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Three-Dimensional Morphometric Analysis of Craniofacial Shape in the Unaffected Relatives of Individuals With Nonsyndromic Orofacial Clefts: A Possible Marker for Genetic Susceptibility

Seth M. Weinberg,[1,2]* Katherine Neiswanger,[1,2] Joan T. Richtsmeier,[3,4] Brion S. Maher,[5] Mark P. Mooney,[2,6,7,8] Michael I. Siegel,[6,8] and Mary L. Marazita[1,2,9,10]

[1]Center for Craniofacial and Dental Genetics, School of Dental Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania
[2]Department of Oral Biology, School of Dental Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania
[3]Department of Anthropology, Pennsylvania State University, University Park, Pennsylvania
[4]Center for Craniofacial Development and Disorders, School of Medicine, The Johns Hopkins University, Baltimore, Maryland
[5]Department of Psychiatry, Virginia Commonwealth University, Richmond, Virginia
[6]Department of Orthodontics, School of Dental Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania
[7]Division of Plastic Surgery, Department of Surgery, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania
[8]Department of Anthropology, University of Pittsburgh, Pittsburgh, Pennsylvania
[9]Department of Human Genetics, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania
[10]Department of Psychiatry, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania

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Numerous studies have described altered patterns of craniofacial form in the unaffected relatives of individuals with nonsyndromic clefts. Unfortunately, results from such studies have been highly variable and have failed to provide a reliable method for differentiating "at-risk" relatives from controls. In the present study, we compared craniofacial shape between a sample of unaffected relatives (33 females; 14 males) from cleft multiplex families and an equal number of age/sex/ethnicity-matched controls. Sixteen *x,y,z* facial landmark coordinates derived from 3D photogrammetry were analyzed via Euclidean Distance Matrix Analysis, while 14 additional linear distances were analyzed via *t* tests. A subset of variables was then entered into a discriminant function analysis (DFA). Compared to controls, female unaffected relatives demonstrated increased upper facial width, midface reduction and lateral displacement of the alar cartilage. DFA correctly classified 70% of female unaffected relatives and 73% of female controls. Male unaffected relatives demonstrated increased upper facial and cranial base width, increased lower facial height and decreased upper facial height compared with controls. DFA correctly classified 86% of male unaffected relatives and 93% of male controls. In both sexes, upper facial width contributed most to group discrimination. Following DFA, unaffected relatives were assigned to risk/liability classes based on the degree of phenotypic divergence from controls. Results indicate that craniofacial shape differences characterizing unaffected relatives are partly sex-specific and are in broad agreement with previous reports. These findings further suggest that a quantitative assessment of the craniofacial phenotype may allow for the identification of susceptible individuals within nonsyndromic cleft families. © 2008 Wiley-Liss, Inc.

Key words: anthropometry; nonsyndromic clefting; CL/P; three-dimensional surface imaging; stereophotogrammetry; morphometrics; EDMA; discriminant function analysis; phenotype

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*Correspondence to: Seth M. Weinberg, 200 Hawkins Drive, W278 GH, Iowa City, IA 52242. E-mail: seth-m-weinberg@uiowa.edu

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INTRODUCTION

Despite decades of research into the underlying causes of nonsyndromic orofacial clefts, the etiological factors responsible for this widespread birth defect remain largely unknown. There is abundant evidence that the most common form of nonsyndromic clefting, cleft lip with or without cleft palate (CL/P), has a substantial genetic basis [Wyszynski et al., 1996; Schutte and Murray, 1999; Marazita, 2002; Mitchell, 2002; Cobourne, 2004; Lidral and Moreno, 2005]. CL/P is probably still best understood as a complex multifactorial disease since susceptibility is believed to be influenced by perhaps 3–14 putative genetic loci coupled with environmental risk factors [Schliekelman and Slatkin, 2002]. Furthermore, CL/P is characterized by both allelic and locus heterogeneity, variable expression and reduced penetrance.

Given the complex multifactorial nature of CL/P, the identification of unaffected individuals within CL/P families who may be harboring susceptibility factors is problematic. Knowledge of the risk status of unaffected relatives in CL/P families is important for a number of reasons. First, the ability to predict offspring recurrence is based on an assessment of risk. Second, the identification of unaffected but genetically predisposed individuals has the potential to increase the power of gene mapping and association approaches. In current studies of CL/P that utilize traditional phenotypic definitions (overt cleft = affected), a large proportion of genetically "at-risk" individuals are probably misclassified [Bixler, 1991], which disrupts the statistical relationship between phenotype and genotype.

One way to improve our ability to predict genetic risk is to develop reliable phenotypic markers, which can be conceptualized as proxies for latent genetic liability. An established method for identifying phenotypic markers is to compare individuals known to be at elevated risk for the disease (e.g., the biological relatives of affected individuals) to population-based controls with no family history of the disease on some measurable aspect(s) of their phenotype. Using this general approach, a broad array of phenotypic markers has been reported in the unaffected relatives of individuals with CL/P [Weinberg et al., 2006a]. For example, mounting evidence suggests that unaffected relatives within cleft families are characterized by a suite of distinctive craniofacial features [Fukuhara and Saito, 1962, 1963; Mills et al., 1968; Niswander, 1968; Fraser and Pashayan 1970; Coccaro et al., 1972; Figalová and Šmahel, 1974; Kurisu et al., 1974; Shibasaki et al., 1978; Nakasima and Ichinose, 1983, 1984; Sato, 1989; Ward et al., 1989, 1994, 2002; Blanco et al., 1992; Raghavan et al., 1994; Mossey et al., 1998a; AlEmran et al., 1999; Suzuki et al., 1999; McIntyre and Mossey, 2003, 2004; Perkiomaki et al., 2003; Yoon et al., 2004; Weinberg et al., 2006b]. Furthermore, numerous studies have confirmed the presence of differences in facial growth and morphology between mouse strains susceptible to teratogen-induced or spontaneous clefting and nonsusceptible strains [Trasler, 1968; Juriloff and Trasler, 1976; Trasler and Machado, 1979; Millicovsky et al., 1982; Trasler and Leong, 1982; Wang and Diewert, 1992; Hallgrímsson et al., 2004; Young et al., 2007]. The presence of craniofacial differences in these "at-risk" populations provides compelling support for the hypothesis that certain heritable aspects of craniofacial form represent either a predisposing factor to isolated orofacial clefting or a subclinical manifestation of the anomaly.

Nevertheless, in humans, inconsistent and contradictory findings across studies have led to confusion as to which craniofacial features are best capable of distinguishing unaffected relatives from the general population. In a recent meta-analysis of the relative-control literature, Weinberg et al. [2006b] identified several potentially characteristic craniofacial features in unaffected first-degree relatives: reduced head width, increased interorbital distance, increased nasal cavity and upper face width, reduced upper face height and increased lower face height. However, the differences between relatives and controls were generally small and characterized by significant between-study heterogeneity. Consequently, a reliable method for distinguishing potentially "at-risk" relatives from controls is still lacking.

Part of the difficulty in achieving consensus likely relates to a number of methodological shortcomings present in earlier studies, including: (1) a reliance on cephalometry, which can only provide the most rudimentary information on craniofacial form and precludes the analysis of many potentially important soft tissue structures; (2) a lack of standardization of variables used to summarize craniofacial form; (3) the use of statistical methods that are incapable of separating size differences from shape differences; (4) a failure to account for sex differences; (5) the inclusion of a large number of simplex families, where the etiology is more likely to be nongenetic in origin; and (6) a failure to utilize analytical methods capable of assigning risk to individual relatives on a case-by-case basis.

In an attempt to overcome many of these limitations, the present study compares the craniofacial morphology of unaffected individuals from multiplex nonsyndromic orofacial cleft families to matched controls by employing state-of-the-art three-dimensional surface imaging technology in conjunction with statistical methods capable of a more rigorous assessment of shape differences. We argue that this approach allows for a more detailed understanding of the facial features that characterize unaffected relatives. In addition, we attempt to develop a viable method for assigning unaffected relatives to risk/liability classes, based on information gathered from morphometric analysis.

METHODS

Sample Recruitment and Selection

Two main groups comprised the study sample: unaffected first-degree relatives of individuals with nonsyndromic orofacial clefts and unaffected controls with no family history of the disease. Following IRB approval, unaffected relatives of individuals with orofacial clefts were identified through nonsyndromic CL/P index cases treated by the Cleft Craniofacial Center at Children's Hospital of Pittsburgh as part of the Pittsburgh Orofacial Cleft Study [Weinberg et al., 2006a]. Multiplex families of Caucasian ancestry were included in the current study, in order to enrich the sample for genetic effects and minimize etiologic heterogeneity. It was not necessary that the second affected individual be a first degree relative of the proband or that they have the same type of cleft as the proband. Although the majority of families contained individuals affected with CL/P, a small number of mixed cleft families (CL/P and CP) and isolated CP only (CPO) families were also included. All families were screened by a board-certified medical geneticist and any families with syndromic forms of clefting were excluded.

Unaffected Caucasian controls were ascertained primarily from the Ohio Valley region, either via a recruitment referral service (Campos, Inc., Pittsburgh, PA) or through solicitation. Controls with a personal or family history of orofacial clefting or any other disease associated with craniofacial dysmorphology, or with a personal history of facial plastic or reconstructive surgery, were not eligible to participate. In general, controls were ascertained as unrelated individuals; however, occasionally control families were recruited.

In order to maximize the chances of identifying anthropometric craniofacial variables that might distinguish unaffected relatives from controls, samples of unaffected relatives were created from the available multiplex families. Separate samples of unaffected male and female relatives were selected, to eliminate the potentially confounding influence of sex on craniofacial differences [Kurisu et al., 1974; Weinberg et al., 2006b]. For each sex, unaffected relatives were selected based on the following criteria: (1) they were a first-degree relative of an affected individual; (2) they had complete anthropometric data; and (3) they appeared to be at elevated risk of carrying cleft susceptibility genes based on a visual inspection of the pedigree. If two or more related individuals within a family met all of the above criteria, for example, full sibs of the proband, the oldest one was chosen. Thus, both male and female samples were comprised only of unrelated individuals. After the samples of unaffected relatives were selected, healthy, unrelated controls with complete anthropometric data were matched to them by sex and age (to within 1 year). If multiple controls were available for a given relative, the control individual closest in height to the relative was chosen.

Anthropometric data was initially available for 124 unaffected relatives (54 males; 70 females) from 41 multiplex cleft families. Thirty-one unaffected relatives had unusable or missing anthropometric data, and were excluded from further analysis. Of the remaining 93 relatives, 46 (22 males; 24 females) did not meet the selection criteria, that is, they were not a first-degree relative of an affected individual, they did not appear to be at elevated risk from pedigree inspection, or they were related to an individual already selected. This left a total of 47 unaffected relatives from 36 families (28 CL/P, 7 mixed cleft, 1 CPO) available for analysis—14 unrelated males and 33 unrelated females. In 11 of the 36 families, both a male and female member was included. The average age of the male relatives was 26.9 ($\pm$18.2) years; female relatives were slightly older at 27.8 ($\pm$14.9) years. Based on the matching criteria outlined above, 14 unrelated healthy males and 33 unrelated healthy females were selected from a possible 227 controls (84 males; 143 females). No significant differences in mean age or height were present between the male or female unaffected relatives and their respective controls.

Anthropometric Data Collection

For each subject, quantitative data on the soft tissues of the head and face were obtained by a combination of 3D photogrammetry-based indirect anthropometry and caliper-based direct anthropometry. Three-dimensional facial surface captures were obtained with a FaceCam 250 digital stereophotogrammetry imaging system (Genex Technologies, Inc., Bethesda, MD). The FaceCam system is a completely noninvasive imaging device, capable of obtaining geometrically accurate, photo-realistic 3D surface captures in under 400 msec. For a detailed description of the Genex system and its validation see Weinberg et al. [2004, 2006c].

Three sequential facial captures were obtained for each subject: frontal, 45-degree-left and 45-degree-right. Multiple captures were required to obtain full facial coverage due to the Genex system's limited horizontal field of view. The capture process begins with the subject seated on an adjustable stool placed against a wall. Positioned directly in front of the FaceCam system, the subject was asked to rest the back of their head against the wall in order to minimize involuntary movement. The operator then adjusted the tilt of the subject's head to approximately 10 degrees above Frankfort horizontal to ensure that the subnasal/submental regions were within the camera's visual range. Following the frontal capture, with the subject remaining in the same position, the camera was then repositioned to obtain the 45-degree-left and

45-degree-right captures. This was accomplished by sliding the camera system along a custom-made dolly track designed to maintain a constant distance between the FaceCam unit and the subject. Prior to 3D capture, landmark positions were marked directly on each subject's face with a black liquid eyeliner pencil, facilitating precise and rapid landmark localization from the 3D captures [Weinberg et al., 2004]. In order to reduce invasiveness, selected landmarks around the eyes (*endocanthion*) and mouth (*chelion* and *stomion*) were left unmarked.

From the frontal 3D capture of each subject, a set of 16 standard soft tissue facial landmarks [Farkas, 1994; Kolar and Salter, 1997] was extracted using the program *3D Surgeon*, Genex's proprietary 3D measurement software (Fig. 1A). The corresponding *x,y,z* coordinate locations for the set of 16 landmarks was saved as a text file for subsequent analysis (see below). From the left and right 3D captures, a set of eight linear distances was obtained: left and right upper face depth (*t-n*), midface depth (*t-sn*), lower face depth (*t-gn*) and ala length (*ac-prn*; Fig. 1B). Six additional linear distances were also obtained directly on subjects with spreading calipers (GPM, Switzerland): maximum head width (*eu-eu*), forehead width (*ft-ft*), upper face width (*zy-zy*),

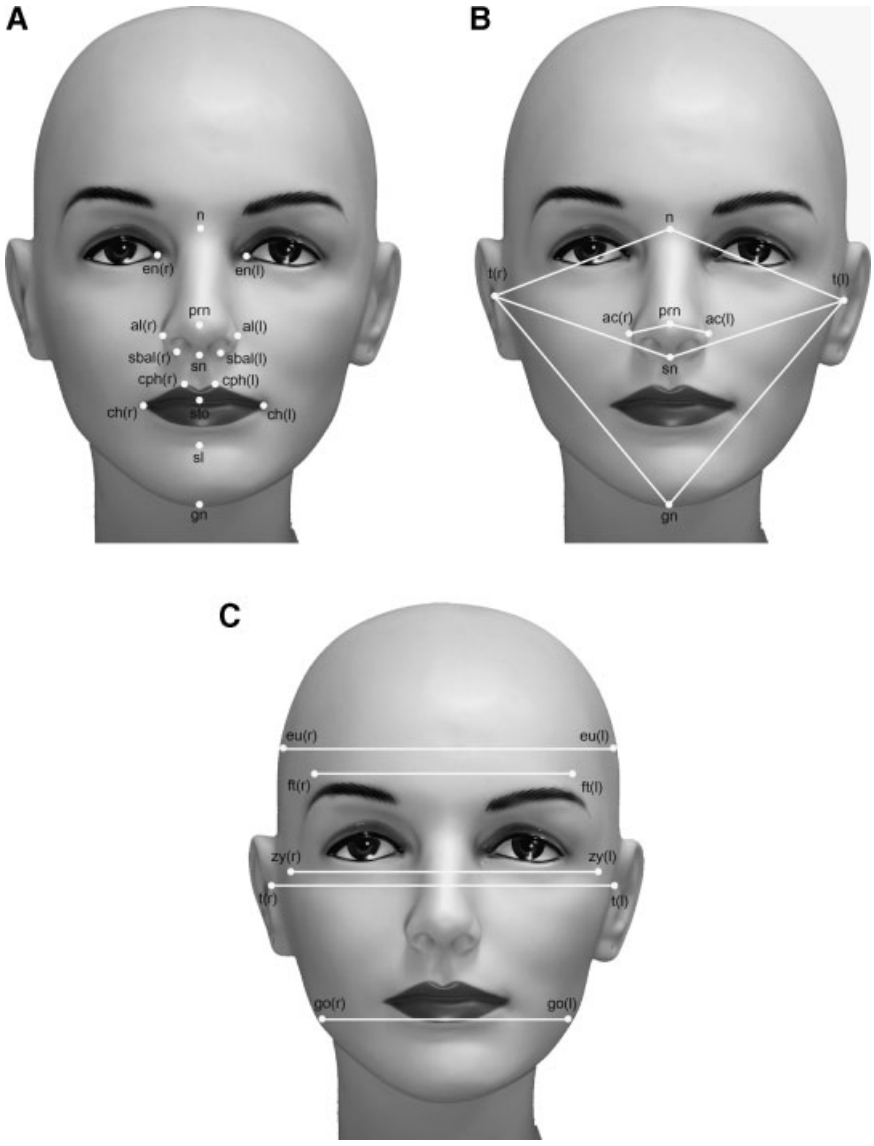

FIG. 1. **A**: The sixteen surface landmarks derived from the frontal 3D capture and subjected to Euclidean distance matrix analysis. **B**: Set of eight linear distances derived from the left and right 3D captures: upper face depth (t-n), midface depth (t-sn), lower face depth (t-gn) and ala length (ac-prn). **C**: Additional linear distances derived through caliper-based direct anthropometry: maximum head width (eu-eu), forehead width (ft-ft), upper face width (zy-zy), cranial base width (t-t) and mandible width (go-go). Maximum head length (g-op) is not depicted. Landmark definitions: n, nasion; en, endocanthion; prn, pronasale; al, alare; sbal, subalare; sn, subnasale; cph, crista philtri; ch, chelion; sto, stomion; sl, sublabiale; gn, gnathion; t, tragion; ac, alar curvature point; eu, euryon; ft, frontotemporale; zy, zygion; go, gonion; g, glabella (not depicted) and op, opisthocranion (not depicted).

cranial base width (*t-t*), mandible width (*go-go*) and maximum head length (*g-op*) (Fig. 1C). In general, these additional caliper measurements involved landmarks that could not be located using indirect methods (e.g., due to hair coverage) and/or were too large to be obtained from any single 3D capture. Intraobserver measurement error (intraclass correlation coefficients) for the caliper-derived measurements was excellent (mean = 0.984; range = 0.965 to 0.992). Furthermore, linear distances obtained from calipers and 3D captures using the same landmarks have been shown to be highly congruent [Weinberg et al., 2004, 2006c].

Statistical Methodology

Data analysis was divided into two phases. In the first phase, the aim was to identify a subset of anthropometric variables that differ significantly between unaffected relatives and matched controls. To accomplish this goal, two different statistical approaches were utilized based on the type of data available (3D landmark coordinates or linear distances). The 16 raw *x,y,z* landmark coordinates derived from the frontal 3D captures were subjected to Euclidean Distance Matrix Analysis (EDMA), an exploratory morphometric approach designed to facilitate the comparison of form/shape between groups of organisms [Lele and Richtsmeier, 2001]. In EDMA, the form of an object is defined by the complete set of linear distances between all possible landmark pairs; thus, for a form comprised of 16 landmarks, there will be 16(16−1)/2 or 120 linear distances. Form variables are converted into shape variables by scaling an object's set of linear distances by its geometric mean. Mean shapes are then constructed by averaging across all scaled homologous distances within a given group. The statistical comparison of shape is based on the arithmetic differences between the mean set of scaled homologous linear distances for each group. Statistical testing with EDMA is performed in both an omnibus and element-wise fashion. In the former test, the goal is to determine whether two mean shapes are equivalent overall. In the latter test, the goal is to facilitate the discovery of regions where shape differences are most apparent between groups. For both types of tests, statistical significance is determined by generating empirical confidence intervals via random bootstrap re-sampling routines. Based on the recommendations set forth by Lele and Richtsmeier [1995], 90% confidence intervals were used for all statistical tests. Importantly, because EDMA is based on a definition of form that is invariant to the nuisance factors of translation, rotation and reflection, it does not require the registration of landmark data for all objects into a common coordinate system prior to the comparison of shape, unlike superimposition-based approaches. All calculations and tests were performed using the shape module within the EDMA statistical package for Windows, WinEDMA 1.0.1 [Cole, 2003]. For a complete discussion of EDMA and its statistical methodology see Lele and Richtsmeier [2001] and Richtsmeier et al. [2002].

The additional set of 14 linear distances derived from the left/right 3D captures and direct anthropometry were compared between relatives and controls with standard univariate statistics. Prior to analysis, the raw linear distances for each subject were scaled by their geometric mean. The scaled data were then checked for normality and screened for outliers. Fourteen separate independent-sample *t* tests were performed, with an alpha level of at least 0.05 required to attain statistical significance. No correction for multiple testing was applied, given the exploratory nature of the analysis. For both EDMA and the *t* tests, the comparison between unaffected relatives and matched controls was carried out separately for each sex. This decision was based on both the findings of previous studies and preliminary results showing that relative-control craniofacial differences are influenced to some degree by sex [Kurisu et al., 1974; Weinberg et al., 2006b].

The goal of the second phase of the analysis was to develop a multivariate model to facilitate the classification of unaffected relatives into risk categories. To this end, a two-group discriminant function analysis (DFA) was carried out on the same relative-control samples utilized in phase one. Based on the recommendations of Duarte Silva and Stam [1966] and the methods used in previous discriminant analyses [Suzuki et al., 1999; McIntyre and Mossey, 2003], variables found to differ significantly between unaffected relatives and controls were entered into the DFA (EDMA, $P \leq 0.10$; *t*-test, $P \leq 0.05$). Limiting the number of variables for inclusion in the DFA both ensures a more parsimonious model and substantially increases the subject to variable ratio. Separate discriminant functions were generated for males and females, each utilizing the set of sex-specific variables identified as statistically significant in phase one. In each discriminant analysis, all predictor variables were entered simultaneously as a single block. Because only two groups were involved, a single discriminant function for each sex was estimated. Prior to performing the DFA, all variables were checked for normality, multicollinearity, equality of variance-covariance matrices and outliers.

The assignment of relatives to risk classes was based on the classification results and Mahalanobis distance statistics generated from the DFA. In the present context, the Mahalanobis distance statistic provides a quantitative estimate of how divergent each unaffected relative is from the control group mean or centroid, based on a combination of informative craniofacial features. Because distance is

measured in standard deviation units, a threshold for risk allocation can be established on statistical grounds; risk status would be determined by an individual's location relative to the threshold. A Mahalanobis distance greater than 1.96 from a given centroid would indicate a less than 5% chance of an individual belonging to that particular group [Meyers et al., 2006]. Therefore, an unaffected relative had to meet two criteria from the DFA to be considered "at-risk": (1) they had to be correctly classified as a relative, and (2) they had to have a Mahalanobis distance greater than 1.96 from the control group's centroid. This dual criterion helps ensure that only those unaffected relatives with facial features substantially different from controls will be assigned elevated risk status. All statistical analyses were carried out in SPSS v11.5 (Chicago, IL).

RESULTS

Female Relatives Versus Controls

EDMA revealed that the shape of the face differed between female unaffected relatives and female controls and that these shape differences were localized to a particular facial region. Figure 2A shows a subset of 40 variables, along with their respective 90% confidence intervals, demonstrating the greatest difference between relatives and controls out of a possible 120 shape variables. Only three of these 40 scaled linear distances were significantly ($P < 0.10$) larger in female unaffected relatives compared with controls: (1) the distance between the right and left *alare* points, what is traditionally called soft tissue nose width; (2) the distance between the left *alare* and the left *subalare*; and (3) the distance between the right *alare* and the right *subalare* (Fig. 3A). The magnitude of the relative-control difference for these three linear distances was 3, 2, and 2 mm, respectively. All three dimensions involve the landmark *alare* and, in combination, suggest that lateral displacement of the alar cartilage (relative to controls) may be a characteristic facial feature of the female unaffected relatives of CL/P individuals.

Regarding the 14 additional scaled linear distances from the left and right 3D captures and direct

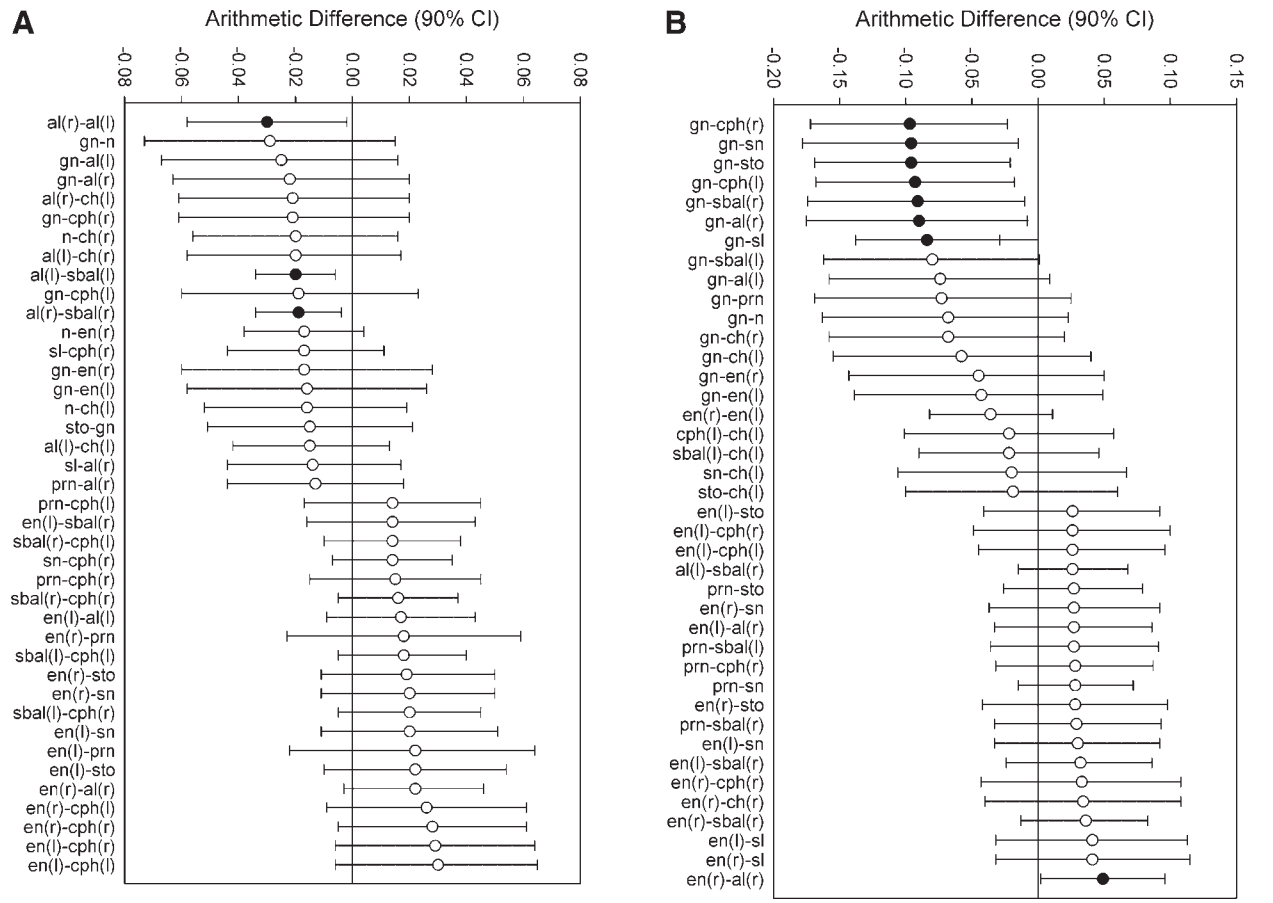

FIG. 2. Arithmetic difference between mean scaled linear distances for unaffected relatives and controls accompanied by 90% confidence intervals for (**A**) males and (**B**) females. The difference estimates are sorted according to magnitude, with only the upper and lower 20 values represented out of a possible 120 variables. Values to the left of the baseline (marking zero or no difference) represent linear distances larger in relatives compared to controls, whereas values to the right of the baseline represent linear distances smaller in relatives compared to controls. Filled circles indicate a significant difference between relatives and controls.

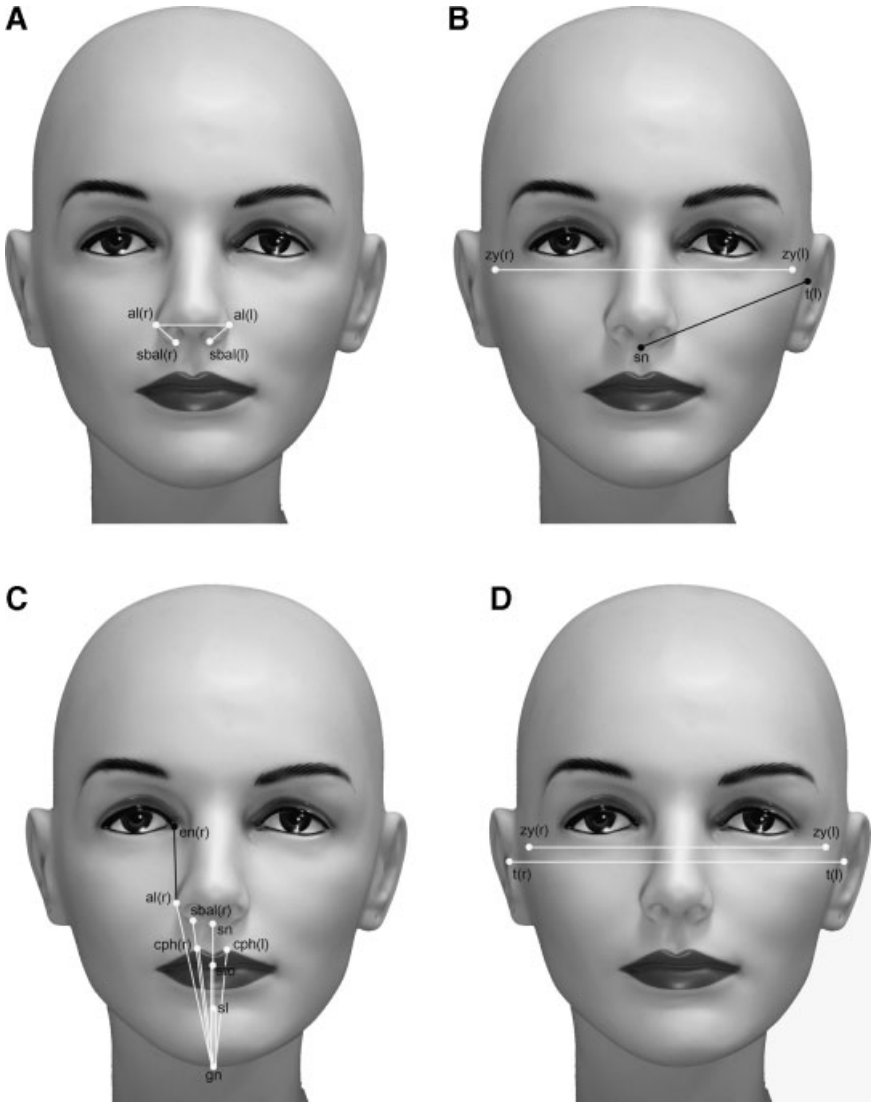

FIG. 3. Significant craniofacial differences between unaffected relatives and controls. **A**: EDMA results for females (**B**) univariate *t*-test results for females (**C**) EDMA results for males (**D**) univariate *t*-test results for males. See Figure 1 caption for landmark definitions.

anthropometry, two were found to differ significantly ($P < 0.05$) between female unaffected relatives and controls from *t* tests (Fig. 3B). Specifically, female unaffected relatives possessed significantly greater upper facial width ($P = 0.02$) and significantly reduced left midfacial depth ($P = 0.04$). The effect sizes (Hedges *g*) for these two dimensions were moderate at 0.57 and −0.51, respectively. Effect sizes for the remaining 12 variables were in the small (0.20–0.49) to very small (0–0.20) range. Nonsignificant trends ($0.05 > P > 0.10$) toward increased mandibular width, reduced head length and reduced right midfacial depth were also observed in the unaffected relative group.

In phase two of the analysis, one variable (left *alare-subalare* distance) was dropped prior to performing the DFA due to excess multicollinearity. Each of the remaining four variables was observed to be distributed normally. Moreover, the assumption of homogeneity of the variance-covariance matrices between the unaffected relative and control groups was met (Box's *M* test; $P = 0.583$) and no multivariate outliers were detected. Using these four variables, a single statistically significant discriminant function was derived ($\Lambda = 0.81$; $\chi^{2\ (4df)} = 12.76$; $P = 0.01$), indicating that the combination of these four predictor variables was capable of differentiating between female unaffected relatives and female controls. A canonical correlation of 0.43 was observed, indicating that the discriminant function was able to account for 18.5% (squared canonical correlation) of the variance in the outcome variable. Overall, 71% of relatives and controls were classified correctly. Broken down by group, 70% of relatives and 73% of controls were classified correctly (Table I). Empirical cross-validation (Jackknife routine) of the

TABLE I. Classification Accuracy of Discriminant Functions

	Predicted group			
	Female		Male	
	Relative	Control	Relative	Control
Actual group				
Relative	23 (69.7%)	10 (30.3%)	12 (85.7%)	2 (14.3%)
Control	9 (27.3%)	24 (72.7%)	1 (7.1%)	13 (92.9%)
	Overall correct classification = 71.2%		Overall correct classification = 89.3%	
	Jackknife reclassification = 67.0%		Jackknife reclassification = 86.0%	

classification procedure resulted in a slight reduction in the overall classification accuracy to 67%. The ability of the discriminant function to classify cases into their correct group was significantly better than expected by chance (Press's $Q = 29.33$; $P < 0.001$). The mean biological distance (Mahalanobis D^2) of female relatives from the centroid of the control group was 1.83 standard deviations. Evaluation of the structure matrix coefficients revealed that maximum face width had the highest loading on the discriminant function ($r = 0.61$), followed in decreasing order by right *alare-subalare* length ($r = 0.57$), left midface depth ($r = -0.55$) and nose width ($r = 0.53$). The narrow range of values for these coefficients suggested that all four predictor variables approximated one another in terms of their substantive importance to the discriminant function.

Based on the DFA results, 11 out of 33 female unaffected relatives (33%) were classified as potentially "at-risk" individuals (Fig. 4).

Male Relatives Versus Controls

As with females, EDMA indicated that face shape differed between male unaffected relatives and male controls. However, these shape differences were generally of greater magnitude and were localized to different facial regions. Figure 2B shows those of variables demonstrating the greatest difference between male relatives and controls. Out of a possible 120 shape variables, eight linear distances were identified as significantly ($P < 0.10$) different: *al(r)-gn*, *sbal(r)-gn*, *cph(r)-gn*, *cph(l)-gn*, *sn-gn*, *sto-gn*, *sl-gn* and *al(r)-en(r)* (Fig. 3C). Seven of these distances were larger in unaffected relatives and represent aspects of lower facial height; all involve the landmark *gnathion*. The magnitude of the relative-control difference for these seven distances ranged from 8 to 10 mm. A single linear distance was significantly smaller (5 mm) in male relatives—the distance between *endocanthion* and

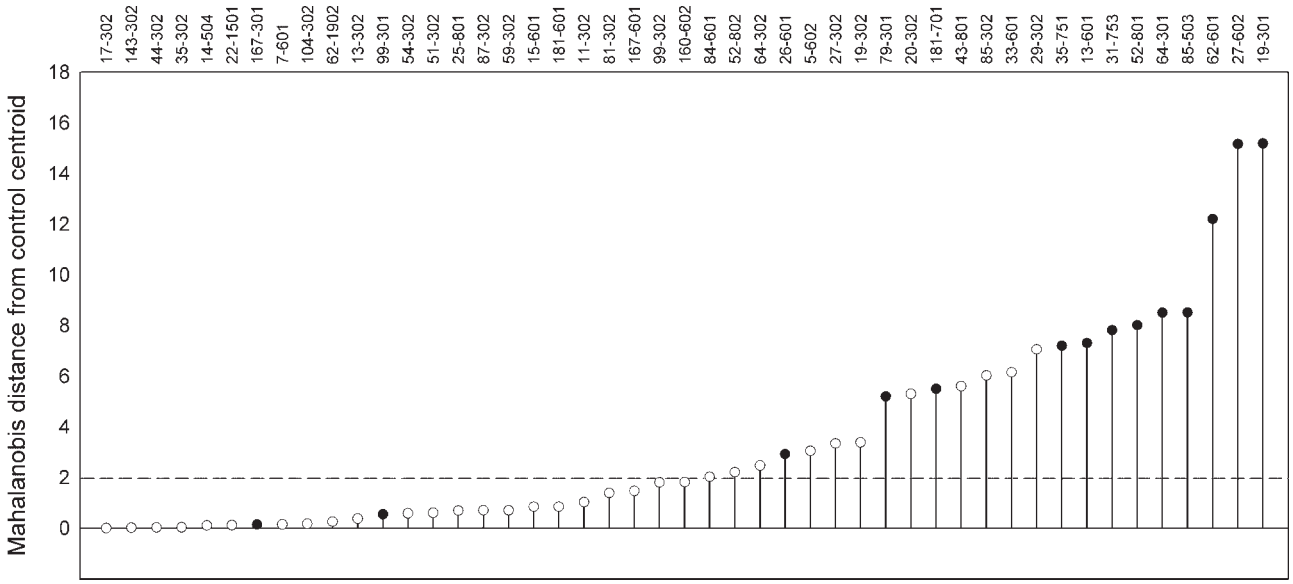

FIG. 4. Mahalanobis distances of all 47 unaffected relatives (33 females and 14 males) from their sex-specific control centroids as determined from discriminant analysis. The unbroken baseline at zero represents the control group centroid, with each relative sorted along the *x*-axis according to their distance in standard deviations from the centroid. The broken line represents the threshold marking 1.96 standard deviations units. Relatives beyond this threshold were considered particularly "at-risk" based on their craniofacial features. Filled circles represent males and open circles signify females.

alare on the right. Although not statistically significant, several additional linear distances involving *endocanthion* (mostly right) were also observed to be reduced in unaffected relatives (Fig. 2B). These distances vertically span the upper/midface region, indicating a strong trend toward reduction in upper/mid facial height, possibly with a more marked expression on the right side of the face.

For the 14 additional linear distances, similar to females, male unaffected relatives were characterized by significantly increased upper face width ($P < 0.001$) and cranial base width ($P = 0.04$) compared to male controls (Fig. 3D), however, unlike female relatives, there were no observed differences in midfacial depth. The effect sizes for these two variables were quite large: 1.47 for upper face width and 0.79 for cranial base width. In general, effect size magnitudes were greater for males than females.

Prior to performing the DFA, 5 out of 10 of the initially informative variables were dropped due to excessive multicollinearity: *al(r)-gn*, *sbal(r)-gn*, *cph(r)-gn*, *cph(l)-gn*, and *sto-gn*. All remaining variables—upper face width, cranial base width, lower face height (*sn-gn*), chin height (*sl-gn*) and right *alare-endocanthion* length—were observed to be distributed normally. Homogeneity of the variance-covariance structure across groups was verified (Box's *M* test; $P = 0.632$) and no multivariate outliers were detected. Based on these five predictor variables, a single statistically significant discriminant function was derived ($\Lambda = 0.38$; $\chi^{2\ (5df)} = 23.05$; $P < 0.001$). A large canonical correlation of 0.79 was observed, indicating that the discriminant function was able to account for as much as 62.6% of the variance in the outcome variable. The classification accuracy was very high; 86% of male relatives and 93% of male controls were classified correctly, with an overall correct classification rate of 89% (Table I). The Jackknife cross-validation classification procedure resulted in an overall accuracy of 86%. As with the female sample, the ability of the discriminant function to assign male cases to their correct group was significantly better than expected by chance (Press's $Q = 17.29$; $P < 0.001$). The average Mahalanobis distance of male relatives from the centroid of the control group was 7.44 standard deviations. Evaluation of the structure matrix coefficients revealed that upper face width had by far the highest discriminant function loading ($r = 0.61$), followed in decreasing order by chin height ($r = 0.34$), cranial base width ($r = 0.33$), right *alare-endocanthion* length ($r = -0.32$) and lower face height ($r = 0.30$). The similarity in coefficient values for the four latter variables suggests that their substantive importance to group discrimination was more or less equivalent.

Based on the DFA results, 12 out of 14 male unaffected relatives (86%) were classified as potentially "at-risk" individuals (Fig. 4).

DISCUSSION

Using a combination of 3D photogrammetry and direct anthropometry, the present study compared aspects of craniofacial shape between unaffected first-degree relatives from multiplex nonsyndromic cleft families and demographically matched unaffected controls. Both unaffected male and female relatives were observed to differ in overall craniofacial shape compared to sex- and age-matched controls. However, these shape changes were localized to specific regions of the face in a partially sex-specific manner. Compared with controls, female relatives were characterized by increased soft tissue nose width, increased upper face width and excess midface retrusion. Based on these characteristics, discriminant analysis was able to classify over 70% of female relatives and controls correctly. In males, unaffected relatives demonstrated increased lower face height, decreased upper face height (mostly right side), and increased upper face and cranial base width compared to controls. Discriminant analysis indicated substantial divergence in craniofacial morphology between male relatives and controls, which was reflected in the 89% overall correct classification rate. For both males and females, upper face width had the largest impact on group discrimination.

An attempt was made to distinguish between those relatives with elevated susceptibility to CL/P and those at nominal risk. A central assumption of this risk allocation approach was that those relatives with the highest liability to CL/P will also have the most divergent craniofacial phenotype from controls. One-third of the female relatives were classified as potentially "at-risk," compared to over 80% of male relatives. This disparity was not surprising given the magnitude of the differences in craniofacial morphology between male relatives and controls. Moreover, the higher proportion of males in the "at-risk" group also makes sense in light of the roughly 2:1 male bias in CL/P observed in most populations [Mossey and Little, 2002].

On the whole, the main findings of the present study largely agree with earlier reports comparing craniofacial morphology in unaffected relatives of CL/P individuals to controls. For example, numerous previous studies report excess upper face width [Fraser and Pashayan, 1970; Nakasima and Ichinose, 1983; Blanco et al., 1992; Suzuki et al., 1999; McIntyre and Mossey, 2004; Yoon et al., 2004; Weinberg et al., 2006b], increased soft tissue nose or nasal cavity width [Fraser and Pashayan, 1970; Figalová and Šmahel, 1974; Nakasima and Ichinose, 1983; Sato, 1989; Raghavan et al., 1994; AlEmran et al., 1999; Suzuki et al., 1999; Yoon et al., 2004; Weinberg et al., 2006b], excess lower face height [Shibasaki et al., 1978; Nakasima and Ichinose, 1983; Sato, 1989; Ward et al., 1989; Weinberg et al., 2006b], reduced upper

face height [Coccaro et al., 1972; Kurisu et al., 1974; Shibasaki et al., 1978; Nakasima and Ichinose, 1983; Raghavan et al., 1994; McIntyre and Mossey, 2003; Perkiomaki et al., 2003; Chatzistavrou et al., 2004; Weinberg et al., 2006b] and increased midface retrusion [Dixon, 1966; Fraser and Pashayan, 1970; Coccaro et al., 1972; Figalová and Šmahel, 1974; Kurisu et al., 1974] in unaffected relatives compared with controls. Although disagreements still exist, such concordance across studies of varying design provides compelling evidence that the phenotypic consequences of increased liability to orofacial clefting can be reliably detected in the unaffected relatives of affected individuals through a quantitative assessment of craniofacial shape.

Even though the morphogenetic factors underlying variation in human craniofacial shape are complex and still poorly understood, many of the features present in unaffected relatives are biologically plausible as risk markers for orofacial clefting. It has been hypothesized, for example, that excess width of the face during embryogenesis could lead to a scenario whereby adjacent facial prominences would be less likely to contact one another [Smiley et al., 1971; Chung and Kau, 1985; Siegel and Mooney, 1986]. However, many reports describing embryonic facial morphology in cleft susceptible mouse strains actually find evidence of *reduced* facial and frontonasal width [Trasler, 1968; Millicovsky et al., 1982; Jacobson and Trasler, 1992; Wang and Diewert, 1992]. To more confuse matters, studies of face shape in *adults* from these same mouse strains confirm the presence of excess face width, similar to that seen in studies of unaffected relatives in CL/P families [Hallgrímsson et al., 2004]. Clearly more research is needed to sort out the root mechanisms underlying variation in facial width across ontogeny.

Documented changes in the growth, shape and/or position of other facial regions in cleft susceptible mice may offer additional clues. For example, A/WySn mice are reported to show reduced outgrowth of the maxillary prominences [Wang and Diewert, 1992; Young et al., 2007]. Such a reduction would likely cause the presumptive maxillary and nasal components to be more divergent from one another during the critical period of primary palate formation, ultimately decreasing the likelihood of contact and fusion [Diewert and Lozanoff, 2002]. Interestingly, Trasler and Machado [1979] reported a decrease in the anterior growth of the maxilla in both *newborn* and *adult* mice susceptible to CL, suggesting that midface reduction may persist as a risk marker. These results are consistent with the hypothesis that intrinsic mesenchymal deficiency is a primary factor in the etiopathogenesis of CL/P. An analogous reduction in the maxillary prominences may take place in humans susceptible to CL/P; this could help explain some of the observed phenotypic differences in unaffected relatives such as reduced midface advancement and/or reduced upper face height.

Any interpretation of the present results must be considered within the context of this study's limitations. One major caveat relates to the method used to assign unaffected relatives to risk classes. The "at-risk" designation was based on two criteria from the DFA: (1) whether relatives were correctly classified and (2) the degree of phenotypic divergence from the control sample. An important feature of this method of risk allocation is that each relative is able to be assigned to a class on a case by case basis. However, the distinction between these two classes is somewhat arbitrary since it is based partly on the dichotomization of a continuously distributed variable (Mahalanobis D^2) on statistical grounds. It is also important to keep in mind that the designation "at risk" is based entirely on an assessment of *phenotypic* features. Because the relationship between genotype and facial phenotype is extremely complex, it is uncertain exactly how these risk categories relate to genetic susceptibility for CL/P. It is only by incorporating this phenotypic information into formal genetic analyses that we can even begin to evaluate such claims.

Additional major limitations of the current study were directly related to the small sample sizes available. This factor likely resulted in a significant loss of power to detect more subtle craniofacial differences and precluded the independent validation of the discriminant function models. Traditionally, validation is accomplished by splitting the total sample into an initial model building group and a holdout validation group. We attempted to mitigate this problem by employing an empirical validation technique using Jackknife re-sampling. However, this method is suboptimal compared with split sample or independent sample validation approaches. Furthermore, the statistical models were likely affected by the fact that the choice of traits to enter into the DFA was based on prior statistical testing in the same samples. This in no way violates the integrity of the discriminant models, per se, but can affect the generalizability of the results. Independent sample validation is required to test whether or not the discriminant model parameters obtained here are over-fitted to the data.

Despite these limitations, we believe this study represents a major advance through its use of advanced 3D imaging technology and statistical shape analysis. Goals for future studies will include the validation and replication these findings in an independent sample of unaffected relatives from multiplex cleft families, extension these methods to simplex families, and development of methods to incorporate these morphometric findings into genetic association and linkage analyses [Mossey et al., 1998b].

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