Type of Award: Orthodontic Faculty Development Fellowship Award

Name(s) of Principal Investigator(s): **Siddharth Vora**

Title of Project: MORPHOGENESIS DURING HUMAN FETAL CRANIOFACIAL GROWTH AND DEVELOPMENT

Period of AAOF Support: 07-01-18 to 06-30-19

Amount of Funding: 20,000

Abstract:
Growth, development of the human craniofacial region is multifaceted and challenging to study. Animal models have proved significant in this regard, yet they pose several limitations particularly when exploring topics from an ontogenetic perspective. The majority of existing human skull ontogeny studies have analyzed postnatal stages. However, it is recognized that ontogenetic divergence between humans and even closely related non-human primates occurs early, during fetal stages. The few studies that have evaluated prenatal stages, historically relied on the use of photos, 2D x-rays or measurements on dissected and disarticulated post-mortem skeletons, not preserving spatial relationships between bones. Indeed, there is a lack of high quality, comprehensive, 3D information on prenatal growth of the human skull, specifically in the second trimester, when adult head morphology is being established and when craniofacial anomalies can develop. This proposal aimed to investigate unexplored areas of 3D ontogeny of human craniofacial development during the fetal period, utilizing a large and well-preserved collection of human fetal heads at the University of British Columbia and the Congenital Anomalies Research Center at Kyoto University. High resolution micro-CT scan imaging techniques were employed and analyzed using geometric morphometric methods and deformation-based shape analysis methods. Growth trajectories of craniofacial bones regions and integration patterns between craniofacial regions were compared between the samples from different populations (Japan and north America). Comprehensive asymmetry analyses of the developing craniofacial skeleton was also assessed. Ongoing studies using contrast-enhancement methods that allow visualization of non-mineralized tissues in μCT scans, are evaluating the relationship between the soft and hard tissues of the oro-facial region, in particular the tongue, hyoid cartilage and mandible. The relationship between the developing primary dentition and its surrounding alveolar bone is also being assessed. Together, data collected from these specimens in enhancing our understanding of prenatal craniofacial morphogenesis.

Response to the following questions:
1.Were the original, specific aims of the proposal realized?
Educational: I completed a 5-day workshop on *Geometric Morphometrics and R*, hosted by Transmitting science, in Calgary AB. In addition, I attending courses/workshops aimed at training in grantsmanship and academic career development, hosted by the University of British Columbia's Support Programs to Advance Research Capacity.
Teaching: I co-directed the Craniofacial Growth and Development module for the Year 1 DMD students. For this, I re-developed several of the lectures, moving them from the ppt. format to a flash-based animated format which enables improved visual learning and online access for reference. I also continued my teaching of Year 3 DMD students, graduate clinical and didactic teaching as well as supervision of several MSc students. I also attended courses offered by the Center for Teaching, Learning and Technology at UBC to enhance my teaching skills.
Clinical Skills: I continue to practice 3-days/month at a Medicaid office in Bellingham, WA
Research: The research project aimed at investigating unexplored areas of 3D ontogeny of human craniofacial development during the fetal period, utilizing a large and well-preserved collection of human fetal heads. The project had two specific aims. Aim1focused on evaluating fetal craniofacial growth patterns through ontogeny while Aim2 focused on studying asymmetry patterns during early development. Results from our analysis are provided below. Not all of the aims of the research project have been completed. Specifically, we had planned to perform analysis of dental tooth germs during the fetal stage of human development by studying contrast-enhanced CT scans (parts of Aim 1 and 2). We are still in the process of completing these scans since they are highly technique sensitive and time consuming. However, we have scanned all the specimen (and more) using standard microCT and analyzed the data (presented below). Data from Aim 2 has been published (see attached). We anticipate two more publications from this study in the next calendar year.

Methods

Conceptuses without any distinct congenital anomalies and artificial deformities of the face were obtained from the British Columbia Women's Hospital, Vancouver Canada (UBC, N=20). In addition, we also obtained specimen from the Congenital Anomaly Research Center at Kyoto University (KU, N=22). Their use was approved by the Ethics Committee at Kyoto University Graduate School and Faculty of Medicine, Kyoto, Japan (R0316, R0347, and R0989) and University of British Columbia, Vancouver, Canada (H08-02576). All specimens were using a combination of foot length and crown-rump length (CRL). The estimations were made by the pathologist at the time of termination and were between 12-19 weeks gestational age (i.e. ~ 99-198mm CRL). Specimens have been fixed in formalin upon collection, preserving soft and hard tissue. Historical comparisons between fresh fetuses and formalin-fixed fetuses have shown a very slight but uniform distortion. Specimen were imaged using TOSCANER-30000 μFD-Z Ⅱ (Toshiba, Tokyo Japan) or the Scanco Medical μCT100 (Scanco, Switzerland) at 38-50 μm resolution.

Threshold based semi-automated segmentation was accomplished in Amira®6.0 using the Magic Wand and Paintbrush Tools by selecting voxels with an intensity exceeding a specified minimum masking value (**Figure 1**). Optimal masking values (between 3500-4500) for each bone were chosen and kept constant between the left and right antimeres bone pairs (**Figure 2**). Subsequently, surface meshes were generated for the segmented bones and utilized for volumetric analysis and landmark annotation. In addition, individual mandibular tooth crypts (pseudo teeth) were segmented by tracing the bone margins on slices followed by volume measurement.

Manual landmarks were annotated on the 3D surface files of segmented cranioskeleton for each specimen using CheckpointTM (Stratovan Corporation, Davis, CA, USA) using a combination of the multiplanar (boundary) and 3D rendering views (**Figure 3**). In addition, thirty-nine landmarks were chosen for the 5 teeth in each mandibular quadrant (9 per molar and 7 per incisor/canine crypt). Landmarks on the left side of the arch were reflected resulting in 50 teeth for each morphotype (**Figure 4** A,B). Repeat landmarks were placed on all the specimen by the same investigator 1 week apart. Intra-investigator error was calculating by measuring the Euclidean distance between the Cartesian coordinates from the original and repeated landmarking attempts for all specimen. Error values were low indicating good reliability for landmark recognition. Volume data was extracted from Amira®6.0 and analyzed in Microsoft Excel. One-way ANOVA was performed using XL-STAT™.

We assessed growth of individual bones (example of mandible provided here; **Figure 6b**) using deformation-based morphology analysis. Averaged 12-week-old and 19 week old right mandible meshes were first rigidly aligned to each other using the annotated landmarks followed by a thin plate spline (TPS) deformation and a closest-point deformation (CPD) to obtain point correspondences at each mesh node. For each point, a vector was defined that represented the transformation between the corresponding points and the magnitude of this vector was extracted and analyzed. A scaled color gradient was calculated between the maximum and minimum bounds of the magnitudes scores and applied to a template mandible mesh to visualize the local growth changes between 12-19 weeks.

Geometric morphometrics (GM) was employed to evaluate and compare growth trajectories of the midface and mandibular regions of the fetal craniofacial skeletons between the UBC and KU specimen. All configurations were superimposed using generalized Procrustes analysis (GPA) and then Principal component (PC) analysis was performed. Group differences were evaluated using a multivariate analysis of variance (MANOVA), performed on PC 1–19 of the midface and the mandible, respectively. To visualize the difference, linear discriminant analysis (LDA) was performed. The allometric shape (AS) vectors for each population were calculated from the multivariate regression of PCs on the CRL to analyze growth trajectories. All GM analyses and visualization was performed using the Geomorph package in R 3.4.1 and MATLAB 9.0.1 (Mathworks, Natick, MA, USA).

Figure 1: Visual depiction of segmentation

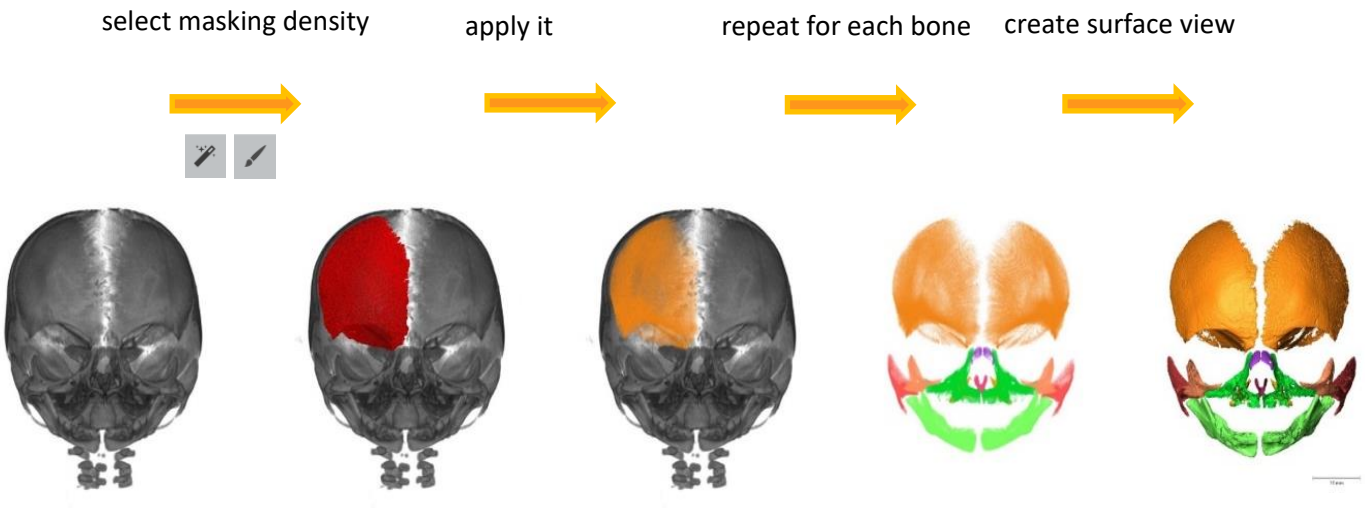


Figure 2: Segmented maxilla, palatal bone, vomer bones of 12 and 19wk fetuses

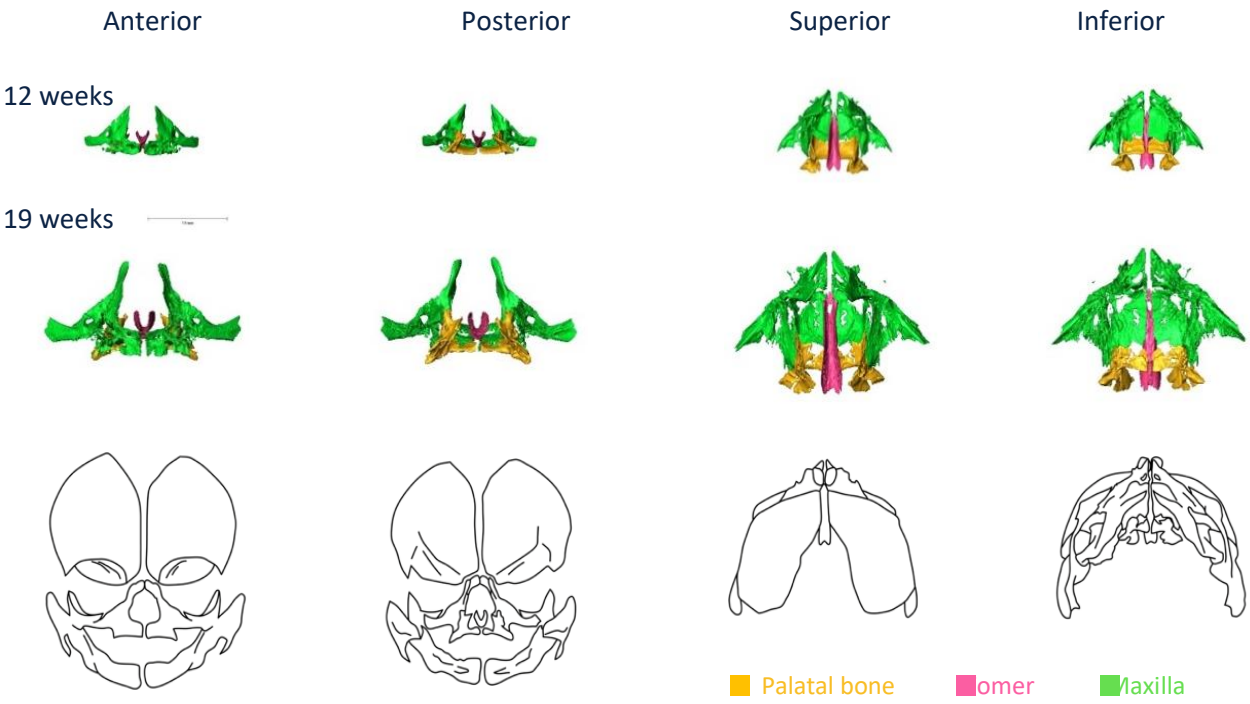
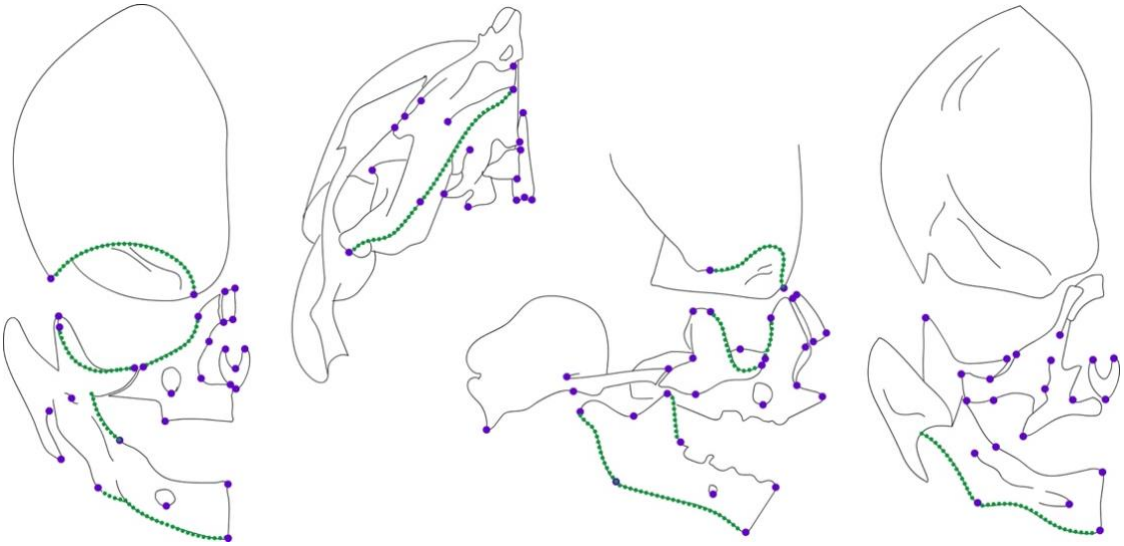


Figure 3: Schema of landmarks (purple) and semi-landmarks (green) used for morphometric.



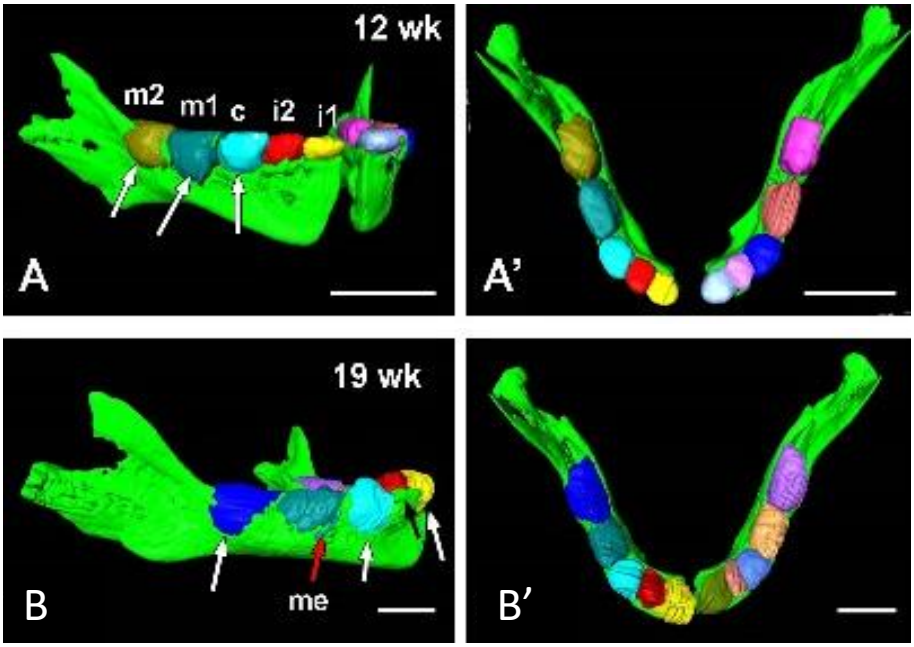
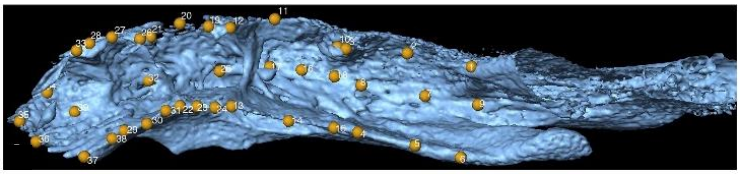


Figure 4. Lateral (A, B) and occlusal (A', B') views of an isosurface of a 12 and 19 week hemi-mandible segmented manually from the skull shown together with segmented pseudo teeth. The absence of buccal and occlusal bone is striking at 12 weeks (white arrows) adjacent to the c, m1. From 13-19 weeks apposition of bone adjacent to m1 is creating the mental foramen and the bony prominence. Occlusal (C) and lateral (D) views of an isosurface showing landmarked dental crypts (i1-m2). The deepest internal surface of the crypt was captured, as well as the buccal and lingual margins.

C



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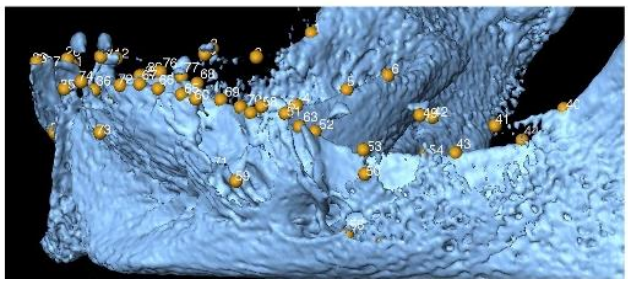


Results and Discussion:

Figure 5 depicts the volumetric growth data which reveals significant growth in the craniofacial bones during the 2nd trimester ($p<0.05$, ANOVA). Fold changes in volumetric growth are different for each bone. Within the timeframe studied here, the maxilla increased in volume 6.2-fold, the mandible 4.9-fold, the palatal 4.3-fold, and the zygomatic 8.9-fold, indicating that the individual bones have distinct growth trajectories from each other. Additionally, the bar graphs depict the grouping summary of extremes of pairwise comparisons for age (Fig 4, #=Tukey HSD post-hoc test). These groupings are not consistent between bones, and supports the notion that individual bone growth trajectories are unique. Notably, right and left bone pairs were analyzed independently and reveal remarkable symmetry.

We also assessed growth of the individual cranioskeletal bones using conventional morphometrics and deformation-based morphology (DBM) analyses. **Figure 6A** revels that most of the mandibular growth between these timepoints occurs in the antero-posterior dimension (**Figure 6A**, yellow arrow). Vertical growth can be noted in the ramus of the mandible (**Figure 6A**, green arrow), with minimal growth changes recorded in the transverse or vertical dimension in the body of the mandible. This suggests allometric as opposed to isometric growth and indicates that the shape of the mandible is dramatically changed during this growth phase. DBM analyses identified local areas of growth change in the mandible between 12 – 19 wks gestation (**Figure 6B,C**). The magnitude of growth at each mesh point between 12 and 19 wk specimen were applied as a scaled color gradient to visualize growth.

Our data indicate that craniofacial bones undergo significant volumetric growth in the 2nd trimester, and different craniofacial bones have unique growth trajectories. Additionally, during this period, most of the growth in the mandible occurs in the condyle and coronoid process, with AP and vertical growth predominating. Overall, paired craniofacial bones demonstrate considerable right-left symmetric volumetric development during the fetal growth period (See publication cited in **Section 2a** below), indicating intrinsic genetic control and good buffering of potential environmental developmental stresses.

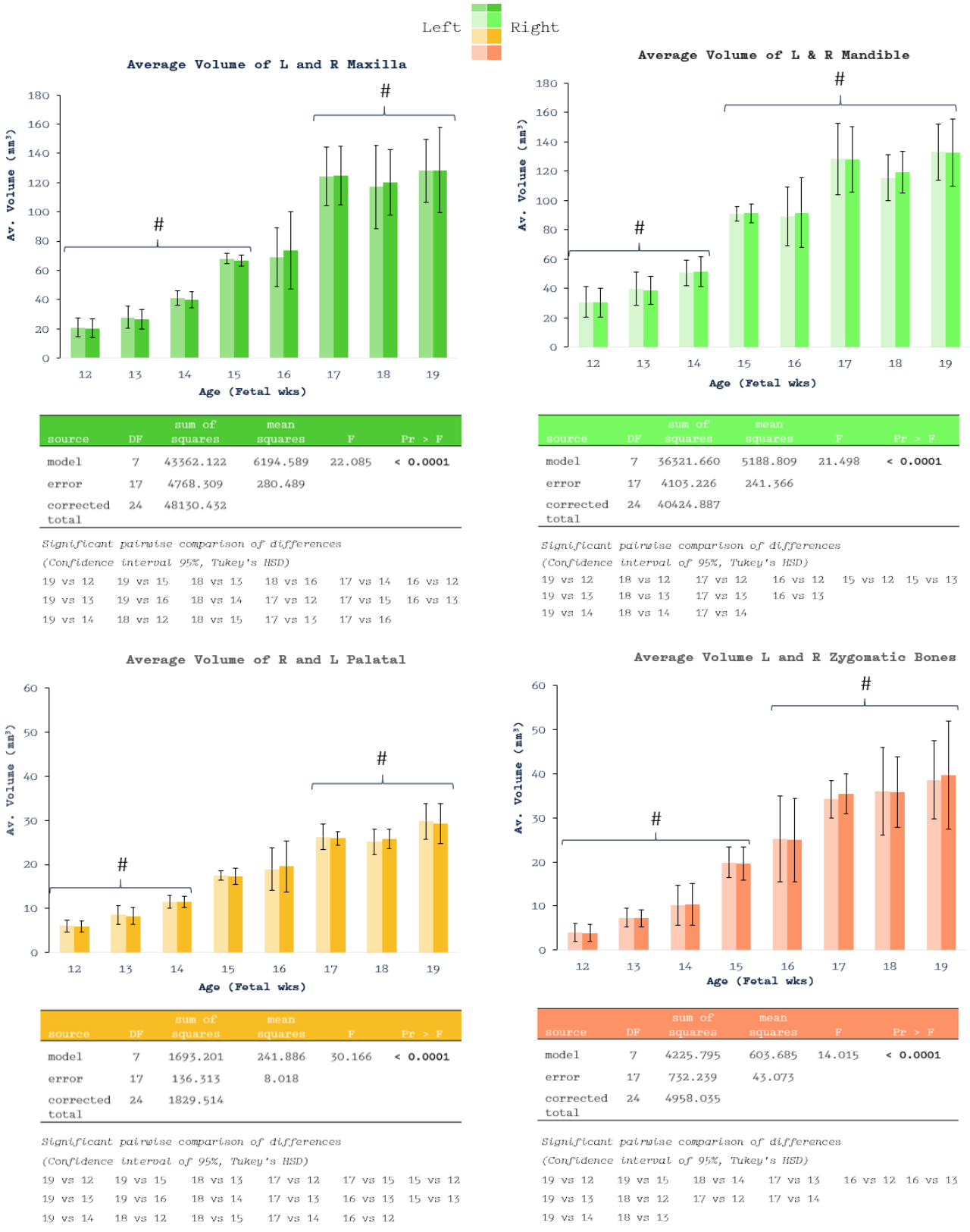
We further utilized GM analysis to evaluate growth of the fetal facial skeleton and assess population differences between the KU and UBC specimen using principle component analysis (**Figure 7**) and a multiple analysis of variance (MANOVA). We found that the mandible did not display a statistically significant difference in shape between the two populations, whereas the midface did. Linear discriminate analysis between the KU and UBC samples indicated that the shifted landmarks was most distinct in the orbital area (**Figure 7,** bottom panel). The growth trajectories of KU and UBC (**Figure 8**, plots) were slightly different. According to the AS vector, the orbital shape of KU fetuses became more round comparing with that of UBC (**Figure 8**, bottom panel).

Together, the GM analysis demonstrates that differences in facial skeletal shape and growth trajectories amongst ethnic populations occurs early during gestation, before the middle trimester. Anthropological studies have identified the orbital region of the face to be distinctly different amongst populations and the orbits of individuals of Asian descent are reported to be more rounded, which was confirmed in our study.

We also examined tooth development during the second trimester and tested the hypothesis that teeth lacking buccal bone (m1 and c) and teeth surrounded by bone (i1 and i2), differ in their growth allometry. **Figure 9** depicts the volumetric changes in individual dental crypts in the second trimester (see Figure 4). Volume measurements indicate two growth spurts with regards to mandibular and tooth crypt volume; one between 14-15 weeks and one between 16-17 weeks. In general the 12-14 week data clustered together as did the 17-19 week data. The intermediate period 15-16 weeks was not significantly different than the early or later timeframes. The allometric shape vector (ASV, blue arrow), indicates the size-related shape variation in the dental crypts (**Figure 10**). This vector is calculated from the multivariate regression (ordinary least-squares [OLS]) of all PCs on the mandibular volume. When all the dental crypts are combined in the analysis, allometric growth can be observed (**Figure 11**, middle wireframes). However, when a paired analysis is performed for the teeth which are encased in buccal bone (i1 and i2), the occlusal view shows minimal shape change (**Figure 11**, top and bottom wireframes). Landmarks adjacent to m1 and c show significant bucco-lingual expansion, with the lack of constraint by bone on those surfaces. Allometric growth in the occlusal gingival direction was also observed with increased depth to the crypts for all teeth over the developmental period (**Figure 11**). This is consistent with the presence of greater resorptive cells on the internal surface of the crypts as reported in literature.

These data reveal that teeth surrounded by bone buccolingually (i1 and i2) show maximal shape change in the apical direction with almost isometric growth when viewed occlusal. By contrast, teeth with buccal fenestra (m1 and c) display dramatic allometric growth in the bucco-lingual dimension during the second trimester of gestation. Ongoing work using contrast-enhancement prior to CT scanning will enable us to study the developing tooth germ (soft tissue) and compare the growth of individual enamel organs to their encasing bony crypts and provide insight into the regulation of the tooth-bone interface (TBI).

Figure 5: Graphs represent average volume of the paired left-right craniofacial bones of 12-19 week old fetuses from the UBC sample. One-way ANOVA was performed with averaged R/L volume for each specimen (below graph) indicating significant difference. Pairwise comparisons which show significant ($p<0.05$) are listed below table. # Denotes grouping summary of extremes of pairwise comparisons for Age (using Tukey HSD post-hoc test)



source	DF	sum of squares	mean squares	F	Pr > F
model	7	43362.122	6194.589	22.085	< 0.0001
error	17	4768.309	280.489		
corrected total	24	48130.432			

Significant pairwise comparison of differences
(Confidence interval 95%, Tukey's HSD)

19 vs 12	19 vs 15	18 vs 13	18 vs 16	17 vs 14	16 vs 12
19 vs 13	19 vs 16	18 vs 14	17 vs 12	17 vs 15	16 vs 13
19 vs 14	18 vs 12	18 vs 15	17 vs 13	17 vs 16	

source	DF	sum of squares	mean squares	F	Pr > F
model	7	36321.660	5188.809	21.498	< 0.0001
error	17	4103.226	241.366		
corrected total	24	40424.887			

Significant pairwise comparison of differences
(Confidence interval of 95%, Tukey's HSD)

19 vs 12	18 vs 12	17 vs 12	16 vs 12	15 vs 12	15 vs 13
19 vs 13	18 vs 13	17 vs 13	16 vs 13		
19 vs 14	18 vs 14	17 vs 14			

source	DF	sum of squares	mean squares	F	Pr > F
model	7	1693.201	241.886	30.166	< 0.0001
error	17	136.313	8.018		
corrected total	24	1829.514			

Significant pairwise comparison of differences
(Confidence interval of 95%, Tukey's HSD)

19 vs 12	19 vs 15	18 vs 13	17 vs 12	17 vs 15	15 vs 12
19 vs 13	19 vs 16	18 vs 14	17 vs 13	16 vs 13	15 vs 13
19 vs 14	18 vs 12	18 vs 15	17 vs 14	16 vs 12	

source	DF	sum of squares	mean squares	F	Pr > F
model	7	4225.795	603.685	14.015	< 0.0001
error	17	732.239	43.073		
corrected total	24	4958.035			

Significant pairwise comparison of differences
(Confidence interval of 95%, Tukey's HSD)

19 vs 12	19 vs 15	18 vs 14	17 vs 13	16 vs 12	16 vs 13
19 vs 13	18 vs 12	17 vs 12	17 vs 14		
19 vs 14	18 vs 13				

Figure 6. (A) Changes in anteroposterior (yellow arrow and bars) and vertical (green arrow and bars) dimensions of the mandible from age 12-19wks gestation. One-way ANOVA performed with averaged R/L lengths (# denotes grouping summary of extremes of pairwise comparisons for age using Tukey HSD post-hoc test). **(B)** Overlay of representative 12 week (red) and 19 week (blue) mandible. (**C**) Heatmaps depict magnitude of growth for each point on the template mesh from a lateral, superior and posterior view. Areas in red show the highest magnitude of growth while areas in blue show the least magnitude of growth (scale bar)

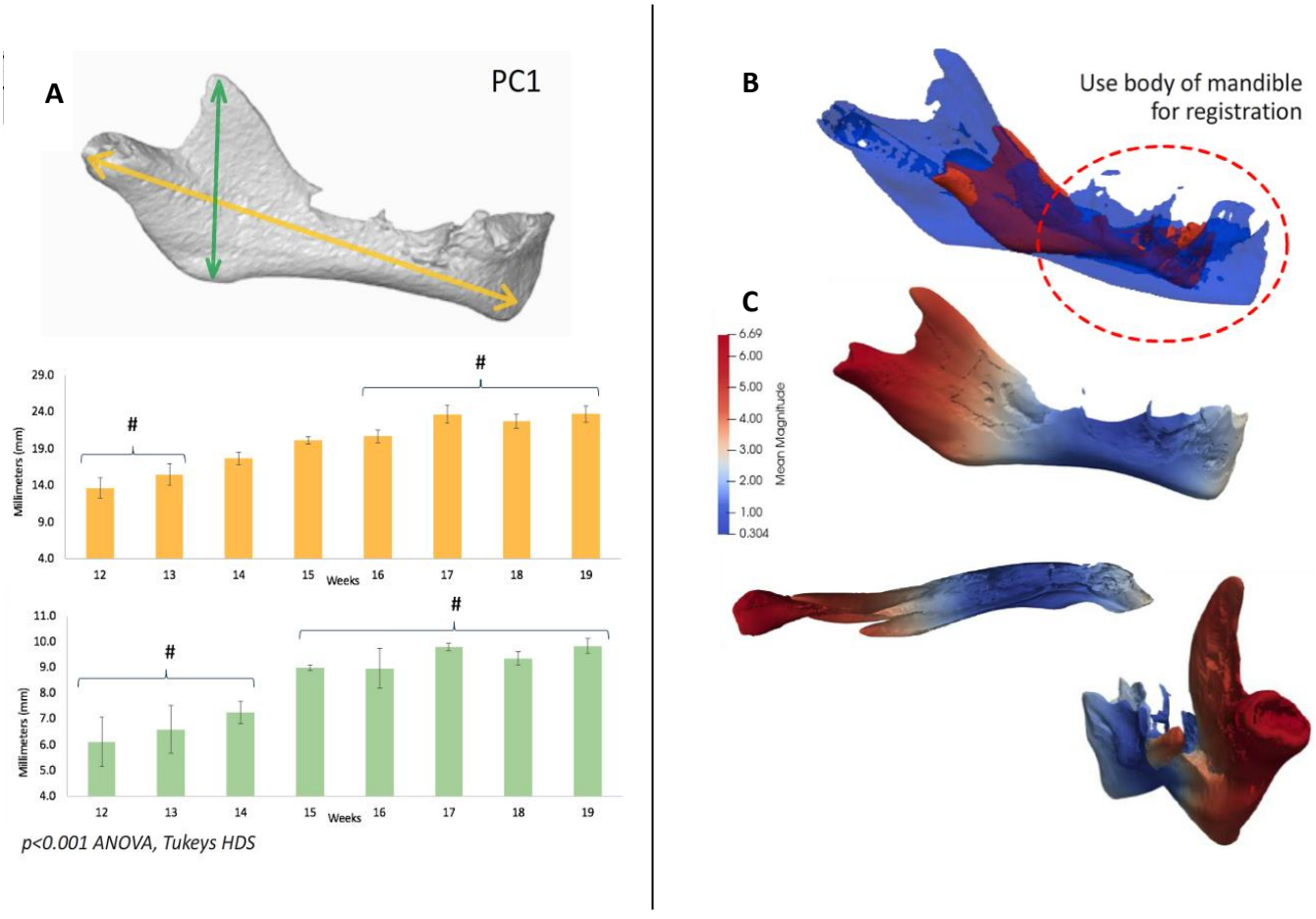
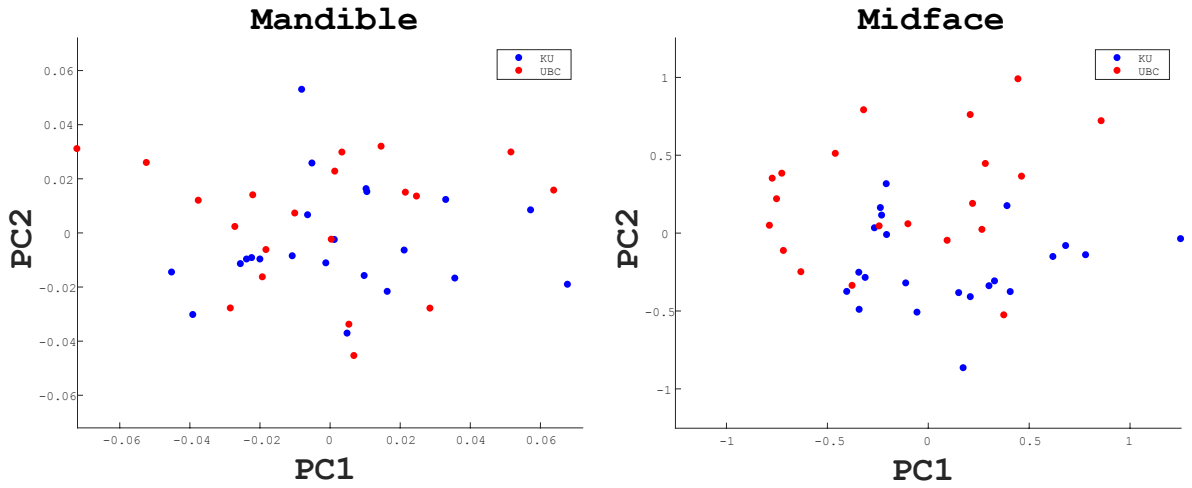


Figure 7: Scatter plots of principal component (PC) analysis of the mandible (left) and the midface (right). Specimens of Kyoto University (KU) and University of British Columbia (UBC) were colored in blue and red, respectively. Table shows the results of the MANOVA on PC 1–19. Bottom diagrams show that the landmarks were shifted along the linear discriminate axis. Landmarks of Kyoto University (KU) and University of British Columbia (UBC) were colored in blue and red, respectively. From *left* to *right*, anterior, inferior, lateral and top views.



	Wilks' Lambda	Approximate F Value	p Value
Mandible	0.4005	1.73	0.11
Midface	0.0817	13.01	6.39e-8

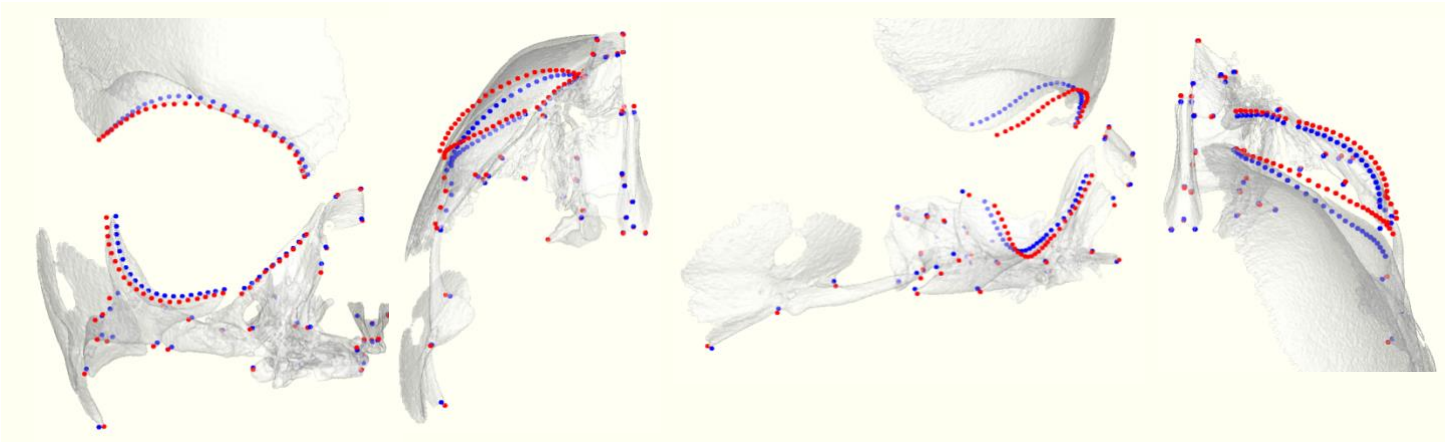
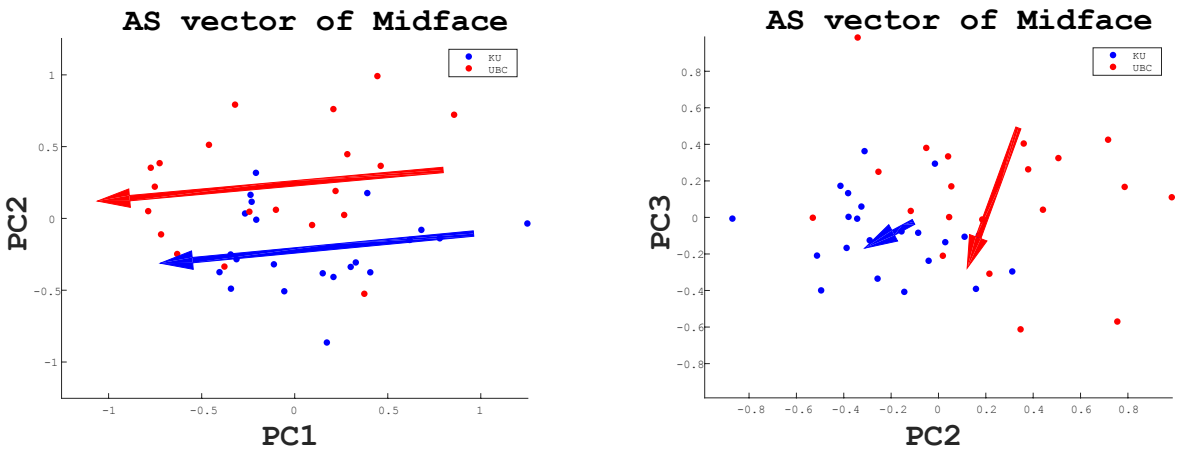


Figure 8 shows the allometric shape (AS) vectors for PC 1 v/s 2 (left) and PC 2 v/s 3 (right) for the midfacial cranioskeletal landmarks of specimens from the Kyoto University (KU, blue) and University of British Columbia (UBC, red) collections. Bottom diagram shows the shift of landmarks along the AS vector for the Kyoto University (KU, blue) and University of British Columbia (UBC, red) specimen (Clockwise from top left anterior, inferior, top and lateral views).



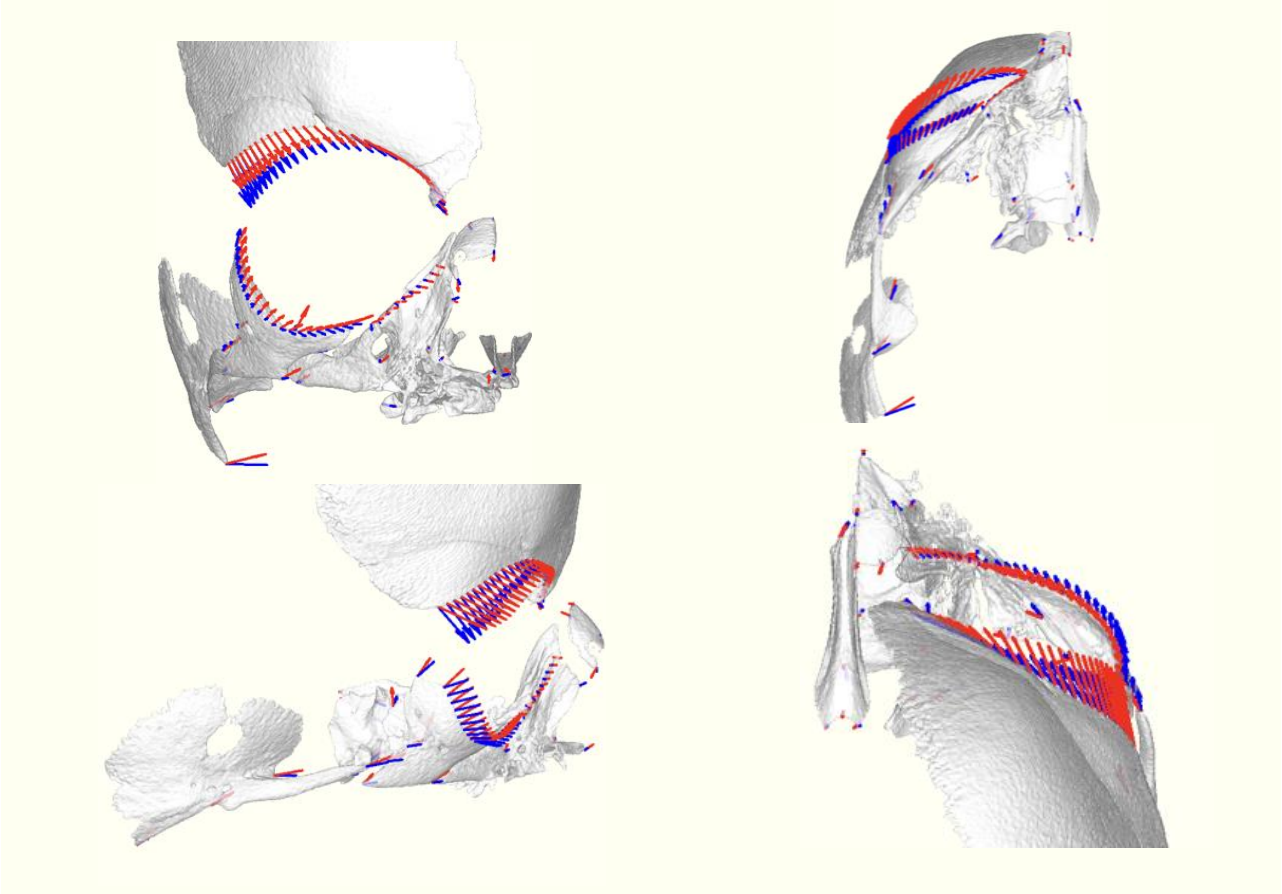


Figure 9. Volumetric measurements of the segmented mandible and pseudo teeth. Values were compared using ANOVA and Tukey's post-hoc testing.

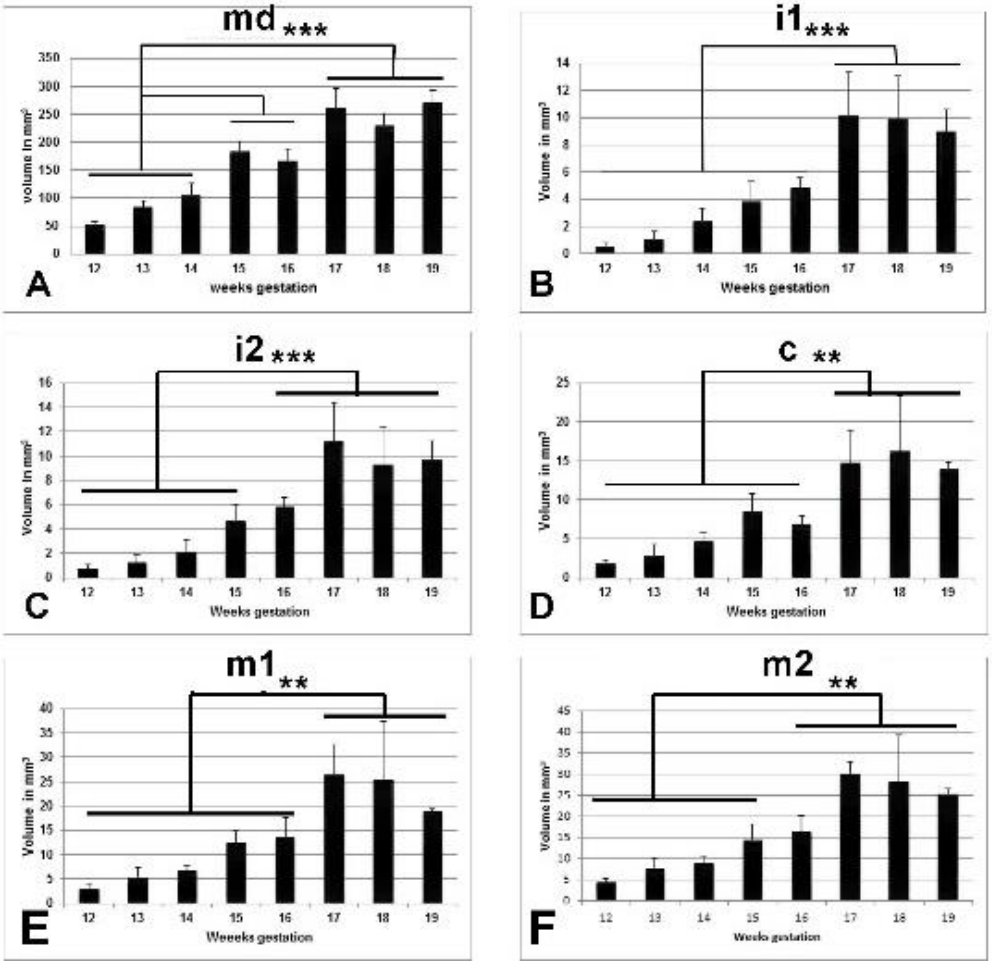
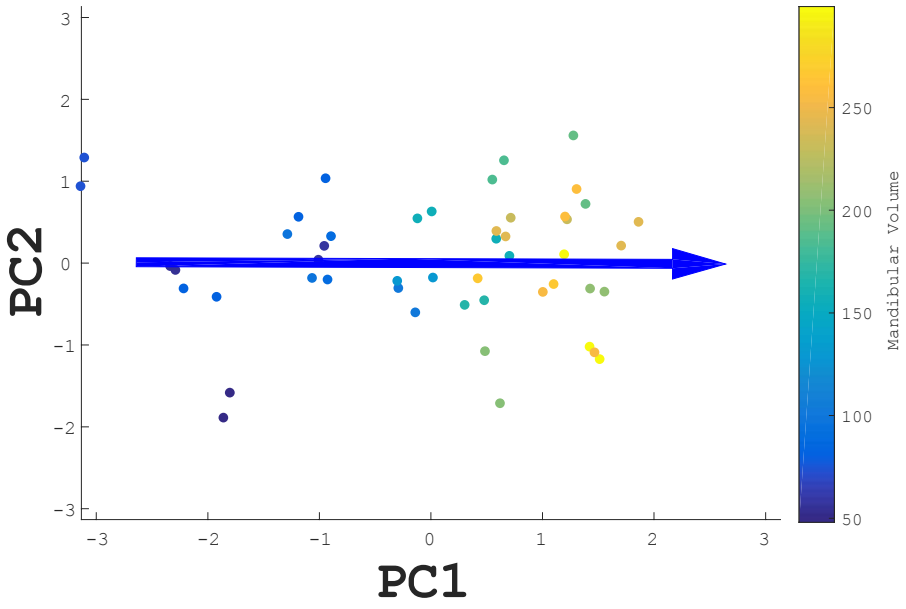


Figure 10. Plot of PC1 vs PC2 (-2 S.D. to +2 S.D.) for all crypt landmarks analyzed together. Individual specimen are depicted as colored dots based on mandibular volume (color bar on right). As is evident, PC1 captures growth allometry.



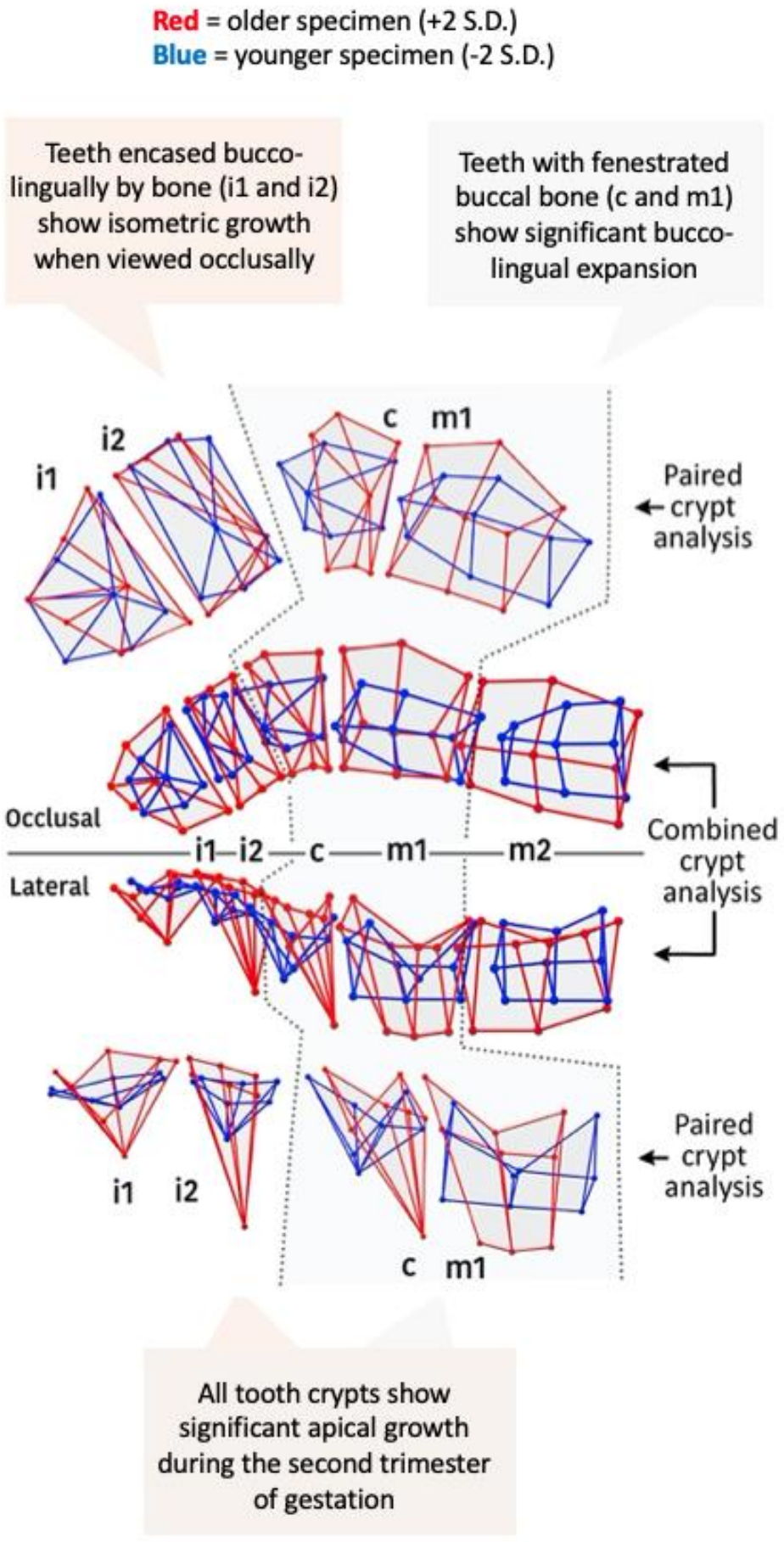


Figure 11: Wireframes based on the ASV depict landmark shifts with growth from 12 – 19 weeks - within the second trimester (Red = older specimen (+2 S.D.), Blue = younger specimen (-2 S.D.); top= occlusal view, bottom = lateral view).

2.Were the results published? **Partly**

a. If so, cite reference/s for publication/s including titles, dates, author or co-authors, journal, issue and page numbers:

- **Analysis of facial skeletal asymmetry during foetal development using µCT imaging. *Katsube M, Rolfe SM, Bortolussi SR, Yamaguchi Y, Richman JM, Yamada S, Vora SR*. Orthodontics and Craniofacial Research, 2019 May 22 Suppl 1:199-206. doi: 10.1111/ocr.12304.**

b. Was AAOF support acknowledged?
Yes

c. If not, are there plans to publish? If not, why not?
We anticipate two more publications from this work in the next calendar year.

3.Have the results of this proposal been presented? **Yes**

a. If so, list titles, author or co-authors of these presentation/s, year and locations

- **3D Imaging. Analysis. A New View into Human Craniofacial Development. *Vora S*, Katsube M, Bortolussi S, Yamada S, Richman J*. Sept 2018. Consortium for Orthodontic Advances in Science and Technology, Scottsdale, AZ.**
- **Comparative Study of Fetal Facial Shape Between Japanese and Canadian Populations. *Katsube M, Vora S*, Bortolussi S, Yamaguchi Y, Richman J, Diewert V, Yamada S*. March 2019. The 124th Annual Meeting of the Japanese Association of Anatomists, Niigata, Japan.**
- **Landmark-based 3D Morphometrics of Human Dental Crypts During the Middle Trimester. *Pham D, Katsube M, Korada A, Diewert V, Vora S* & Richman J*. June 2019, International Association of Dental Research, Vancouver, BC Canada.**

b. Was AAOF support acknowledged?
Yes

c. If not, are there plans to do so? If not, why not?
N/A

4.To what extent have you used, or how do you intend to use, AAOF funding to further your career?

Funding from the AAOF over the past many years has enabled me to pursue an academic career. In a financially challenging research environment, the AAOF awards have afforded me the ability to follow an organized period of research development during my post-doc, and is supporting me in my initial years as a junior faculty member at UBC. In this regard, these awards from the AAOF are not only encouraging but more importantly, empowering. This support will be crucial to my future goals of obtaining independent research funding. I thank the foundation, the PARC committee and all of its champions and hope to continue a positive relationship with this organization.