

Supplementary data

Subphenotyping and classification of cleft lip and alveolus in adult unoperated patients: a new embryological approach

Embryological model

Normal embryonic development of the primary and secondary palate

Fusion processes of the primary and secondary palate and their directions

It is generally accepted that the formation of the secondary palate is based on fusion of the palatal shelves (processes) (Mossey et al., 2009). By contrast, the formation of the primary palate is thought to result from fusion (Gaare and Langman, 1977; Hinrichsen, 1985; Johnston and Millicovsky, 1985; Jiang et al., 2006; Jugessur et al., 2009; Szabo-Rogers et al., 2010; Som and Naidich, 2013; Kurosaka et al., 2014; Young et al., 2014, Hu et al. 2015), merging (Patten, 1961; Andersen and Matthiessen, 1967; Yoon et al., 2000; Gritli-Linde, 2007; Liu et al., 2007) or a combination of fusion and merging of the facial swellings (prominences or processes) that surround the nasal placode (Diewert and Wang, 1992; Schwartz et al., 1993). However, when the formation of the primary palate and secondary palate are compared with each other, then both can be subdivided into three phases: 1) outgrowth of the facial swellings or palatal shelves, which are mesenchymal cores covered with epithelium; 2) opposition of the swellings or shelves followed by adhesion of their epithelium and subsequent epithelial plate or seam formation; and 3) programmed cell death (apoptosis) followed by epitheliomesenchymal transformation and migration, resulting in disruption and subsequent disappearance of the epithelial plate (seam) and fusion of the mesenchymal cores of the swellings or shelves. The presence of all three phases during formation of the primary palate (Gaare and Langman, 1977; Vermeij-Keers et al., 1983; Vermeij-Keers, 1990; Sun et al., 2000; Cox, 2004; Kurosaka et al. 2014) proves that the primary palate is formed by fusion and not by merging, as the last two phases do not occur in the process of merging (Vermeij-Keers, 1990).

Although the fusion processes of the primary and secondary palate are roughly similar, there are also some differences. First, the facial swellings and palatal shelves fuse in opposite directions (Fig. 1: 6). The development of the primary palate starts with widely separated nasal placodes, which are located on the lateral sides of embryonic head (four weeks development). These placodes are transformed into nasal pits, grooves, and tubes, respectively, by the three

outgrowing facial swellings (medial nasal process, maxillary process, and lateral nasal process) (Fig. 1: 1-3). These outgrowing facial swellings, which are separated by grooves, adhere from occipital to frontal and form an epithelial plate known as the nasal fin (Vermeij-Keers, 1972; Hinrichsen, 1985; Johnston and Bronsky, 1995; Cox, 2004). Within this plate, cell death occurs before, during, and after formation, which results in a disruption halfway the epithelial plate (Vermeij-Keers, 1990; Diewert and Wang, 1992; Sperber, 2002; Kurosaka et al. 2014). Then cell death continues within the remnants of the epithelial plate, the three swellings fuse gradually, and the primary palate is formed (human embryos 11-17 mm crown-rump length (CRL)) (Vermeij-Keers, 1990; Sperber, 2002). The medial nasal process fuses first occipitally with the maxillary process and then frontally with the lateral nasal process (Sulik et al., 1979; Vermeij-Keers, 1990; Johnston and Bronsky, 1995; Hu et al. 2015). Consequently, the external nasal aperture (nostril) is formed by the medial nasal and lateral nasal processes (Fig. 1: 1-3). In this period a continuous arch-shaped band of thickened epithelium develops in the fused medial nasal and maxillary processes indicated by a groove —named labial groove— on the inferior border of these processes, i.e., the first indication of the presumptive dental and labial laminae (Arey, 1965; Vermeij-Keers, 1967; Nanci, 2012). This band is called primary epithelial band (Nanci, 2012).

In contrast to the primary palate, the secondary palate is formed from frontal to occipital, starting with adherence and fusion of the frontal borders of the palatal shelves to the primary palate (Fig. 1: 3-6). Note that fusion of the secondary palate occurs after fusion of the primary palate; the facial swellings fuse during the *early embryonic period*, 4-7 weeks of development (≤ 17 mm CRL), while the palatal shelves fuse during the *late embryonic period*, 7-12 weeks of development (≥ 17 mm - ≤ 60 mm CRL) (Vermeij-Keers, 1990; Luijsterburg et al., 2014).

A second difference between the primary and secondary palate is that the formation of the primitive choanae is based on the development and subsequent rupture of the oronasal membranes (bucconasal membranes), while the secondary choanae are formed by the openings into the nasopharynx after formation of the secondary palate. During the development of the primary palate (*early embryonic period*), the two nasal pits, nasal grooves and nasal tubes, respectively, have newly formed cavities, which belong in fact to the amniotic cavity and not to the stomodeum (oral cavity) (Vermeij-Keers, 1972; Hinrichsen, 1985). The floors of these two blind ending cavities are formed by the fusing facial swellings, which enclose

the epithelial plates. From the occipital part of these epithelial plates, the oronasal membranes develop and subsequently rupture by cell death, thereby forming the primitive choanae (12-17 mm CRL; Fig. 1: 3-4) (Hinrichsen, 1985; Vermeij-Keers, 1990; Diewert and Wang, 1992). Occipitally, the nasal tubes now open into the stomodeum. Then the secondary palate develops, and the incisive canals demarcate the primitive choanae frontally. Finally, the definitive nasal cavities are formed from the nasal tubes and from a part of the stomodeum (captured through fusion of the palatal shelves), which then can communicate with the pharynx by the secondary choanae (Arey, 1965).

Formation of the lip and alveolus & mesenchymal differentiation of the lip, alveolus, hard and soft palate

During the *late embryonic period*, the grooves between the swellings in the embryonic face are eliminated by merging (Hinrichsen, 1985; Vermeij-Keers, 1990; Hinrichsen, 1991; Sperber, 2002). The lip and alveolar process including the teeth develop from the primary palate. The presumptive lip and alveolar process are separated from each other by the labial groove and grow out in caudal direction (Fig. 1: 3-6). The primary epithelial band located on the primary palate splits into two sheets, the dental lamina and labial or vestibular lamina; the latter in continuity with the labial groove becomes the vestibule (Arey, 1965; Nanci, 2012). Then, the dental lamina differentiates via dental placodes and mesenchyme of the primary palate into the various teeth (Nanci, 2012). Additionally, the mesenchyme of the primary and secondary palate differentiates into several bone centers and musculature. The number of bone centers and their timing in the development of the premaxilla and maxilla forming the alveolar process are still controversial (Barteczko and Jacob, 2004). According to Vermeij-Keers (1990) the first bone centers, which form the alveolus and secondary palate, arise in the mesenchyme of each maxillary process, i.e., the presumptive maxilla (17 mm CRL). Each maxilla and each palatal bone (anlage at 23 mm CRL) develops from a single bone center, while the premaxilla —bearing both incisors— develops from two centers in the medial nasal process, i.e., the frontal center at 23 mm CRL and occipital center at 50 mm CRL. In addition, Barteczko and Jacob (2004) distinguish a small third center that fuses with the nasal septum and vomer. All bone centers grow out and a) fuse with each other (the frontal premaxillary center fuses with the maxillary center and with the occipital premaxillary center, respectively) or b) form a suture between the occipital premaxillary center and maxillary center (the incisive suture), and between both

maxillary and palatine centers (the median and transverse palatine sutures) (Vermeij-Keers, 1990).

Abnormal embryonic development and classification of clefts of the primary and secondary palate

Disturbances in the fusion phases of the primary and/or secondary palate, including the formation of the primitive choanae, may cause various fusion defects along the respective fusion lines (Table 1). For example, interrupted fusion of the primary palate during the *early embryonic period* may result in a complete CL with (in)complete CA, while defective fusion of the secondary palate during the *late embryonic period* may give rise to a(n) (in)complete hard CP with complete soft CP. Differences in anatomical extent and morphological severity are explained by the fusion direction of the facial swellings (occipito-frontal) and palatal shelves (fronto-occipital), and by the development of the primitive choanae (primordial oronasal membranes) during fusion of the facial swellings (Luijsterburg et al., 2014). If the disturbance occurs at a later stage during the fusion process, less of the primary and secondary palate will be defect.

Similarly, disturbances in the formation of lip, alveolus and teeth —after the completed fusion process— and in the subsequent mesenchymal differentiation (into bone and musculature) of the primary and secondary palate may cause differentiation defects (Table 1). More specifically, insufficient merging, outgrowth, or differentiation of the lip±alveolus may result in incomplete CL±A. These subphenotypes always show a tissue bridge at the base of the nostril, as the fusion process of the lip and alveolus has been completed at this stage (Luijsterburg et al., 2014). The height of this bridge is related to timing in embryonic development; that is, the earlier the disturbance, the lower the tissue bridge.

Additionally, disturbances in differentiation of bone anlage/musculature may cause bony defects, such as submucous clefts or hypoplastic bone/musculature in the primary or secondary palate.

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Figure legend S1

Figure S1. Notch of the vermillion with a normal alveolus and rotated lateral incisor, persistent milk canine, and supernumerary teeth.