ONTOGENY OF THE HUMAN FETAL TONGUE, MANDIBLE, AND HYOID

CARTILAGE

by

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Abstract

BACKGROUND: Growth characteristics of the human craniofacial region during the fetal stages of development remain largely unexplored. This project investigates the growth of the human tongue, mandible and hyoid cartilage, during the early fetal stage, using a rare collection of preserved fetal head tissues. These structures differ in the tissue types that they are comprised of, however, they share common developmental origins. We hypothesized that the growth of these tissues exhibits a strong positive correlation during prenatal human life.

MATERIALS AND METHODS: Human fetal heads were obtained from elective terminations between nine and nineteen postconceptional weeks (n=16). They were contrast-enhanced with phosphotungstic acid (PTA) and imaged with high-resolution micro-CT. Segmentation of the tongue, mandible, and hyoid cartilage was performed, three-dimensional models were constructed, and their volumes were calculated. To assess the relationship between the variables, correlations between different tissues were determined. Additionally, regression analyses were performed after normalization of data to permit comparisons between the different tissues. A single-rater interclass correlation coefficient was performed for 5 randomly selected samples, to study the measurement reliability. **RESULTS:** PTA contrast enhancement provides excellent visualization and allows for accurate digital segmentation of hard and soft tissues of the craniofacial regions in the fetal head samples. Pearson's correlation coefficients of 0.96, 0.85, and 0.91 were calculated between the growth of the tongue – hyoid, tongue – mandible, and mandible-hyoid, respectively. The mandibular bone showed a similar growth trajectory as the tongue and the hyoid cartilage. However, the growth of the tongue was found to precede the mandibular growth, albeit slightly, at day 109.6 pc. Interclass correlation coefficient for all repeated measurements were > 0.9 with a P < .001.

CONCLUSION: Contrast-enhancement followed by high-resolution 3D scanning provides an important resource to study hard-soft tissue correlations within the fetal craniofacial region during development. Our data indicate a strong positive correlation between the tongue, mandible, and hyoid cartilage, during the early fetal stage of human growth. These structures seem to begin a growth spurt between the end of the 13th to the 15th postconceptional weeks.

Lay Summary

This study utilizes a collection of human fetal heads that were gathered from elective terminations around 30 years ago at the University of British Columbia. Our goal was to examine the growth of the soft tissue and compare it to the neighboring hard tissues using a 3D analysis software. We have measured and analyzed the tongue, lower jaw, and hyoid cartilage and found a strong association between their growth between 9 and 19 weeks of post conceptional weeks.

Preface

This thesis is an original intellectual work of the author; Raid Khayat, under the supervision of Dr. Siddharth Vora as per the requirements of a Master of Science in Craniofacial Science. Access to sample population, and ethical approval (Human ethics approval H08-02576-A014) was obtained by Drs Virginia Diewert and Joy Richman. PTA staining of specimen was performed at UBC and at the University of Vienna by Dr. Brian Metscher. Scans of the specimen were performed at the Laboratory of X-ray Micro CT and Nano CT- CEITEC Brno, Czech Republic. Literature review, methodology, data collection, statistical analysis, writing, and preparation of the thesis's script, tables, and figures was completed by the author. The study was designed by the supervisor and the author. Modifications of the study design, input for statistical analysis, and editing of the script was done by the supervisor and the research committee members.

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List of Abbreviations

2D: two dimensions

3D: Three dimensions

BC: British Columbia

CFM: Craniofacial microsomia

CHL: Crown-heel length

CRL: Crown-rump length

CT: Computed tomography

ICC: Interclass correlation

NCCs: Neural crest cells

PC: Post-conception

PRS: Pierre Robin Sequence

PTA: Phosphotungstic acid

TCS: Treacher Collins syndrome

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Dedication

To my mother; Azzah Ashour, and my father Faisal whom I will be forever in debt to them for everything in my life, my prayers and thoughts are always with you. To my wife Samar, and my son Mikael the delight of my life and the companions during my journey. To my sister Rafeef, and my brothers Abdulaziz, and Abdullah for all their love, and support. To all my teachers, professors, and friends I dedicate this effort to you.

Chapter 1: Introduction

A considerable amount of research has been conducted to study human craniofacial growth. However, most of this research is focused on studying post-natal growth, with relatively lesser information begin available with regards to prenatal growth. Continued morphometric and developmental biological research is providing significant input for a better understanding of craniofacial growth, aided by the study of human disease progression and the use of animal disease models. With regards to the latter, however, there is limited information which animal models can provide as it pertains to human-specific growth characteristics. Hence, access to human fetal samples can provide a significant resource. The main objective of this research project was to investigate the growth of the human tongue, mandible, and hyoid cartilage during the early fetal period of development, using human samples and advanced 3D imaging modalities.

1.1 Overview of orofacial development

Human prenatal development is constituted by three major phases; the first seven days are the preimplantation phase, followed by the embryonic period that includes most of the tissue differentiation and this phase ends by the 8th week post-conception (pc) (1). Finally, by 9th week, early fetal movements characterize the initiation of the fetal stage which ends at birth, this stage has minor new tissue formation yet marked rapid growth (1).

During the early embryonic period ~ the 14th day of development the prechordal plate appears and features the first sign of oral development (1). Just caudal to this, the ectoderm, and the endoderm directly contact each other, forming the oropharyngeal membrane, where the primitive mouth; the stomodeum forms as a shallow depression (1). By the 4th week of development, pharyngeal arches develop around the stomodeum, as segmentations in the mesodermal ventral foregut area. In vertebrates, the five distinctive mesenchymal swellings – the pharyngeal archesare invaded by neural crest cells (NCCs) (1). NCCs originate from the neuroectoderm, as the neural folds lift up and fuse. The ectodermal cells undergo epithelial-mesenchymal transition, ultimately displaying mesenchymal properties, including the ability to migrate. All three germ layers contribute to the formation of the pharyngeal arches; endoderm makes up the lining of each arch, while the core is composed of mesoderm and migrated NCCs and the outer surface is covered with ectoderm (2,3). The NCCs that populate each of the pharyngeal arches originate from specific locations; the first pharyngeal arch is invaded by cells derived from the mid, and hindbrain (1).

With continued development, the stomodeum is bounded by the frontonasal prominence (FNP) superiorly, the maxillary prominences laterally, and the mandibular prominences of the first pharyngeal arch inferiorly (1). Nasal placodes develop within the FNP, with medial and lateral nasal prominences (MNP and LNP respectively) developing around them. Interestingly, around the 5th week, the FNP narrows, resulting in the two MNPs moving towards each other and merging in the midline. The maxillary process of the first arch meets the MNP, eventually fusing with it to give rise to the upper lip around week 6.

As this is occurring, the midface continues to narrow and the developing eyes move more medially, towards each other. The MNP gives rise to most of the midline structures of the midfacial region including the nasal septum, bridge of the nose, columella, philtrum of the upper lip, the primary palate as well as the maxillary incisors. The LNP forms the alae of the nose. Besides its contribution to the upper lip, the maxillary process of the first pharyngeal arch contributes to the formation of the maxilla, palatine bones, zygomatic bones as well as the remainder of the maxillary dentition. The skeletal components of the mandibular process of the first arch consist of the Meckel's cartilage and the mandible (see 1.1.2). The skeletal components of the second and third arch arise from the Reichert's cartilage, namely the hyoid (see 1.1.3). Needless to say, the contributions of the pharyngeal arches are vast, much beyond the brief description provided in this overview. These include the tongue, thyroid, and parathyroid glands, various muscles in the orofacial and neck regions, as the neural, vascular elements associated with them (1). A more detailed description of the development of the tissues under investigation in this study is provided below.

1.1.1 Tongue development

Embryonic development of the tongue begins with the appearance of lingual swellings within the floor of the first pharyngeal arch during the 4th week pc (1,2,4). One median and two lateral lingual swellings grow and merge forming the anterior two-thirds of the tongue (1,2,4). Swellings from the floor of the third, and fourth pharyngeal arches grow and merge to form the posterior one-third of the tongue, joining the anterior part at the sulcus terminalis (1,2). There is no addition from the second pharyngeal arch to the tongue as any initial contribution is lost by the overgrowth of the swellings from the third, and the fourth pharyngeal arches (2).

The mesenchyme is derived from NCCs arriving from the midbrain, and hindbrain (1). Mice studies showed that migrated NCCs invaded the epithelium and mesenchyme of the tongue primordium at the early stages of embryonic development (5). In conjunction with the swellings in the floor of the pharyngeal arches, the tongue is invaded by migratory myoblasts that arise from occipital somites that contribute to the formation of the intrinsic muscles of the tongue (1,2,4). Then, myoblasts contact the cranial NCCs derived mesenchyme and establish a close connection (6). Determination factors (MyoD, MRF4, myogenin) are expressed in the future tongue muscles earlier than other muscles that are somites-derived reflecting the early demand of the tongue's functional activity (1). The tongue rapidly enlarges occupying the stomodeum which will form the future mouth (1). It doubles in size in adolescence compared to its size at birth, and it reaches its final size around the age of sixteen years old (1).

The intrinsic muscles of the tongue include the superior longitudinal, inferior longitudinal, vertical, and transverse muscles (6). The extrinsic muscles of the tongue are the genioglossus that arises from the mental spine of the mandible, the palatoglossus that originates from the inferior surface of the palatine aponeurosis, the styloglossus arises from the styloid process, and hyoglossus which originates from the superior border of the greater horns of the hyoid (6-8). Taste buds are mainly present on the dorsal surface of the tongue and they are observed after the 13th week pc (1). Given the varied origins of the tongue and the different functions it performs, it has a very complex nerve supply. The hypoglossal nerve supplies the tongue muscles, while sensory supply to the anterior two-thirds comes from the trigeminal nerve. The posterior one-third is supplied by the glossopharyngeal nerve. Interestingly, the taste sensation is supplied by the chorda tympani, branch of the facial nerve.

1.1.2 Mandible development

The first pharyngeal arch, from which the anterior two-thirds of the tongue develops, also gives rise to the mandible, with the contribution of embryonic NCCs (1,2). The mandibular branch of the trigeminal nerve is the first structure to develop in the region of the mandible (1). The cartilage of the first pharyngeal arch is Meckel's cartilage (1), and its formation is initiated by the ectomesenchymal cells that are derived from NCCs (9). The cartilage's primordium is observed as early as day 32 (week 5) in human embryos (10).

Curiously, however, the Meckel's cartilage has a limited role in the development of the mandible, which ossifies primarily via intramembranous ossification, similar to most of the bones of the craniofacial region (maxilla, zygomatic, nasal, palatine, etc.). This is in contrast to the bones that form the base of the skull (ethmoid, sphenoid, basioccipital) which develop via endochondral ossification of the chondrocranium, much like the long bones of the body (11).

Hence, by the 6th week pc; a single ossification center appears on the inferior surface of Meckel's cartilage on each hemi-mandible, close to the distal branching of the inferior alveolar artery into the incisal and mental branches (11). While the lower jaw eventually appears as a single bone, yet it is composed of distinct developmental skeletal units (1). During the 7th embryonic week, ossification is seen the rami area, and the inferior alveolar nerve lies between the lateral surface of Meckel's cartilage and the interior surface of the ramus until the nerve gradually becomes encapsulated within the bone (11).

Meckel's cartilage does not transform into mandibular basal bone, except in the rostral process where the cartilage next to the midline undergoes endochondral ossification (1,12). Most of Meckel's cartilage is resorbed except for the posterior segment that forms the sphenomandibular ligament and the retroarticular process that forms the malleus and incus, two of the middle ear ossicles (13). Additionally, the sagittal growth of the mandible is found to be highly affected by the growth of Meckel's cartilage, as it provides the initial "blueprint" for bone deposition around it (11).

During the 8th week, the condylar primary formative mesenchyme can be seen, with attachments of the lateral pterygoid muscle (14). This portion of the mandible undergoes endochondral ossification and as condylar growth continues in an upward and backward direction along with the pterygoid muscle attachment, the ramus length and the overall anteroposterior mandibular lengths increase (14).

The temporalis muscle precedes the development of the secondary cartilage of the coronoid process and is closely associated with it (1). Later as the ramus is expanding, it joins with the coronoid processes (1). The mandibular symphysis remains unfused during human prenatal life, fusion occurs in the first year post-nataly (15).

After birth, the mandible enlarges by appositional growth as it grows in a forward, and downward displacement, which also impacts the direction of the mental foramen from forward to backward direction when viewed at birth, and adulthood respectively (1). Moreover, the vertical position of the foramen changes from midway of the lower border until it ascends near the upper border in the aged mandible (1).

Bone resorption occurs on the anterior surface of the ramus, in contrast accompanying bone deposition takes place at the condylar head as well as the posterior surfaces of the mandible and the coronoid process, resulting in an upward and backward growth of the ramus (1). The alveolar process growth responds to teeth eruption which adds to the mandibular overall dimensions (1). The sagittal growth of the mandible was described to be prognathic up to the 10th week pc as it exceeds the upper jaw until the maxilla catches up at a later stage during fetal development (1). Furthermore, disruption in mandibular anteroposterior growth in relation to the maxillary growth results in skeletal malocclusion (1).

Animal studies have been conducted to study the relationship between mandibular skeletal units and their soft tissue attachments in post-natal life. These studies have aimed to establish the effect of mandibular function on its growth. In a study that was carried out on new-born rats, the temporal muscle was resected. The result was that the coronoid process has either completely resorbed, or a tiny tubercle with some muscle fibers attached to it remained on the same operated side (16). In 1951, Horowitz, and Shapiro reported on the alteration of the mandibular architecture and the absence of the coronoid process following the unilateral excision of the temporalis muscle in a series of one-month-old rats, indicating that these observations correspond to the loss of the functional stimuli (17). Rodrigues et al., reported atrophic changes in the angular process and shortening of the mandible following the complete unilateral excision of the masseter muscle in growing rats (18). While these studies highlight the importance of muscle function on mandibular bony maintenance and growth in post-natal time points, such relationships have not been comprehensively assessed during prenatal periods.

1.1.3 Hyoid development

The hyoid bone is located in the midline of the neck, it is a floating bone in the human body that does not articulate with any other bone (19). The hyoid is a horse-shoe shaped structure that is composed of the body, greater horns, and lesser horns that provides support for the laryngeal complex thereby playing a key role in deglutition and phonation.(20). It varies in size and shape as well as the ankylosis of its horns to the body (7,20). During early development, the hyoid appears as a cartilaginous tissue that is derived from the 2^{nd} and 3^{rd} pharyngeal arches (1,2). The second pharyngeal arch which is also known as Reichert's arch appears between the 45^{th} to 48^{th} days pc (1). It gives rise to the cranial part of the body of the hyoid and the lesser horns, where the caudal part of the body of the hyoid and the greater horns are derived from the third pharyngeal arch (1,2).

Unlike the mandible, the hyoid develops via endochondral ossification (19). Ossification into bone starts around the thirtieth fetal week and advances after birth (19). However, greater, and lesser horns complete ossification through puberty (19). The hyoid is the site of attachment for the suprahyoid and the infrahyoid muscles. The suprahyoid muscles that elevate the hyoid include the geniohyoid, stylohyoid, mylohyoid, and digastric (7). On the other hand, the infrahyoid muscles that depress the hyoid include the omohyoid, thyrohyoid, sternohyoid, and sternothyroid (7). Moreover, the hyoglossus muscle and the middle pharyngeal constrictor muscle originate from the greater horn of the hyoid and are the largest muscles that attach to the hyoid (7,20).

Ligaments that are attached to the hyoid are stylohyoid that connects the lesser horns to the styloid process, and the thyrohyoid membrane that connects it to the thyroid cartilage (20). The hyoepiglottic ligament connects the hyoid to the epiglottis, given its involvement in airway patency; it is one of the most important ligaments in the human body (21). During infancy, the hyoid is located opposite to the 2nd cervical vertebrae, however during adulthood, it is found against the 4th, and 5th cervical vertebrae (7).

By about the first year of the post-natal human life up to 18 months old, the hyoid descends, and the tongue falls more posteriorly towards the pharyngeal direction (20). Consequently, infants are no longer obligate nose-breathers during feeding and swallowing; a dynamic afforded previously due to the close proximity of the epiglottis and soft palate (20). The exact timing of the descent of the epiglottis remains unclear; earlier reports based on clinicians' observations estimated it to be around four to six months of age (22).

1.2 The relationship of the tongue, hyoid, and mandible

The developmental, functional, or structural association between composite tissues is identified as integration (23). The concept of integration perhaps is exhibited well in craniofacial anomalies where the failure in the development of a single structure or its malfunction, negatively impacts the configuration of an interrelated network. On the other hand, dysmorphogenesis of a single part in an organization may be tolerated to a certain degree by other segments which suggests their modularity. Hence, modularity refers to the self-determine of each separate unit within an integrated partnership (23). The extent of autonomy in a tissue may reflect its hierarchy.

In other words, the greater the tolerance to an insult the more sovereignty over self-dominance, and/or maturity, i.e. modularity. The tongue, mandible and hyoid cartilage studied here are histologically and developmentally variant structures. However, they are functionally related, share the oropharynx, and may have a significant influence on each other. Hence many studies have attempted to study the integration and/or modularity within these structures and assess the intricate relationship between them.

1.2.1 The tongue and the mandible

The lower jaw is the skeletal component that provides housing, and protection for the tongue. As mentioned before, the mandible and anterior two-thirds of the tongue share the origin of the first pharyngeal arch (1,2,4). Much of the lingual mass is made up of the genioglossus muscle which connects it to the mandible (24) and tongue muscles were found to be attached to the perichondrium of Meckel's cartilage before the mandibular bone differentiation during the embryonic stage 23 (25).

In an experimental study on 12-week old Yucatan miniature pigs, the volume of the anterior twothirds of the tongue was reduced by approximately 25%, then pressure transducers were placed on the pre-maxillary area and on the lingual surface of the mandible anteriorly to illustrate the masticatory pressures and strains that were produced by the tongue (26). The authors reported that loads on the lingual surface on the mandible were greater than the loads measured on the premaxilla (26). Moreover, the functional loads measured in the surgically excised group were lower compared to the sham animals' group (26) indicating the strong link between the tongue and the lower jaw. Additionally, Liu et al. surgically reduced the volumes of the anterior two-thirds of the tongue in 12-week-old Yucatan minipigs by approximately 15% after placing mini stainless-steel implants for the purpose of growth tracing (27). Next, they measured linear and angular changes on a series of longitudinally obtained cephalograms over the period of four weeks in both the surgical reduction and the sham group (27). They reported that the tongue-reduced volume group showed a significant negative impact on craniofacial growth but mainly on the anterior mandible and the symphysis area (27).

Similar attempts have been made to explore the relationship between the human mandible, and tongue. Yılmaz et al., studied the deglutitive tongue movements in two skeletally variant groups in terms of malocclusion (28). Their study groups consisted of skeletal class II patients in comparison to the control group which consisted of skeletal class I patients (29). Using dynamic MRI, they observed the tongue and reported that its position, and its movements during deglutition differ between skeletal class I, and Class II patients supporting the fact that the behavior of the tongue correlates with distinct maxillomandibular relationships (29).

Moreover, the linkage between the tongue and the mandibular size was assessed using various methods that established a significant relationship. Hren & Barbic, investigated the tongue volumes in two groups of adult patients (30). The first group consisted of skeletal class III deformity patients with a mean age of 24 years old whom all presented with negative overjet (30). Their control group consisted of skeletal class I patients with normal overjet, and overbite with a mean age of 25.3 years old (30).

The measurements of the volume of the tongue were carried out by using a software program that analyzed the images that were acquired by extraoral ultrasound imaging (30). Their results were significant for both males and females; they reported that patients with skeletal class III had larger tongue volumes than their control group, relating their findings to effects of the surgical mandibular set-back on the backward re-positioning of the tongue and its subsequent possible airway obstruction consequences (30).

Cone-beam computed tomography has also been used in the aforementioned analysis (31). Iwasaki et al., analyzed the images acquired by CBCT of sixty children with a mean age of 9.2 years old dividing them into three groups based on their maxillomandibular skeletal relationship (31). They measured the tongue volumes of their pediatric subjects and reported that children with skeletal class III malocclusion had larger tongue volumes than their peers with skeletal class I, and class II relationships (31). While, the average tongue volume in class II patients is smaller than Class I patients, the difference was not found to be statistically significant (31).

The findings in patients diagnosed with Pierre Robin Sequence (PRS; OMIM: % 261800) demonstrates yet another example of the relationship between the tongue and the mandible. In 1923, the French stomatologist Pierre Robin described a state of an oropharyngeal obstruction mainly due to a displaced tongue (32,33). In his classical document, Dr. Robin introduced the term glossoptosis that referred to the downward, and backward position of the base of the tongue. Moreover, he theorized that it is a direct consequence of an underdeveloped mandible since there is an absence of any local lesion that could rationalize the presentation of the tongue in such manner (32,33).

Lenstrup et al. reported three cases of infants who suffered from choking, cyanotic attacks, among other different symptoms (34). These patients suffered from a cleft palate, displaced tongue against the pharyngeal wall, and under-developed mandible (34). Likewise, Eley and Farber reported four cases of infants who were admitted for complaints such as failure to gain weight and vomiting, but mainly due to cyanotic attacks (35). All of those infants exhibited a displaced tongue, cleft palate, and mandibular hypoplasia (35). Furthermore, Pierre Robin acknowledged the work of Eley, and Farber (35), and published a second article confirming the observation of the cleft palate in such patients (36). Again, he maintained that the hypotrophic mandible to be the direct cause of glossoptosis, leading to the physical obstruction of the airway and respiratory insufficiency in infants (36). Nowadays, there is a general consensus among most authors that the exact cause of PRS is unknown (37-40). The working theory of the sequence of events is that improper intrauterine growth of the mandible failed to provide sufficient space for the tongue to fall in the right place. Later, the tongue becomes the physical element of obstruction in between the palatal shelves, preventing them from fusing together; cleft palate may be present (12,37-41). However, these theories have not been substantiated with evidence. Yet, PRS patients present a unique population in which to study the integration of tongue position with mandibular size.

1.2.2 The tongue and the hyoid

Parts of the posterior third of the tongue and the hyoid share the third pharyngeal arch origin (1,2,4). Undoubtedly, these two structures are functionally closely related, in terms of swallowing and speech (20). The largest muscle that attaches to the hyoid is the hyoglossus muscle which originates from the greater horns of the hyoid and inserts in the tongue (20).

In literature, the association between the size of the tongue and the size of the hyoid has not received much attention. Instead, some studies have investigated the changing position of the hyoid in relation to age.

1.2.3 The mandible and the hyoid

The mandible and the hyoid are attached by the geniohyoid, anterior belly of digastric, and mylohyoid (42). Attention has been drawn to the position of the hyoid in relation to the mandibular skeletal occlusal behavior. Adamidis & Spyropoulos analyzed a set of lateral cephalometric radiographs of skeletal class I and class III patients and reported different positions and orientations of the hyoid bone in the two groups (28).

According to Iwasaki et al., the vertical position of the hyoid in children with skeletal class II malocclusion was lower, while its position in children with class III malocclusion was higher compared to the skeletal class I individuals (31). Moreover, the hyoid bone and the tongue seem re-position posteriorly after mandibular set-back via intraoral vertical ramus osteotomy in patients with skeletal class III malocclusion (43,44). Once again, the position of the hyoid in relation to the mandible was the main area of investigation rather than exploring their size association.

In the light of these findings, we can appreciate that there is an established growth influence, and a developmental impact between the tongue, hyoid, and mandible on each other in the fetal, and post-natal life.

1.3 Study rationale

Since the tongue, mandible, and hyoid cartilage, which are different types of tissues but share function, spatial territory, and arise from a mutual origin; the first four pharyngeal arches, it is likely that they would share a common developmental growth trajectory.

1.3.1 Research questions

- Is there an integrated growth pattern between the tongue, hyoid cartilage, and mandible during the early fetal stage of prenatal human growth?
- Is there a growth spurt in these structures during this stage?

1.3.2 Study aims

Aim 1: Quantify the development of the human mandibular bone, tongue, and hyoid between 9 and 19 postconceptional weeks using 3D micro-CT imaging of contrast-enhanced fetal skull samples.

Aim 2: Determine whether growth of the mandible correlates with that of the tongue and the hyoid cartilage during the early fetal growth stage.

1.3.3 Hypothesis

1 The null hypothesis (H0) of this study was that there is no relationship between the growth of the tongue, the hyoid cartilage, and the mandible during the early fetal stage of prenatal human life.

2 The alternative hypothesis (H1) of this study is that the growth of the tongue has a strong positive correlation to the growth of the mandible, and the hyoid cartilage during the early fetal stage of prenatal human life.

Chapter 2: Materials and methods

2.1 Sample

The study population consisted of human fetal heads that were obtained between 1986 to 1988 from elective terminations carried out at BC Women's Hospital and later collected by Dr. Virginia Diewert (Human ethics approval H08-02576-A015). Conceptions were determined to be normal by a pathologist although no genetic analyses were performed. The samples have been fixed in formalin since collection, preserving soft and hard tissues. The ages were determined by postconceptional days. Gender, crown-rump length, crown-heel length, the weight of the specimens, and head circumference measurements were registered. The crown-rump lengths of 3 out of 16 samples were missing, and they were estimated by an expert based on available data (Table 1, CRL*).

2.2 Contrast enhancement and CT imaging

Specimens have been imaged with conventional CT scanning at UBC, showing excellent visualization of ossified tissues of the fetal skull as well as the dental crypts and the developing bone around each crypt (Figure1). Selected samples without obvious deformation from different ages between 9 to 19 weeks pc were stained using a contrast-enhancement agent - phosphotungstic acid (PTA, see below), and re-scanned with high-resolution micro-CT (28-58) μ at the Central European Institute of Technology, Czech Republic (Table 1). PTA allows the visualization of soft tissue, giving the ability to recognize facial skeleton, vessels, and muscles (Figure 2).

No.	Trimester	Months	Weeks PC	Days PC	Sex	CRL	CHL	Voxel size /micron
1		3 rd	9	67	-	5.5*	6.8*	17.2
2				67	-	5.7*	6.9*	17.2
3			10	75	М	7.0	9.1	17.2
4	First		11	76	-	7.2*	9.3*	17.2
5			12	85	М	9.0	12.0	17.2
б				90	М	10.0	14.3	17.2
7			13	96	М	11.2	16.6	24.6
8				94	F	10.7	14.5	25
9		4 th Second	14	103	М	12.5	17.0	24.6
10			15	108	М	13.5	20.0	29
11				107	М	13.3	18.2	27
12	Second		16	114	М	14.6	22.1	32
13				122	F	15.9	23.5	26
14			17	124	F	16.4	24.2	24.9
15				123	М	16.1	24.8	24.9
16		5 th	19	133	F	17.0	25.2	35

Table 1. List of all the samples, with corresponding ages in weeks, and days of post conception, sex, crown rump length, crown heel length, and resolution of each scan. (Asterisk) stands for estimated.



Fig. 1. Fetal heads were imaged with highresolution micro-CT without contrast enhancement. Lateral view of a 12-week-old fetus.



Fig. 2. 45 degree of a CT scan of a 14-weeksold PTA stained fetal head.

The fetal heads were first stained using PTA; this stain is prepared as a 1% mixture of PTA in 70% ethanol. Samples are immersed in the stain solution for long periods depending on the thickness of the tissues. Finally, the specimens are scanned in 70-100% ethanol (45,46). which helps to inhibit air bubbles formation (47). High-resolution micro-CT scans were performed either at the Center for High Throughput Phenogenomics at the University of British Columbia or the Laboratory of X-ray Micro CT and Nano CT- at the Central European Institute of Technology (CEITEC) Brno, Czech Republic.

2.2.1 Visualization of scans and defining the anatomy of the tongue, mandible and hyoid

When 3D scanning was performed, the ideal sample orientation was not possible. Hence, our first step involved the reorientation to the true sagittal, coronal, and axial planes using 3D Slicer software (48). All tracings and segmentations were performed by a single, and unblinded rater. The tongue was the reference structure, as we were looking for symmetry in the multi-planner viewer. This step was carried out using the *Reformat* module (Figure 3). Spacing of slices was checked to ensure isometry and accurate analysis output.



Fig. 3. The tongue was used as a reference for re-orientation. (A) sagittal, (B) coronal, and (C) axial view of a 16-week-old fetus. (a) anterior, (p) posterior, (G) geniohyoid, (H) body of the hyoid cartilage, (M) mandible, (S) soft palate, (T) tongue.

The anteroposterior anatomy of the segmented tongue extended from the tip of the tongue anteriorly (Figure 4), followed the natural curvature of the dorsum surface all the way to the roots of the tongue posteriorly at the level of the vallecula (Figure 4). The cranio-caudal anatomy of the tongue included the dorsal surface superiorly, while the inferior level extended to the superior surface of the geniohyoid muscle, and the body of the hyoid cartilage (Figure 4).

The genioglossus muscle and the body of the tongue's intrinsic muscles were included (Figure 4). The extensions of the hyoglossus muscle outside the body of the tongue along the greater horns of the hyoid were not included (Figure 9-C). The sublingual salivary gland area was excluded (Figure 5). The transverse extension of the segmented tongue included all the borders to the sagittal plane of the palatoglossus and the styloglossus insertions within the tongue.

In the mid-sagittal view, the body of the hyoid cartilage appears as a kidney-shaped radiolucent structure surrounded by more radiopaque boundary; its perichondrium, it is located at the base of the caudal end of the tongue (Figures 3-A, 4). The anatomy of hyoid cartilage included the body of the hyoid cartilage and the greater horns.

The mandible had the highest contrast among the tissues in study. The calcified body, ramus, angle, coronoid, condylar area, teeth crypts, and the interior surface of the mandible with the projecting Meckel's cartilage were included in the segmentation.



Fig. 4. (A,B) Sagittal view in a 9-week-old fetus. (C,D) Sagittal view in a 17-week-old-fetus. Anatomy of the segmented tongue is highlighted in red in (B,D). (a) anterior, (p) posterior, (E) epiglottis, (G) geniohyoid, (GG) genioglossus, (H) body of the hyoid cartilage, (M) mandible, (S) soft palate, (T) tongue, (V) vallecula. All sections are para sagittal.


Fig. 5. Coronal view of the anterior tongue in a 19-week-old fetus. (A) Before segmentation, (B) after segmentation (highlighted in red), the sublingual salivary glands area was excluded. (G) geniohyoid, (GG) genioglossus, (M) mandible, (SL) sublingual salivary glands area, (T) tongue.

2.2.2 Scheme of segmentation

Next, the application of *Gaussian noise filters* from the *simple filters* module helped to reduce blur, and noise from the images. After determining the appropriate contrast range, the intensity *threshold* was increased further beyond the borders of the tongue and the hyoid to verify the inclusion of all the internal voxels within these structures that otherwise may be missed and miscounted as voids. Meticulous segmentation of the tongue muscles and the hyoid was performed by going through the slices in the sagittal, coronal, and axial planes using *paint* module. Critical areas of the tongue to be differentiated from the surrounding structures like the root of the tongue posteriorly, and the inferior surface of the segmented area are best viewed in the sagittal plane (Figure 6). The differentiation between the borders of the sublingual salivary gland area and the tongue in order to exclude the former was mostly clear in the coronal (Figure 5), and the sagittal planes (Figure 6). The fine details of the inferior (Figures 5,7) and anterior extensions of the genioglossus muscle borders (Figure 7) as well as, the insertions of the styloglossus muscle and the palatoglossus muscle in the lateral borders of the tongue are best determined in the axial plane (Figure 8).



Fig. 6. Sagittal view of the tongue in a 12-week-old fetus. (A) Before segmentation of the tongue; the dotted line with white arrow represents the plane between the genioglossus and the upper border of the geniohyoid. Posteriorly, the white line with the blue arrow represents the plane of the vallecula. (B) After segmentation. (a) anterior, (p) posterior, (E) epiglottis, (G) geniohyoid, (GG) genioglossus, (H) body of the hyoid cartilage, (M) mandible, (S) soft palate, (SL) sublingual salivary gland area, (T) tongue, (V) vallecula.

The hyoid body and the greater horns with their perichondrium were traced in all planes at the base of the tongue (Figure 9). Perichondrium of the hyoid was also included; it was clearly recognized given its relative radio-opacity in comparison to the surrounding structures. Each segment or section embodies a different tissue separately, as the tongue and the hyoid were counted into two different segments.

The following step was developed in our lab for the addition of an extra segment to paint all the areas, and the dead space around the borders of the tongue and the hyoid to be counted as a separate structure by the software. This step was performed to include all the fluids and formalin proteins around the tongue and the hyoid that were stained with the PTA, thus they appear as noise and under these circumstances would distort the final output. Next, the 3D models were constructed using *grow from seeds* module, and the extra segment that contains all the boundaries was deleted (Figure 10). The final 3D model was edited based on the 3D view and the multi-planner viewer.

2.2.2.1 Segmentation of the mandible

Segmentation of the mandible was done using multiple automated, and manual tools. *Scissors and paint* modules were employed after determining the appropriate calcified bone threshold on the same images of the PTA stained scans (Figure 11). To ensure that less ossified tissues were included, the intensity was increased beyond the required threshold, then additional noise was removed in the 3D view after the construction of the 3D model.

Due to the variation in PTA staining intensity between samples, it was not possible to maintain a specific threshold for all samples. Volumes of all the tissues were calculated in mm³ using *segment statistics* module.

The body of the mandible was separated from the rami area based on a drawn line from the meeting point of the body of the mandible and the ascending ramus cranially and extended obliquely to the midpoint of the angle of the mandible throughout the buccolingual thickness.



Fig. 7. Axial view of the inferior extensions and borders of the genioglossus muscle (highlighted in red) in a 9-week-old fetus. (a) Anterior, (M) mandible.



Fig. 8. The axial view is best to visualize the lateral borders of the tongue. (A,B) 13-week-old fetus. (A) Before segmentation, the dotted lines represent the plane of the styloglossus muscle insertions in the tongue. (B) After segmentation, highlighted in red. (C,D) 19-week-old fetus. (C) Before segmentation, the dotted curves represent the palatoglossus muscle insertions in the tongue. (D) After segmentation, highlighted in red. Note the pressed area on the left side of the tongue in (C,D). (a) Anterior, (p) posterior, (SG) styloglossus, (PG) palatoglossus, (T) tongue.



Fig. 9. The body of the hyoid cartilage, and the greater horns are traced in all planes in a 16-week-old fetus. (A) Axial view. Sagittal views of (B) the body of the hyoid and (C) the greater horn, and the extensions of the hyoglossus muscle. Coronal views of (D) the body of the hyoid and (E) the bilateral greater horns. (a) anterior, (p) posterior, (E) epiglottis, (G) geniohyoid, (H) body of the hyoid cartilage, (HG) hyoglossus, (HH) greater horn of the hyoid, (M) mandible, (T) tongue, white arrows point at the perichondrium of the hyoid.



Fig. 10. (A,B) 3D-model construction, followed by deleting the extra segment that includes all the boundaries to bring off a noise-free/minimally distorted tongue and hyoid.



Fig. 11. (A,B,C) Mandibular axial, coronal, and sagittal views, respectively. After determining the threshold's intensity that is exclusive to the mandible (highlighted in yellow) to segment it from a 15-week-old fetal head' CT, that was previously stained with PTA. (a) Anterior, (p) posterior.

2.3 Statistical analysis

Statistical analysis was performed using IBM® SPSS including descriptive statistics, Pearson's correlation analysis, and regression analyses of the growth of the different structures.

Volumes' fold increase of each tissue was calculated by subtracting the largest volume value, minus the lowest value (increase), and then dividing the outcome by the lowest value (original number). Interclass correlation analysis (two-way mixed-effects model) and absolute agreement definition testing (49), was performed for intra-rater reliability on five samples that were randomly, and blindly selected. Re-segmentation was done after approximately two months from the initial segmentation Correlation of the tongue and the body of the mandible was further analyzed.

Using Microsoft Excel, standardization of the volume values by subtracting the mean from each score then dividing it by the standard deviation to obtain the z-score. This allowed for the comparison of the patterns of the growth trajectories between the different structures. Statistical differences in gender were not explored.

Chapter 3: Results

3.1 Observations from constructed 3D models of the tongue, hyoid, and mandible



Fig. 12. Lateral view of the segmented tongue, hyoid, and mandible after construction of the 3D models. Sample: 15-week old male fetus with a crown-rump length of 13.5 cm.



Fig. 13. (A,B,C) Superimposition of the tongue, hyoid, and mandible in different views in a 15-week old male fetus. (A) 45-degree view, (B) frontal view, (C) lateral view. (D) dorsal view of the tongue with the body of the mandible after cutting out the ramus in a 16-week old male fetus with a crown-rump length of 14.6 cm.



Fig. 14. The body of the mandible was segregated from the ramus. Multiview of a 16-week old fetal mandible. Blue highlights represents the body of the mandible in (A,B). (C) Lateral view of the body of the mandible after cutting out the ramus area.

The 3D constructed models of the different tissues resembled the post-natal morphological features to some extent on a petite scale. Several interesting features were noted, during the segmentation process.

The tongue had a papillary-like surface morphology and it occupied most of the space of the oral cavity in all samples. Also, the tip of the tongue was found to rest at or extended beyond the mandibular ridge in almost all samples (Figures 4,6). The concavity of the palatal surface seemed to follow the shape of the dorsal surface of the tongue in the sagittal view (Figures 3A, 4, 6).

In terms of development, the dense geniohyoid muscle fibers (Figure 9A) seemed to have more bulk in comparison to the neighboring genioglossus muscle. The gap between the insertions of the bilateral genioglossus muscles was wider in young samples (Figure 7), as the age increases; the thickness of the muscle augments, and this gap is found to be reduced. The sublingual salivary gland areas were distinguishable primarily by their position and the lack of condensed muscular mass as found in the remaining tongue muscles. The extrinsic tongue muscles in the youngest samples (9, 10, 11) weeks were relatively easy to identify as regards to segmentation, in comparison to the more differentiated, and more mature older samples.

However, unlike the tongue, the mandibular ossified tissues and the posterior extensions of Meckel's cartilage were challenging to distinguish in the younger samples, possibly due to the different range of calcification within the partially ossified areas.

In our youngest samples (67 days pc) both the condyle and the coronoid processes were partially present and interconnected by the immature sigmoid notch to the ramus. The lingual and buccal periosteal surfaces were porous which may suggest the incomplete, yet ongoing ossification in this region, along with the continuous remodeling of bone. The angle between the body of the mandible and the ascending ramus, where the future retromolar area is located, was noticed to be obtuse and started to decrease in the following weeks in older samples.

In general, the interior zone within the head of the condyle was scooped and fairly filled with bone. Furthermore, the gap in the lower end of the symphysis was relatively wider than the cranial end.

Interestingly, we were unable to detect the lesser horns in any of the early fetal hyoid cartilage samples in the sagittal, axial, and coronal views as well as after three-dimensional reconstruction. The lesser horns were reported to start ossification in the first or second year after birth (20), and according to Baker et al., they complete ossification around puberty (19). The lesser horn is the site of attachment for the stylohyoid ligament, that connects the hyoid to the styloid process which is a derivative of the second pharyngeal arch as well as the lesser horns (1).

Based on our scans, we estimate the cartilage of the lesser horns to develop considerably later than the 19th week pc; our eldest sample. This is supported by the fact that the styloid process appears in the late stages of gestation, while the timing of its complete ossification is around the 8th year of life and varies widely amongst individuals (50).

The greater horns proximal heads were not completely ankylosed with the lateral sides of the body of the hyoid; according to Irvine et al., this connection may remain fibrous until middle age (20) however, they are reported to complete ossification around puberty (19). The posterior surface of the body of the hyoid was concave (Figure 9B, D). Notably, there is a high amount of symmetry in the segmented structures. This is in line with the observations made in previous studies in our lab using the same samples (51).

3.2 Descriptive statistics

The minimum volume of the tongue (n=16) was 10.1 mm³ and the maximum volume was 982.8 mm³. The hyoid's minimum volume (n=16) was .8 mm³ and the maximum was 36.1 mm³. The mandibular volumes that were segmented from the same images of the PTA stained samples had a minimum of 4.8 mm³ and a maximum of 254.8 mm³. Descriptive statistics are shown in (Table 2). The complete list of volumes is found in (Table 3). There was a (96, 41.86, 43.0) fold increase in the tongue, hyoid, and mandible respectively. Fold increase was calculated based on the differences between the lowest, and the largest volumes registered.

Descriptive Statistics					
		Volume	Volume (mm ³)		
	N	Min	Max		
Tongue	16	10.1	982.8		
Hyoid	16	0.8	36.1		
Mandible	16	4.8	254.8		

Table 2. Descriptive statistics.

3.3 Post-hoc power

Observed power was calculated using SPSS for the extracted volumes of each tissue and the age in days pc as a fixed factor. Computed using alpha = .05 and confidence intervals of 95%, the least observed power reported was .89 with a significance of (.031).

Sample	Days post	Sex	Tongue	Hyoid	Mandibular	Mandibular body
	fertilization		volume mm ³	volume mm ³	volume mm ³	volume mm ³
9 weeks	67	-	10.1311	0.841068	4.83571	3.1797
9 weeks	67	-	22.3785	1.06097	9.49598	5.51865
10 weeks	75	М	50.1421	2.98905	10.4682	6.41658
11 weeks	76	-	30.2532	2.55208	16.3847	9.75642
12 weeks	85	М	116.829	7.60097	27.078	18.116
12 weeks	90	М	131.988	10.1987	19.9942	13.6064
13 weeks	96	М	191.394	8.69601	32.4757	17.2717
13 weeks	94	F	148.019	9.18842	58.8557	39.5518
14 weeks	103	М	280.032	10.9842	76.3339	45.9814
15 weeks	108	М	347.413	21.4592	115.232	68.9484
15 weeks	107	М	299.158	14.505	82.6348	54.4967
16 weeks	114	М	524.14	28.4791	254.793	149.185
17 weeks	122	F	656.759	28.9296	164.534	110.006
17weeks	124	F	689.402	36.0529	153.54	100.026
17 weeks	123	М	982.808	36.0471	164.689	98.7462
19 weeks	133	F	611.155	35.4823	212.962	130.371

Table 3. Complete list of volumes of the segmented tissues.

3.4 Interclass correlation

ICC correlation coefficients of a single rater based on the two-way mixed effects and the absolute agreement definition were all > 0.9 with a P < .001 for all repeated measurements of the three different tissues (Table 4). Average error, and maximum error in mm³, and in percentages for each tissue of the selected samples, are as shown in (Table 5).

		99% Confidence Interval		F Te	F Test with True Value 0			
	Intraclass Correlation ^b	Lower Bound	Upper Bound	Value	df1	df2	Sig	
Single Measures	1.000ª	.997	1.000	11619 803	4	4	.000	Tongua
Average Measures	1.000°	.998	1.000	11017.805	4	4	.000	Tongue
Single Measures	.999ª	.985	1.000	3447 054	4	4	.000	IIwoid
Average Measures	1.000°	.993	1.000	5447.054	4	4	.000	пуош
Single Measures	.999ª	.977	1.000	1208 142	4	4	.000	Mandible
Average Measures	.999°	.988	1.000	1508.145	4	4	.000	Manufole

Table 4. Intraclass correlation coefficient for the repeated measurements of the tongue. Two-way mixed effects and absolute agreement. Results are shown for a single rater.

Unit / Variable	Tongue	Hyoid	Mandible
Average error (mm ³)	1.84	0.27	1.88
Max. error (mm ³)	4.71	0.44	3.94
Average error (as % of measured value)	1.51	5.79	6.32
Max. error (as % of measured value)	4.12	15.82	10.73

Table 5. Average error, and maximum error in mm³, and in percentages for repeated measurements for each tissue.

3.5 Correlation coefficients between the growth of the tongue, hyoid, and mandible

Pearson's correlation coefficients of .960, and .853 were calculated between the increased volumetric measures of the tongue and of the hyoid and the mandible, respectively. A correlation of .918 was found between the growth of the mandible and the hyoid (Figure 15), (Table 6). While Pearson's *r* increased to .864 when the volume of the tongue was correlated to the body of the mandible with a *P*-value < 0.001 for all correlations.



Fig. 15. Correlations between the growth of the tongue, mandible, and hyoid.

Correlations		Tongue volume (mm ³)	Hyoid volume (mm ³)
Hyoid volume (mm ³)	Pearson Correlation	Pearson Correlation .960**	
	Sig. (2-tailed)	.000	-
	Ν	16	-
Mandible volume (mm ³)	Pearson Correlation	.853**	.918**
	Sig. (2-tailed)	.000	.000
	Ν	16	16

Table 6. Pearson correlation coefficients of the growth of the tongue, hyoid, and mandible and mandibular body. **. Correlation is significant at the 0.01 level (2-tailed).

3.6 Grouped scatter of the volumes of different tissues, and regression analysis

The spread of the volumes of the tongue, hyoid, and mandible in relation to the age in days pc is illustrated in (Figure 16). An exponential function was found to best-fit the data obtained.



Grouped Scatter of Total volume of the Segmented Tongue in mm³, of Total volume of the Segmented Hyoid in mm³, of Total volume of the Segmented Mandible in mm³...

Fig. 16. Grouped scatter of the volumes of different tissues.

Exponential curve estimation regression analysis of each individual tissue shows near-identical growth patterns of soft, and hard tissue, age in days pc as a predictor. R Square for the curve of the tongue was .919, and .917 for both curves of the hyoid and the mandible with the significance of < .001 for all analyses (Figure 17). We also performed a correlation analysis of the volumes with the CRL as a predictor of fetus' size and obtained R-Square values of .941, .933, and .930 for the tongue, hyoid, and mandible respectively with the significance of < .001 for all analyses (Figure 18).



Fig. 17. Exponential curve regression analysis. (A) tongue, (B) hyoid, and (C) mandible. Age in days pc as a predictor and model summery and parameter estimates.



Fig. 18. Exponential curve regression analysis. (A) tongue, (B) hyoid, and (C) mandible. CRL as a predictor and model summery and parameter estimates.

3.7 Standardizations of volume outputs and comparison of growth trajectories

To compare the growth trajectories of the tongue and the mandible we standardized the volume values by calculating their z-score based on the output fit on the exponential curves. To ensure positive numerals we added a constant value "1.1" to each z-score. Having positive values will allow us to fit an exponential model for the standardized outputs. Results show that both curves show a related behavior until day 109.6 pc (Figure. 19), which point, the tongue (orange curve) accelerates by a slight measure and precedes the mandibular growth (blue curve).



Fig. 19. Superimposition of growth curves of the tongue, and the mandible after standardization of the volumes using z-scores fitted to the curve equations (inset). The tongue (orange), precedes the mandible (blue) at day 109.6 pc (red arrow)

Next, we compared the hyoid cartilage growth with the growth trajectory of the tongue, since it was shown to mimic the mandibular curve up to a certain point In a similar method, z-scores were obtained with the addition of "1.2" as a constant value to the actual outputs ensure a positive numeral. Results show that, when age is < 95.9 pc days, all three curves behave similarly but the tongue (orange) and the mandible (blue) precede the hyoid's trajectory (grey). Then the hyoid accelerates its growth rate, this acceleration continues until it surpasses the growth velocity of the tongue at day 111.8 pc (Figure 20).



Fig. 20. Superimposition of growth curves after standardization of the volumes using z-scores of the actual outputs. The hyoid (grey) accelerates its growth rate at day 95.9 pc (red arrow, left), and then precedes the tongue at day 111.8 pc (red arrow, right).

Chapter 4: Discussion

4.1 PTA as a contrast enhancement agent

The methodology for quantification of the tongue volume has advanced throughout the years and the use of 3D analysis is perhaps a more robust technique compared to previous conventional approaches. Here, we used PTA contrast-enhanced micro CT images that provided excellent visualization of the hard and soft tissue of the craniofacial regions of developing fetal heads. Soft tissue does not absorb x-ray very well (52), hence the need for a satisfactory approach for increasing soft tissue contrast is essential. The most commonly used contrast-enhancement stains with preserved specimens are iodine, osmium, and phosphotungstic acid (52). As they penetrate the sample, soft tissue absorbs the heavy metals which will result in a better contrast as x-ray will be absorbed well (52).

PTA is an acidic stain, that is stable, and easy to handle (45,46). In comparison to osmium; PTA has much lower toxicity, and it is cheaper to dispose (46). It is compatible with formalin-fixed structures, yet it penetrates tissues slowly and requires longer incubation time with limited tissue penetration capability compared to iodine (45,46). In animal studies, the penetration depth of PTA in mice paws after staining for 24 hours was only 2.08 mm compared to 4.74 mm achieved by mercury chloride (HgCl₂), but after one week of staining; results showed an 8.88 mm, and 9.61 mm penetration depths for the same stains, respectively (53).

Metscher, stained non-mineralized animal tissues using different contrast agents. He reported that muscles and nerves were distinguishable with PTA staining in micro-CT images animal heads as it provided high contrast, this allowed to visualize soft tissue clearly in order to apply quantitative studies on small biological samples (46). Moreover, PTA-stained differentiated tissues showed to have better contrast than immature embryonic mesenchyme (46). In a quantitative comparative study between iodine, and PTA it was reported that PTA staining provided superior inter-tissue details (47).

PTA does not stain cartilaginous tissue very well, which may affect its appearance in volume rendering, yet in high-resolution sections, chondrocytes can be viewed clearly (46). In fact, micro-CT images of PTA stained bovine articular cartilages have been utilized to objectively analyze collagen content (54). Another study has been able to visualize and reconstruct 3D models from high resolution micro-CT images of the inner ear in fish including the sensory epithelia after staining their samples with PTA (55).

In humans, Netzer et al., analyzed CT images of samples of lumbar facet joints after complete decalcification, and PTA staining (56). They reported that the maximum penetration depth of PTA was 5mm and that the contrast enhancement achieved was enough to identify chondrocytes and the microenvironment around them, in an effort to investigate ex vivo osteochondral diseases (56). Inside tissues, PTA remains fixed, but some surface precipitation may take place (53), which was clearly visible on the skin surfaces of our samples, after volume rendering.

In our sample, we were able to detect non-ossified tissues such as the tongue and the hyoid from CT scans. We examined the tongue's intrinsic and extrinsic muscles clearly and defined the exact area to be segmented. Also, the hyoid cartilage and the greater horn with their perichondrium were traced and reconstructed efficiently. The ossified tissue of the mandible was unmistakably visible with a higher contrast compared to its surrounding tissues even as early as 9 weeks pc. Our goal was to be precise and allow for the implemented segmentation methods to be reproducible, which we were able to achieve due to the excellent contrast provided by the PTA staining process.

4.2 The scale of measurement

Researchers have been trying to standardize human fetal measurements by measuring heights, cranial circumference, and even weights of internal organs such as the heart, spleen, lung, brain, and kidneys on fetal and neonatal autopsies (57-59). In our study, the volume was chosen as a scale of measurement to compensate for any presence of dysmorphic distortion among the different tissues, mainly the tongue due to storage, staining procedure, or handling. To elaborate more, if one side of the tongue was pressed the muscular volume would build up somewhere else as shown in (Figure 8 C, D). Hence, if we relied on landmarks and linear distances or angles, this type of deformation due to sample storage may have proved problematic.

4.3 Methods of measurement of the tongue and mandible in the literature

Only a few have studied the human skull in a comprehensive 3D analysis. The physical measurement of the tongue is quite difficult because of the tongue's contractility, limited accessibility, challenging reproducibility, and inability to reach or define all the borders of the tongue. For example, Tamari et al., have measured the tongue with alginate impressions on a population of Japanese adults in an attempt to correlate its size with the lower arch's size (60).

Alternatively, Hutchinson et al., examined the tongue and the mandible in 174 human cadavers from the age of twenty gestational weeks to three years of age post-nataly (61). Using an immersion microscribe, they first registered a set of landmarks on the mandible and then measured the dorsal surface of the tongue using a sliding caliper (61). They were able to assess the size and shape changes by applying principal component analysis to investigate morphometrical growth changes (61). Their results showed that the tongue and the mandible have a positive association in terms of size and shape until the age of two years old post-nataly (61). During the 3rd year of life, the tongue enlarged in size only as opposed to the lower jaw that still changed in both size, and shape (61).

Another method of measuring of the tongue is as simple as subjective visual inspection. Usually, in a clinical setting macroglossia or microglossia is judged based on the subjective evaluation of the tongue relative to the surrounding maxillomandibular complex.

However, a more structured approach is Mallampati score (62,63). The authors developed a simple classification to clinically evaluate the size of the tongue in relation to the faucial pillars, soft palate, and base of the uvula to help anesthesiologists predict the difficulty of endotracheal intubation (62,63). Yet, non-invasive 3D analysis visualization and quantification of the tongue provides a more robust analytical design (30,31).

Ultrasound medical sonography has been used extensively to examine the fetal mandible (64). In 1993, Watson & Kats measured the fetal mandibular anteroposterior, and the transverse lengths on 204 pregnant women using medical sonography (65). The ages of the fetuses ranged from 14 to 40 weeks of gestation (65). They made an effort to establish normal fetal mandibular linear measurements to aid in the prenatal diagnosis of micrognathia that is particularly associated with PRS (65).

Rotten et al., measured the inferior facial angle in normal, and syndromic fetuses (i.e. those with PRS, hemifacial microsomia, postaxial acrofacial dysostosis, Down syndrome, and cleft lip and palate) using ultrasound to assess retrognathia (66). Their results indicated that a retruded chin which may affect the angle being measured can be seen in a micrognathic as well as normally sized mandibles (66). In addition, fetal head volumes were obtained and calculated from three-dimensional transabdominal ultrasonography (67). Furthermore, fetal mandibular landmarking for geometric morphometric analysis was carried out using an immersion-microscribe as previously described (61).

4.4 Craniofacial growth integration

Our results suggest a very strong positive correlation of the volumetric growth between the tongue, and the hyoid, as well as the relation between the mandible and the hyoid, while the tongue and the mandible had a high positive association (68) in the early fetal stage. Growth trajectories of the tongue, hyoid, and mandible may seem inconsistent in the grouped scatterplot mainly because of the considerable differences in volumes of each tissue. Nevertheless, if each one of them was explored individually, it seems that muscle, cartilage, and bone which are derived from the first four pharyngeal arches show a near-identical growth pattern.

To allow for a more normalized comparison, we standardized the volume outputs in relation to the age in days. The growth curve of the tongue outpaces the mandible by day 109.6 pc, albeit very slightly. This suggests that the growth of soft tissue is increased relative to its skeletal component at this stage.

The explanation that both the tongue and the mandible were behaving similarly before day 109.6 is perhaps justified by the fact that our capture of the growth timeline started from the 9th pc week; likely when the mandible just started to grow rapidly (69), apparently to catch up with the growth of the tongue. Then after the tongue growth preceded the mandible around the 15th week pc, it is possible that the mandible may catch up to the tongue again in the following stage or continue to lag behind. Histological examination of the tongue in embryos and fetuses revealed that the tongue development preceded other maxillofacial structures (70).

On the other hand, the hyoid cartilage was found to match the curves of the tongue and the mandible until day 95.9, beyond which it begins to outgrow the other tissues, crossing them by day 111.8 pc. This could be attributed to the combined effect of multiple suprahyoid and infrahyoid muscles (10 pairs), tendon, and ligaments that are attached to the hyoid (20).

Overall, the regression curves and the values of the correlation coefficients found in our study indicate a strong integration between various tissues in the craniofacial complex in the early fetal stage of human growth, which is in line with previous data that investigated late fetal and postnatal growth integration (61).

The mechanisms leading to such integration are not completely understood. Undoubtedly, much of this integration may be genetically driven. The growth of bone, muscle and cartilage may be intrinsically programmed and growth factors concentrations that promote growth may be important in regulating individual tissue sizes. However, shared factors may also contribute to the muscular growth at the same time as they influence cartilage and bony growth, an example of molecular integration of growth. Many of the genes, transcription factors, growth factors, etc. are indeed shared between various craniofacial structures, and hence these tissues may respond synchronously to genetic regulation during development. Another level of integration may be derived from the functional movements in these tissues. For example, it is possible that the mechanosensors in the hyoid chondrocytes or developing bony spicules of the mandible may respond to muscle pull with higher proliferation, influencing its rate of growth.

Indeed, the 11th gestational week is the initiation of the early fetal period (71); a phase that is marked by the earliest movements of the fetus (1). One of the earliest functions of the tongue in the womb is the facilitation of the swallowing of the amniotic fluid (72). In fact, the fetus is constantly swallowing amniotic fluid (72). Such swallowing movements would involve not only the tongue but the mandible as well as the hyoid cartilage, via all the associated muscles. During these functions, it is possible that the tongue exerts forces on the mandible as well as the surrounding tissues in pre and post-natal life (73-76). Increasing data show that frequent force loading induces osteogenesis (110). Hence, normal development of the tongue, mandible, and hyoid may be in response to accommodate the functional demands at that stage, such as the amniotic fluid homeostasis (77,78).

The functional matrix theory has been quoted as an explanation for cranioskeletal growth (79). The term "functional" describes the integration of the soft, and hard tissues that serve a task, such as vision or olfaction (80). A *functional component* refers to the composite unit of the soft tissue that executes the function and termed the *functional matrix*, and its hard tissue vehicle that is called the *skeletal unit*, which safeguards the functional matrix and/or adds to it (80,81). According to Moss, bone tissue responds to stimulus generated by the functional matrix, by adapting proportionally to the "load" applied by the stimulus (81). On the cellular level, osteocytes and osteoblasts transduce this stimulus between them which will be translated as bone remodeling (81). In simple words, as the matrix grows the bone will follow (81), wherein, active transformation and passive translation complete each other (81,82). Based on this assertion, the mandible is regarded as a complex of several functional components composed of independent skeletal units, each having its own functional matrix (83,84).

Indeed, animal experiments (as described in the Introduction) have demonstrated the link between the coronoid process and the temporalis muscle, supporting such a theory. However, most of the data that support this theory come from post-natal animal studies. However, it is possible that such "functional" relationships are important during the developmental stages as well. For example, the alveolar bone growth is stimulated by the eruption of teeth; its functional matrix in this case, while the development of the coronoid process may relate to the temporalis muscle, and the angular process is affected by the masseter (83,84).

4.5 Growth spurt

At the earliest stage of the fetal period; the ninth week, half of the CRL is constituted by the fetal head length, after which point the fetal body grows at a rapid rate (12). There are many growth spurts during human prenatal and post-natal life which are often organ-specific. For example, Dobbing and Sands studied brain growth in different species and identified a beginning of a brain growth spurt in mid-pregnancy in humans (85,86). Moreover, different growth peaks for different tissues, and measurements of biparietal diameter, head circumference, femur length, and abdominal circumference, in the 13th ,14th ,15th, and 16th gestational weeks respectively, have been reported by Grantz et al. (87).

Our data suggest a significant volumetric growth increase by each week as shown in (Table 3). (Figures 19, 20). Yet, using our growth curve data, we were unable to find an inflection point, which would have objectively signified a potential growth spurt. However, subjectively, our curves have a "knee" in the zone between the end of the 13th week to the 15th week pc, suggestive of a growth spurt. The timepoints that precede or follow our studied interval may have a more significant spurt in these tissues.

4.6 Congenital anomalies affecting the craniofacial region

One of the areas where our study of normal craniofacial development is particularly relevant is in applying its findings to understanding the pathogenesis of craniofacial abnormalities. Many genetic inherited disorders, developmental abnormalities, or teratogens can lead to growth disturbances that affect the mandibular phenotype along with other related structures, in terms of size and/or morphology.

Severe dysmorphology that affects the craniofacial regions includes PRS, Treacher Collins syndrome (TCS), and craniofacial microsomia (CFM). PRS is classified as a syndrome of the first pharyngeal arch (88,89), while TCS and HFM involve anomalies of derivatives of the first and second pharyngeal arches (89). In milder forms of craniofacial growth disturbances, the size of the mandible in relation to the maxilla may be affected. These are commonly classified into three categories: either normal-sized known as skeletal class I, a skeletal class II in the form of a mandibular retrognathia, or a prognathic large mandible, known as skeletal class III (90).

Individuals diagnosed with TCS, also known as mandibulofacial dysostosis, are found to have severe craniofacial distortion that includes ocular, periorbital, and auricular abnormalities, as well as mid-facial hypoplasia (91). The mandibular development is severely disturbed and presents as underdeveloped, and distinctly retrognathic or even malformed (91). On the other hand, CFM which is usually a unilateral malformation (hence also referred to as hemifacial microsomia) that involves ocular, auricular, facial soft tissue, and jaws abnormal development (92).

In CFM, the mandibular malformation is classified based on the extent of developmental disturbance of the condyle, ramus, glenoid fossa, and temporomandibular joint in terms of presence or size (92). The tongue is usually overlooked in both TCS, and CFM, with only a few reports that describe its involvement in these syndromes as it presents in a PRS-like manner in TCS (93), or with mild dysmorphology in CFM (94).

Macrostomia and macroglossia are among the clinical manifestations in both Simpson-Golabi-Behmel and Beckwith-Wiedemann syndromes; as the abnormal large mouth is sometimes explained to accommodate the accompanying oversized tongue (95,96). Other conditions that take into account mandibular abnormality as a manifestation are achondroplasia (97), congenital syphilis (98,99), Nager syndrome (100,101), Miller syndrome (101), cerebrocostomandibular syndrome (102), and many more. PRS patients present with various degrees of mandibular size deficiency, with a posteriorly positioned tongue, frequent involvement of a cleft palate, and potential correlation of the hyoid (36). The prevailing theory for the pathogenesis of PRS is that the retrognathic mandible fails to grow adequately to provide a sufficient compartment for the tongue, which subsequently encroaches into the airway. Under these circumstances, the mandible has been deemed as the root of the sequence of events. However, this theory has not been completely substantiated with evidence.

Indeed, this conclusion by Pierre Robin (36) was based on the fact that he did not find any lesion that would result in the abnormal position of the tongue in those patients. Hence a dysmorphic mandible seemed to be a credible cause. Moreover, the therapeutic advancement of the mandible relieved the symptomatic patients by helping to reposition the tongue and clearing the airway as it was described in the early reports (32-36), thus offering additional support for the theory.

In our study, we divided the mandible into ramus and body portions and repeated the correlation analysis. We found the tongue to be highly correlated with the mandible as a whole, and the segregated body as well by correlation coefficients of .853, and .864 respectively. This may suggest that the mandibular growth at this stage is isometric, rather than markedly disproportionate. In a study in 2018 by Liu et al., it has been reported that 18 patients who were diagnosed with PRS with significant micrognathia had a normal temporomandibular joint when they were screened before surgical intervention (103). Their results indicate that specific parts of the mandible may be disturbed in PRS patients rather than describing the whole mandible as affected.

Interestingly, among the original reports by Pierre Robin (32,33,36), Eley and Farber (35), as well as Lenstrup (34), the later was the only one to note that the size of the body of the mandible is proportionally small in comparison to the ramus. The confusion between micrognathia or severe retrognathia as criteria in PRS triad has not been cleared and accurately reported. With attention to establishing the average normal lengths of the ramus and the mandibular body in healthy fetuses would help to define the dysmorphology of PRS mandibles.

The mandible was found to grow rapidly in the 11th gestational week (69). Humphrey (104), described an immediate rapid growth of the mandible after the tongue descends into the floor of the mouth and suggested that the presence of the tongue in the correct position drives the acceleration in the mandibular growth. Hong et al., suggested that the development of maxillofacial structures may be regulated by the role of the tongue based on histological analysis of different stages of prenatal human development (70). Since the tongue size is considered normal in PRS, while its position is impacted, this incorrect position of the tongue may be driving the formation of the cleft palate (105) as well as improper mandibular growth. Although our data cannot shed light on what may be driving the pathogenesis of PRS or other anomalies, it may help in providing normative data for volumes of craniofacial structures, which can prove useful defining dysmorphologies in future studies.
4.7 Sonographic evaluation and quantification

Many studies are trying to establish the effectiveness of prenatal imaging for identification of abnormal growth (66,106,107). Paladini et al., have developed an index to diagnose prenatal micrognathia from ultrasound images, in the act of measuring the antero-posterior diameter of the mandible divided by biparietal diameter (108). Similarly, many attempts were made, for diagnosing PRS in intra-uterine life using medical sonography (109). Most of the focus was directed towards the early detection of micrognathia, for example, linear mandibular measurements at different time points were determined to estimate mandible lengths in healthy pregnancies (64,65,110).

Here, we have studied the mandibular volume measurement rather than 2D measurements as an approach to evaluate the size of the lower jaw in the early stages of pregnancy. To be able to identify an abnormality, our data may prove useful in establishing normative growth volumetric values during fetal development and be helpful when trying to determine future diagnostic indicators, and comparative studies of congenital developmental anomalies such as PRS from medical sonography images (111,112). For this to be fully applicable however, further laboratory investigation would be required to assess the validity of volumetric measurements obtained from newer 3D ultrasound imaging methods. One possible way to test this out would be to mimic the in-utero environment and use the contemporary ultrasound imaging on our fetal samples, followed by comparing the obtained volumetric values to those found here.

Ideally, the initial approach to predict micrognathia by means of volume from ultrasound images is a retrospective study of the fetal mandible in both healthy, and syndromic fetuses such as PRS because the definitive diagnosis in only reached after birth. Ultrasound examination is the most accessible and clinically applicable approach. After establishing the average volumes of the mandibles in different gestational stages in a large sample, and preferably in a diverse ethnicity; a prospective study would be the next step to establish the sensitivity and specificity of the volumetric approach as a laboratory diagnostic tool.

4.8 Limitations

The main limitations of this study were the pursuit to find the appropriate methodology, the unrevealed ethnicity data of the aborted fetuses, and the small sample size.

Even though PTA provides excellent visualization, perhaps it stained the proteins within the surrounding fluids of the tissues. It diminished the software's ability to accurately differentiate between adjacent structures, therefore the utmost drawback was the inability to use the fully automated tools for segmentation. Several variations were detected among different age samples that were related to imaging. Some scans had a clearer overall view than the others as they were richer in detail such as the orientation of muscle fibers, and the radiopacity of the perichondrium of the hyoid. Since the segmentation step entails a large amount of subjectivity, we wanted to ensure that our protocols and methodology were repeatable.

This was analyzed by the interclass correlation coefficient by comparing the volumes that were obtained after repeating the segmentation steps. We found coefficients with values > .9 which indicate excellent reliability for single-rater. However, to establish the validity of our methodology; inter-observer reliability will be tested by different individuals in the future.

The fetal heads that were collected from elective terminations from Women's BC hospital in Vancouver, Canada for scientific investigation are of anonymous ethnicity. Moreover, the medical history and the pharmaceutical intake of pregnant individuals and the wellbeing of their gestation condition are undisclosed.

The process of acquiring a large collection of cadavers of human fetuses along with ethical approvals and putting them through the process of contrast-enhancement staining and high-resolution CT imaging besides other logistics is a prolonged procedure. As a result, the sample size is considerably small, despite the high observed power.

Furthermore, some shape distortion and configuration alteration may be present in a few samples that could be due to the following reasons: abortion operation, storage, staining process, or handling. As a result, the development of a methodology for shape analysis is challenging for soft tissue.

Differences in growth between male and female samples were not explored for a few reasons including the unequal distribution of samples based on gender among different ages, and the sex of a few samples was unknown.

Moreover, dissimilarities of the tongue, mandible, and hyoid sizes are not expected to be significant especially during the early fetal stage. However, one out of three 17-week-old fetuses in our study was a male and it had a much larger tongue volume in comparison to the other female samples in the same age group as well as larger than the 19-week-old female sample. Such variation could not be justified by gender but might correlate with his longer crown-heel length (CHL) rather than the crown-rump length (CRL) in his age group.

It is possible that formalin and PTA staining may have a minor shrinkage effect mostly on soft tissue that was not accounted for. Comparing measurements from two different sources of scans would be quite challenging as regards to correlating contrast-enhanced high-resolution micro-CT outputs to ultrasound images.

Primarily, different types of ultrasound imaging techniques such as transvaginal, and transabdominal sonography were found to produce some discrepancy in linear measurements when compared to each other (113). Other factors such as the position of the fetus during imaging (113), shrinkage effects of fixatives, as well as contrast-enhancement stains such as formalin and PTA respectively, or any potential confounding variable are expected to have an impact on the values extracted from different sources. In a study that compared CT and ultrasound measurements, it has been found that there is considerable disagreement between them concerning the measurement of the aortic diameter in axial plane images from both sources (114), as well as substantial inter-observer variability (115).

4.9 Future directions

While we have focused on some oral structures in this study, our samples can provide other areas of investigation into craniofacial integration. For example, volumetric size changes of the ocular mass with the eye socket size in the early fetal stage can provide information about soft tissue-hard tissue integration. Similarly, we can study the growth relationships between the chondrocranium and the other, connected, craniofacial structures.

With regards to the tongue and its integration to the mandible and hyoid, another future investigative theme is to assess the cross-sectional areas of different intrinsic and extrinsic tongue muscles as well as suprahyoid muscles. Muscle cross-sectional area has been correlated to its functional capacity and hence, such analysis may prove informative with regards to functional maturation and its link to bone/cartilage growth.

Chapter 5: Conclusion

3D analysis of PTA contrast-enhanced micro-CT images of fetal head scans provides a robust, and reproducible methodology to study craniofacial structures. Our data indicate a strong positive correlation between the growth of soft and hard tissue components of the craniofacial structures, namely the mandible, tongue, and hyoid cartilage during the early fetal stage of human growth, indicating integration of these structures throughout the timepoints studied here. Comparing growth trajectories after standardization of data indicated that the tongue although, very slightly, grows earlier than the lower jaw; in the time frame studied here. We have detected a potential beginning of a growth spurt in the tongue, hyoid, and mandible between the end of the 13th to the 15th postconceptional week. Additionally, the volumetric data we have obtained may provide normative values with which to study and diagnose craniofacial abnormalities in utero.

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Appendix

Normality testing

For the data as a whole, skewness and kurtosis were normal (Table 1). Z (skew), and z (kurtosis) were calculated for each variable individually as shown in (Table 2). A normally distributed data refers to the number of samples that are closer to the mean rather than away from it. Z-scores that are calculated by dividing the skewness by its standard error and the kurtosis by its standard error should be within the range of +1.96 and -1.96 for the data to be judged as normally distributed (116). The standard errors for both skewness and kurtosis are dependent on the sample size as the error becomes smaller when the sample increases. Based on these z-test scores, our data appear to be parametric and normally distributed.

Normality assessment					
	Skewness		Kurtosis		
Total volume of the tongue in mm ³	Statistic	Std. Error	Statistic	Std. Error	
	.906	.564	043	1.091	
Total volume of the hyoid in mm ³	.515	.564	-1.336	1.091	
Total volume of the mandible in mm ³	.768	.564	593	1.091	

Table 1. Statistic skewness and its standard error, and statistic kurtosis and its standard error.

	Skewness z	Kurtosis z
Tongue	1.6	.03
Hyoid	.9	1.2
Mandible	1.3	.54

Table 2. Normality outcome: Normally distributed parametric data.

Regression analyses of the tongue, hyoid, and mandible without the outliers, show similar

growth curves as shown below.

