

# Shortened Telomeres in Families With a Propensity to Autism

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**Objective:** Shortened telomeres have been linked to poorer health outcomes. Exposure to psychological stress is associated with accelerated telomere shortening, and a well-established body of evidence indicates that families with a child with autism spectrum disorder (ASD) experience heightened levels of psychological stress. Also, alterations in a number of biological processes implicated in telomere length dynamics (i.e., oxidative stress, DNA methylation) have been linked to ASD susceptibility. We examined whether families of children with ASD who have an infant show shortened telomeres.

**Method:** Saliva samples were collected from infants, their older sibling (proband), and parents in families with or without a child with ASD. Infants and their families were designated as high-risk for ASD (HRA;  $n = 86$ ) or low-risk for ASD (LRA;  $n = 118$ ) according to the older siblings' diagnostic status. We used the real-time polymerase chain reaction (PCR) telomere assay to determine relative average telomere length for each participant.

**Results:** HRA families demonstrated significantly shorter telomere length relative to LRA families. This effect was observed at the individual family member level, with infants, probands, and mothers in HRA families showing reduced relative telomere length compared to individuals in LRA families; although not significant, fathers of high-risk infants showed a similar pattern of decreased telomere length.

**Conclusion:** Families of children with ASD who have an infant show shortened telomeres relative to families with no history of ASD. These results suggest that such "high-risk" families should be monitored for the physical and mental health consequences that are often associated with accelerated telomere shortening.

**Key Words:** autism spectrum disorder, telomeres, biomarker, psychological stress

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Telomeres are DNA protein structures that cap the ends of linear chromosomes.<sup>1</sup> Telomere length represents a biological marker of cellular aging.<sup>1</sup> Telomere shortening occurs with each cell division; when telomeres shorten to a critical length, the cell enters a state of senescence and can no longer divide.<sup>2</sup> Shortened telomere lengths have been reported in a variety of medical conditions, including cardiovascular disease and cancer,<sup>3–5</sup> as well as psychiatric disorders, including mood disorders, schizophrenia, and, recently, autism spectrum disorder (ASD).<sup>6–8</sup>

Although telomere length is highly heritable,<sup>9</sup> a variety of demographic variables and health-related behaviors have been linked to telomere dynamics. For example, age, sex, body mass index, ethnicity, smoking status, and physical activity have demonstrated contributions to telomere length.<sup>10–13</sup> In addition, psychosocial factors have been linked to telomere length. Specifically, an abundance of evidence links psychological stress to accelerated telomere shortening. In particular, Epel *et al.*<sup>14</sup> identified an association between telomere length and psychological stress among 20- to 50-year-old women who served as primary caregivers to children with chronic health issues or

disabilities (including ASD) and women with healthy children. The greatest telomere shortening was observed among those women with more years of caregiving and, importantly, among those with the greatest levels of perceived stress (notably, women whose children had a health issue or a disability). Thus, telomere length, at least partially, represents a biological marker of both genetic predisposition and environmental influence.

It is well established that familial caregivers of children with ASD experience considerable perceived and actual stress.<sup>14–18</sup> Indeed, caregivers of children with ASD often report experiencing levels of stress that are higher than both caregivers of typically developing children and caregivers of children with other developmental disabilities.<sup>15</sup> In addition to self-reported levels of stress, there is evidence that both mothers and fathers of children with ASD experience dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis.<sup>19–21</sup> Studies have also reported increased rates of psychiatric problems and poorer health outcomes in caregivers of children with neurodevelopmental disorders, including ASD.<sup>22,23</sup> Considering that telomere shortening has been linked to increased levels of perceived stress, poorer health outcomes, and psychiatric disorders, it is highly likely that shortened telomeres would be observed in caregivers of children with ASD and, importantly, in families with a new infant sibling.

Recent evidence suggests that shorter telomere length is present in children with ASD. Li *et al.*<sup>8</sup> recently reported that



This article is discussed in an editorial by Dr. Stacy S. Drury on page 539.

children with ASD (at approximately 4 to 5 years of age) show significantly shorter leukocyte telomere length than typically developing children of the same age. It remains to be delineated whether decreased telomere length in this clinical group plays a causal role in ASD or is the result of an unrelated variable that is independently associated with both ASD and telomere length.<sup>8</sup> Furthermore, a number of biological mechanisms and genes linked to telomere length dynamics have been implicated in ASD and ASD susceptibility, including increased oxidative stress,<sup>24,25</sup> abnormalities in DNA repair mechanisms,<sup>26</sup> DNA methylation,<sup>25</sup> and chromatin remodeling.<sup>27</sup> These lines of evidence, in association with links between perceived stress and telomere length, suggests that there may be multiple and variable genetic and environmental factors that contribute to telomere dynamics in families with a child with ASD who also have an infant. Establishing whether shortened telomere length is specific to children with ASD or also extends to family members (including the at-risk infant) has implications for how we conceptualize treatment in families who have a child with ASD.

As part of our prospective study of infant siblings of children with ASD, we collected saliva samples from parents, infant siblings, and the affected proband with ASD. We aimed to determine whether families with at least 1 child diagnosed with ASD and a younger infant (whose risk of developing autism is 20 times greater than that of the general population<sup>28</sup>) show reduced telomere length compared to a comparison sample of families with no affected family members. Based on Li *et al.*'s evidence for shortened telomeres in children with ASD,<sup>8</sup> we anticipated telomere reductions in older siblings with ASD compared to those without the disorder. We also predicted decreased telomere length in parents of children with ASD compared to those without a child with ASD, likely operating through a mechanism of heightened stress. It was less clear, however, as to what to expect of telomere length in infant siblings of children with ASD compared to the infant siblings of typically developing children; although at high risk for ASD, the majority of these infants will not develop the disorder (however, an additional 20%–30% will likely develop another disorder or be viewed as possessing the broader autism phenotype).<sup>29</sup> If shortened telomere length is specific to individuals with ASD, overall reductions in telomere length in infant siblings of children with ASD would not be expected. If, however, shortened telomeres in families with ASD reflects an inherited or acquired familial vulnerability, we would anticipate telomere reductions in infant siblings of children with ASD.

## METHOD

### Study Design

Participants in the current study formed part of an ongoing, longitudinal investigation of neurodevelopment in infant siblings of children with ASD over the first 3 years of life. Infants and their families were designated as high-risk for ASD (HRA) or low-risk for ASD (LRA) according to their older siblings' (proband) diagnostic status. For HRA families, infants had at least 1 older sibling with a

community diagnosis of ASD that was not attributable to a known genetic disorder (e.g., Fragile X, tuberous sclerosis complex). Proband diagnoses of ASD were made according to expert community clinicians before the family's enrollment in the study. After enrollment, diagnoses were verified using the Autism Diagnostic Observation Schedule (ADOS)<sup>30</sup> ( $n = 12$ ) or the Social Communication Questionnaire (SCQ)<sup>31</sup> ( $n = 5$ ). For 1 HRA proband, grouping was made according to expert community diagnosis, as ADOS and SCQ information were missing. For LRA families, infants had an older sibling that did not have ASD (confirmed using the SCQ [ $<12$ ] and/or the ADOS) and no first-degree relatives with a diagnosis of ASD or other neurodevelopmental disorder. Inclusion criteria for infant siblings included a gestational age of at least 36 weeks, no known perinatal or prenatal complications, and no known genetic disorders (e.g., Fragile X syndrome, tuberous sclerosis complex).

For the current study, saliva samples were obtained from consenting families as an optional portion of the larger, longitudinal study. Participating families received \$10 compensation for each individual providing a sample. Institutional review board (IRB) approval was obtained from Boston Children's Hospital (IRB# X10-02-0082). Written, informed consent was obtained from each parent before his or her and their children's participation in the study. Written assent was obtained for siblings more than 8 years of age.

### Participants

Saliva samples were collected from 205 individuals from HRA or LRA families. Data from 1 infant (HRA) were excluded due to a methodological error (with relative telomere length more than 3 standard deviations above the mean of the entire sample). The final sample comprised data for 204 individuals from 66 families, of whom 37 provided samples for all family members (infant sibling, proband, mother, father), and 29 provided data for some family members. Table 1 outlines the sample size and descriptive characteristics for each group.

Age was calculated from each individual's birth date. Age at saliva sample collection was missing for 8 infant siblings (4 HRA; 4 LRA), 7 mothers (4 HRA; 3 LRA), and 3 fathers (2 HRA; 1 LRA). For 14 HRA infants, their older siblings were diagnosed with ASD before the infants' conception (mean length of ASD diagnosis until the infant's birth = 42.46 months, standard deviation [SD] = 20.95 months). Seven older siblings were diagnosed while their mother was pregnant with the infant (mean length of ASD diagnosis until the infant's birth = 4.37 months, SD = 2.07 months). Seven older siblings received an ASD diagnosis after the infant's birth (mean age of infant sibling at proband diagnosis = 13.06 months; SD = 9.21 months).

### Telomere Length

Saliva was collected from participating families using Oragene-assisted DNA collection kits (OGR-575; DNA Genotek Inc., Ottawa, ON, Canada). For parents, 2 mL of saliva was collected. For infants and probands, Oragene saliva sponges (CS-2) were used in conjunction with DNA collection kits (OGR-575). Sponges were placed in the cheek pocket of the mouth and then transferred to the collection kit; 5 saliva sponges were taken for each infant/proband. All individuals were requested not to have eaten within 30 minutes of saliva collection.

We used the real-time polymerase chain reaction (PCR) telomere assay<sup>32</sup> for use in a high-throughput, 384-well format with an Applied Biosystems 7900HT PCR System to determine the relative average telomere length. Briefly, 5 ng of buffy coat-derived genomic DNA was dried down in a 384-well plate and resuspended in 10  $\mu$ L of either the telomere or 36B4 PCR reaction mixture for 2 hours at 4°C. The telomere reaction mixture consisted of 1x QiagenQuantitectSybr Green Master

**TABLE 1** Demographics of High-Risk for Autism Spectrum Disorder (HRA) and Low-Risk for Autism Spectrum Disorder (LRA) Groups for Each Category of Family Member

	HRA (n = 86)				LRA (n = 118)			
	Infant (n = 28)	Proband (n = 18)	Mother (n = 23)	Father (n = 17)	Infant (n = 35)	Proband (n = 31)	Mother (n = 30)	Father (n = 22)
Mean age (mo)	26	87	416	453	27	81	411	449
Age range (mo)	6–53	42–166	334–551	378–563	6–56	52–145	315–499	332–547
Sex (M:F)	16:12	16:2	—	—	22:13	14:17	—	—
Race/ethnicity								
White	25	15	21	16	30	27	27	20
Black	1	0	1	0	1	1	2	0
Asian	0	0	1	0	1	1	1	1
Native Hawaiian/ Other Pacific Islander	0	0	0	0	0	0	0	1
Hispanic/Latino	5	2	1	2	0	0	0	0
More than 1 race	2	3	0	0	3	2	0	0
Not reported	0	0	0	1	0	0	0	0
Median relative TL <sup>a</sup>	0.84 (0.67, 0.97)	0.66 (0.52, 0.79)	0.59 (0.46, 0.72)	0.55 (0.49, 0.74)	0.95 (0.80, 1.12)	0.79 (0.58, 1.05)	0.66 (0.55, 0.83)	0.73 (0.53, 0.93)

Note: F = female; M = male; TL = telomere length.  
<sup>a</sup>Telomere length refers to telomere length to single copy gene number (T/S) ratio value, unadjusted for age (interquartile range: 25th, 75th percentile).

Mix, 2.5mmol/L of DTT, 270 nmol/L of Tel-1 primer (GGTTTTT GAGGGTGAGGGTGAGGGTGAGGGT), and 900 nmol/L of Tel-2 primer (TCCCGACTATCCCTATCCCTATCCCTATCCCTATCCCTATCCCTA). The reaction proceeded for 1 cycle at 95°C for 5 minutes, followed by 40 cycles at 95°C for 15 seconds, and 54°C for 2 minutes. The 36B4 reaction consisted of 1x QiagenQuantitectSybr Green Master Mix, 300 nmol/L of 36B4U primer (CAGCAAGTGGGAAGGTG-TAATCC), and 500 nmol/L of 36B4D primer (CCCATTCTATCAT-CAACGGGTACAA). The 36B4 reaction proceeded for 1 cycle at 95°C for 5 minutes, followed by 40 cycles at 95°C for 15 seconds, and 58°C for 1 minutes 10 sec. All samples for both the Telomere and single-copy gene (36B4) reactions were performed in triplicate on different plates. In addition to the samples, each 384-well plate contained a 6-point standard curve from 0.625 ng to 20 ng using pooled buffy coat-derived genomic DNA. The purpose of the standard curve is to assess and compensate for interplate variations in PCR efficiency. The slope of the standard curve for both the telomere and 36B4 reactions were  $-3.30 \pm 0.15$ , and acceptable linear correlation coefficient (R2) values for both reactions were 0.97 and 0.98, respectively. Blinded quality control samples were interspersed throughout the dataset in order to assess inter-plate and intra-plate variability of threshold cycle (Ct) values. The telomere to single copy gene (T/S) ratio ( $-dCt$ ) for each sample was calculated by subtracting the average 36B4 Ct value from the average telomere Ct value. The relative T/S ratio ( $-ddCt$ ) was determined by subtracting the T/S ratio value of the 5 ng standard curve point from the T/S ratio of each unknown sample. The percent coefficient of variation of Ct values for the telomere and 36B4 PCR reactions was below 0.75% and 0.60%, respectively. The percent coefficients of variation of the Exponential ddCt values were 12%.

### Statistical Analysis

Distributions of telomere length, within groups and family member categories, were non-normal based on their respective

histograms or Shapiro–Wilk tests. Nonparametric Wilcoxon Mann–Whitney *U* test was used to test for any group differences in age. Spearman's rho correlations were used to examine the association between telomere length and age. Point-biserial correlations were used to test for any associations between telomere length and gender and telomere length and race/ethnicity (coded as 0 for white and 1 for African American, Native Hawaiian, or other Pacific Islander, Asian, more than 1 race, and/or Hispanic or Latino). Spearman's rho correlations were used to examine associations in telomere length between family members when telomere length was unadjusted for age. Partial correlations were used to examine associations in telomere length, between family members, adjusting for age. Our ability to examine the effects of family group status on relative telomere length using complex mixed-model procedures that account for the nested design of our data was limited by our sample size. For this reason, we used multiple linear regression to examine the contribution of family groups (HRA versus LRA) on T/S ratios. The association between family grouping (HRA versus LRA) and relative telomere length was tested using linear regression with age, race/ethnicity, and gender as covariates. The association between family grouping and relative telomere length for individual family members was tested using 4 separate regressions (i.e., 1 for each category of family member: infant siblings, probands, mothers, fathers) with age as a covariate. Participants with missing age values were excluded within each regression analysis. All analyses were conducted using SPSS version 21.0 (IBM Corp., Armonk, NY).

## RESULTS

### Demographic Variables and Telomere Length

There were no significant age differences between HRA (median = 114.50 months) and LRA groups (median = 92.50

months),  $U = 4.241$ ,  $z = 0.17$ ,  $p = .866$ , collapsed across all family members. Similarly, at the family member level, there were no group differences in age between infant siblings ( $U = 395.00$ ,  $z = 0.39$ ,  $p = .696$ ), probands ( $U = 257.00$ ,  $z = -0.46$ ,  $p = .648$ ), mothers ( $U = 276.00$ ,  $z = 0.44$ ,  $p = .663$ ) or fathers ( $U = 157.00$ ,  $z = -0.02$ ,  $p > .999$ ). Consistent with prior research demonstrating telomere shortening as a function of age, telomere length was inversely related to age in our sample ( $r_s = -0.448$ ,  $p < .001$ ). Race/ethnicity was associated with telomere length across the entire sample, with longer T/S ratios in individuals identifying as African American, Native Hawaiian or other Pacific Islander, Asian, more than 1 race, and/or Hispanic or Latino ( $r_{pb} = 0.159$ ,  $p = .023$ ). However, this association was only observed at the group level, with no significant associations found within family member categories (infants:  $r_{pb} = 0.132$ ,  $p = .302$ ; probands:  $r_{pb} = 0.066$ ,  $p = .653$ ; mothers:  $r_{pb} = 0.232$ ,  $p = .094$ ; fathers:  $r_{pb} = 0.205$ ,  $p = .210$ ). Furthermore, there was no association between telomere length and gender either across the entire sample ( $r_{pb} = 0.019$ ,  $p = .790$ ), or within the infant sibling ( $r_{pb} = 0.193$ ,  $p = .130$ ) or proband categories ( $r_{pb} = 0.189$ ,  $p = .194$ ).

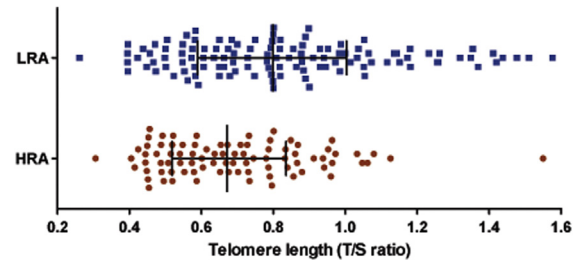
#### Regression Analyses: Group Status (HRA Versus LRA) and Telomere Length

We next considered the contribution of group status (HRA versus LRA) to telomere length at the family level. Given the significant associations with telomere length, age and race/ethnicity were included as covariates in the group level (HRA versus LRA) regression analyses. Adjusting for these variables, HRA family status was associated with shorter relative telomere length ( $\beta = 0.138$ ,  $p < .001$ ) (Figure 1). Of note, this same effect was observed when we confined our analyses to only those families that provided samples for all four family members; specifically, HRA family status was significantly associated with shorter T/S ratios ( $\beta = 0.108$ ,  $p = .012$ ). Although gender was not associated with telomere length in our sample, based on evidence for longer average T/S ratios in females compared to males,<sup>10</sup> we ran the same models with gender included as an additional covariate. Incorporating all individuals, group status predicted telomere length ( $\beta = 0.136$ ,  $p < .001$ ); however, gender was not associated with T/S ratios ( $\beta = 0.021$ ,  $p = .558$ ). When constraining our analyses to complete quads, group status was associated with telomere length ( $\beta = 0.106$ ,  $p = .015$ ) but not gender ( $\beta = 0.027$ ,  $p = .529$ ).

#### Regression Analyses: Individual Family Members and Telomere Length

We next considered whether shortened telomeres in the HRA families were observed across all family members, or instead, whether this effect was specific to certain members of the family (e.g., parents). Because of the association with age within each family member category, all models were performed with age as a covariate. For mothers, being a part of a HRA family was associated with shorter relative telomere length ( $\beta = 0.110$ ,  $p = .039$ ). For infant siblings, there was a marginal association between HRA family

**FIGURE 1** Average relative telomere length (T/S ratio) for individuals in high risk for autism spectrum disorder (HRA) and low risk for autism spectrum disorder (LRA) family groups (medians and interquartile ranges plotted).



status and shorter T/S ratios ( $\beta = 0.141$ ,  $p = .051$ ). Similarly, for probands, we observed a marginal association with HRA family status ( $\beta = 0.159$ ,  $p = .050$ ). Fathers presented with a similar pattern of results (Figure 2); however, the effect of both age ( $\beta = -0.001$ ,  $p = .100$ ) and family grouping ( $\beta = 0.132$ ,  $p = .127$ ) were nonsignificant in this subgroup; notably, we had fewer samples from fathers than other categories of family member, limiting our power at this level of analysis.

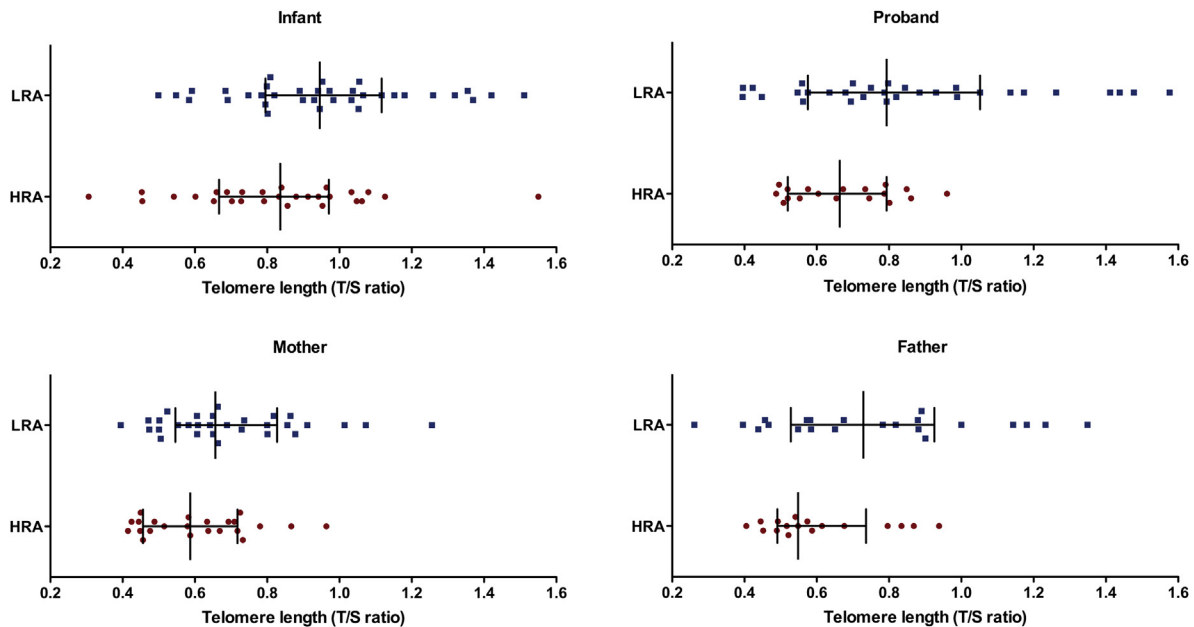
## DISCUSSION

Overall, we found that in families with 1 child with autism and a younger sibling, the mother, infant, and affected sibling all showed shortened telomeres relative to families with no history of the disorder (fathers showed a nonsignificant trend in the same direction as other family members). These findings extend prior research demonstrating shorter telomere length in caregivers of children with a chronic illness,<sup>14</sup> and children with ASD<sup>8</sup> to suggest that infants at high risk for ASD also have shortened telomeres.

It is well known that families raising a child with autism can experience considerable stress,<sup>14,21</sup> which would be one putative mechanism underlying shortened telomere length. In addition, genetic alterations and metabolic processes linked to telomere length dynamics have also been implicated in ASD and/or family members of individuals with ASD. Indeed, increased oxidative stress, which has been linked to telomere erosion, has been reported in individuals with ASD,<sup>24</sup> as well as their parents.<sup>33</sup> Relatedly, DNA hypomethylation has been found in children with ASD (but not their unaffected siblings), compared to typically developing children,<sup>25</sup> and post mortem studies have identified aberrant DNA methylation of MeCP2<sup>34</sup> and oxytocin receptor genes<sup>35</sup> in ASD; this is pertinent in lieu of evidence suggesting that DNA methylation is integral in telomere biology.<sup>36,37</sup> Furthermore, genetic variations in pathways associated with DNA repair mechanisms and chromatin remodeling, processes involved in telomere structure and function,<sup>38</sup> have been implicated in ASD susceptibility.<sup>26,27</sup> In addition, shortened telomeres have been reported in individuals who carry the full FMR1 mutation<sup>39</sup> (responsible for Fragile X syndrome, a genetic disorder linked to increased risk of ASD) as well as those who carry the



**FIGURE 2** Individual plots of average relative telomere length (T/S ratio) between high risk for autism spectrum disorder (HRA) and low-risk for autism spectrum disorder (LRA) infant siblings, probands, mothers, and fathers (medians and interquartile ranges plotted).



permutation<sup>40</sup> compared to age-matched controls. Together, these lines of evidence suggest that a number of genetic mutations and variants implicated in ASD susceptibility are also linked to alterations in telomere length dynamics. It is also well known that telomere length is highly heritable, with a recent meta-analysis<sup>9</sup> reporting a heritability estimate of 0.7. The 20-fold increased prevalence of autism among infant siblings<sup>28</sup> clearly points to a genetic liability among such families. At the same time, it is plausible to postulate that such families are also under considerable stress due to the uncertainty of whether the infant sibling will subsequently develop the same disorder as the older sibling. Such stress would affect not only the parents but might reasonably filter down to the children as well. Overall, then, high-risk families carry 2 risk factors for shortened telomeres: a genetic liability and a familial (experiential) liability. Such high-risk families clearly require close monitoring.

An additional consideration for future research is whether decreased telomere length in the infancy and early childhood period can serve as a biomarker for predicting autism risk. In this context, it is extremely promising that in using a different and less invasive method of DNA extraction (i.e., saliva), our findings in probands and parents replicate previous work by Li *et al.*<sup>8</sup> and Epel *et al.*,<sup>14</sup> both of whom assessed telomere length from leukocytes. This is an especially important consideration for future studies that aim to examine telomere length as a predictive biomarker of ASD in young infants. It will also be important to consider whether telomere length may serve as a marker of risk for developing other physical (e.g., gastrointestinal) or psychological (e.g., anxiety) sequelae. The marginal effects of shortened telomeres in probands with ASD and high-risk

infants, while requiring replication with larger sample sizes in each group, highlights a need for studies to examine physical health outcomes in ASD and ASD susceptibility, particularly with regard to rates of obesity, cardiovascular disease, and diabetes, in lieu of their associations with shortened telomere length.

A limitation of the current study was the lack of an independent assessment of family stress, although a substantial body of literature supports the plausibility of this hypothesis.<sup>14-22,41-47</sup> Future studies would benefit from larger sample sizes that would allow more detailed examinations of telomere length, stratified by gender and race/ethnicity. The effect of gender on telomere length was unable to be adequately examined in this sample, due to the skewed gender distribution between the groups, particularly for HRA probands. Larger samples would also allow analytic approaches that could account for possible correlated data (such as hierarchical linear modeling). In addition, because telomere length is partially heritable, it is impossible to ascertain what portion of the variance in our findings can be accounted for by genetics versus experience and the interaction thereof. Nevertheless, the facts that the parents share no genes and the siblings share only 50% of their genes with each other and with the parents makes it likely that our findings reflect a genetic vulnerability that interacts with perceived or real family stress. Although additional research will be required to tease apart these variables, it seems that there is a potential biological cost to family members raising an infant in a family that already has 1 sibling with ASD; how this plays out in terms of long-term outcome as well as its influence on rearing environment remains to be determined. &

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