ΔΙΠΛΩΜΑΤΙΚΗ ΕΡΓΑΣΙΑ

ΣΥΣΧΕΤΙΣΗ ΤΟΥ ΣΧΗΜΑΤΟΣ ΤΟΥ ΚΡΑΝΙΟΠΡΟΣΩΠΙΚΟΥ ΣΥΜΠΛΕΓΜΑΤΟΣ ΜΕ ΤΟ ΛΟΓΟ ΤΟΥ ΜΗΚΟΥΣ ΤΩΝ ΔΑΚΤΥΛΩΝ (2Δ:4Δ) ΣΕ ΠΑΙΔΙΑ ΠΡΟΕΦΗΒΙΚΗΣ ΗΛΙΚΙΑΣ

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ΕΠΙΒΛΕΠΩΝ ΚΑΘΗΓΗΤΗΣ: Δημήτριος Χαλαζωνίτης

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CORRELATION OF 2nd to 4th DIGIT RATIO (2D:4D) AND CRANIOFACIAL SHAPE IN PREPUBERTAL CHILDREN

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... στη μνήμη της μητέρας μου

... στο Φίλιππο

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ΠΕΡΙΛΗΨΗ

Ο λόγος 2Δ:4Δ αναφέρεται ότι αποτελεί ένδειξη των επιπέδων των ανδρογόνων κατά την εμβρυϊκή περίοδο, εμφανίζοντας πιθανώς συμμεταβολή με το σχήμα του κρανιοπροσωπικού συμπλέγματος. Σε αυτήν την εργασία τη συσχέτιση αυτή σε 58 αγόρια και 59 κορίτσια προεφηβικής ηλικίας χρησιμοποιώντας την μέθοδο της γεωμετρικής μορφομετρίας. Τα δάκτυλα μετρήθηκαν με την χρήση ηλεκτρονικού υπολογιστή με μέθοδο που απαιτούσε την ιχνογράφηση του περιγράμματος του δακτύλου από τον ερευνητή, ενώ ο προσδιορισμός του επιμήκη άξονα και ο υπολογισμός του μήκους έγινε αυτόματα από τον υπολογιστή. Η μέθοδος αυτή μείωσε το σφάλμα μέτρησης στο ένα τρίτο σε σχέση με προηγούμενες δημοσιευμένες εργασίες. Για τον υπολογισμό του σχήματος του κρανιοπροσωπικού ιχνογραφήθηκαν 15 σκελετικά σημεία σε πλάγιες κεφαλομετρικές ακτινογραφίες στις οποίες στη συνέχεια έγινε αλληλεπίθεση Προκρούστη και ανάλυση κυρίων παραγόντων. Για τη συσχέτιση του λόγου με το μέγεθος και το σχήμα του κρανιοπροσωπικού χρησιμοποιήθηκε η μέθοδος της πολυπαραγοντικής παλινδρόμησης. Ο λόγος 2Δ/4Δ στα αγόρια βρέθηκε μικρότερος από αυτό των κοριτσιών (Cohen's d: 0.275 για το αριστερό χέρι, 0.126 για το δεξί χέρι), η διαφορά όμως δεν ήταν στατιστικά σημαντική. Κατά την αξιολόγηση του σχήματος του κρανιοπροσωπικού μετά την αφαίρεση της επίδρασης του μεγέθους, δε διαπιστώθηκε διμορφισμός του φύλου, ενώ στα αγόρια το μέγεθος του κρανιοπροσωπικού βρέθηκε μεγαλύτερο σε σχέση με τα κορίτσια. Δε βρέθηκε συσχέτιση μεταξύ του λόγου των δακτύλων και του κρανιοπροσωπικού στο δείγμα συνολικά, αλλά ούτε και σε κάθε φύλο ξεχωριστά.

Λέξεις-Κλειδιά: γεωμετρική μορφομετρία, συμμεταβολή, κεφαλομετρική ακτινογραφία,

ηλεκτρονική μέτρηση μήκους

ABSTRACT

The 2D:4D digit ratio may reflect fetal sex-hormone levels, potentially showing covariation with craniofacial skeleton shape. We studied this correlation in a group of 58 male and 59 female prepubertal children, aged 7 to 12 years, using geometric morphometric analysis. Fingers were measured with a computer-assisted procedure that involved tracing the finger outline by the user, and subsequent automatic calculation of the finger long axis and length by the computer. This method reduced errors of measurement to one third or less of previously reported values. Craniofacial shape was evaluated using 15 skeletal landmarks on lateral cephalometric radiographs. Procrustes alignment and principal components analysis was applied to the craniofacial landmarks and multivariate regression between digit ratios and craniofacial shape was computed in shape space and form space. The male 2D:4D ratio was smaller than the female ratio (Cohen's d: 0.275 left hand, 0.126 right hand), but the difference was not statistically significant. Craniofacial shape did not show sexual dimorphism, but males were larger than females. No correlation was found between digit ratio and craniofacial shape, either for the whole sample or for any of the two sex groups.

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REVIEW OF THE LITERATURE

150 years finger length research

The comparative length of the second digit (2D or index finger) to the fourth digit (4D or ring finger) has received attention for over 150 years. In the article "Anthropological notes on the human hand", Frank Baker [1] described not only the myths and the superstitions about hand and finger length but also all the scientific conclusions until 1888. He presented the second finger length as "a reversive character", a trait common to biologically lower forms. He noticed that the index finger was usually shorter than the ring finger, as in the anthropoid apes, sometimes equals it and rarely exceeded it. In this article he referred to Ecker's findings who was the first in 1875 who pointed out that there are three formulas of relative index and ring finger length and presented the unusual longer index finger as a "progressive character" more frequent in women with a high type of mind.

Many researchers measured the relative finger length of index and ring finger (in relation to the middle finger) in human hand as well as many others just looked at the difference in fingertip extent. Relative finger length was measured (in mm) from tip of finger to skin in gap between fingers and absolute length from metacarpal-phalangeal joint to fingertip. Some measurements have also been made directly on finger bones [2].

In 1892, Pfitzner [3] in a morphological study on human skeletons (115 males, 462 females), trying to explain the anatomical basis of the digit extent difference, found that the absolute length of the index digit (2D) is to some degree smaller than the ring digit (4D) but the index ray (metacarpal and digit bones) may be shorter, equal or longer than the ring ray. His conclusion was that the hand type is determined by the relative finger ray lengths and not by the relative digit lengths. In 1920, Wooden–Jones [4] in his special book for hand anatomy, termed the "digital formula" which expressed the relative projection of the tips of the digits and not the relative finger lengths and he proposed three different types (3 > 4 > 2, 3 > 4 = 2 and 3> 2 > 4). In all cases, the middle finger (3rd digit) was the longest whereas the formula with the index finger (2nd digit) shorter than the ring finger (4th digit) seemed to be a distinctly simian trait. He also dealt with the "metacarpal formula" which was obvious in a clenched human fist, examining which knuckle projected more. He noted three different types of metacarpal formula (3 > 2 = 4, 3 > 2 > 4 and 2 > 3 > 4) where the primitive type was the 3 > 2 = 4 and the most common type in man was the prominence of the index knuckle (2 > 3 > 4). The most notable finding was that the metacarpal formula 2 > 3 > 4 can be combined with a 3 > 4 > 2 digit formula which meant that the elongation of the index metacarpal did not necessarily create a longer index finger as a whole.

For more than a century, the relative finger length and the three different hand types with the index finger equal, shorter or longer than ring finger was an anthropological and anatomical aspect.

Digit Ratio: The new period of finger length measurement

In 1998, a psychologist, Professor Manning from the University of Liverpool and his colleagues [5] introduced a new way of measuring the absolute finger length on palmar skin from the middle of the ventrally located most proximal metacarpophalangeal flexion

crease that divides the finger from the palm region to fingertip, the most prominent point in the contour of the finger. They proposed the ratio of the second to fourth digit length (2D:4D) as a biomarker of prenatal testosterone, responsible for sex differentiation as they found in their research that the ratio 2D:4D was sexually dimorphic: it was lower in adult men and negatively related (only for the right hand) with testosterone concentrations, while in adult females the ratio was higher and positively correlated with estrogen concentrations.

Since then, more than 500 studies (conducted mainly at psychology departments) have been published in biosciences with the number of publications growing faster than linear where Manning and his colleagues, in the early of 2009, accounted for about a quarter of them [6].

2D:4D and methods of measurement

In 2010, a meta-analysis [7] confirmed sex difference in 2D:4D ratio concluding that females have larger ratio than males in both hands but the right hand reveals larger sex differences which also depends on the method used for the finger length measurement. Many different methods were used such as direct measurement on the ventral surface of the palm with calipers and indirect measurement on photocopies, scanned images, digital photographs or radiographs with ruler, caliper or computer assisted analysis which is claimed to be the most accurate [8]. Inconsistency there is also on the conclusions that direct measurements lead to higher ratios than indirect measurements of finger length [8,9,10] as many researchers failed to replicate these findings [11,12]. Radiographs only for the left hand produced homogeneous results with small sex difference in 2D:4D ratio and a size effect $d = 0.27$ whereas direct and indirect methods produced heterogeneous results with higher effect size (direct methods: right hand $d=0.35$ and left hand $d = 0.28$, indirect methods: d was 0.13 higher in both hands) [7].

2D:4D as a sexual dimorphic trait

The "organizational hypothesis" in behavioral endocrinology that exposure to androgens such as testosterone (T) during fetus development permanently masculinizes the brain and behavior [13], turned the researchers to look for links between 2D:4D ratio and a great number of sex related traits ranged from sexual orientation [14,15,16,17] and psychological disorders such as autism [18], depression [19], neuroticism and psychoticism [20] to diseases like heart disease [21], breast and prostate cancer [22,23] and sporting abilities [24,25,26] as well as musical ability [27], fertility [28,29,5] spatial ability [30,32] and behavioral characteristics like aggression [33,34] success in financial and social risk-taking [35] and a great number of other phenotypic traits.

As in most cases the results were controversial and attempts of many authors to replicate significant results failed in a great number of trials [36], the digit ratio 2D:4D remains the most popular, still debated prenatal androgen biomarker [37,38,39] without any clinical application.

In the majority of introductions, 2D:4D is presented being established as early as prenatal stages or by the second year of childhood with a reference to findings of Manning et al. (1998) [5].

Some years later than 1998, Manning [40] with another scientific team, in a longitudinal study, found that in a four year mean time, children's 2D:4D ratio increased with growth and the direction of change was similar in males and females. In the first measurement in 1998, statistical significant sex differences were noted in children aged from 7 to 11 years which were not replicated in measurements in the same sample four years later (children aged from 11 to 17), leading to confounding conclusions. The ratio 2D:3D was also found to increase with age, in contrast to the 3D:4D ratio which showed no appreciable change. They concluded that as 2D:4D is age dependent, ratio comparisons should take age into

account. This last conclusion seemed not to have become so widely known, while the results that the ratio 3D:4D remained stable over time and the ratio 2D:3D was more sexually dimorphic, were dealt as of no importance. McIntyre et al. [41] concluded that the ratio 3D:4D may be a better descriptor of childhood sex differences, even if it is less sex dimorphic in adults because it is less variable than 2D:4D especially between different ethnicities [42]. From all the above, it could be supposed that second finger lengthens more relatively to third and fourth finger causing age dependent increase in digit ratios, in growing hands. This was the conclusion of Galis et al. [43] in a research of 169 male and 158 female fetuses who found that 2D:4D ratio sexual dimorphism was present by the 14th week post gestation whereas the ratio increased in both sexes and slightly more in females. As a result, the index finger seemed to grow more and faster relatively to median finger, especially in females, indicating that the final 2D:4D ratio was defined by procedures not only during intrauterine life but also after birth. Many years ago, in 1926, Schultz [44] made mention of the same conclusions reporting that the differences in index finger length formula could be found as early as in the third month of intrauterine life. He suggested that the long index finger was progressive because it was rare in the first weeks of gestation and approached the adult frequency at the end of fetal life and after birth when just little if any change happens in the digit formula. Peters at al. [2] also claimed that the index finger made the difference between sexes, presenting that the sex difference in finger extent derived largely from sex differences in index finger extent where in females the index finger tip extended more than ring finger relatively to middle one. In a meta-analysis, right hand showed greater sex differences in 2D:4D ratio [7].

2D:4D and allometry

 In contrast to age-dependent increase in the 2D:4D ratio in the longitudinal study, Manning [45] observed age-dependent decrease in the big self-measured BBC internet cross-sectional study where in a sample of 12-20 year-old persons there was evidence for allometry. This is in agreement with Kratochvil and Flegr [46] findings who first introduced the aspect of allometry in the use of digit ratio concluding that the 2D:4D is influenced by the different finger lengths and necessarily tended to decrease as the finger length increased. He suggested the use of full factorial ANCOVA for the examining of covariance of the length of 2D and 4D and not ANOVA or t-tests which investigate the effect of a single factor on the digit ratio.

 The "ratio method" has been criticized for more than 60 years in biosciences, because it does not take into account the non zero y intercept, introduces mathematical bias and spurious results, inserting the effect of different size [46,47,48,49]. In ratios, the random measurement error is multiplied in contrast with averages where they are mutually cancelled out, making ratios much less precisely measurable [50].

Digit Ratio and Heredity

 In 1952 Phoelps [51] tried to find if the digit formula was hereditary, conducting families investigation and population analysis and he concluded that the phenotype occurrence between parents and children was in agreement with the theory of sex influence inheritance where the differences in formula were due to variations in the length of the index finger. The different formulas were independent of metacarpal or ray length and could be observed as early as in seventh week of intrauterine life when hormones could not regulate the index finger length as fetal gonads were still in the process of sex

differentiation. Garn et al. [52] showed that by the seventh intrauterine week "adult" metacarpal-phalangeal length rankings were attained and by the thirteenth week the bone to bone analogy was "near" to adult. At the beginning, the hand parts presented a relative elongation which was followed by a relative reduction to adult proportions by the 90-104 mm Crown-Rump Length, after the thirteenth week. This reduction was stated also by Huizinga in 1924 [53] who concluded that at fourth month of fetus life all fetuses have a long index finger formula but during the late fetal period the frequency of the type 2 > 4 (formula, not ratio) decreased until maturity, and this decrease was more obvious in males than females.

Digit ratio and Distal Finger Extent

A semantic issue which arose during literature reading is the confusion between two different terms such as digit formula and digit ratio, which many times are confronted as synonymous but they aren't. For many years, the research interest was focused on anthropologists' and anatomists' observation that there were three different hand types based on the relation of the distal tip extent of the 2nd and the 4th finger in relation to the middle (3rd) finger. The first type was the 2 < 4 where the index finger extended less than the ring finger, the second type was the 2 = 4 where both fingers extend equally and the last type 2 > 4 where index extended more than the ring finger and appeared more often in women. It should be noted that the distal finger extent of one type e.g. 2 > 4 does not necessarily mean that the second finger is longer than fourth finger and it is not synonymous to the ratio 2D:4D. The placement of the distal tip of the finger is the result of a coordination of digit bones as well as metacarpal bones. What exactly do we want to measure with the ratio 2D:4D and which trait is the one we want to investigate?

It is clear that across the bibliography the difference is not so well-defined. The most representative example is the Fig. 1 (pg 16143) in Manning's paper [54] "Resolving the

role of prenatal sex steroids in the development of digit ratio", where under a "female" type palm is presented the respective "female" relation 2D > = 4D. After careful observation, we can easily estimate that the length of the index finger (2D) measured from the proximal palmar crease is smaller than the length of the ring finger (4D). In this case the digit ratio would be of "male type", < 1.However the equation of the distal finger extents confirms a more "female type". In 2008, Voracek et al. [55], trying to replicate the findings - of the unpublished throughout life research- of Hans-Dieter Rosler, a German psychologist with PhD thesis in psychology and anatomy, concluded that the correspondence between hand type (optical categorization) and 2D:4D ratio was very low and even in the group with the most "female-type" (index finger tip more distal than ring finger tip) hand there were some individuals with a "male-ratio" < 1 hand, while in the group with the most "male-type" hand (index finger tip more proximal than ring finger tip) there were some individuals with a "female-ratio" > 1. Significant sex differences were found with both methods but without a comparison between them.

In a study of Peters and al in 2002 [2], the only one who measured the distal finger extent as well as the digit ratio of absolute finger length in both hands, it was found that the distal extent of finger tips expressed more strongly the sexual dimorphism in finger measures than the length of fingers with the use of ratio. It was also suggested that the small effect size did not mean that the variable was unimportant but it could not be used as a clinical predictor

 In a trial [56] where digit bones as well as metacarpal bones were measured, the optical categorization of the hands in one of the three known types via radiographs (2 > 4, 2 = 4, 2 < 4) was in compliance with the results of the radiograph ratio measurements. The bone digit ratio 2D:4D as well as the metacarpal ratio 2D:4D was smaller in men than in women. Metacarpal bones ratio may be more useful as they are less sensible to injuries and osteoarthritis than digit bones.

Palmar creases development and skeletal relations

In 1998, Manning and his colleagues [5] used a new way of measuring the absolute finger length on the ventral surface of the palm, from the distal palmar crease to the distal finger tip with the comment that " this measurement is known to show high degree of repeatability" giving for reference two previous papers of his own.

The creases development is determined primarily by genetic factors and secondarily by developmental function. Early genetic and environmental factors may affect the developing flexion creases. The main crease lines are developed before any sign of active movement in early fetus life and before the eighth week of gestation when the hand is presented as a "digitate member" and may be modified by action of muscles on the developing joints [4,57,58].

As it is well known for many years, the distal palmar crease does not correspond to the metacarpophalagian joint. "None of these basal digital lines has the least pretension to marking the site of the metacarpophalangeal joints. The basal creases of the palm are situated almost three-quarters of an inch nearer to the tips of the fingers than the line of the joints" [4]. Such external measurement using palmar creases may be an imperfect indicator of skeletal finger length. Manning et al. [59] found a significant correlation of r = 0.47 for the left hand and r = 0.46 for the right hand between measurements of finger skeleton on radiographs and measurement on photocopies of ventral surfaces of palms. This statistical significance does not make the new method (ratio of 2D:4D) clinically sufficient and the two methods interchangeable. This kind of correlation is inappropriate for the study of the agreement between two different methods of measurement [60]. All the literature has been based on the findings of this research. But how reliable are these findings as the photocopies were made approximately 2.5 years after the X rays? How accurate are these findings when six years later, almost the same scientific team,

concluded for the same sample (Jamaican children) that the ratio increases with age? [40].

Sex hormones and digit development

It is well known that sex hormones play the most important role on bone and muscle development, greater than that of genetic or environmental factors. There is evidence that androgens have a direct effect on bone as suggested by the presence of Androgen Receptors (AR) in human osteoblast–like cells *in vitro* [61,62,63] and in normal developing human bone *in situ* [64]. Like all steroid hormones, androgens and estrogens produce effects by bonding with receptors on the cell's membrane surface or inside the cell in the liquid cytoplasm. In humans, the actions of androgens may be mediated either directly as testosterone or indirectly via the estrogen receptor (ER) after aromatization to estrogens by the action of aromatase [64]. Two different types of ER have been described which are encoded by different genes [65]. The estrogens are regulators of bone modeling and remodeling not only in women but also in men as it was recently reported by studies in humans [66].

Zheng and Cohn [67] using male and female mice demonstrated that the developing digits in uterine were rich in androgen (AR) and estrogen (ER) receptors, particularly in the fourth digit and AR activity is higher in 4th digit than in 2nd digit. According to previous researches this was something expectable [68] as the increase in receptor levels reflects maturation and growth of the fetus skeleton with a concomitant increase in cell number. The different number of AR in second and fourth digit may be the result of differential growth and ossification in a particular time of observation. It has also been reported about children's fingers that digits were developed at different rates where the 4th digit was presented more immature relatively to the 2nd digit [69]. In postnatal life, the

ossification process is earlier in girls than in boys with the maximum difference among children from 5 to 11 years old with the distal epiphysis of metacarpal bones showing the highest correlation between chronological age and bone age [70].

A positive covariance among 50 healthy men of the ratio 2D:4D and a polymorphism in CAG repeat sequence (number of polyglutamine CAG repeats in exon1) in the gene coding the AR, supported the view that 2D:4D ratio may be a proxy for the fetal androgen exposure [71]. This finding could not be replicated by other genetic researches with larger samples which failed to find a significant association [72,73,74,75], making the relation unreliable.

Craniofacial development and morphology

While the chondrocranium and Meckel's cartilage form the skeleton, the cranial base angulation and the position of the maxilla to the cranial base which are developed during the late embryonic period remain unchanged prenatally and postnatally. The lower face region become more prominent between the 7th and 10th weeks post-conception where facial structures grow more in the sagittal plane and little in height.

Meckel's cartilage grows more rapid and present more sensitivity to inhibitions. The differential growth of cartilage results in changes in facial morphology and by the 10th week post conception, the face has a typical human appearance [76,77,78].

The midline cranial base attains adult shape at 7-8 years while the adult shape of lateral cranial floor is achieved by the 12 years and that of the facial shape at 15-16 years [79,80]. In a longitudinal serial cephalometric study from infancy to young adulthood Enlow [81] described the growth of the facial skeleton. He noticed the elongation of the entire face vertically, the increase in the anterior posterior dimension and the increase of the width in the transverse plane. The most pronounced changes occured with growth were the increase of the glabella protrusion, the lateral translation of the orbits and the

expansion of the supraorbital ridges, the increase in all dimensions of the nose, the increase in the depth and width of the cheeks as well as the vertical increase in the alveolar process area with increase in chin prominence. Craniofacial growth and differential changes continued throughout life.

Facial sexual dimorphism in shape and form is already present at the 3rd year of age but not in the newborn. Age dependent changes alter the nature of dimorphism during development where four factors seem to contribute to craniofacial sexual dimorphism during postnatal development: a. prenatal differences in shape b. differences in size and shape c. male hypermorphosis and d. different growth direction between sexes [82]. The shape variations among adults which are already established at the age of 5 are attributed to ontogenetic allometry where size related shape changes are found [83]. The genetically determined skeletal structure of craniofacial complex can be influenced by various different environmental and epigenetic factors such as climate and latidude [84,85], function [85,86,87,88] and activity [89].

The disturbance of androgen and estrogen secretion immediately after birth differentiates craniofacial growth through altering cortical bone density. Sex hormones deficiency may disturb endochondrial ossification, produce degenerative changes in temporomandibular joint and lower peak in bone mass during puberty [90,91,92,93].

In a longitudinal study, Verdonck et al. [94] examined the effect of testosterone on craniofacial growth in boys with constitutional delay of growth and puberty (CGDP). They concluded that low dose testosterone treatment accelerated the craniofacial growth rate without signs of disproportional growth in all facial dimensions.

Only few researches have been carried out examining the correlation between face shape and digit ratios, all of them using frontal facial photographs.

Neave et al. [95] trying to find the relationship between organizational effects and activational levels of testosterone with the perceived male facial characteristics concluded

that perceived masculinity and dominance presented significant correlation with 2D:4D supporting the ratio as a pointer for prenatal and actual level of testosterone. They noted that "features developed under the influence of testosterone do not directly account for attractiveness but rather for male dominance and masculinity, both of which are features of perceived behavioural social status rather than mate value".

The sensitive periods for organizational effect of androgen in craniofacial complex and fingers are about the same (from 7th to 10th or 13th respectively). As craniofacial complex is presented to be sexually dimorphic and masculinization is a result of androgen effect during development and growth, correlation of low 2D:4D with more masculine craniofacial skeletal pattern would be expected, in the view that 2D:4D is a potential proxy for fetus prenatal exposure to androgens.

The null hypothesis of our research is that there is no correlation between the ratio 2D:4D and the craniofacial complex in a growing population of prepubertal children between 7 and 12 years old.

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THE RESEARCH

Introduction

The relative length of the second digit (2D, or index finger) to the fourth digit (4D, or ring finger) has received extensive attention for over 150 years. Although there is considerable overlap between the sexes, men tend to have a lower second to fourth digit length ratio (2D:4D) than women [1]. Sexual dimorphism in the 2D:4D ratio is already present by the 14th gestational week [2] and remains relatively constant with age [1], although the ratio itself increases in postnatal life [3,4,5]. The sexually dimorphic finger ratio has been attributed to different levels of prenatal androgens [1,6,7]. Androgen and estrogen receptors seem to be involved in chondrocyte development and differentiation [8]. They are present in fetal cartilaginous tissues in fingers [9], and most probably throughout the body in cartilage and bone [10,11], including calvarial bone during embryogenesis [12]. Consequently, factors that contribute to 2D:4D dimorphism might also operate on craniofacial cartilaginous or osseous structures creating morphological co-variation.

Although appealing, recent evidence has thrown doubts on the fetal androgen hypothesis [13,14,15]. Genome wide association studies have revealed that digit ratio is linked to polymorphisms in the *LIN28B* and *SMOC1* genes [14,16], the latter being associated with ocular development. The common regulation of digit and oculofacial development by *SMOC1* raises the possibility that digit and facial features cosegregate in human populations. The craniofacial complex is certainly sexually dimorphic after puberty

[17,18,19,20,21]. Facial sexual dimorphism in shape and size may even be present since the 3rd year of age, showing age dependent changes during development [22]. The mandible has been shown to exhibit shape dimorphism immediately after birth, but this gradually disappears, to reappear at the pubertal growth spurt [23]. Surprisingly, only a few studies have investigated the association between 2D:4D ratio and craniofacial shape [24,25,26,27], and these used frontal photographs, limiting the analysis to the transverse and vertical dimensions of the soft-tissues. No study could be found to examine the correlation between the lateral skeletal craniofacial pattern and digit ratio. Our aim was to investigate this relationship, the null hypothesis being that there is no correlation between the 2D:4D ratio and the shape of the craniofacial skeleton in a population of prepubertal children.

Material and Methods

Ethics Statement

The study was conducted according to the principles and guidelines of Declaration of Helsinki. The protocol of the study was approved by the Ethics Committee of School of Dentistry, National and Kapodistrian University of Athens and parental and patient written consent was obtained prior to hand scanning.

Participants

Due to wide ethnic and country variation of the digit ratio [28,29,30,31], we computed the sample size using data from a Greek study [32]. Based on this, a sample size of 30 subjects in each group would be sufficient to provide a power of 80% at 5% alpha, but because that study showed an unusually high effect size, we decided to double the number of subjects.

The sample initially consisted of 123 Greek (both parents), prepubertal children (61 boys and 62 girls), aged from 7 to 12 years, who were selected from patients attending the Department of Orthodontics, School of Dentistry, University of Athens, for orthodontic treatment. Patients with syndromes, finger and craniofacial congenital malformations or injuries, impacted or congenitally missing teeth and previous orthodontic treatment were excluded. A lateral cephalometric radiograph was already available for each subject as part of the pre-treatment orthodontic records and a scan of both hands was procured for the purposes of this study.

Cephalometric Radiographs and Measurements

The pre-treatment lateral cephalometric radiographs were used for measurement of the craniofacial complex; only radiographs of good quality with a reference ruler for exact measurement of the magnification were included. The radiographs were scanned at a resolution of 150 dpi using an Epson 1600 scanner (Seiko Epson Corporation, Nagano, Japan) and digitized using Viewbox 4 software (dHAL software, Kifissia, Greece). A comprehensive number of skeletal and dental landmarks were manually digitized but the following 15 skeletal landmarks [33] were used for the purposes of this study (Figure 1): basion (Ba), sella (S), sphenoethmoidale (SE), nasion (N), porion (Po), orbitale (O), posterior nasal spine (PNS), anterior nasal spine (ANS), A point (A), B point (B), pogonion (Pg), menton (Me), antegonial notch (Ag), gonion (Go) and articulare (Ar).

The tracings were superimposed using generalized Procrustes superimposition, a best-fit procedure that minimizes the sum of squared distances between all corresponding points [34,35,36]. We used partial Procrustes alignment (i.e. size was set to 1) and orthogonal projection on the tangent plane. The average craniofacial shape was calculated and principal component analysis (PCA) was used to extract the most significant shape components (principal components of shape in shape-space, PCs). Craniofacial size was

computed as the centroid size (CS) of the landmark configuration and comparison between the sexes was based on the natural logarithm of CS (lnCS). PCA analysis was also conducted in form-space, where shape-space is augmented by lnCS [37]. All calculations were carried out using the Viewbox 4 software.

Hand Images and Measurements

Finger length was measured on scanned images of children's right and left hands. All hands were scanned at a resolution of 150 dpi using an Epson 1600 scanner (Seiko Epson Corporation, Nagano, Japan). Children placed their palms lightly on the surface of the scanner with the second to fifth fingers roughly parallel to each other, but not in contact, in line with the wrist and elbow. All scanned images were analyzed using the Viewbox 4 software, specifically configured for this research.

Our aim was to measure finger length from the tip of the finger (excluding fingernails) to the most proximal metacarpophalangeal flexion crease that divides the finger from the palm region [38]. This measurement presents with various difficulties: a) the crease may not be a straight line or a simple curve and is usually not perpendicular to the long axis of the finger, b) the middle of the crease may not be aligned to the long axis of the finger, c) skin folding may be present at the crease area, d) the finger may bend, e) the most distal point of the finger is difficult to locate due to the smooth curvature at the tip. In order to circumvent these problems, we designed a computer-assisted procedure that limited user interaction to the tracing of the finger outline and delegated all remaining calculations of length measurement to the computer. The procedure was as follows (Figure 2):

User procedure

1. On the scanned image, a curve was fitted to the most proximal crease that divided the finger from the palm. This base curve was a cubic spline, freely adjustable by

5 or more control points using the mouse cursor on screen, and could be fitted to any of the observed shapes (Figure 2b)

2. A perimeter curve (cubic spline with 25 or more control points) was similarly fitted along the whole finger outline (Figure 2b).

Computer calculations

1. Five equally spaced points were computed on the base curve (Figure 2c).

2. The principal inertial axis of the perimeter curve was computed and the most distal point (d) of the curve along this axis was located (Figure 2c).

3. The middle point of the base curve and point d defined a provisional long axis. The portion of the finger corresponding to the central 70% section of this axis was considered more representative, as it did not include the tip, which may bend, or the proximal finger root, which may exhibit skin folds (Figure 2d).

4. Eleven equally spaced points were located on the perimeter curve on each side of the finger, within the 70% central part of the provisional axis (Figure 2d).

5. A least-squares best-fit line was computed for the 11 points on each side of the finger (lines b1, b2) (Figure 2e).

6. The bisector of the angle formed by lines b1 and b2 was computed. This was considered the true long axis of the finger.

7. A least-squares best-fit line was computed for the 5 points of the base curve (line b3). This was considered the base of the finger (Figure 2e).

8. Finger length was computed as the distance between two points, the intersection of the long axis with the base (point B), and the most distal point of the perimeter curve along the long axis direction (point A) (Figure 2e). Point A was therefore on

a perpendicular to the long axis of the finger, but not necessarily on the long axis itself; point B was not necessarily in the middle of the proximal crease.

Lengths of the second and fourth fingers of the left and right hands (L2D, L4D, R2D, R4D) were used to compute the second-to-fourth digit ratio (2D:4D) for the left (L2D:4D) and the right (R2D:4D) hand. Additionally, we measured left and right distal finger extent [39] (LDFE, RDFE) as the calculated distance between the projection of the 2nd and 4th finger distal points on the bisector of the angle formed by the axes of the two fingers (Figure 3).

During digitization, the images could be freely enlarged on-screen. In order to ensure accurate fitting of the perimeter curve, brightness and contrast were adjusted, if needed, and inversion of the image colors was used, to better visualize the dark edges of the finger perimeter (Figure 4).

All measurements were carried out by one observer blinded to children's sex and age. In order to avoid measurement bias, the second and fourth fingers of each hand were digitized at separate occasions. A second observer digitized a sub-sample for error estimation.

Error Estimation

As errors may arise both during finger placement and imaging, as well as in point identification, method error was evaluated with repeated digitization of the same images, as well as with repeated scanning. Both hands of 6 randomly selected patients were scanned and redigitized after a period of one month, providing a second set of images. The first set was digitized twice at an interval of at least one week independently by two observers, thus providing 24 (six pairs of hands, two fingers each) double measurements for estimation of intra-observer digitization error, as well as inter-observer error. The

second scanning set was digitized by one observer and was used to evaluate intra-observer scanning and digitization error combined.

We calculated the technical error of measurement (TEM) as the square root of the sum of the squared differences of repeated measurements divided by double the number of objects measured, and the relative TEM (rTEM) as TEM divided by the grand average of the measurements and multiplied by 100 [38,40].

All cephalometric radiographs were checked for errors by the second investigator. Twenty four cephalometric radiographs were also selected at random and redigitized a month later. Intra-observer error was assessed by calculating the Procrustes distance between repeated digitizations.

Sample Homogeneity

After performing principal component analysis of the cephalometric data, we visually inspected the PCA plots in order to identify potential outliers, as our subjects were in need of orthodontic therapy and had potentially extreme morphological patterns. We identified three outliers in each group and the initial sample was reduced to 58 boys and 59 girls. We used histograms of the 2D:4D ratio to inspect for outliers in the finger measurements and found none. The Shapiro-Wilk W test did not reveal any evidence of non-normality of the 2D:4D ratios, or of the distal finger extents.

Results

Method Error

Intra-observer technical error of measurement (TEM) for repeated measurements on the same images was 0.03 mm for observer 1 and 0.11 mm for observer 2 (Table 1). Rescanning of hands on a second occasion resulted in an error of 0.26 mm. Inter-observer TEM was 0.28 mm.

The average Procrustes distance between repeated digitizations of the 24 cephalograms was 7.9 % of the total variance of the sample.

Hand Measurements

In 58 male children, age ranged from 8.1 to 12.0 years whereas in 59 females the range was from 7.0 to 11.9 years (Table 2). Females had, on average, larger fingers than males (Cohen's d effect size ranged from 0.12 to 0.24), but the difference was not statistically significant.

Digit ratio followed the expected pattern of being larger in females than males but the difference was not statistically significant(Table 2, Cohen's d effect size: left 0.275, right 0.126). The ratio was more pronounced in the left hand than the right, but only in females did the difference between hands reach statistical significance (95% CI of the difference = 0.001 to 0.013, t = 2.25, P = 0.03). Distal finger extent (DFE) was larger in males than females and approximately equal for both hands (effect size: left: -0.34, right -0.32). Although not statistically significant, both 2D:4D and DFE had 95% confidence intervals that bordered close to zero. DFE was very close to the conventional significance level of 0.05.

Cephalometric Measurements

The first three principal components (PC) of shape space described 56.4% of the total variance of craniofacial shape. Figure 5 shows the patterns of shape variability, as described by the first three PC axes, using thin plate spline grids, and Figure 6 shows the subjects in shape space, plotted on the first three PCs. The first PC described variability in the vertical direction, differentiating between hyper- and hypo-divergent subjects. The second PC was mainly related to anteroposterior position of the mandible relative to the

craniomaxillary complex and the third PC described variability in the angle of the mandible.

Sexes were compared in shape space using a permutation test (10,000 permutations, without replacement) comparing Procrustes distance between means. No sexual dimorphism in craniofacial shape was found (P = 0.291). However, males had larger size (lnCS) than females (males: 5.187, females: 5.152, t-test: 4.75, P < 0.0001, 95% CI: 0.021 to 0.050).

Covariation

Regression of Procrustes coordinates on digit ratio revealed no statistically significant correlation, either for each sex separately or for the whole sample combined. The shape variance predicted by digit ratios was less than 1% of the total variance for the total sample and slightly larger for females than males. Similar results were obtained for DFE (Table 3).The same analysis was conducted in form space, which includes lnCS, without any appreciably different results (Table 4). We also analyzed craniofacial shape as two separate landmark clusters, one including the mandibular landmarks only, and the other including the remaining landmarks (maxilla and cranial base). No significant correlation was found between these shapes and the 2D:4D ratio or DFE; the percent variance explained was similar to that computed for the whole shape (results not shown).

Discussion

A recent model explaining the sexual dimorphism of the 2D:4D ratio is based on differential androgen receptor (AR) and estrogen receptor (ER) activity between the two fingers, as studied in mice [9]. These receptors respond to circulating sex hormones; the higher androgen/estrogen hormonal ratio of males increases AR and decreases ER activity, promoting higher chondrocyte proliferation during gestation and leading to

longer fingers. The effect is more pronounced in the 4th finger than the 2nd, due to higher activity of both receptors in the 4th finger, thus creating the observed dimorphic digit ratio. We hypothesized that AR and ER proteins are also active in the craniofacial region and regulate skeletogenic genes even before birth, potentially creating sexual dimorphism in that region as well.

In this study we confined the age of the subjects to prepuberty in order to evaluate potential effects before the pubertal hormonal surge might overshadow any previous covariation. Age range was limited because craniofacial shape is known to vary with age [17,18,19,20,21]. Digit ratio also increases with age [3,4,5], although the difference between males and females seems independent of age [1].

The selected cephalometric landmarks describe the cranial base, maxilla and mandible. No points were placed on teeth, as these might be more affected by environmental or local factors. Similar landmark configurations have been used previously [33,41]. Shape analysis was performed using the tools of geometric morphometrics for comprehensive description of shape without the confounding effect of size [34,35,36].

Hands were measured indirectly from scanned images. Indirect measurements have been shown to reveal larger differences between sexes [1,42] and may include bias due to distortion of soft tissues by pressure on the glass plate [43]. We minimized measurement error by delegating most of the measurement steps to the computer and evaluated error by computing differences between repeated scannings of the same hands, in addition to the usual inter- and intra-observer differences on the same images. The results showed that technical error of measurement was more than three times lower than that reported by other investigators. Intra-observer error was less than 0.2% and inter-observer error was approximately 0.4% compared to e.g. 1.3% reported by Voracek et al. [38] for finger length measurements. More importantly, combined intra-observer error and repeated

scanning error was also around 0.4%. Part of the error reduction may be due to the use of a computer [44] but we believe a significant part to be due to the specific method of identifying the relevant landmarks. There are only two landmarks involved in finger length measurement but the potential for error is high in both. The proximal landmark is located on a crease that may be curved and is frequently inclined relative to the long axis of the finger. Due to this inclination, a small error in locating the landmark along the crease may translate to a large difference in finger length. The distal landmark may also be difficult to locate because the curvature is smooth at the finger tip, but also because the tip (or the whole finger) may be curved to one side. To overcome these problems we considered the middle part of the finger to be representative of its long axis and used best-fit lines to represent both the proximal crease and the finger sides.

Digit ratios have been found to vary between ethnic groups and countries [28,29,31,45], a finding attributed to latitude or population genetic variations [30, 31,46]. A study on Greek subjects [32] reported larger 2D:4D ratios for both males and females than those found here, and a surprisingly high effect size (d = 0.65). Our digit ratio effect sizes were much lower (d = 0.13 and 0.27 for the right and left hand, respectively), even lower than the average values reported by Hönekopp et al. [1] in their meta-analysis (d = 0.48 and 0.41 for the right and left hand, respectively, when using indirect measurements). However, our 95% confidence intervals (Table 2) include the Hönekopp et al. [1] values. We could not find a statistically significant sexual dimorphism in digit ratio. Similarly, no difference in craniofacial shape was found between the sexes. Although the craniofacial region is known to be sexually dimorphic in the adult stage [20], there are conflicting findings for children. Bulygina et al. [22] reported significant dimorphism on 14 male and 14 female subjects who were studied longitudinally from one month to 21 years of age with cephalometric radiographs, but no landmarks on the mandible were used. Studies using larger, but cross-sectional, samples and similar landmark configurations to

the present study could not detect prepubertal shape differences between the sexes, although males are consistently larger [41,47]. Interestingly, the mandible may be dimorphic both in shape and size immediately after birth, but shape differences disappear by 4 years of age, to re-appear at puberty [23].

Although the 2D:4D digit ratio has been extensively studied, very few papers have examined its association to craniofacial shape [24,25,26,27]. These papers limit their scope to soft-tissue facial shape as evaluated in the frontal view from photographs; we decided to focus on skeletal craniofacial shape from the lateral aspect using cephalometric radiographs, because we considered that vertical and sagittal mandibular size and position might be a strong indicator of potential covariation between chondrocyte proliferation in the fingers and the mandibular condyle. Indeed, mandibular growth, as measured from the lateral aspect, has been shown to be affected by AR and ER antagonists in mice both shortly after birth and up to 8 weeks of age, which corresponds to skeletal maturation [48,49]. In addition to the mandibular condyle, which is considered secondary cartilage exhibiting different growth responses [50,51], the synchondroses of the cranial base (spheno-occipital and spheno-ethmoidal) are also strong candidate sites that might be affected by fetal hormones and would produce shape dimorphism in the anteroposterior and vertical direction.

Our results could not provide evidence for any association between digit ratio and craniofacial shape or size, for the age group studied. The percentage of variance predicted barely reached the level of 2.6% for the female group and was below 1% for the whole sample. The low correlations cannot be attributed to low variance in the sample; our digit ratio variance was equal to or larger than that reported by a meta-analysis [1] and the variance of craniofacial shape was comparable to previous studies including a wide range of craniofacial skeletal patterns [52,53]. Such low correlations, even if of statistical

significance, are of dubious biological interest. Fink et al. [24] reported a correlation between digit ratio and frontal facial shape, but their sample was of adults and the relation was significant only in the male group (no data were given as to the strength of the correlation). Interestingly, Schaefer et al. [25] studied the facial shape of 46 adult males using similar geometric morphometric methods but could not find a statistically significant result. The proportion of facial shape variance explained by the digit ratio was only 2.5%, which is comparable to our results. Meindl et al. [27] continued this research on a sample of boys and found that 14.5% of shape variation was accounted for by the digit ratio, but their sample was small (17 boys), and the age range extended from 4 to 11. Burriss et al. [26] studied an adult sample using direct and indirect hand measurements and reported some statistically significant correlations between digit ratio and facial measurements on frontal photographs. However, these correlations are of low confidence because they were found in females only, were significant for some, but not all, of the measurement methods, were inconsistent between hands, were computed using one-tailed tests and finally, they did not survive a Bonferroni correction. This study is interesting in that the male digit ratios tended to be larger than the female ratios.

The model of [9] mentioned above is based on research on mice and it might not be directly applicable to humans, where genetic studies have not provided expected results, as predicted by the fetal androgen exposure hypothesis [7]. Estrogen receptor α (ER1) TA allele repeats were found to correlate with 2D:4D digit ratio in men [52], but the effect was weak ($r^2 = 0.026$) and only present in the left hand. Recent analysis of CAG/GGN repeat polymorphisms of the AR gene, which are associated with AR activity, could not show any association to digit ratios [15,53,54]. In addition to AR and ER, other genes may function to control morphology both in fingers and the craniofacial skeleton, homeobox genes being an obvious example. *Hox* genes, although active in fingers [28,55,56], are probably not related to facial morphology, as the cranium and the 1st

pharyngeal arch are considered *Hox* negative [57,58]; however, *Hoxd13* has recently been implicated in mandibular shape variation in mice [59]. *PAX3* may also be a factor of normal facial variation [60] but it is not known if, and to what extent, its effect is controlled by sexual hormones. Interestingly, a genome-wide association study (GWAS) [14] could not find strong associations between 2D:4D ratio and the AR gene, or the *HOXA* and *HOXD* clusters, but a strong signal was found for the *LIN28B* gene, whose role in finger development is yet unclear. These results were supported in another GWAS [16], but, by applying a meta-analysis of both GWASs, the signal for the *SMOC1* gene was found even stronger. The *SMOC1* gene is known to play a significant role in limb development, being associated with 'microphthalmia with limb anomalies' syndrome (Waardenburg anophthalmia, OMIM 206920) [61,62]. In addition to syndactyly, fusions of metacarpals and other distal limb abnormalities, the syndrome presents with microphalmia, anophalmia, facial asymmetry and orofacial clefts. Other factors linking limb development to craniofacial structures are Gja1 (ocular and limb development) [63] and the FGF/FGFR system (craniofacial modularity and limb development) [64], but the effect of these on digit ratio has not been investigated. In view of the association of *SMOC1* and Gja1 to ocular development and clefting, we examined the correlations between digit ratios (or DFE) and the shape of the cranial base and maxilla (i.e. after removing the cephalometric landmarks of the mandible) but obtained the same non-significant results.

In conclusion, no correlation was found between craniofacial shape and digit ratios or relative lengths. Although several factors might be involved in the development and growth of both these structures, such factors are probably unable to impose a measurable effect within the variation of a normal population. Future research needs to examine an adult sample for potential covariation arising after the pubertal growth spurt.

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TABLES

Table 1. Measurement error of absolute finger lengths.

	Observer 1	Observer 2	Inter-observer
Redigitize same images	0.03 (0.05%)	0.11 (0.17%)	0.28 (0.42%)
Rescan hands and digitize	0.26 (0.39%)		

Measurements on 6 subjects (repeated measurements on 24 fingers). TEM and rTEM (in parenthesis).

Table 2. Descriptive statistics of age and hand measurements and comparison between sexes.

| Measurement | Males (n = 58) | | Females (n = 59) | | t-test | | |
	Mean (SD)	Range	Mean (SD)	Range	t (P value)	95% CI	Cohen's d
Age (years)	10.25 (0.98)	8.1 to 12.0	10.43 (1.05)	7.0 to 11.9	0.970 (0.334)	-0.56 to 0.19	0.18
L2D (mm)	62.73 (5.16)	49.2 to 73.4	63.93 (4.59)	50.6 to 73.7	1.323 (0.189)	-2.98 to 0.59	0.24
L4D (mm)	65.32 (5.32)	52.0 to 78.0	65.94 (5.06)	50.3 to 81.4	0.648 (0.518)	-2.52 to 1.28	0.12
R2D (mm)	62.78 (5.19)	50.3 to 73.0	64.06 (4.35)	53.1 to 76.6	1.443 (0.152)	-3.03 to 0.48	0.27
R4D (mm)	65.50 (5.19)	53.5 to 77.9	66.54 (4.73)	53.0 to 82.4	1.136 (0.258)	-2.86 to 0.78	0.21
L2D:4D	0.961 (0.033)	0.88 to 1.05	0.970 (0.036)	0.89 to 1.04	1.489 (0.139)	-0.022 to 0.003	0.275
R2D:4D	0.959 (0.036)	0.89 to 1.07	0.963 (0.035)	0.88 to 1.04	0.682 (0.497)	-0.018 to 0.009	0.126
LDFE (mm)	1.05 (3.34)	-4.4 to 10.5	-0.25 (4.19)	-10.1 to 8.0	1.849 (0.067)	-0.09 to 2.68	-0.34
RDFE (mm)	1.42 (3.56)	-6.3 to 9.4	0.19 (4.10)	-9.4 to 9.4	1.730 (0.086)	-0.18 to 2.64	-0.32

SD: standard deviation, CI: confidence interval.

Table 3. Multivariate regression of shape-space PCs on digit ratios and DFE.

Measurement	Whole sample (n = 117)		Females (n = 59)		Males (n = 58)	
	% variance predicted	P value	% variance predicted	P value	% variance predicted	P value
L2D:4D	0.93	0.342	1.68	0.420	1.62	0.461
R2D:4D	0.90	0.356	2.57	0.145	0.75	0.945
LDFE (mm)	0.83	0.427	1.70	0.407	1.06	0.793
RDFE (mm)	0.71	0.570	1.93	0.310	0.31	0.999

Permutation test: 10,000 permutations, without replacement.

Table 4. Multivariate regression of form-space PCs on digit ratios and DFE.

Measurement	Whole sample (n = 117)		Females (n = 59)		Males (n = 58)	
	% variance predicted	P value	% variance predicted	P value	% variance predicted	P value
L2D:4D	0.68	0.560	1.23	0.617	1.06	0.738
R2D:4D	0.62	0.617	1.66	0.412	0.81	0.877
LDFE (mm)	0.57	0.676	1.15	0.678	1.32	0.585
RDFE (mm)	0.48	0.793	2.32	0.210	0.22	0.999

Permutation test: 10,000 permutations, without replacement.

FIGURES
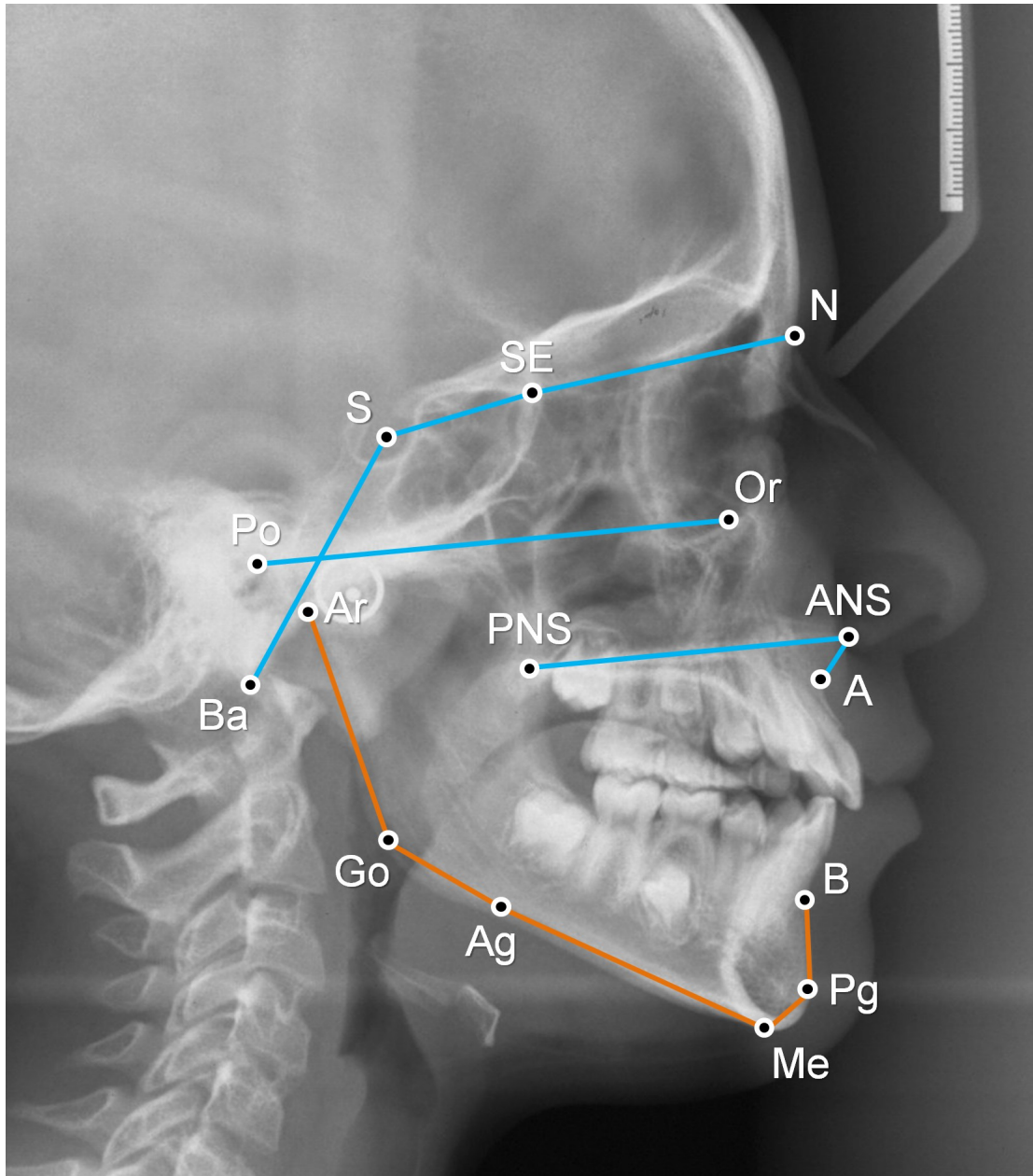
Figure 1. Cephalometric landmarks.

Cephalometric points: basion (Ba), sella (S), sphenoethmoidale (SE), nasion (N), porion (Po), orbitale (O), posterior nasal spine (PNS), anterior nasal spine (ANS), A point (A), B point (B), pogonion (Pg), menton (Me), antegonial notch (Ag), gonion (Go), articulare (Ar).
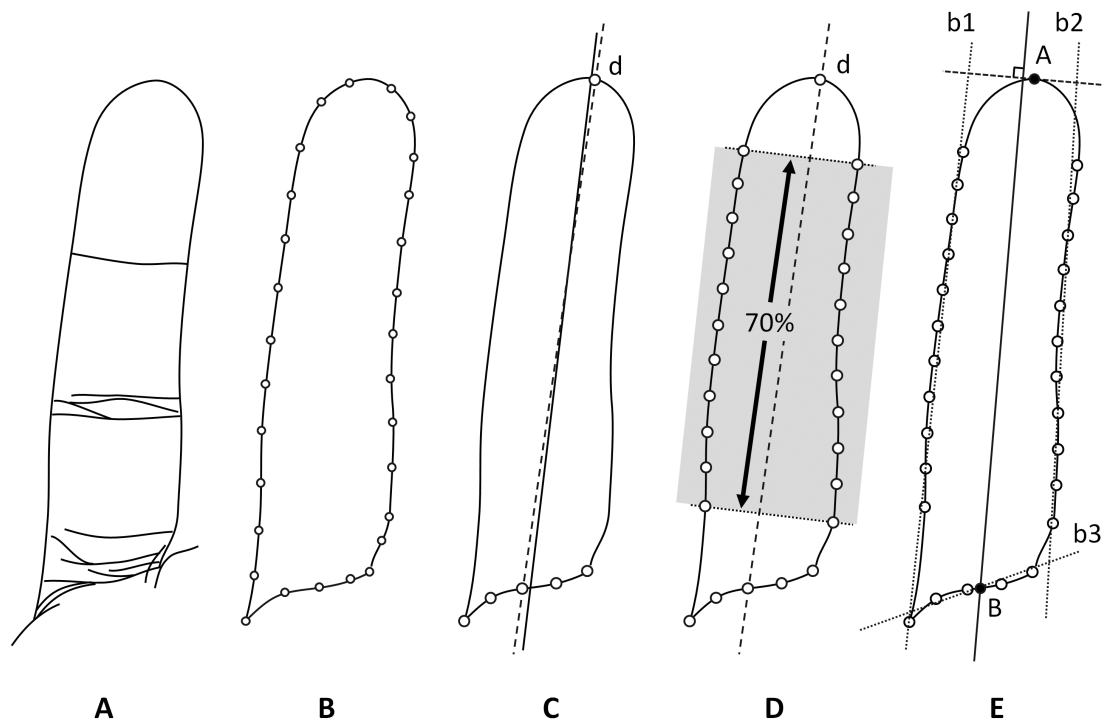
**Figure 2. Method of finger length measurement.**

a) Fourth finger of the right hand. b) Cubic spline curves and their control points. c) Five equidistant points on base curve. Principal axis (solid line) of perimeter curve was used to define most distal point of finger (point d). Provisional long axis (broken line) defined by point d and middle point of base curve. d) Middle section of finger and 11 equidistant points on either side. e) Best-fit lines (b1, b2, b3). Solid line is the bisector of the angle formed by b1 and b2. Point A: most distal point, in the direction of the bisector, on perimeter curve. Point B: intersection of bisector and b3. Finger length is distance between points A and B.
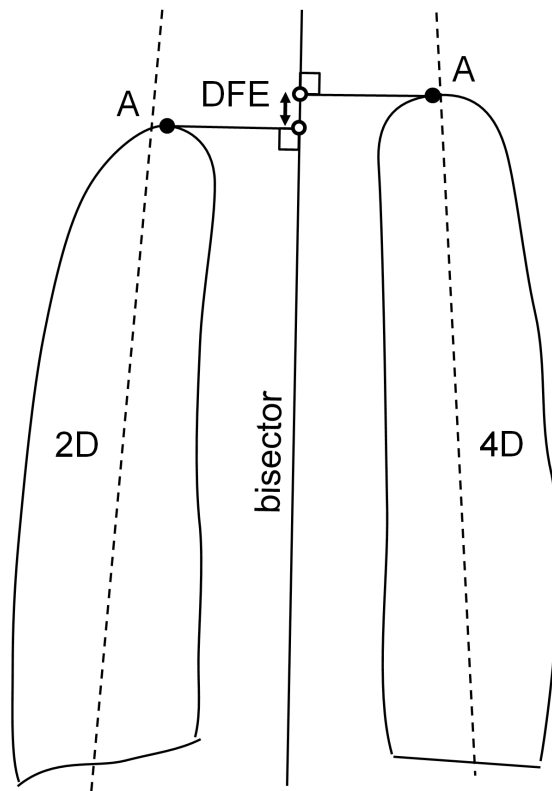
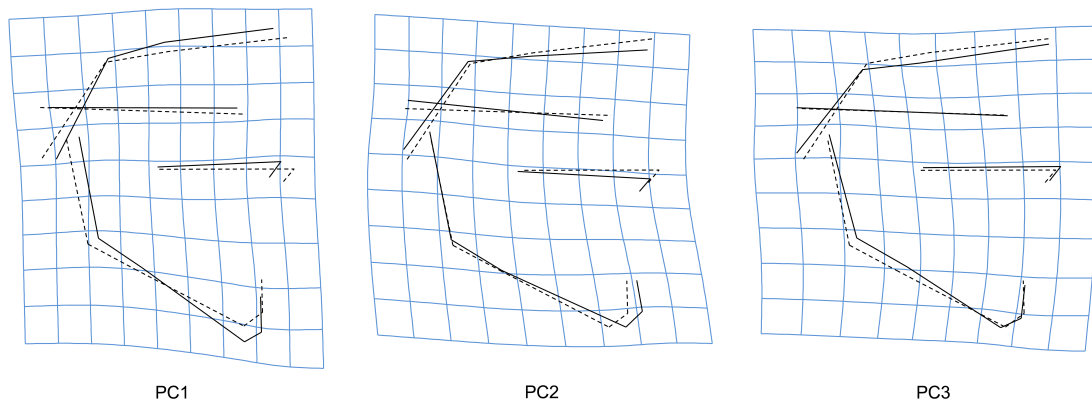**Figure 3. Measurement of DFE.**

Distal finger extent (DFE) was measured on the bisector of the long axes of the two fingers,

as the distance between the projections of the most distal point of each finger.
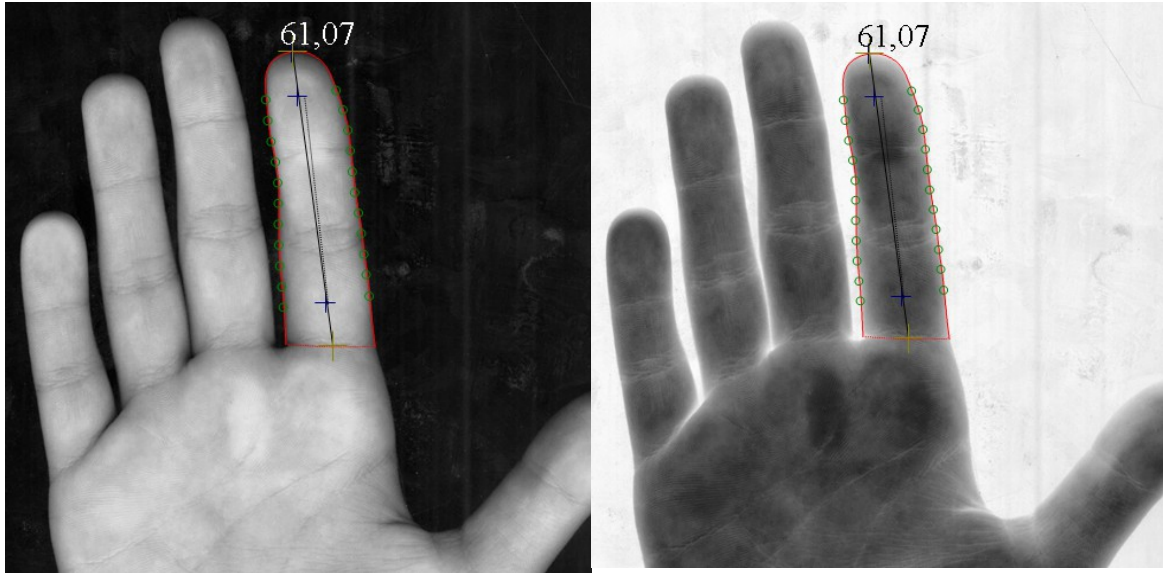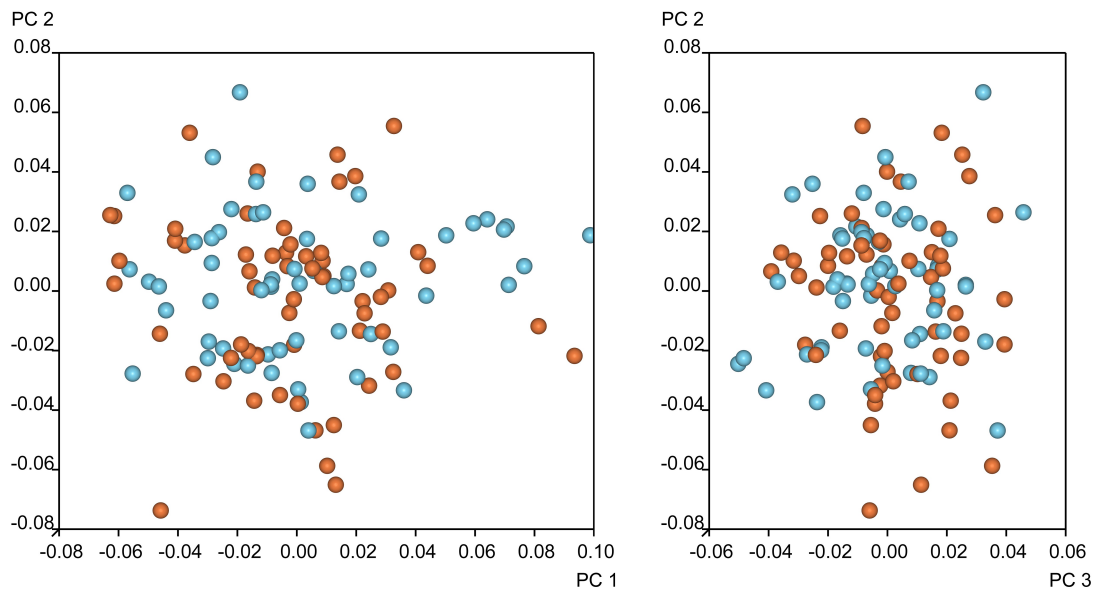
Figure 4. **Inversion of the image colors**

Inversion of the image colors was used to better visualize the dark edges of the finger perimeter

Figure 5. Craniofacial shape variability patterns.

Thin plate spline grids showing deformation from average shape (broken line) to three

standard deviations along each of the first three principal component (PC) axes.

Figure 6. Subjects in craniofacial shape space.

Plot of the 117 subjects on the first 3 PCs in shape space. Blue spheres: males, orange spheres: females.

APPENDIX