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**ACQUIRED CYTOGENETIC CHANGES IN ADULT TWINS DISCORDANT FOR A  
HISTORY OF CHILDHOOD SEXUAL ABUSE**

**A dissertation submitted in partial fulfillment of the requirements for the degree  
of Doctor of Philosophy at Virginia Commonwealth University.**

**by**

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**Virginia Commonwealth University, 2011  
Richmond, Virginia  
September, 2011**

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## **Abstract**

### **ACQUIRED CYTOGENETIC CHANGES IN ADULT TWINS DISCORDANT FOR A HISTORY OF CHILDHOOD SEXUAL ABUSE**

**Jenni Rebecca Brumelle, B.S., B.A.**

**A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.**

**Virginia Commonwealth University, 2011**

**Director: Colleen Jackson-Cook, Ph.D.  
Department of Pathology**

The primary study aim was to evaluate the latent biological effect of childhood sexual abuse (CSA) on adults by quantifying acquired cytogenetic changes and cortisol levels in identical twins who were discordant (N=22) or concordant (N=2) for a history of CSA. Although the difference scores for cortisol values between discordant identical co-twins were not significantly different from zero, a trend was observed for the twins exposed to intercourse, the most severe form of CSA, to have a blunted cortisol awakening response. Acquired cytogenetic changes were assessed by scoring telomere lengths and somatic cell abnormality frequencies via a cytokinesis-block

micronucleus (MN) assay. No significant difference in overall telomere intensity values was observed between co-twins, but chromosome-specific telomere differences were observed in the individuals exposed to intercourse compared to their unabused co-twins ( $[ \chi^2(45) = 62.88; p = 0.040$  and  $\chi^2(45) = 73.72; p = 0.004$ ). Specifically, shortened telomeres were observed on the short arms of chromosomes 3, 5, & 6, and long arms of chromosomes 11 & 13. A significant increase in MN frequencies was observed in the abused twins compared to unabused twins ( $t=2.65; df=16; p=0.009$ ). A significant interaction between micronuclei frequencies and age was also observed, suggesting that the biological effects of stress are cumulative (coefficient [SE] = 0.030 [0.009];  $p=0.0006$ ). However, the pattern of chromatin present in MN, which was assessed using spectral karyotyping methodologies, was not limited to the subset of chromosomes with telomeric attrition.

In summary, this is the first assessment of acquired chromosomal abnormalities, chromosome-specific telomere lengths and cortisol levels in identical adult twins discordant for exposure to CSA. Given that a portion of biological changes were most pronounced in the intercourse discordant twins, these findings support a possible dose-response relationship with CSA severity. Our data also suggest that the MN assay is a superior tool in assessing the latent effects of stress compared to either cortisol profiling or the measurement of telomere lengths. Collectively, application of the information gained from these studies may allow for novel screening techniques to identify individuals who are most at risk for developing stress-associated disease states.

## **Chapter 1**

### **Background and Significance**

Beginning with Walter Cannon who, in the early 1930's referenced the role of stress in the disruption of homeostasis, it has been acknowledged that stress can have deleterious physiological effects (1932). Cohen et al. (1997) defined a theoretical model of stress as "a process in which environmental demands tax or exceed the adaptive capacity of an organism, resulting in psychological and biological changes that may place persons at risk for disease". Stress is a known risk factor for cardiovascular disease, the leading cause of morbidity and mortality in industrialized nations (Yusef et al., 2004; American Heart Association, 2005) and has been linked to autoimmune diseases, infectious diseases, psychiatric illness and decreased immune function (reviewed in Cohen et al., 1997; Yang and Glaser, 2000). Although several human disease states have been associated with stress, it is not known how stress causes disease because the mechanisms of stress-induced pathology are multi-faceted, complex and include physical as well as psychological response patterns.

In a provocative 2004 study, Epel et al. reported a correlation between stress and accelerated telomere shortening. Telomeres are non-coding repetitive (TTAGGG) sequences at the ends of linear chromosomes that act as a buffer for semi-conservative replication and the end replication problem. Telomeres protect the ends of linear

chromosomes and shorten with normal cellular aging, which was found by Epel et al. to be accelerated in caregivers of sick children (2004). Accelerated telomere shortening has also been associated with pathogenic processes. Telomere attrition and dysfunction have been speculated to be a causal factor in the acquisition of many age-related diseases including, but not limited to, cardiovascular disease; hypertension; atherosclerosis; diabetes and Alzheimer's disease (Aviv and Aviv, 1998; Effros et al., 2005; Zanni et al., 2011; reviewed in Zhu et al., 2011). Attrition of telomeres has been associated with diminished cellular proliferative capacity and chromosomal instability (de Lange et al., 1990). This chromosomal instability is thought to arise, at least in part, as a result of end-to-end chromosome fusion (de Lange et al., 1990; Hemann et al., 2001; Verdun et al., 2007) and has been associated with chromosomal structural abnormalities, aneuploidy (Harley, 1991; Counter et al., 1992; Sandell and Zakian, 1993; Day et al., 1998; Filatov et al., 1998; Fagagna et al., 1999; Schwartz et al., 2001; Leach and Jackson-Cook, 2004) and mutations that lead to cancer (Surrallés et al., 1999; O'Sullivan et al., 2002; Aida et al., 2010).

Epel has referred to telomeres as a “psychobiomarker”, or a biological measure influenced by psychological parameters, which chronicles an individual's recent and cumulative health and/or stress history (2009). In addition to disease, loss of telomere length homeostasis has been linked to psychological factors. Psychological parameters, including an individual's perception of the duration and severity of a stress experience, have been implicated in telomere shortening (Epel et al., 2004, 2006). An attrition of 550 base pairs, or an estimated 17 years of accelerated aging, has been associated

**Table 1. Summary of studies linking stress to telomere attrition**

<b>Author</b>	<b>Date</b>	<b>Cohort</b>	<b>Method</b>	<b>Results</b>
<b>Chronic stress in adults<sup>1</sup></b>				
Epel et al.	2004	19 controls 39 caregivers to chronically ill children	qPCR	Chronicity and perceived stress associated with telomere attrition (up to 17 years accelerated aging)
Cherkas et al.	2006	749 twin pairs, subset discordant for low SES <sup>2</sup>	Southern blot	Telomere attrition in those exposed to low SES
Simon et al.	2006	44 controls 44 with chronic mood disorders	Southern blot	Telomere attrition in those with mood disorders (10 years accelerated aging)
Damjanovich et al.	2007	41 controls 41 caregivers to patients with AD <sup>3</sup>	Southern blot	Telomere attrition in caregivers (loss of 6.2-6.4 kb <sup>4</sup> ) compared to controls
Parks et al.	2007	647 sisters of breast cancer patients	qPCR	Perceived stress “modestly associated” with telomere attrition and stress hormones
Humphreys et al.	2011	41 controls 61 exposed to IPV <sup>5</sup>	qPCR	Telomere attrition in those exposed to IPV
<b>Stress in childhood measured in children/adolescents</b>				
Drury et al.	2011	136 children in institutionalized care	qPCR	Telomere attrition by middle childhood in females exposed to institutionalized care. Duration of stress also correlated to telomere shortening
<b>Stress in childhood measured in adults</b>				
Tyrka et al.	2009	21 controls 10 exposed to neglect	qPCR	Telomere attrition in those exposed to childhood neglect

**Table 1 (continued). Summary of studies linking stress to telomere attrition**

<b>Author</b>	<b>Date</b>	<b>Cohort</b>	<b>Method</b>	<b>Results</b>
Glass et al.	2010	1751 controls 123 exposed to CSA <sup>6</sup> or physical abuse	Southern blot	Childhood maltreatment not linked to telomere attrition
Kananen et al.	2010	653 controls 321 with anxiety disorder	qPCR	Telomere attrition in those with multiple childhood adversities
Entringer et al.	2011	49 controls 45 exposed to prenatal stress	qPCR	Telomere attrition in those exposed to prenatal stress (loss of 178 bp compared to controls )
Kiecolt-Glaser et al.	2011	74 controls 58 caregivers to patients with dementia (a subset also experienced childhood adversity)	qPCR	Telomere attrition in those exposed to childhood adversity (7-15 yrs accelerated aging) and increased inflammation following later life stress
O'Donovan et al.	2011	43 with PTSD <sup>7</sup> and childhood trauma	qPCR	Telomere attrition in those exposed to childhood trauma and with PTSD

<sup>1</sup>all female participants

<sup>2</sup>socio-economic status

<sup>3</sup>Alzheimer's disease

<sup>4</sup>kilobases

<sup>5</sup>Intimate partner violence

<sup>6</sup>childhood sexual abuse

<sup>7</sup>post traumatic stress disorder



with perceived high levels of stress and chronicity of stress (Epel et al., 2004). Additionally, it is believed that telomere maintenance confers an ability to recover from, or may provide increased resistance to, environmental stress (Epel et al., 2004; Strub, et al., 2008). One mechanism of telomere length maintenance is via telomerase, a reverse transcriptase that functions to extend the 3' end of chromosomes. Reduced telomerase activity has been measured in individuals with stress-related phenotypes (Epel et al., 2004). The results of these and additional studies have demonstrated that telomere shortening and reduced telomerase activity is associated with stress from a variety of situations including low socio-economic status (Cherkas et al., 2006), primary caregiver status to patients diagnosed with Alzheimer's disease (Damjanovic et al., 2007), and mood disorders (Simon et al., 2006). These results indicate that chronic and perceived life stress play a role in stress-induced premature telomere shortening (Table 1).

While several investigators have evaluated telomere attrition in response to chronic stress experienced in adulthood, few researchers have examined telomere length in individuals who experienced childhood adversity. Telomere attrition has been observed in adults who experienced stressful events in childhood (Kananen et al., 2010; Tyrka, 2010), but this association has not been universally observed. For example, Glass et al. (2010) reported no correlation between childhood physical or sexual abuse and telomere shortening, and suggested that alternative environmental exposures such as smoking and obesity confounded prior observations linking childhood stress to telomere attrition.

Historically, the gold standard for telomere length assessment is the terminal restriction fragment (TRF) technique. Quantitative polymerase chain reaction (PCR)-based techniques have also been utilized for telomere length analysis, particularly to characterize stress-induced shortening (Table 1). However, these approaches are limited because the data yield an average of genomic telomere length, with no measure of chromosome specific length. While these methods are efficient for detecting telomere attrition that involves a majority of chromosomes, they do not allow for the recognition of a small subset of chromosomes with shortened telomeres and provide no information about the specific chromosome(s) with potentially dysfunctional telomeres. In the first study to report chromosome-specific telomere length assessments, Lansdorp et al. (1996) observed a 6-fold difference in telomere lengths between chromosomes, independent of tissue type. It is important to know if one, or a combination of a particular set of chromosomes, is involved in a phenotypic characteristic, particularly if the trait is pathogenic. Modifications of Landdorp's et al (1996) fluorescence *in-situ* hybridization (FISH)-based method have been employed by a small number of investigators to gain precision in the assessment of chromosome-specific telomere lengths. While chromosome-specific telomere length varies from chromosome to chromosome, between metaphase spreads and between tissues, a common telomere profile has emerged, with chromosome 17 demonstrating short, if not the shortest, telomere. (Table 2). This high resolution approach to telomere length characterization has been used in heritability and cancer studies but has not, to date, been applied to evaluate chromosome-specific lengths in a stressed cohort.

The observation that psychosocial factors, including chronic stress from a variety of modalities, are associated with telomeric shortening supports the idea that stress leads to alterations in the DNA of somatic cells. Additionally, telomere attrition in peripheral blood leukocytes has been associated with age-related aneuploidy, especially acquired chromosomal loss (Surralles et al., 1999; Leach and Jackson-Cook, 2004). It has been suggested that shortened telomeres may be unable to stabilize chromosomes and may therefore compromise the fidelity of replication and mitotic segregation leading to acquired chromosomal abnormalities (Aviv and Aviv, 1998). The cytokinesis-block micronucleus assay allows information regarding chromosome

**Table 2. Review of human chromosome-specific telomere length studies**

<b>Author</b>	<b>Date</b>	<b>Tissue</b>	<b>N</b>	<b>Results</b>
Lansdorp et al.	1996	Fetal liver Umbilical cord Adult bone marrow	16	6-fold variation in telomere lengths between metaphase spreads; 17p shortest; 4q longest; 19p, 22q and 20q also short
Martens et al.	1998	Lymphocytes	16	17p shortest
Martens et al.	2000	Fetal skin fibroblasts	2	2q, 16p, 17p, 20q shortest; 3p, 5p, 6p/q longest
Graakjaer et al.	2003	B Lymphocytes, amnion cells, fibroblasts	26 9 3	Common telomere profile between tissues in an individual; 13p shortest; Xp longest
Perner et al.	2003	PB Lymphocytes UC Lymphocytes	70 108	17p among but not shortest; Xq shorter in boys compared to girls
Graakjaer et al.	2004	T Lymphocytes	7 twin pairs	Aging impacts telomere lengths of homologous chromosomes in twins (ie: maternally derived homologue) equally
Leach et al.	2004	Lymphocytes	6	16p/q and 17p/q shortest
Mayer et al.	2006	Lymphocytes	205	19p shortest; 17p also short; Gender specific differences for 21p/q
Zheng et al.	2011	Lymphocytes	240	Short telomeres on 9p, 15p and 15q associated with increased breast cancer risk

breakage and loss to be evaluated. A micronucleus is a small chromatin-containing structure that forms when whole chromosomes or chromosome fragments fail to migrate to spindle poles during anaphase. The lagging chromosome(s) or fragment(s) are enclosed in their own nuclear envelope and are excluded from the daughter nuclei (Fenech, 2000). A high throughput and robust method for estimating acquired chromosomal abnormalities is to analyze the frequency and chromosomal contents of micronuclei in cytokinesis-blocked cells. Twin studies have revealed that micronuclei frequencies are impacted by environmental factors (Jones et al., 2011, Surowy et al., 2011) and an increased frequency of micronuclei has been shown to be an indication of genomic instability (Fenech, 2000). The cytokinesis-block micronucleus (CBMN) assay is an attractive tool for estimating genomic damage associated with environmental insults such as stress (Battershill et al., 2008). To date, no investigators have evaluated the effect of psychosocial stress on micronucleus frequency in humans.

Although little is known about the biological mechanism(s) linking psychosocial stress and pathologic processes, the relationship between cortisol and telomere length is an emerging area of research. Cortisol is a corticosteroid hormone (glucocorticoid) released from the adrenal cortex which functions in a largely catabolic manner to restore homeostasis after stress (Baum et al., 1995). Cortisol is secreted when the hypothalamic-pituitary adrenocortical (HPA-) axis is stimulated by a stressor. *In vitro* hydrocortisone (synthetic cortisol) levels, comparable to *in vivo* physiologic plasma cortisol concentrations reached during stress, reduce telomerase activity by 50% (Choi

et al., 2008). *In vivo*, thymic murine telomeres were shortened in response to glucocorticoid injection (Ichiyoshi et al., 2003). In humans, reduced genomic telomere lengths have been associated with an elevated nocturnal urinary cortisol level (Epel et al., 2006). To date, the aforementioned is the only analysis of cortisol and telomere length in humans *in vivo*. It is not known if cortisol, as part of the stress response, impacts acquired chromosomal changes and chromosome specific telomere length.

The increased activation of the HPA-axis and elevated cortisol levels that occur during chronic stress have been associated with poor antibody response to immunization and several disease states including depression, coronary heart disease, obesity and diabetes (Vedhara et al., 1999; Koertege et al., 2002; Innes et al., 2007). Low socioeconomic status is associated with an increased cortisol response to awakening and high basal cortisol levels independent of race, age, or gender (Kunz-Ebrecht et al., 2004, Cohen et al., 2006). Similarly, elevated cortisol levels have been linked to stress from caregiver status, work strain and anticipation of stressful situations (Pruessner et al., 1999; Vedhara et al., 1999; Kunz-Ebrecht et al., 2004; Kim et al., 2008). Further illustrating the ambiguous nature of the stress response, chronic stress conditions, such as post-traumatic stress disorder, are associated with lower cortisol levels in normal (Olf et al., 2006) and abused populations (Bremner et al., 2007). These findings suggest that chronic stress leads to perturbations of the normal activity of the stress response system. However, it is not known if the effects of stress on allostatic systems are cumulative, or if there is a threshold before stress “gets under the skin” to become biologically embedded.

If the effects of stress are cumulative, the greatest sequelae may be observed by studying adults with a history of stressful events in childhood. One source of childhood stress is abuse, and although a biological response to acute stress occurs to maintain homeostasis after an environmental insult, chronic stress has complex effects and a large impact on the allostatic system (reviewed in Miller et al., 2007). Stressful life events (SLE) in childhood lead to dysregulation of allostatic systems that have long-term consequences and/or potential latent effects (reviewed in Miller et al., 2007). Childhood sexual abuse (CSA) is an early and extreme SLE, with an incidence of 1 in 4 in females (National Center for Victims of Crime, 2011). CSA is linked to psychological disorders; sexual dysfunction; neurological and musculoskeletal problems; cardiovascular disease; respiratory, gastrointestinal and metabolic disorders; chronic fatigue syndrome; criminal and risk taking behaviors including smoking, prostitution, suicide and substance abuse (Kendler et al., 2000; Miller et al., 2009; Wegman et al., 2009; American Psychological Association, 2011).

When evaluating the long-term impact of CSA on biological measures in adults, most investigators have focused on assessments of cortisol. Perturbations in cortisol levels have been observed in adults with a history of CSA. Longitudinal studies of cortisol levels in individuals exposed to CSA reveal a shift from hypercortisolism in childhood following abuse exposure(s) to hypocortisolism in adolescence (Putnam, 1997; reviewed in Tarullo and Gunnar, 2006; Trickett et al., 2010). Although there are many reports regarding cortisol and the neuroendocrine response to stress in

individuals exposed to childhood abuse, varying responses have been reported depending on the type of abuse evaluated (sexual, physical and emotional), the severity of the abuse and the testing methodology utilized. HPA axis function can be assessed at basal levels and in response to an applied stressor (stress reactivity). There are two methods used in published studies to measure stress reactivity. These methods include pharmacological challenge and application of a psychological stressor. A pharmacological challenge is an exogenous administration of a hormone to stimulate cortisol production, and a psychological stressor is a stressful situation, such as a public speaking task. With respect to adult subjects, a number of investigators observed cortisol response to a laboratory stressor, but not baseline, representative daily levels. Some studies were conducted using patients with co-morbid mood or anxiety disorders, or in populations with adverse life events, but without sexual abuse. Although these confounders make comparing the results from the current literature difficult, it is evident that disruption of the normal activity of the HPA axis in early childhood has complex and long-term effects.

It is not known if cortisol levels, perhaps altered by an early SLE, impact acquired chromosomal changes and chromosome-specific telomere lengths. Furthermore, measurement of telomere lengths and acquired chromosomal abnormality frequencies may allow elucidation of how stress impacts cellular function and may have promise to be used as biomarkers for the identification and early detection of susceptibility to stress-related disease states. Before optimizing an assay for predictive or screening purposes, the contributory factors to and variability of the measured trait need to be



evaluated. One powerful method to fully appreciate the role of complex influences, such as stress in humans, while avoiding potentially confounding effects from heritable genetic and common environmental factors, is to evaluate that trait in identical (MZ) twins. Using MZ co-twins who are discordant for a trait or for an environmental exposure is a robust approach to determining the effect of environmental factors on biological outcomes (Wong et al., 2005). Because MZ co-twins are genetically identical, if they are discordant for an environmental exposure, any trait difference observed between them is attributable to the environmental exposure. Such a design has been used to assess the effect of bullying on cortisol (Ouellet-Morin et al., 2011), psychiatric disorders in combat veterans (Orr et al., 2003; May et al., 2004; Pitman et al., 2006; Metzger et al., 2009; Gilbertson et al., 2010), depression risk and social support in a stressed cohort (Coventry et al., 2009), and epigenetic profiles in individuals with psychiatric disorders (Kaminsky et al., 2008; McGowan and Kato, 2008).

In conclusion, given the documented links between: 1) stress and telomere shortening; 2) telomere shortening and acquired chromosomal changes; and 3) stress and cortisol levels, we will directly test the association between CSA, chromosome-specific telomere length, acquired chromosomal changes and cortisol levels. This study is the first to assess all of these factors and the results will help elucidate a possible mechanism between stress and biologic dysfunction. A powerful identical (MZ) twin design will allow us to evaluate the utility of potential novel biomarkers for stress that may have clinical application in personalized medicine. The data obtained will allow us to directly test the following 4 main hypotheses:

1. There are differences in salivary cortisol levels in adults who were exposed to a major stressor (childhood sexual abuse) at an early age.
2. There is a decrease in chromosome-specific telomere lengths in adults who were exposed to a major stressor (childhood sexual abuse) at an early age.
3. There is an increase in micronuclei frequencies in adults who were exposed to a major stressor (childhood sexual abuse) at an early age.
4. There are differences in the chromosomes included in micronuclei in adults who were exposed to a major stressor (childhood sexual abuse) at an early age.

Collectively, the application of information gained from these studies may allow for novel and advanced screening techniques for stress-associated disease states and/or disease susceptibility.

## **Chapter 2**

### **Salivary Cortisol in Twins Discordant for Childhood Sexual Abuse**

#### **Introduction**

Although stress is an adaptive response that allows an individual to respond to acute external threats, prolonged periods of stress are deleterious. Exposure to chronic or early life stress overburdens allostatic systems, which strive to maintain homeostasis, and leads to dysregulation of metabolic systems and commensurate disease states (Cohen et al., 1997; Yang and Glaser, 2000; Yusef et al., 2004). Children who experience traumatic, early-life events have consistently been shown to have an increased incidence of behavioral and health problems in adulthood. These problems include depression, anxiety, fear, sexual dysfunction, self-destructive behaviors, neurological and musculoskeletal problems, cardiovascular disease, and respiratory, gastrointestinal and metabolic disorders (Kendler et al., 2000; Miller et al., 2009; Wegman et al., 2009; American Psychological Association, 2011). The biological basis for this increased latent morbidity risk is not known, but it has been suggested to reflect a biological cascade of events mediated by the hypothalamic-pituitary adrenocortical (HPA) axis (Baum et al., 1995). Briefly, in response to a stressor, the release of corticotropin releasing hormone (CRH) from the hypothalamus initiates systemic arousal and stimulates the anterior pituitary to release adrenocorticotrophic hormone (ACTH). ACTH acts on the adrenal cortex to trigger release of glucocorticoid (cortisol), which

functions in a largely catabolic manner by increasing gluconeogenesis and decreasing the activity of the immune system (Baum et al., 1995). The anti-inflammatory actions of cortisol include the suppression of pro-inflammatory cytokines and repression of the transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and activator protein-1 (AP-1 [reviewed in Butcher and Lord, 2004]). The resultant perturbation in gene activity may contribute to biological changes in several systems (Lupien et al., 2007). However, because many of the molecules involved in these processes are relatively short-lived, the mechanism(s) for how this response leads to a latent effect on health has been enigmatic.

Given that cortisol levels are thought to serve as an objective marker of stress-induced HPA-axis activity (Kirschbaum et al., 1989), it has been the major focus of research on stress-related conditions. Assessment of cortisol levels has allowed researchers to gain insight about how stress contributes to an associated increase in morbidity risk. The diurnal rhythm of cortisol secretion varies among individuals and is impacted by physiological stressors (illness and physical exertion) and by psychological stressors (pain and fear [Levine, 1993]). For most people, the highest levels of cortisol are measured 30 minutes after awakening (cortisol awakening response [CAR]) followed by the progressive reduction to a nadir at bedtime (Linkowski et al., 1993). During chronic stress, prolonged cortisol secretion (hypercortisolism) and increased activation of the HPA-axis have been associated with poor antibody response to immunization, as well as the acquisition of several disease states, including depression,

coronary heart disease, obesity and diabetes (Vedhara et al., 1999; Koertege et al., 2002; Innes et al., 2007).

Perturbations of the normal activity of the HPA axis in early childhood are thought to have complex and long-term effects that persist into adulthood (Heim and Nemeroff, 2000). Thus, the greatest effect of stress in adults may arise from extreme stress situations experienced in childhood. One source of extreme stress in childhood is abuse, which has been the focus of many investigators. Varying and sometimes opposite cortisol responses have been reported depending on the type of maltreatment studied (emotional neglect, physical neglect, sexual abuse, physical abuse, emotional abuse) and the testing methodology utilized. Furthermore, the independent effects specific to abuse can be difficult to identify when, as is often the case, more than one type of abuse is reported and when a comorbid psychological disorder is present (Olf et al., 2006; Bremner et al., 2007). Therefore, interpreting and comparing the results from many studies in the current literature regarding the long-term effects of childhood abuse can be problematic.

When focusing on cortisol levels in individuals with a history of childhood sexual abuse (CSA), one of the most traumatic forms of early-life stress, investigators have consistently shown measurable perturbations in baseline cortisol levels, beginning as early as 7 years after the CSA exposure (Shenk et al., 2010), that are maintained for decades after the abuse incident(s) (Brewer-Smyth and Burgess, 2008). These perturbations are thought to arise due to higher basal levels of neuroendocrine

hormones commensurate with hyperactivity of the HPA axis and increased production of CRH (deBellis et al., 1994; Nemeroff, 1996; Liu et al., 1997). Longitudinal studies of sexually abused girls revealed a shift from hypercortisolism in childhood to hypocortisolism by adulthood (Putnam, 1997; reviewed in Tarullo and Gunnar, 2006; Trickett et al., 2010). This hypoactivation of cortisol levels, which has been termed “HPA burnout”, is thought to occur when chronically elevated cortisol levels during childhood neurodevelopment exhaust allostatic systems and lead to permanent alterations (blunted responses) in HPA axis activity later in life. The attenuation hypothesis posits that to prevent a chronic stress response, there is a down regulation of the stress response system to compensate for the development of an allostatic load that would lead to morbidity and mortality. This down regulation of the neuroendocrine response to stress is thought to result in a hypoarousal state with lower circulating levels of cortisol (Susman, 2006), and thus the hypocortisol baseline state observed in adolescents and adults with a history of CSA. In addition to directly influencing cortisol, counter-regulatory mechanisms lead to a resistance to glucocorticoid receptor (GR)-mediated signaling by downregulation and desensitization of GR to cortisol (reviewed in Miller et al., 2009).

As is evident, the relationship between CSA and HPA-axis activity is not static or straightforward. Using psychosocial stress tests to evaluate cortisol reactivity in adults with a history of CSA, investigators have shown varied responses. Heim and Nemeroff (2000) observed increased cortisol levels following a stress challenge, but other investigators observed no such effect (Carpenter, 2010). One explanation for these

disparate results is that long-term cortisol response may be mediated by one's psychological assimilation of the abuse experience. Pierrehumbert et al. (2009) reported that individuals with unresolved trauma had the highest perceived levels of stress and suppressed cortisol reactivity to applied laboratory stressor. In addition, heritable genetic effects have also been shown to influence glucocorticoid levels (Bartels et al., 2003), as well as the regulation of baseline cortisol levels (Franz et al., 2010) and are likely to account for some of the variation observed in laboratory-induced or non-induced cortisol measures. To avoid the confounding influences on cortisol levels that are inherently present due to genetic effects, an efficient research design for assessing cortisol patterns would be to study adult identical (monozygotic or MZ) twins discordant for a history of stress (Wong et al., 2005). To date, no studies evaluating cortisol levels and CSA have been conducted in twins. Therefore, to gain insight about the long-term effects of CSA on non-laboratory stress-induced cortisol levels, we measured baseline, salivary cortisol values in adult MZ twin pairs who are discordant for a history of CSA. The primary aim of this study was to determine if there are differences in salivary cortisol levels in individuals exposed to CSA compared to their identical, unabused co-twins.

## **Materials and Methods**

### **Sample Ascertainment**

Female twin pairs with a history of childhood sexual abuse (CSA) were ascertained through the Mid-Atlantic Twin Registry at Virginia Commonwealth University. These individuals were recruited for this project as a result of their participation in a study completed by Kendler et al. (2000), which focused on understanding genetic and social influences impacting the propensity to develop psychiatric and substance abuse disorders. Self-reported history of CSA was assessed via mailed questionnaire and was co-twin confirmed (Kendler et al., 2000). CSA categories included: 1) no genital contact (sexual invitation, sexual kissing and exposing; 2) genital contact without intercourse (fondling and sexual touching; and 3) intercourse (Kendler et al., 2000). Discordance for abuse exposure was used to prioritize the twins selected for recruitment in this project. Twin pairs concordant for exposure to CSA were included as positive controls.

After providing informed consent (VCU IRB protocol HM12407), participants were instructed to collect 6 ml of saliva at home over two consecutive, non-weekend days prior to providing a blood sample, the latter of which was used for confirmation of zygosity status. Saliva samples were collected on each of the two days: 1) upon



awakening; 2) 30 minutes after awakening; and 3) at bedtime. If needed, participants were encouraged to chew Trident original sugarless gum, which was provided to them to stimulate saliva production. Previous testing demonstrated that this particular gum does not interfere with cortisol concentration measurement (Franz et al., 2010). Participants were also instructed to refrain from smoking or drinking for at least 30 minutes prior to sample collections. At the completion of each collection, the participants noted the time of day and answered a series of questions regarding their food or drink intake (if consumed 30 minutes prior to sample collection); mood (sleepy, happy, depressed, frustrated, anxious, excited, or angry); and planned daily activities (working in or outside of the home; relaxing at home). At the awakening collection, the participants were also asked to describe their sleep experience including time and quality (slept well, fairly well, fairly poorly, or very poorly), and if the previous night's sleep was typical (better than usual or worse than usual). Participation in moderate or vigorous exercise, yoga, meditation or prayer was queried at the bedtime collection. After venipuncture for collection of the blood specimen on day 3, the saliva specimens were shipped to our laboratory via overnight courier and upon receipt were stored at – 20°C.

### **Health History Questionnaire**

In addition to the queries completed at the time of saliva collection, participants completed a paper-based questionnaire which assessed factors regarding their medical and personal experiences including histories of disease diagnosis and treatment;

alcohol and cigarette use; medication or hormone replacement therapy use; dietary intake of micronutrients and supplements; height and weight; and self-assessments of attitudes regarding health and vitality.

### **DNA Isolation and Zygosity Determination**

In order to confirm the zygosity of the twins, genomic DNA was isolated from whole blood using the Puregene DNA Isolation Kit (Qiagen). Twins were classified as monozygotic if the marker data for the co-twins matched at 13 highly polymorphic short tandem repeat sequences evaluated (AmpFISTR Profiler Plus and Cofiler kits, Applied Biosystems, Foster City, CA).

### **Salivary Cortisol Immunoassay**

As noted above, upon receipt in the laboratory, all specimens were stored at -20°C until they were shipped to the Endocrine Core Lab at the University of California Davis (site of assay performance), which is under the direction of Dr. Sally Mendoza. To reduce potential experimental influences, the specimens were pooled for evaluation in 2 batches, and all samples collected from a participant were included in the same batch. Upon receipt in the reference lab, the aqueous salivary components were separated from mucins and other suspended particles by centrifugation (3000 rpm for 20 minutes). Concentrations of salivary cortisol were then estimated in duplicate using a commercial radioimmunoassay kit (Siemens Medical Solutions Diagnostics, Los

Angeles, California). Assay procedures were modified to accommodate overall lower levels of cortisol in human saliva relative to plasma as follows: 1) standards were diluted to concentrations ranging from 2.76 to 345 nmol/L; 2) sample volume was increased to 200  $\mu$ l; and 3) incubation times were extended to 3 hours. To meet quality control standards for the modified assay, serial dilutions of samples were required to display a linearity of 0.98 and a least detectable dose of 1.3854 nmol/L. Respectively, intra- and inter-assay coefficients of variation were 6.36 and 4.72%. Tests completed on blank tubes showed no interference from the tube used to collect and store the samples. At the time of cortisol assay, investigators were blinded to the CSA status of the participants.

## **Statistical Analysis**

The statistical analysis package Prism (GraphPad) was used for all statistical assessments. The area under the curve (AUC) was calculated using the trapezoid rule (GraphPad). The total value from both days of collection were included in the calculation of AUC, following the recommendation of Hellhammer et al. (2006). Cortisol awakening response (CAR) was calculated by subtracting the first (awakening) value for each day from the second (30 minute after awakening) value. Co-twin difference scores were calculated for each time point across both days of collection (awakening, 30-minute after awakening and bedtime) as well as for AUC and CAR. Difference scores for discordant twins were calculated by subtracting cortisol values of the unabused co-twin from the abused co-twin. Difference scores for concordant twins were calculated by

randomly selecting one co-twin to be subtracted from her co-twin for each of the pairs. If there is no effect of CSA on cortisol values, the difference between identical twins is expected to be zero. To evaluate potential differences in cortisol values between co-twins, the non-parametric Wilcoxon signed rank test was used, which alleviates the need to transform data that might not be normally distributed, while also providing a safeguard against biases that can be present with small sample sizes. For all statistical comparisons, an alpha level of 5% was used in order to limit type I errors.

## **Results**

### **Sample Distribution and CSA Class Assignment**

Of the 19 female twin pairs studied in which at least one twin had a history of CSA, the average age was 49.61 (SD 9.39) years and ranged from 35-70 years. All CSA twin pairs were determined to be monozygotic and all participants described their ethnicity as Caucasian. For the full cohort evaluated in the Kendler et al study (2000), the abused twins had an average age of 10.2 (3.5) years when they experienced their first CSA incident. The twins participating in this current study included a subset of 17 pairs from the original study who were discordant for CSA using a narrow definition of discordance (“one twin reported CSA and the co-twin reported no CSA” [Kendler et al., 2000]). The remaining 2 pairs of twins in this current study were concordant for a history of CSA. The discordant twin pairs were divided into three hierarchical groups based on the abused twin’s exposure. These categories included: 1) no genital contact (sexual invitation, sexual kissing and exposing [n=3 pairs]); 2) genital contact without intercourse (fondling and sexual touching [n=8 pairs]); and 3) intercourse (n= 6 pairs [Kendler et al., 2000]).

As recommended by Helhammer et al. (2009), a cut-point of 50 nmol/L was set for the salivary cortisol assay, and any samples exceeding this value were considered beyond physiological range and excluded from analysis (outliers). This quality control criterion resulted in the exclusion of one intercourse discordant twin pair because the

affected co-twin had extremely high cortisol levels (3-fold higher than physiologic range). Among the remaining 18 CSA pairs (16 discordant, 2 concordant), all awakening (n=72) and 30-minutes after awakening (n=72) samples met inclusion criteria. Of the possible 72 bedtime samples, 3 were missing, resulting in 69 total bedtime samples. Overall, only 4.2% (n=9) of 213 total samples failed to meet assay criteria, indicating that the twins had a high compliance with the collection protocol.

### **Salivary Cortisol Concentrations in Twin Pairs Discordant for CSA**

Among the 16 narrow discordant pairs and 2 concordant pairs who met the cortisol assay inclusion criteria, no significant differences were detected between each of the 3 collection time points (awakening, 30 minutes after awakening, bedtime) on day 1 and day 2. For example, the mean of the awakening samples for the narrow discordant CSA group on day 1 did not differ from the mean of the awakening samples for the narrow discordant CSA group on day 2. Therefore, the data points were averaged over the 2 days for further analyses. Among the discordant pairs, the mean (SD) concentrations of the awakening, 30 minute after awakening and bedtime samples were, respectively, 12.73 (6.59) nmol/L, 17.99 (8.61) nmol/L, and 3.87 (5.23) nmol/L. The cortisol secretion pattern displayed the normal diurnal rhythm with a peak cortisol level observed in the 30 minutes after awakening sample and a nadir at the bedtime sample.

**Table 3. Mean values of salivary cortisol among CSA concordant pairs (positive controls) and pairs discordant for 2 CSA classes.**

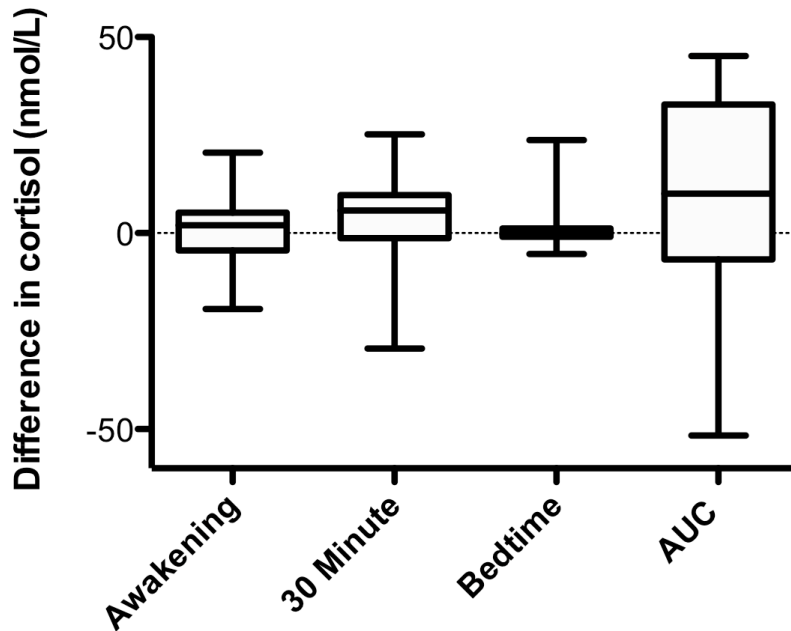
	<u>Narrow Discordant</u>		<u>Intercourse Discordant</u>		<u>Concordant</u>
	CSA	No CSA	CSA	No CSA	
N	16	16	5	5	4
Mean Age (SD)	49.63 (9.54)	49.44 (9.35)	40.8 (3.49)	40.8 (3.49)	57.0 (0.00)
Mean AUC (SD) Range	39.69 (19.05) 29.54-49.84	30.71 (15.49) 22.46-38.97	26.61 (10.76) 13.25-39.97	32.58 (12.22) 17.41-47.65	61.73 (19.01) 31.49-91.98
Mean Awakening <sup>1</sup> (SD) Range	12.73 (6.59) 10.35-15.1	12.11 (5.36) 10.17-14.04	9.47 (3.55) 6.93-12.01	11.82 (4.54) 8.58-15.07	16.94 (6.27) 11.70-22.18
Mean 30-minute (SD) Range	17.99 (8.61) 14.89-21.10	14.08 (7.33) 11.44-16.72	10.48 (4.25) 7.44-13.52	14.13 (7.15) 9.02-19.25	19.98 (7.51) 13.61-26.26
Mean Bedtime (SD) Range	3.87 (5.23) 1.91-5.82	2.59 (1.78) 1.93-3.24	2.65 (1.74) 1.31-3.98	2.75 (2.17) 1.24-4.33	2.81 (1.53) 1.54-4.10
Mean CAR (SD) Range	5.27 (5.17) 2.51-8.02	1.98 (4.91) -0.64-4.59	1.01 (1.70) -1.10-3.12	2.31 (6.21) -5.40-10.01	3.04 (3.65) -2.77-8.85

<sup>1</sup>Values for day 1 and day 2 were averaged for the awakening, 30-minute after awakening, and bedtime categories

To determine if salivary cortisol concentrations differed between identical twins discordant for a history of CSA, within-twin pair analyses were completed. As mentioned above, difference scores for discordant twins were calculated by subtracting cortisol values of the unabused co-twin from the abused co-twin. Wilcoxon signed rank tests were performed on averaged (day 1 and day 2) values. Comparisons of the differences between co-twins from the 16 discordant twin pairs were not significantly different from zero for the AUC ( $W=54$ ;  $df=15$ ;  $p=0.17$ ), awakening ( $W=44$ ;  $df=15$ ;  $p=0.69$ ) or bedtime ( $W=6$ ;  $df=15$ ;  $p=0.95$ ) samples (Figure 1). Difference scores between co-twins were significantly different for the 30-minutes after awakening samples ( $W=260$ ;  $df=15$ ;  $p=0.02$ ). Evaluation of this time point by CSA class revealed different trends based on CSA severity. The intercourse discordant group displayed a negative difference score, indicating the cortisol levels of the intercourse-exposed individuals were lower than their unexposed co-twins (Figure 2). As expected, there was no significant difference between co-twins concordant for CSA for the awakening ( $W=4$ ;  $df=1$ ;  $p=0.625$ ), 30 minutes after awakening ( $W=-2$ ;  $df=1$ ;  $p=0.875$ ), bedtime samples ( $W=-8$ ;  $df=1$ ;  $p=0.25$ ), CAR ( $W=-4$ ;  $df=1$ ;  $p=0.625$ ) or AUC ( $W=1$ ;  $df=1$ ;  $p=1.0$ ). In contrast, the difference in CAR between discordant co-twins was significantly different from zero ( $W=252$ ;  $df=15$ ;  $p=0.019$ ). However, the comparison of intercourse discordant twins also showed that the averaged values for the unabused co-twins lacked a CAR on day 2 of collection for 4 of the 5 unabused co-twins participating in the study. The reason for this unexpected observation is not clear. However, one possible explanation is that the initial sample may not have been collected immediately upon awakening, but several minutes later. If this scenario occurred, this potential breach in compliance with the collection



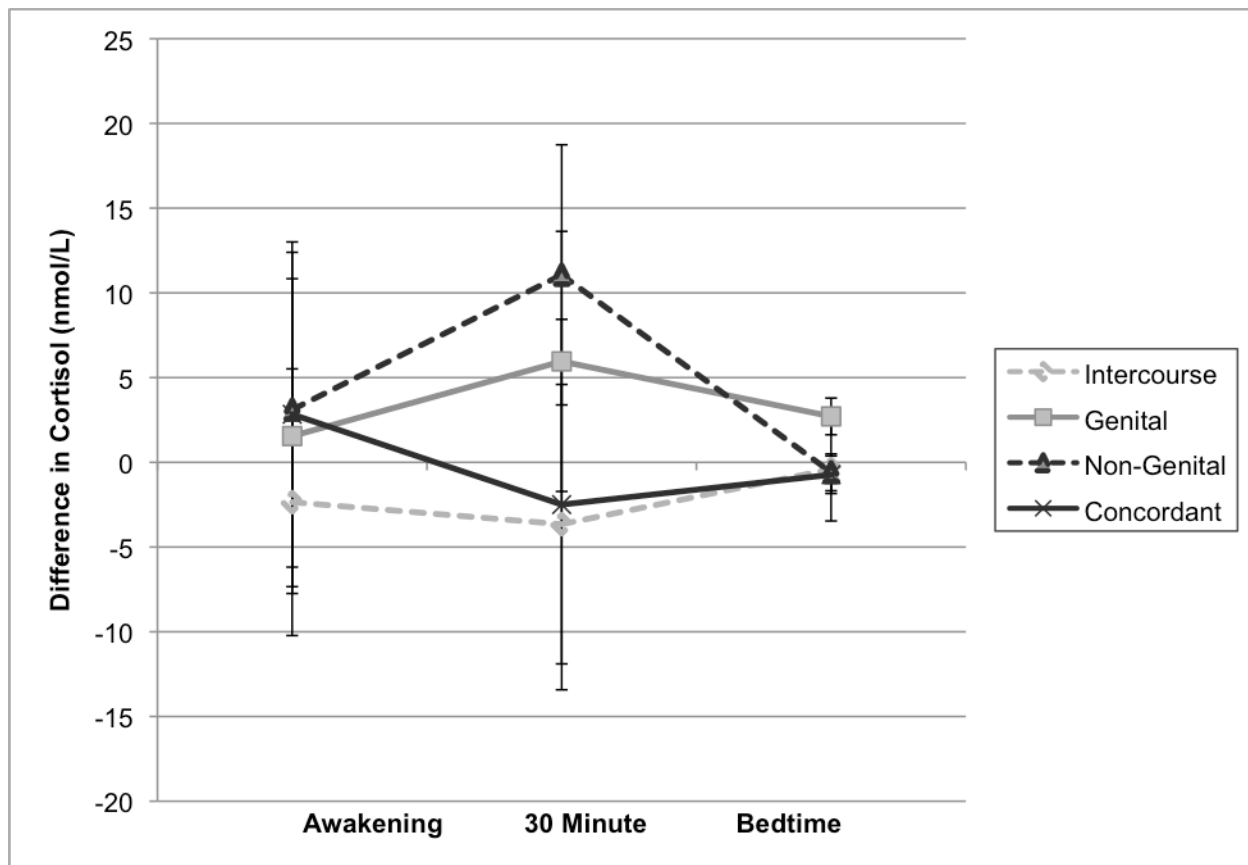
protocol could skew the resulting data. Therefore, taking a conservative approach to the data analysis, the comparisons were recalculated with these individuals removed, resulting in 11 discordant pairs. When the 5 individuals and their co-twins who did not demonstrate a CAR were removed, the difference values at the 30-minutes after awakening sample were no longer significantly different from zero for any of the CSA exposure groups (Intercourse  $W = -13$   $df = 5$ ;  $p = 0.219$ ; Genital  $W = 50$ ;  $df = 11$ ;  $p = 0.062$ ; Non-genital  $W = 10$ ;  $df = 3$ ;  $p = 0.125$ ). Similarly, the co-twin difference values for CAR for the subset of 11 twin pairs were not significantly different from zero ( $W = 38$ ;  $df = 10$ ;  $p = 0.102$ ). Difference scores for all pairs in the intercourse discordant group were negative because the unabused co-twins had higher values for CAR than their abused co-twins. Difference scores for co-twins in the genital/non-intercourse and non-genital groups were positive because the abused twin had higher values for CAR than their unabused co-twin (Figure 3).



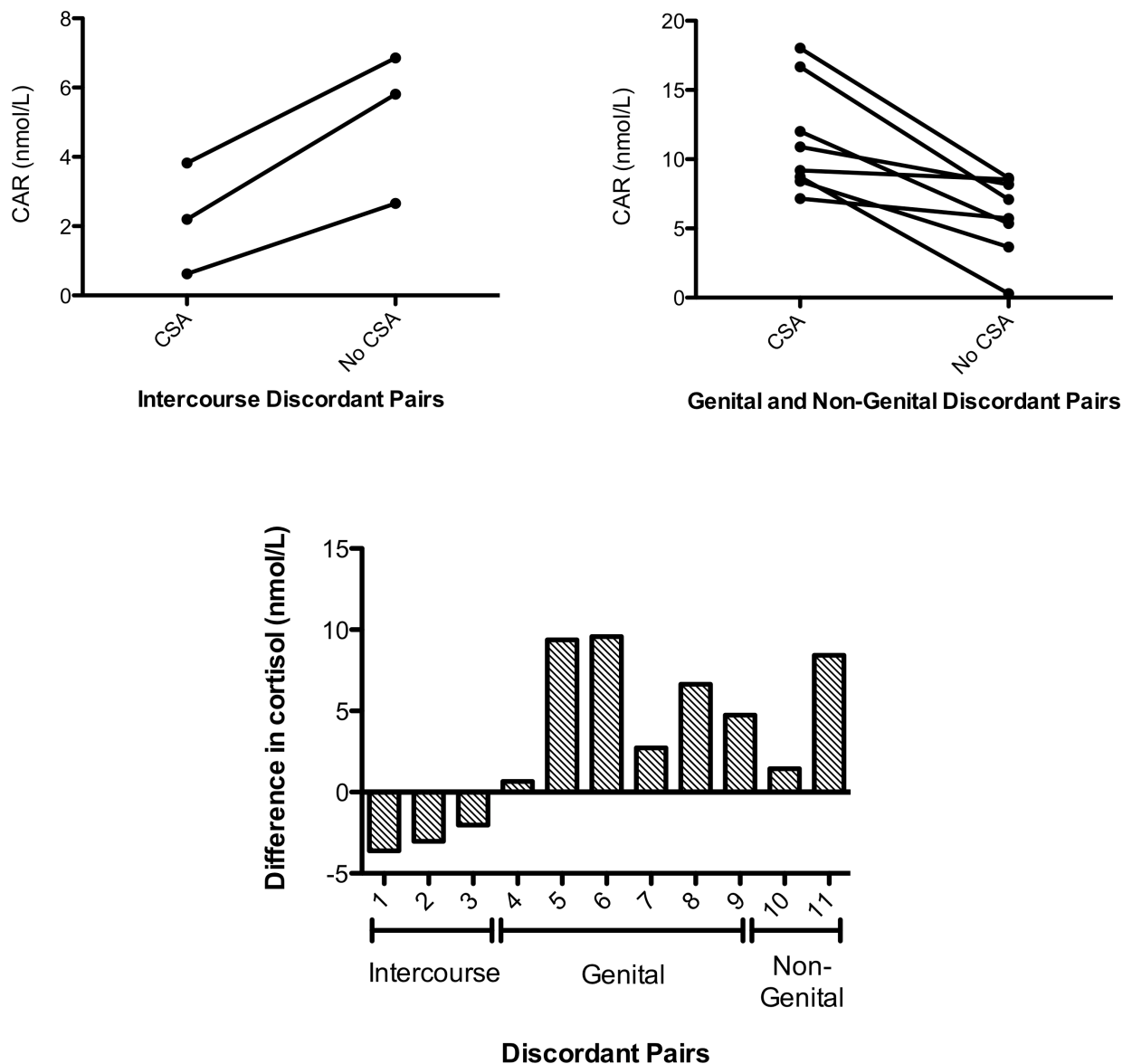
**Figure 1. Differences in salivary cortisol concentrations in identical twins discordant for CSA.** Cortisol values measured on Day 1 and Day 2 are averaged for each time point. Difference scores were obtained by subtracting the value of the unabused co-twin from that of the abused co-twin. If there was no effect of CSA on adult cortisol values, the difference in genetically identical co-twins ( $n=16$  pairs) would be expected to be zero if this trait is not primarily controlled by unique environmental influences. Co-twin difference values in cortisol levels at the awakening and bedtime time points, as well as AUC, were not significantly different from zero ( $p=0.69$ ,  $0.95$ ,  $0.17$ ). Differences in cortisol levels between co-twins for the 30 minutes after awakening sample were significantly different from zero ( $p=0.02$ ) for the complete cohort of twins prior to the exclusion on outliers. The whiskers of the box and whiskers plot represent the minimum and maximum.

## **Impact of Lifestyle Factors on Salivary Cortisol Concentrations**

Because stress-relieving activities impact cortisol levels (Michalsen et al., 2005; Rapaprt et al., 2010; Gopal et al., 2011), the twins' self-reports of prayer, moderate and vigorous exercise and yoga/meditation were evaluated. Among the 16 discordant pairs, the practice of stress relieving activities (prayer, exercise, yoga/meditation were reported for abused co-twins and unabused co-twins. Specifically, yoga/meditation was reported by 4 individuals with a history of CSA and 2 unabused individuals. Across the two days of collection, prayer was reported 16 times by both unabused and abused individuals, but these individuals were not predominately co-twins. Respectively, moderate and vigorous exercise was reported 17 times and 3 times by abused individuals and 16 times and 4 times in unabused individuals. Hormone replacement therapy (HRT) and oral contraceptive use, which impact cortisol secretion, were not commonly reported. The use of HRT was reported by 2 co-twins (a discordant pair) and 1 unabused individual, and only 2 individuals in the CSA affected group reported the use of oral contraceptives. The use of psychopharmaceuticals was also evenly reported among the affected and unaffected individuals, with anti-depressants used most frequently (n=8; 4 abused and 4 unabused). The use of other psychopharmaceutical drugs including benzodiazepines (n=1), non-benzodiazepine hypnotics (n=2), anti-psychotics (n=3) and a psychostimulant (n=1) was also reported. None of these lifestyle factors were statistically evaluated due to sample size limitations.



**Figure 2. Differences in salivary cortisol concentrations between co-twins discordant for CSA and positive controls (concordant pairs), based on the type of CSA experienced by the abused twin.** Difference scores were obtained by subtracting the value of the unabused co-twin from that of the abused co-twin. The mean co-twin difference scores of cortisol values measured on Day 1 and Day 2 are pooled for each time point. Negative difference values reflect a larger cortisol concentration in the co-twin not exposed to CSA and were observed in the intercourse discordant co-twins.



**Figure 3. Cortisol awakening response (CAR) in twin pairs discordant for CSA severity.** CAR in abused (CSA) individuals exposed to intercourse (top left panel; n=3) or genital and non-genital types of CSA (top right panel; n=8) and their identical, unabused co-twin (No CSA). Although the CAR for all intercourse-exposed individuals was lower than their unabused co-twin, the same trend was not observed for less severe forms of CSA. To evaluate CAR within twin pairs discordant for CSA, difference values were calculated (bottom histogram [n=11 pairs]). Difference scores for pairs in the intercourse discordant group were negative because the unabused co-twins had higher values for CAR than their abused co-twins. Difference scores for co-twins in the genital/non-intercourse and non-genital groups were positive because the abused twin had higher values for CAR than their unabused co-twin.

## Discussion

Early life stress events have been shown to modulate HPA axis function, particularly morning values and CAR (Heim and Nemeroff, 2001; Elzinga et al., 2008; Brewer-Smyth, 2008; Carpenter et al., 2010; Trickett et al., 2010). In the present study, the first to be conducted on identical twins discordant for CSA, we found no significant difference in cortisol baseline or awakening responses between MZ co-twins discordant for exposure to this early life stress. Additionally, across the full study cohort, which also included twins concordant for abuse as positive controls, we saw no clear evidence of a latent stress effect on cortisol values.

Interpreting and comparing the literature assessing cortisol values in individuals exposed to childhood adversity is problematic because the studies differ in methodology and in the populations evaluated. A meta-analysis by Miller et al. (2007) revealed that in adults under non-stress (non-laboratory) conditions, a history of chronic stress was associated with lower morning baseline cortisol levels and an increased output. We did not observe this effect in our discordant MZ twins. However, our findings are consistent with the results of other investigators (Heim et al., 2000 and Elzinga et al., 2008) who, although evaluating response to a laboratory stressor, also reported no difference in baseline cortisol values. While we did observe a significant difference in the 30-minute after awakening samples and CAR between the discordant co-twin pairs studied, this effect was eliminated when removing those twin pairs for whom at least one co-twin failed to demonstrate a CAR. Interestingly, we saw opposite trends of CAR between twins in the intercourse class and those in the genital and non-genital classes. Although

not significant, the CAR in the 3 co-twins who experienced intercourse tended to be lower when compared to their co-twin. Given that the morning (baseline) difference between twin pairs was not significantly different, this result indicates that the abused co-twins tended to have a lower or blunted CAR when compared to their unaffected co-twins. The reverse pattern was observed for the genital and non-genital twin pairs analyzed. Because a blunted CAR was observed only for those individuals who were exposed to the most severe form of CSA, and not others who experienced a less extreme form, this may suggest a possible latent dose-response effect of stress on cortisol and the CAR and/or a threshold that must be surpassed before childhood stress causes measureable biological effects that persist into adulthood. However, our sample size was small and, as noted above, the observed trend in CAR failed to reach statistical significance when individuals not displaying a CAR were removed from analysis. Investigations into CAR have produced conflicting results. A blunted CAR was observed by Heim et al. (2009) in individuals with a history of CSA, but the participants in the study had also been diagnosed with chronic fatigue syndrome. Smeets et al. (2007) reported that the CAR was not blunted in women with a history of CSA, although some participants had recovered memories of CSA and others had continuous memories of CSA. The severity of CSA (intercourse, genital, non-genital exposure) has been largely ignored by current HPA-axis investigators. Risks for developing CSA-associated psychological disorders has been shown to increase with CSA severity, with the highest risks noted for individuals exposed to intercourse (Kendler et al., 2002, 2004; Cong et al., 2011). Furthermore, a dose-response relationship between CSA severity and psychiatric disorders was observed in prior studies in this cohort (Kendler

et al., 2000). Given this, and that our data suggests that the effects of CSA may vary with the severity of the abuse, CSA exposure severity is an important factor to consider when comparing stress-induced effects. More studies need to be completed in stressed cohorts, particularly those exposed to varying severities of CSA, with corrections for confounding factors, to more clearly define the presence or absence of stress-induced alterations to cortisol and HPA axis function.

HPA axis function can be assessed at basal levels, which reflect a non-stimulated homeostatic state, and in response to an applied stressor, which reflects stress responsivity. A majority of investigators studying adults who were maltreated as children have focused on analyzing stress responsivity rather than baseline, non-stimulated cortisol levels. In contrast, we elected to characterize the cortisol profile of adult female MZ twins discordant for a history of early life stress (CSA) in a non-laboratory setting because specimens collected under normal conditions, in the comfort of the participants' home, have been suggested to improve compliance with collection protocols (Kudielka et al., 2003). One weakness of this study was that, although the mean of the different groups analyzed displayed a normal diurnal rhythm, which suggests that timing of sample collection was adequate, 5 individuals failed to demonstrate a CAR on both days of sample collection. A negative CAR (30-minute after awakening samples lower than awakening) has been suggested to indicate non-compliance and could result from an inaccurately timed collection of either the awakening samples or 30-minute after awakening samples (Kudielka et al., 2003). Although participants were instructed to collect samples immediately upon awakening



while still in bed and 30 minutes after the first sample, and not to eat drink or smoke before sample collection, if these instructions are ignored, inaccuracies in cortisol measurement are possible. To circumvent the problem with timing, track caps could be used to monitor the time span between the first and second collection. However, there are no interventions that would ensure complete compliance in the privacy of a participant's home. Lastly, while it is most likely that the failure to observe a CAR reflects collection time variables, one cannot rule out the possibility that other factors (potentially biological) influenced the CAR value.

As an alternative to CAR, AUC has been suggested to be a better and more reliable measure of free cortisol (Hellhammer et al., 2007). However, although carried out in a chronically stressed caregiving population, Jeckel et al. (2010) revealed no difference in AUC between the caregivers and nonstressed controls. Similarly, in the current study, within twin pair analyses revealed that AUC did not differ in MZ co-twins discordant for CSA or in MZ co-twins concordant for abuse. We cannot discount that no differences were observed between co-twins because the unabused twin in the CSA discordant pairs could have been stressed due to knowledge of her co-twin's abuse. Alternatively, the effect of stress in the abused twin could have been prevented by coping mechanisms and psychological assimilation of the abuse, which is aided by close interpersonal relationships and adult attachment (Pierrehumbert et al., 2009; Dimitrova et al., 2010). Future studies should consider the effect of the home or rearing environment and whether emotional support was received by an individual following the abuse. Additional considerations for future studies include the possible need for

simultaneous assessment of the HPA axis and the autonomic nervous system (ANS), which responds to stress by releasing catecholamines (epinephrine and norepinephrine) and with HPA axis, comprise the “fight or flight response.” Bauer et al. (2002) postulated that an asymmetric response pattern might occur in maltreated individuals. Specifically, they stated that although the ANS and HPA axis are separate systems that respond to stress, there is an inter-play between them such that one may continue to respond to stress and one may become blunted.

The major strength of this study design included the use of a discordant MZ twin population, thereby controlling for heritable genetic influences and shared environmental influences that might contribute to observed variations in cortisol values. Another strength was that the sample collection occurred on 2 consecutive non-weekend days. Measurement on 2 non-weekend days has been shown to provide greater sensitivity of cortisol measurement due to the routine observed by participants in their normal, daily schedule (Kunz-Ebrecht et al., 2004) and also to provide sufficient replicates to evaluate total AUC (Hellhammer et al., 2006). Oral contraceptives have been shown to influence cortisol secretion (Pruessner et al., 1997; Kirschbaum et al., 1999; Kudielka and Kirschbaum, 2005). However, the effect of these potentially confounding influences was minimal in our study participants, as only 2 women reported using oral contraceptives.

The biggest weakness of this study is the small sample size, which was further complicated by the need to exclude 5 individuals/twin pairs from the analyses due to

concerns regarding protocol compliance. Although the current design was within cost constraints, adding additional days for specimen collection (replicate measures), as well as additional study participants, could enhance the accuracy of cortisol measurements since it would allow the participants to become familiar with the collection protocol. Transient environmental stressors are another concern with this type of sampling. Although we attempted to assess any non-routine stressors by asking the participants to evaluate events in a personal log which they completed at the time of saliva collection, and no self-reports of increased (“a lot” or “extremely”) feelings of depression, frustration or anger were documented by the participants, there are multiple psychological and physiological factors, including depression, that we could not control for.

Measurement of cortisol in saliva is a convenient and non-invasive way to profile free, unbound cortisol levels. Nonetheless, there are many psychological, physiological and environmental factors that act transiently upon cortisol. These factors may prevent the accurate profiling of cortisol patterns and illustrate the equivocal nature of the stress response. Inherent problems with sampling and measuring cortisol point to the need for an easier and more reliable method to assess stress, and underscore its limitations as a robust biomarker for evaluating the biological effects of stress.

The results reported herein, the first to use a powerful CSA discordant identical twin design, although with a small sample size, have not supported the current literature demonstrating alterations in cortisol in individuals exposed to early life stress or CSA.

We did observe a trend for abused twins to have a blunted CAR when exposed to intercourse, the most severe form of CSA, which may suggest a biological dose-response relationship because this trend was observed only in those individuals exposed to the most severe form of CSA. In light of the numerous studies that have used cortisol as a biomarker for stress and which have yielded varied results, there is a clear need for a more stable and efficacious biomarker to not only assess possible latent effects of childhood stress on adults, but also one with utility as a biosurveillance tool.

## **Chapter 3**

### **Chromosome-specific Telomere Attrition in Co-twins Discordant for Childhood Sexual Abuse**

#### **Introduction**

Stressful life experiences are known risk factors for an adult's likelihood to develop a diversity of health problems, including cardiovascular, respiratory, and infectious diseases, but the underlying biological changes that occur to elicit these health consequences remain largely unknown (Cohen, et al., 1997). In their paradigm-shifting study, Epel et al. (2004) reported a correlation between the perceived stress levels reported by parents providing care for their chronically ill children and accelerated telomere shortening, thereby establishing that measurable DNA-based changes could be acquired in response to an adverse psychosocial experience. Since that initial report, additional investigators have observed telomere shortening associated with chronic stress from a variety of situations including (but not limited to), low socio-economic status (Cherkas et al., 2006); primary caregiver status to patients with Alzheimer's disease (Damjanovic et al., 2007); mood disorders (Simon et al., 2006); and intimate partner violence (Humphreys, 2011).

Referencing pioneering work by Barbara McClintock and Hermann Muller, Lansdorp (2008) wrote, “Telomeres used to be obscure functional elements at the chromosome ends studied by a few eccentric scientists.” Much is now known about telomeres, which are the specialized nucleoprotein structures of non-coding, repetitive TTAGGG/CCCTAA DNA sequences located at the terminal ends of chromosomes. Telomeres function to protect the chromosome from genomic instability. The telomere acts as a buffer for semi-conservative (incomplete) DNA replication and prevents recognition of the 3’ strand overhang as a double stranded break (Blackburn, 2000). Telomeres are particularly susceptible to oxidative damage, which induces single and double strand breaks, due to the high content of guanine bases in the telomeric repeat sequence. Oxidation of the guanine base causes transversions and has been linked to cancer (reviewed in Klaunig et al., 2010). Telomere shortening occurs when DNA damage is induced by oxidative damage and also with successive rounds of DNA replication (Oikawa et al., 1999). The rate of telomere shortening in normal adults has been estimated at 19-71 base pairs per year (Rufer et al., 1999; Hodes et al., 2002, Huda et al., 2007). When telomere lengths reach a critical threshold, replicative senescence and cell cycle arrest, or programmed cell death, may be triggered (Hayflick and Moorhead, 1965; Harley et al., 1991). Maintaining telomere length, which is crucial to preserve telomere integrity, is the function of telomerase, a reverse transcriptase that extends the 3’ end of chromosomes. However, it is also possible for cells to escape the “telomere checkpoint” and fuse with other chromosomes, leading to breakage, break-fusion-bridge cycles, translocations and aneuploidy (reviewed in Lansdorp and Auber, 2008). It has been postulated that signaling from the shortest telomere may begin the

process of senescence, but the chromosome-specific aspects leading to senescence and cell growth arrest/death are not fully known, as few investigators have studied chromosome-specific telomere length patterns (Lansdorp et al., 1996; Martens et al., 1998 [Table 2]).

Most of the studies evaluating the impact of stress on telomere length have centered on assessments in adults. However, it has been proposed that “during critical developmental periods, unfavorable environmental circumstances can reprogram biological systems in ways that persist across the lifespan” (Miller et al., 2009). Therefore, if the effects of stress are cumulative, one may expect stress to have its greatest impact during childhood, with biological changes potentially contributing to latent health consequences in adulthood. The results of the few studies that have been completed on adults exposed to early-life stress are analogous to those seen in response to chronic stress experienced by adults, with telomere attrition and reduced telomerase activity observed in response to a variety of childhood adversity conditions. Specifically, childhood trauma, including physical and emotional neglect, as well as a history of chronic or severe childhood illness, has been associated with shortened telomeres (Kananen et al., 2010; Tyrka et al., 2010; O'Donovan et al., 2011). Interestingly, exposure to childhood adversity has been reported to enhance the telomere-shortening effects of chronic stress in later life (Kiecolt-Glaser et al., 2011). Prenatal stress exposure has also been correlated to telomere attrition in adults (Entringer et al., 2011), and the telomere shortening effect of stress in early childhood has been observed as early as middle childhood (Drury et al., 2011). However, an

association between early-life stress and telomere shortening has not been universally observed. In a recent study of 123 adults reporting a history of childhood abuse (physical or sexual) and 1751 negative control subjects, no correlation between childhood maltreatment and telomere length was detected. The authors suggest that the observed correlations reported by other investigators could reflect the influence of confounding environmental exposures such as, “smoking, obesity, lack of exercise, and social class, as well as genes” (Glass et al. 2010).

One of the most efficient ways to tease apart the influences of environmental or genetic effects on a trait is to study identical twins who are discordant for an exposure/trait. Because the DNA of monozygotic (MZ) twins differs only for induced changes, they provide a unique opportunity to study the impact of stressful events in childhood. Although Glass et al. (2010) collected their samples from twins, they elected to report their data, to date, as individual results rather than co-twin findings. Thus, to our knowledge, there has not yet been a study with a discordant twin design that has reported the impact of childhood adversity on adult telomere length. However, telomere lengths have been evaluated in normal twins (primarily healthy adults). The results of the majority of these twin studies have suggested that the observed variation in length is largely attributable to heritable genetic influences (Slagboom et al., 1994; Rufer et al., 1999; Graakjaer et al., 2003, 2006). Studies of twins have also shown that telomere length is associated with lifespan, with the co-twins possessing shorter telomeres dying earlier than their co-twins with longer telomeres (Graakjaer et al., 2006; Kimura et al., 2008). However, when studying telomere lengths in 306 older twin pairs (ages 73 to



85), who were ascertained because they served in either World War II or the Korean War, Huda et al. (2007) determined telomere lengths to be most heavily influenced by shared environmental components. For this cohort, a major shared or common environmental factor would include the traumatic experience of serving in the Armed Forces during a war. Thus, while this study did not have a primary aim of measuring the effects of a potentially traumatic stress experience on telomere length, their conclusions are consistent with this association.

Telomeres are now regarded as a “psychobiomarker”, or a biological measure influenced by psychological parameters, which chronicles an individual’s recent and cumulative health and/or stress history (Epel, 2009). This association is particularly relevant given the strong association between telomere attrition and cardiovascular disease (Cawthon et al., 2003; reviewed in Serrano and Andres, 2004; Fitzpatrick et al., 2011) and stress and cardiovascular disease (Yusef et al., 2004). Given the potential for using telomere length as a biomarker for monitoring the biological consequences of stress, it is important to better understand the association between childhood adversity and telomere length. Therefore, we have elected to study the impact of one of the most extreme forms of early-life stress, childhood sexual abuse (CSA), by evaluating the telomeres of identical twins discordant for a history of CSA. A limitation of most studies that have evaluated associations between stress and telomere lengths is that they have used methodology (terminal restriction fragment or quantitative polymerase chain reaction) that provides an overall “average” telomere length (Aubert et al., 2011). While this approach has proven useful for detecting large and consistent effects on telomere

attrition, it has the potential to miss changes that are limited to a subset of chromosomes (Epel et al., 2006). It also provides no information regarding any skewing in chromosome-specific telomere length shortening that might be present. To overcome these technological restrictions, we elected to use a method that provides semi-quantitative estimates of chromosome-specific telomere lengths (Leach et al., 2004). The primary aim of this study was to determine if there are differences in the chromosome-specific telomere lengths of adults who experienced CSA when compared to their genetically identical co-twins who were not abused.

## **Materials and Methods**

### **Sample Ascertainment**

A total of 21 female twin pairs (42 individuals) with a history of CSA were ascertained through the Mid-Atlantic Twin Registry at Virginia Commonwealth University. Discordance for abuse exposure was used to prioritize the twins selected for recruitment in this project. Childhood sexual abuse was self reported and co-twin corroborated and was assessed via mailed questionnaire in a previous study completed by Kendler et al. (2000). CSA was categorized based on the type of CSA experienced and included: 1) no genital contact (sexual invitation, sexual kissing and exposing; 2) genital contact without intercourse (fondling and sexual touching; and 3) intercourse (Kendler et al., 2000). Discordance between twin pairs was further classified as narrow or broad. Specifically, narrow discordance was defined as “one twin reported CSA and the co-twin reported no CSA” (Kendler et al., 2000). Broad discordance was defined as “one twin reported CSA and her co-twin reported no or a less deviant form of CSA” (Kendler et al., 2000). Twin pairs who were concordant for childhood sexual abuse were also identified in the Kendler study and were recruited for the current investigation as positive controls.

After providing their informed consent (VCU IRB protocol HM12407), the participants in this current study submitted peripheral blood specimens, which were drawn by the participant’s health care provider and shipped to our laboratory via

overnight courier. These peripheral blood samples were used to obtain metaphase chromosomes for the telomere studies and to confirm the zygosity of the twin pairs.

### **Health History Questionnaire**

In addition to providing blood samples, the participants completed a paper-based questionnaire which assessed factors regarding their medical and personal history including, disease diagnosis and treatment; alcohol and cigarette use; over-the-counter and prescription medication use; dietary intake of micronutrients and supplements; height and weight; and self-scores of attitudes regarding health and vitality. As mentioned above, approximately 15 to 18 years ago, the participants also completed questionnaires assessing their childhood family environment, CSA history and feelings about the abuse (for a detailed description, see Kendler et al. [2000]).

### **DNA Isolation and Zygosity Determination**

In order to confirm the zygosity of the twins, genomic DNA was isolated from whole blood using the Puregene DNA Isolation Kit (Qiagen). Twins were classified as monozygotic if the marker data for the co-twins matched at all of the 13 highly polymorphic short tandem repeat sequences evaluated (AmpFISTR Profiler Plus and Cofiler kits, Applied Biosystems, Foster City, CA).

## **Cell Culture**

Following collection of the blood samples, lymphocytes were isolated using Histopaque-1077 (Sigma) and lymphocyte cultures were established using standard procedures (RPMI 1640 media supplemented with 15% fetal calf serum and the mitogen phytohemmagglutinin [Moorhead et al., 1960]). Twenty minutes prior to harvest, colcemid (final concentration 0.1 $\mu$ g/ml) was added to the cultures. Colcemid limits microtubule formation, thereby inactivating spindle fiber formation, and arresting cells in metaphase. A total of 72 hours after initiation, the cultures were harvested as previously described (Leach and Jackson-Cook, 2001) using standard techniques, including a 20-minute incubation in hypotonic solution (0.075 M KCl) and serial fixation (three times in 3:1 methanol: acetic acid solution). Slides were made following standard procedures (Leach and Jackson-Cook, 2001) and aged at room temperature for two weeks before beginning the fluorescence in situ hybridization (FISH) procedure.

## **Chromosome-specific telomere FISH**

Telomeres of metaphase chromosomes were visualized using a pantelomeric peptide nucleic acid (PNA) probe specific for the telomeres of all chromosomes, which has been shown to demonstrate better hybridization efficiency than DNA probes (Landsdorp et al., 1996). PNA probes differ from DNA probes because the backbone, which is composed of repeating N-(2-aminoethyl) glycine units linked by peptide bonds (Egholm et al., 1993), is uncharged and therefore can overcome electrostatic repulsion

of the target DNA during hybridization. PNA probes also demonstrate an increased thermal stability of the probe-target duplex. The aged slides with target metaphase spreads were immersed in 2xSSC for 30 minutes at room temperature. Following dehydration in an ethanol series (70%, 85%, and 100% for 2 minutes each), the slides were fixed (3:1 methanol: acetic acid) for 1 hour and air dried. Slides were washed for 5 minutes in PBS and then immersed in 4% formaldehyde (in PBS) for 2 minutes, dehydrated in an ethanol series and air dried. The PNA probe solution (70% formamide, 0.38  $\mu\text{g/mL}$  FITC-(CCCTAA)<sub>3</sub> [Applied Biosystems], 1% blocking reagent [Roche]) was applied to the slides. The probe and metaphase chromosomes were then simultaneously heat denatured at 80°C for 3 minutes and allowed to hybridize overnight in a humidified chamber at 37°C. Excess unbound probe was removed by washing (70% formamide/2xSSC solution at 30°C for 15 minutes, 2xSSC at room temperature for 5 minutes, 2xSSC/0.1%NP-40 solution at room temperature for 5 minutes). After washing, the slides were air dried and the chromosomes stained with 20  $\mu\text{L}$  of a 1:5 propidium iodide (PI): DAPI II (Abbott) mix.

### **Telomere Image Analysis**

For each individual, 10 metaphase spreads were analyzed using comparative genomic hybridization (CGH) software on an Applied Imaging Cytovision system (Genetix). Telomere signal intensity values were obtained following standard CGH protocols. Briefly, 3 images were captured on the Cytovision system for each metaphase. The first image was captured using a DAPI filter to identify chromosomes

based on their reverse DAPI banding pattern. The second “test” image was captured using a FITC filter to enable the telomeric probe to be visualized. The third “reference” image was captured using a single band filter for red fluorophores to visualize the PI stain (Figure 4). Each metaphase was karyotyped and the position of the centromere and central axis placed for each homologue. Chromosomes were omitted from analysis if the telomere or near-telomeric region was overlapped by another chromosome. Chromosome-specific telomere intensity measurements were determined by comparing the relative fluorescent intensities of each telomere (test image) to the fluorescence of the PI staining of the chromosome body (reference image [Krejci and Koch, 1998]). The CGH software generated a quantitative ratio profile for each chromosome based on a comparison of the intensities of the test and reference images. Because maternally- and paternally- derived homologs cannot be distinguished for all chromosomes, homologues of a chromosome were averaged for each of the metaphase spreads scored. At the time of slide scoring, investigators were blinded to participant CSA status.

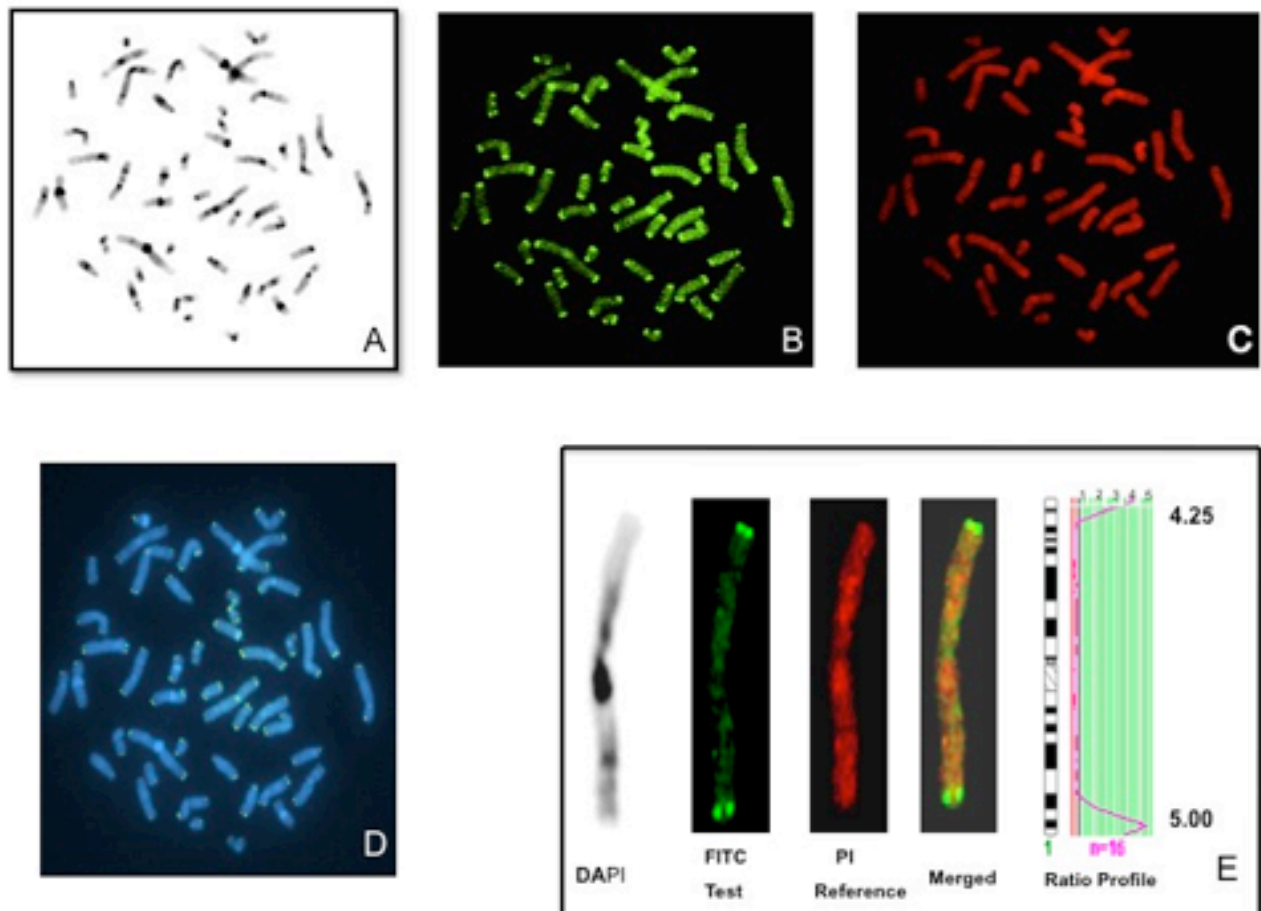
### **Salivary Cortisol Immunoassay**

To determine if telomere lengths were influenced by cortisol levels, salivary cortisol was assayed over two consecutive, non-weekend days as described elsewhere (Chapter 2). The total cortisol area under the curve (AUC) was calculated for assessment with telomere intensity levels because the AUC provides an accurate measure of total hormone concentration (Pruessner et al., 2003).

## Statistical Analyses

To determine if telomere length in adults was impacted by exposure to CSA, comparisons in overall telomere signal intensities were completed, along with chromosome-specific telomere intensity comparisons. The statistical analysis package Prism (GraphPad) was used for all statistical assessments. Because the twins were genetically identical, within-pair differences in telomere length were calculated to assess acquired changes in telomere length over time in the abused compared to unabused twins. For the narrow discordant twin pair analyses, the telomere intensity values of the unabused individual were subtracted from the values observed in her abused co-twin. For twin pairs in any broad discordance category, the telomere value for the less severely abused twin was subtracted from her more severely abused co-twin. Difference scores for twin pairs concordant for abuse were calculated by subtracting the telomere intensity values of one twin (randomly selected) from those of her co-twin. The difference in genomic (average) telomere signal intensities between co-twins discordant for exposure to childhood stress and concordant pairs was examined using a one-sided t-test on square root transformed data. The nonparametric Friedman test was used to determine if chromosome-specific differences in telomere intensity values were present. Spearman's correlation coefficients were used to examine the relationship between age and telomere length as well as the effect of cortisol on telomere length. Cortisol area under the curve was calculated using the trapezoid rule (GraphPad). In order to limit type I errors, an alpha level of 5% was used.





**Figure 4. Determination of relative telomere signal intensities using a comparative fluorescence approach (Cytovision, Genetix).** A representative metaphase spread with: (A) reverse DAPI bands; (B) FITC-labeled PNA probe (test); (C) propidium iodide (reference); and (D) merged PNA probe and DAPI counterstain images. The intensity of the telomere signals is shown as a ratio profile of the test and reference images (E). In this example, the chromosome 1 long arm telomere has a relative fluorescence intensity value of 5.00 compared to a 4.25 value on the short arm. Ten metaphase spreads were analyzed per individual.

## **Results**

### **Sample Distribution and CSA Class Assignment**

The twins participating in this current study included 2 concordant pairs and 14 pairs who were discordant for CSA using a narrow definition of discordance (one twin experienced abuse and her co-twin did not experience any type of abuse) as summarized in Tables 2 and 3. The remaining pairs of twins (n=5 pairs) were classified as broad discordant pairs because they shared exposure to abuse, but differed for the severity of CSA, experienced. The number of pairs in the three hierarchical CSA groups included: 1) no genital contact (sexual invitation, sexual kissing and exposing [n=2 pairs]); (2) genital contact without intercourse (fondling and sexual touching [n=8 pairs]); and 3) intercourse (n=9 pairs [Kendler et al., 2000]). Of the 21 female twin pairs with a history of CSA, the average age was 51.74 (SD= 9.99) years and ranged from 35-70 years. All twin pairs were determined to be monozygotic, had chromosomal complements within normal limits (46,XX) and described their ethnicity as Caucasian.

### **Mean Telomere ‘Length’ in Twins with a History of CSA**

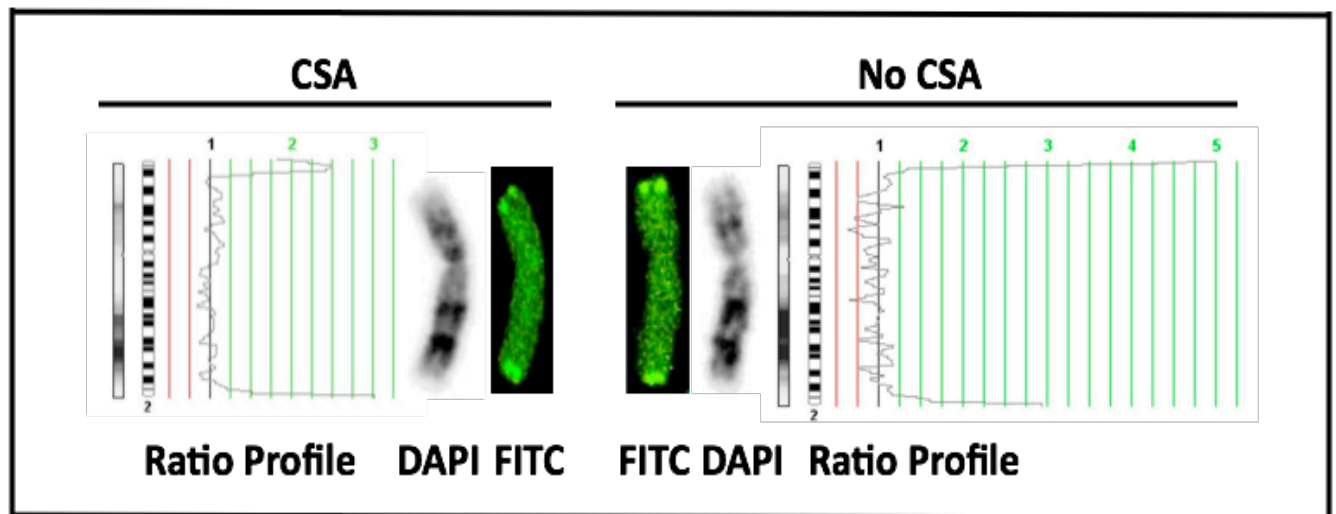
Over the entire cohort (n=21 pairs), the average telomere ‘length’ measured in relative fluorescent units was 2.86 (SD=1.07). When evaluating the data on the basis of the type of CSA exposure experienced by the twin(s), the telomere intensity values were noted to range from a low of 2.01 (0.41) for the non-genital group to a high of 3.77

**Table 4. Mean telomere length of MZ twins discordant and concordant for CSA.**

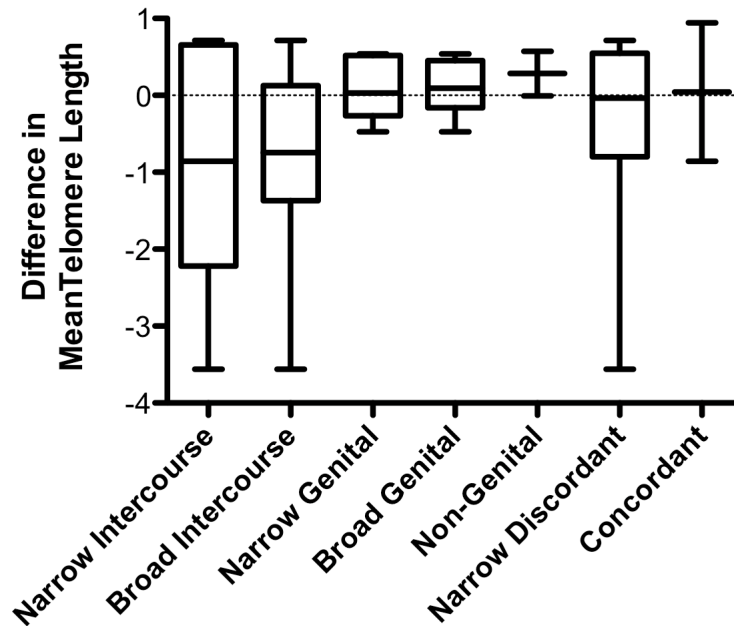
Twin Pairs	CSA Type (more severe)	<i>Narrow</i>		<i>Broad</i>	
		No CSA	CSA	CSA (less severe)	CSA Type (less severe)
Discordant					
1	Intercourse	4.07	4.70		
2	Intercourse	5.50	1.94		
3	Intercourse	4.14	3.17		
4	Intercourse	4.39	2.62		
5	Intercourse	3.33	4.05		
6	Intercourse	2.71	1.97		
7	Intercourse		2.91	3.54	Genital
8	Intercourse		1.97	2.72	Genital
9	Intercourse		1.73	2.11	Genital
10	Genital	1.92	2.05		
11	Genital	1.85	1.65		
12	Genital	2.41	2.34		
13	Genital	2.62	3.14		
14	Genital	2.77	2.29		
15	Genital	1.92	2.45		
16	Genital		2.32	2.26	Non-Genital
17	Genital		3.84	3.59	Non-Genital
18	Non-Genital	2.31	2.34		
19	Non-Genital	1.43	2.00		
Concordant		CSA	CSA		
1	Intercourse	2.78	1.83		
2	Genital	4.80	5.65		

(1.76) for the concordant CSA exposure group (Table 4). To determine if there was a difference in the mean telomere intensity values (averaged over all chromosomes) between the discordant co-twin pairs, the average value of the unabused/less severely abused twin was subtracted from the average intensity value of the abused/more severely abused twin for each pair. A value of zero would be expected between the identical co-twins if this trait was strictly under genetic control or shared environmental influences. The largest difference values were observed for the twin pairs with intercourse exposure (Figure 5 and 6). Both narrow and broad exposure groups showed a trend toward shorter telomeres in the abused/most severely abused twin. Although these values were not significantly different from zero, they approached significance for the broad intercourse CSA class ( $t=2.13$ ;  $df=8$ ;  $p=0.066$  [Figure 6]). As expected, the difference in average telomere intensity values in the co-twins who were concordant for abuse was not significantly different from zero ( $t=0.05$ ;  $df=1$ ;  $p=0.986$  [Figure 6]).

Given that telomere length is inversely correlated with age in individuals from the general population, the effect of age on telomere length was assessed. As expected, age was negatively correlated to average telomere length ( $r= -0.387$ ;  $df=40$ ;  $p=0.011$  [Figure 7]) in the entire cohort. Among twin pairs discordant for CSA ( $n=19$  pairs), telomere length was negatively correlated with age in the unabused co-twins ( $r= -0.682$ ;  $df=17$ ;  $p=0.0013$ ), but not in the abused co-twins ( $r= -0.131$ ;  $df=21$ ;  $p=0.552$  [Figure 7]). However, because a discordant twin design was used for this study, age influences on telomere length do not confound the interpretation of the telomere intensity values observed



**Figure 5. Ratio Profiles of chromosome 2 from an MZ twin pair discordant for intercourse exposure.** The short arm telomere of the chromosome 2 from an unabused twin (No CSA- right panel) is brighter (longer; value of 5) than that of her abused co-twin (CSA- left panel; value of 2.5). The chromosome 2 long arm telomere ratio profile showed no shortening between co-twins, as demonstrated by a value of 3.0 for both individuals



**Figure 6. The difference in average telomere intensity values in co-twins exposed to childhood stress.** Although the mean difference values calculated for co-twins in each exposure group were not significantly different from zero, the value for the co-twins who were discordant for intercourse (broad) approached significance ( $p=0.066$ ). Whiskers of the box and whiskers plot represent the minimum and maximum values observed.

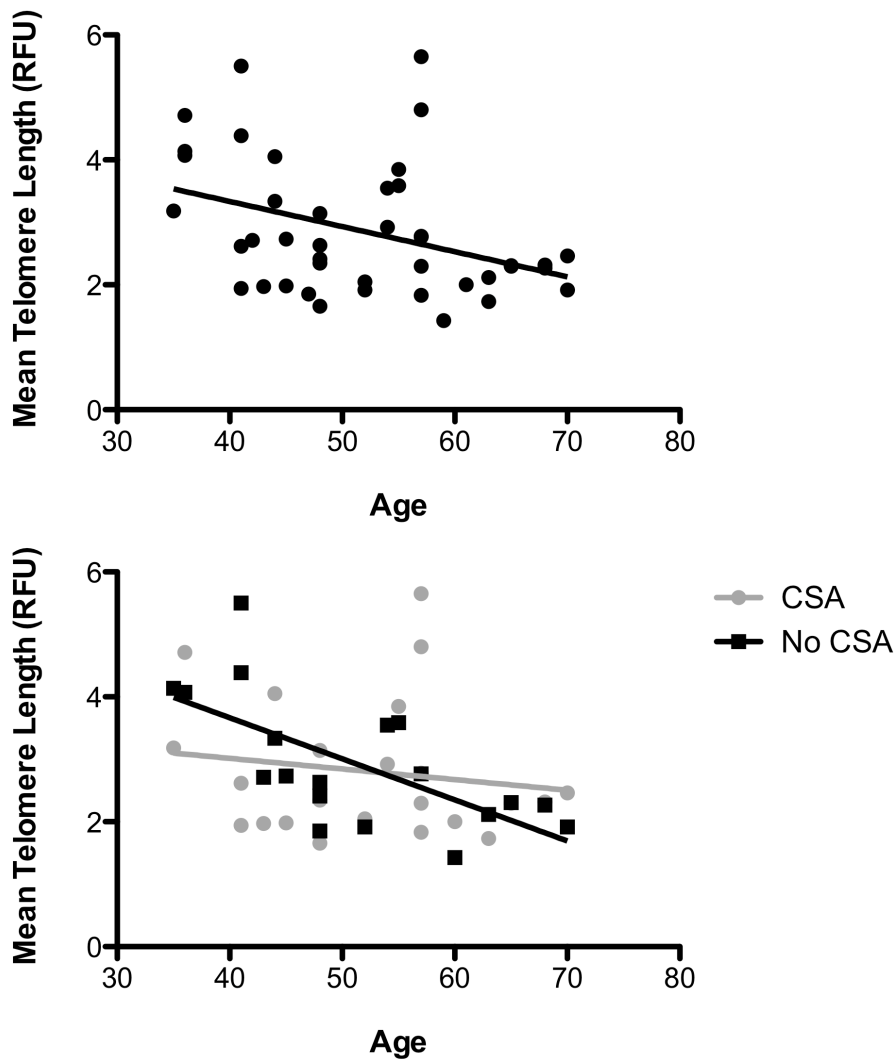
between the abused and unabused co-twins. To determine if cortisol levels were correlated with telomere length (potential HPA association), the relationship between telomere length and cortisol area under the curve (AUC) was assessed in all individuals for whom both telomere data and cortisol AUC data were available (n=35). Telomere lengths were not significantly correlated to cortisol AUC values ( $r = 0.442$ ;  $df=33$ ;  $p=0.137$  [Figure 8]).

### **Chromosome-specific Telomere Intensity Values in Twins Discordant for CSA**

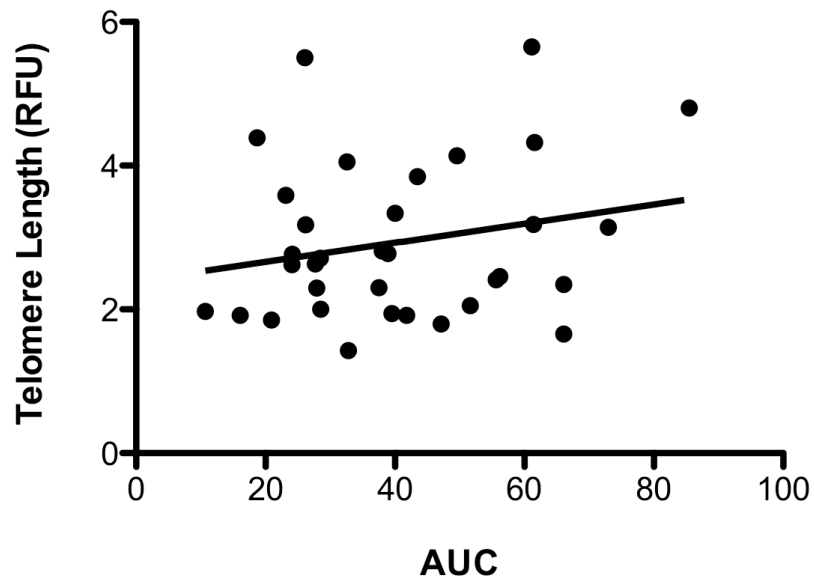
A non-random pattern of chromosome intensity values was observed among all 21 twin pairs analyzed (Friedman  $\chi^2(45)= 984.2$ ;  $p<0.0001$ ). The telomeres of the short (p) arms of chromosomes 1, 4, 16 and 17 and the long arm (q) of chromosomes 2, 9, 16 and 17 were the shortest and 3p and 21p the longest observed (Figure 9). To determine if chromosome-specific telomere 'lengths' differed between identical co-twins who were discordant for their exposure to childhood stress, within-pairs analyses were completed by subtracting the telomeric intensity value of each chromosome arm in the unabused twin from that of her abused co-twin. A negative difference score, which indicates that the telomere lengths of the abused twin were shorter than the telomere lengths of the unabused twin, was observed for all chromosome arms in the co-twins who were discordant for intercourse exposure (Friedman tests for the narrow intercourse and broad intercourse [which includes narrow] CSA classes [ $\chi^2(45)= 62.88$ ;  $p= 0.040$  and  $\chi^2(45)= 73.72$ ;  $p= 0.004$ , respectively] (Figure 10 and Table 5). As expected, due to the overlap of twins represented in the broad and narrow discordant cohorts, there was

agreement between these sub-groups for chromosome-specific telomere shortening. The greatest differences in telomere value intensities between identical co-twins in the broad intercourse subgroup were for telomeres on chromosomes 2p, 3p, 5p, 6p, 13p and 11q, 13q, Xq. In the narrow intercourse discordant subgroup, the greatest chromosome-specific telomere value differences within twin pairs were seen for 3p, 5p, 6p, 8p, and 1q, 3q, 4q, 11q, 13q (Figure 10).

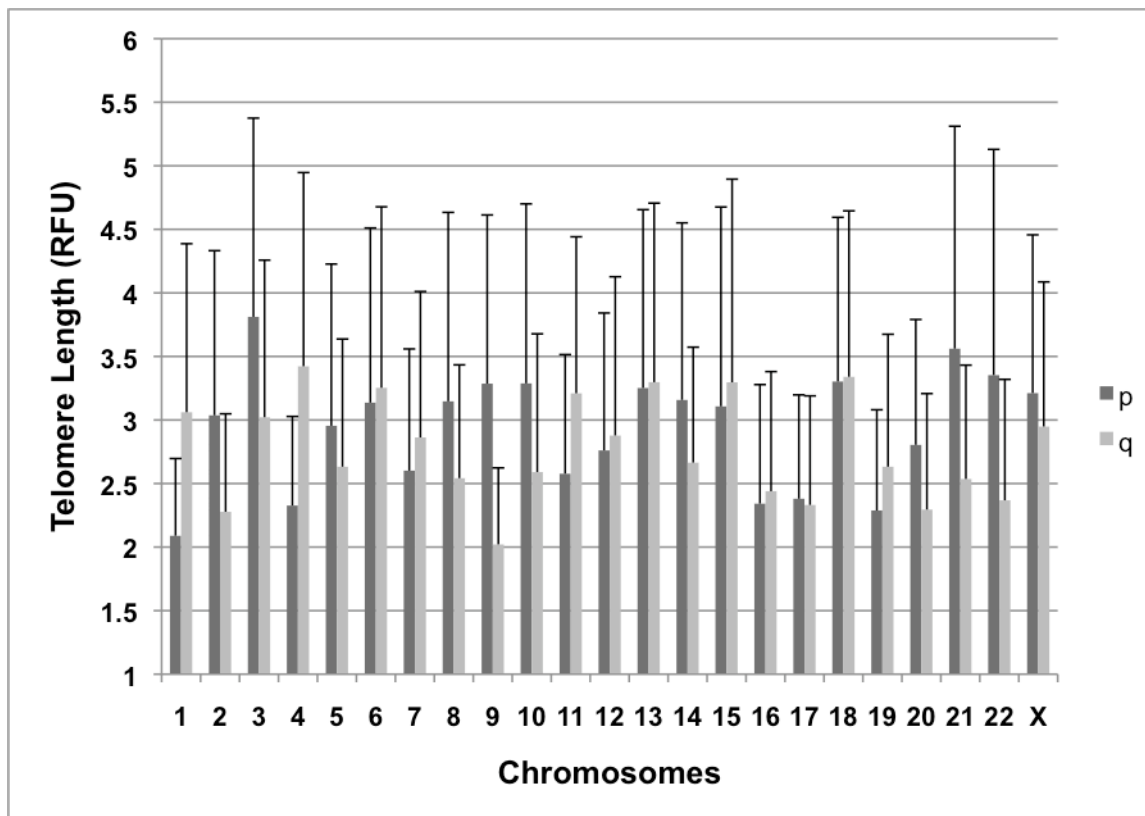




**Figure 7. Spearman's correlation of mean (overall) telomere length with age.** The average of all chromosome-specific telomere values was negatively correlated with age ( $r = -0.387$ ;  $df=40$ ;  $p=0.011$  [top panel]) in the entire study cohort ( $n=21$  pairs). Interestingly, when evaluated on the basis of CSA exposure, telomere length was negatively correlated to age in the unabused co-twins ( $r = -0.682$ ;  $df=17$ ;  $p=0.0013$ ), but this relationship was not observed in the abused co-twins ( $r = -0.131$ ;  $df=21$ ;  $p=0.552$  [bottom panel]).



**Figure 8. Correlation of mean (overall) telomere intensity values with cortisol area under the curve (AUC).** The average of all chromosome-specific telomere values was not significantly correlated with cortisol area under the curve values ( $r= 0.137$ ;  $df=33$ ;  $p=0.442$ ).



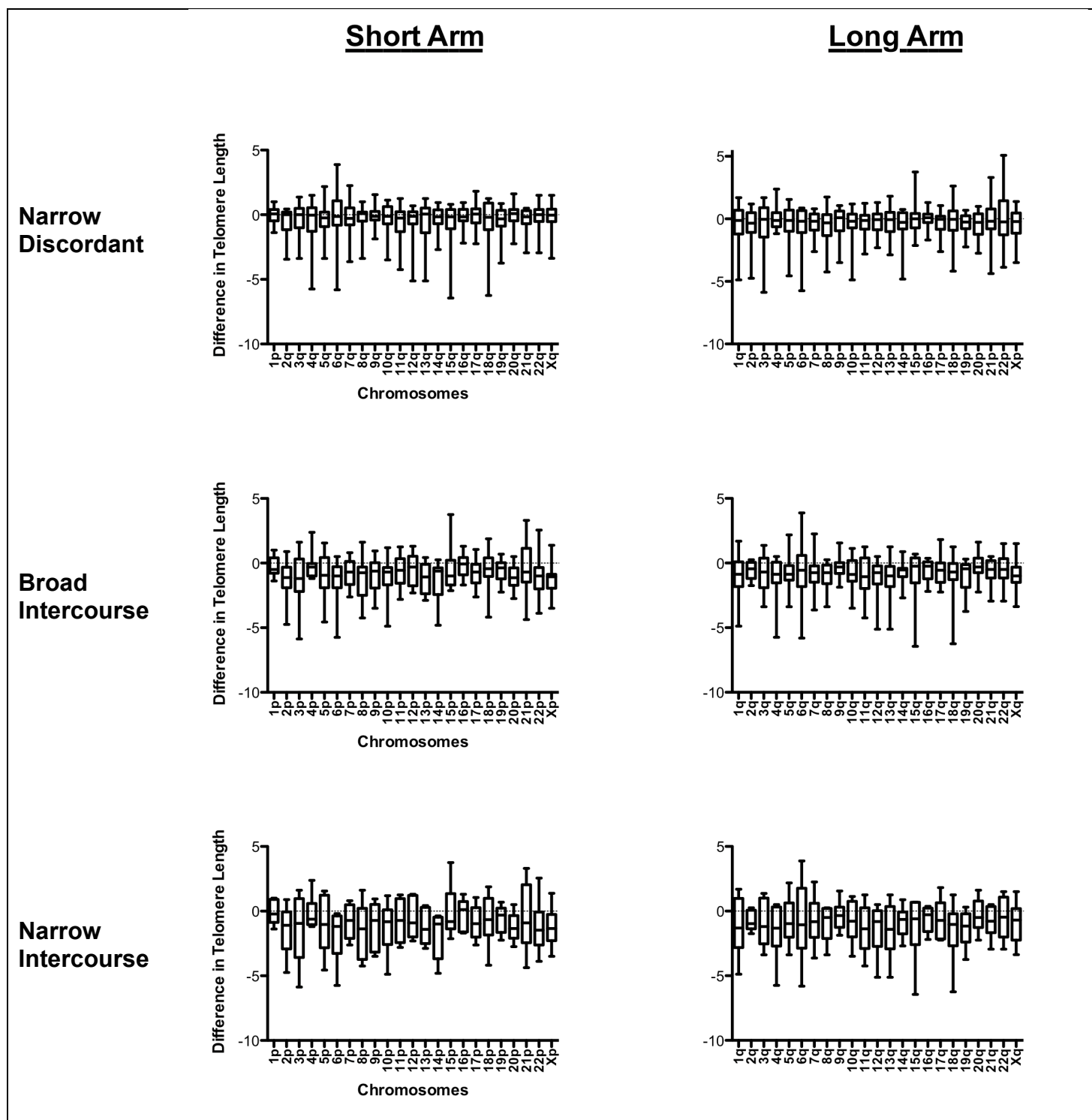
**Figure 9. Distribution of chromosome-specific telomere intensity values in co-twins discordant for CSA.** Average telomere intensity values of all participants (n=42 individuals) for the chromosomal short (p) and long (q) arms. Over all chromosomes analyzed, 1p, 2q, 4p, 9q, 16p, 16q, 17p, 17q, 19p and 20q had the smallest values and 3p and 21p had the highest intensity values.

**Table 5. Freidman test of chromosome-specific telomere intensity values in co-twins discordant for CSA.**

<b>CSA Class</b>	<b>Friedman <math>\chi^2</math></b>	<b>df</b>	<b>p-value</b>
Narrow Intercourse	62.88	45	0.040*
Broad Intercourse	73.73	45	0.004*
Narrow Genital	37.34	45	0.780
Broad Genital	36.79	45	0.803
Non-Genital	55.72	45	0.131
Narrow Discordant	51.30	45	0.240

df=degrees of freedom

\*statistically significant (p<0.05)



**Figure 10. Within-pair differences in chromosome-specific telomere intensity values.** Significant differences in chromosome-specific telomere values were observed in co-twins from the narrow and broad intercourse CSA classes (see Table 3). These differences involved both chromosome short arms (left panel) and long arms (right panel). Specific telomeres that had the greatest difference values in both intercourse discordant groups included 3p, 5p, 6p, 11q, and 13q.

## Discussion

A semi-quantitative, chromosome-specific FISH-based approach was utilized to determine if exposure to childhood stress influences chromosome-specific telomere length in adults. Telomere length values between identical co-twins discordant for exposure to CSA, as well as between concordant co-twins, were compared. Given that most previous reports associating stress with telomere attrition have been performed using methods that quantified an overall (genomic) mean telomere length (Epel et al., 2004; Glass et al., 2010; Kananen et al., 2010; Tyrka et al., 2010; Drury et al., 2011) we first examined the data from our discordant twins by comparing average (genomic) telomere intensity values and calculating difference scores for each twin pair. As noted previously, a value of zero would be expected between identical co-twins if genomic telomere length was under heritable genetic or common environmental influences and if stress (or another unique environmental influence) was not impacting telomere length. While we found no significant difference in the largest group of twins discordant for exposure to CSA, a trend for abused individuals to have shorter telomeres than their unabused co-twins was observed for the most severe form of CSA (intercourse). This finding may suggest a threshold for the deleterious biological effect of childhood adversity that is not exceeded until one is exposed to the most severe stress or a possible dose-response relationship between stress and telomere length. As expected, because both co-twins were exposed to abuse, there was no difference in telomere intensity values for the concordant pairs (positive control group). Unexpectedly, this positive control group, which included one pair exposed to intercourse, also showed a

trend for having longer telomeres, but this observation likely reflects the small sample size for this group (n=2 pairs). It is also possible that these 2 pairs inherited comparably longer telomeres than the other participants in this study, making any stress-induced attrition more difficult to detect.

Based on this semi-quantitative genomic approach to the estimation of telomere length, our results do not fully support those of some investigators who reported telomere attrition in children exposed to stress (Drury et al., 2011), in adults with a history of childhood stress (O'Donovan et al., 2011), or in adults with a history of chronic stress or high perceived stress (Epel et al., 2004; Cherkas et al., 2006; Simon et al., 2006; Damjanovic et al., 2007; Parks et al., 2009; Humphreys et al., 2011). However, the aforementioned studies differ not only in the type of stress evaluated (physical, emotional and sexual abuse, low socio-economic status, diagnosis of a mood disorder, caregiver to the chronically ill), but also in the age of the population measured and the age when the stressful event was experienced (chronic adult exposure versus acute childhood exposure). Most relevant to our study are the studies in which adults with a history of childhood stress were examined. One such study by Glass et al. (2010) evaluated twins who were physically and sexually abused and reported no difference in either abuse category compared to controls. We have corroborated these findings, although within a powerful discordant MZ design.

To expand upon studies in which only average genomic telomere length was evaluated in stressed populations or those with a history of CSA, we elected to utilize a

chromosome-specific approach. The data presented herein is the first report on the chromosome-specific telomere lengths in an adult population with a history of childhood stress. Among all 42 participants (21 twin pairs), the telomere values showed a non-random pattern, with the smallest telomere values observed for 1p, 2q, 4p, 9q, 16p, 16q, 17p, 17q, 19p and 20q. Telomeres on 3p and 21p had the largest observed values. These findings agree, at least in part, with the results of the few previous reports of chromosome-specific telomere lengths. Specifically, the telomere for 17p has been reported to be one of the shortest observed by other investigators profiling human chromosome-specific telomere lengths (Lansdorp et al., 1996; Graakjaer et al., 2003; Perner et al., 2003; Mayer et al., 2006) and the shortest by Martens et al. (1998). The telomeres of 19p and 20p are also among the shortest observed in previous reports (Martens et al., 1998; Graakjaer et al., 2003; Mayer et al., 2006). Consistent with Graakjaer et al. (2003), whose findings are particularly relevant because their study design utilized twins, we found longer telomeres on 3p. These observations underscore the fact that although there is heterogeneity among different chromosomes, a common telomere profile in humans is appreciable. As suggested by Lansdorp (2006), because there is telomere length heterogeneity among different cell types, future studies evaluating different populations of leukocytes and/or utilizing flow-FISH to evaluate stress-induced effects on different cells of the hematopoietic system, might provide additional insight as to the presence of telomeric attrition in adults who experienced CSA.



To determine if a chromosome-specific shortening pattern was appreciable in individuals exposed to childhood stress, we performed within-pair analyses of MZ twins discordant for CSA. As mentioned above, difference scores were calculated for each twin pair and Friedman tests revealed significant differences in the chromosome-specific telomere lengths of co-twins in both the broad and narrow intercourse CSA classes. Interestingly, the greatest differences did not involve the chromosomes with overall shorter telomere value profiles (1p, 2q, 4p, 9q, 16p/q, 17p/q, 19p and 20q). Instead, the largest differences that were consistent across both groups were for shorter telomeres on 3p, 5p, 6p, 11q and 13q in the abused twins. Given that shortening of the telomeres leads to a reduction of heterochromatin on these chromosomes, which, in turn, could lead to changes in the activity of nearby telomeric/subtelomeric genes (Mefford and Trask, 2002), we reviewed genetic maps (Human Genome Build 18) of the chromosomes with telomere attrition to determine if there might be genes localized to the distal, near-telomeric bands that could contribute to the various morbidity conditions that have been reported in adults who experienced CSA. This search revealed several genes involved in cell signaling pathways and mitochondrial function (Table 6), including TERT on 5p, which encodes one of the critical components (the catalytic subunit) of telomerase; and succinate dehydrogenase complex, sub-unit A, flavoprotein (SDHA), which is a member of the mitochondrial respiratory chain that plays a role in oxidative stress.

**Table 6. Subtelomeric genes on chromosomes with telomere attrition.**

<b>Chromosomal Location</b>	<b>Gene</b>	<b>Name</b>	<b>Function</b>
3p26.1-p25.1	GRM7	Glutamate receptor, metabotropic 7	Receptor for L-glutamate, excitatory neurotransmitter in Central Nervous System
3p26-p24	IL5RA	Interleukin 5 receptor, alpha	Signaling activates eosinophils and the allergic response
5p15.33	PDCD6	Programmed cell death 6	Calcium binding protein that participates in t-cell receptor-, Fas- and glucocorticoid-induced cell death
5p15.33	TERT	Telomerase reverse transcriptase	Polymerase that maintains telomere ends/length
5p15	SDHA	Succinate dehydrogenase complex, subunit A, flavoprotein	Member of mitochondrial respiratory chain
11q25	NCAPD3	Non-SMC condensing II complex, subunit D3	Mitotic chromosome assembly and segregation
11q25	THYN1	Thymocyte nuclear protein 1	Induction of apoptosis
11q25	ACAD8	Acyl-CoA dehydrogenase family, member 8	Catabolism of valine, fatty acid metabolism
13q34	RASA3	RAS p21 protein activator 3	GTP-ase activating protein that inactivates GDP-bound RAS to allow cellular proliferation and differentiation
13q34	TFDP1	Transcription factor Dp-1	Forms a heterodimer with E2F proteins to control transcriptional activity of genes involved in cell cycle progression from G1 to S phase
13q34	ADPRHL1	ADP-ribosylhydrolase like 1	Posttranslational modification of protein function

**Table 6 (continued). Subtelomeric genes on chromosomes with telomere attrition.**

<b>Chromosomal Location</b>	<b>Gene</b>	<b>Name</b>	<b>Function</b>
13q34	ARHGEF7	Rho guanine nucleotide exchange factor 7	Cytoplasmic protein that exchanges bound GDP for GTP and activates Rho proteins
13q34	ING1	Inhibitor of growth family, member 1	Tumor suppressor that can induce growth arrest and apoptosis
13q34	COL4A2	Collagen, type IV, alpha 2	Structural component of basement membrane. C-terminus of protein is canstatin, an inhibitor of angiogenesis and tumor growth

Neuroendocrine status has been shown to modulate the effect of stress on telomere attrition (Parks et al., 2009), and stress hormones have been speculated to be the intermediary between stress and telomere shortening. Accelerated telomere attrition and dampened telomerase activity have been linked to elevations of the stress hormones cortisol and norepinephrine (Epel et al., 2006; Choi et al., 2008). Furthermore, the biological cascade resulting from perturbations in cortisol levels have been conjectured to contribute to increases in oxidative stress and reactive oxygen species that could directly damage the telomere and cause disruption of the protective t-loop structure (Griffith et al., 1999; von Zglinicki et al., 2002; Sharma et al., 2003). However, we did not observe a correlation between cortisol AUC and mean telomere intensity values in this study. This lack of a correlation could reflect the fact that cortisol levels were not significantly altered in this study cohort. Alternatively, the relationship between cortisol values and telomere length may be mediated by an intermediary in the biological cascade that is not apparent from direct assessments of cortisol. For example, one such intermediary could be tumor necrosis factor-  $\alpha$  (TNF- $\alpha$ ), which is elevated following stress (Damjanovic et al., 2007) and acts directly and indirectly (through decreasing insulin activity) to set up a pro-inflammatory state with increased oxidative stress and subsequent telomere shortening. TNF- $\alpha$  also acts through c-Jun N-terminal kinase (JNK) pathway to decrease telomerase (Beyne-Rauzy et al., 2004) and its actions to maintain telomere integrity.

Given that chronic stress has been correlated with elevated telomerase activity (Damjanovic et al., 2007), it is also feasible that telomerase could be activated in

lymphocytes in response to stress, thereby maintaining telomere length and integrity. Longitudinal studies evaluating telomere length and telomerase in a stressed cohort are lacking. It has been suggested that telomere lengths and the state of telomere capping have plasticity and that change in telomere length may be bidirectional, with lengthening and shortening occurring in a dynamic relationship (Weng et al., 1998) dependent on telomere binding proteins and the capping status of the telomere (McEachern et al., 1995; Smith and Blackburn, 1999). We did, however, observe chromosome-specific telomere shortening in a subset of our cohort that was exposed to intercourse, suggesting the presence of a biological threshold, which might be surpassed with stress from the most severe form of CSA and/or a dose response relationship with CSA severity.

There are several reasons why the data reported herein is unique when compared to prior studies on stress-induced telomere attrition. Firstly, Glass et al. used Southern blot methodology to assess telomere length, which has also been used by Cherkas et al. (2006) and Damjanovic et al. (2007). The use of PCR-based techniques has also been employed to assess average telomere lengths in stressed populations (Epel et al., 2004, 2006; Parks et al., 2009; Tyrka et al., 2010; Drury et al., 2011). Although these approaches are valid, it is possible that telomere attrition involving a specific chromosome(s) would evade detection because the DNA-based assays yield an average of all telomeres. Secondly, to date, in the extant literature on telomere length and CSA, consideration has not been given to the severity of sexual abuse or the type of sexual abuse experienced. Data reported thus far could be skewed by the type

and severity of abuse reported by participants, as our data suggests a threshold before stress impacts telomere length, with clear effects only recognized for individuals experiencing intercourse, the most severe form of CSA. As mentioned above, participants were recruited because they participated in a study characterizing the determinants for development of psychiatric and substance abuse disorders (Kendler et al., 2000, 2004). The risk of these disorders was “modest and nonsignificant with non-genital CSA and increased with genital CSA and especially intercourse” (Kendler et al., 2000, 2004). Using the same cohort, the fact that we observe telomere attrition in individuals with intercourse exposure is further evidence of the latent health effects of early childhood stress and strongly supports either a threshold or “dose-response” phenomenon between childhood stress and latent biological changes. Although we were unable to discriminate between the two aforementioned phenomena, this is an important area of future research. Future studies should consider that the type of abuse experienced by an individual may mediate a measureable biological effect.

In conclusion, we observed significant chromosome-specific telomere length patterns for the subgroup of individuals exposed to intercourse, although when averaged over all telomeres, the observed values did not reach statistical significance in this or any CSA discordant cohort evaluated. However, as illustrated by our study, evaluating only average telomere length may overshadow a chromosome-specific effect and is a drawback to the use of PCR-based and Southern blot methodologies. Indeed, as mentioned above, in intercourse-exposed individuals, the most severe CSA class, chromosome-specific telomere shortening was observed for 3p, 5p, 6p, 11q and 13q.

That stress-induced telomere shortening was observed only in the individuals that experienced the most severe childhood stress suggests a possible biological threshold that is strongly supported by a dose-response relationship between CSA severity and psychiatric disorders in prior studies in this cohort. Collectively, these results suggest that telomere length measurement as a psychobiomarker may have value when assessed on a chromosome-specific level, but the strength of its utility as a biomarker for latent effects in adults exposed to CSA remains equivocal.

## **Chapter 4**

### **Chromosomal Instability in Identical Co-twins Discordant for a History of Childhood Sexual Abuse**

#### **Introduction**

Early-life stress events have been associated with an increased incidence of behavioral and health problems in adulthood (Kendler et al., 2000; Miller et al., 2009). In addition to contributing to significant morbidity, Brown et al. (2009) found that adults who experienced early-life stress events die nearly 20 years earlier than adults without childhood adversity. However, little is known about how these early stress experiences become biologically embedded to result in pathological manifestations later in life. One possible means for adverse childhood social experiences to be biologically “remembered” would be if that event resulted in a change in the individual’s DNA. In adults, the observation that psychosocial factors, including chronic stress from a variety of modalities, are associated with telomeric shortening supports the idea that stress leads to alterations in the DNA of somatic cells (Epel et al., 2004; Cherkas et al., 2006; Simon et al., 2006; Damjanovich et al., 2007; Humphreys, 2011). It is not known if the effects of stress are cumulative. However, if an individual’s stress history is chronicled in DNA, the greatest sequelae from psychosocial events may be observed by studying adults with a history of childhood adversity.



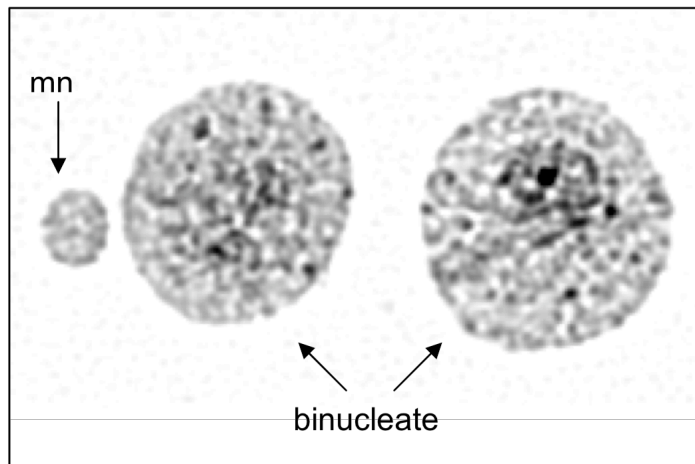
Evidence that early-life stress can lead to DNA-based alterations comes from reports of shortened telomeres in children exposed to adverse rearing settings (Drury, et al., 2011). Further support for childhood stress-induced DNA-based alterations comes from studies showing telomeric attrition in adults with a history of chronic or severe childhood illness (Kananen et al., 2010) or childhood maltreatment (Tyrka et al., 2010). However, Glass et al. (2010) failed to detect a correlation between childhood maltreatment and telomere length and others have suggested that the association between stress and telomere attrition in both adults and children may be merely correlative in nature, with telomere length simply serving as a “chronicle” to document that other biologically relevant changes have occurred which may lead to an increased health risk (Effros et al., 2005).

Given that stress has been shown to affect telomere length, and that telomere shortening has been associated with an increased frequency of chromosomal abnormalities (Harley, 1991; Counter et al., 1992; Day et al., 1998; Filatov et al., 1998; Fagagna et al., 1999; Schwartz et al., 2001; Leach and Jackson-Cook, 2004), an alternative and/or additional biological effect of stress could be the acquisition of somatic cell chromosomal instability. To test this hypothesis, one could estimate the frequency of chromosomal abnormalities in somatic cells from individuals exposed to childhood stress. These estimates would be optimally scored in cells without the influences of potential *in vitro* selective growth alterations, the latter of which might skew the proportion of chromosomally normal and abnormal cells present for evaluation.

Therefore, the cytokinesis-block micronucleus (CBMN) assay, which provides information regarding the previous interphase and mitotic division of a somatic cell prior to the presence of selective pressure on the resultant daughter cells, is ideal for this type of study. The data obtained from the CBMN assay, a high throughput methodology, is comparable to metaphase chromosomal analyses (Miller et al., 1998; Bonassi et al., 2001). However, because the CBMN assay is less labor intensive and less prone to producing artifacts than classical chromosomal studies, it is an attractive tool for estimating genomic damage associated with environmental insults, such as early life stress (Battershill et al., 2008). Briefly, a micronucleus is a small chromatin-containing structure that can be visualized juxtaposed to the main daughter nuclei following the completion of mitosis in cells blocked at telophase by cytochalasin B (Figure 11). Micronuclei are thought to form when whole chromosomes or chromosomal fragments fail to correctly migrate to spindle poles during mitosis (Fenech and Morley, 1985; Savage, 1988; Lindberg et al., 2007). The lagging chromosome(s) or fragment(s) are excluded from the daughter nuclei and are encased in their own nuclear envelope, forming a micronucleus (Fenech and Morley, 1985). Micronuclei frequencies increase with age (Miller et al., 1998; Bonassi et al., 2001; Jones et al., 2011) and are elevated in patients with several chronic health conditions including (but not limited to), cancer (Bonassi et al., 2007); cardiovascular disease; Alzheimer's disease; and Parkinson's disease (Petrozzi et al., 2002; Murgia et al., 2007; Federici et al., 2008).

Micronuclei frequencies are influenced by both heritable genetic and environmental influences (Jones et al., 2011), but the extent to which social experiences

influence micronuclei frequencies is not known. One of the most robust approaches to determine the role of non-genetic influences on trait variation is to study identical (monozygotic [MZ]) twins who are discordant for exposure histories (Wong et al., 2005). Because the DNA of MZ twins discordant for childhood abuse differs only for induced changes, they provide a unique opportunity to study the biological impact of childhood stress events on adults. One of the most traumatic forms of childhood adversity is childhood sexual abuse. Hence, if early-life stress elicits DNA-based changes that persist into adulthood, one might best detect these effects by studying adult identical twins discordant for a history of childhood sexual abuse. To date, there have been no studies evaluating micronuclei frequency and childhood sexual abuse, with or without this powerful discordant twin design. Therefore, the primary aim of this study was to measure the frequency of spontaneously occurring micronuclei in adult MZ twins discordant for a history of childhood sexual abuse to evaluate the biologic impact of stress on age-related, acquired somatic cell chromosomal abnormalities.



**Figure 11. A Giemsa stained micronucleus (mn) and corresponding daughter binucleates.** By definition, a micronucleus is no larger than 1/3 the size of the parental nuclei and appears adjacent to the binucleate.

## **Materials and Methods**

### **Sample Ascertainment**

A total of 24 female twin pairs (48 individuals) with a history of childhood sexual abuse (CSA) were ascertained for this study due to their participation in a study completed by Kendler et al. (2000). Discordance for CSA exposure was used to prioritize twin pairs for recruitment, although 2 twin pairs who were concordant for CSA (positive controls) and 7 female control twin pairs who did not experience CSA (negative controls) were also recruited. All twins were ascertained through the Mid-Atlantic Twin Registry at Virginia Commonwealth University. Discordance for CSA was classified as narrow ("one twin reported CSA and the co-twin reported no CSA" [Kendler et al., 2000]) or broad ("one twin reported CSA and her co-twin reported no or a less deviant form of CSA" [Kendler et al., 2000]). CSA exposure was further classified as 1) no genital contact (sexual invitation, sexual kissing and exposing); 2) genital contact without intercourse (fondling and sexual touching); or 3) intercourse (Kendler et al., 2000). As part of the previous investigation, which focused on understanding genetic and social influences impacting one's propensity to develop psychiatric and substance abuse disorders, these twins were extensively interviewed and administered the Structured Clinical Interview for DSM-III-R and -IV (American Psychological Association). For the purposes of the current study, participants consented to grant access to the archived DSM-III-R and -IV scoring results obtained in the prior study, as well as data

obtained from questionnaires assessing the participants' CSA experience(s) and self-reported stress levels (Kendler et al., 2000). After providing informed consent (VCU IRB protocol HM12407), participants submitted peripheral blood specimens, which were drawn by the participant's health care provider and shipped to our laboratory via an overnight courier. These blood samples were used to evaluate chromosomal instability levels and to confirm the twins' zygosity status.

### **Health History Questionnaire**

In addition to providing blood samples, the participants completed a paper-based questionnaire which assessed factors regarding their medical and personal history, including; disease diagnosis and treatment, alcohol and cigarette use, over-the-counter and prescription medication use, dietary intake of micronutrients and supplements, height and weight (to calculate body mass index), and self-scores of attitudes regarding health and vitality. As mentioned above, approximately 15 to 18 years ago, the participants also completed questionnaires assessing their childhood family environment, CSA history and feelings about the abuse (for a detailed description, see Kendler et al. [2000]).

### **DNA Isolation and Zygosity Determination**

In order to confirm the zygosity of the twins, genomic DNA was isolated from whole blood using the Puregene DNA Isolation Kit (Qiagen). Twins were classified as

monozygotic if the marker data for the co-twins matched at all of the 13 highly polymorphic short tandem repeat sequences evaluated (AmpFISTR Profiler Plus and Cofiler kits, Applied Biosystems, Foster City, CA).

## **Cell Culture**

To ensure that erythrocytes did not confound the recognition and scoring of micronuclei, leukocytes were isolated using Histopaque-1077 (Sigma) and then established in culture according to standard procedures (RPMI 1640 media supplemented with 15% fetal calf serum and the mitogen phytohemmagglutinin [Fenech, 2000]). Forty-four hours after initiation of the cultures, cytochalasin-B was added at a final concentration of 3µg/ml. Binucleate interphase cells were harvested at 72 hours using standard techniques, including a 10-minute incubation in hypotonic solution (0.075 M KCl), and serial fixation (three times in 3:1 methanol: acetic acid solution [Fenech, 2000]). Slides were made following standard procedures (Leach and Jackson-Cook, 2001).

## **Metaphase Analysis**

Metaphase chromosomes were harvested and GTG-banded according to standard techniques (Moorhead et al., 1960). It was necessary to determine if any of the twins carried a balanced chromosomal rearrangement because the presence of a constitutional chromosomal finding may influence the frequency of chromosomal aberrations observed in an individual's somatic cells. A minimum of 10 metaphase

spreads per participant were evaluated. Of these 10 metaphase spreads, 2 were selected for karyogram preparation using a Cytovision Imaging system (Genetix).

### **Micronuclei Analysis**

Binucleates and micronuclei were stained in a 4% Harleco Giemsa solution for 4 minutes at room temperature. The frequency of micronuclei was determined by replicate scoring of the number of micronuclei observed per 1,000 cytochalasin-B blocked binucleates (2 separate areas of the same slide), according to the protocol of Fenech (2006 [Figure 12]). The frequency of micronuclei was calculated by averaging the values obtained from the 2 replicate scores (2,000 total binucleates). Investigators were blinded to the CSA status of the participants at the time of slide scoring.

### **Statistical Analysis**

The statistical analysis package Prism (GraphPad) was used for all statistical assessments unless otherwise noted. Because it is reasonable to assume that the distribution of micronuclei frequencies follow a Poisson distribution, the micronuclei frequencies were square root transformed to approximate a Gaussian distribution. Paired student's, one-sided t-tests were used to evaluate support for the directional hypothesis of higher micronuclei formation due to CSA-elicited stress and to assess differences in mean micronuclei frequencies in abused co-twins compared to their unabused co-twins. Because co-twins may share adverse familial factors not including



CSA and/or because knowledge of a co-twin's abuse may serve as a stressor for the unabused co-twin, age-matched controls were also included for assessment. To this end, generalized mixed-effects models were performed with Poisson error distribution adjustments for covariance within families and the effect of age. This approach was also used to evaluate potential interaction influences due to diet and tobacco/alcohol usage. To specify the relevant contrasts among the different CSA exposure classes, 2 fixed effect terms were established. Unabused co-twins and age-matched controls were coded negative for a CSA exposure term, while abused co-twins were coded positive. To investigate the potential influence of an adverse familial environment, an additional term was created in the generalized mixed effects model. For this term, CSA discordant pairs were coded as positive and control pairs as negative. A significant coefficient for the second term would indicate an influence of family adversity while controlling for any influence from CSA exposure.

Because the discordant twins evaluated in this study were genetically identical, within-pair differences in micronuclei frequencies were calculated by subtracting the micronuclei frequency of the unabused twin from that of the abused twin. Difference scores for twin pairs concordant for abuse were calculated by subtracting the micronucleus frequency of an individual (randomly selected) from the micronucleus frequency of her co-twin. One-sample t-tests were used to determine if differences in micronuclei frequencies were present within twin pairs discordant and concordant for CSA. Statistical tests evaluating mean frequencies in abused compared to unabused groups (unabused co-twins and age-matched controls), as well as personal history

indices, were assessed by Dr. Timothy York using the statistical analysis package R. An alpha level of  $p < 0.05$  was set to limit type I (false positive) errors for all statistical analyses.

## **Results**

### **Sample Distribution and CSA Class Assignment**

Of the 24 female twin pairs with at least one co-twin exposed to CSA, the average age was 50.0 (SD=9.5) years, with a range of 35-70 years. The average age and range of the 7 healthy female control twin pairs was 51.57 (8.59) years, with a range of 40-69 years. All twin pairs were determined to be monozygotic, had chromosomal complements within normal limits (46,XX) as assessed by GTG-banding, and described their ethnicity as Caucasian. The childhood sexual abuse event(s) was self-reported and also co-twin corroborated (Kendler et al., 2000). The 24 twin pairs participating in the current study included 2 concordant pairs, 17 pairs discordant for CSA using a narrow definition of discordance ("one twin reported CSA and the co-twin reported no CSA" [Kendler et al., 2000]), and 5 pairs who were classified as broadly discordant for CSA ("one twin reported CSA and her co-twin reported no or a less deviant form of CSA" [Kendler et al., 2000]). The number of study participants in the hierarchical exposure groups was: 1) no genital contact (sexual invitation, sexual kissing and exposing [n=3 pairs]); 2) genital contact without intercourse (fondling and sexual touching [n=11 pairs]); and 3) intercourse (n= 10 pairs [Kendler et al., 2000]).

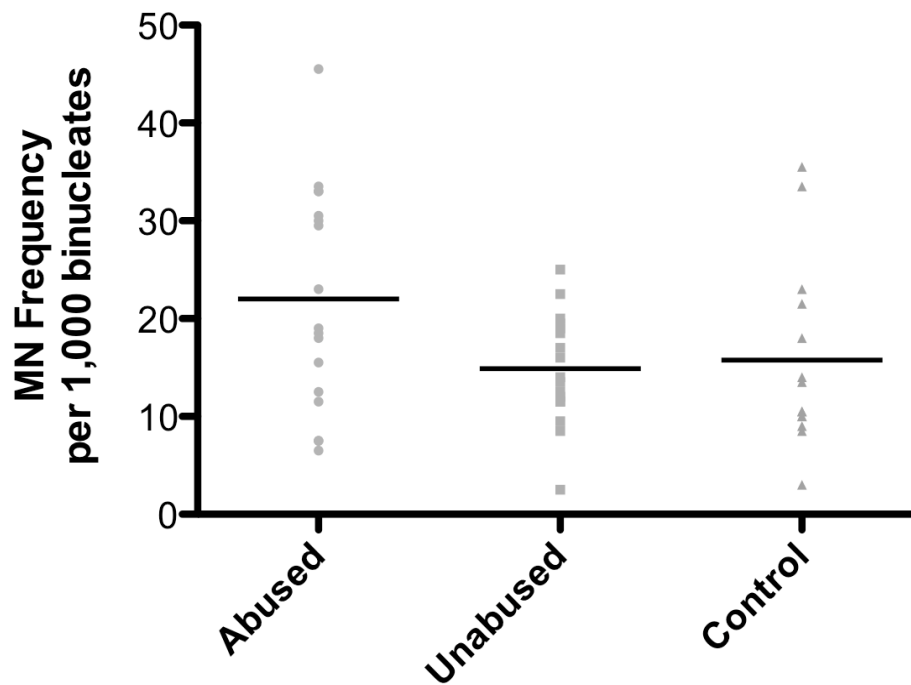
## **Micronuclei Frequencies in Twins Discordant for CSA**

Mean MN frequencies in the abused twins (22.03 [11.35]) from the narrow discordant class (n=17), which included participants exposed to intercourse, genital and non-genital CSA, were significantly higher than the frequencies observed in their non-abused co-twins (14.88 [5.62];  $t=2.65$ ;  $df=16$ ;  $p=0.009$ ). [Table 7, Figure 12]). Based on results of generalized mixed-effect models, there was no indication of an effect from familial environment ( $p=0.406$ ), which suggests that CSA is the determining factor for the observed increase in micronuclei frequencies in the abused individuals. To determine if micronuclei frequencies differed between identical co-twins, within-pair analyses were completed and difference scores calculated by subtracting the micronuclei frequency of the unabused twin from that of her abused co-twin. A positive difference score reflects that the micronuclei frequencies of the abused individuals were larger than that of their unabused co-twins. A trend toward increased micronuclei frequencies in abused individuals was observed for the co-twins assigned to the broad genital sub-category, although this failed to reach significance ( $t=2.05$ ;  $df=9$ ;  $p=0.07$ ). The difference in micronuclei frequencies between co-twins from the other CSA sub-categories; narrow intercourse, broad intercourse, narrow genital and non-genital, were also not significantly different from zero ( $t=1.01$ ,  $df=5$ ,  $p=0.36$ ;  $t=1.45$ ,  $df=8$ ,  $p=0.19$ ;  $t=1.69$ ,  $df=7$ ,  $p=0.13$ ;  $t=2.24$ ,  $df=2$ ,  $p=0.15$ , respectively). As expected, no significant difference in MN frequency was observed in co-twins concordant for CSA ( $t=3.00$ ;  $df=1$ ;  $p=0.205$  [Figure 13]).

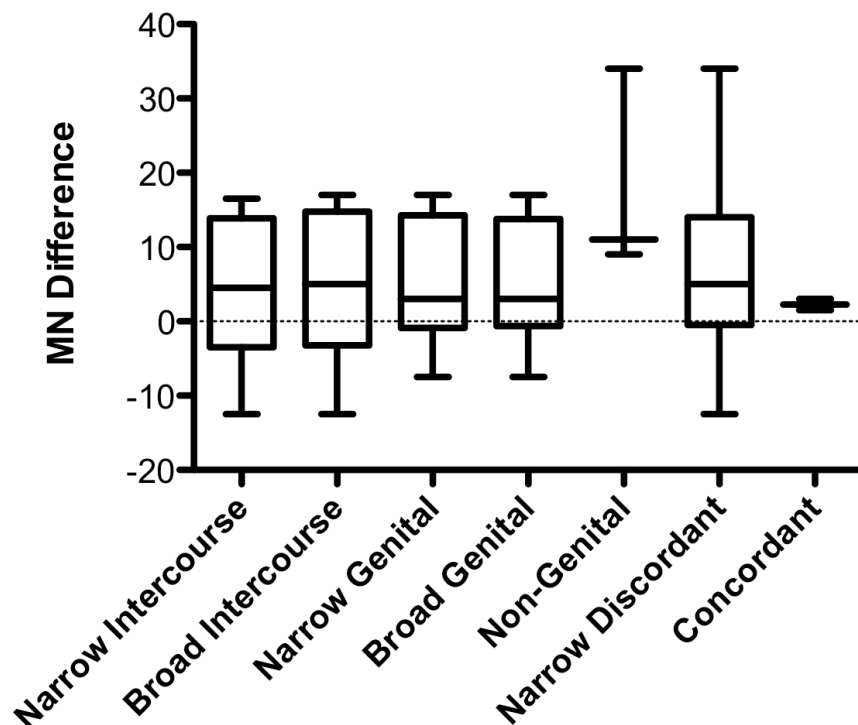
**Table 7. Distribution of MN frequencies (per 1,000 binucleates) among CSA classes.**

Discordant Pairs	CSA Type <sup>1</sup>	<i>Narrow</i>		<i>Broad</i>	
		No CSA	CSA (more severe)	CSA <sup>2</sup> (less severe)	CSA Type
1	Intercourse	11.5	15.5		
2	Intercourse	19.5	19		
3	Intercourse	25	12.5		
4	Intercourse	20	33		
5	Intercourse	2.5	7.5		
6	Intercourse	13.5	30		
7	Intercourse		20.5	13.5	Genital
8	Intercourse		22.5	5.5	Genital
9	Intercourse		16.5	22.5	Genital
10	Genital	8.5	7.5		
11	Genital	14	6.5		
12	Genital	19	23		
13	Genital	16	18		
14	Genital	12	11.5		
15	Genital	18.5	33		
16	Genital	12.5	29.5		
17	Genital	17	30.5		
18	Genital		15	15	Non-Genital
19	Genital		26.5	15.5	Non-Genital
20	Non-Genital	9.5	18.5		
21	Non-Genital	11.5	45.5		
22	Non-Genital	22.5	33.5		
Concordant Pairs	CSA Type	CSA	CSA		
1	Intercourse	17	20		
2	Genital	30	31.5		

<sup>1</sup>per Kendler et al., 2000



**Figure 12. Micronuclei (MN) frequencies in individuals who did (abused co-twins) or did not (unabused co-twins and controls) experience childhood sexual abuse.** The mean frequency of micronuclei in twins with a history of CSA (n=17) was significantly higher than the mean frequency observed in the unabused co-twin group (n=17 [p=0.027]). While a trend toward higher micronuclei frequencies was noted for the abused twins compared to the negative control group (n=14), this value was not significantly different (p=0.11). Similarly, the mean micronuclei frequency of the unabused co-twin group was not different from that observed in the age-matched controls (p=0.75).



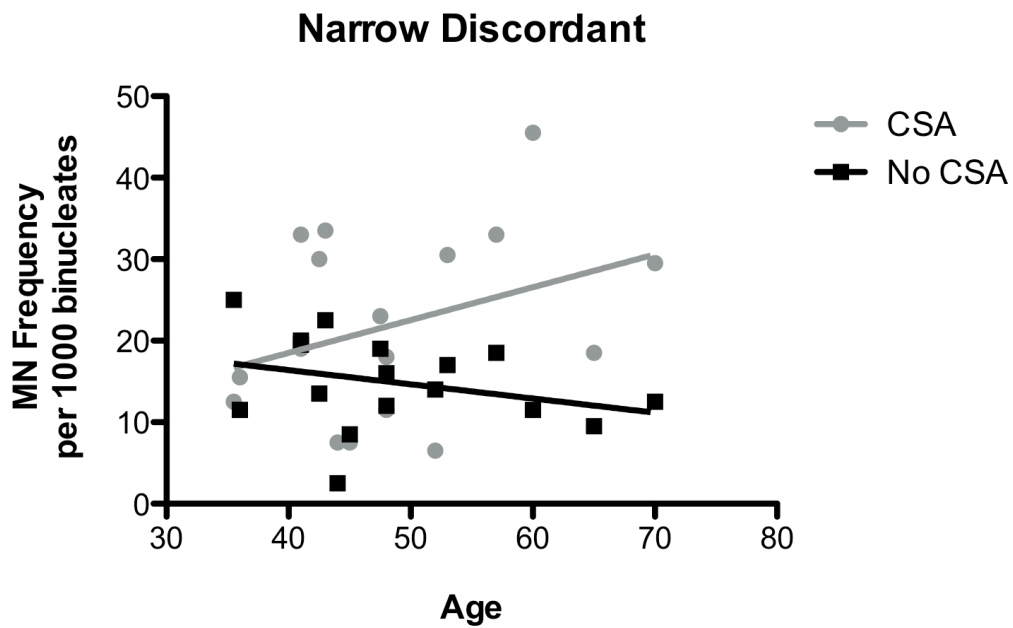
**Figure 13. The difference in micronuclei (MN) frequencies in co-twins with a history of childhood stress.** If micronuclei frequencies were fully determined by heritable genetic or common environmental influences, one would expect the difference between genetically identical co-twins to be zero. However, the difference in micronuclei frequencies of the co-twins in the 17 narrow discordant pairs was significantly greater than zero ( $p=0.015$ ). While a trend for an increased difference value was also observed in the abused compared to non-abused twins (when sub-divided based on the type of abuse experienced), these values were not significantly different from zero. As expected, the difference values of concordant twins were not different from zero ( $p=0.20$ ). Whiskers of the box and whiskers plots represent the minimum and maximum of observed frequencies.

In the narrow discordant group, the largest group studied, age was not predictive of mean micronucleus frequency (mixed-effects models  $p=0.803$ ) across the entire group (complete twin pairs). However, separate evaluations of the abused individuals and unabused individuals indicated a significant interaction between age and CSA exposure (coefficient [SE] = 0.030 [0.009]:  $p=0.0006$  [Figure 14]), with this interaction being driven primarily by a increase in micronuclei frequencies in abused individuals, rather than a decrease in micronuclei frequencies in the unabused co-twins (coefficient [SE] = -0.012 [0.007];  $p=0.072$ ).

### **Micronuclei Frequencies and Personal Health History**

Participants' answers to personal health history questionnaires, as well as information obtained from interviews with the participants (Kendler et al, 2000), were used to determine if lifestyle factors impacted micronuclei frequencies. Among all 24 pairs analyzed, there were no self-reports of Parkinson's disease, Alzheimer's disease, lupus, rheumatoid arthritis, or ulcerative colitis. Diabetes and cancer were reported by 6 of 48 (12.5%) and 5 of 48 individuals (10.4%), respectively. Heart disease and high blood pressure, a risk factor for heart disease, were reported by 4 of 48 (8.3%) and 14 of 48 individuals (29%), respectively. Interestingly, 10 of 14 (71.4%) individuals with high blood pressure were abused. Of these 10 individuals, 4 were exposed to intercourse, 4 were exposed to CSA involving genital contact but not intercourse, and 2 were exposed to non-genital CSA. Because the frequencies of most of the queried diseases were too small to allow for unbiased statistical assessment, the frequencies of





**Figure 14. The relationship between age and micronuclei (MN) frequency.** A significant interaction was observed for the difference values in micronuclei frequencies and age using mixed-effects models (coefficient [SE] =0.030 [0.009];  $p=0.0006$ ).

these variables are reported, but formal statistical comparisons were not completed. Among the narrow discordant group, which is the largest group studied, no clear difference in smoking, alcohol use, body mass index (BMI), or intake of green leafy vegetables was observed between abused and unabused twins (Table 8). Furthermore, no significant differences in the DSM-III-R and- IV diagnoses (Kendler et al, 2000) of depression, panic disorder and alcohol or drug dependence were detected between the discordant co-twins. Because these factors were not differently distributed among the co-twins, additional analyses to assess potential correlates between these lifestyle factors and micronuclei frequencies were not completed.

**Table 8. The distribution of personal health history factors and psychological disorders among narrow discordant co-twins (n=17 pairs).**

	<b>Narrow Discordant Co-twins</b>		
	<u>CSA</u>	<u>No CSA</u>	<u>p-value</u>
<b>Self-Report Indices</b>			
Age (SD)	48.7 (9.7) <sup>1</sup>	48.7 (9.7)	
BMI (kg/m <sup>2</sup> )	28.3 (6.5)	26.5 (7.5)	0.538
Tobacco Use			
Lifetime (>50 cigarettes)	6/14	4/13	0.801
Last month (>2/wk)	1/8	0/7	0.945
Alcohol Use			
Lifetime (>50 drinks)	13/14	10/13	0.533
Time when drinking most	9/15	7/11	0.826
Medication (Rx and non-RX) >1	6/14	7/12	0.736
Green leafy vegetables (5/wk)	4/14	6/12	0.474
Depressed for 2+ weeks	7/14	7/13	0.853
Nervous person	2/16	2/13	0.751
<b>DSM-IIIR Diagnosis</b>			
Lifetime depression	10/17	7/17	0.493
Lifetime GAD <sup>2</sup> (narrow 1mo)	1/17	0/17	1
Lifetime GAD (broad 1mo)	5/17	1/17	0.178
Panic (narrow)	2/14	0/15	0.433
Panic (least restrictive)	3/14	3/15	0.716
<b>DSM-IV Diagnosis</b>			
Alcohol dependence	3/17	1/17	0.595
Any drug abuse or dependence	2/17	0/17	0.466

<sup>1</sup>Mean (standard deviation)

<sup>2</sup>GAD- Generalized anxiety disorder

## Discussion

To determine if exposure to childhood stress influences chromosomal instability, the micronuclei frequencies of identical twins discordant for exposure to CSA were compared. In the narrow discordant group, micronuclei frequencies observed in individuals who experienced CSA were compared to those of their identical co-twins, who did not experience any form of CSA (n=17 pairs). For these studies, all narrow discordant twins with a history of CSA (intercourse, genital and non-genital exposure) were evaluated and showed an increased frequency of micronuclei in the twins exposed to CSA compared to their identical co-twins who did not experience CSA. This is the first study to report an increase in micronuclei frequency, or chromosomal instability, in adults who were exposed to childhood stress.

Maximizing the power of our discordant MZ twin design, we further evaluated differences within identical twin pairs, which would be expected to be zero if micronuclei frequencies are influenced by genetic or common environmental factors. The differences in micronuclei frequency within twin pairs were significantly increased from zero for the narrow discordant group. However, there was no significant difference between co-twins in the other CSA groups (narrow and broad intercourse, narrow and broad genital and non-genital), which is likely due to a smaller sample size for each of these sub-groups. The small sample size of our cohort is the major limitation of this study.

An significant interaction between differences in co-twin micronuclei frequencies and age was observed, with this interaction appearing to reflect an increase in frequency with age in the twins exposed to CSA rather than a decrease in their unabused co-twins. This observation suggests that an deleterious environmental factor such as stress in childhood can have a biological effect that becomes more pronounced with advancing age (cumulative). Compared to adults, children are thought to be more sensitive to DNA damaging agents, presumably because their cells are experiencing rapid growth as they transition through developmental periods, making cellular processes more easily disrupted (Perera, 1996; Landrigan et al., 2003; Suk et al., 2003). Many studies on micronuclei frequencies in children focus on genotoxic agents. However, similar to environmental toxins, it is possible that stress initiates a cascade of biological changes, including increased micronuclei frequencies. The accumulation of DNA damage initiated by stressful events in childhood may lead to an earlier biological manifestation than that which occurs with normal aging in individuals not exposed to stressful events in childhood. Because this study was not longitudinal in scope, the time point at which these biological changes occurred is unknown. To date, there have been no reports on micronucleus frequency in children following CSA exposure, but that is an important area of future research.

Thus far, an unresolved question is how to connect stress from an experience(s) in childhood to the increased micronuclei frequencies observed in adults in this study. A stressed state increases the cytokine tumor necrosis factor-alpha (TNF- $\alpha$ ) [Damjanovic

et al., 2007]), which acts indirectly, by decreasing the function of insulin, and directly, to set up a pro-inflammatory state (reviewed in Innes et al., 2007). An increase in downstream effectors, which include transcription factors and other cytokines, play a role in a positive feedback loop to further increase TNF- $\alpha$  and inflammation (reviewed in Zhang et al., 2009). The commensurate increase in oxidative stress and reactive oxygen species causes direct DNA damage (Yan et al., 2006, 2009), which may increase the frequency of micronuclei. Additionally, TNF- $\alpha$  has been shown to increase micronuclei frequency *in vitro* (Yan et al., 2009), presumably through induction of oxidative stress and DNA damage. Oxidative stress and a pro-inflammatory state in the vasculature are associated with high blood pressure (Zhang et al., 2009; DeLoach et al., 2011), which is a risk factor for cardiovascular disease that has been shown to be increased by 20% in other individuals who experienced CSA (Riley et al., 2010). Another gene in the biological network involved in micronuclei formation is interleukin-6 (IL-6), which encodes a protein that acts as an inflammatory mediator (van Leeuwen et al., 2011). In addition, several genes involved in mitotic spindle assembly, cell cycle checkpoints, and epigenetics have also been identified in the formation of micronuclei (van Leeuwen et al., 2011). Changes in any of these genes could result in checkpoint dysregulation, spindle assembly dysfunction, and/or replication errors, all of which could lead to the malsegregation of chromosomes at anaphase and the formation of micronuclei. Whether originating via oxidative damage or malsegregation events, it is obvious that increased micronuclei frequencies reflect latent biological changes in adults exposed to childhood stress.

Because alternative environmental or lifestyle factors may influence micronuclei frequency, exposure-related variables that have been demonstrated to influence micronuclei frequencies were assessed via a questionnaire format. These factors, including dietary nutrient intake, the use of alcohol, tobacco, prescription and over-the-counter medication, showed no significant difference in their distribution between abused and unabused groups. Additionally, the distribution of DSM-III-R and -IV diagnoses, assigned in the original study on this cohort (Kendler et al., 2000), did not significantly differ between co-twins. Given that environmental factors were not significantly different between CSA co-twin groups, the observed differences in micronuclei frequencies seem unlikely to be attributable to other environmental factors, and instead, seems most likely to reflect the discordant experience of childhood sexual abuse.

Measurement of cortisol levels is regarded as the gold standard for estimating the physiological impact of stress. However, measurement of cortisol levels is inherently problematic due to the sensitivity of cortisol to a myriad of factors including, but not limited to, oral contraceptive use and menstrual cycle phase (Kirschbaum et al., 1999); use of corticosteroids and nicotine (Wilson, 1999); and obesity (Heim et al., 2000). Additionally, methodological problems including sample collection and storage procedures can interfere with accurate measurement of cortisol concentrations. We profiled cortisol patterns in identical twins discordant for CSA (Chapter 2) and our results demonstrate the challenges of salivary cortisol collection and measurement and point to the need for a more reliable biosurveillance tool. The findings from the current

study demonstrate the relevance and utility of the CBMN assay, used to estimate chromosomal instability, to evaluate the biological effects of stress.

In conclusion, an increase in micronuclei frequencies in twins exposed to early childhood stress was observed when compared to their co-twins. Chromosomal instability/DNA damage has been associated with stress and is also predictive for the development of cancer and cardiovascular disease (Tucker and Preston, 1996; Andreassi and Botto, 2003, Bonassi et al., 2004; Yusef et al., 2004; American Heart Association, 2005). Based on the results of this study, the frequency of micronuclei appears to be a potential novel biomarker for psychosocial stress, and the CBMN assay may be an attractive screening tool to assess an individual's risks for acquiring a subset of stress-related health conditions. Collectively, information gained from this study provides an important clue as to how early life stress leads to biological manifestations and seemingly "gets under the skin".



## **Chapter 5**

### **Contents of Micronuclei in Co-Twins Discordant for Childhood Sexual Abuse**

#### **Introduction**

Stress is recognized as a risk factor for a broad spectrum of complex diseases and age-related conditions including cardiovascular disease, which is the leading cause of morbidity and mortality in industrialized nations (Yusef et al., 2004; American Heart Association, 2005); autoimmune diseases; infectious diseases; psychiatric illness; and decreased immune function (reviewed in Cohen et al., 1997; Yang and Glaser, 2000). However, the biological basis for stress-related health risks has not been well resolved. Given the observed similarities in morbidity associated with aging and stress, as well as evidence to support the proposal that acquired chromosomal imbalances are an early initiating step in the cascade of biological changes leading to age-related conditions (Fenech, 1998; Aviv et al. 2001), we hypothesized that stress may lead to an increased rate of acquired somatic cell chromosomal aberrations. One could further speculate that, akin to the circumstances arising with age-related diseases, stress-related chromosomal instability could be an initiating step for latent disease development.

A high throughput and robust method for estimating acquired somatic cell chromosomal abnormality rates is to analyze the frequency of micronuclei in

cytokinesis-blocked cells. A micronucleus is a small chromatin-containing structure that forms when whole chromosomes or chromosome fragments fail to migrate to spindle poles during anaphase. The lagging chromosome(s) or fragment(s) become encased in their own nuclear envelope and are excluded from the daughter nuclei (Fenech, 2000). Our studies of identical twins who are discordant for an extreme form of early-life stress, childhood sexual abuse, showed a significant increase in the frequency of micronuclei in the abused twins compared to their identical, non-abused co-twins (Chapter 4). However, the results of these exciting studies provided no information about the specific chromosomes that acquired abnormalities. Wojda and Witt (2003) observed that several of the chromosomes with high frequencies of age-related acquired anomalies harbor genes that play roles in cellular senescence or premature aging syndromes. Thus, recognition of the specific chromosomes involved in stress-related acquired aberrations might lead to insight about the biological consequences of stress.

Given that the micronucleus/cytome assay requires assessment of interphase nuclei, the chromosomes present in micronuclei cannot be directly visualized. However, their chromatin content can be inferred through the use of fluorescence in situ hybridization (FISH) methodologies (Norppa and Falck, 2003). Combining the use of pancentromeric and pantelomeric FISH probes, investigators have shown that the majority of spontaneously occurring micronuclei in lymphocytes contain a single, intact chromosome (Leach and Jackson-Cook, 2001; Wojda and Witt, 2003; Lindberg et al., 2008). Sex chromosomes have been observed most often in micronuclei from older individuals, and the frequency of sex chromosome exclusion into micronuclei increases

with age (reviewed by Norppa and Falck, 2003). However, to date, the majority of studies completed to determine the chromosomal contents of micronuclei have been restricted to inferences about a small subset of chromosomes. A limitation of using chromosome-specific, FISH-based studies to infer micronuclei content is that the probes used are often targeted to only centromeric chromatin. Therefore, micronuclei containing acentric fragments cannot be characterized using this approach. Spectral karyotyping (SKY), which allows for the simultaneous detection of all 24 chromosomes, is traditionally used to assist in the identification of complex rearrangements in metaphase chromosomes. However, Leach and Jackson-Cook (2001) utilized this technology on interphase chromatin to assess the chromatin contents of micronuclei and to avoid the methodological problems inherent with conventional FISH methods. When applied to cytokinesis-blocked interphase preparations, SKY allows for the recognition of the chromosomal contents of micronuclei that contain a single chromosome or chromosome fragment (Figure 16).

Utilizing the SKY assay, Jones (2009) observed that there is a non-random pattern of chromosomes excluded into micronuclei that spontaneously occur in healthy individuals. In these studies, which were completed on healthy, normal twins in our laboratory, sex chromosomes and autosomes 4, 8 and 9 were observed in micronuclei most frequently and chromatin from autosomes 17 and 22 least frequently (Jones 2009). However, to date, no investigators have reported the chromosome-specific contents of micronuclei present in adults with a history of childhood stress. Therefore, the primary aim of this study was to expand upon our previous analyses of micronuclei

frequencies in identical twins discordant for a history of childhood stress (sexual abuse) by determining the frequencies of specific chromosomes excluded into the micronuclei of this discordant twin population. Because the DNA of monozygotic (MZ) twins differs only for induced changes, they provide a unique opportunity to study the DNA-based impact that stressful events experienced in childhood have on adults. In addition, because chromosomes with shortened telomeres, (the ends of linear chromosomes that serve a protective function) are predisposed to acquire abnormalities (Filatov et al., 1998; Leach and Jackson-Cook, 2004), a secondary aim of this study was to determine if telomere length was correlated with chromosome-specific micronuclei frequencies.

## **Materials and Methods**

### **Sample Ascertainment**

A total of 17 female twin pairs (34 individuals) discordant for a history of childhood sexual abuse (CSA) were recruited for this project as a result of their participation in a study completed by Kendler et al. (2000). The study participants were ascertained through the Mid-Atlantic Twin Registry at Virginia Commonwealth University. Discordance for abuse exposure was used to prioritize the twins selected for recruitment in this project. After providing informed consent (VCU IRB protocol HM12407), participants submitted peripheral blood specimens, which were drawn by the participant's health care provider and shipped to our laboratory via an overnight courier. The blood specimens were used to obtain cells for the cytokinesis-block micronucleus (CBMN) assay and to obtain metaphase chromosomes to allow for the assessment of chromosome-specific telomere lengths. The blood sample was also used to confirm the zygosity of the twin pairs.

### **DNA Isolation and Zygosity Determination**

In order to confirm the zygosity of the twins, genomic DNA was isolated from whole blood using the Puregene DNA Isolation Kit (Qiagen). Twins were classified as monozygotic if the marker data for the co-twins matched at all 13 of the highly

polymorphic short tandem repeat sequences evaluated (AmpFISTR Profiler Plus and Cofiler kits, Applied Biosystems, Foster City, CA).

## **Cell Culture**

Because erythrocytes can confound the scoring of micronuclei frequencies, leukocytes were isolated using Histopaque-1077 (Sigma), and then established in culture according to standard procedures (RPMI 1640 media supplemented with 15% fetal calf serum and the mitogen phytohemmagglutinin [Fenech, 2000]). Cytochalasin-B was added to the cultures 44 hours after culture initiation at a final concentration of 3µg/ml. Binucleate interphase cells were harvested at 72 hours using standard techniques, including a 10-minute incubation in hypotonic solution (0.075 M KCl) and serial fixation in a 3:1 methanol: acetic acid solution (one ten-minute incubation in fix, followed by two brief rinses [Fenech, 2000]). Slides were made following standard procedures (Leach and Jackson-Cook, 2001) and aged at room temperature for four days prior to initiating the SKY procedure.

## **Metaphase Analysis**

Given that the presence of a constitutional chromosomal abnormality could skew the observed frequency of chromosomal aberrations present in an individual's somatic cells, GTG-banding chromosomal studies were performed according to standard techniques (Moorhead et al., 1960) for each twin. A minimum of 10 metaphase spreads

were evaluated for each participant with 2 cells selected for karyogram preparation using a Cytovision Imaging system (Genetix).

## **Spectral Karyotyping**

The chromosomal contents of micronuclei were determined using SKY according to the manufacturer's (Applied Spectral Imaging) protocol. The hybridization process was initiated by denaturing the SKY probe (75°C for 10 minutes), followed by suppression hybridization of the highly repetitive DNA sequences with Cot1 DNA (37°C for 60 minutes). During suppression hybridization of the SKY probe, target chromatin on the slides was denatured at 73°C for 2 minutes in a 70% formamide/2xSSC solution. Following denaturation, the slides were briefly rinsed in cold water and then dehydrated in an ethanol series (70%, 85%, and 100% for 2 minutes each). The denatured/suppression hybridized probe was added to slides containing denatured chromatin and allowed to hybridize for 44 hours in a humidified chamber at 37°C. Excess unbound probe was removed by washing (2 minute wash in 0.4xSSC/0.3% NP-40 at 73°C followed by a 1 minute wash in 2xSSC/0.1% NP-40 at room temperature). Probes not conjugated directly to fluorochromes, those with biotin and/or digoxigenin labels, were visualized using fluorescently labeled avidin (for biotin probes) or antibodies (digoxigenin probes [Applied Spectral Imaging]). The indirect detection was achieved through a series of incubations and wash steps. DAPI/Antifade (Applied Spectral Imaging) was applied as a nonspecific stain for chromatin to allow for

visualization of micronuclei and binucleates. Following identification of micronuclei (Zeiss Axioskop), the chromatin (DAPI filter), and probe (custom triple-band pass filter [Chroma]) images were captured using a SpectraCube system (Applied Spectral Imaging). The images were then processed and analyzed using the proprietary SKY software, which utilizes an algorithm to assign a spectra-specific classification to all pixels in the image (Applied Spectral Imaging [Figure 15]). To verify that the SKY classification met laboratory quality control standards (no errors in SKY chromosome assignments as determined using the reverse DAPI banding pattern of the chromosomes), at least one metaphase spread was analyzed per slide. Per participant, a total of 100 micronuclei, or every micronucleus on the slide if less than 100, and their respective binucleates were captured and analyzed.

### **Assessment of SKY Chromosomal Classification Using CEP FISH**

Following completion of the SKY assay, fluorescence in situ hybridization (FISH) was performed on a subset of randomly selected slides ( $n= 3$ ) to verify the classification of chromatin in the micronuclei by the SKY software. For each verification slide, a three-color FISH experiment was completed using independent chromosome-specific probe combinations. The probes selected for use in these studies were based on the three most frequently observed chromosomes that were identified for each participant following the SKY classification. This approach toward probe selection was used to enhance the probability of having a sufficient number of micronuclei available for the verification analyses. Prior to these FISH verification studies, the SKY probe, antibodies



and DAPI counterstain were removed using a 2 minute wash in 70% formamide/2xSSC solution (pH 7.0). Once complete removal was confirmed by viewing the slide using a Zeiss Axioskop, FISH was performed using centromere enumeration probes (CEP [Abbott Molecular]) following standard procedures. Briefly, slides were washed in 2xSSC followed by co-denaturation of the probe and slide at 73°C for 2 minutes. The probe was allowed to hybridize overnight in a humidified chamber at 37°C. Following hybridization, the unbound probe was removed by a 2 minute wash in 0.4xSSC/0.3% NP-40 at 73°C and a 1 minute wash in 2xSSC/0.1% NP-40 at room temperature. The chromatin in the micronuclei was counterstained using DAPI II (Abbott Molecular). The same micronuclei/binucleates that were evaluated with SKY were then relocated with vernier coordinates recorded during the SKY analysis. To further confirm localization of the micronuclei/binucleate under the microscope, comparisons were made to the captured SKY images. These cells were then re-evaluated using triple-band and single-band pass filters to determine the presence or absence of probe signals. To ensure that a lack of signal was not attributable to compromised hybridization, only cells demonstrating FISH signals in the accompanying binucleates were used to evaluate the presence or absence of the FISH probes in the micronuclei.

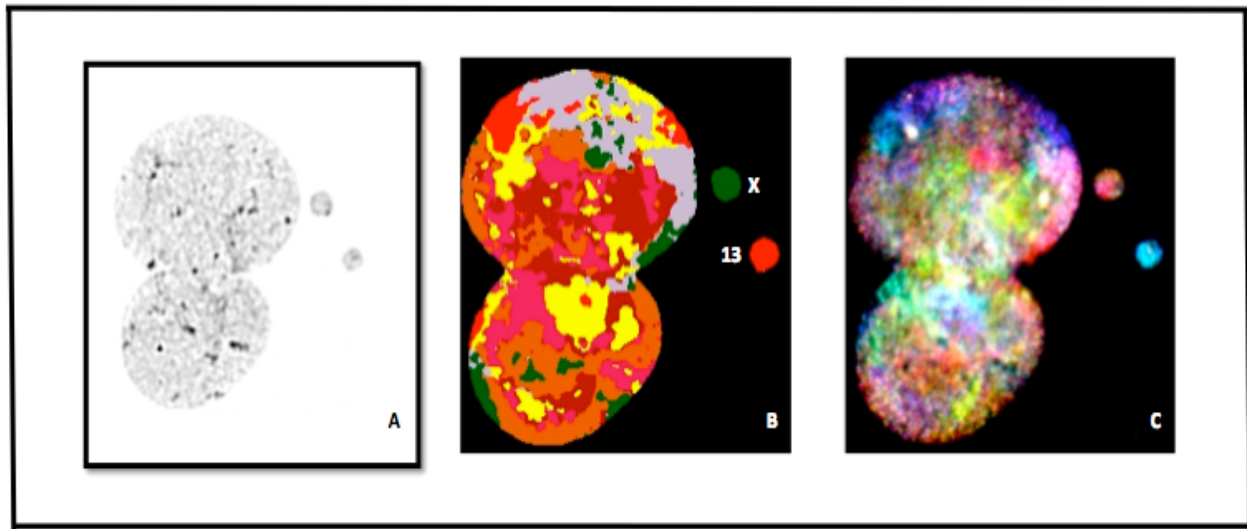
### **Chromosome-specific Telomere FISH and Image Analysis**

Because chromosomes with short telomeres have been associated with the acquisition of somatic cell chromosomal abnormalities (de Lange et al., 1990; Filatov et al., 1998; Fagagna et al., 1999; Leach and Jackson-Cook, 2004), the telomere lengths of co-twins discordant for childhood stress were also examined. Semi-quantitative FISH

with telomere-specific probes was applied to metaphase chromosomes to determine chromosome-specific telomere intensity values as described elsewhere (Chapter 3).

## **Statistical Analyses**

The statistical analysis software package Prism (GraphPad) was used for all statistical assessments unless otherwise noted. A contingency Chi-square test was used to determine if there was a non-random pattern of chromatin, as classified by SKY, present in micronuclei. Because the discordant twins evaluated in this study were genetically identical, the presence of potential within-pair differences in micronuclei contents could be calculated by subtracting the proportion of micronuclei containing a specific chromosome in the unabused twins from that of the abused co-twins. The difference in micronuclei content between co-twins discordant for exposure to childhood stress was calculated using the nonparametric Friedman test on arcsin-square root transformed data (proportions) using The Statistical Package for the Social Sciences (IBM; Version 19). Friedman tests were also used to derive ranks for telomere length (longest to shortest) and chromosomes excluded into micronuclei (most often excluded to least often excluded). These ranks were used in a Spearman correlation to determine if chromosomes with short telomeres were more frequently excluded into micronuclei. Because sentinel, short telomeres have been shown to induce senescence and are conjectured to be the primary chromosomes involved in aberrations (Zou et al., 2004), the shortest telomere from each chromosome was identified for this rank order analysis. In order to limit type I errors, an alpha level of 5% was used for all statistical analyses.



**Figure 15. Micronuclei (MN) as seen using SKY.** Reverse DAPI image (A); classified image (B); and spectral image (C) of a binucleate with 2 MN. Using SKY, the contents of the MN were determined to originate from chromosomes X and 13.

## **Results**

### **Sample Distribution and CSA Class Assignment**

The average age and range of the study participants was 50.9 and 35-70 years, respectively, and included 17 Caucasian female twin pairs who were discordant for abuse (one twin experienced abuse, the other twin did not). These 17 pairs included 3 pairs in which the abuse experience(s) involved no genital contact (sexual invitation, kissing and exposing), 8 pairs exposed to genital contact without intercourse (fondling and sexual touching), and 6 pairs in which the childhood abuse involved intercourse. Zygosity testing confirmed that all twin pairs were monozygotic and GTG-banding confirmed that all participants' chromosomal complements were within normal limits (46,XX).

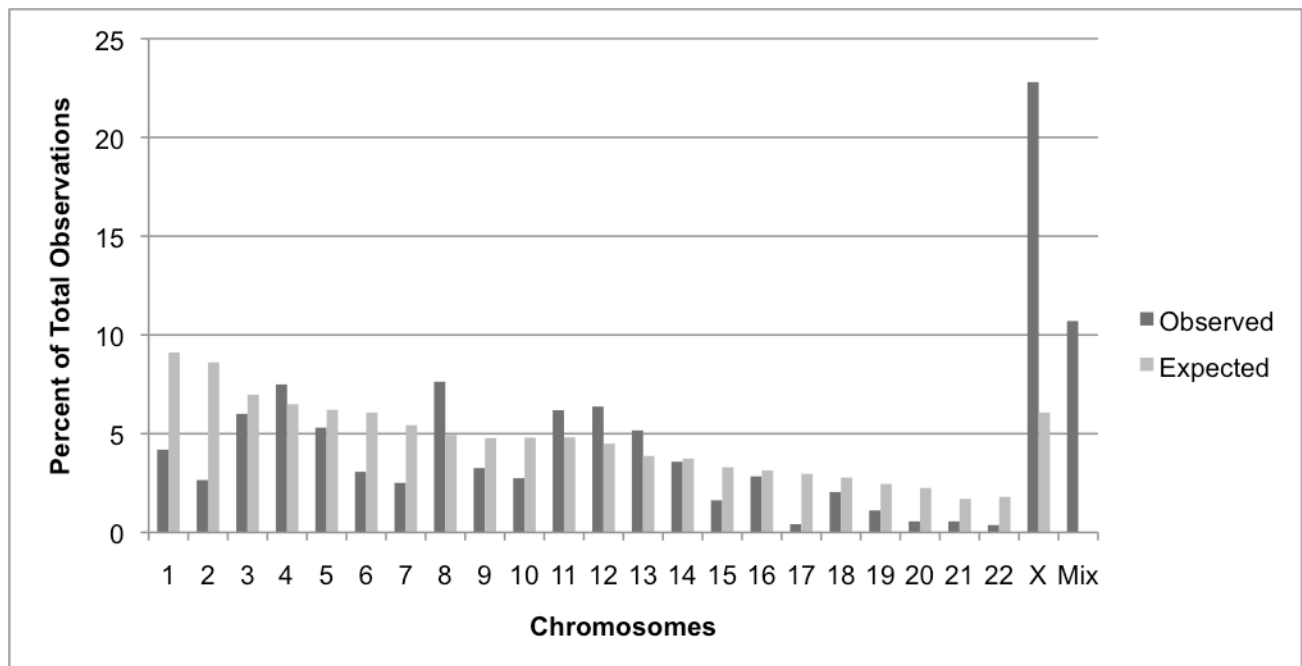
### **Chromatin Content of Micronuclei**

A total of 2,149 micronuclei were analyzed with SKY. A mix of chromatin from 2 or more chromosomes was observed in 10.7% of micronuclei. The remaining 89.3% of micronuclei contained chromatin from a single chromosome. To determine if there was a non-random pattern of chromosome exclusion into micronuclei, the observed proportions for each chromosome were compared to the expected proportions. The latter values were calculated based on the percentage of the total mitotic chromosome length contributed by each chromosome (ISCN, 1978). A non-random pattern was

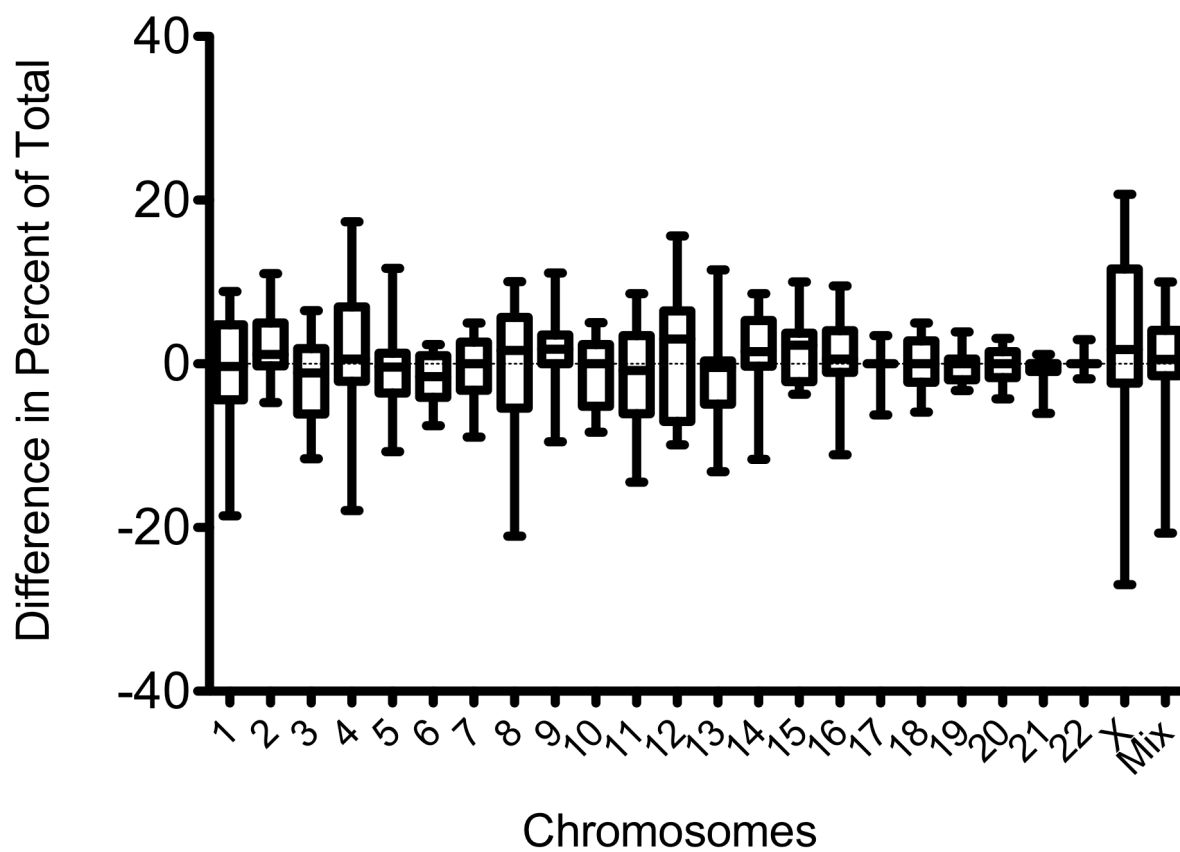
detected (Figure 16), with significant increases observed for the total proportion of micronuclei containing chromatin from chromosomes 8 ( $\chi^2(23)= 31.51$ ;  $p<0.001$ ) and X ( $\chi^2(23)= 71.22$ ;  $p<0.001$ ). Significant decreases were noted for chromosome 1 ( $\chi^2(23)= 57.15$ ;  $p<0.001$ ); chromosome 2 ( $\chi^2(23)= 88.59$ ;  $p<0.001$ ); chromosome 6 ( $\chi^2(23)= 31.84$ ;  $p<0.001$ ); chromosome 7 ( $\chi^2(23)= 33.70$ ;  $p<0.001$ ); and chromosome 17 ( $\chi^2(23)= 47.09$ ;  $p<0.001$ ).

### **Effect of CSA on Micronuclei Content**

To determine if there was a difference in the chromosome-specific patterns of micronuclei between the discordant identical co-twins, the proportion of micronuclei containing each chromosome in the non-abused twin was subtracted from the proportion observed for the abused twin. If this trait was strictly influenced by genetic or common environmental factors, one would expect to observe a value of zero between identical co-twins. The difference values that were calculated for the discordant co-twins were not significantly different from zero (autosomes only: [Friedman  $\chi^2(21)= 32.05$ ;  $p=0.058$ ]; autosomes and the X chromosome pooled: [Friedman  $\chi^2(22)= 34.06$   $p=0.064$ ]), but showed a trend toward different patterns in the abused compared to non-abused co-twins (Figure 17).



**Figure 16. SKY analysis of chromatin present in micronuclei.** The distribution of chromosomes excluded into micronuclei (dark histograms) and their expected distribution based on mitotic chromosome length (light histograms) are shown. The X chromosome had the highest rate of exclusion into micronuclei. Non-random autosomal patterns were also detected. An increased frequency, compared to random expectations, was observed for chromosomes 8 and X. Decreases were observed for chromosomes 1, 2, 6, 7 and 17. A mixture of chromatin (Mix) was observed in 10.7% of micronuclei, suggesting that these micronuclei originated from a mechanism other than exclusion of a single, intact chromosome [possibly a structural aberration(s)].



**Figure 17. The difference in chromatin content of micronuclei in co-twins discordant for exposure to childhood stress.** The difference values for chromosome-specific micronuclei frequencies between co-twins were not significantly different from zero for any of the autosomes, the X chromosome, or the mixture categories. A trend toward a difference was observed for chromosomes 8, 12, 15, and X. The whiskers of the box and whisker plot represent the minimum and maximum of observed data.

## **Confirmation of SKY Assignment**

FISH with chromosome enumeration probes (CEP-FISH) was used to determine the accuracy of the SKY assay for classifying the chromatin present in micronuclei (Table 9). Over all probes evaluated, 82.4% of the micronuclei scored contained the centromeric probe for the chromatin identified with the SKY analysis. When evaluating only the X chromosome, which accounted for 22.8% of all micronuclei evaluated, a 90.9% agreement level was observed.

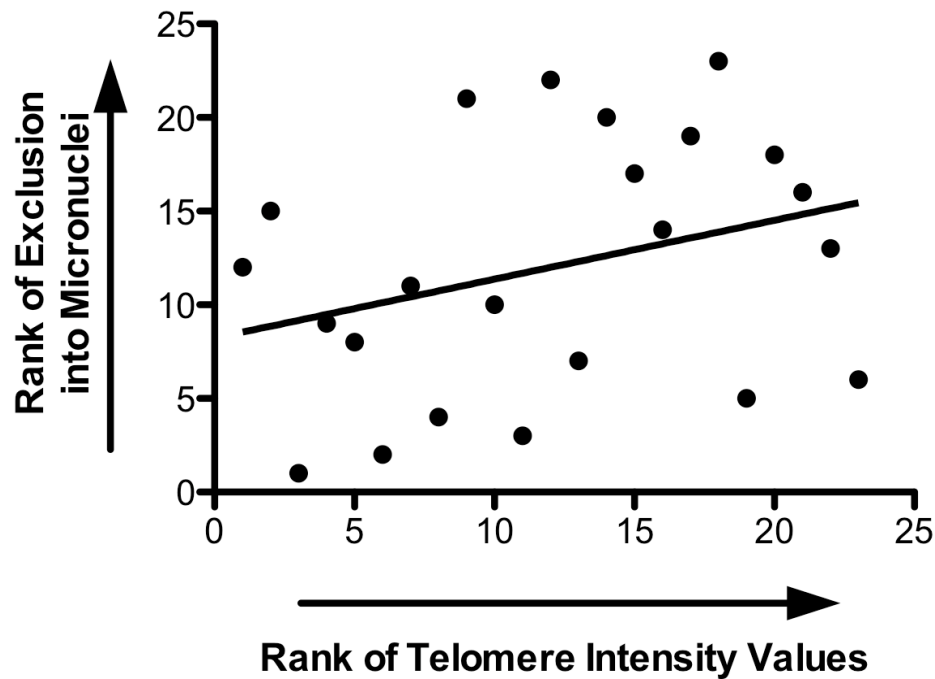
## **Chromatin Content of Micronuclei and Telomere Length**

Ranks derived from Friedman tests were used to determine if chromosomes with the shortest telomeres had a higher frequency of exclusion into micronuclei. If a shortened telomere length predisposes a chromosome to undergo malsegregation/structural alterations, then one would expect to observe an inverse correlation between these chromosomal attributes (chromosomes with shorter telomeres excluded more often in micronuclei). Because this analysis is not dependent on a discordant identical twin design, all individuals with data from both the chromosome-specific telomere length assay and SKY assay were used for this comparison (n=45). No significant correlation was detected between chromosome-specific telomere length and chromosome-specific exclusion into micronuclei ( $r=0.314$ ;  $df=21$ ;  $p=0.144$  [Figure 18]).



**Table 9. Confirmation of SKY chromosomal classification by CEP FISH.**

<b>Chromosomes (locus)</b>	<b>SKY CLASSIFICATION</b>	<b>FISH SIGNAL PRESENT</b>
<b>X</b> (DXZ1)	66	60
<b>3</b> (D3Z1)	2	1
<b>8</b> (D8Z2)	9	5
<b>9</b> (alpha satellite)	5	4
<b>12</b> (D12Z1)	6	2
<b>18</b> (D18Z1)	3	3
<b>Total</b>	<b>91</b>	<b>75</b>



**Figure 18. Correlation between chromosomes excluded into micronuclei and chromosome-specific telomere intensity score rankings.** Friedman tests were used to derive ranks for the average telomere intensity scores for the short and long arms of each of the 23 chromosomes (autosomes and X). The shortest telomere per chromosome was identified and telomeres were then ranked from 1 (“shortest”) to 23 (“longest”). Chromosome-specific exclusion into micronuclei was ranked from 1 (observed least frequently) to 23 (observed most frequently). Ranks from the telomere data and ranks from the SKY data were then compared to determine if chromosomes with smaller telomere intensity values were more often excluded into micronuclei. No significant correlation between chromosome-specific telomere length and exclusion into micronuclei was detected ( $r=0.314$ ;  $df= 21$ ;  $p=0.144$ ).

## Discussion

The use of SKY methodology provides the potential to recognize the chromatin contents of a micronucleus in a single hybridization experiment, assuming the micronucleus contains chromatin from only one chromosome. To determine if exposure to childhood sexual abuse influences the distribution of chromatin contained in micronuclei in adults, SKY analyses of identical twins discordant for exposure to CSA were completed. This study is the first to report the chromosomal contents of micronuclei in a population of adult females who have a history of childhood sexual abuse. The majority (89.3%) of all micronuclei contained chromatin from a single chromosome. The remaining 10.7% of micronuclei contained chromatin from 2 or more chromosomes, which is a value similar to that observed in healthy adults (10.4%; Jones, 2009). Micronuclei containing chromatin from 2 or more chromosomes would arise if a structural aberration involving more than one chromosome occurred, or if more than one structurally normal chromosome was excluded into a micronucleus. Given that micronuclei can contain chromatin from either the malsegregation of an intact chromosome or chromatin resulting from a structural alteration(s) (deletions, inversions, translocations, duplications, etc), one would expect the potential for a chromosome's exclusion into a micronucleus to occur at a frequency that is proportional to its length (if this reflects a random process). However, chromosomes 8 and X were found to have a higher frequency than expected by chance and chromosomes 2, 6, 7, and 17 were found less frequently than expected. With respect to autosomes, an increase in chromosome 8 and a decrease in chromosome 17 are consistent with the finding of

Jones (2009), whose studies focused on evaluations of the micronuclei content of healthy individuals. Contrary to previous reports (Sawyer et al., 1995; Stacey et al., 1995; Tucker et al., 1996; Fauth et al., 1998, 2000), in general, chromosomes with large blocks of heterochromatin were not preferentially excluded into micronuclei in this study. In our prior studies of healthy individuals, the only chromosome with a large block of heterochromatin that was observed in micronuclei at an increased frequency was chromosome 9 (Jones, 2009). This discrepancy in findings may reflect the small sample sizes evaluated in all studies (3 participants in Tucker et al. and also in Fauth et al.), as well as differences in methodology. The aforementioned studies utilized FISH methodology to assess micronucleus contents and were limited in statistical assessments. Although Fauth et al. (1998, 2000) used whole painting probes in a series of experiments to evaluate all chromosomes (3 at a time), the Sawyer et al. (1995), Stacey et al. (1995) and Tucker et al. (1996) studies were limited to dual or triple color FISH experiments and focused on evaluations and statistical comparisons between chromosomes 1, 9 and 16. In two of the above noted studies (Sawyer et al. and Stacey et al.), micronuclei were biased toward having aberrations involving chromosomes 1, 9 and 16 because the cells targeted for analyses were isolated from patients with immunodeficiency, centromeric region instability, and facial anomalies syndrome ICF, a condition caused by a mutation in a DNA methyltransferase that preferentially targets pericentromeric heterochromatin-rich chromosomes. The methylation status of the discordant co-twins in the current study was not assessed, but epigenetic regulation of heterochromatin may be an explanation for the disparate findings regarding the micronucleation of chromosome 9. Additionally, given that the non-random,

chromosome-specific patterns observed did not result in an over- or under-representation of the large chromosomes (from group A or B) or the small chromosomes (from groups F and G), chromosome size (beyond random expectations of chromatin involvement) did not seem to have a significant impact on micronucleation in this cohort.

When comparing the difference values for chromosome-specific micronuclei frequencies between identical twins discordant for exposure to CSA, no clear differences were observed. Therefore, although an overall increase in micronuclei frequencies was observed in individuals with a history of CSA compared to their unaffected co-twin (Chapter 4), this increase did not appear to be specific to one chromosome or a subset of chromosomes.

To confirm the chromosome-specific classifications of micronuclei, FISH was performed on a subset of micronuclei following SKY. An overall agreement rate of 82.5% was observed between CEP FISH and SKY. When assessing the X chromosome, the most commonly observed chromosome in micronuclei, a 91% agreement rate was observed. One possible explanation for the observed discrepancy in classification between the methodologies is that the CEP probes will only detect chromatin from the pericentromeric region. Therefore, if the micronuclei contained acentric fragments, these micronuclei would lack a signal when evaluated with the pericentromeric probe for that chromosome. To determine if this discrepancy reflects the presence of structural versus numerical aberrations, one could evaluate the

micronuclei using pan-centromeric and pan-telomeric probes. Using such methodology, Lindberg et al. (2008) reported that 86% of micronuclei in women were positive for a centromeric and telomeric signal. The majority of micronuclei that did not contain a single, intact chromatid contained acentric terminal (lack of centromere signal and presence of telomere signal) fragments. Given these results, when considering our 82.5% FISH validation rate of SKY classification, it is plausible that the 17.5% of micronuclei that failed to demonstrate a FISH validation signal were comprised of acentric fragments. This finding emphasizes the utility of the SKY assay in the detection and identification of chromosome fragments that lack a centromere and which would otherwise not be detected with CEP-FISH.

Because chromosomes with shortened telomeres have been shown to be predisposed to acquire abnormalities (Harley, 1991; Counter et al., 1992; Sandell and Zakian, 1993; Day et al., 1998; Filatov et al., 1998; Fagagna et al., 1999; Schwartz et al., 2001; Leach and Jackson-Cook, 2004), a secondary aim of this study was to determine if telomere length was correlated with chromosome-specific micronuclei frequencies. In this cohort, the chromosomes with shorter telomeres did not appear to be preferentially excluded into micronuclei. However, because micronuclei that contained chromatin from two or more different chromosomes (Mix) were excluded from this chromosome-specific analysis, it is possible that short telomeres play a role in the formation of these 'Mix' micronuclei. This effect could potentially arise through an end-to-end fusion mechanism (deLange et al., 1995), which may lead to malsegregation of a dicentric chromosome (comprised of two different chromosomes) at anaphase or to the

formation of micronuclei with more than one chromosomal fragment(s) (breakage). However, since 89.3% of the micronuclei in this study contained chromatin from a single chromosome, the contents of the majority of micronuclei did not appear to be influenced by telomere length.

In conclusion, while there was a non-random pattern of chromosomes excluded into micronuclei, this pattern did not appear to differ between co-twins discordant for a history of childhood sexual abuse. Thus, the increase in micronuclei frequencies observed in individuals exposed to childhood stress does not appear to be targeted to a specific chromosome or subset of chromosomes and does not seem to be directly influenced by telomere length.

## **Chapter 6**

### **Conclusions**

To assess the biological impact of childhood stress on adults, cortisol levels and cytogenetic factors were evaluated in a twin population discordant for a history of childhood sexual abuse (CSA). CSA was self-reported, co-twin corroborated and classified according to severity (Kendler et al., 2000). In addition to being discordant for CSA, to maximize the conclusions that could be drawn from collected data and to minimize potentially confounding genetic and common environmental effects, the twins selected for inclusion in this study were also monozygotic (identical). Salivary cortisol, chromosome-specific telomere length, overall micronuclei frequencies, and chromosome-specific micronuclei frequencies were evaluated.

**A.** To determine if there were differences in salivary cortisol levels present in adults who were exposed to a major stressor (CSA) at an early age, salivary cortisol was measured at 3 time points over 2 consecutive days and assessed via a radioimmunoassay. Comparisons were made between discordant co-twins, as well as CSA concordant twin pairs evaluated as positive controls. These studies led to the following primary conclusions:



1. There were no significant differences in cortisol levels between identical co-twin pairs discordant or concordant for CSA for the individual sample collection time points (awakening, 30-minutes after awakening, bedtime), AUC or CAR.

2. Although not statistically significant, individuals exposed to intercourse, the most severe form of CSA, showed a trend toward having a blunted CAR.

3. While measurement of cortisol in saliva is a convenient and non-invasive way to profile free, unbound cortisol levels, many factors may prevent the accurate profiling of cortisol patterns. These contingency factors compromise the use of cortisol values as a robust biomarker for evaluating the long-term biological effects of stress arising from childhood adversity.

**B.** To determine if there is a decrease in chromosome-specific telomere lengths in adults who were exposed to a major stressor (CSA) at an early age, Q-FISH with a peptide nucleic acid (PNA) probe was completed on metaphase chromosomes from identical twins discordant for a history of CSA. These studies led to the following primary conclusions:

1. For all discordant groups evaluated, which were classified according to CSA severity, genomic (average) telomere values, 'lengths', were not significantly different between abused and unabused individuals.

2. When compared to their unexposed co-twins, the twins who experienced CSA that involved intercourse demonstrated significantly smaller intensity values (attrition) for the telomeres localized to 3p, 5p, 6p, 11q and 13q.

3. The observation that telomeric attrition occurred only in the subgroup of twins exposed to the most severe form of CSA (intercourse), and not in other groups exposed to less extreme forms of CSA, suggests that there may be a dose-response relationship between CSA and the induction of latent biological changes in adults.

4. The utility of telomere length measurements as a psychobiomarker appears to have value for a subset of cases, especially when telomere lengths are evaluated on a chromosome-specific level. However, additional studies are needed to better assess the limitations of this assay for quantifying the potential cumulative and/or latent effect(s) of childhood adversity on adults.

**C.** To determine if there is an increase in genomic instability in adults who were exposed to a major stressor (CSA) at an early age, micronuclei frequencies were determined. Comparisons were made between abused and unabused groups, as well as between co-twins. CSA concordant twin pairs and age-matched controls were included as positive and negative controls, respectively. These studies led to the following primary conclusions:

1. There is a significant increase in micronuclei frequencies in adults exposed to early childhood stress (CSA), suggesting that adverse social events experienced in childhood may have a latent effect on biological processes in adulthood.

2. There appears to be a significant interaction between age and the difference in micronuclei frequencies within genetically identical discordant twin pairs. This interaction, which was primarily attributable to increases with age in the abused twins, rather than decreases in their unabused co-twins, is consistent with the hypothesis of an accelerated aging phenotype in the twins who experienced CSA.

3. The cytokinesis-block micronucleus (CBMN) assay appears to be an attractive screening tool for assessing the long-term effects of childhood adversity on adults. Based on this promising preliminary data, additional studies are warranted to determine if this assay might provide a means for recognizing individuals who are most at risk for developing stress-related morbidity and mortality.

**D.** To determine if there are differences in the chromosomal contents of micronuclei in adults who were exposed to a major stressor (CSA) at an early age, spectral karyotyping (SKY) methodology was used to characterize the contents of micronuclei in identical twins discordant for a history of CSA. The influence of telomere length on micronucleation was also evaluated. These studies led to the following primary conclusions:

1. There is a non-random pattern of chromosomes excluded into micronuclei.
2. The increase in micronuclei frequencies observed in individuals who experienced CSA, compared to their non-abused co-twins, does not appear to be targeted to a specific chromosome or subset of chromosomes.
3. Chromosomal inclusion into micronuclei does not appear to be directly influenced by chromosome-specific telomere lengths.

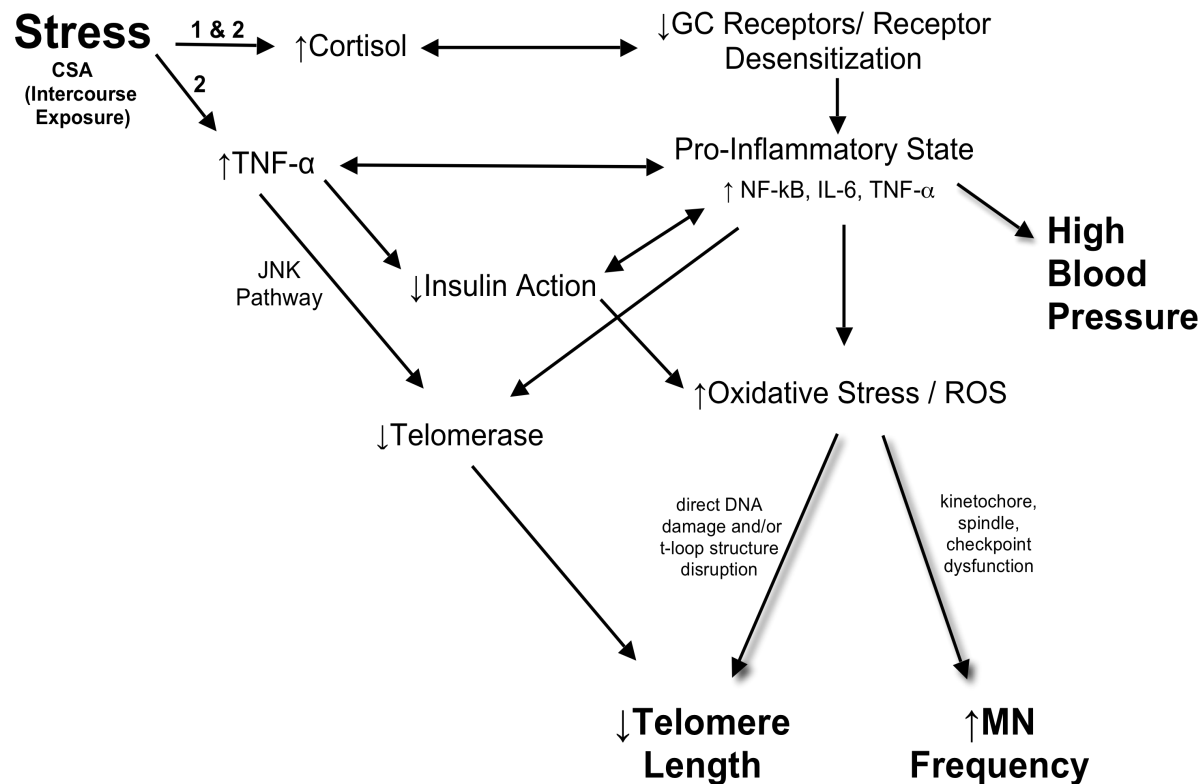
In summary, we observed a decrease in telomere 'lengths' in a subset of twins who experienced severe CSA (intercourse), and an increase in micronuclei frequency over all abused twins when compared to their unabused co-twins. Furthermore, the difference in micronuclei frequencies between identical co-twins increased with time, suggesting that the effects of an adverse event experienced in childhood (CSA), can exert a strong influence on acquired chromosomal instability frequencies in adulthood. This influence may override those attributable to heritable genetic or common environmental influences increasing the risk of morbidity and mortality. Individuals who experience CSA have a 20% increased risk for developing hypertension (Riley et al., 2010), a risk factor for cardiovascular disease. A diagnosis of hypertension was disclosed by 14 participants. Among these, 71.4% (10/14) were abused and 40% (4/10) were exposed to intercourse. CSA also strongly predicts the development of adult psychiatric and health disorders including major depression, generalized anxiety disorder, panic disorder, bulimia nervosa, alcohol and drug dependence, chronic fatigue

syndrome, irritable bowel syndrome, asthma, and fibromyalgia (Kendler et al., 2000, 2004; Arnow, 2004; Riley et al., 2010; Wilson, 2010; Cong et al., 2011; Young et al., 2011). With respect to psychiatric and substance abuse disorders, the association between the prevalence and risk factors for disease increases with CSA severity, with the highest risk noted by some investigators among those individuals with intercourse exposure (Kendler et al., 2002, 2004; Cong et al., 2011).

As mentioned above, we observed increased micronuclei frequencies in abused individuals, as well as decreased telomere lengths in intercourse-exposed individuals and a trend toward a blunted cortisol CAR in intercourse-exposed individuals. The observation that the greatest level of biological changes was observed in the adults who experienced the most severe form of CSA suggests a possible threshold and/or dose-response relationship between childhood stress and biological changes in adults that is strongly supported by a dose-response relationship observed between CSA severity and psychiatric disorders in prior studies in this cohort (Kendler et al., 2000, 2004). Because this study was not longitudinal in scope, the time point at which these biological changes occurred is unknown. The over-arching question is; How do psychological constructs, which may not be immediate, observable or readily measurable (Miller et al., 2009) impact pathological processes? Based on a thorough review of the extant literature, one can speculate that severe stress, initiated by intercourse CSA, results in a 2-pronged cascade at different time intervals (childhood and adulthood) leading to the observed biological endpoints (increased micronuclei frequency, decreased telomere length) that persist across the life span and increase the

risk for diseases of aging (high blood pressure). We propose that the CSA experience(s) initiates an acute/traumatic stress, which develops into a chronic stress throughout adolescence, perhaps through repeat victimization, social stigma, inadequate psychological coping or social support, and/or memories of the event(s); and that the burden of this experience(s) induces an emotional toll that persists as chronic psychosocial stress into and across adulthood (Figure 19). One can further speculate that both the acute and chronic stress phases produce lasting biological changes, which were observed in the current study in the most severely abused (stressed) cohort.

Firstly, serious acute stress from CSA results in elevations of cortisol and a state of hypercortisolism, which is observed in children exposed to CSA and which typically resolves by adolescence (reviewed in Miller et al., 2009) or results in a switch to lower levels of cortisol in adolescence (Susman, 2006; reviewed in Tarullo and Gunnar, 2006). This state may persist in some individuals, consistent with our observation of trend toward a blunted CAR in the adults exposed to intercourse in this study. The high levels of cortisol initially induced following abuse in childhood trigger counter-regulatory mechanisms, which lead to a resistance to glucocorticoid receptor (GR)-mediated signaling by downregulation and desensitization of GR to cortisol (reviewed in Bauer et al., 2009 and Hansel et al., 2010). Consequently, cortisol may not be able to initiate and/or deliver anti-inflammatory signals at the cellular level. At the molecular level, this leads to a decrease in the activity of genes in the biological cascade that are influenced by GR, and an increase in response elements to nuclear factor



**Figure 20. A 2-pronged mechanism to explain the biological endpoints observed in identical twins discordant for CSA.** 1. CSA induces a serious acute stress that persists across childhood and adolescence and which is mediated by cortisol and its receptors to result in telomere attrition. 2. CSA induces a moderate chronic psychosocial stress “burden” mediated by TNF- $\alpha$ , which ultimately results in increased micronuclei frequencies. Both processes increase oxidative stress and may play a role in the development of high blood pressure (see text for detailed description).

kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B [Miller et al., 2008]). NF- $\kappa$ B, interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF- $\alpha$ ) are pro-inflammatory mediators leading to systemic inflammation and oxidative stress (Yan et al., 2009). TNF- $\alpha$  also reduces the anti-inflammatory effect of insulin (Houben et al., 2007), thereby leading to increases in oxidative stress and signaling from inflammatory cytokines, which have been shown to cause direct DNA damage to telomeres as well indirect damage to the protective t-loop structure (Jennings et al., 2000; reviewed in Houben et al., 2007). Additionally, direct DNA damage could also lead to increased micronuclei frequencies, which were observed in our adult CSA cohort.

Alternatively, it is possible that the burden of the CSA experience induces an emotional toll that persists as a chronic psychosocial stress into and across adulthood. This chronic stress state is mediated by TNF- $\alpha$  (Damjanovic et al., 2007). As mentioned above, TNF- $\alpha$  acts indirectly and directly to set up an inflammatory state (reviewed in Innes et al., 2007). An increase in downstream effectors, including transcription factors NF- $\kappa$ B, and the cytokine IL-6 play a role in a positive feedback loop to further increase TNF- $\alpha$  and inflammation (reviewed in Zhang et al., 2009). The commensurate increase in oxidative stress and reactive oxygen species in the vasculature are associated with high blood pressure (Zhang et al., 2009; DeLoach et al., 2011), cause direct DNA damage (Yan et al., 2006, 2009) and increase the frequency of micronuclei (Yan et al., 2006). In addition, TNF- $\alpha$  also acts to decrease telomerase (Beyne-Rauzy et al., 2004),



which plays an integral role in maintaining telomere integrity. A decrease in telomere length following childhood adversity has been measured in children as early as middle childhood (Drury et al., 2011). The proposed telomeric damage that is initiated in childhood may be resolved by adulthood for individuals with less severe forms of CSA, but may persist, especially if telomerase activity is inhibited, in adults who were exposed to more severe forms of CSA as children.

Another biological change that might arise in response to stress, either as part of the above noted biological cascade or through an independent mechanism, is an alteration to heterochromatin or other epigenetic changes including (but not limited to) those that might induce a functional decline in damage sensors and repair pathways (reviewed in Chen et al., 2007). Damage to this repair system may lead to an increase in chromosomal damage. Furthermore, because normal aging alters the expression of several genes involved in centromere function, spindle assembly and spindle checkpoints leading to age-related chromosomal instability (Geigl et al., 2004), atypical perturbations in the activity of genes controlling these functions would be expected to result in an increased level of micronuclei (Fenech et al., 2011). These types of changes may underlie our observation of accelerated aging in those individuals who experienced abuse.

Finally, although the psychosocial impact of stress is difficult to quantify, the effect of stress on human health is well documented as a contributory factor to a large number of age-related conditions. This is the first report on cortisol profiles, micronuclei content

and frequency, and chromosome-specific telomere values (lengths) in individuals with a history of CSA. Furthermore, we chose to utilize a powerful discordant identical twin design to minimize confounding genetic influences. The data reported herein has potential clinical application in personalized medicine and also offers practical comparisons of screening tools to assess psychosocial stress-induced biological changes, stress-associated disease states and/or disease susceptibility.

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### **Vita**

Jenni Rebecca Brumelle was born on November 25, 1977, in Beaumont, Texas. She graduated from Odessa High School, Odessa, Texas in 1996. In 2000, she graduated with a Bachelor of Arts in Psychology from Texas Tech University, Lubbock Texas. She obtained a Bachelor of Science in Cell and Molecular Biology from Texas Tech University in 2006 and then began graduate school in Pharmacology and Neuroscience at Texas Tech University Health Sciences Center before transferring to Virginia Commonwealth University in 2007.