

# What have genetically modified animals told us about the genetics and aetiology of the human lower limb abnormality, ‘clubfoot’?

Paul Shepherd

February 10, 2023

‘Clubfoot’, or congenital talipes equinovarus (CTEV), is a lower limb defect that occurs during development. It is characterised by persistent plantar flexion and medial rotation of the foot (seen on Figure 1 in the appendix), which if left untreated leads to permanent disability. CTEV is among the most common paediatric orthopaedic conditions effecting 1-3 in every 1,000 live births (Lupo et al. 2019; Dobbs et al., 2012; Cartlidge, 1984). The majority of CTEV is idiopathic, meaning that clubfoot is the only problem in an otherwise healthy child, but it can also be associated with other syndromes including spina bifida (Wynne-Davies 1964). Clubfoot has been a part of human history through the ages, and the fate of individuals with clubfoot depended on the attitude of their society, ranging from casting aside to die at birth all the way to Gods (Ramachandran, 2006; Strach, 1986). Hippocrates (born approximately 460BC) established the foundations for modern clubfoot treatment urging repeated manipulation and fixation with strong bandages as soon as possible after birth (Strach, 1986). Today’s Ponseti method is not too dissimilar, which offers a low-cost treatment involving a treatment phase, including serial; manipulation, casting and tenotomy, and a maintenance phase, using foot abduction braces (Ponseti 1997). However, despite centuries for development this ‘modern’ method still results in 10-15 percent relapsing and the majority experiencing leg fatigue (Ippolito 2009). Human population genetics and twin studies, with identical twins having a higher concordance rate (at 33 percent) than fraternal twins (3 percent), strongly suggest a genetic basis for CTEV (Lupo et al., 2019; Miedzybrodzka, 2003), but there is an incomplete understanding of CTEV’s genetic factors and aetiology. This genetic basis can be seen with inbreeding probably increases the probability of clubfoot both human and horses (Comparini et al. 2019; Hawass et al., 2010). Genetically modified animals are a valuable research tool for knowing the causes and genes involved in CTEV through which we develop the possibility to future human treatment options. This essay will discuss the hypotheses for the causes of human clubfoot before looking at genetically modified animal models to explain the cause and genetics of human clubfoot, then it will finally touch on the future genetic based treatment options for clubfoot.

There are six hypotheses for the cause of human clubfoot with it long being hypothesised that ‘Clubfoot’ could be due to muscle weakness caused by malformations in the musculoskeletal and/or nervous system (Feldbrin et al., 1995; Yoshimura et al., 1988; Nonaka et al., 1986; Handelsman and Badalamente, 1981). The following have been established in humans and can be broken down into; (1) The bone/joint hypothesis, which is where bone abnormalities, specifically endochondral and perichondral ossification disruption, are the cause (Fritsch and Eggers 1999). (2) The connective tissue hypothesis, which is the belief that increased fascia, ligaments, tendon sheaths and fibrous tissue in muscle causing retracting fibrosis could be the cause (Ippolito and Ponseti 1980), but there is conflicting evidence with no observed abnormality in connective tissue in humans (Atlas et al., 1980). (3) The vascular hypothesis, which suggests that a reduction in perfusion, specifically through the anterior tibial artery, can cause calf muscle atrophy in humans (Merrill et al., 2011; Atlas et al., 1980). (4) The neurological hypothesis, which could be due to nerve abnormalities observed in 18 out of 44 idiopathic CTEV, 8 of which had abnormalities at the spinal level (Nadeem et al. 2007). The other two theories for the cause of human clubfoot that are more based on observation of traits are; (5) The ‘mechanical forces’ or ‘positional’ hypothesis, which suggests that it is the restriction of foetal foot movement in the uterus possibly due to reduced amniotic fluid volume (which on its own causes neurological problems) that causes clubfoot (Farrell et al., 1999; Hoffa 1902). (6) The failure to progress in development

hypothesis, which is just the idea that the foot in severe clubfoot resembles the embryonic foot two months into development (Kawashima and Uhthoff 1990; Böhm 1929). Now that we have established the hypothesised causes of human clubfoot we will focus on the genetically modified animals that have been studied to put forward evidence to actually define the cause with respect to the first four hypotheses. The bone/joint hypothesis was reproduced in animals using retinoic acid as a teratogen to cause induced foetal growth retardation and reduced ossification of the same hindlimb bones as in congenital clubfoot (Liu et al., 2010; Delgado-Baeza et al., 1999). But as it is teratogen induced, it is probably not as useful of a model as genetically modified peroneal muscular atrophy (pma) mutant mice whose skeletal changes were clearly seen to resemble human CTEV using micro-magnetic resonance imaging, the results of which can be seen in Table 1 and Figures 2 and 3 in the appendix (Duce et al., 2010). This evidence establishes, in this essay, that pma mice are a good model for human CTEV as the pma mice show the same persistent plantar flexion and medial rotation of the ankle and toes, as well as the mid-foot inversion and adduction. They also showed that it occurred from embryonic day 14.5 with the exception of the rotation which took longer, and therefore supports the failure to progress in development hypothesis, thus linking hypothesis 1 and hypothesis 6. This same study also showed that muscle volume of shank muscle was significantly reduced by approximately 70 percent, the specific muscles being the fibularis (peroneus) tertius, extensor digitorum longus, and extensor hallucis longus muscles, which make up the antero-lateral muscle group. This was slightly different to what has been seen to happen in humans which was mainly the posterior compartment muscles (Ippolito et al., 2009) as opposed to this antero-lateral muscle group observed in pma mice. So, although not entirely consistent with human CTEV they both the pma mice and humans showed that muscle changes are a key feature, and just for clarity this does not point to the connective tissue being the route course so doesn't support hypothesis 2. Ultimately this study showed numerous anatomical similarities (specifically in bone and muscle) between humans and pma mice.

The pma gene has been mapped to chromosome 5 in mice with the gene order being: centromere-D5Mit263-[2.65 cM]-D5Mit141-[2.56 cM]-pma-[5.13 cM]-D5Mit97-telomere in pma mouse (Katoh et al., 2003), opens up the possibility to fully understand the genetic basis and developmental mechanisms behind CTEV. Figure 4 in the appendix was taken from this paper and clearly shows the morphological characteristics of the twisted foot, the reduced muscle volume, previously discussed, and the underdeveloped peroneal nerve. A recent human clubfoot genome wide association study showed that no SNP reached genome-wide level significance, but the strongest evidence pointed to an intergenic SNP on chromosome 12q24.31 between NCOR2 and ZNF664 (Lupo et al., 2019). Which doesn't support the causing gene being on chromosome 5 but on 12, but with it not being significant and PITX1 being on chromosome 5 and showing clubfoot symptoms in both humans (when microdeletions occur) and in Pitx1 knockout mice (Alvarado et al., 2011). These between both PITX1 haploinsufficient human and Pitx1 knockout mice similarities in morphology (with the twisted foot), reduced muscle volume as well as reduced artery perfusion can be seen in Figures 5, 6, 7, and 8, in the appendix, all taken from Alvarado et al., 2011. Since PITX1 is a known transcriptional target for TBX4, the T-box transcription factor known to be associated with human clubfoot (Alvarado et al., 2010) it further supports the involvement of PITX1 in human clubfoot. Alvarado et al. does raise an interesting point in their 2011 paper suggesting that the Pitx1<sup>+-</sup> mouse is a better model for human clubfoot than pma mice, as it is missing the common peroneal nerve, while it is present in both Pitx1<sup>+-</sup> mice and humans. This linked with the slight difference in the affected muscles, does raise concerns about the validity of being a human clubfoot model, but there are still too many similarities for it not to be considered as a decent model, in my opinion. Gammy mice, in which the GRIT gene which encodes Rho-GTPase is deleted was another proposed mouse model, but it showed severe brain abnormalities (Sangha et al., 2003) that are not present in human's with CTEV. This example is important for establishing what animal models are probably useful because gammy mice displaying symptoms that are completely absent in human clubfoot makes it a much less accurate tool than that of pma mice who display very similar traits, just not exactly the same. I believe through 'genetic tweaking' of the many genes that are involved in bone, muscle, and nerve development in the foot we can engineer pma mice, or the Pitx1<sup>+-</sup> mice, to have exactly the same traits as human clubfoot, therefore better understanding the genetics/causes of human clubfoot.

Having previously mentioned some neurological defects, this section will examine the neurological hypothesis. Decamethonium Bromide (DB) is a neuromuscular blocking agent that has previously been used to investigate muscle development in paralysed chicken limbs (Macharia et al., 2001; Germiller et

al., 1998), but DB can be used to teratogenically induce idiopathic clubfoot, possibly neurogenically (Kilby and Vargesson, 2009). This DB induced clubfoot showed reduced amniotic fluid volume, absent tendons, reduced muscle volume, and stunted muscle-nerve branches (Kilby and Vargesson, 2009). The nervous system malformations have been observed in genetically modified EphA4 homozygous mutant mice to produce clubfoot like deformities, see figure 9 in the appendix (Helmbacher et al., 2000). When the EphA4 gene is inactivated it causes abnormal navigation during the early stages of limb development, specifically of the lateral motor column (LMC) in the hindlimb and at later stages causes an overall reduction in the amount of motor neurons, seen in human CTEV. However, like the gammy mouse, there have been past experiments that implicate this gene with hindbrain making it likely to cause defect (Nieto et al., 1992; Gilardi-Hebenstreit et al., 1992), calling into question this genetically modified animals' reliability in accurately representing clubfoot, but there was no sign of these defects in the Helmbacher et al. experiment. A 2018 study using pma mice by Collinson et al. has pointed to this growth reduction in this sciatic nerve LMC, involving EphA4, to be the primary development defect. Upon mapping the implicated region, they found overexpression of LIM-domain kinase 1 (Limk1), then using molecular and genetic analysis they found that it acts in the EphA4-Limk1-Cfl1/cofilin-actin pathway, which controls nerve growth cone collapse and extension. This reduction in nerve growth, see figure 10 in the appendix, was then shown to cause the reduced muscle growth, see figure 11, as previously mentioned, and increased apoptosis in the dorsal muscles mentioned previously. This study also ruled out neural tube patterning and showed that it was the reduced extension of motor that causes the neurological defects. They also went further than using pma mice through the electroporation of plasmids expressing LIMK1 into chicken neural tube, which caused the same axon loss as in pma mice, and subsequent clubfoot. I agree with their conclusion that this supports a neuromuscular aetiology for clubfoot, and this indicates Limk1 as one of the genetic components. This therefore means that pma mice are a good model for human clubfoot, and only through more experimentation will we be able to understand the full genetic mechanisms of clubfoot, but it is clearly complex.

We now understand that the EphA4-Limk1-Cfl1/cofilin-actin pathway is involved along with PITX1-TBX4 pathways (which are important in early limb development), but these complex also involve lateral mesoderm HOX signalling (Alvarado 2016). HOXD, HOXC, and HOXA clusters due to their involvement in limb and muscle patterning and has been shown that errors in these can lead to oxidative damage, inflammation and apoptosis (Wang 2018). NAT2 is involved in smoking associated TEV, as it causes less acetylation, which could lead to the build-up of possibly toxic amines and adducts. MYH8, MYH3, TNNI2, TNNT3, and TPM2 are all components of the contractile complex for muscles, also associated with CTEV (Basit and Khoshhal 2017). All of these associated genetic factors and more (including how they were identified), along with the one environmental risk factor of smoking, can be seen in figure 13 in the appendix. These all add to the to the genetic factors above when trying to explain CTEV in humans. A main gene that causes clubfoot has not been found using these methods (genome wide association studies, copy number variations and linkage analysis), and as such, it is my opinion that we must use genetically modified animal models to work towards fully understanding the genetics behind clubfoot.

With this progress of understanding of the genetic mechanisms and causes of clubfoot, a very important question is what we are going to do with this knowledge, it is ultimately to help and treat those who are born with clubfoot. There has been research into developing personalised treatment for patients with clubfoot specifically those resistant to standard treatment by using not only molecular genetic engineering of mouse models of clubfoot but also human gene sequencing with MRI of clubfoot (Dobbs and Gurnett 2017). This means that those with 'treatment resistant clubfoot' can be identified early at or before birth and as such their treatment can be the more extreme initial surgical intervention, instead of starting on the Ponseti method only to have it fail leaving the new-born older and consequently less responsive to treatment (Dobbs and Gurnett 2017). Beyond increasing the initial and subsequent manipulation, as well as surgical interventions, it is important to consider the future preventative methods with pre-implantation genetic screening, or human genetic editing to irradiate clubfoot, as well as the ethical implications. This would mean that blastocysts with the genetics for clubfoot could be selected either to not be implanted, or have their genome edited, and through these two methods eradicate clubfoot. Past societies like ancient nomads and later Spartans viewed 'deformed' children as a burden and would 'lay them out to die' (Strach, 1986). However, within other societies people with clubfoot have been accepted even becoming figureheads from the Greek

god Hephaestus who was depicted with twisted feet (Strach, 1986), and inbred Egyptian pharaohs, including Tutankhamun (Hawass et al., 2010). with the progress of our understanding, and therefore treatment/screening methods, of CTEV it will be up to future societies to determine the fate of those with clubfoot. In conclusion through the research of genetically modified mice and chickens, we have come to develop a genetic understanding of clubfoot in these animals, and we understand that it is extremely complex including many genes across multiple chromosomes. We now understand The PITX1-TBX4 pathway from Pitx1<sup>+/−</sup> mice and humans involved in CTEV. We also know from pma mice (and chickens) that the EphA4–Limk1–Cfl1/cofilin–actin pathway is involved and points to neuromuscular aetiology. But we understand from that in humans there are many more implicated genes, but through the use of genetically modified organisms we can fully understand the genetics and causes of human clubfoot. Finally, it will be up to future societies to determine the chosen course of action for individuals with clubfoot, whether it be advancement in physical treatment, genome editing or blastocyst selection, genetically modified animals are allowing us to understand the genetics and aetiology needed for clubfoot interventions.

## References

- Alvarado, D., Aferol, H., McCall, K., Huang, J., Techy, M., Buchan, J., Cady, J., Gonzales, P., Dobbs, M. and Gurnett, C. (2010). Familial Isolated Clubfoot Is Associated with Recurrent Chromosome 17q23.1q23.2 Microduplications Containing TBX4. *The American Journal of Human Genetics*, 87(1), pp.154-160.
- DOI: <https://doi.org/10.1016/j.ajhg.2010.06.010>
- Alvarado DM, McCall K, Aferol H, Silva MJ, Garbow JR, Spees WM, Patel T, Siegel M, Dobbs MB, Gurnett CA. (2011) Pitx1 haploinsufficiency causes clubfoot in humans and a clubfoot-like phenotype in mice. *Hum Mol Genet*. Oct 15;20(20):3943-52.
- DOI: <https://dx.doi.org/10.1093/hmg/ddq411>
- Alvarado, D., McCall, K., Hecht, J., Dobbs, M. and Gurnett, C. (2016). Deletions of 5HOXCgenes are associated with lower extremity malformations, including clubfoot and vertical talus. *Journal of Medical Genetics*, 53(4), pp.250-255.
- DOI: <https://dx.doi.org/10.1136/jmg.2015.103700>
- Basit, S. and Khoshhal, K. (2018). Genetics of clubfoot; recent progress and future perspectives. *European Journal of Medical Genetics*, 61(2), pp.107-113.
- DOI: <https://doi.org/10.1016/j.ejmg.2017.09.006>
- Böhm M. (1929) The embryologic origin of club-foot. *JBJS*; XI:229–259 Collinson JM, Lindström NO, Neves C, Wallace K, Meharg C, Charles RH, Ross ZK, Fraser AM, Mbogo I, Oras K, Nakamoto M, Barker S, Duce S, Miedzybrodzka Z, Vargesson N. (2018) The developmental and genetic basis of 'clubfoot' in the peroneal muscular atrophy mutant mouse. *Development*. Feb 8;145(3):dev160093.
- DOI: <https://dx.doi.org/10.1242/dev.160093>
- Comparini L., Podestà A., Russo C., and Cecchi F. (2019) Effect of inbreeding on the “Club Foot” disorder in Arabian Pureblood horses reared in Italy, *Open Veterinary Journal*, Vol. 9(3): 273–280
- DOI: <http://dx.doi.org/10.4314/ovj.v9i3.14>
- Delgado-Baeza E., I. Santos-Alvarez, A. Martos-Rodríguez (1999) Retinoic acid-induced clubfoot-like deformity: pathoanatomy in rat foetuses. *J Pediatr Orthop B*, 8, pp. 12-18
- Link: <https://www.ncbi.nlm.nih.gov/pubmed/10709591>
- Dobbs, M. and Gurnett, C. (2012). Genetics of clubfoot. *Journal of Pediatric Orthopaedics B*, 21(1), pp.7-9.
- DOI: <https://dx.doi.org/10.1097/BPB.0b013e31825a2a2c>
- Dobbs, M. and Gurnett, C. (2017). The 2017 ABJS Nicolas Andry Award: Advancing Personalized Medicine for Clubfoot Through Translational Research. *Clinical Orthopaedics and Related Research®*, 475(6), pp.1716-1725.
- DOI: <http://dx.doi.org/10.1007/s11999-017-5337-2>
- Duce, S., Madrigal, L., Schmidt, K., Cunningham, C., Liu, G., Barker, S., Tennant, G., Tickle, C., Chudek, S. and Miedzybrodzka, Z. (2010). Micro-magnetic resonance imaging and embryological analysis of wild-type and pmamutant mice with clubfoot. *Journal of Anatomy*, 216(1), pp.108-120.
- DOI: <https://dx.doi.org/10.1111/j.1365-2796.2009.01922.x>
- Farrell, S. A., Summers, A. M., Dallaire, L., Singer, J., Johnson, J. A., Wilson, R. D. (1999). Club foot, an adverse outcome of early amniocentesis: disruption or deformation? CEMAT. Canadian Early and Mid-Trimester Amniocentesis Trial. *Journal of medical genetics*, 36(11), 843–846.
- Link: <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1734259/>
- Feldbrin, Z., Gilai, A. N., Ezra, E., Khermosh, O., Kramer, U. and Wientroub, S. (1995). Muscle imbalance in the aetiology of idiopathic club foot. An electromyographic study. *J. Bone Joint Surg. Br.* 77, 596-601.
- Link: <https://www.ncbi.nlm.nih.gov/pubmed/7615605>
- Fritsch, H. and Eggers, R. (1999). Ossification of the Calcaneus in the Normal Fetal Foot and in Clubfoot. *Journal of Pediatric Orthopaedics*, 19(1), pp.22-26.
- Link: <https://www.ncbi.nlm.nih.gov/pubmed/9890281>
- Germiller, J., Lerner, A., Pacifico, R., Loder, R. and Hensinger, R. (1998). Muscle and Tendon Size Relationships in a Paralyzed Chick Embryo Model of Clubfoot. *Journal of Pediatric Orthopaedics*, 18(3), pp.314-318.
- Link: <https://www.ncbi.nlm.nih.gov/pubmed/9600555>
- Gilardi-Hebenstreit, P., Nieto, M. A., Frain, M., Mattei, M. G., Chestier, A., Wilkinson, D. G. and Charnay, P. (1992). An Eph-related receptor protein tyrosine kinase gene segmentally expressed in the

- developing mouse hindbrain. *Oncogene* 7, 2499-506. Link: <https://www.ncbi.nlm.nih.gov/pubmed/8455939>
- Handelsman, J. E. and Badalamente, M. A. (1981). Neuromuscular studies in clubfoot. *J. Pediatr. Orthop.* 1, 23-32.
- DOI: <https://doi.org/10.1097/01241398-198101010-00004>
- Hawass Z, Gad YZ, Ismail S, et al. (2010) Ancestry and Pathology in King Tutankhamun's Family. *JAMA*. 2010;303(7):638–647.
- DOI: <https://doi.org/10.1001/jama.2010.121>
- Helmbacher F., Schneider-Maunoury S., Topilko P., Tiret L. and Charnay P. (2000). Targeting of the EphA4 tyrosine kinase receptor affects dorsal/ventral pathfinding of limb motor axons. *Development* 127, 3313-3324.
- Link: <http://www.ncbi.nlm.nih.gov/pubmed/10887087>
- Hoffa A. Lehrbuch der Orthopadischen Chirurgie. Stuttgart: Ferdinand Enke; 1902. Ippolito Ernesto, De Maio F., Mancini F., Bellini D., and Orefice A. (2009) Leg muscle atrophy in idiopathic congenital clubfoot: is it primitive or acquired? *Journal of Children's Orthopaedics*, 3:3, 171-178.
- DOI: <https://doi.org/10.1007/s11832-009-0179-4>
- Ippolito E, Ponseti IV. (1980) Congenital club foot in the human fetus. A histological study. *The Journal of Bone and Joint surgery. American Volume*. Jan;62(1):8-22.
- Link: <https://europepmc.org/article/med/7351421>
- Katoh, H., Watanabe, Y., Ebukuro, M., Muguruma, K., Takabayashi, S. and Shiroishi, T. (2003). Chromosomal Mapping of the Peroneal Muscular Atrophy (pma) Gene in the Mouse. *Experimental Animals*, 52(5), pp.433-436.
- DOI: <https://doi.org/10.1538/expanim.52.433>
- Kawashima, T. and Uhthoff, H. (1990). Development of the Foot in Prenatal Life in Relation to Idiopathic Club Foot. *Journal of Pediatric Orthopaedics*, 10(2), pp.232-237.
- Link: <https://www.ncbi.nlm.nih.gov/pubmed/2312708>
- Kilby E., and Vargesson N. (2009). 06-P034 Determining the developmental basis of idiopathic clubfoot. *Mechanisms of Development*, Volume 126, Supplement, August 2009, Page S13
- DOI: <https://doi.org/10.1016/j.mod.2009.06.260>
- Liu, Z., Li, X., Chen, B., Zheng, C., Zhong, Y., Jia, Y. and Du, S. (2010). Retinoic acid retards fetal and hindlimb skeletal development asymmetrically in a retinoic acid-induced clubfoot model. *Experimental and Toxicologic Pathology*, 62(6), pp.663-670.
- DOI: <https://doi.org/10.1016/j.etp.2010.05.003>
- Lupo, P., Mitchell, L. and Jenkins, M. (2019). Genome-wide association studies of structural birth defects: A review and commentary. *Birth Defects Research*, 111(18), pp.1329-1342.
- DOI: <https://doi.org/10.1002/bdr2.1606>
- Macharia, R., McKinnell, I., Christ, B., Patel, K. and Otto, W. (2004). Decamethonium bromide-mediated inhibition of embryonic muscle development. *Anatomy and Embryology*, 208(1), pp.75-85.
- DOI: <https://doi.org/10.1007/s00429-003-0362-1>
- Merrill, L. J., Gurnett, C. A., Siegel, M., Sonavane, S., Dobbs, M. B. (2011). Vascular abnormalities correlate with decreased soft tissue volumes in idiopathic clubfoot. *Clinical orthopaedics and related research*, 469(5), 1442–1449. doi:10.1007/s11999-010-1657-1
- DOI: <https://dx.doi.org/10.1007>
- Miedzybrodzka, Z. (2003). Congenital talipes equinovarus (clubfoot): a disorder of the foot but not the hand. *Journal of Anatomy*, 202(1), pp.37-42.
- DOI: <https://dx.doi.org/10.1046>
- Nadeem, R., Brown, J., Lawson, G. and Macnicol, M. (2007). Somatosensory evoked potentials as a means of assessing neurological abnormality in congenital talipes equinovarus. *Developmental Medicine Child Neurology*, 42(8), pp.525-530.
- DOI: <https://doi.org/10.1111/j.1469-8749.2000.tb00708.x>
- Nieto, M. A., Gilardi-Hebenstreit, P., Charnay, P. and Wilkinson, D. G. (1992). A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. *Development* 116, 1137-50
- Nonaka, I., Kikuchi, A., Suzuki, T. and Esaki, K. (1986), Hereditary peroneal muscular atrophy in the mouse: an experimental mode for congenital contractures (arthrogryposis). *Exp. Neurol.* 91, 571-579.
- DOI: [https://doi.org/10.1016/0014-4886\(86\)90053-1](https://doi.org/10.1016/0014-4886(86)90053-1)

- Ponseti, I. (1997). Common errors in the treatment of congenital clubfoot. International Orthopaedics, 21(2), pp.137-141.  
DOI: <https://dx.doi.org/10.1007>
- Ramachandran, M., Aronson, J. K. (2006). The diagnosis of art: diastrophic dysplasia and Hephaestos. Journal of the Royal Society of Medicine, 99(11), 584–585.  
DOI: <https://dx.doi.org/10.1258>
- Sangha HK, Robson JC, Bowen S, et al. (2003) Deletion studies in the gammy mouse. J Pathol 201, 55A.
- Strach E.H. (1986) Club-foot Through the Centuries. In: Rickham P.P. (eds) Historical Aspects of Pediatric Surgery. Progress in Pediatric Surgery, vol 20. Springer, Berlin, Heidelberg.  
DOI: [https://doi.org/10.1007/978-3-642-70825-1\\_6](https://doi.org/10.1007/978-3-642-70825-1_6)
- Yoshimura, N., Fukuhara, N. and Noguchi, T. (1988). Sensori-motor neuropathy associated with congenital bilateral club feet: histological and ultrastructural study of the sural nerve. No To Shinkei 40, 857-861.  
Link: <https://www.ncbi.nlm.nih.gov/pubmed/3190934>
- Wang, Y. (2018). Relationship between HOX gene and pediatric congenital clubfoot. Experimental and Therapeutic Medicine. 2018 Jun; 15(6): 4861–4865.  
DOI: <https://dx.doi.org/10.3892>
- Wynne-Davies, R. (1964). FAMILY STUDIES AND THE CAUSE OF CONGENITAL CLUB FOOT. The Journal of Bone and Joint Surgery. British volume, 46-B(3), pp.445-463.  
Link: <https://www.ncbi.nlm.nih.gov/pubmed/14216453>

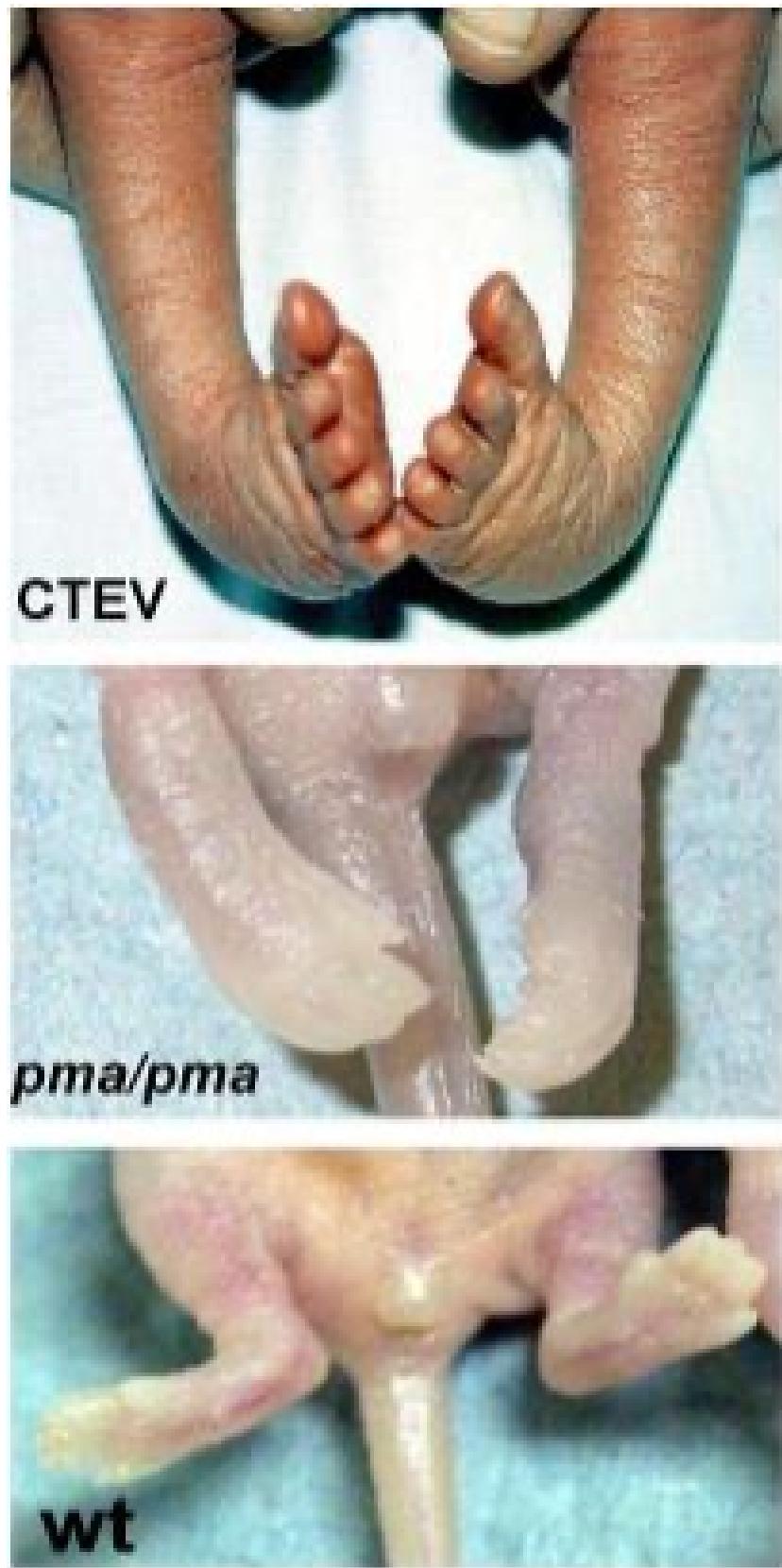


Figure 1: *Clubfoot phenotype in pma/pma mice*. Human newborn with congenital talipes equinovarus (CTEV, commonly known as clubfoot) (top) compared with newborn pma/pma mouse (middle) and newborn wild-type mouse (bottom). From Collinson et al., 2018

Anatomical feature	<i>pma</i> mouse hind limb anatomy	Human CTEV leg anatomy
Tibia and fibula	<i>pma</i> bones same size and shape as wild-type. Distal end of the <i>pma</i> bones not distorted	Not affected
Shank muscle	Reduced	Overall shank muscle reduced
Anterior lateral shank muscles	Hypoplastic in <i>pma</i>	Relatively unaffected
Anterior medial shank muscles	Similar total muscle volume	Relatively unaffected
Posterior shank muscles	Similar total muscle volume	Most affected
Orientation of the foot in sagittal plane	Plantar flexion at the ankle in <i>pma</i>	Equinus deviation
Orientation of the foot in the frontal plane	<i>pma</i> foot shows inversion and adduction of the mid foot and forefoot	Inversion and adduction
Flexor digitorum longus tendons	Appear shorter in <i>pma</i>	May be shorter than normal
Calcaneus, talus, centrale	<i>pma</i> bones same size and shape as wild-type, slight inversion in <i>pma</i>	Variable evidence for changed shape/size
Tarsals	Supination in the <i>pma</i>	Supination
Metatarsal	<i>pma</i> bones are the same size and shape as wild-type, supination with clear adduction in the <i>pma</i>	Adductus
Phalanges	Curled and inverted in <i>pma</i>	May demonstrate latent curling during correction due to shortened flexors

Figure 2: Anatomical characteristics of the hind limb in the *pma* mouse mutants and in human CTEV. From Duce et al., 2010

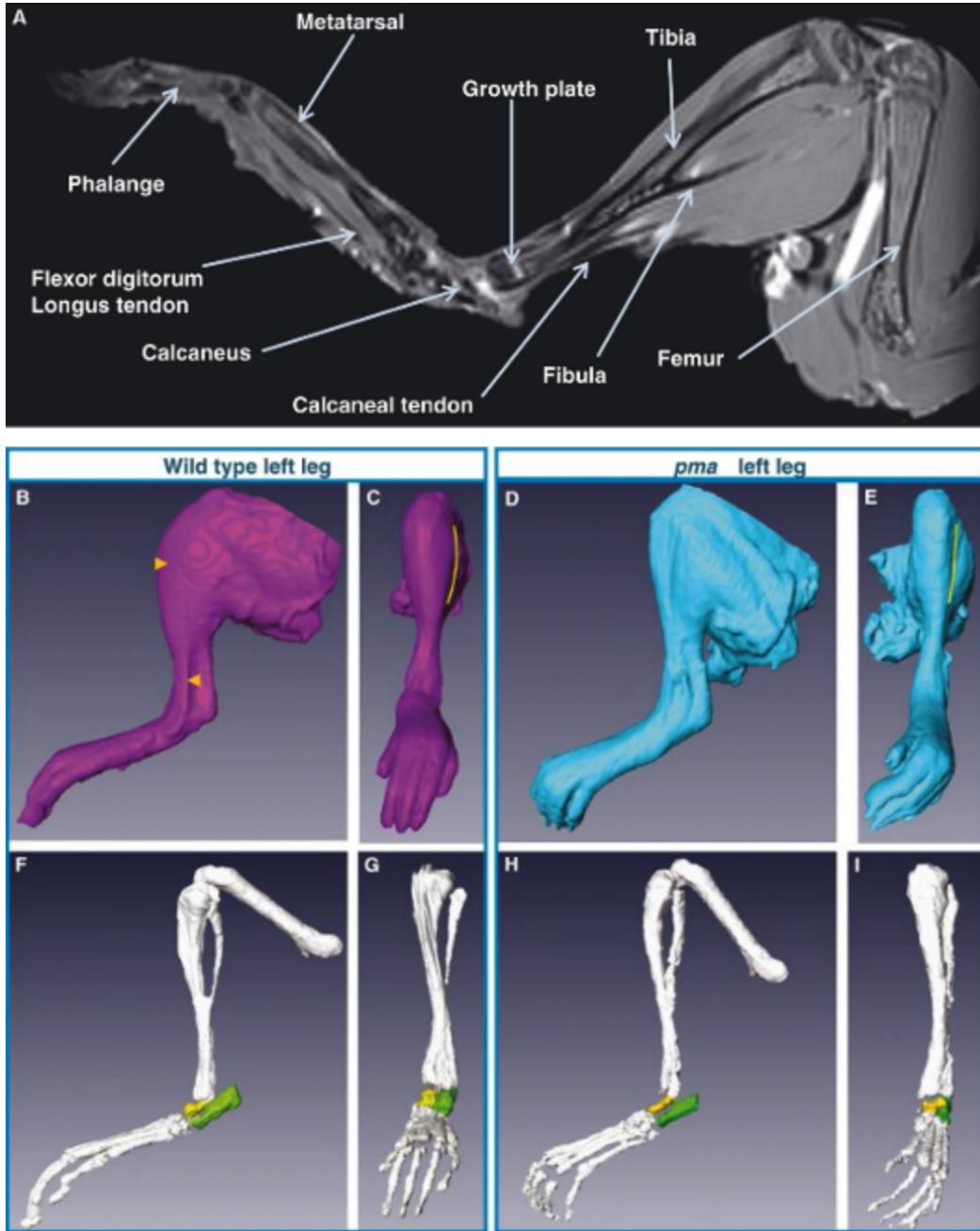


Figure 3: MRI spin-echo images of left legs of 3-week-old wild-type and pma mice; five pairs of limbs were studied. (A) 2D slice in sagittal plane from a TR/TE = 1000/40 ms spin-echo 3D MRI data set of wild-type leg showing main anatomical features. (B,C) 3D surface reconstruction of outer surface of wild-type leg from TR/TE = 1000/6 ms spin-echo image data set. (D,E) 3D surface reconstruction of outer surface of pma mouse leg from TR/TE = 1000/6 ms spin-echo image. (F,G) 3D surface reconstruction of mineralized bones of wild-type leg from TR/TE = 1000/40 ms and TR/TE = 1000/6 ms spin-echo image data sets. (H,I) 3D surface reconstruction of mineralized bones of pma mouse leg from TR/TE = 1000/40 ms and TR/TE = 1000/6 ms spin-echo image data sets (matrix size,  $256 \times 256 \times 256$ ; field of view,  $25 \times 25 \times 25$  mm; voxel dimensions,  $97 \times 97 \times 97$  m). (B,D,F,H) Lateral views of sagittal plane; (C,E,G,I) anterior views of frontal plane. Dashed line in C and E outlines anterior lateral side of leg (i.e. shank); note reduction in mutant. Calcaneus shown in green and talus in yellow in F,G,H and I. From Duce et al., 2010

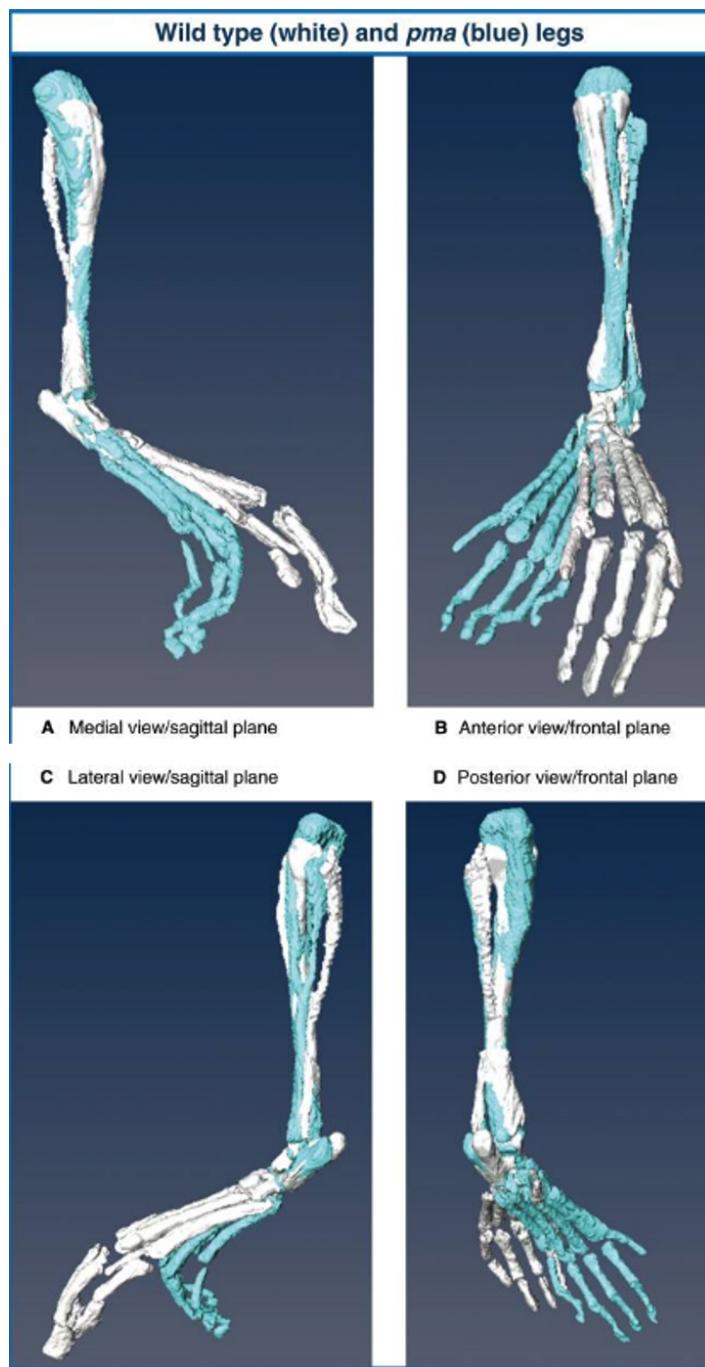


Figure 4: 3D surface reconstructions of mineralized bones from MRI of left legs of 3-week-old mice [wild-type (white) and pma (blue)]. Images aligned relative to the tibial mechanical axis to allow direct comparison of anatomy. Three pma and wild-type hind limbs were acquired and overlaid; all comparisons showed the same trends. (A) Medial view of sagittal plane, (B) anterior view of frontal plane, (C) lateral view of sagittal plane and (D) posterior view of frontal plane. Mineralized bones of wild-type and pma legs reconstructed from TR/TE = 500/6 ms and TR/TE = 500/40 ms spin-echo image data sets and a TR/TE = 250/2.3 ms gradient-echo image data set (matrix size,  $256 \times 256$ ; field of view,  $25 \times 25 \times 25$  mm; voxel dimensions,  $97 \times 97 \times 97$  m). From Duce et al., 2010

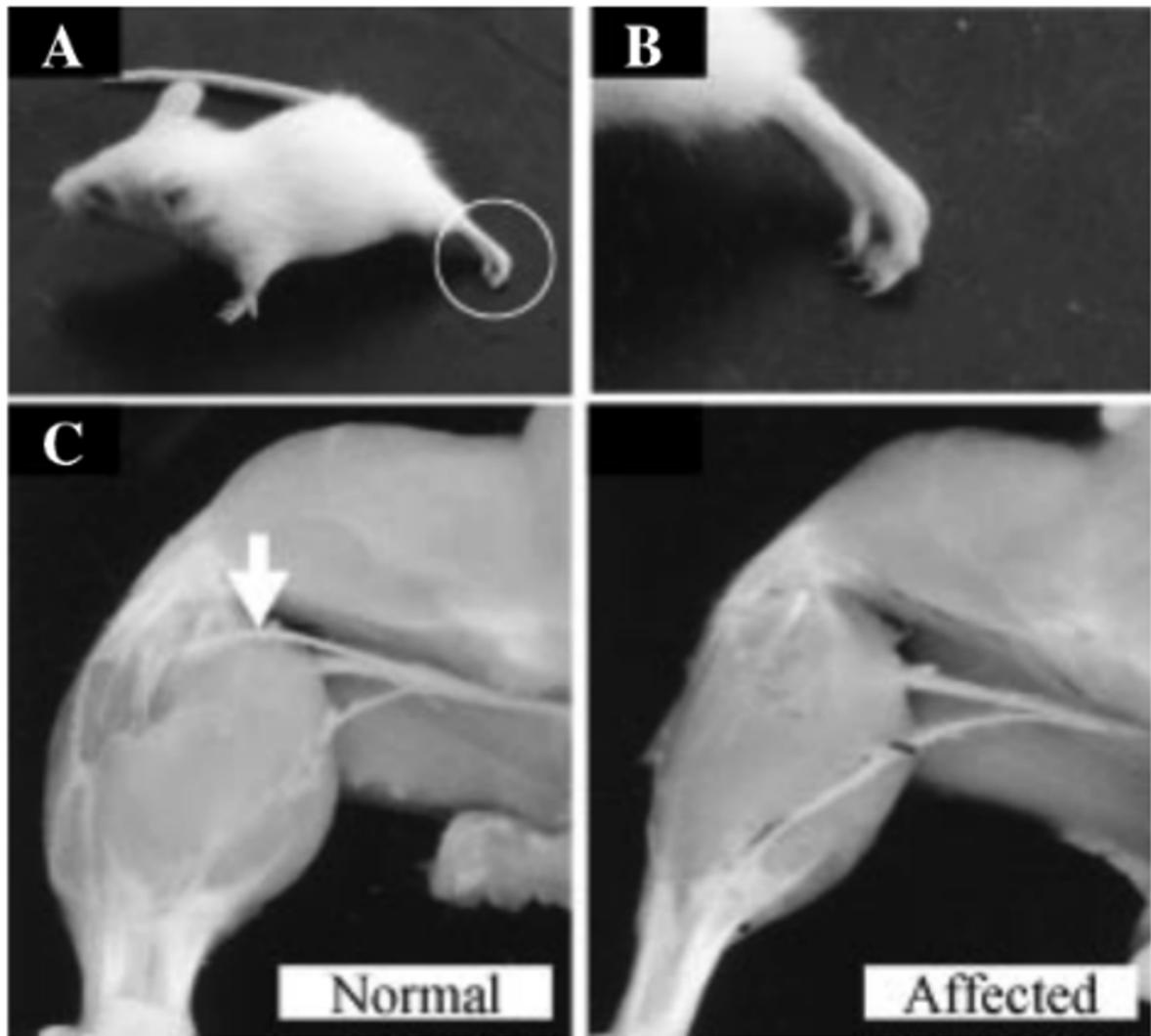


Figure 5: Morphological characteristics of the PMA (peroneal muscular atrophy) mouse. A: A PMA mouse with peroneal muscular atrophy in hind limbs, B: Clubfoot caused by absence of the common peroneal nerve, C: Developed common peroneal nerve (shown by white arrow) in the normal mouse, and D: Underdeveloped common Peroneal nerve in the abnormal mouse. From Katoh et al., 2003

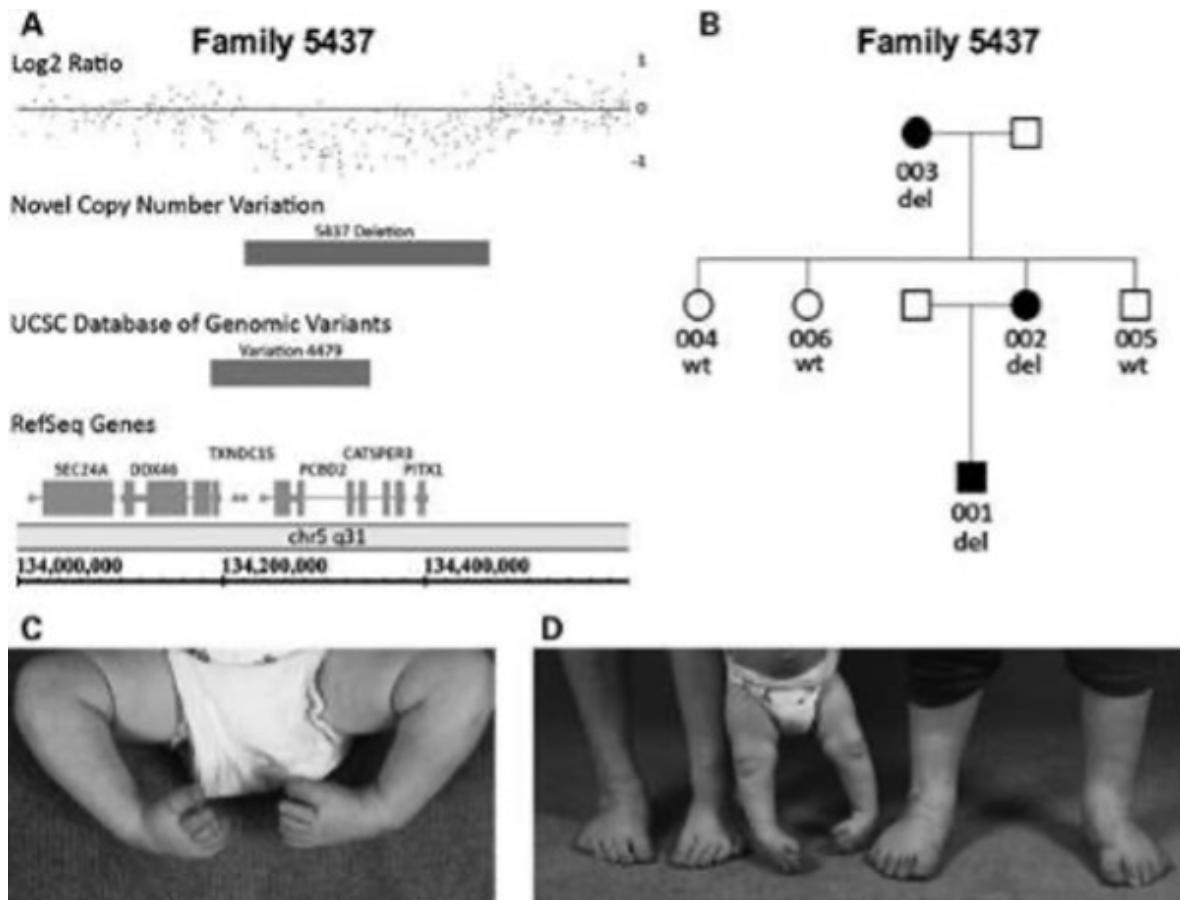


Figure 6: Chromosome 5q31 microdeletion containing PITX1 is present in family with isolated clubfoot. (A) Chromosome 5q31 region showing 241 kb deletion at chr5:134222383–134463022 (hg18 build of the UCSC genome browser), involving 124 markers with decreased log<sub>2</sub> ratios that was detected in the proband. Four RefSeq genes are located within the interval, including PITX1. (B) The chromosome 5q31 microdeletion segregates with clubfoot in family 5437. Black affection status indicates isolated clubfoot, del indicates deletion and WT indicates normal copy number. (C) Proband from family 5437 with untreated bilateral clubfoot. (D) Three generations of family 5437 with the chromosome 5q31 microdeletion showing surgically treated clubfoot in mother (left), untreated proband (middle) and surgically treated maternal grandmother (right). From Alvarado et al., 2011

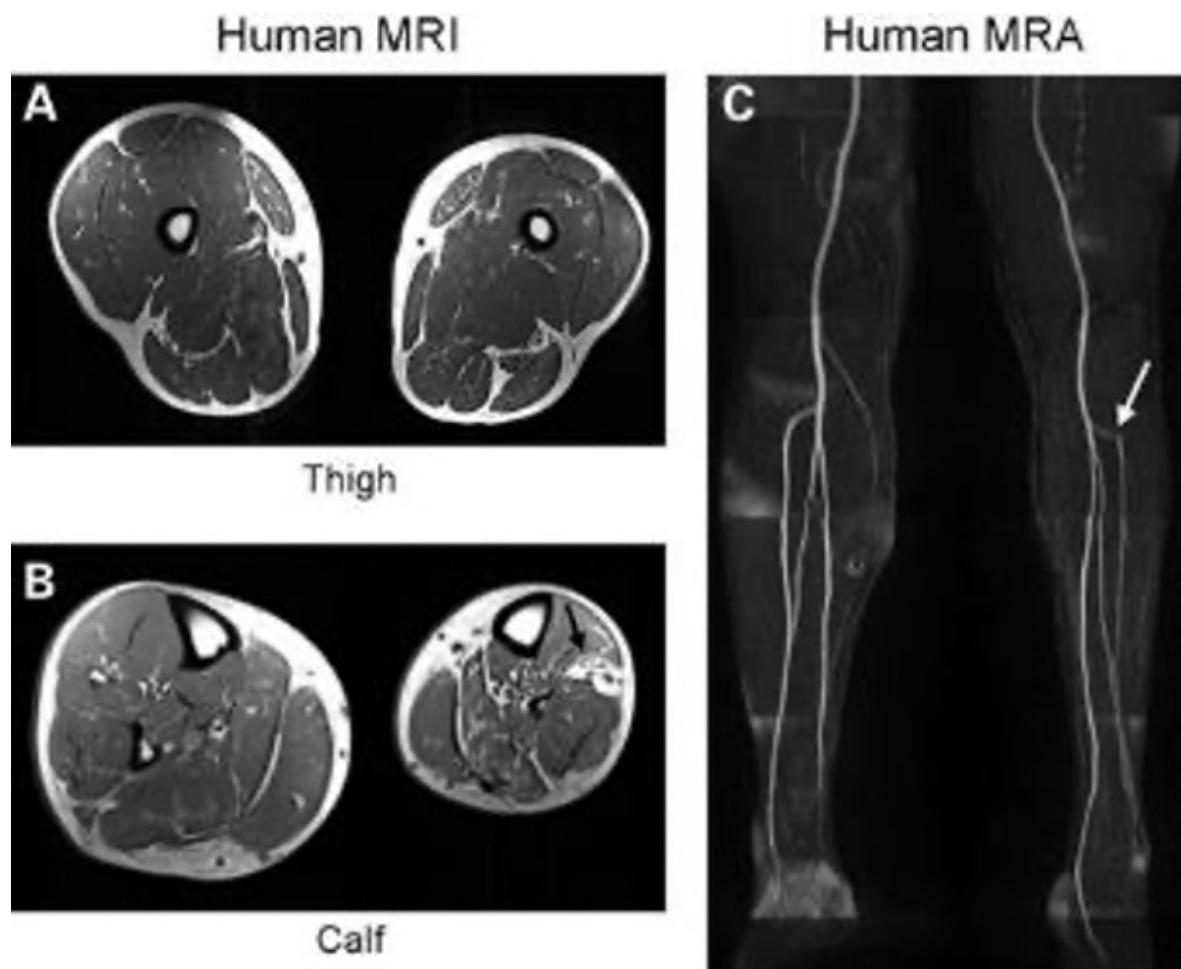


Figure 7: Muscle volume and vascular abnormalities in the affected clubfoot limb of an adult patient with PITX1 E130K mutation. (A) MRI shows hypoplasia of the left clubfoot limb (shown on the right) compared with the unaffected right leg on transverse sections. (B) Images obtained at calf level show more severe involvement than those taken at the thigh. Muscle hypoplasia is present throughout but is most prominent in the lateral and anterior compartments that also show a corresponding increase in fat tissue (black arrow). (C) Magnetic resonance angiograph of same patient showing reduced perfusion of the anterior tibial (white arrow) and peroneal arteries on the affected left leg compared with the right. From Alvarado et al., 2011

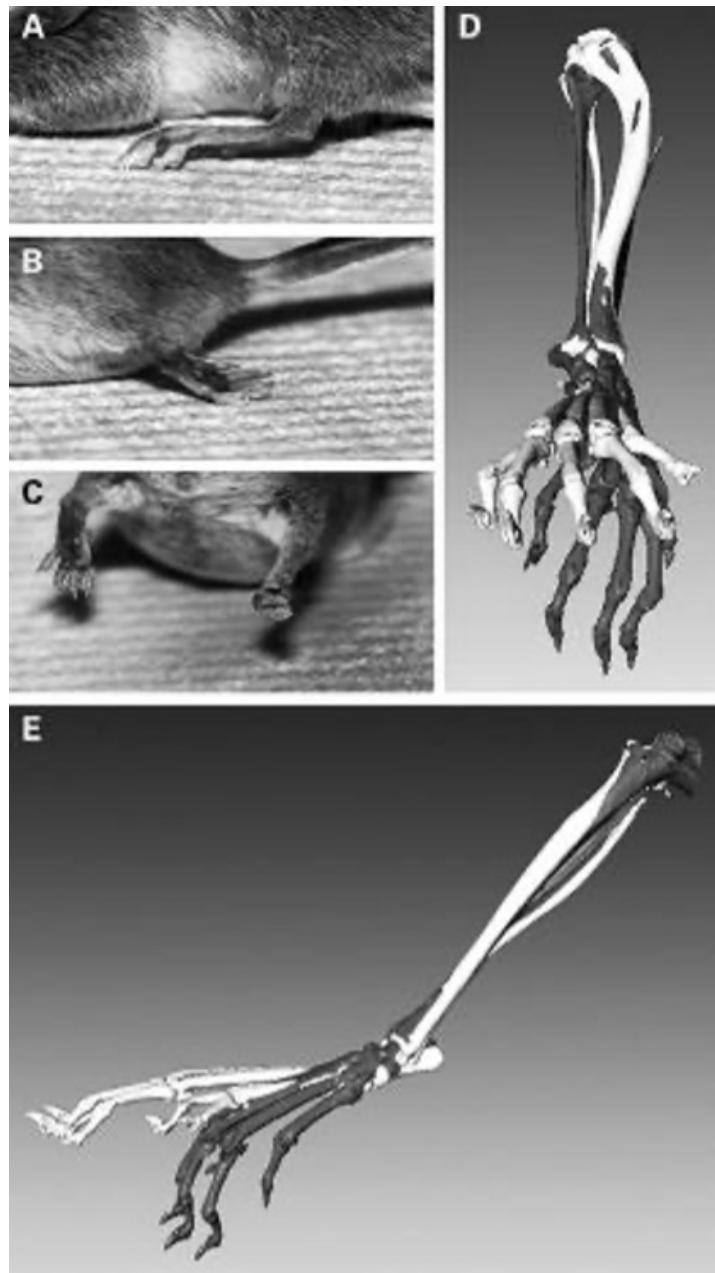


Figure 8: Clubfoot-like phenotype is present in some *Pitx1* haploinsufficient mice. (A) Lateral view of unaffected hindlimb of *Pitx1+/-* mouse. (B) Lateral view of affected hindlimb of *Pitx1+/-* mouse. (C) Dorsal view of unaffected (left) and clubfoot-affected (right) hindlimbs. (D) MicroCT image comparing clubfoot-like right limb of an affected mouse (gray) to unaffected right limb of a control mouse (white) demonstrating forefoot cavus in the frontal plane and (E) hindfoot equinus in the sagittal plane. From Alvarado et al., 2011

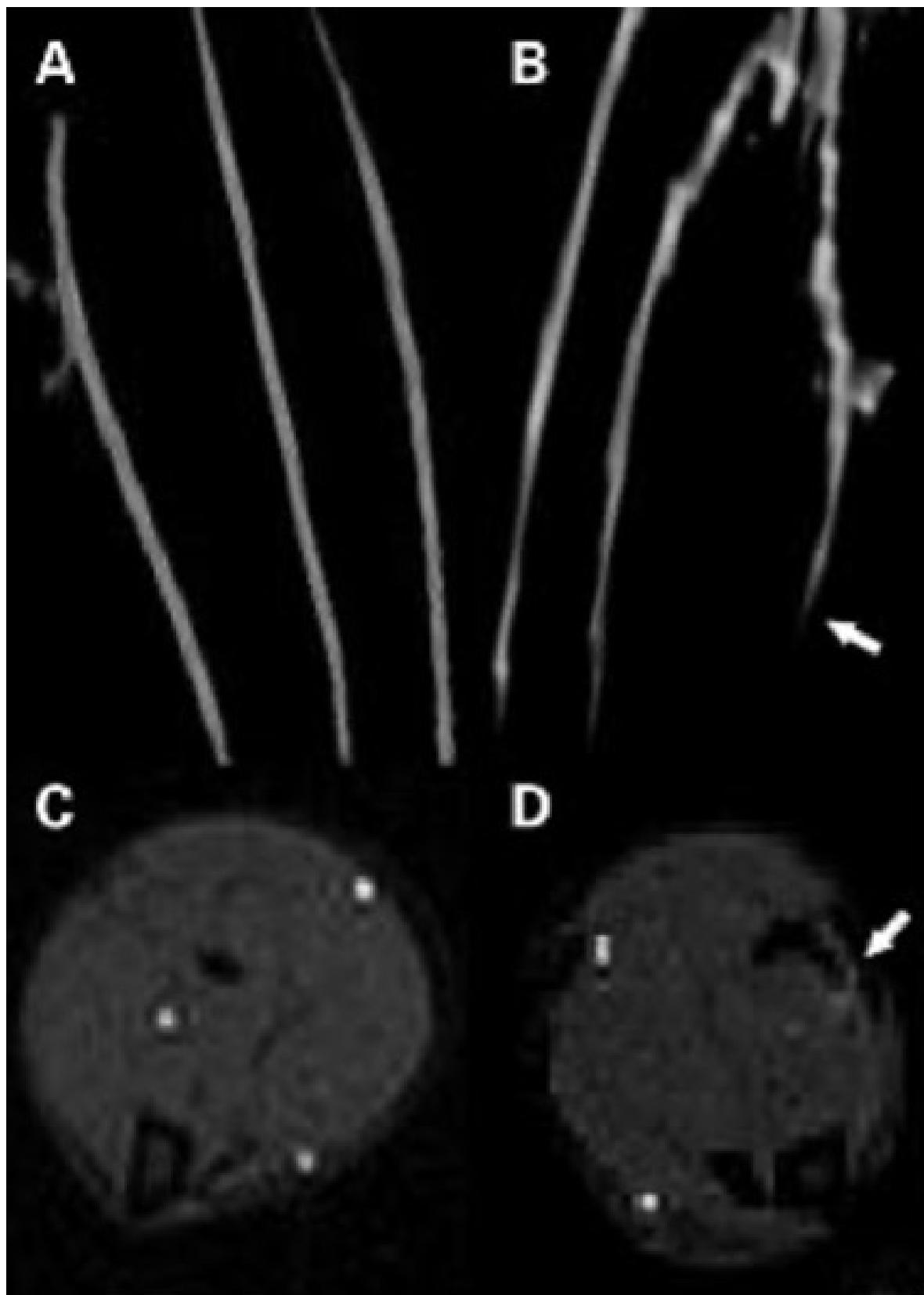


Figure 9: Peroneal artery hypoplasia is present in the clubfoot-like limb of Pitx1<sup>+/−</sup> affected mice. Magnetic resonance angiograph of unaffected (A and C) and affected (B and D) Pitx1<sup>+/−</sup> hindlimbs. The peroneal artery (white arrows) is hypoplastic in affected hindlimbs. There are no apparent anomalies in the anterior tibial or posterior tibial arteries. From Alvarado et al., 2011



Figure 10: (F) EphA4<sup>+/−</sup> P5 mouse showing an abnormal positioning of the left hindlimb (arrow). From Helmbacher 2000

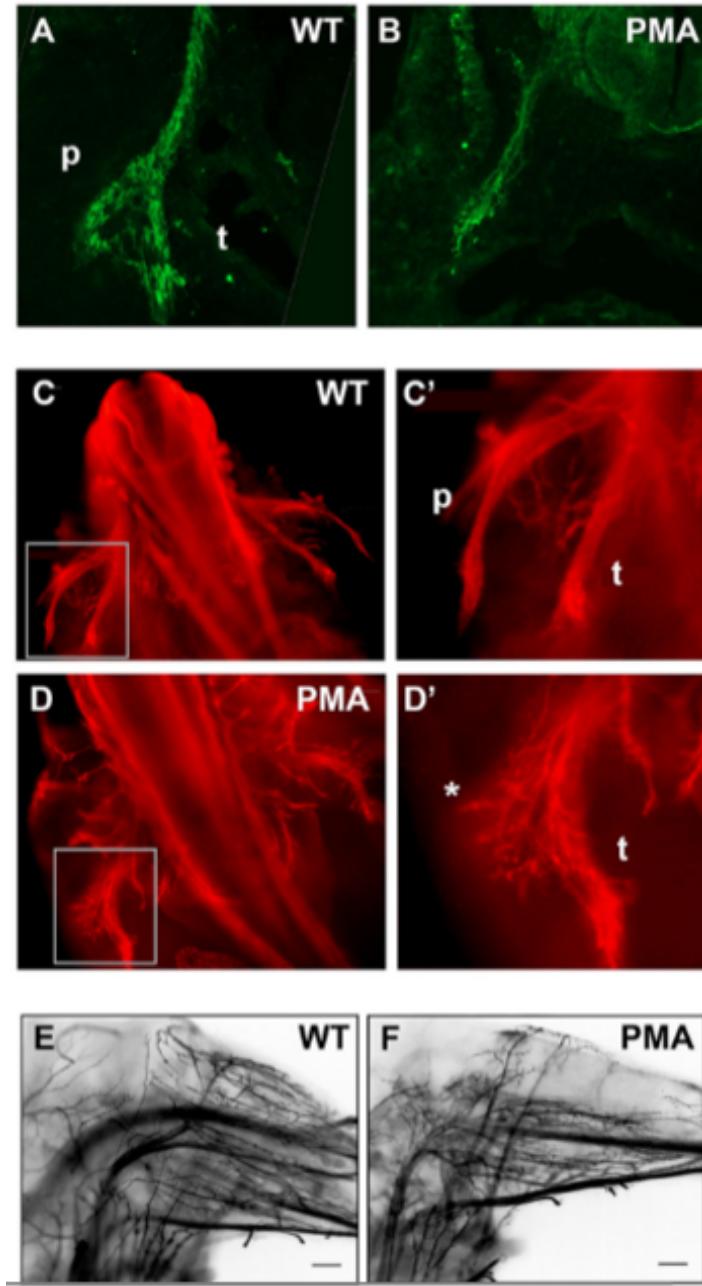


Figure 11: Retardation of nerve growth and abortive innervation of dorsal muscles in PMA mice. (A,B) -III-tubulin immunohistochemistry (green) in transverse sections of stage-matched E11.75 wild-type (WT; A) and pma/pma mice(B). The wild-type sciatic nerve has projected further than that in the pma/pma mice and, unlike the PMA nerve, started to branch into discrete dorsal (peroneal, p) and ventral (tibial/sural, t) components. (C,D) Whole-mount -III-tubulin immunohistochemistry (red) on wild-type (C,C) and pma/pma (D,D) embryos. C and D show magnifications of the boxed areas over the left-hand side nerves in C and D, respectively. The peroneal (p) and tibial/sural (t) components are labelled. In pma/pma embryos, the tibial/sural branch is grossly normal, but only a few defasciculated axons are observable (asterisk) in place of the peroneal nerve, presenting a feather-like appearance. (E,F) Whole-mount -III-tubulin immunohistochemistry on lower hindlimbs of E16.5 wild-type (left) and pma/pma embryos (right). Dorsal is to top: the dorsal muscles of the pma/pma foetuses are completely aneural, suggesting that the putative peroneal axons noted at E12.5 have not survived. Scale bars: 50  $\mu$ m. From Collinson et al., 2018

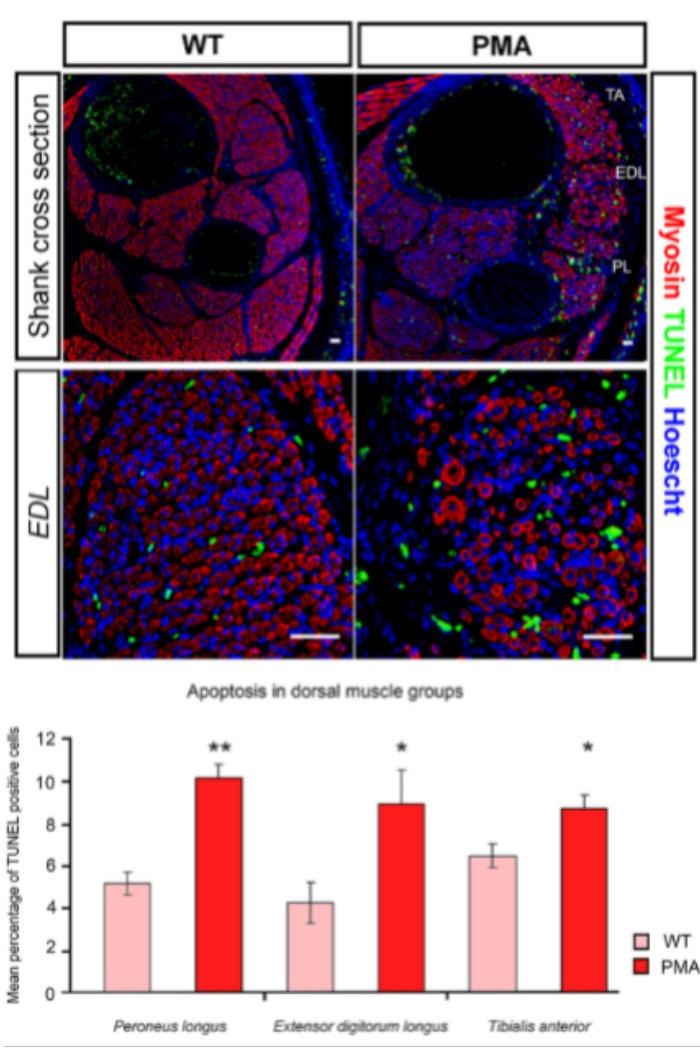


Figure 12: Increased apoptosis in dorsal muscleblocks of the pma hindlimb. Top: TUNEL labelling (green) to visualise apoptotic cells in cross-sections of E16.5 wild-type (WT; left) and pma/pma foetuses (right), combined with immunohistochemistry for myosin heavy chain (red) and Hoechst nuclear stain (blue). Higher magnification of the one dorsal muscle, the extensor digitorum longus, is shown. Bottom: Although apoptosis occurs in all muscles, the percentage of TUNEL-positive cells was significantly greater in the three major dorsal muscle blocks of pma/pma foetuses than in wild-type controls ( $n=8$  for both groups). \* $P<0.05$ ; \*\* $P<0.01$ . Error bars represent s.e.m. Scale bars: 50  $\mu$ m. From Collinson et al., 2018

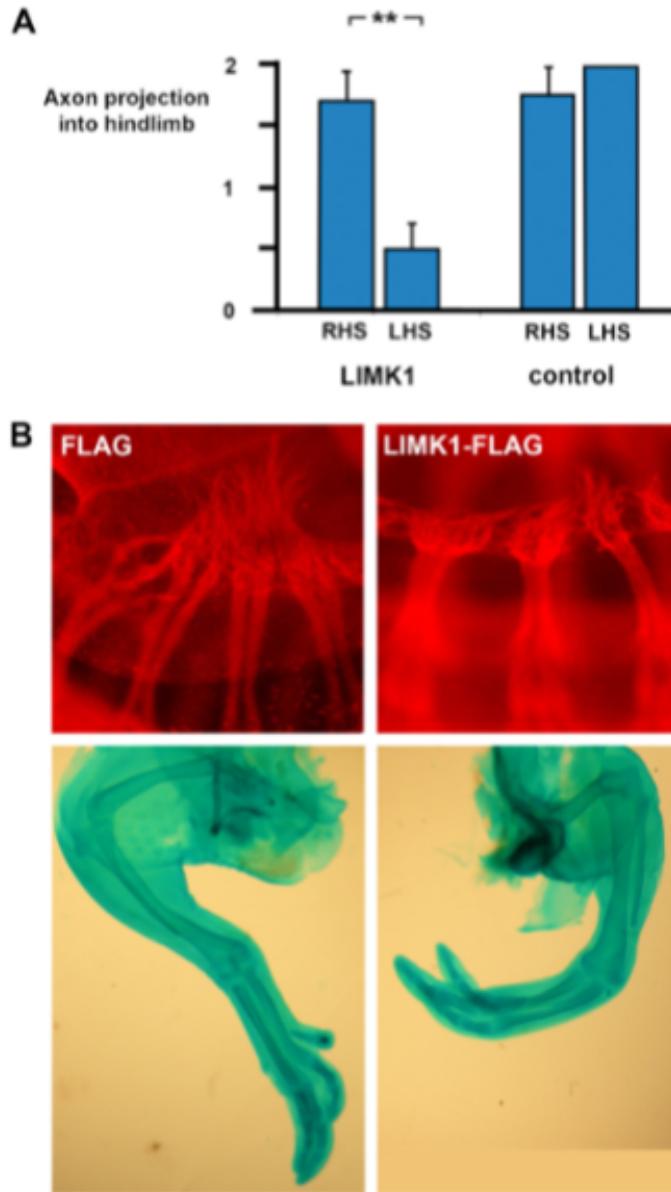


Figure 13: Electroporation of LIMK1 into chicken neural tube causes axon loss and clubfoot. Electroporation of plasmids expressing LIMK1 or empty vector controls into HH stage 11 chickens followed by immunohistochemistry for -III-tubulin 72 h later or Alcian Blue cartilage staining after a further 5 days. (A) Nerve projection scored 0-2 as described in the Materials and Methods for electroporated (left-hand side, LHS) and non-electroporated contralateral sides (right-hand side, RHS) of each embryo, for LIMK1 and control vectors, and shows significant inhibition of axon growth in LIMK1-treated nerves, but not in empty-vector electroporations. LIMK1 electroporation: RHS nerve score=1.67±0.25, n=9; LHS score=0.5±0.22, n=8 (one embryo damaged cf. RHS). Control FLAG electroporation: RHS nerve score=1.75±0.25, n=4; LHS score=2±0.00, n=3. \*\*P=0.001 (paired t-test). Error bars represent s.e.m. (B) (Top) Whole-mount -III-tubulin immunohistochemistry (red) on chicken embryos showing normal sciatic plexus formation and axon projection (score 2) after an empty 'FLAG' vector transfection compared with representative failure of nerve plexus formation (score 0) after LIMK1 transfection. (Bottom) Alcian Blue cartilage preparation of (left) a control FLAG-electroporated chicken limb and (right) limb of one of the chickens (3/12) that exhibited a mild clubfoot-like phenotype after transfection with LIMK1. From Collinson et al., 2018

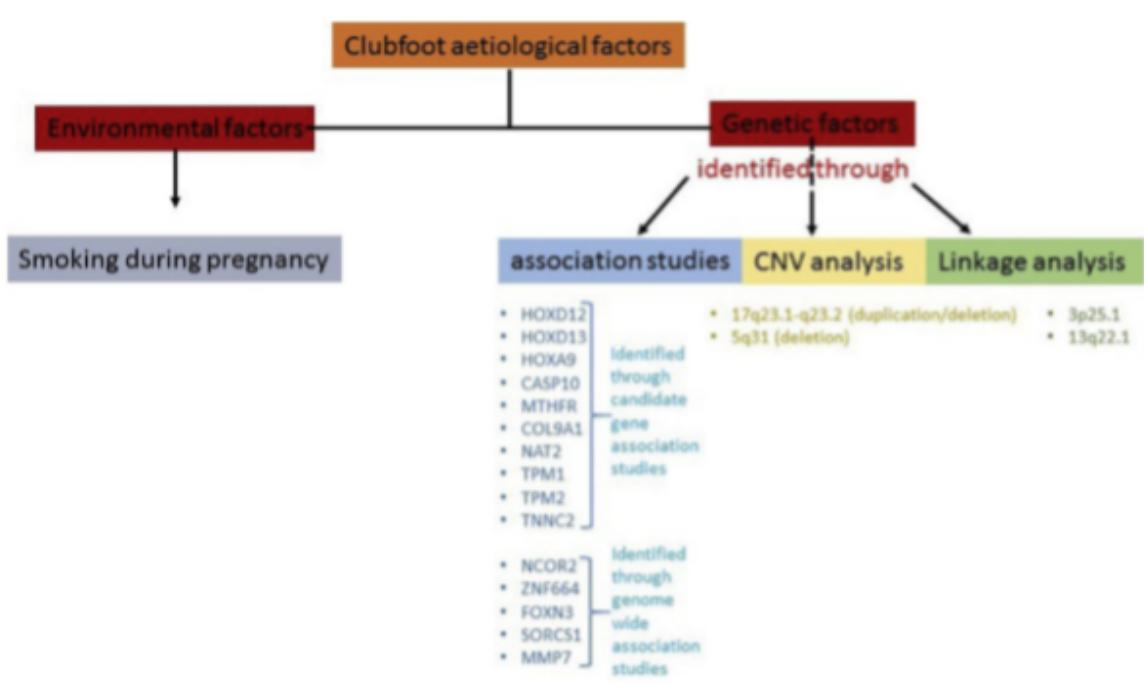


Figure 14: Overview of environmental and genetic factor associated with [human] clubfoot. From Basit and Khoshhal 2017

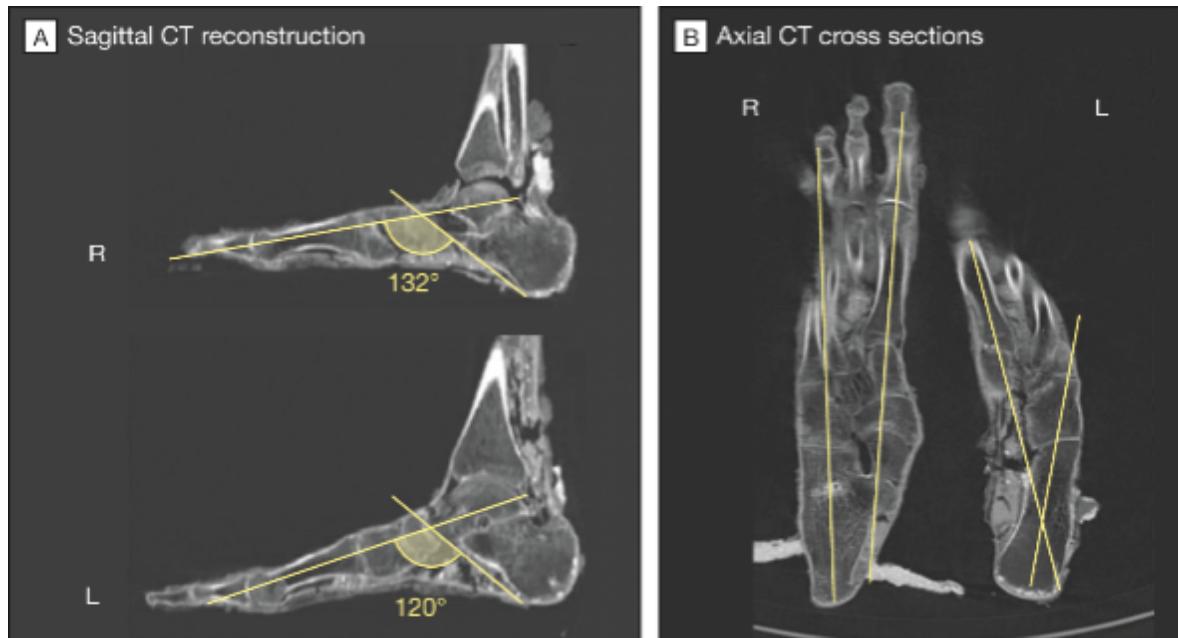


Figure 15: Analysis of malformations in the feet of Tutankhamun A, As indicated by the angle between the axis of the first metatarsal and the line between the lowest point of the calcaneal tuberosity to the lowest point of the calcaneocuboid articulation (Rocher angle), the arch of the right foot is flat (132°) compared with that of the left (120°). The Rocher angle of a normal foot is 126°. B, The supine and inwardly rotated position of the left foot are further features of clubfoot. From Hawass et al., 2010



Figure 16: Hephaistos (mounted) with Dionysus (left) as depicted on a sixth century BC hydria (water-vase). (Image reproduced in colour online.). From Ramachandran, M., Aronson, J. K. (2006)