Family Aggregation of Upper Airway Soft Tissue Structures in Normal Subjects and Patients with Sleep Apnea

Richard J. Schwab, Michael Pasirstein, Laura Kaplan, Robert Pierson, Adonna Mackley, Robert Hachadoorian, Raanan Arens, Greg Maislin, and Allan I. Pack

Center for Sleep and Respiratory Neurobiology; Pulmonary, Allergy, and Critical Care Division; and Division of Sleep Medicine, Department of Medicine, University of Pennsylvania Medical Center, Philadelphia, Pennsylvania

Rationale: **Sleep apnea is believed to be a genetic disorder. Thus, we hypothesized that anatomic risk factors for sleep apnea would demonstrate family aggregation.**

Objectives: **We used volumetric magnetic resonance imaging in a sib pair "quad" design to study the family aggregation of the size of upper airway soft tissue structures that are associated with increased risk for obstructive sleep apnea.**

Methods: **We examined 55 sleep apnea probands (apnea–hypopnea index [AHI]: 43.2 ± 26.3 events/h), 55 proband siblings (AHI: 11.8 ± 16.6 events/h), 55 control subjects (AHI: 2.1 ± 1.7 events/h), and 55 control siblings (AHI: 4.2 ± 4.0 events/h). The study design used exact matching on ethnicity and sex, frequency matching on age, and statistical control for visceral neck fat and craniofacial dimensions.**

Measurements and Main Results: **The data support our *a priori* hypothesis that the volume of the important upper airway soft tissue structures is heritable. The volume of the lateral pharyngeal walls (h^2 = 36.8%; p = 0.001), tongue (h^2 = 36.5%; p = 0.0001), and total soft tissue (h^2 = 37.5%; p = 0.0001) demonstrated significant levels of heritability after adjusting for sex, ethnicity, age, visceral neck fat, and craniofacial dimensions. In addition, our data indicate that heritability of the upper airway soft tissue structures is found in normal subjects and patients with apnea. Thus, it is not simply a consequence of the prevalence of apnea.**

Conclusions: **This is the first time family aggregation of size of the upper airway soft tissue structures has been demonstrated.**

Keywords: family aggregation; genetics; magnetic resonance imaging; obstructive sleep apnea; upper airway

Obstructive sleep apnea is a serious public health disorder that affects at least 4% of middle-aged men and 2% of middle-aged women and is associated with significant cardiovascular and neurophysiologic morbidity (1–4). Although obstructive sleep apnea is an important clinical problem, we presently possess only fragmentary knowledge about the genetic risk factors for this disorder. Evidence is accumulating, however, that there are genetic risk factors for sleep apnea. There are several disorders with single-gene, Mendelian genetic abnormalities, or chromosomal defects, in which there is an increased prevalence of sleep-disordered breathing (5). Even in the absence of a Mendelian disorder, obstructive sleep apnea has been shown to cluster in families such that family members of patients with sleep apnea have an increased relative risk of obstructive sleep apnea (5, 6). This increased risk is not simply explained by obesity, a known risk factor for sleep apnea, because the increased relative risk is found even after controlling for body mass index (BMI) as a covariate and for relatives of nonobese subjects with apnea (6, 7). Inheritance patterns of obstructive sleep apnea in whites and African Americans have demonstrated a recessive mode of inheritance with a single major gene accounting for about 20% of the variance (8). There are also preliminary linkage studies showing specific areas of the genome that are linked to sleep apnea as a quantitative trait (9, 10). Thus, there is persuasive evidence that there are genetic risk factors for sleep apnea.

If sleep apnea has a genetic component, what are the intermediate traits associated with this condition? Several studies have demonstrated family aggregation of craniofacial morphology in patients with sleep apnea (7, 11). Although there are likely to be a number of other intermediate traits associated with sleep apnea, the focus of this investigation was on factors related to upper airway structure. We previously identified in a case-control study (12) using novel three-dimensional volumetric analysis techniques with magnetic resonance imaging (MRI) that the volume of several of the upper airway soft tissue structures is larger in subjects with obstructive sleep apnea than in control subjects. In this case-control study (12), our validated MRI and computer-based analysis techniques (13) quantified the volume of the tongue, soft palate, parapharyngeal fat pads, and lateral pharyngeal walls. The volume of a number of upper airway soft tissue structures, specifically the tongue, lateral pharyngeal walls, and total soft tissue, emerged as being statistically significantly associated ($p < 0.0001$) with the presence of apnea, even after controlling for age, race, sex, amount of visceral neck fat, and overall craniofacial dimensions (12).

Although our previous investigation (12) identified anatomic differences in upper airway soft tissue structures in patients with obstructive sleep apnea, it did not determine if the size of these upper airway soft tissue structures demonstrate family aggregation and hence might contribute to the genetic risk for sleep apnea. To examine this question, we performed volumetric MRI of the upper airway in probands with obstructive sleep apnea, proband siblings, control subjects, and control siblings, all matched on sex and ethnicity. We hypothesized that the size of the upper airway structures (volume of the tongue, lateral pharyngeal walls, and total soft tissue) would demonstrate significant family aggregation. We also examined intraclass correlations of the size of the upper airway soft tissue structures independently for the probands/proband siblings and control subjects/control siblings to determine if the family aggregation of these structures is different in normal subjects than in patients with apnea. Some of the results of our study have been previously reported in the form of an abstract (14). Some of the data

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Correspondence and requests for reprints should be addressed to Richard J. Schwab, M.D., Center for Sleep and Respiratory Neurobiology, 893 Maloney Building, University of Pennsylvania Medical Center, 3600 Spruce Street, Philadelphia, PA 19104-4283. E-mail: rschwab@mail.med.upenn.edu

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from the probands and control subjects have been previously reported (12).

METHODS

See the online supplement for additional methods.

Subjects

We used a sib pair "quad" design with four subject groups (*see* Figure 1): (*1*) probands (patients with obstructive sleep apnea); (*2*) same-sex siblings of proband within 10 yr of the age of the proband; (*3*) control subjects (normal subjects), matched to the proband for sex, ethnicity, and living in the neighborhood (same school district) of the matched proband; and (*4*) same-sex siblings of control subjects within 10 yr of the age of the control subject. Age was also included as a covariate in all analyses to account for the effect of any residual differences related to age. To qualify as a "proband," patients had to have an apnea–hypopnea index (AHI) greater than 15 events/h and have a same-sex sibling within 10 yr of age. Control subjects, matched for sex, ethnicity, and school district, were required to have an AHI of less than 5 events/h, confirmed with an overnight sleep study. We report data on 220 subjects (55 probands and their siblings and 55 control subjects and their siblings). Forty-eight of these probands and 48 control subjects were the basis for our previously reported case-control study (12). *See* online supplement for additional information about subjects.

Polysomnography

Standard polysomnography procedures and scoring were performed, as previously described from our laboratory (15). *See* online supplement for additional information about sleep study methodology and definitions of events.

MRI

Upper airway imaging was performed identically in all subjects using a 1.5-Tesla MR scanner to obtain spin-echo axial and sagittal images.

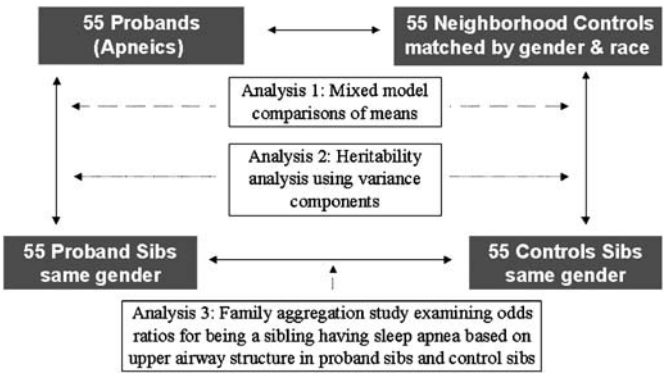

Figure 1. A schematic of the sib pair "quad" design with four subject groups: (*1*) probands (patients with obstructive sleep apnea); (*2*) same-sex siblings of proband within 10 yr of the age of the proband; (*3*) control subjects (normal subjects), matched to the proband for sex and ethnicity and had to live in the neighborhood (same school district) of the matched proband; (*4*) same-sex siblings of control subject within 10 yr of the age of the control subject. Family aggregation of the airway and soft tissue risk factors was assessed with three analysis strategies. The first analytic approach compared mean values across subject groups (proband, proband sibling, control subject, control sibling) taking into account the sampling by family within quad using mixed-model analyses of variance (ANOVA). The second analysis approach used an analogous mixed-model ANOVA but focused on the variance components to quantify the degree of familial aggregation (heritability) for each measurement. The third analysis approach examined odds ratios for being a sibling having sleep apnea based on upper airway structure in proband sibs and control sibs.

The volumetric upper airway reconstructions were performed from the axial images using techniques previously described by us (12). The imaging protocols were identical to those used in our previous MRI studies (12–15).

Anatomic Definitions, Measurements, and Analysis

The primary analysis of the MR dataset involved examining three-dimensional volumes of the following structures: lateral pharyngeal walls (analysis of the lateral walls was only performed in the retropalatal and retroglossal regions); soft palate; tongue (genioglossus muscle and then, separately, the entire tongue, including genioglossus, geniohyoid, hyoglossus, myohyoid, digastric, and myohyoideus muscles); parapharyngeal fat pads; and finally, total soft tissue, which includes all the measured soft tissues (Figure 2). We also performed secondary analyses on the heritability of measurements of the size of the upper airway itself (volume, minimum cross-sectional area, lateral and anteroposterior dimensions), and two-dimensional soft tissue measurements. The upper airway anatomic definitions, measurements, and analysis strategies were identical to those used in our previous MRI study (12) and our analysis techniques have been validated previously (13).

Statistical Analysis

Our design used exact matching on sex and race, frequency matching on age, and statistical control for craniofacial structure and visceral (i.e., parapharyngeal fat in the neck). We controlled for craniofacial size in the analysis by measuring mandibular width (lateral head measurement) and by measuring from the teeth (at the occlusal plane of the teeth) to the posterior subcutaneous tissue (an anteroposterior head measurement). For measurements of nonfat structures, we controlled for visceral fat in the neck (volume of parapharyngeal fat), because we believed this would be a superior measure of adiposity in the neck than BMI, which can be affected by fat in other locations.

Our primary *a priori* hypothesis for this investigation was that the volume of upper airway soft tissue structures would demonstrate heritability. Therefore, the volumes of the soft tissue structures were selected as the primary analysis variables (volume of the soft palate, tongue, lateral pharyngeal walls, and total soft tissue). The significance levels of these four primary endpoints were adjusted using a Bonferroni-corrected p value of $0.05/4 = 0.0125$ in the heritability analysis. The airway measurements and two-dimensional measurements were assessed in secondary exploratory analyses. Findings for the remaining variables in the other analyses were considered hypothesis-generating and required confirmation in an independent dataset.

Family aggregation of the airway and soft tissue risk factors were assessed using three complementary analysis strategies (*see* Figure 1). The first analytic approach focused on comparing mean values across subject groups (proband, proband sibling, control subject, control sibling) taking into account the sampling by family within quad using mixed-model analyses of variance with parameters estimated by restricted maximum likelihood (16). All models included the following variance components: (*1*) between-quad matches, (*2*) families within quads, and (*3*) residual error. Group differences in mean values were estimated with and without controlling for age, sex, craniofacial dimensions, and ethnicity, and then adding adjustment for visceral neck fat in volume of parapharyngeal fat pads. If the group differences were significant ($p < 0.05$), then pairwise contrasts between subject groups (proband vs. proband sib and control vs. control sib [within-family comparisons], proband vs. control and proband sibling vs. control sibling [between-family comparisons]) were assessed.

The second analysis approach used an analogous mixed-model analysis of variance but focused on the variance components to quantify the degree of familial aggregation (heritability) for each measurement. The variance components (between-quad matches, families within quads, and residual error) were used to estimate (broad-sense) heritability as $h^2 = 100\% \times \sigma^2_{family(quad)}/(\sigma^2_{family[quad]} + \sigma^2_{quad} + \sigma^2_{error})$. This is the percentage of total variance around the mean of the phenotype measure explained by systematic variance between families, taking into account the matching of families by quads. Then, sex, age, craniofacial size, ethnicity, and visceral neck fat were added as fixed effects and h^2 recomputed.

The third analysis approach used a reconstituted cohort design (17) in which we compared proband siblings with control siblings. Multiple

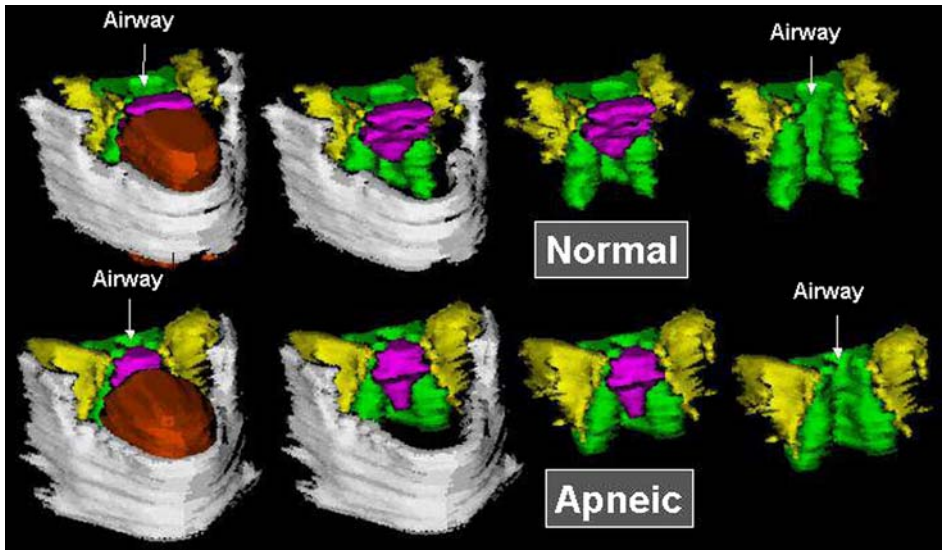

Figure 2. Volumetric reconstructions from a series of 3-mm contiguous axial magnetic resonance (MR) images of the mandible (*gray*), tongue (*orange/rust*), soft palate (*pink/purple*), lateral parapharyngeal fat pads (*yellow*), and lateral/posterior pharyngeal walls (*green*) in a normal subject (*top panel*) and in a patient with sleep apnea (*bottom panel*). The upper airway is larger in the normal subject than in the patient with apnea. In addition, the tongue, lateral parapharyngeal fat pads, and lateral pharyngeal walls are larger in the patient with apnea.

logistic regression models were used to obtain adjusted odds ratios (ORs) for having a sibling with sleep apnea to quantify the relative magnitudes among soft tissue structures, proband sibling versus control sibling differences. For each soft tissue measure, an adjusted OR and 95% confidence interval (CI) were computed that expressed the relative likelihood of having a sibling with sleep apnea for each one (control sibling) standard deviation (SD) increase in the size of soft tissue measurements. The reconstituted cohort analyses were performed to both confirm the familial aggregation findings and also to facilitate assessment of the association between soft tissue structures and apnea risk among individuals who were not selected on the basis of sleep apnea. This third analysis approach can provide evidence that the observed soft tissue changes are likely a cause and not a consequence of apnea.

RESULTS

See online supplement for additional results.

Demographics of Probands, Proband Siblings, Control Subjects, and Control Siblings

Our quad study design consisted of 55 sets each containing a proband, a sibling of this proband, a matched control subject, and a control sibling (*see* Figure 1). Quads were 49.1% male. In addition, they were 45.5% white, 49.1% African American, 3.6% Asian, and 1.8% Hispanic. As a consequence of matching by age, group differences in ages were relatively small. Although there were significant group age differences ($p = 0.02$; *see* Table 1), examination of the paired contrasts for age did not demonstrate significant differences in the age of probands and proband sibs ($p = 0.64$), probands and control subjects ($p = 0.08$), or control subjects and control sibs ($p = 0.30$); however, there were significant differences between the age of the proband sibs and control sibs ($p = 0.02$). Nonetheless, all major analyses included age as a covariate to control for residual age differences.

Probands were required to have an AHI of 15 or greater (mean AHI: 43.2 ± 26.3 events/h) and control subjects an AHI of less than 5 (mean AHI: AHI: 2.1 ± 1.7 events/h; *see* Table 1). Proband siblings had a mean AHI of 11.8 ± 16.6 events/h and the control siblings had a mean AHI of 4.2 ± 4.0 events/h. Thus, proband siblings had an intermediate AHI between the probands and control subjects. There was a significant group difference in BMI across groups (*see* Table 1), although many subjects in all groups were overweight. The BMI of the proband siblings was intermediate between that of the probands and the control subjects/control siblings. BMI, however, is not an ideal surrogate for the amount of adipose tissue surrounding the upper airway, because BMI can be affected by fat in other locations. Therefore, we used the volume of the parapharyngeal fat pad to adjust for obesity. Figure 3 demonstrates the parapharyngeal fat pad volumes in all subjects. There is sufficient overlap in the parapharyngeal fat pad volume distributions in the four groups to control for parapharyngeal fat pad volume as a covariate in our analyses.

Polysomnography

There were significant differences across groups for arousal index ($p = 0.0001$), amount of REM sleep ($p = 0.03$), and amount of non-REM sleep ($p = 0.009$; *see* Table E1 of the online

TABLE 1. DEMOGRAPHICS FOR AGE, BODY MASS INDEX, AND APNEA–HYPOPNEA INDEX IN PROBANDS, PROBAND SIBS, CONTROL SUBJECTS, AND CONTROL SIBS

	Probands		Proband Sibs		Control Subjects		Control Sibs		
Factor	Mean	SD	Mean	SD	Mean	SD	Mean	SD	p Value
Age	44.5	9.70	43.6	10.6	41.0	10.2	38.9	11.3	0.023*
BMI	35.5	8.5	29.6	5.8	25.9	4.6	25.8	4.3	< 0.001*
AHI	43.2	26.3	11.8	16.6	2.1	1.7	4.2	4.0	< 0.0001†

Definition of abbreviations: AHI = apnea–hypopnea index; BMI = body mass index.
For all groups, n = 220; 55 in each group.
* Analysis of variance between all four subject groups.
† Wilcoxon scores (rank sum) test between all four subject groups.

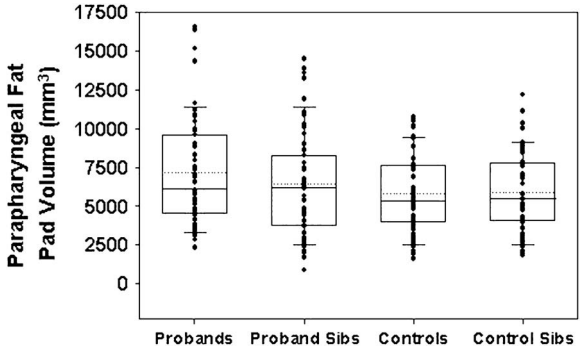

Figure 3. Comparisons of distributions of parapharyngeal fat volumes in the four subject groups. The boundary of the *box* closest to zero indicates the 25th percentile, a *line* within the box marks the median, and the boundary of the *box* farthest from zero indicates the 75th percentile. *Whiskers above* and *below* the *box* indicate the 90th and 10th percentiles. The mean is designated with a *dotted line*. Individual points are also plotted. There is substantial overlap in the distributions of this measurement in the four subject groups.

supplement). Probands had the highest arousal index, the least amount of REM and delta sleep compared with the other subject groups. *See* the online supplement for the complete results.

Comparisons of Upper Airway Soft Tissue Volumes between and within Families

Comparisons of mean values between the four subject groups for the upper airway volumetric soft tissue measurements are displayed in Table 2. We found significant group differences for the retropalatal lateral pharyngeal wall ($p = 0.04$), retroglossal lateral pharyngeal wall ($p < 0.0001$), total lateral pharyngeal wall ($p < 0.0001$), soft palate ($p = 0.03$), genioglossus ($p < 0.0001$), total tongue ($p < 0.0001$), and total soft tissue ($p < 0.0001$), even after controlling for sex, ethnicity, age, craniofacial size, ethnicity, and visceral neck fat. The volumes of the upper airway soft tissue structures were largest in the probands, intermediate in size in the proband siblings, and smallest in the control subjects and control siblings (Table 2). In Figures 4 through 6, we show within- and between-family differences for the size of these different upper airway structures. There are much smaller within-family differences than between-family differences for the volume of the lateral pharyngeal walls, tongue, soft palate, and total soft tissue. We found no significant differences between control and control sibs (within family comparison) for the volume of the lateral walls (retropalatal/retroglossal/total lateral walls), tongue (genioglossus/total tongue), soft palate, and total soft tissue (Figures 4–6). There were, however, significant differences between proband and proband sibs for the total lateral pharyngeal wall volume (Figure 4C; $p = 0.04$), genioglossus volume (Figure 5A; $p = 0.008$), total tongue volume (Figure 5B; $p = 0.0001$), and total soft tissue volume (Figure 6B; $p = 0.0001$), but not for the retropalatal lateral wall (Figure 4A; $p = 0.16$), retroglossal lateral pharyngeal wall volume (Figure 4B; $p = 0.15$), and soft palate volume (Figure 6A; $p = 0.26$). These data indicate that the volume of the upper airway soft tissue structures is similar within families (probands and proband siblings or control subjects and control siblings) but different between families (larger in the probands compared with control subjects and the proband sibs compared with control sibs).

Although the volume of the parapharyngeal fat pad was larger in the probands than in the other subject groups, these data were not statistically significant after controlling for age, sex, ethnicity, and craniofacial size (*see* Table 2 and Figure 7). The difference between the volume of the parapharyngeal fat in the probands compared with the control subjects after controlling for the covariates almost achieved statistical significance ($p = 0.06$; Figure 7).

Family Aggregation of Size of Upper Airway Structures

The similar size of the upper airway soft tissue structures in families suggests family aggregation of the size of these structures. To more directly assess the magnitude of family aggregation, we directly calculated heritability for each of these measures. This is the primary analysis on which this study is based. For the subjects studied, we found a heritability index for BMI of $h^2 = 39.4\%$. Review of population studies indicates that heritability accounts for approximately 40% of the variance in BMI (18). Our value was remarkably consistent with this *a priori* expectation and this consistency should be taken as evidence of the validity of our analysis approach.

For the volumetric soft tissue measurements, the size of the retropalatal lateral pharyngeal wall ($h^2 = 28.2\%$; $p = 0.02$), retroglossal lateral wall ($h^2 = 26.0\%$; $p = 0.03$), total pharyngeal lateral wall ($h^2 = 36.8\%$; $p = 0.001$), genioglossus ($h^2 = 27.1\%$; $p = 0.002$), total tongue ($h^2 = 36.5\%$; $p < 0.0001$), and total soft tissue ($h^2 = 37.5\%$; $p < 0.0001$) demonstrated heritability, even after adjusting for sex, ethnicity, age, craniofacial size, and visceral

TABLE 2. COMPARISONS OF SOFT TISSUE VOLUMES IN PROBANDS, PROBAND SIBS, CONTROLS AND CONTROL SIBS

	Probands		Proband Sibs		Control Subjects		Control Sibs		
Soft Tissue Volumes	Mean	SD	Mean	SD	Mean	SD	Mean	SD	p Value*
Parapharyngeal fat pad, mm^3	7,164.2	3,477.5	6,407.9	3,373.0	5,792.9	2,452.8	5,888.4	2,546.9	0.281†
RP lat. pharyn. wall, mm^3	**10,305.6**	**3,644.4**	**9,428.2**	**2,857.1**	**8,442.4**	**2,890.9**	**8,466.6**	**3,263.3**	**0.036**
RG lat. pharyn. wall, mm^3	**6,541.1**	**3,873.0**	**5,338.0**	**2,675.1**	**4,244.1**	**2,303.4**	**3,989.0**	**1,955.3**	**< 0.0001**
Total lat. pharyn., wall mm^3	**16,846.7**	**5,222.6**	**14,766.2**	**4,537.7**	**12,686.4**	**4,359.6**	**12,455.6**	**4,339.5**	**< 0.0001**
Soft palate, mm^3	**5,304.6**	**2,493.0**	**4,750.7**	**1,708.6**	**4,278.2**	**1,874.3**	**3,957.3**	**1,499.7**	**0.034**
Genioglossus, mm^3	**86,490.1**	**15,607.5**	**78,433.9**	**16,824.3**	**69,902.8**	**16,569.1**	**73,002.5**	**14,541.6**	**< 0.0001**
Total tongue, mm^3	**11,4928.4**	**18,304.1**	**101,721.9**	**18,529.1**	**94,287.9**	**20,525.0**	**93,290.3**	**21,721.5**	**< 0.0001**
Total soft tissue, mm^3	**144,243.9**	**22,456.5**	**127,646.7**	**22,896.1**	**117,045.4**	**25,519.9**	**115,591.6**	**24,829.0**	**< 0.0001**

Definition of abbreviations: lat. = lateral; pharyn. = pharyngeal; RG = retroglossal; RP = retropalatal.

Significant differences ($p < 0.05$ or 95% confidence interval for odds ratios excluding 1.0) are presented in bold.

* Analysis of variance (ANOVA) between all four subject groups adjusting for age, sex, craniofacial size, ethnicity, and visceral neck fat.

† ANOVA between all four subject groups adjusting for age, sex, craniofacial size, and ethnicity.

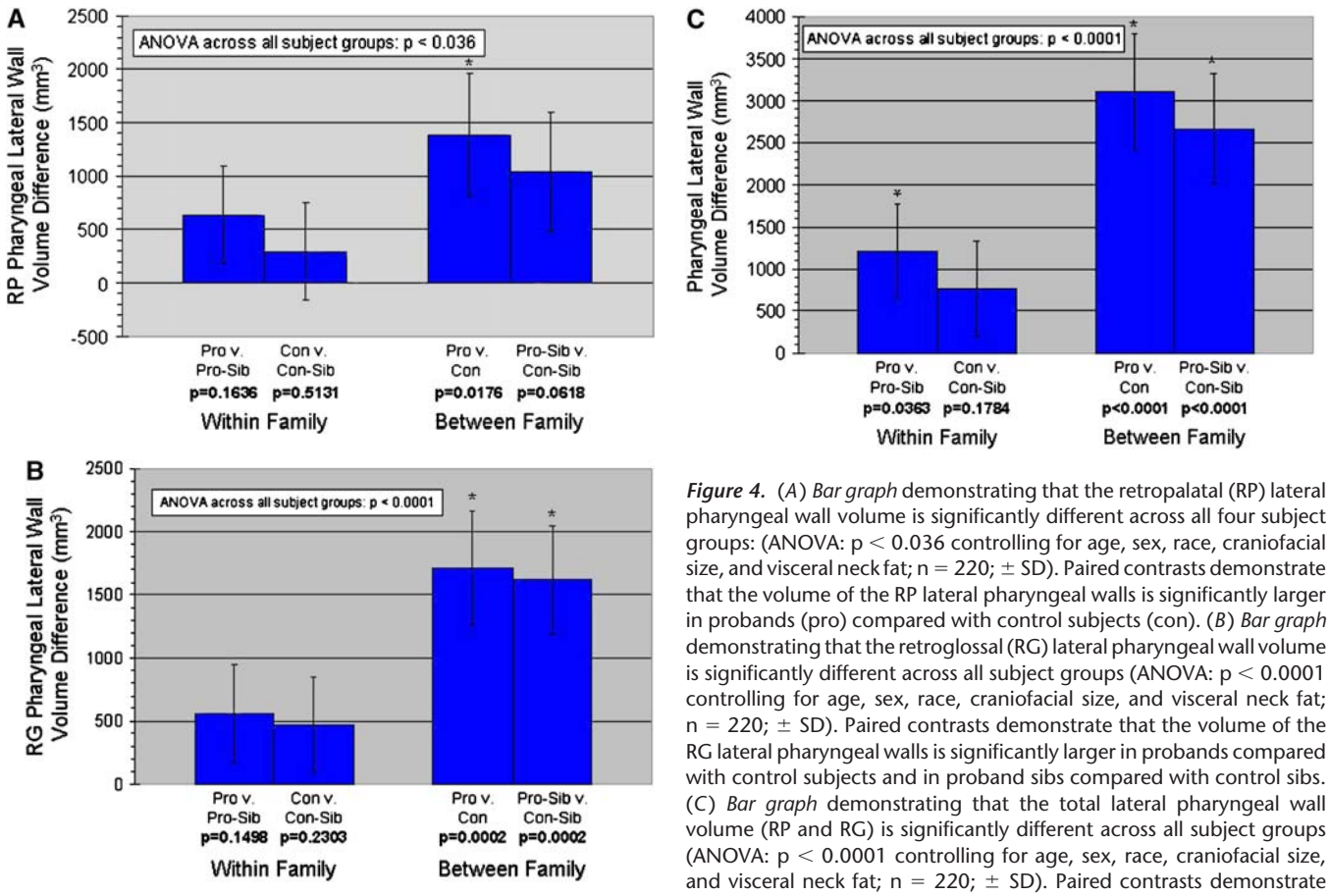

Figure 4. (*A*) *Bar graph* demonstrating that the retropalatal (RP) lateral pharyngeal wall volume is significantly different across all four subject groups: (ANOVA: $p < 0.036$ controlling for age, sex, race, craniofacial size, and visceral neck fat; $n = 220$; $\pm$ SD). Paired contrasts demonstrate that the volume of the RP lateral pharyngeal walls is significantly larger in probands (pro) compared with control subjects (con). (*B*) *Bar graph* demonstrating that the retroglossal (RG) lateral pharyngeal wall volume is significantly different across all subject groups (ANOVA: $p < 0.0001$ controlling for age, sex, race, craniofacial size, and visceral neck fat; $n = 220$; $\pm$ SD). Paired contrasts demonstrate that the volume of the RG lateral pharyngeal walls is significantly larger in probands compared with control subjects and in proband sibs compared with control sibs. (*C*) *Bar graph* demonstrating that the total lateral pharyngeal wall volume (RP and RG) is significantly different across all subject groups (ANOVA: $p < 0.0001$ controlling for age, sex, race, craniofacial size, and visceral neck fat; $n = 220$; $\pm$ SD). Paired contrasts demonstrate that the volume of the total lateral pharyngeal walls is significantly larger in probands compared with control subjects, in proband sibs compared with control sibs, and in probands compared with the proband siblings. In general, the within-family differences are significantly smaller than the between-family differences. No significant differences are noted between control subjects and control siblings for any of the measurements in *A*–*C*.

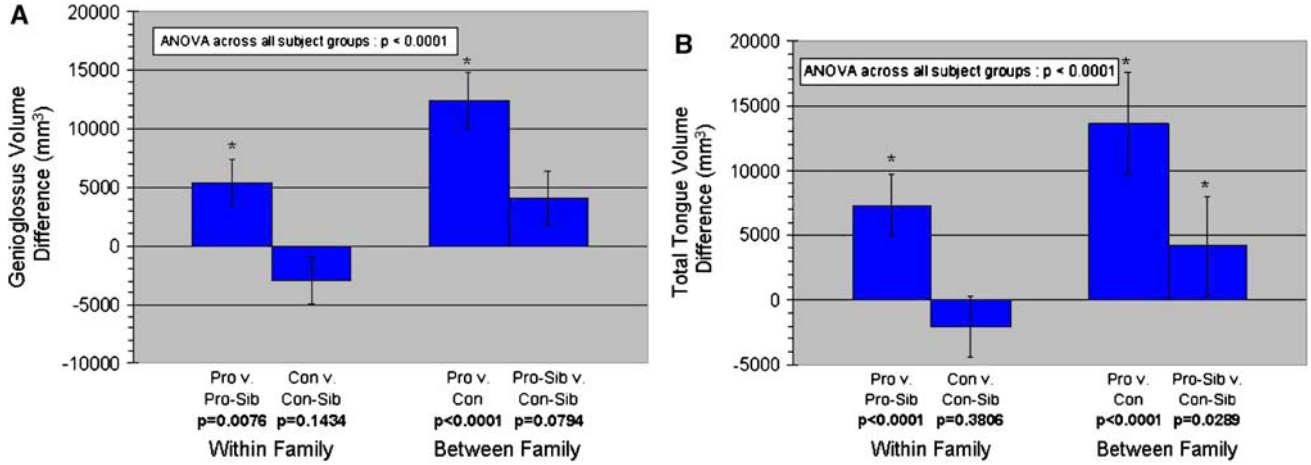

Figure 5. (*A*) *Bar graph* demonstrating that the genioglossus volume is significantly different across all four subject groups (ANOVA: $p < 0.0001$ controlling for age, sex, race, craniofacial size, and visceral neck fat; $n = 220$; $\pm$ SD). Paired contrasts demonstrate that the volume of the genioglossus is significantly larger in probands (pro) compared with control subjects (con) and in the probands compared with the proband siblings. (*B*) *Bar graph* demonstrating that total tongue volume is significantly different across all four subject groups (ANOVA: $p < 0.0001$ controlling for age, sex, race, craniofacial size, and visceral neck fat; $n = 220$; $\pm$ SD). Paired contrasts demonstrate that the total tongue volume is significantly larger in probands compared with control subjects, in proband sibs compared with control sibs, and in the probands compared with the proband siblings. In general, larger differences are noted between families than within families and there are no significant differences between control subjects and control siblings for any of the comparisons in *A*–*B*.

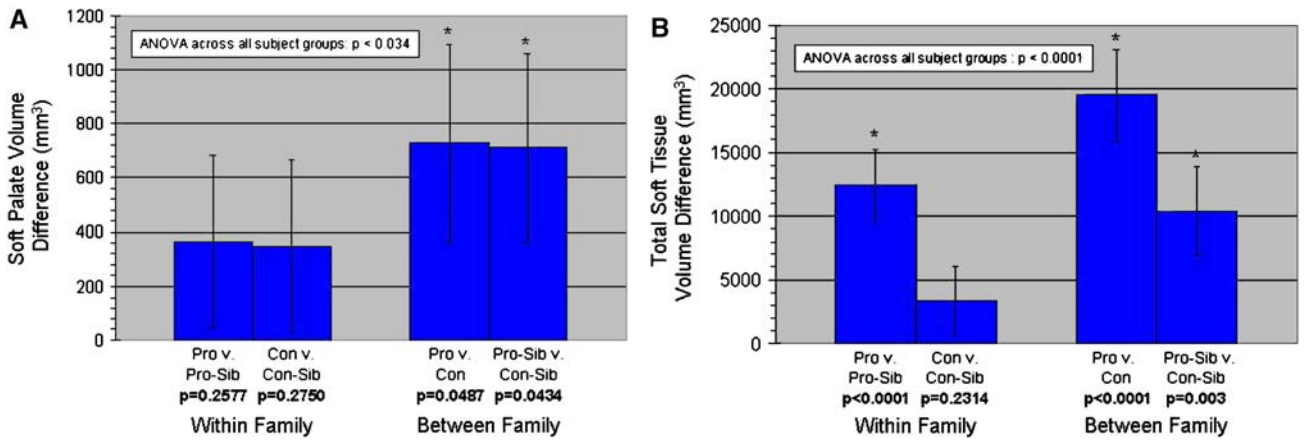

Figure 6. (*A*) *Bar graph* demonstrating that the soft palate volume is significantly different across all four subject groups (ANOVA: $p < 0.034$ controlling for age, sex, race, craniofacial size, and visceral neck fat; $n = 220$; ± SD). Paired contrasts demonstrate that the soft palate volume is significantly larger in probands (pro) compared with control subjects (con) and in the probands compared with the proband siblings. (*B*) Comparisons of differences in total soft tissue in all four subject groups. *Bar graph* demonstrating that total soft tissue volume is significantly different across all four subject groups (ANOVA: $p < 0.0001$ controlling for age, sex, race, craniofacial size, and visceral neck fat; $n = 220$; ± SD). Paired contrasts demonstrate that the total soft tissue volume is significantly larger in probands compared with control subjects, in proband sibs compared with control sibs, and in the probands compared with the proband siblings. In general, significant differences are noted between families but there are no significant differences between control subjects and control siblings for any of the comparisons in *A–B*.

neck fat (Table 3). The heritability estimates for the total lateral walls, tongue, and total soft tissue maintained their significance after controlling for multiple comparisons (Bonferroni-corrected α value of $0.05/4 = 0.0125$). The heritability estimate for the volume of the soft palate was not significant with or without the Bonferroni correction. These data provide evidence that size of upper airway soft tissue structures demonstrates family aggregation and will likely contribute to the genetic basis of sleep apnea. The unadjusted heritability estimate for the volume of the parapharyngeal fat pads was 29.1%. After adjustments for age, sex, ethnicity, and craniofacial size, this estimate was 14.9%, which did not reach statistical significance ($p = 0.08$).

Tables 4 and 5 show the intraclass correlations comparing probands and proband siblings and control subjects and control siblings independently for volume of different upper airway soft tissues. Most of the intraclass correlations for the volumes of the upper airway soft tissue structures are greater in the pairs of normal subjects than in the patients with apnea and their siblings. The only intraclass correlations that are larger in the probands and their siblings are for the retroglossal lateral pharyngeal wall and genioglossus volume. The remaining intraclass correlations (parapharyngeal fat pads, retropalatal pharyngeal wall, total lateral pharyngeal wall, soft palate, total tongue, and total soft tissue volumes) were greater in the control subjects and control siblings.

ORs between Proband Siblings and Control Siblings

We next examined unadjusted and adjusted relative risks of a sibling having sleep apnea by estimating ORs for the effects of 1-SD increases in the measurements of the soft tissue structures using data only from proband siblings and control siblings (Table 6). The standard deviations were taken from the control sibling distributions and the specific values used are provided in Table 6. Changes in several of the volumetric structures were associated with increased likelihood of having a sibling with apnea, even after adjusting for sex, ethnicity, age, craniofacial size, and visceral neck fat (Table 6). Increased size of the retropalatal lateral pharyngeal wall (OR, 2.04; 95% CI, 1.07–4.21), retroglossal pharyngeal wall volume (OR, 3.07; 95% CI, 1.69–6.30), total lateral pharyngeal wall (OR, 4.43; 95% CI, 2.04–11.23), soft palate (OR, 1.79; 95% CI, 1.11–3.02), and total soft tissue volume (OR, 1.98; 95% CI, 1.02–4.49) were associated with an increased risk of having a sibling with sleep apnea. Thus, increased volume of several of the upper airway soft tissue structures was shown to be associated with having a family member who has sleep apnea among individuals not specifically selected, because they presented

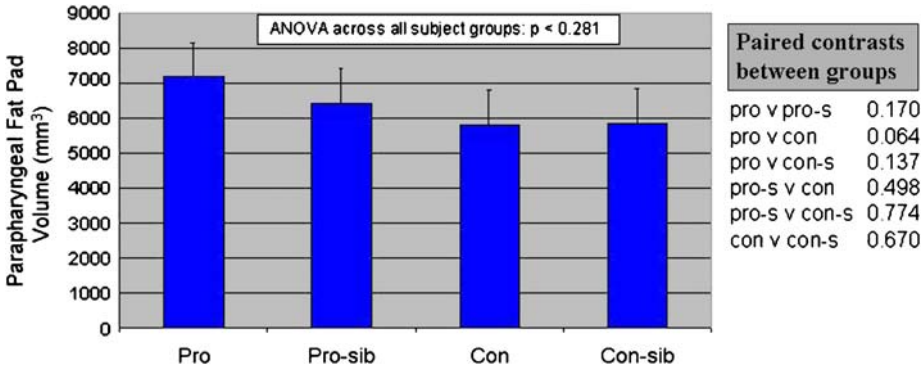

Figure 7. *Bar graph* demonstrating that the parapharyngeal fat pad volume is not significantly different across all four subject groups (ANOVA: $p < 0.281$ controlling for age, sex, race, and craniofacial size; $n = 220$; ± SD). The parapharyngeal fat pad volume is largest in the probands but the differences between subject groups are not statistically significant.

TABLE 3. HERITABILITY INDICES FOR THREE-DIMENSIONAL SOFT TISSUE VOLUMES

	Unadjusted (%)	Adjusted for Age, Sex, Craniofacial Size, Race (%)	Adjusted for Age, Sex, Craniofacial Size, Race, and Visceral Neck Fat	
Soft Tissue Volumes	h^2	h^2	h^2 (%)	p Value*
Parapharyngeal fat pad, mm^3	29.1	14.9	—	0.083†
Retropalatal lateral pharyngeal wall, mm^3	**25.2**	**30.8**	**28.2**	**0.018**
Retroglossal lateral pharyngeal wall, mm^3	**17.7**	**25.2**	**26.0**	**0.034**
Total lateral pharyngeal wall, mm^3	**25.6**	**36.4**	**36.8**	**0.001‡**
Genioglossus, mm^3	**30.8**	**28.6**	**27.1**	**0.002**
Soft palate, mm^3	11.7	10.4	9.3	0.218
Total tongue, mm^3	**37.8**	**34.9**	**36.5**	**< 0.0001‡**
Total soft tissue, mm^3	**41.3**	**31.6**	**37.5**	**< 0.0001‡**

Definition of abbreviation: h^2 = heritability estimate.
Significant differences ($p < 0.05$ or 95% confidence interval for odds ratios excluding 1.0) are presented in bold.
* p value adjusting for age, sex, craniofacial size, ethnicity, and visceral neck fat.
† p value adjusting for age, sex, craniofacial size and ethnicity.
‡ A significant Bonferroni-corrected α value of 0.05/4 = 0.0125.

with manifest sleep apnea even after controlling for obesity and other parameters. This provides evidence that at least some of the increased size of upper airway soft tissue structures observed in individuals with apnea is likely to precede apnea onset as opposed to being a consequence of the disease itself.

Airway and Two-Dimensional Soft Tissue Measurements (Secondary Analyses)

The upper airway was smallest in the retropalatal region, and there were significant group differences in airway volume ($p = 0.015$), airway area per slice ($p = 0.0004$), and minimum airway area ($p < 0.0001$), and the lateral ($p < 0.0001$) and anteroposterior dimensions ($p = 0.0002$) of the retropalatal airway after adjusting for age, sex, ethnicity, craniofacial size, and visceral neck fat (Table 7). However, differences were not demonstrated in the retroglossal region. In the retropalatal region, airway area per slice ($h^2 = 35.0\%$), minimum airway area ($h^2 = 46.0\%$), and lateral airway dimensions ($h^2 = 17.0\%$) demonstrated significant heritability after adjusting for the covariates (Table 8). There were no significant airway heritability estimates in the retroglossal region. For the two-dimensional soft tissue measurements, only retropalatal lateral pharyngeal wall thickness demonstrated significant group differences after controlling for the covariates (Table E2). There were no significant heritability estimates for the two-dimensional measurements of soft tissue size (Table E3). *See* online supplement for the complete results (*see* Tables E2 and E3).

DISCUSSION

Volumetric MRI is a powerful modality to phenotype the upper airway. We previously demonstrated in a case-control study that an increase in volume of the lateral pharyngeal walls, tongue, and total upper airway soft tissue were significant risk factors for sleep apnea (12). We have shown in the present investigation that these same anatomic risk factors (or intermediate traits) also demonstrate family aggregation and heritability. We demonstrated this with different analysis strategies. In particular, we have shown heritability of the size of the lateral pharyngeal walls, tongue, and total soft tissue, even after controlling for important covariates, including amount of visceral neck fat and overall craniofacial size. This is the first time that heritability of the size of these upper airway soft tissue structures has been demonstrated. The demonstration of family aggregation of the size of the upper airway structures provides the basis for future investigations to identify genes associated with these intermediate traits for sleep apnea.

TABLE 4. INTRACLASS CORRELATIONS COMPARING PROBANDS AND PROBAND SIBLINGS FOR UPPER AIRWAY SOFT TISSUE VOLUMES

	Unadjusted	Adjusted for Age, Sex, Craniofacial Size, Race	Adjusted for Age, Sex, Craniofacial Size, Race, and Visceral Neck Fat	
Soft Tissue Volumes	ICC	ICC	ICC	p Value*
Parapharyngeal fat pad, mm^3	0.13	0.03	—	0.407†
Retropalatal lateral pharyngeal wall, mm^3	0.18	0.13	0.13	0.169
Retroglossal lateral pharyngeal wall, mm^3	0.43	0.25	0.25	0.055
Total lateral pharyngeal wall, mm^3	0.47	0.19	0.21	0.066
Genioglossus, mm^3	**0.45**	**0.24**	**0.24**	**0.042**
Soft palate, mm^3	0.25	0.03	0.06	0.270
Total tongue, mm^3	0.36	0.47	0.07	0.278
Total soft tissue, mm^3	0.35	0.0	0.07	0.278

Definition of abbreviation: ICC = intraclass correlation.
Significant differences ($p < 0.05$ or 95% confidence interval for odds ratios excluding 1.0) are presented in bold.
* p value adjusting for age, sex, craniofacial size, ethnicity, and visceral neck fat.
† p value adjusting for age, sex, craniofacial size, and ethnicity.

TABLE 5. INTRACLASS CORRELATIONS COMPARING CONTROL SUBJECTS AND CONTROL SIBLINGS FOR UPPER AIRWAY SOFT TISSUE VOLUMES

	Unadjusted	Adjusted for Age, Sex, Craniofacial Size, Race	Adjusted for Age, Sex, Craniofacial Size, Race, and Visceral Neck Fat	
Soft Tissue Volumes	ICC	ICC	ICC	p Value*
Parapharyngeal fat pad, mm^3	0.54	0.45	—	0.004†
Retropalatal lateral pharyngeal wall, mm^3	0.71	0.56	0.52	0.002
Retroglossal lateral pharyngeal wall, mm^3	0.49	0.00	0.00	—
Total lateral pharyngeal wall, mm^3	0.68	0.28	0.28	0.049
Genioglossus, mm^3	0.61	0.17	0.14	0.129
Soft palate, mm^3	0.47	0.13	0.11	0.247
Total tongue, mm^3	0.61	0.42	0.42	0.004
Total soft tissue, mm^3	0.67	0.41	0.45	0.003

Significant differences ($p < 0.05$ or 95% confidence interval for odds ratios excluding 1.0) are presented in bold.
* p value adjusting for age, sex, craniofacial size, ethnicity, and visceral neck fat.
† p value adjusting for age, sex, craniofacial size, and ethnicity.

Study Design and Methodology

We used MRI upper airway paradigms and analysis strategies to study the family aggregation of size of the upper airway structures; however, some limitations of our imaging approach need to be reviewed. Each spin-echo MR dataset was acquired in approximately 3 min. Thus, the MR images used in the present investigation represent averaged values for the upper airway and upper airway soft tissue structures over several respiratory cycles. Nonetheless, we have demonstrated in a previous study that the same volumetric MRI analysis algorithms that were used in this investigation are reproducible, reliable, and accurate (13). We have shown that the volumes of upper airway soft tissue structures measured at two different times with the same temporal imaging sequences used in this investigation are equivalent (13). Moreover, the accuracy of our imaging techniques has been confirmed by demonstrating that the volume of a phantom measured with our volumetric imaging algorithms is very similar to its known volume (13). Thus, we do not believe that our volumetric analysis of tissue structures was adversely affected by the acquisition of MR images over several respiratory cycles.

There were several confounding variables that we specifically controlled for in our recruitment of subjects for each quad. Ethnicity and sex were controlled in each quad. Environmental differences were partially controlled for by matching the probands and control subjects to the same school district. However, within a school district, variability in the environment may have still existed. Age was partially controlled: each sibling was within 10 yr of the proband or control. We then frequency matched the proband/sib pairs to control/sib pairs so that, in general, they were not more than 10 yr apart. The frequency matching was largely successful, although there were small overall group differences in terms of the mean ages of the four subject groups (Table 1). Similarly, we were able to control for visceral neck fat in the analysis as a covariate because there was sufficient overlap in visceral neck fat between the subject groups (*see* Figure 3).

We did not examine craniofacial structure in this investigation, apart from controlling for overall craniofacial size. Studies have demonstrated that changes in craniofacial morphology are an important risk factor for sleep-disordered breathing (19, 20) and studies with standard cephalometrics have also demonstrated family aggregation of these structures (7, 11). We are currently developing new methods to analyze craniofacial structures in three dimensions and plan to apply these to this dataset in the future. Nonetheless, we controlled for craniofacial form in this investigation by measuring mandibular width (lateral head measurement) and by measuring from the teeth to the posterior subcutaneous tissue (an anteroposterior head measurement). It is important to control for head size because it has been shown to affect airway caliber (21).

TABLE 6. ODDS RATIOS WITH 95% CONFIDENCE INTERVALS (LOWER BOUND, UPPER BOUND) FOR SLEEP APNEA FOR 1-SD INCREASE IN SOFT TISSUE VOLUMES FOR PROBAND SIBS AND CONTROL SUBJECT SIBS

		Unadjusted			Adjusted for Age, Sex, Craniofacial Size, Race			Adjusted for Age, Sex, Craniofacial Size, Race, and Visceral Neck Fat		
Soft Tissue Volumes	SD*	OR	LB	UB	Adj. OR	LB	UB	Adj. OR	LB	UB
Parapharyngeal fat pad, mm^3	2,546.9	1.16	0.84	1.62	1.00	0.69	1.44	1.01	0.67	1.53
RP lateral pharyngeal wall, mm^3	3,263.3	1.41	0.94	2.15	**1.65**	**1.01**	**2.83**	**2.04**	**1.07**	**4.21**
RG lateral pharyngeal wall, mm^3	1,955.3	**1.65**	**1.18**	**2.40**	**2.49**	**1.57**	**4.20**	**3.07**	**1.69**	**6.30**
Total pharyngeal lateral wall, mm^3	4,339.5	**1.68**	**1.15**	**2.54**	**2.56**	**1.51**	**4.74**	**4.43**	**2.04**	**11.23**
Soft palate, mm^3	1,499.7	1.39	0.98	2.02	**1.86**	**1.15**	**3.13**	**1.68**	**0.93**	**3.24**
Genioglossus, mm^3	14,541.6	**1.59**	**1.11**	**2.33**	**2.02**	**1.30**	**3.28**	**1.79**	**1.11**	**3.02**
Total tongue, mm^3	24,558.7	1.40	0.93	2.19	1.71	0.99	3.12	1.45	0.75	3.23
Total soft tissue, mm^3	21,721.5	**1.60**	**1.05**	**2.52**	**2.09**	**1.19**	**3.96**	**1.98**	**1.02**	**4.49**

Definition of abbreviations: LB = lower bound; OR = odds ratio; RG = retroglossal; RP = retropalatal; UB = upper bound.
Significant differences ($p < 0.05$ or 95% confidence interval for ORs excluding 1.0) are presented in bold.
* Standard deviation from control sibling sample (n = 55).

TABLE 7. COMPARISONS OF UPPER AIRWAY MEASUREMENTS IN PROBANDS, PROBAND SIBS, CONTROL SUBJECTS, AND CONTROL SIBS

	Probands		Proband Sibs		Control Subjects		Control Sibs		
Airway Measurements	Mean	SD	Mean	SD	Mean	SD	Mean	SD	p Value*
RP airway volume, mm^3	**3,044.7**	**1,424.6**	**3,488.0**	**1,843.1**	**3,473.2**	**1,778.1**	**3,376.8**	**1,831.8**	**0.015**
RG airway volume, mm^3	6,442.9	3,345.6	5,593.1	3,701.4	5,174.8	3,148.8	5,236.2	2,776.1	0.821
Total airway volume, mm^3	9,487.6	3,911.8	9,081.1	4,941.9	8,648.0	4,119.8	8,613.0	4,148.8	0.877
Airway area per slice RP, mm^2	**89.3**	**36.0**	**106.8**	**46.9**	**119.1**	**54.6**	**116.0**	**63.1**	**0.0004**
Airway area per slice RG, mm^2	201.4	92.1	173.3	77.8	164.2	71.4	171.7	70.6	0.516
Minimum area in RP region, mm^2	**26.3**	**18.1**	**39.4**	**26.2**	**54.4**	**36.0**	**50.3**	**45.1**	**< 0.0001**
Minimum area in RG region, mm^2	108.5	60.1	87.4	51.8	82.5	52.2	86.7	54.9	0.292
RP anteroposterior dimensions, mm	**4.6**	**3.7**	**7.2**	**6.5**	**7.1**	**4.8**	**6.2**	**4.4**	**0.0002**
RP lateral dimensions, mm	**7.9**	**5.2**	**11.0**	**6.1**	**12.4**	**5.4**	**13.5**	**6.0**	**< 0.0001**
RG anteroposterior dimensions, mm	11.3	4.7	10.2	5.1	9.2	4.9	9.4	4.20	0.515
RG lateral dimensions, mm	19.1	7.9	17.6	7.2	18.5	6.4	19.3	6.7	0.08

Definition of abbreviations: RG = retroglossal; RP = retropalatal.
Significant differences ($p < 0.05$ or 95% confidence interval for odds ratios excluding 1.0) are presented in bold.
* Analysis of variance between all four subject groups adjusting for age, sex, craniofacial size, ethnicity, and visceral neck fat.

The increased size of upper airway structures in patients with sleep apnea may not be genetic but may be secondary to the sleep apnea itself. Trauma associated with airway closure may increase the size of the upper airway soft tissue structures (i.e., through edema). We do not believe that this explains the family aggregation of the size of these structures that we are describing because we showed family aggregation of the upper airway soft tissue structures in normal subjects as well as in patients with apnea. Moreover, the reconstituted cohort analysis demonstrated that the increased volume of several of the upper airway soft tissue structures is associated with having a family member who has sleep apnea among individuals who do not have evidence for significant sleep apnea. These data suggest that the increased volume of the upper airway soft tissue structures observed in individuals with apnea likely precedes apnea onset as opposed to being a consequence of the disease itself.

Volumetric MRI: A New Standard to Phenotype the Upper Airway

We believe MRI is an ideal modality to phenotype the upper airway. Although we were able to show family aggregation of volumetric soft tissue measurements, we were not able to show the same robust findings with two-dimensional MRI measurements. None of the two-dimensional soft tissue measurements demonstrated significant levels of heritability. These data indicate that volumetric MRI is an important advance for phenotyping the soft tissues of the upper airway. Two-dimensional soft tissue measurements (the standard used in most imaging studies examining the upper airway) evaluating the thickness/dimensions or cross-sectional area of a structure are not as powerful an approach for quantifying anatomic risk factors for sleep apnea (12, 15). This is not surprising because a two-dimensional approach only provides partial characterization of the anatomy. We propose that future studies in this area should use three-dimensional volumetric imaging.

Family Aggregation of the Size of Upper Airway Soft Tissue Structures

The central hypothesis of this investigation is that the size of the upper airway soft tissue structures is at least partially determined by genetic factors. Our data support this hypothesis. We have shown with complementary analysis strategies that intermediate traits for sleep apnea (increased volume of the tongue, lateral walls, and total soft tissue) demonstrate family aggregation, independent of obesity. First, we demonstrated that the size of these upper airway soft tissue structures was intermediate in the siblings of the probands compared with the probands and control subjects (Table 2). Second, we showed that, in general, the between-family differences (proband vs. control, proband sib vs. control sib) were greater for the size of these structures

TABLE 8. HERITABILITY INDICES FOR AIRWAY MEASUREMENTS

	Unadjusted (%)	Adjusted for Age, Sex, Craniofacial Size, and Race (%)	Adjusted for Age, Sex, Craniofacial Size, Race, and Visceral Neck Fat	
Airway Measurements	h^2	h^2	h^2 (%)	p Value
Airway RP volume, mm^3	14.1	18.3	16.8	0.058
Airway RG volume, mm^3	9.6	0.0	0.0	—
Airway total volume, mm^3	14.2	5.8	0.6	0.273
Airway area per slice RP region, mm^2	**40.8**	**35.8**	**35.0**	**0.001**
Airway area per slice RG region, mm^2	21.6	12.6	9.7	0.132
Minimum area in RP region, mm^2	**47.5**	**46.3**	**46.0**	**< 0.0001**
Minimum area in RG region, mm^2	9.9	5.6	3.2	0.402
Anteroposterior dimension RP, mm	0.0	0.0	0.0	—
Lateral dimension RP, mm	**31.3**	**23.4**	**17.0**	**0.014**

For definition of abbreviations, *see* Table 7.
Significant differences ($p < 0.05$ or 95% confidence interval for odds ratios excluding 1.0) are presented in bold.

than the within-family differences (proband vs. proband sib, control vs. control sib; Figures 4–6). Third, we directly demonstrated increased heritability estimates for the volume of the lateral pharyngeal walls, tongue, and total soft tissues after adjusting for sex, ethnicity, age, craniofacial size, and visceral neck fat (Tables 3–5). Fourth, we compared the anatomic risk factors in proband siblings and control siblings. We demonstrated that increased volume of the soft palate, lateral pharyngeal walls, and total soft tissue was associated with increased likelihood of having a sibling with apnea, even after adjusting for sex, ethnicity, age, craniofacial size, and visceral neck fat (Table 6). We believe these complementary analysis strategies provide strong evidence for family aggregation of the size of upper airway structures.

A somewhat surprising result from our studies is that the intraclass correlation coefficient for the size of upper airway structures was greater within control pairs than in those where one sib had apnea. This indicates that, even in normal subjects, siblings have very similar-sized soft tissue structures of the upper airway (tongue/lateral walls/total tissue/parapharyngeal fat pads). The most parsimonious explanation that the intraclass correlation for the size of these structures is less in sibs where one sib has apnea is that the disease itself alters upper airway size, causing larger differences between proband and the proband sib. It has previously been argued that enlargement of the upper airway structures in patients with sleep apnea may be a consequence of the disease (secondary to remodeling from trauma, recurrent apneas, vibratory effects, edema) rather than the primary cause of the disorder (22, 23). Recurrent apneas with associated large negative intraluminal pressure swings, and repeated bouts of upper airway vibration secondary to snoring are believed to produce traumatic changes in the airway (22, 23). In support of this hypothesis are the following observations: upper airway sensation is reduced in patients with apnea compared with control subjects (23); there is denervation of afferent nerve fibers to the muscles of the pharynx in patients with apnea (22); inflammatory cell infiltration and edema have been demonstrated in the upper airway mucosa and muscular layer of the pharynx (24). Because these changes were not be present in normal subjects, this may explain why there is greater family aggregation in the size of the upper airway soft tissue structures in this group compared with patients with apnea.

The parapharyngeal fat pads did not demonstrate as robust heritability estimates across the four subject groups, as did the other upper airway soft tissue structures, after controlling for sex, ethnicity, age, and craniofacial size. In addition, the volume of the parapharyngeal fat pads was not significantly different across the four subject groups after controlling for sex, ethnicity, age, and craniofacial size, although the volume of these fat pads was larger in the patients with apnea than in the other subject groups. Previous investigations (15, 25) have demonstrated enlargement of the parapharyngeal fat pads in patients with apnea compared with control subjects but most of these investigations used two-dimensional measures of parapharyngeal fat (thickness of the lateral walls or cross-sectional area) and did not control for sex, ethnicity, age, and craniofacial size. The data on parapharyngeal fat pad volumes in this study are similar to the data that we published in our recent case-control study (12). In the case-control study, the volume of the parapharyngeal fat pads was significantly larger in patients with apnea compared with control subjects ($p = 0.009$) using unadjusted data; however, after adjustments for sex, ethnicity, age, and craniofacial size, this difference was no longer statistically significant ($p = 0.058$; although it almost reached significance). These data suggest that the known increased risk of sleep apnea with obesity may not be solely mediated through enlargement of the parapharyngeal fat pads.

Genetic Basis for the Soft Tissue Risk Factors for Obstructive Sleep Apnea

Our data demonstrating family aggregation of the upper airway soft tissue structures suggest that the size of these structures is at least partially genetically determined. Do genes exist that explain the enlargement of the tongue or lateral pharyngeal walls (both structures largely composed of skeletal muscle)? We believe that the answer to this question is likely to be yes and it is likely that genes associated with muscle development play an important role in determining the size of these structures. Unfortunately, there is little information in humans on the genetics of the normal development of upper airway muscles such as the tongue and lateral pharyngeal walls. However, congenital forms of macroglossia have been described (26) and macroglossia has been reported in patients with Trismony 21 (27) and Wiedemann-Beckwith syndrome (28). Both Down's syndrome and Wiedemann-Beckwith syndrome are the result of chromosomal abnormalities.

Information is also accruing about the genetics of skeletal muscle development and some information specifically about tongue development. Muscle-specific genes coding for proteins (desmin, myosin, actin, troponin, and tropomysin) involved with structure and contraction have been described (29–31). Studies in animals have examined skeletal muscle–specific genes and how they are regulated in different anatomic tissues (32). Genes for muscle creatine kinase (which is transcribed at high levels in skeletal muscle) and the myogenic regulatory factors (MyoD, myogenin, Myf-5, MRF4) have been shown to play an important role in muscle development (32). MyoD and Myf-5 are believed to play an important role in early myogenesis, whereas myogenin and MRF4 are believed to be involved with terminal differentiation of the muscle cell (29–33). Hepatic growth factor and genes associated with hepatic growth factor stimulate muscle precursors in the development of the mouse tongue (34). Inactivation of the hepatic growth factor genes results in hypoplasia of the murine tongue (34). These studies may provide a number of plausible candidate genes that may be used in future association studies to determine genes related to enlargement of the tongue and lateral pharyngeal walls in patients with obstructive sleep apnea.

Conclusions

In conclusion, we have used volumetric MRI to show that size of upper airway soft tissue structures shows family aggregation after controlling for age, sex, ethnicity, craniofacial size, and visceral neck fat. In particular, the size of the tongue, lateral pharyngeal walls, and total soft tissue demonstrates family aggregation. The demonstration of family aggregation of the size of upper airway structures provides an important step for future investigations to determine genes associated with these intermediate traits. Such studies will require the phenotyping strategies used here.

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