolds K, Zhang S, Sun B, Garland MA, Ji Y, Zhou CJ (2020) Genetics and signaling mechanisms of orofacial clefts. *Birth Defects Res* 112:1588–1634. <https://doi.org/10.1002/bdr2.1754>
47. Richtsmeier JT, Sack IGH, Grausz HM, Cork LC (1994) Cleft palate with autosomal recessive transmission in Brittany spaniels. *Cleft Palate Craniofac J* 31:364–371. https://doi.org/10.1597/1545-1569_1994_031_0364_cpwart_2.3.co_2
48. Roman N, Carney PC, Fiani N, Peralta S (2019) Incidence patterns of orofacial clefts in purebred dogs. *PloS One* 14:e0224574. <https://doi.org/10.1371/journal.pone.0224574>

49. Sadler TW, Rasmussen SA (2010) Examining the evidence for vascular pathogenesis of selected birth defects. *Am J Med Genet A* 152A:2426–2436. <https://doi.org/10.1002/ajmg.a.33636>
50. Scott WJ Jr (1983) Caffeine-induced limb malformations: description of malformations and quantitation of placental transfer. *Teratology* 28:427–435. <https://doi.org/10.1002/tera.1420280314>
51. Stoll C, Alembik Y, Dott B, Roth M-P (2010) Associated malformations in patients with limb reduction deficiencies. *Eur J Med Genet* 53:286–290. <https://doi.org/10.1016/j.ejmg.2010.07.012>
52. Sweat YY, Sweat M, Mansaray M et al (2020) Six2 regulates Pax9 expression, palatogenesis and craniofacial bone formation. *Dev Biol* 458:246–256. <https://doi.org/10.1016/j.ydbio.2019.11.010>
53. Tickle C, Towers M (2017) Sonic hedgehog signaling in limb development. *Front Cell Dev Biol* 5:14. <https://doi.org/10.3389/fcell.2017.00014>
54. Villagómez DAF, Alonso RA (1998) A distinct Mendelian autosomal recessive syndrome involving the association of anotia, palate agenesis, bifid tongue, and polydactyly in the dog. *Can Vet J* 39:642–643
55. Webster S, Abela D (2007) The effect of hypoxia in development. *Birth Defects Res C Embryo Today* 81:215–228. <https://doi.org/10.1002/bdrc.20102>
56. Wehby GL, Cassell CH (2010) The impact of orofacial clefts on quality of life and healthcare use and costs. *Oral Dis* 16:3–10. <https://doi.org/10.1111/j.1601-0825.2009.01588.x>
57. Wolf ZT, Leslie EJ, Arzi B et al (2014) A LINE-1 insertion in DLX6 is responsible for cleft palate and mandibular abnormalities in a canine model of Pierre Robin sequence. *PLoS Genet* 10:e1004257. <https://doi.org/10.1371/journal.pgen.1004257>
58. Wolf ZT, Brand HA, Shaffer JR et al (2015) Genome-wide association studies in dogs and humans identify ADAMTS20 as a risk variant for cleft lip and palate. *PLoS Genet* 11:e1005059. <https://doi.org/10.1371/journal.pgen.1005059>
59. Wyszynski DF, Beaty TH (1996) Review of the role of potential teratogens in the origin of human nonsyndromic oral clefts. *Teratology* 53(199605):309–317. [https://doi.org/10.1002/\(sici\)1096-9926\(199605\)53:5%3C309::aid-tera5%3E3.0.co;2-w](https://doi.org/10.1002/(sici)1096-9926(199605)53:5%3C309::aid-tera5%3E3.0.co;2-w)

Figures

Figure 1

Pedigree of a dog family with high frequency cleft palate and transverse limb deficiency. The ancestry of dogs 170 and 173 is curly coated retriever from the sire side and mixed lineage from the dam side, including giant schnauzer, beagle and mongrel. Phenotype labeling is shown in the key. The numbers inside the symbols represent the number of individuals for that phenotypic class. The number above a symbol is the identification number for that dog, as appear in the text. An asterisk is placed next to the identification numbers of the four affected dogs whose cleft palate was surgically repaired, including one male (248) and three females (286, 329, and 335).

Figure 2

Morphology of dog palates. Newborn pup with typical palate (a) and littermate with cleft palate (b).

Figure 3

Micro-computed tomography (μ CT) of dog heads. Newborn pup with typical palate (a-c) and littermate with cleft palate (d-f). Panels (a) and (d) are 3D image views of the ventral aspect of the palate after removing the mandible with visualization software. Panels (b) and (e) are 2D greyscale images of the representative slice that shows the palate ventrally. Panels (c) and (f) are 2D greyscale images from the transverse view. All greyscale 2D images are one slice at $144\mu\text{m}$ resolution. The normal pup had a narrow longitudinal gap between mineralized bones of the hard palate (a-c), but the gap was filled entirely by soft tissue fused at the midline (c). The pup with cleft palate had a longitudinal midline gap between bony edges of the bilateral palatine processes of the maxillae and horizontal laminae of palatine bones (d-f). The gap is reduced, but not filled by soft tissues (f). Orientation markers signify right (R), left (L), rostral (ROS), caudal (C), ventral (V), dorsal (D). The Hounsfield Unit (HU) scale bar was used to standardize all images in histogram brightness from 0-5000 HU. The horizontal scale bar represents the length of 1 cm.

Figure 4

Morphology of cleft palate in fetal dog. Coronal sections of fetal day 39 dog heads showing an individual with no cleft (a-c) and an individual with cleft palate (d-f). Sections from three planes were stained for each fetal pup with hematoxylin and eosin (H&E). Planes were defined by the anterior of the eye (a, d), middle of the eye (b, e) and posterior of the eye (c, f).

Figure 5

Immuno-fluorescent staining of epithelial layers of fetal dog palate. Coronal sections of gestational day 39 fetal dog heads showing an individual with no cleft at 4x (a-d) and 40x magnification (e-h) and an individual with cleft palate at 4x (i-l) and 40x (m-p). The areas used in the high magnification images are represented by the white box in (a) for the no cleft fetus and in (i) for the fetus with cleft palate. The labeled structures in the low magnification (panels a, i) are palatal shelves (P) and vomer (V). DAPI stained all cell nuclei (blue). The signal for p63 (green) specifically marked nuclei of cells in the basal epithelial layer, and the signal for K17 (red) specifically marked the cytoplasm of cells in the periderm layer. The three individual signals were digitally merged (d, h, l, p).

Figure 6

Variable expressivity of limb defects. Newborn dog with typical forelimbs (a) and hindlimbs (b). Newborn dogs with variable transverse limb deficiencies in the forelimbs (c, e). All dogs with forelimb deficiencies were also missing the toenails for digits 3 and 4 in the hindlimbs (d). Images of forepaws (f and g) and hind paws (h and i) of a dog born without cleft palate, but with limb abnormalities. The right forepaw is normal (f), but in the left forepaw (g), digits 4 and 5 are fused and digit 5 is missing the toenail. Like all dogs with cleft palate and limb defects, digits 3 and 4 on both hindlimbs are missing the toenails (h, i).