



## Review

# The pharyngeal pouches and clefts: Development, evolution, structure and derivatives

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## ABSTRACT

The pharyngeal arches form the face and neck of the developing embryo. The pharyngeal tissue is divided into distinct arches by the formation of clefts and pouches in between the arches. These clefts and pouches form at the juxtaposition between the ectoderm and endoderm and develop into a variety of essential structures, such as the ear drum, and glands such as the thymus and parathyroids. How these pouches and clefts between the arches form and what structures they develop into is the subject of this review. Differences in pouch derivatives are described in different animals and the evolution of these structures are investigated. The implications of defects in pouch and cleft development on human health are also discussed.

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## 1. The emergence and development of the pharyngeal arches

In vertebrates, the pharyngeal apparatus develops from a transient series of segmental structures appearing as bulges on the cranial lateral side of the embryo and named the pharyngeal, or branchial, arches. The pharyngeal arches form successively in a cranial to caudal way during ontogeny. Five pairs of arches emerge between the second and fourth week of gestation in man, between Theiler's stages St13 and -16 in the mouse, and between Hamburger and Hamilton stages HH14 and -19 in the chick [1–3]. Notably, in all amniotes, the fifth arch does not form or regresses. Consequently, the most caudal arch corresponds to the sixth arch, which remains rudimentary. In non-amniotes, the fifth arch is maintained and an

additional posterior arch develops [4]. Thus, in the zebrafish, from the second to the third day after fertilization, a total of seven arches emerge [5]. Eight and nine arches are also observed in some chondrichthyans, while the lamprey, a jawless agnathan, has nine arches [6].

During evolution there appears to be a general trend towards loss of pharyngeal arches and pouches. In keeping with this, many fossil fish have high numbers of arches, with ostracoderm fossils discovered with as many as 30 arches [7]. This general trend in reduction in the number of posterior arches may reflect the changing function of the caudal pharyngeal regions, as the requirement for multiple gill supports was lost during the move from water to land.

In the jawed vertebrates the first arch divides into two clearly identifiable subunits termed the maxillary and the mandibular arches, which expand ventrally to form the upper and lower jaw. In agnathans (jawless vertebrates), the mouth forms between the first arch (mandibular) crest and the more anterior pre-mandibular

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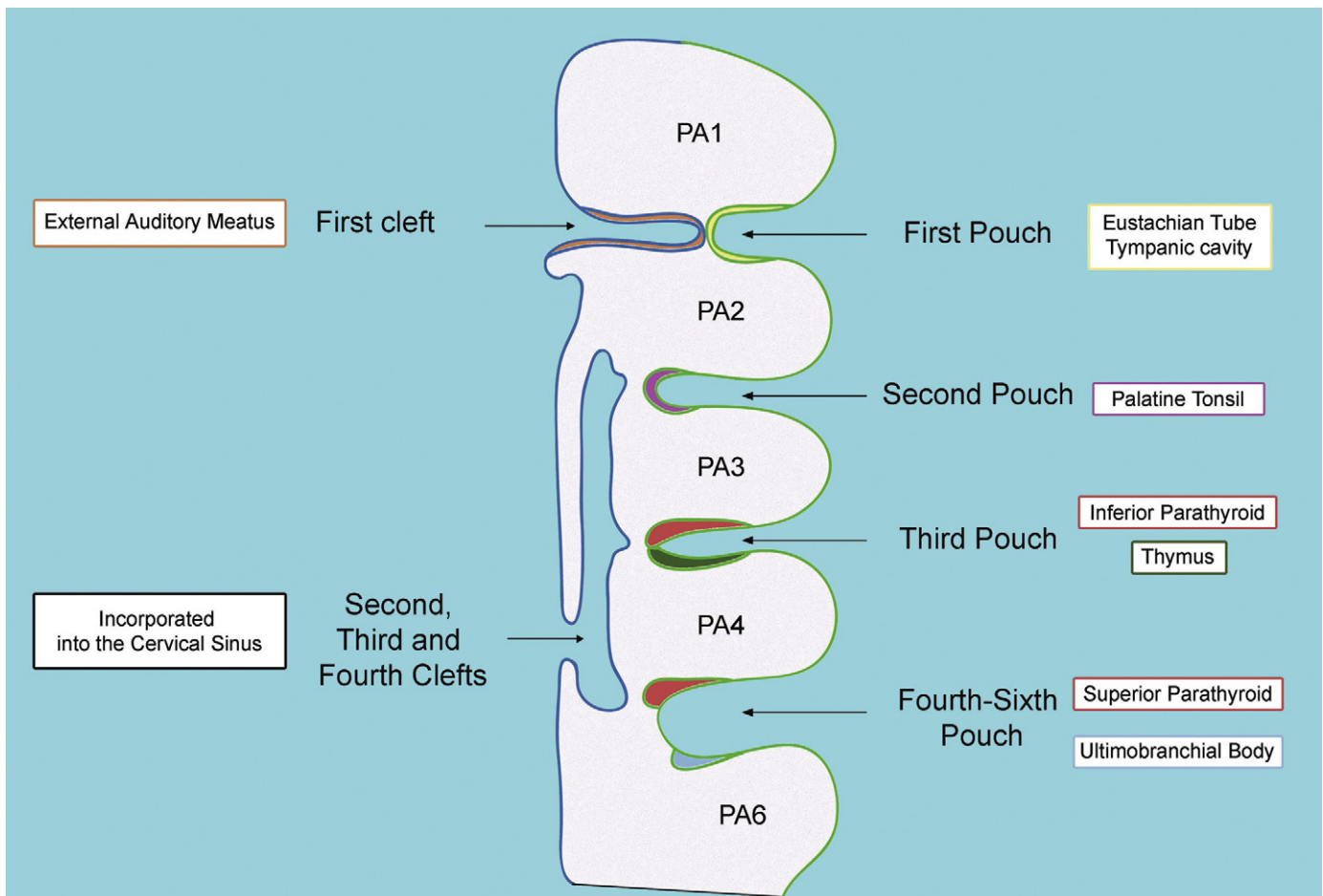
crest [8]. The upper and lower lips of a lamprey are therefore not homologous to the upper and lower jaw of a jawed vertebrate. This change in position of the mouth appears to have occurred through changes in the area over which signalling molecules, such as *Fgfs* (Fibroblast growth factors) and *Bmps* (Bone morphogenetic proteins), act to define the initial mouth region, and as such is a good example of how a relatively small early heterotopic shifts can lead to major changes in resulting structures [8].

The pharyngeal arches are composed of tissues derived from the three embryonic germ layers. Indeed, each arch is covered on the outside by ectoderm, and on the inside by endoderm. Its core is of mesoderm origin and is surrounded by neural crest-derived mesenchyme. As development proceeds, in between the arches, the lateral wall of the pharynx evaginates forming an out-pocketing known as the pharyngeal pouch, whereas, externally, the overlying ectoderm depresses forming a groove termed the pharyngeal cleft. As a result of these movements, the endoderm and ectoderm physically contact forming a closing membrane [3,4,9]. Cells within the pouch endoderm have high levels of actin fibres that form a web of supra-cellular actin cables. These cables appear to direct expansion of the pouch thereby controlling pouch morphogenesis, and, in keeping with this, disruption of the cables leads to abnormal pouch formation [10].

Over the course of embryogenesis, all the arch components differentiate into distinct derivatives. Specifically, the neural crest develops into the skeleton, the connective tissues, and part of the neurosensory ganglia of the cranium [11,12]; the mesoderm forms the head and neck muscles, skeletal elements, such as the cranial base, and blood vessels [13,14]; the arch ectoderm gives rise to

the oral epithelium, the keratinized epidermis of the face and the throat, as well as the sensory neurons of the epibranchial ganglia [11,15] and the arch endoderm forms the pharynx epithelium and glands [3,9,16]. The pharyngeal pouches and clefts are also regionalized to generate specific tissues and organs (Fig. 1).

The segmentation of the pharyngeal region appears to be driven by the endoderm [6]. Pharyngeal segmentation is a general feature of chordates and is found in animals without apparent neural crest, as observed in the cephalochordate amphioxus. The segmented pharynx of cephalochordates, hemichordates and urochordates is homologous to that of vertebrates, as shown by their shared expression of genes such as the paired motif transcription factors *Pax1/9* [17]. From developmental studies, ablation of the neural crest does not affect the initial formation and patterning of the endodermal pouches [18], again suggesting that the first stages of pouch development are independent of the crest that migrates into them. In zebrafish mutants where the endoderm is defective the pouches fail to form and as a consequence the pharyngeal region is severely disrupted [19]. At these early stages, the endoderm plays a role in patterning the surrounding arches. In the chick, excision of the foregut endoderm leads to a failure in development of specific parts of the pharyngeal skeleton, while ectopic grafts of foregut endoderm leads to duplication of skeletal elements [20]. In the zebrafish the endoderm has been shown to be essential for the identity, survival and differentiation of chondrogenic neural crest [21]. Many key signalling molecules are found expressed in the endoderm of the forming pharyngeal pouches, including *Shh* (*Sonic hedgehog*), *Fgfs*, and *Bmps* [18]. *Shh* from the foregut endoderm has been shown to play a key role in inducing expression of *Fgf8* in



**Fig. 1.** Derivatives of the pharyngeal clefts and pouches in humans. PA = pharyngeal arch.

the pharyngeal arch epithelium, which then in turn signals to the underlying crest to pattern the proximo-distal and rostral–caudal axis of the developing arch [22–24]. The initial patterning of these regions is therefore thought to involve signals from the endoderm, however these are combined with patterning information from the neural crest [25,26], with the *Hox* genes specifying individual arch identity [27]. The relative roles of the endoderm and neural crest are reviewed in [6,28].

## 2. The pharyngeal pouch and cleft derivatives in birds and mammals

### 2.1. The first pharyngeal pouch and cleft derivatives

In all jawed vertebrates the first arch forms the jaw, while the second arch forms the hyoid apparatus. These two arches are separated by the first pharyngeal pouch and cleft. During amniote embryogenesis, the pharyngeal arches grow differentially in size, with the first and second arches becoming much bigger in volume than the third to fourth arches. The first pouch and cleft are crucial contributors to the external and middle ear [29,30]. In mammals, the first pouch gives rise to the tubotympanic recess, which later elongates forming, in its distal part, the epithelium of the tympanic cavity, and in its proximal part, the eustachian tube. Indeed, the first cleft develops into the external auditory meatus, the lateral portion of which differentiates into the entrance of the external auditory canal. The proximal edge of the external auditory meatus invaginates towards the distal end of tubotympanic recess until only a thin mesenchymal fibrous layer separates them. The apposition of these two epithelia, together with the mesenchymal layer, constitutes the tympanic membrane, also referred to as the eardrum. The tympanic membrane is essential for converting sound waves into mechanical vibrations that are subsequently transmitted via the ossicles to the inner ear [29]. The eustachian tube joins the tympanic cavity to the nasopharynx. Its inner surface is covered by a mucosal layer of ciliated cells, supporting cells, secretory cells, and connective tissue [31]. The eustachian tube is primarily involved in evacuating middle ear secretions into the nasopharynx via the movements of the eustachian tube ciliated cells [31]. In addition, the eustachian tube is required for balancing pressure between the middle ear and the ambient air pressure by permitting the passage of air into the middle ear cavity. A dysfunction of the ciliated cells, or incorrect pressure adjustments may lead to the accumulation of middle ear fluids in the tympanic cavity, resulting in a condition termed otitis media with effusions, which is notably characterised by partial conductive hearing loss [31].

In mammals, *Tbx1*, *Eya1*, and *Eya4* have been associated with first pouch and cleft development [32–34]. *TBX1* is a T-box containing transcription factor, and a candidate gene for causing DiGeorge syndrome (also known as 22q11 deletion syndrome or Velo-cardio-facial syndrome) [35]. During embryonic mouse development *Tbx1* is expressed in the pharyngeal endoderm, mesoderm and otic vesicle. In mice with *Tbx1* conditionally knocked-out in the endoderm, the tympanic membrane is missing due to the failure of the first pouch to grow towards the external auditory canal, and the external auditory canal is dysplastic or missing [36]. *Eya1* is a transcription factor, homologous to the *Drosophila Eyes Absent* gene. In humans, mutations in *EYA1* lead to Branchio-Oto-Renal (BOR) syndrome [32]. In mutant mice the external auditory canal is dysplastic and the middle and inner ears are disrupted [32]. BOR and DiGeorge syndrome patients display conductive hearing loss with defects in the external, middle and inner ears.

Mutations in *Eya4*, another member of the *Eya* family, result in mice with an undersized eustachian tube and tympanic cavity and partial conductive deafness, and as such these mice have been

proposed as models for the study of otitis media with effusions [31,34].

Interestingly, defects in the first pouch and cleft have knock on effects on other tissues of the first arch. For example loss of *Tbx1* in the endoderm leads to aplasia of the mesenchymal tympanic ring, a tissue that does not express *Tbx1* [36]. In normal development, therefore, the cleft and pouch play an important role in signalling to other tissues, aiding the co-ordinated development of key structures such as the outer and middle ear. This link between the external and middle ear is also shown by clinical research, with 98% of patients with severely defective external ear development also having a middle ear defect [37]. Mutations in the *Tbx1* gene, have been shown to be responsible for the *van gogh* mutation in zebrafish, where a similar disruption of the development of the pharyngeal arches is observed [38].

### 2.2. The second pharyngeal pouch derivatives

In amniotes the second arch enlarges caudally, forming a deep depression called the cervical sinus, which completely covers the third and fourth arches. Ultimately, this sinus is obliterated by the fusion of its walls [2]. Failure in this fusion process leads to the formation of branchial cysts and fistulas [39].

The mammalian second cleft ectoderm reportedly generates no derivatives. On the other hand, the dorsal elongation of the second pouch endoderm of all mammals, with the exception of the rodents, gives rise to the palatine tonsil epithelium [40–42].

Like the spleen and the lymph node, the palatine tonsil is a secondary lymphoid organ. The palatine tonsils provide the immune system antigenic information about the foreign microbial species entering the body through the oral cavity [43]. Nonetheless, at odds with their role in activating immune responses, the tonsils are frequently colonised by potentially invasive micro-organisms, the accidental spread of which may cause recurrent throat infections [44].

Structurally, the tonsils differ substantially from the lymph nodes. In particular, they do not have afferent lymph vessels, and are not enclosed into a capsule. In some mammals, the tonsil epithelium is characterised by epithelial folds referred to as crypts that invaginate from the oropharynx into the underlying lymphoid tissue [41,45]. The number of crypts that are eventually formed, however, may vary between species, with just one for instance being present in the rabbit or the cat [41,46,47]. The crypts are thought to play a major role in the initiation of the palatine tonsil immune response [43]. In rodents, the ventral portion of the second pouch appears to degenerate whereas the remaining part is incorporated into the lateral border of the pharynx [40]. It appears that rodents no longer require tonsils as their function is carried out by the well-developed NALT (Nose/Nasal-Associated Lymphoid Tissue) system in the upper respiratory tract [48].

In humans, the second pharyngeal pouch starts displaying canalization and branching around the eight week after fertilization [42,46]. Lymphoid infiltration of the tonsil occurs at seven months of development, and primary follicle are visible during late gestation, however, the tonsil immune response is not fully functional until the beginning of postnatal life [42]. During the first year after birth, intense proliferation of the tonsil lymphoid elements is observed. As a result, the tonsil increases in size, thereby displacing the neighbouring soft tissues and expanding into the oropharyngeal cavity [42]. At puberty, the palatine tonsil involutes so that little tonsillar tissue remains at adulthood.

Since the non-mammals do not have tonsils, what structure if any does the second pouch gives rise to?

Limited data is available regarding the derivatives of the second pouch in avians. Interestingly, in the pigeon second pouch, the appearance of large mucous glands that might be reminiscent

of salivary glands have been reported, and in keeping with this, structural similarities have been made between salivary glands and tonsils [46,49]. Based on these observations, it has been suggested that over the course of mammal evolution, the palatine tonsils have been acquired as a modification of a salivary gland [47,49]. In accordance with this idea, in the dog, the cat and rabbit, the presence of salivary glands was evidenced in the palatine tonsil surface epithelium or lymphoid tissue [47]. Therefore, the avian second pouch might give rise to salivary glands; and the potency to generate this structure might be shared with mammals.

Because the second pouch yields no obvious derivative in the most commonly used avian and mammal models, namely the chick and the mouse, no genes involved in the early patterning and differentiation of this pouch have so far been uncovered.

The thyroid gland also develops from the pharyngeal endoderm at the level of the second arch. This gland, however, is not a derivative of the pharyngeal pouch but originates medially from the endodermal tissue in contact with the aortic sac. The early stages of development of the thyroid are similar to those of the pharyngeal pouch derived glands, with the formation of a bud like structure from the endoderm [50]. The thyroid primordium then migrates into the neck mesenchyme and in most mammals fuses with the ultimobranchial bodies (see Section 3) [50].

### 2.3. The third and fourth pharyngeal pouch derivatives

The third and fourth pouches develop into the thymus and parathyroids. Fish form thymic tissue derived from the pharyngeal arches, but parathyroids are only found in tetrapods. The evolution of the parathyroids is proposed to have played a pivotal role in the move to land, allowing regulation of extra-cellular calcium. This freed the tetrapods from relying on calcium uptake from water by providing internal regulation of calcium levels. No evidence of a thymus is found in jawless vertebrates, such as the lamprey [51].

In mammals the cranial dorsal aspect of the third pouch generates the inferior parathyroid (or parathyroid III), whereas the caudal ventral part of the pouch gives rise to the thymus epithelium [16,52–54]. Similarly in the chick, the third pouch differentiates into the thymus epithelium and parathyroid.

In avians and most mammals, including humans, the anterior fourth pouches yield a second pair of parathyroids, referred to as the superior parathyroids or parathyroids IV [9,16,54,55]. In the chick, the posterior fourth pouch differentiates into a second thymus rudiment, designated as 'thymus IV', which later fuses with the thymus originating from the third pouch [55]. In mammals, the formation of a 'thymus IV' has been described in some species such as the calf, the cat and human [56–58]. However, these observations, and their interpretations, are disputed by many authors. Thus, at least in most mammals, the posterior fourth pouch probably does not give rise to any thymic derivatives [9]. In the mouse, but also in the rat and hamster, the anterior fourth pouch does not generate a parathyroid [9,59]. Consequently, rodents develop only one pair of parathyroid glands.

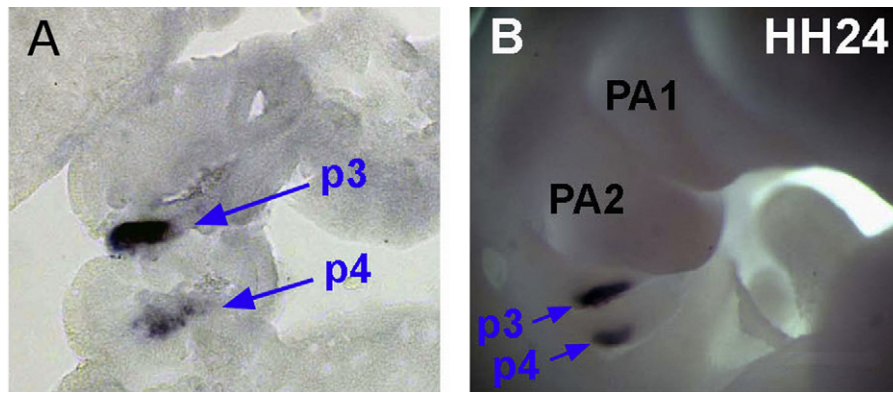
The difference in origin of thymic and parathyroid tissue in different animals may reflect loss of the ability to form these derivatives as the individual arches became more specialised during evolution. An ancestral form would therefore be predicted to have more widespread development of these gland types in the pharyngeal arches. This is backed up by the finding that in some species of fish every pouch develops thymic tissue [60]. It would be of interest to try and identify molecular differences that might have led to changes in developmental origin of the glands, for example, the loss of parathyroid development in the rodent fourth pouch. Differences in *Shh* signalling between mouse and chick has been suggested as one possible change that might be responsible for such species-specific difference [61].

The parathyroids are endocrine glands, the main function of which is to secrete a peptidic hormone essential for the regulation of calcium and phosphate homeostasis named parathyroid hormone (PTH) [62]. The parathyroid glands consist of pararenchyma surrounded by a dense and irregular connective tissue capsule. The PTH is produced by the chief cells, which form the major cell type of the glands. These cells appear arranged in clusters (or 'cords') encircled by numerous capillaries [63]. During development the parathyroids are closely linked to the thymus [52]. The thymus provides an area for T lymphocyte maturation and is essential in protecting against autoimmunity. Loss of the thymus, as observed in DiGeorge syndrome, thus results in severe immunodeficiency [64].

It was originally proposed that the thymus epithelium derived both from the ectodermal cleft and from the endodermal pouch (reviewed in [53]). However, the endoderm is able to give rise to the thymus alone and lineage tracing of the third cleft shows no contribution of the ectoderm to the thymus [65]. The thymus epithelium, therefore, appears to be derived solely from the pouch. Importantly, whereas the parathyroid chief cells and thymus epithelial cells derive from the endoderm, the connective tissues that surround them originate from the neural crest [66]. Moreover, neural crest cell ablation prior to migration results in parathyroid and thymus dysgenesis or agenesis [67]. In the *Pax3* (*Splotch*) mutant the neural crest is disrupted leading to a deficit of mesenchymal cells in the pharyngeal arches. This reduction in neural crest in turn influences the size of the parathyroid and thymus domains set up in the third pouch [68]. The parathyroid domain is reduced while the thymus domain expands, changing the relative size of the final glands. Thus although the pouches and glands are derived from endoderm, and the initial patterning of the pouches is controlled by the endoderm, the neural crest plays an essential later role in regulation of the pouch derivatives.

Aplasia of the thymus and parathyroids is found in a number of mouse mutants where the patterning of the third pouch is disrupted. The *Hox* gene, *Hoxa3*, plays an important regulatory role in setting up the identity of the third and fourth pouches. In *Hoxa3* mutants, the identity of the third pouch is lost and no thymus or parathyroid primordia form [69]. A similar loss of third arch identity and gland primordia is observed in the *Eya1* mutant [70]. In *Tbx1* mutants the third pouch primordium forms but does not develop resulting in aplasia or dysplasia of the glands [36] (reviewed in [64]). A similar phenotype is generated in the *Pax9* mutant [71]. *Pax9* is a transcription factor, closely related to *Pax1*, containing a 128 amino acid paired motif. *Pax 9* and *1*, unlike other *pax* genes, do not contain a homeodomain. In humans loss of *PAX1* or *PAX9* lead to multiple defects but thyroid defects have not been described. *Pax* and *Eya* genes interact in the same pathway, playing a highly conserved role in eye development in both *Drosophila* and mouse [72].

The earliest marker of the parathyroid is *Gcm2* (*Glial cells missing 2*). The *Drosophila* homolog of *Gcm* acts as a switch determining glial or neuronal cell fate [73]. In vertebrates, however, *Gcm2* is largely involved in non-neuronal cell types. In the mouse, *Gcm2* is first expressed in the third pouch endoderm from E9.5. As development proceeds, genes involved in calcium homeostasis, such as *PTH* (parathyroid hormone) and *CasR* (calcium sensing receptor) are upregulated in the developing parathyroid and, with *Gcm2*, remain transcribed in the parathyroid rudiments as they migrate towards the midline and into adulthood [74]. In mouse, the null mutation of *Gcm2* leads to agenesis of the parathyroids from E12 onwards [74,75]. At E11.5, the *Gcm2* mutant mice glands do not initiate *PTH* expression and they fail to maintain the transcription of *CasR*. Loss of the parathyroid is thought to be caused by increased cell death [74]. In the chick *Gcm2* is expressed in the third and fourth pouch at HH24, reflecting the development of parathyroids from both of



**Fig. 2.** *Gcm2* expression in the chick pharyngeal pouches at HH stage 24. DIG *in situ* hybridisation. Expression is observed in the third and fourth pouches which form the parathyroids. (A) Section through developing pouches. (B) Sagittal view of embryo in wholemount. PA = pharyngeal arch; p = pouch.

these pouches [76] (Fig. 2). Interestingly, the chick also has transient expression of *Gcm2* in the second pouch [76], but this is later lost, perhaps reflecting a more widespread expression of *Gcm2* in the pouches of an ancestral form. The *Gcm2* promoter contains *Pax1* and *Pax9* binding sites, and so acts downstream of these transcription factors [74].

In humans, homozygous inactivating mutations in *GCM2* have been related to the familial autosomal recessive and dominant isolated forms of hypoparathyroidism. Typically, the *GCM2* mutated individuals exhibit undetectable or residual PTH levels. Nevertheless, whether the parathyroids are unformed or hypoplastic in the patients has not been reported [77–80]. Evidence suggests that some parathyroid adenomas might be associated with a dysregulation of *GCM2* expression. Indeed, both reduced and enhanced *GCM2* expression levels were found in some human parathyroid adenoma [81,82]. It was speculated that *GCM2* might be required in the mature glands in order to maintain the cells in a fully differentiated state.

As has been mentioned a key event in the transition to land was the development of the parathyroids. Although fish do not have parathyroids, recent research has indicated that in fact the gill buds are homologous structures that play a similar role in controlling calcium levels [76]. *Gcm2* is expressed in the pharyngeal arch epithelium starting in the second pouch before extending to the other pouches, and later in the internal gill buds in both zebrafish and dogfish [76]. Importantly, loss of *Gcm2* resulted in loss of the gill buds [76,83]. Loss of *Gcm2* also led to cartilage defects [84]. It was therefore proposed that the internalization of the *Gcm2* positive region during the move to land led to the formation of the tetrapod parathyroid gland. The tissue origin of the *Gcm2* expression domain, however, is in debate, as both an endodermal and ectodermal expression has been reported [76,83]. In amniotes the *Gcm2* domain is clearly endodermal, thus the issue of which tissue expresses *Gcm2* is of particular importance if homology is to be assigned.

The *Gcm2* expression domain in the mouse third arch is complementary to *Foxn1* in the thymus primordium at E11.5 [52]. *Foxn1* is the earliest known thymus marker. It is a transcription factor of the winged helix/forkhead class and is the gene mutated in the *Nude* mouse strain [85]. *Nude* mice and rats display abnormal hair growth, and failure in thymus development leading to immunodeficiency [86]. *FOXN1* mutations have also been discovered in humans [87]. Similarly to the *Nude* mice, the *FOXN1*-mutated individuals display T-cell immunodeficiency, nail dystrophy, and congenital alopecia. *Foxn1* is required for initial thymic epithelial cell differentiation into cortical and medullary epithelium [88]. Loss of *Foxn1* after birth results in thymus degeneration and reduced T-

cell production, suggesting that *Foxn1* is also required for functional maintenance of the postnatal thymus [89].

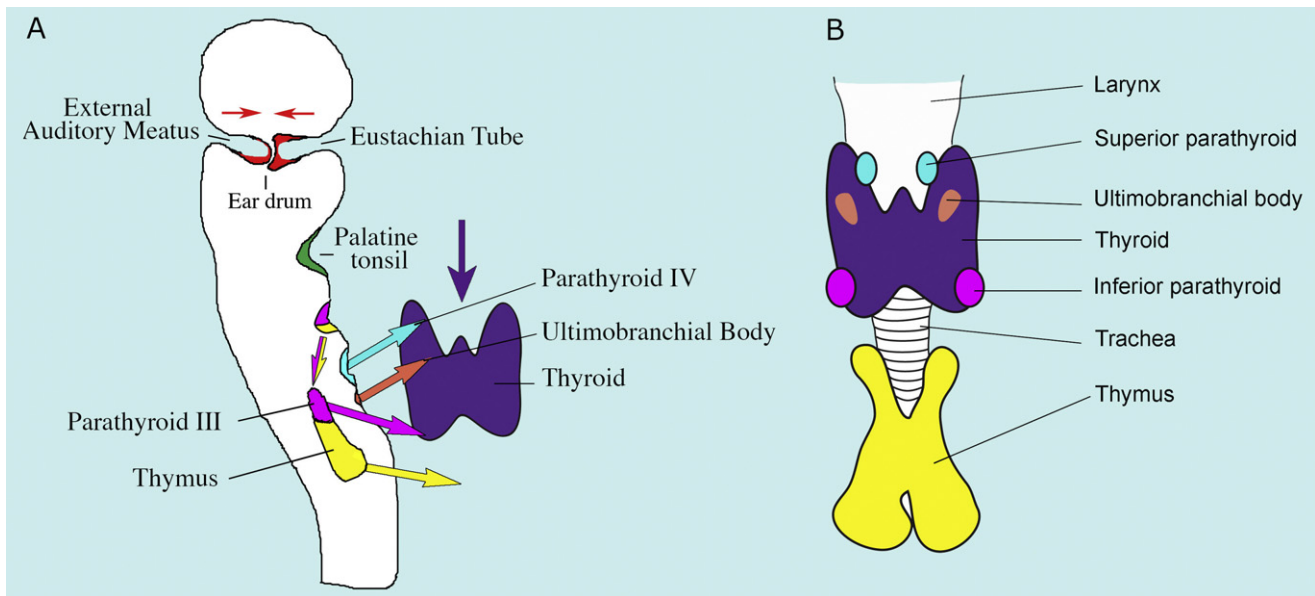
#### 2.4. The most caudal pharyngeal pouch derivatives

In mammals and birds, the fifth and sixth pouches are vestigial or absent. Still, the most lateral caudal part of the pharynx, which is interpreted as a sixth pouch in avian [54,55,90], a fifth pouch in human [16] or a fourth pouch portion in mouse [9], generates the ultimobranchial bodies. Notably, according to some authors, in human and avians, the fifth and sixth pouches are included within the fourth pouch, forming a so-called ‘caudal pharyngeal complex’. The ultimobranchial bodies develop into calcitonin producing cells (or C-cells), which function in calcium homeostasis [91]. In most mammals these bodies fuse with the endodermally derived follicular cells of the thyroid, while in avians and lower vertebrates the bodies remain distinct, indicating that fusion is a new adaptation [92]. The ultimobranchial bodies fail to develop, along with the thymus and parathyroids in *Pax9* mutant mice [71]. In *Hoxa3* and *Eya1* mutants, the ultimobranchial bodies do form but fail to migrate correctly and do not fuse with the thyroid [70,93]. Intriguingly, the ultimobranchial body phenotype in the *Hoxa3* and *Eya1* mutants is reminiscent of that found in echidna, a monotreme where the ultimobranchial bodies naturally do not fuse with the thyroid (discussed in [70]).

### 3. The migration of the pharyngeal glands

After emerging from the caudal endodermal out-pocketings, the avian and mammalian thymus, parathyroids and ultimobranchial bodies, migrate towards the midline in order to gain their final position in the adult (Fig. 3). A similar migration of the thyroid gland from the pharyngeal endoderm also occurs. In bony fish the thymic epithelium does not completely separate from the gill epithelium but evaginates inwards [94]. Migration of such tissues is therefore associated with the move to land and the need to internalise these organs.

Among mammals, the pharyngeal organ movements have been particularly well characterized in rodents [69,95]. In the mouse, between E11.5 and E12.0, the common thymus/parathyroid primordium significantly increases in size, with the thymus starting to expand caudally. Later, from E12.5 to E13.5, the third pouch primordium detaches from the pharynx and begins to move in a caudal–ventral–medial direction towards the anterior thoracic cavity. Notably, throughout this period, the parathyroid and thymus progress together, with the parathyroid being attached to the



**Fig. 3.** Migration of the caudal glands and final position in humans. (A) Migration of the caudal glands. (B) Final position in midline.

thymus on its cranial pole. Nonetheless, at E14.0–E14.5, while the organ rudiments are passing the lateral side of thyroid, they separate from each other. Subsequently, by E15.5, the parathyroid contacts the adjacent thyroid lobe, with which it remains connected until adulthood. The thymus descends further caudally and medially until reaching its final location above the heart, and at the rear of the sternum in the midline. There, it eventually joins its symmetric thymic lobe, thereby forming a complete thymic organ. Similarly to the thymus and parathyroids, the murine ultimobranchial bodies separate from the pharynx and migrate caudally and ventrally from the fourth pouch towards the midline. By E13.5, they approach the anterior thyroid lobe, with which they fuse by E14.5. The ultimobranchial body cells spread throughout the thyroid lobe and gradually become integrated into the thyroid follicular tissue by birth [96].

In mammals, the position of the parathyroids at adulthood varies considerably [97]. In humans, the inferior parathyroids (parathyroid III) are generally found between the thyroid lower pole and the tongue of the thymus. However, a number of them are also detected laterally to the thyroid caudal end, or inside the mediastinal thymus [97]. Unlike the inferior parathyroids, the superior parathyroids (parathyroid IV) are set at less variable body sites, reflecting their shorter migration. Remarkably, both the inferior and superior parathyroids may be ‘extracapsular’ or ‘subcapsular’, meaning that they may be positioned outside the thyroid connective tissue capsule (extracapsular) or within the organ parenchyma (subcapsular) [97].

Interestingly, in humans, a significant proportion of parathyroid III and IV (2–16% depending on studies) are ectopic, i.e. situated away from their normal locations [97,98]. When ectopic, the inferior glands may be lying anywhere from the angle of the mandible to the pericardium, whereas the superior glands are mostly seen next to the pharynx, trachea or oesophagus. Notably, in addition to be ectopic, the parathyroid III may remain attached to the thymus [97–99]. Then, the complex formed by both organs is sometimes designated as ‘parathymus’ [97,99]. In addition to these defects, supernumerary parathyroids associated with thymic tissue lobules have been described [97,98], and *Gcm2* expressing tissue was also detected within the thymus of a patient [100]. Reminiscent of these observations, in the chick, accessory parathyroid tissues may be found in diverse locations, including in the thymus [90].

In the mouse, the joint mutation of several *Hox3* paralogs was shown to block the common parathyroid–thymus primordium migration at around E12.5–E14, and to impair the fusion of the ultimobranchial bodies with the thyroid [95]. Interestingly, apart from *Hoxa3*, the *Hox3* paralogs are expressed in the mesenchyme but not in the organ rudiments. Therefore, gland migration may be partially controlled in a non-cell autonomous fashion by mesenchymal factors. Ectopic thymic glands have also been reported in the *Hoxa3*<sup>+/-</sup>;*Pax1*<sup>-/-</sup> compound mutants, and in *Pax9* and *Pax3* mutants [101,102]. *Pax1* and *Pax9* are downstream targets of *Hoxa3* [93,101] [68], hence, it is possible that a common *Hox3*–*Pax1/9* signalling pathway contributes to the regulation of thymus progression to the mediastinum.

In addition to the *Pax* and *Hox* factors, the *Fgfs* (Fibroblast growth factors) appear to be required for pharyngeal gland relocation in mammals. In the mouse, *Fgf10* and *Fgf7* are expressed in the thymus, as the organ is en route towards the midline. In addition, aberrantly positioned thymi have been observed in mice heterozygote for a null or hypomorphic allele of *Fgf8* [103,104]. In keeping with this, genetic disruption of the docking protein *FRS2α*, which plays an important role in FGF-induced intracellular signal transduction, leads to a failure in pharyngeal gland primordia separation from the pharyngeal epithelium [105].

Finally, the study of thyroid organogenesis may suggest some roles for blood vessels in directing pharyngeal gland migration. Interestingly, in zebrafish, the ventral aorta was shown to provide guidance cues to the thyroid tissue in order for it to elongate along the anteroposterior axis [106]. Likewise, in the mouse, data support that signalling factors coming from the carotid arteries control the directionality of the thyroid lobe expansion during lobulation [106]. A close association between the migrating pouch glands and the carotid artery has also been noted in the chick [107]. Therefore, based on these observations, a role for the blood vessels in guiding the gland movements remains an intriguing possibility.

#### 4. Conclusions

The pharyngeal pouches, and first arch cleft, form a number of diverse structures and glands that play essential roles in the body, allowing for hearing, immune responses, and calcium homeostasis. The exact derivatives of the second, third and more caudal

pouches vary between different species indicating some flexibility with regard to which pouches form what derivative. In amniotes the caudal derivatives migrate and with the thyroid are positioned in the midline, forming part of the mediastinum. In most species investigated, the control of pharyngeal organ migration during embryogenesis appears quite loose. In humans, an ectopic gland usually goes unnoticed, so that the genetic underlying this anomaly is unknown. The flexibility of origin and migration path indicates that each pharyngeal pouches has the potential to form a variety of gland types, and that the ability of these glands to function correctly is not dependent on a defined end position. This has allowed for a range in compliment and position of pharyngeal pouch derivatives throughout the vertebrates.

In humans defects in formation or development of the pharyngeal pouches and clefts leads to a number of disorders. Defects in the first pouch and cleft lead to abnormalities in the external ear, which in turn affect the formation of the neural crest-derived middle ear. Defects in the second pouch lead to defects in palatine tonsil development, while defects in the third and fourth pouch lead to abnormalities in the thymus and parathyroid. Often several pharyngeal pouches are affected by a single mutation. For example loss of *Tbx1* (DiGeorge syndrome) or *Eya1* (Branchio-Oto-Renal syndrome) affects all the pouches resulting in defects in the ear and third pouch glands. An understanding of how the clefts and pouches form during development and the genes involved in these processes is key to understanding such human disorders.

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