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Telomere length variation in preschool children: Associations with chronic family stress, maternal depression, and cortisol reactivity

Aditi Thakur
The University of Western Ontario

Supervisor
Dr. Shiva M. Singh
The University of Western Ontario

Graduate Program in Biology

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Abstract

Telomeres are repetitive nucleotide repeats of TTAGGG sequences located at the two ends of each chromosome. Telomeres protect DNA from degradation and maintain genomic stability. Shorter telomeres are implicated in different diseases and disorders including psychiatric disorders. I measured buccal cell telomere length (TL) in 384 pre-school children (3-4 years), from Southwestern Ontario using monochrome multiplex qPCR method. I assessed the associations between TL and family, child-specific variables. I did not find a significant correlation between TL and maternal depression, chronic family stress, or age and sex of the children. However, I found a significant positive correlation between TL and cortisol levels (AUC_I (increase) r=0.186, p=0.000 and AUC_G (ground) r=.159, p=0.002). This is a novel study assessing gene-environment interactions between TL and stress related variables in a large sample of very young children. However, further studies will be necessary to establish this relationship.

Keywords: telomere length, psychiatric disorders, monochrome multiplex qPCR, maternal depression, chronic family stress, cortisol levels
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Finally, I would like dedicate this work to my parents and my lovely sister. Without their endless love and constant support, this would not have been possible.
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Abbreviations

aTL          Absolute telomere length
AUC_B        Area under the curve with respect to baseline cortisol levels
AUC_I        Area under the curve with respect to increase in levels of cortisol from baseline
AUC_G        Area under the curve with respect to ground (baseline and increase in) levels of cortisol
DNA          Deoxyribonucleic acid
Q-FISH       Quantitative fluorescence in-situ hybridization
qPCR         Quantitative (Real-time) polymerase chain reaction
MDD          Major depressive disorder
MMqPCR       Monochrome multiplex quantitative polymerase chain reaction
RNA          Ribonucleic acid
S or scg     Single copy gene
STELA        Single telomere length analysis
T            Telomere
TERT         Telomere reverse transcriptase
TERC         Telomere RNA component
TRF          Telomere restriction fragment
Chapter 1: Introduction

A telomere is a region of nucleotide repeats present at each end of the eukaryotic chromosome (Blackburn, 2001). Telomere structure has evolved to accommodate replication problems expected at the end of a double helix. It serves as a cap at the end of the chromosome, protecting its end from deterioration or fusion with neighboring chromosomes. In addition, telomeres preserve the ends of chromosomes during semi-conservative DNA replication. In doing so, telomeres play a vital role in maintaining chromosomal accuracy, integrity and stability (Blackburn, 2001). Any deterioration of telomeres may lead to cellular apoptosis, aging, and cancer, as well as other disorders.

1.1 Telomere structure, function and evolution

Telomeres were first discovered by Elizabeth Blackburn (Blackburn, 2001). Structurally, telomeres consist of double-stranded repetitive TTAGGG DNA repeats, conserved among most eukaryotes, including humans (Blackburn, 2001). Telomerase, a ribonucleoprotein enzyme that includes a reverse transcriptase and RNA template, synthesizes the G-rich strand of the telomeres. The RNA sequence of telomerase (AAUCCC) provides the template for telomere replication.

Telomerase is expressed in embryonic stem cells and in adult germ line cells (Blackburn, 2001). It is undetectable in almost all normal somatic cells, with the exception for proliferative cells of tissues like hematopoietic stem cells, basal cells of the epidermis, activated lymphocytes, proliferative endometrium and intestinal crypt cells. During DNA replication, telomeres are shortened in successive cell divisions (Blackburn, 2001). The daughter cells inherit the shortened telomeres and the process repeats itself. Consequently, normal human cells are left with a limited capacity to proliferate (Hayflick & Moorhead, 1961). When the telomeres shorten to a critical
length, the ends of telomeres are exposed and in turn initiate DNA damage, which leads to genomic instability or apoptosis (Blackburn, 2001).

At birth, human telomeres are approximately 10,000 nucleotides long and lose approximately 50-100 nucleotides per cycle of DNA replication (Okuda et al., 2002). This loss of nucleotides occurs due to the “end replication problem” which arises because the DNA polymerase can synthesize DNA only in one direction (5’-3’) during DNA replication. This method of replication is continuous on the leading strand (5’-3’), but on the lagging strand (3’-5’), it is discontinuous. The DNA synthesized in fragments (Okazaki fragments) require an RNA primer molecule to provide a 5’ initiation point. With the completion of each fragment on the lagging strand, the RNA primer moves further to initiate the synthesis of the next fragment and the chain grows. Since the RNA primer always has to attach itself prior to the synthesis of the DNA fragments on the lagging strand and base pair to the complementary nucleotides on the leading strand, the 5’ end of the lagging strand is always shorter as compared to the 3’ end of the leading strand. The enzyme telomerase replenishes shortened telomeres. Telomerase is comprised of the enzyme, telomerase reverse transcriptase (TERT) and a telomerase RNA component (TERC) that functions as a template for the synthesis of a new complementary telomeric DNA along the leading strand (Figure 1.1). As the telomerase grows forward, new nucleotides are added along the leading strand of the telomeric DNA, helping in further extension of the lagging strand (Chakhparonian & Wellinger, 2003). Telomere maintenance is a highly regulated process and is extremely important for maintaining the length of telomeres.
Figure 1.1: Telomere repeat synthesis by the enzyme telomerase at chromosomal ends (Lindqvist et al., 2015).
The special feature of the replication of chromosomal ends using telomerase is shared by all eukaryotes (Greider & Blackburn, 1985; Yu et al., 1990). Telomerase-based maintenance of chromosomal ends is likely very old, since it is found in almost all major lineages including animals, plants, fungi and ciliates. Interestingly, every telomerase contains GT-rich nucleotide repeat sequence consisting of mostly 6-8 nucleotides (Greider & Blackburn, 1985; Yu et al., 1990). However, the sequence of this repeat may vary across different groups of organisms (Telomerase database. (n.d.). Retrieved from telomerase.asu.edu), with vertebrates containing the sequence 5’CCCUAA3’ to complement 5’TTCAGGG3’. Approximately 300-8000 repeats (2-50 kb) of these sequences are present at the ends of each human chromosome. A loss of telomerase is catastrophic, causing shortening of the chromosomal ends and allowing double-stranded ends to join, leading to an unstable aberrant karyotype.

1.2 Techniques used for measuring telomere length

Terminal restriction fragment (TRF) analysis was the first technique to measure telomere length (Moyzis et al., 1988; Southern, 1979). Multiple restriction enzymes that did not recognize telomeric and sub-telomeric regions digested genomic DNA and therefore kept the telomeres intact. These telomeres were then resolved (based on size) using agarose gel electrophoresis (where DNA fragments can be visualized by either Southern blotting or in-gel hybridization). The major strengths of this method were that the results acquired from one study were comparable to the results from another study, the protocol was convenient and cost effective, and the size of telomeres were measured in kilobases. However, the major limitations of this method included requirement of large quantities of DNA (in micrograms) to estimate telomere length and the ability of restriction enzymes used that lead to the inclusion of sub-telomeric DNA, attached to the telomeres. Hence, this method overestimated the telomere length in an individual and was unable
to measure very short telomeres. The data generated from this method could not be compared across different studies as the technique was not standardized with respect to the restriction enzyme collection (Aubert et al., 2012).

Another technique used for assessing telomere length was the Single Telomere Length Analysis (STELA) performed for a subset of chromosomes using polymerase chain reaction (PCR) method (Baird et al., 2003). This method involved the amplification of telomeres from a single chromosomal end by using primers that were specific to the sub-telomeric sequences on a single chromosome. The biggest advantage of this technique was that it required limited starting material to generate telomere length measurements. This method used the fact that the ends of all telomeres have a single stranded 3’ overhang (G-rich) and involved annealing and ligating an oligonucleotide linker to the 5’ end (of the telomere) using the overhang as a specific template (Aubert et al., 2012). STELA was ideal for measuring short telomeres but was not appropriate for measuring very long telomeres. Another limitation to this technique was that it was restricted to a small subset of chromosomes (like XpYp, 2p, 11q, 12q and 17p) that met the criteria for designing the primers (for yielding chromosome-specific telomere amplification). This occurred due to the lack of specificity and complexity of individual chromosomal sub-telomeric regions (Britt-Compton et al., 2006).

Recent techniques used to assess telomere length overcome the limitations of the previous two methods listed above. These techniques include quantitative (or real-time) PCR (qPCR) (Cawthon, 2002), monochrome multiplex qPCR (MMqPCR) (Cawthon, 2009), and absolute telomere length (aTL) quantitation (Callaghan & Fenech, 2011). Amplification of the DNA sequence of interest occurs using specifically designed primers during 20-40 cycles in a PCR. The quantity of the PCR product (amplicon) keeps increasing 2-fold with each cycle. There are two
ways to amplify the sequence of interest in a qPCR. The first one involves using a fluorophore (emits a fluorescent signal) that intercalates with the DNA (double-stranded) like SYBR green; and the second is to use a probe with an attached fluorophore that releases only when the sequence of interest amplifies like TaqMan probes. Small quantities of DNA (nanograms) are required to initiate the reaction. As telomeres are a repeat quantity of the sequence “TTAGGG” on the ends of all chromosomes, specific primers are required to amplify these regions accurately without forming a primer-dimer (gives a false fluorescent signal for the telomere amplicon). Cawthon (2002) designed a qPCR method that required a set of telomere primers: telg (forward primer) and telc (reverse primer) that could efficiently amplify the telomeres without the formation of a primer-dimer (Cawthon, 2002). This technique compared the amount of telomere amplification product (T) to that of a single-copy gene (S), amplified in separate tubes. The ratio of T/S was an estimate of the average telomere length in an individual. However, the concentration of DNA varied in separate tubes that could lead to inconsistent results. To overcome this limitation, Cawthon revised this protocol and called it monochrome multiplex quantitative PCR. In this method, the amplification of both the telomere and the single copy gene (albumin) products was generated in the same well (tube) to avoid inconsistencies (Cawthon, 2009). Another adaptation of the basic quantitative PCR method is known as the absolute telomere length (aTL) method (Callaghan & Fenech, 2011). It follows the initial (Singleplex) qPCR method with a modification of using a standard curve of known telomere lengths. A synthesized oligo primer standard made of 14 copies of the TTAGGG telomeric sequence (a total of 84 base pairs in length) is serially diluted to construct the standard curve that provides a base-pair length estimation for telomere length in an individual rather than a relative T/S ratio (Callaghan & Fenech, 2011).
There are many advantages of using PCR-based techniques. These techniques require small amounts of DNA (nanograms, instead of micrograms) and are useful in high-throughput testing but the DNA used should be of high quality and not compromised by any sort of degradation. PCR based methods however, also have limitations. There can be some variation observed in the telomere lengths among replicate estimations of the same sample. There can be differences in the DNA quality depending on the method used for genomic DNA extraction, as well as the differences in sample fixation methods in the case of fixed and paraffin-embedded tissue samples which can lead to the inability of comparing the results obtained in different studies (Cunningham et al., 2013; Koppelstaetter et al., 2005).

Some other methods for estimating telomere length include Quantitative fluorescence in situ hybridization (Q-FISH) (Lansdorp et al., 1996) with different adaptations including Metaphase chromosome Q-FISH, Interphase Q-FISH, Flow-FISH; Primed in situ approach, hybridization protection assay and, single-strand 3’ overhang measurement (Montpetit et al., 2015). Quantitative fluorescence in situ hybridization method was developed by Pete Lansdorp and his associates (Lansdorp et al., 1996). In this method, metaphase chromosomes or interphase nuclei were assessed followed by hybridization or labeling with a fluorescent (CCCTAA)$_3$ probe. The substrate for Q-FISH was cells, instead of DNA (used in TRF and PCR-based assays). The cells could be fresh, frozen, formalin-fixed, or paraffin-embedded (Montpetit, 2015). The biggest strength of the metaphase Q-FISH method was that it could recognize individual, chromosome-specific and cell-specific telomere alterations (Montpetit et al., 2015). However, the major drawbacks of using this technique included the inability to measure telomere length in cells that were not mitotically active (like the terminally senescent cells). This method was labor intensive,
costly and technically challenging. Consequently, it was not suited for large epidemiological studies on most human cells available for molecular analysis (Montpetit et al., 2015).

I assessed the feasibility of these methods for this research and came to the conclusion that the monochrome multiplex quantitative polymerase chain reaction (MMqPCR) (Cawthon, 2009) method is most suitable for this research. This conclusion is backed by a number of reports, all relatively recent, that have also used the MMqPCR in their studies on a variety of biological problems in human populations (Cawthon, 2009; Drury et al., 2014).

1.3 Telomere length variations in humans

The length of telomeres is individual specific. Telomere length appears to be genetically determined as monozygotic twins show close concordance (Hjelmborg et al., 2015; Hjelmborg et al., 2015; Strohmaier et al., 2015). There are at least two components to telomere length. The first refers to the length at birth and the second involves attrition over time. Both these components have their own determinants and heritability. It means that a given measurement of telomere length will depend on the telomere length at birth as well as any attrition that may have taken place after birth. The determinants of this attrition are unknown but must depend on variety of environmental and living conditions. These include reactive oxygen species (like peroxides, superoxide) that lead to oxidative stress (Aviv, 2002; Coluzzi et al., 2014; Maeda et al., 2013). Others include smoking (Babizhayev & Yegorov, 2011; Verde et al., 2015), diet (Martínez-gonz et al., 2016; Zhou et al., 2016), socioeconomic status (Needham et al., 2013; Prescott et al., 2012), stress (Epel et al., 2004; Naess & Kirkengen, 2015; Surtees et al., 2011; Wolkowitz et al., 2011), and paternal age (De Meyer et al., 2007; Njajou et al., 2007; Nordfjäll et al., 2010; Prescott et al., 2012; Unryn et al., 2005). Although many telomere length variations may be non-pathogenic and non-symptomatic, any extreme reduction in telomere length may be associated with a variety of ailments.
1.4 Spectrum of telomere length disorders

There are different genetic and environmental factors that can lead to the shortening of telomeres. Apart from genetic factors that may include mutations, potential environmental factors include psychological stress (Kiecolt-Glaser et al., 2011; O’Donovan et al., 2011; Savolainen et al., 2014; Surtees et al., 2011), smoking (Babizhayev & Yegorov, 2011; Ryder et al., 2005; Verde et al., 2015), obesity (Ryder et al., 2005) and diet (Martínez-gonz et al., 2016; Zhou et al., 2016). Reactive oxygen species (ROS) also affect telomere length. ROS are chemically active oxygen species containing oxygen that are generated in cells and result in cumulative damage of the cells (Harman, 2002) (process known as oxidative stress). There are a number of factors affecting ROS including: endogenous sources like mitochondria, peroxisomes, lipoxygenases, NADPH oxidase, and cytochrome P450; enzymatic (CAT, SOD, GPx) and non-enzymatic systems (Glutathione, vitamins A, C and E); exogenous sources like ultraviolet light, ionizing radiation, inflammatory cytokines, chemotherapeutics, and environmental toxins (Finkel & Holbrook, 2000). The effect of any of these factors on telomere length may lead to a variety of diseases or disorders.

Apart from diseases like cancer and psychiatric disorders, telomere length has been implicated in a number of other diseases including diabetes (Adaikalakoteswari et al., 2005; Fyhrquist et al., 2009), cardiovascular diseases like congenital heart disease (Vecoli et al., 2016) and atherosclerosis (Benetos et al., 2016; Samani et al., 2001). Down syndrome (Gruszecka et al., 2015), hypertension (Aviv & Aviv, 1999), and obesity (Ryder et al., 2005) are among the others that affect telomere length. However, the mechanisms that lead to telomere shortening in these diseases remain unknown.
1.5 Telomere length and cancer

Of all the disorders associated with shortening of the telomeres, the case of its involvement in cancer is the most understood. Telomeres shorten every time the cell divides and telomerase is responsible for the maintaining the length of telomeres. However, telomerase is not active in somatic cells (active only in germline cells). Thus, the length of telomeres is shortened after each cell cycle. This limits the number of DNA replications that can occur in a cell (Hayflick & Moorhead, 1961), which leads to critical shortening of the telomeres ultimately leading to DNA degradation, genomic instability and cell death (apoptosis). This phenomenon provides a barrier for tumor progression.

Normal human cells, after undergoing replication a set number of times, enter a growth arrest stage (stop dividing) triggering cellular senescence (Kraemer & Shay, 2003). Thus, telomeres are also known as markers of cellular aging (Blackburn, 2001; Olovnikov, 1996). However, some cells bypass this stage and enter into another senescence stage. In a few cells, when the telomeres critically shorten, the enzyme telomerase reactivates and starts lengthening the telomeres making the normal somatic cells (mortal) malignant/cancerous (immortal). Telomere length has been found to be associated in a number of studies conducted in patients with different types of cancer (Barczak et al., 2016; Falandry et al., 2015; Fernández-Marcelo et al., 2016; Mzahma et al., 2015; Poojary et al., 2016; Reddel, 2014; Sun et al., 2015).
1.6 Stress and telomere length

Stress affects the physiological and psychological well-being of an individual. It is defined as an individual’s response to an external stimulus (Selye, 1973). Stress is a “mind-body interaction” and different people perceive and respond to it differently (McEwen, 2006). Adapting oneself to the external stressors involves the activation of “neural, neuroendocrine and neuroendocrine-immune mechanisms” or “stability through change” (McEwen, 1998). There are individual differences in the extent to which stress can affect these mechanisms. This sensitivity may be linked to many factors (Harkness et al., 2015). It may include an individual’s reaction to stress and cognitive responses, personality traits, psychophysiological reactivity and neural perspectives (Hammen, 2015). A theoretical model, known as the diathesis-stress model, explains the etiology for psychopathology (Zuckerman, 1999). It was developed to describe the risk factors in schizophrenia (Clark and Watson, 1991; Watson and Clark, 1984) that has been extended to study depression and anxiety as the vulnerability-stress model (Brown and Harris, 1978; Goldberg and Huxley, 1992). Also, the activity of the hypothalamic pituitary adrenal (HPA) axis may regulate stress and both hyper- and hypo-reactivity have been associated with mental health problems (Kudielka and Wüst, 2010).

According to the American Psychological Association (APA), there are three different types of stress, which include acute, episodic and chronic stress (www.apa.org). Briefly, the most common kind of stress is acute stress. It lasts for a short period of time and may lead to changes in behavior and emotions. Episodic stress causes hypertension, headaches and migraines and the chronic stress is hard to cope with mentally. The most severe type of stress is chronic stress and long-term chronic stress can increase the risk of depression. Physiologically, all stresses can lead
to cellular stress and cause macromolecular damages (Kultz, 2003) and an increase in stress hormones (Gu et al., 2012). A system that links psychological stress to cellular stress is the hypothalamic-pituitary-adrenal axis which can be assessed by cortisol levels (Selye, 1973), a measure used in this research.

More specifically, stressful life events precede most episodes of major depression (Hammen, 2005; Kendler et al., 1999). Understanding the biological underpinnings of the stress response may shed light on why some individuals are particularly sensitive to psychosocial stress, and hence develop depression in the context of stress. A potentially important biological correlate of stress is telomere length as suggested by emerging literature (Naess & Kirkengen, 2015; Savolainen et al., 2014; Surtees et al., 2011). In addition to age-related changes, telomere length has also been implicated in depression, perhaps by virtue of how psychosocial stress influences telomere shortening.

Continuous exposure to stress can affect an individual’s telomere length later in life. This is reflected in the outcomes of a number of studies. A study conducted in 974 people (more than 30-years-of-age), compared the telomere lengths measured in 321 subjects diagnosed with DSM-IV anxiety disorder to the telomere lengths in 653 age-matched healthy controls. Shorter telomeres were found in older individuals (48-87 years) with anxiety disorder, specifically the ones who reported adverse childhood life events (Kananen et al., 2010). Another study, comparing telomere lengths was conducted in 1486 participants, including 215 individuals who were exposed to early life stressors like separation from both parents during childhood or experienced emotional and physical trauma (Savolainen et al., 2014). Individuals who experienced either one of these stressors did not have any associations with their leukocyte telomere length. However, the participants who experienced both these life stressors had shorter telomeres. Posttraumatic stress disorder (PTSD)
can lead to telomere shortening; this was confirmed by a study in 43 participants with chronic PTSD and 47 control subjects (O’Donovan et al., 2011). They found shorter leukocyte telomere length in participants with PTSD (exposed to childhood trauma) as compared to control subjects (O’Donovan et al., 2011). A number of studies relate childhood stress and/or traumatic experiences (in adults) with shorter telomeres. However, a handful of studies have studied this relationship in young children. Gotlib found shorter telomeres in healthy daughters of women with a history of depression as compared to daughters of mothers without a history of depression. This study suggested that telomeres may be a biomarker for risk for developing depression later in life rather than a consequence of experiencing depression (Gotlib et al., 2014). Another study (Wojcicki et al., 2015), assessed early childhood exposure to maternal depression by measuring telomere length in 203, 3-5-year-old preschool Latino children and found shorter telomeres in 3-year-olds having mothers diagnosed with clinical depression. However, further research is required to confirm such associations in larger cohorts.

Additionally, maternal depression may influence children’s telomere length through a number of mechanisms, including the stress associated with care provided by a mother having depression, as outlined in the model to be tested. Thus, understanding whether maternal depression affects children’s telomere length after addressing associations between telomere length and chronic stress would have implications for understanding the mechanisms that lead to telomere shortening.

The key physiological regulator of stress (environmental) in humans is the hypothalamic-pituitary-adrenal (HPA) axis (Selye, 1973). The HPA axis helps in adapting to a stressful environment and contributes to the pathophysiology when it is dysregulated or overused. Recent research is showing dysregulated HPA axis responses to stress in individuals having major
depressive disorder (Chopra et al., 2009; Weinstein et al., 2010). Cortisol levels, that are a measure of the activity of HPA axis in an individual, are reflecting the dysregulated HPA axis responses. Cortisol is a glucocorticoid steroid hormone synthesized and secreted from the adrenal cortex (Hadley, 1984). Adrenocorticotropic hormone (ACTH) stimulates the production of cortisol. However, the directionality of the effect of HPA axis on depression or vice-versa still remain a topic of debate and require further research to establish a relationship. Researchers believe that the dysregulation of HPA axis plays a role in putting the children of depressed parents at risk of developing MDD later in life (Goodman & Gotlib, 1999; Holsboer, 2000). In fact, according to a study, infants of depressed mothers exhibit higher levels of cortisol in response to a stressor as compared to infants of mothers without a history of depression (Azar et al., 2007). Another study in 9-year-old children found maternal and paternal depression were predictors of greater cortisol reactivity to stress in a 2-year follow-up assessment (Mackrell et al., 2014). In addition to these reports, in a recent study, 10-year-old children, exposed to maternal depression since birth, exhibited higher levels of glucocorticoids as compared to their never-exposed friends (Lupien et al., 2011). All these studies indicate that children of depressed parents exhibit higher levels of cortisol in response to stress and the dysregulation of HPA axis in these children may incline them towards developing depression later in life (Gotlib et al., 2014). Researchers have started studying the associations between telomere length and HPA axis activity (Epel et al., 2006; Kroenke et al., 2011). A recent study (Gotlib et al., 2014), was the first to my knowledge, that studied the associations between telomere length in 10- to 14-year-old daughters of depressed mothers and HPA axis activity, in terms of cortisol response levels to stress. Shorter telomeres were found in the daughters of depressed mothers and they also showed greater cortisol reactivity to stress when compared to daughters of never depressed mothers (Gotlib et al., 2014).
These speculations have led to the formulation of my hypothesis and objectives for the current study in young children from southwestern Ontario.

1.7 Hypothesis

Telomere length varies in the general population of preschool (3- to 4-year-old) children. Further, this variation, if present, may be associated with stress or stress related variables in the early environment.

Objective 1: To assess telomere length in 3- to 4-year-old children (N=409) from Southwestern Ontario using monochrome multiplex quantitative polymerase chain reaction (MMqPCR) (Cawthon, 2009) method.

Objective 2: To assess any associations between telomere length and family and child specific variables including a history of maternal depression, chronic family stress and cortisol levels in response to stress.

This research aims at assessing the variation in telomere length of preschool (3- to 4-year-old) children from Ontario, as a component of an ongoing study on gene-environment interactions in stress response at the University of Western Ontario. The children and families represent the general population from Southwestern Ontario (selection not based on any abnormality). In the original study, a large body of individual and family specific data was collected on these children and their families (Kryski et al., 2011, 2013; Sheikh et al., 2014). The novelty of this research is that it will assess for the first time, the distribution of telomere length at this young age in the general population of Southwestern Ontario, and assess any effect of telomere length on family specific variables including stress responses.
The University of Western Ontario Human Research Ethics Review Panel has approved the protocol for this ongoing study. This study has included 409, 3- to 4-year-old children, representing 201 boys and 208 girls from mostly Caucasian (90.7%) families. The remainder were Asian-American (2.3%), Hispanic (1.7%), African-Canadian (0.05%), or other/mixed race (5.25%) (Kryski et al., 2011). The parents of these children gave their consent for the stress task and the collection of saliva samples from the participating children and provided the family data (Kryski et al., 2011, 2013; Sheikh et al., 2014).

1.8 Nature of Family data

The family data available included life stress measures and lifetime history of depression in mothers. An interview conducted by trained PhD students in clinical psychology was used to record this data. The UCLA Life stress interview was conducted to assess chronic stress in the families. This interview covered stress in the context of intimate relationships, family relationships, close friendships, social life, childcare hassles, finances, work, personal health and health of close family members (Adrian & Hammen, 1993; Hammen, 1991). The interviewer gave a Likert-style rating for the level of stress present in each domain on a scale of low (1) to high (5). These life stress scores for each domain were standardized to produce an average life stress variable. This data has been used in earlier studies (Kryski et al., 2011, 2013; Sheikh et al., 2014).

1.9 The Stress task data

A detailed description of the stress task and saliva sampling procedure is described in previous publications (Kryski et al., 2011, 2013; Sheikh et al., 2014).

The testing took place in children’s homes by a female experimenter, whom the children were familiar with from a previous study. To minimize the effects of diurnal variation on cortisol,
the visits began at 12.00 pm until 3.30 pm in the afternoon. Children were refrained from eating or drinking anything one and a half hours prior to the visits for minimal error in cortisol assays. In the beginning, the experimenter and the child played together with toys (including blocks, books, coloring, etc.) quietly for 30 minutes to allow any increased cortisol levels (due to the arrival of the experimenter) to settle down before collection of baseline samples. The child was encouraged to stay at a place with minimal physical activity, so that it did not affect the cortisol levels (Wellhoener et al., 2004). After 30 minutes, baseline salivary cortisol sample was collected, followed by a stress task (Kryski et al., 2011).

The stress task was adapted from a previous work (Lewis & Ramsay, 2002) conducted in 4-year-old children. It involved matching colored stickers to animals on worksheet using a key. However, this task was difficult for 3-year-old children, suggested by the pilot data. Therefore, they (Kryski et al., 2011) simplified the stress task in order to make it more suitable for 3-year-olds and introduced a few modifications (Dickerson & Kemeny, 2004; Gunnar et al., 2010). For the task, the experimenter and the child were seated at a table, in front of which was a large felt board with numerous bear and frog icons stuck on it. A large toy replica of a traffic stoplight (a modified Yacker-tracker) was placed next to the board. A remote control (controlled by the experimenter) was used to manipulate the traffic light. In the beginning, the child was given a chance to select a prize (from a collection of small toys). The child was asked to get a blue ball for the bear icons and a red ball for the frog icons on the board. The child got many chances to practice matching the animals with the correct color. The traffic light was set-up and the child was told that the traffic light would show the time left for completion of the task. Green light meant plenty of time left to complete the game, yellow meant time was running out, and red meant time out. A loud buzzer sound accompanied the red light. The child had to match all the animals on the board.
with the right colored balls to get the prize. However, the experimenter turned the lights from green to red before the child completed the task. Three trials were completed following this protocol and after that, samples were collected (Kryski et al., 2011).

Cortisol samples were collected at baseline (before the task), and at 10, 20, 30, 40, and 50 minutes after performing the stress task. For collection of the saliva, the child chewed on an absorbent cotton dental roll until it was wet. Saliva expunged in a tube was stored at -20°C. A salivary cortisol enzyme immunoassay kit (Salimetrics, PA, USA) was used for cortisol assays (Kryski et al., 2011).

Specifically, the cortisol response was assessed in the form of base line (AUC\textsubscript{B}), change in levels following stress (AUC\textsubscript{I}) and total (AUC\textsubscript{G}). This data was available to me for additional analysis involving telomere length under this research.
**Figure 1.2:** Cortisol response recorded in children over 3 time-points: Area under the curve with respect to baseline cortisol response ($AUC_B$), area under the curve with respect to increase in cortisol response after performing the stress task ($AUC_I$) and area under the curve with respect to cumulative cortisol response after summing up baseline and increase in cortisol levels ($AUC_G$). Adapted from Fekedulegn et al., 2007.
Chapter 2: Materials and Methods

2.1 DNA extraction

Saliva samples collected from children were stored at -20°C, to protect them from degradation. Genomic DNA was isolated from these samples using QIAGEN kits (Qiagen Micro DNA kit). I optimized the standard protocol for extraction of the DNA from saliva samples. These included the following modifications. First, the frozen samples were thawed (two replicates of each sample mixed in a 1.5 ml tube) to room temperature. 500 µl of PBS (Phosphate-buffered saline) was added to each tube and mixed. This was followed by centrifuging the tubes at 10,000 rpm for 5 minutes. The supernatant was discarded and 500 µl of PBS was added to each tube. The contents were transferred into a new tube. The tubes were centrifuged at 8000 rpm for 5 minutes to collect the pellet. 100 µl of buffer AL (lysis buffer) and 20 µl of proteinase K were added to the pellet and mixed (pulse vortexed for 15 seconds). Then, the tubes were kept in a thermomixer at 56°C at 900 rpm for 1 hour, followed by centrifugation to collect the sample at the bottom of the tubes. The contents of the tubes were then treated with 100 µl of ethanol (96-100%) and kept on ice for 3 minutes. The supernatant from each tube was transferred to QIAamp MinElute columns (in 2 ml collection tubes) and centrifuged at 8000 rpm for 1 minute. Finally, the DNA (eluted in 30 µl of Buffer AE) was stored in the freezer at -20°C. The quantitation of individual DNA samples was assessed using Bio-Rad NanoDrop Spectrophotometer. The readings for the amount of DNA in each tube were obtained in ng/µl. The quality of DNA was tested by the 260/280 ratio. Integrity of selected samples was assessed by agarose gel electrophoresis to assure high quality of genomic DNA with little degradation.
2.2 Measurement of telomere length using real-time PCR

The extracted DNA was used for the amplification of telomeres in relation to a known single copy gene, using the monochrome multiplex qPCR method (Cawthon, 2009). This method allows the amplification of the two target sequences: telomere and albumin (single copy gene) in the same reaction. The two amplicons (telomere and albumin), in the same reaction, are separated by their differential temperature sensitivity. The two quantities were measured and the quantity of the telomere amplicon in relation to the albumin amplicon was assessed. This ratio represented an estimate of the relative telomere length in an individual (Cawthon, 2009). Bio-Rad CFX 96 Real-time PCR system was used for the amplification of telomeres for this study.

The total reaction mixture for the multiplex qPCR consisted of 17 µl of master mix and 3 µl (approximately 20 ng) of the DNA samples. Each sample was loaded in triplicate, with approximately a total of 20-22 samples on a 96-well plate. SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) was used to prepare the master mix. This supermix is a 2X concentrated, highly efficient, ready to use reaction mix, containing an antibody-mediated hot-start Sso7d-fusion polymerase, dNTPs, MgCl2, SYBR Green I dye, enhancers, stabilizers, and a blend of passive reference dyes (including ROX and fluorescein) (Bio-Rad). 17 µl of master mix (per well of a 96-well plate) contained: 10 µl of SsoAdvanced Supermix (Bio-Rad), 0.2 µl of telomere primers (forward primer, telg and reverse primer, telc each), 0.12 µl of albumin (single copy gene) primers (forward primer, albd2 and reverse primer, albu2 each), and 6.36 µl of double distilled water. 17 µl of master mix was added to individual wells and 3 µl (20 ng) of DNA samples were added to the respective wells. A reference DNA was used to generate standard curves from a set of 3-fold dilutions. The dilutions (of the reference DNA) used were as follows: 22.5 ng, 7.5 ng, 2.5 ng, 0.833 ng, 0.277 ng, and 0.093 ng per µl of distilled water (H2O). 3 µl of these dilutions were added to 17
µl of the master mix and added to the wells of the 96-well qPCR plate in triplicates. The plates were then sealed and placed inside the CFX Real-time system. The qPCR cycle was set and the reaction was completed in 2 hours. The cycling profile involved Stage 1: 15 mins at 95°C, Stage 2: 2 cycles of 15 s at 94°C, 15 s at 49°C. Followed by stage 3: 4 cycles of 20 s at 85°C, 30 s at 59°C and 33 cycles of 15s at 94°C, 10 s at 64°C, 15 s at 72°C with signal acquisition, 30 s at 80°C, 20 s at 85°C with signal acquisition.

The cycle in which fluorescence is detected is termed as the Quantification cycle (Cq) according to the MIQE guidelines. In the amplification curves, the 72°C reads provided the Cq values for the amplification of the telomere template (in early cycles, the single copy gene (scg) signal is still at baseline). The 85°C reads provided the Cq values for the amplification of the single copy gene template (at this point, all the telomere PCR product has completely melted, thus giving out no signal). I followed the cycling protocol (Cawthon, 2009) with a few modifications for optimal results. After thermal cycling and collection of raw data, the Bio-Rad CFX Manager software generated standard curves for each plate, one for telomere and one for the albumin (scg) PCR products. The ratio of starting quantities obtained in nanograms for the telomere product (T) to the albumin product (S) provided the average telomere length (in terms of T/S ratio) in each individual.

2.2.1 Telomere primer design and amplification of a fixed-length product

As telomeres are a repeat of the “TTAGGG” sequence on the ends of all human chromosomes, specific primers are required for the amplification of telomeres. The primers used in this study are telg (forward primer) and telc (reverse primer). The sequence of the telg primer is 5’ACACTAAGGTTTGGGTTTGGGTTTGGGTTAGTGT3’ and of telc is 5’TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTATTCCTAAAC3’ (Cawthon, 2009). The
forward primer amplifies the telomeres present on both the ends of all the chromosomes, and the reverse primer makes copies of the telomere PCR product (obtained after the amplification of native telomeres by the forward primer), thus generating a fixed length product. A mismatch on the 3’ end of the reverse primer disables it to prime DNA synthesis along the native DNA strand. To bind the telg primer (extension) product and telc primer, a mismatch is introduced in the telg at the third base from the 3’ end that enables only the last three bases of the telg and telc primers to overlap complementarily (Cawthon, 2009). Thus, telg and telc produce specific and fixed length products (Figure 2.1). The software (CFX Real-time Manager) was used to generate the starting quantity of telomere (T) product in nanograms that was used to calculate the telomere length in an individual (Cawthon, 2009). The telomere PCR products melt around 79°C.
Figure 2.1: Forward (telg) and reverse (telc) telomere primers and their mode of action. In cycle 1, telg binds to the ends of the native DNA strand (telomeres) and starts DNA synthesis. The telc primer attaches itself to the native telomere sequences but is unable to synthesize DNA because of the mismatch on its 3’ end. The telg and telc primers have multiple mismatches included at their 3’ ends that inhibit them to bind with each other and form a primer dimer. In cycle 2, the telc primer amplifies the telg primer extension product obtained from Cycle 1, only in the configuration shown here (all other configurations will show a mismatch at the 3’ end of telc primer). The italicized bases in Cycle 2 represent the telg extension product synthesized in the Cycle 1 while the non-templated sequences at the 5’ ends of the primers prevent the 3’ ends of the telomere product to initiate DNA synthesis in the middle of the other copies of the telomere PCR product. (Adapted from Cawthon, 2009).
2.2.2 Albumin primer design to melt at high temperatures

Only one copy of the albumin gene (used as the single copy gene in this study) is located on chromosome 4 in humans. The primers used for the amplification of the albumin gene, albd2 and albu2 are highly specific. The sequence for the albumin primer albd2 is 5’GCGGGCCCGTGCGAGCGAGCGGGCCCGGaaaagcatgcgtgcctgt3’ and albu2 5’GCCTCGCTCCGGAGCGGCGGCGGGGCCaatgtgctacagaatcttg3’ (Cawthon, 2009). The primers have albumin gene specific bases (lower case) accompanied by a long tail (capitalized bases). The CG-rich capitalized bases represent non-templated 5’ sequences that enable albumin amplicon to melt at a very high temperature as compared to the telomere amplicons (Cawthon, 2009). Therefore, the fluorescent signal from the albumin amplicon is acquired at a temperature higher than the melting temperature of the telomere amplicon (Cawthon, 2009). The software (CFX Manager) gives back the starting quantity of albumin (S) product in nanograms.

2.3 Measurement of telomere length in an individual

The ratio of the starting quantity of telomere products (T) to the starting quantity of the albumin products (S) gives the average telomere length in an individual. The “T” value is the amount of DNA (in nanograms) that matches the individual’s copy number of the telomere template. The “S” value is the amount of DNA (in nanograms) that matches the individual’s copy number of the single copy gene (scg) template (Figure 3.2). This ratio would represent the relative telomere length in an individual. Also shown in the formula:

\[
\text{Telomere length} = \frac{\text{Starting quantity of telomere product (T)}}{\text{Starting quantity of albumin product (S)}}
\]
2.4 Data Analysis

2.4.1 Telomere length estimation

The telomere length (as ratio of T/S) for the three replicates of each sample (child) was calculated. The average of these values provided a global (average) telomere length for each child further used in all analyses. Telomere length was measured for 409 samples. Out of them, 384 samples were selected based on the consistency of the T/S ratios among the triplicates for each sample.

2.4.2 Relationship of telomere length with family and child data

The relationship between average telomere length of each child (dependent variable) and family data variables (including maternal depression, chronic family stress, and child sex) was assessed through bivariate analysis. IBM SPSS Statistics version 24.0 was used to perform bivariate analyses.

2.4.3 Relationship of telomere length with children’s stress features

The stress feature of the children under stress tasks included (i) total observed cortisol levels (baseline and increase) following exposure to stress ($\text{AUC}_{\text{G}}$) and (ii) an increase in cortisol ($\text{AUC}_{\text{I}}$) above the baseline in response to stress. Bivariate analyses were used to assess associations between telomere length and cortisol levels in children.

Multivariate analysis was performed to confirm any associations between these variables (family and child specific) and telomere length. IBM SPSS Statistics version 24.0 was used for multivariate analyses.
Chapter 3: Results

3.1 DNA extraction, quality and quantity

Genomic DNA was extracted from the saliva samples of 409 children. The DNA obtained and eluted in 30 µl of AE buffer was stored at -20°C. The quantity of DNA measured by Thermo Fischer NanoDrop spectrophotometer in all the 409 saliva samples from children ranged from 2.7 to 315.2 ng/µl. The 260/280 ratios obtained from the NanoDrop spectrophotometer, ranged from 1.7 to 2.0, and were used as a measure of the quality of the DNA.

3.2 Monochrome multiplex quantitative PCR data

The monochrome multiplex real-time PCR (or quantitative PCR) method was used to amplify the telomere regions present on the ends of all the chromosomes in each individual of this study. Cawthon developed two methods for the amplification of telomeres: first was a singleplex qPCR protocol (Cawthon, 2002) and the second one was a multiplex qPCR protocol (Cawthon, 2009). Initially, the singleplex qPCR protocol was followed (Cawthon, 2002) for amplifying the telomere and albumin genes. In this method, the telomere and albumin (single copy gene) amplicons were amplified in separate wells (in triplicates) on a 96-well plate. In addition to the DNA samples from the children in this cohort, a reference DNA sample was used and telomere and albumin amplicons (in separate wells) were amplified. The reference DNA generated a standard curve for the telomere and albumin products. However, this method could amplify only 6-8 samples on one plate along with the reference DNA samples and would require large quantities of the master mix. To overcome this limitation, another method was followed that was developed by Cawthon, known as the monochrome multiplex qPCR method (Cawthon, 2009). In this method, both the telomere and albumin amplicons for each sample were amplified in the same well on a
96-well plate. This method is efficient and requires less amount of master mix (as compared to singleplex) and telomere length of around 20 to 22 samples can be determined on a single 96-well PCR plate. Finally, the MMqPCR method (Cawthon, 2009) with a few modifications, was found to be the best fit for this type of study and was adopted for the measurement of telomere length in all the samples from 409 children. The products obtained from both the singleplex and multiplex reactions were subjected to electrophoresis on a 9% Polyacrylamide Gel, in order to compare the reliability of results obtained from the two reactions (Figure 3.1). This assured that the multiplex qPCR conditions established were reliable for the amplification of multi-copy telomere and single copy albumin sequences in the human genome.
Figure 3.1: 9% PAGE results of the telomere and albumin products obtained in the singleplex and multiplex qPCR runs: The DNA ladder (1 kb) was loaded in lane 1. Lane 2 and lane 3 contain the telomere and albumin products obtained after singleplex reactions respectively. Lane 4 and lane 5 contain the telomere and albumin products obtained from the multiplex qPCR runs (shown here as two separate bands, one for the telomere product and one for the albumin product for two different samples.)
3.2.1 Standard Curve

A standard curve gives the estimate for the quantities of the experimental sample amplified in a qPCR. In this study, a reference DNA sample was used to generate standard curves for both amplicons (telomere and albumin), that were used to calculate the starting quantities (for telomere and albumin products in nanograms) of the samples. The ratio of these values (T/S) provided the average telomere length of each individual.

The reference DNA sample was taken from a healthy individual with no previous history of any psychiatric disorder or illness. There was no family history of psychiatric disorder or exposure to chronic family stress. Standard curves were generated (per plate) using the reference DNA sample- shown in Figure 3.2: (a) for telomere and (b) albumin. The samples from children (in triplicates) were plotted on the standard curve (for telomere and albumin). The standard curve provided the starting quantities of the PCR products.
Figure 3.2: Standard curve generated using a reference human DNA sample for (a) telomere and (b) albumin products. The “o” represents the six dilutions of the reference sample, where “x” represents the DNA samples from the cohort. The efficiency of the runs was between 90-100 % for all of the runs was while the $R^2$ value was approximately 0.90 for all of the standard curves.
3.2.2 Melting Curve

A melting curve analysis in a quantitative PCR shows the temperatures at which the products (DNA) start denaturing. The melting temperatures of the telomere and albumin amplicons are quite different from each other (Figure 3.3), which is a major strength of this method.

**Figure 3.3:** Melt curve obtained from the CFX manager showing different melting points of the two products- telomere (melting temp. around 79°C) and albumin (melting temp. around 89°C) for both, a reference sample in triplicates and one of the DNA samples out of the 384 samples referred as “unknown DNA sample”. Heating the product up to 89°C ensures that the telomere product has completely melted and is not interfering with the collection of the rising SYBR Green fluorescence signal for the albumin amplicon.
3.3 Estimation of telomere length

3.3.1 CFX real-time PCR and CFX manager results

CFX real-time manager software was used to measure the telomere length in the children, using the starting quantities (nanograms) for the telomere and albumin PCR products. The telomere length for all of the 409 samples (in triplicates) was calculated by taking the ratio of the starting quantity of the telomere product to the starting quantity of the albumin product (obtained from the standard curve) and the triplicates (of each sample) were compared for consistency. The inconsistent replicates were removed from further analysis. Thus, out of the 409 samples, 384 samples were selected for further analysis.

3.3.2 Telomere length distribution

The telomere length (T/S) measured for 384 children (selected from 409 samples) represents a relative ratio of telomere to albumin gene amplification. The distribution of telomere length in this population is positively skewed (Figure 3.4-b) with most individuals having a T/S ratio of four or less. It follows a long tail that covers a fraction of individuals with comparatively longer telomeres. These estimates are based on three replications of each sample (in multiplex qPCR) and are considered repeatable and reliable within the effectiveness of the most acceptable technology (Cawthon, 2009). The range of telomere length measured (T/S) in this cohort was from 0.04 to 9.89.
**Figure 3.4:** (a) Scatter plot of 384 samples and their telomere length and (b) histogram showing the population distribution of telomere length (T/S ratio) in these samples of 3-4 year-old children from Southwestern Ontario. A polynomial trend line was generated to show the trends in telomere length ratios.
One of the main objectives of this thesis was to assess if the telomere length is related to any of the features of the family and child data collected on these children. In order to accomplish this, the skewness of the distribution of telomere length estimates was tested. As the telomere length data was positively skewed, it was log transformed to near normal distribution. These transformed values of telomere length were used to assess associations with family and child-specific variables using bivariate and multivariate analyses. Our sample was almost entirely Caucasian, and the subset of participants who were not Caucasian were heterogeneous in terms of ethnicity; thus, analyses of whether ethnicity influenced the pattern or findings obtained were limited to re-running all analyses excluding non-Caucasian participants. As these results were virtually identical to those reported on the full sample, we present results based on the full sample throughout.

3.4 Associations of telomere length in children with maternal depression and exposure to chronic family stress

The telomere length in healthy children was statistically tested to assess associations with any of the two variables, lifetime history of maternal depression and chronic family stress through bivariate analyses. Correlation analysis (using Pearson correlations) was conducted to assess any associations present between chronic family stress, age and telomere length in children whereas independent sample t-tests were run for the other variables including lifetime history of maternal depression, and child sex, using IBM SPSS Statistics 24.0. A multiple regression analysis (multivariate) was conducted to observe if any of the independent variables including chronic family stress, maternal depression, child sex and age, or cortisol levels predicted telomere length of children.

3.4.1 Life time maternal depression and telomere length in children
An independent sample t-test was conducted to compare the mean telomere length in children with depressed mothers and those with non-depressed mothers. There was no significant difference found in the mean telomere length of children of depressed mothers (M=1.46, SD=0.311) and those with non-depressed mothers (M=1.39, SD=0.61); t (365) =1.015, p=0.51 (Figure 3.5). The telomere length distribution in the children with non-depressed mothers and those with depressed mothers has been shown in Figure 3.6 (a) and (b) respectively.

**Figure 3.5:** The mean telomere length in children with depressed mothers (on the left) and those with mothers who did not have a history of depression (on the right). The mean telomere lengths of the two groups of children shown are not significantly different from each other.

Note: M= Mean, SD= Standard deviation
Figure 3.6: Distribution of telomere length in children with mothers (a) not having a lifetime history of depression and (b) having a lifetime history of depression. Out of the 367 individuals (for whom the data is available regarding a history of maternal depression and telomere length), there were 270 children with mothers having a history of depression while 97 children had mothers without a history of maternal depression during their lifetime. A polynomial trend line was generated to show the trend in distribution of telomere length.
3.4.2 Chronic family stress and telomere length in children

A Pearson correlation coefficient was used to assess the relationship between chronic family stress and telomere length of children in this cohort. There was no correlation found between the two variables, $r=0.042$, $n=380$, $p=0.414$. Overall, there was no significant difference observed in the telomere length of children due to chronic family stress (Figure 3.7).

![Scatter plot of distribution of telomere length (0.01-10.0) in children and chronic family stress levels. No significant correlation was found between chronic family stress and telomere length in children.](image)

**Figure 3.7:** Scatter plot of distribution of telomere length (0.01-10.0) in children and chronic family stress levels. No significant correlation was found between chronic family stress and telomere length in children.

3.5 Telomere length in relation to child-specific features

3.5.1 Relation of telomere length to age and sex of children
A Pearson correlation coefficient was used to assess the relationship between telomere length and the age of children in this cohort. There was no correlation found between the two variables, $r=0.038$, $n=379$, $p=0.462$ (Figure 3.8). The children in this cohort were roughly aged between 3- to 4-years-of-age at the time of collection of samples.

![Figure 3.8: Scatter-plot showing distribution of telomere length among the children in this cohort.](image)

The children in this cohort belonged to a homogenous age group (aged 3-4 years). Telomere length was not significantly associated with child age, possibly because there was limited variability in age in the sample.

An independent sample t-test was conducted to compare the mean telomere length in boys (186) and girls (197). No significant difference was found in the mean telomere length of boys (M=1.40, SD=0.64) and girls (M=1.47, SD=0.57); t (381) = -1.552, p=0.121. These results suggest
that in this cohort, the telomere length in boys was not significantly different from the telomere length measured in girls (Figure 3.9).

**Figure 3.9:** Mean telomere length of boys (Mean=1.40, Std. Error=0.05) and girls (Mean=1.47, Std. Error=0.04) measured in this cohort. There was no significant difference in the telomere length between boys and girls.

Note: M= Mean, SD= Standard deviation
3.5.2 Children’s telomere length and cortisol reactivity levels to stress

Three aspects of cortisol stress reactivity were studied during the assessment of cortisol response levels (Sheikh et al., 2014). The first one was $\text{AUC}_B$ that was the area under the curve with respect to baseline cortisol levels. The second was $\text{AUC}_I$ that was the area under the curve with respect to the increase in cortisol response recorded in children after performing the stress task. Finally, the third one was $\text{AUC}_G$ that was the area under the curve with respect to the net/ground cortisol response recorded including the baseline cortisol levels ($\text{AUC}_B$) and the increase in cortisol levels in children ($\text{AUC}_I$) after exposure to stress.

A Pearson correlation coefficient was used to assess the relationship between telomere length and the increase in cortisol levels ($\text{AUC}_I$) in this cohort. A positive correlation was found between the two variables, $r = 0.186$, $n=379$, $p=0.000$. The children with lower cortisol levels had shorter telomeres as compared to children who had higher cortisol response to stress levels (Figure 3.11).

A Pearson correlation coefficient was used to assess the relationship between telomere length and the ground cortisol levels ($\text{AUC}_G$) in this cohort. There was a positive correlation found between the two variables, $r = 0.159$, $n=379$, $p=0.002$. The children with lower cortisol levels had shorter telomeres as compared to children who had higher cortisol response to stress levels (Figure 3.12).
Figure 3.10: Average change in cortisol response ($\text{AUC}_1$) in children and their telomere length ratios. The children with lower $\text{AUC}_1$ levels tend to have comparatively shorter telomeres. A polynomial trend line was generated to show the trend in telomere length of these children in relation to their cortisol levels.
Figure 3.11: Average ground cortisol response to stress task (AUC_G) in children and their telomere length. The children with lower AUC_G levels tend to have comparatively shorter telomeres. A polynomial trend line was generated to show the trend in telomere length of these children in relation to their cortisol levels.
3.6 Multiple Regression analysis

Multiple regression (multivariate) analyses was used to assess if any of the independent variables (including maternal depression, chronic family stress, cortisol levels, age, and sex of children) were associated with the telomere length in these children.

Of all the variables tested, $\text{AUC}_I$ (area under the curve with respect to increase in cortisol levels) was significantly correlated with telomere length in children of this study (Table 3.1).

Table 3.1: Increase in cortisol levels post-stress tasks in children associated with their telomere length

<table>
<thead>
<tr>
<th>Variable</th>
<th>Unstandardized coefficients (b)</th>
<th>SE</th>
<th>T</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal depression</td>
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<td>.258</td>
<td>-.052</td>
<td>.157</td>
</tr>
<tr>
<td>Chronic family stress</td>
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<td>.011</td>
<td>.446</td>
<td>.656</td>
</tr>
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<td>$\text{AUC}_I$</td>
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<td>.042</td>
</tr>
<tr>
<td>$\text{AUC}_G$</td>
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<td>.099</td>
<td>.166</td>
<td>.868</td>
</tr>
<tr>
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<td>1.277</td>
<td>.202</td>
</tr>
<tr>
<td>Age</td>
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<td>.073</td>
<td>.699</td>
<td>.485</td>
</tr>
</tbody>
</table>

Note: $\text{AUC}_I$ (Increase in cortisol levels); $\text{AUC}_G$ (Ground cortisol levels). *p<0.05
Chapter 4: Discussion

Telomeres are specialized and protective DNA-protein complexes present on the ends of all chromosomes. In humans, telomeres are a sequence of double stranded TTAGGG repeats, synthesized by a ribonucleoprotein enzyme telomerase (Blackburn, 2001). At birth, human telomeres are approximately 10,000 nucleotides long and lose approximately 50-100 nucleotides per cycle of DNA replication (Okuda et al., 2002). Critical shortening of telomeres affects the cellular lifespan, eventually leading to cell death (apoptosis). There are many genetic and environmental factors that can affect telomere length like mutations, oxidative stress (Coluzzi et al., 2014; Epel et al., 2004), obesity (Ryder et al., 2005), diet (Martínez-gonz et al., 2016; Zhou et al., 2016), smoking (Babizhayev & Yegorov, 2011; Ryder et al., 2005; Verde et al., 2015). Certain diseases affect the length of telomeres as well. Telomere length is implicated especially in different types of cancer (Barczak et al., 2016; Falandry et al., 2015; Fernández-Marcelo et al., 2016; Poojary et al., 2016; Sun et al., 2015; Walsh et al., 2015). Shorter telomere length is also implicated in patients with diabetes (Adaikalakoteswari et al., 2005; Fyhrquist et al., 2009; Liu et al., 2014).

4.1 Psychiatric disorders, stress and telomere length

In addition to diseases, telomere length is a correlate to different types of psychiatric disorders as well. Psychological stress has been associated to telomere length in a number of studies. Prolonged and chronic psychological stress can lead to different psychiatric disorders including anxiety disorder, major depressive disorder (MDD), and post-traumatic stress disorder (PTSD). Strong correlations have been confirmed between mean telomere length and early life stress (Savolainen et al., 2014), childhood abuse and adversities (Kiecolt-Glaser et al., 2011; O’Donovan et al., 2011), childhood stress (Tyrka et al., 2010), anxiety disorder (Kananen et al.,
2010), major depressive disorder (outlined in table 4.2). On the other hand, no such associations have been found in some others (Jodczyk et al., 2014; Surtees et al., 2011).

Mostly, research conducted in the past focused only on the measurement of mean telomere length in individuals with different psychiatric disorders but no study has suggested and established a possible mechanism responsible for these outcomes. However, almost all the studies are different from each other with respect to sample type or size, type of psychiatric disorder, techniques used to measure telomere length and their outcomes. Most of the research focuses on adults with psychiatric disorders and fewer studies assess telomere length in children exposed to a stressful environment. A recent study assessed telomere length in 97, 10- to 14-year-old healthy daughters of mothers either with recurrent episodes of major depression or without a history of psychiatric disorder (Gotlib et al., 2014). They found shorter telomeres in the daughters of depressed mothers when compared to the telomere length measured in daughters of mothers without a history of depression. This study was the first to suggest that children at familial risk of developing depression are characterized by shorter telomeres, a sign of accelerated biological aging and this may incline them towards developing depression later in life along with some other age-related illnesses (Gotlib et al., 2014). Another study conducted in 203, 4- to 5-year-old Latino children (belonging to low-income families) with oppositional defiant behavior and mothers diagnosed with clinical depression found shorter telomeres (by approximately 450 base pairs) as compared to the controls (Wojcicki et al., 2015). This was the first study to link maternal depression and oppositional defiant behavior in children with their telomere length. A more recent study in 70 Latino mother-children pairs, showed that most of the telomere loss occurs until 4 years of age and then it plateaus at 4 years of age in children (Wojcicki et al., 2016). Telomere length measured twice over the course of a year showed that most of the children had longer
telomeres as compared to their mothers after a year. They found telomere maintenance occurring in children from 4- to 5-years-of-age, with minimal telomere attrition (Wojcicki et al., 2016). Maternal psychosocial stress was found to affect the telomere length in their newborn babies, in a study on 27 mother-newborn pairs, with mothers undergoing pregnancy-specific stress (Entringer et al., 2013). This study provided preliminary evidence that maternal stress during pregnancy may affect the telomere biology of the baby, visible by their leukocyte telomere length at birth. Another study in 677 individuals, belonging to a 30-year birth cohort (from birth to 30-years-of-age) found no associations of telomere length with exposure to stress (Jodczyk et al., 2014). Most of the studies here confirmed an association between telomere length in children and exposure to stress and/or maternal depression, but some of them did observe negative results. However, each study is unique in terms of its sample type and outcomes and requires further confirmations. It remains a controversy if maternal stress and depression and exposure to chronic stress during early childhood, affect the cellular state (in terms of telomere length) in a child at that stage of their life. The underlying mechanisms behind the effect of stress and depression on the telomere length of an individual remain a mystery. However, the major limitations to these studies were the small sample size and the measurement of exposure to only one variable, either stress or maternal depression. Further, no measure of stress in children was available in most of the studies. In addition, most of the research was conducted on adults and fewer studies were done in children. A plausible explanation for this bias could be the availability of samples (blood/saliva/buccal swabs/other tissues) that are easier to get in adults as compared to young children. However, it is important to study the effects of a stressful environment on children and observe any signs of cellular damage (telomere length being one of the signatures of cellular damage).
4.2 Methodological considerations: Strengths and limitations

I assessed the effects of different life measures on the cellular state (in terms of telomere length) in young children. I measured telomere length in young and healthy children belonging to a larger cohort and took into account various family and child data measures. To my knowledge, this is a first of its kind study, which targets gene-environment associations that may be present between mean telomere length in 409 pre-school children (3-4 years of age) and family and child data measures through bivariate and multivariate analyses. These measures primarily included children’s exposure to chronic stress, maternal depression (present/absent), cortisol reactivity to stress (based on a stress task performed by children), age, and sex of children.

One of the strengths of this research is that a large body of data has already been collected on the children and their families used in this study. They include factors that may affect upbringing of children including maternal depression and stressful conditions. As outlined in the introduction, childhood stress is a major contributor to personality and behavior. More importantly, a number of recent publications have implied an association between stressful conditions and telomere length (Kananen et al., 2010; Naess & Kirkengen, 2015; Surtees et al., 2011). The data included in these reports however are heterogeneous; some showing weak associations while others showing no association. In addition, these studies have primarily focused on adults. At the time of the planning of this research, there were no reports that dealt with very young children. The telomere length generated under this thesis along with data already collected by Dr. Hayden (Psychology) on the same set of children offer an opportunity to assess if telomere length is in fact related to any of the family and child specific variables.

The method used for assessing telomere length in this study is one of the most recent and effective techniques developed by Cawthon (2009). Monochrome multiplex qPCR method is a
reliable and efficient method of measuring telomere length using small quantities of DNA. The telomere length is obtained as a ratio of the starting quantities of the telomere products to the albumin products. In my study, the range of telomere length obtained as the T/S ratio in children was from 0.04-9.89. The telomere length obtained in my cohort is similar to those obtained in previous studies (Gotlib et al., 2014; Jodczyk et al., 2014; Lindqvist et al., 2015; Needham et al., 2014).

4.3 Study findings

The effects of different family and child measures on the telomere length in these children were assessed through bivariate and multivariate analyses. The first variable I assessed was the effect of maternal depression on the telomere length in children of this cohort through bivariate analysis. In my study, 97 out of the 367 children (for whom the data was available) had mothers with a lifetime history of depression. An independent sample t-test was conducted to compare the telomere length of children having depressed mothers to those with non-depressed mothers. No significant correlation was found between a lifetime history of maternal depression and the telomere length in children. These results support the outcome of a previous study (Wojcicki et al., 2015), that assessed early childhood exposures to maternal depression by measuring telomere length in 203, 3- to 5-year-old preschool Latino children. They did not find any significant correlations between child telomere length and maternal depression in the prenatal, postnatal (4-6 weeks, 6 months and 12 months) and early childhood phase (3, 4 and 5 years), with an exception of a significant association between exposure to maternal depression at 3-years-of-age. They found shorter telomeres in 3 year old children having mothers diagnosed with clinical depression (Wojcicki et al., 2015). Another study by Gotlib and colleagues (Gotlib et al., 2014) mentioned earlier, found significant associations between telomere length in healthy young daughters (10-14
years) and mothers with major depressive disorder. Daughters (n=50) having mothers with MDD had shorter telomeres as compared to daughters (n=47) representing the control group. However, a limitation to this study (Gotlib et al., 2014), was that it did not have a large study sample size (n=97; including 50 girls with depressed mothers and 47 girls with never-depressed mothers). More studies have to be conducted to further confirm the findings of these studies. The cohort in my study is bigger in size (385) and represents a population sample from Southwestern Ontario. Another distinguishing feature of my study is that I have assessed multiple variables including exposure to stress at home, unlike the previous study (Gotlib et al., 2014), that observed only maternal depression and cortisol levels of children in relation to their telomere length.

Another important variable assessed in my study was the exposure to chronic family stress. A Pearson correlation coefficient was computed to assess the relationship between chronic family stress and telomere length of children in this cohort. I found no significant correlation was found between the two variables. These results support a previous study (Jodczyk et al., 2014), in which leukocyte telomere length was assessed in 677 participants (birth- to 30-years-of-age) from New Zealand, exposed to a number of stressors during development from the perinatal period through to young adulthood. No significant associations were found between telomere length and life course adversity or stress (Jodczyk et al., 2014). However, there are other studies that support the notion that exposure to different types of stressors throughout the lifetime can affect the mental health and telomere biology of an individual (Kananen et al., 2010; Kiecolt-Glaser et al., 2011; O’Donovan et al., 2011; Surtees et al., 2011). An important factor in this study is the age of children. These children are pretty young (3-4 years) and even though they are exposed to stress at home, this might not affect them at a cellular level (measured in terms of telomere length here).
Over time, if these children are exposed to stress for a long time, changes in their telomere length might be observed.

The relationship between cortisol levels in children and their telomere length was assessed through bivariate analysis in my study. Three cortisol levels were recorded in these children: first at the baseline level (AUCB), second was the increase (change) in cortisol response after a stress task (AUCI), and the third was a cumulative cortisol response of the baseline and increase in levels (AUCG) (Kryski et al., 2011). Pearson correlation coefficients were computed to assess the relationships between ground cortisol (AUCG) levels and increase in cortisol (AUCI) levels, and telomere length of children in this cohort. I found a positive correlation between cortisol responses to stress levels (both AUCG and AUCI) and telomere length in children in bivariate analyses.

However, in multivariate analysis, only the increase in cortisol levels (AUCI) after the stress task, were found to be associated with the telomere length in these children. Though cortisol is an important measure of HPA axis, more research is required on cortisol levels in children in relation to their telomere length. A study by Gotlib and colleagues (Gotlib et al., 2014) measured cortisol levels and telomere length in 97 healthy adolescent girls (10- to 14-years of age) who had mothers either with/without a history of depression. They found a negative correlation between cortisol levels and telomere length in these girls and inferred that girls with a higher cortisol reactivity to stress had shorter telomeres as compared to the girls with lower cortisol reactivity. The results acquired from this study (Gotlib et al., 2014) are contradictory to the results obtained in my study as it is expected that children who are more stressed would show an inclination towards having shorter telomeres. However, these measures of cortisol recorded are only at a certain day and for a certain task. Recording cortisol levels over a long time would provide better insight.
In addition to these variables, age, and sex of children are also a part of my study. Independent sample t-tests (bivariate analysis) assessed the relationship between these variables and the mean telomere length. Independent sample t-tests did not provide a significant correlation between child sex and telomere length. There were almost an equal number of boys and girls in this study and telomere length was roughly the same among them. A Pearson correlation coefficient computed to assess the relationship between children’s age and their telomere length provided no significant association between these two variables. Although the reason for this result is that, there was not a big age difference in this cohort. All the children were roughly of the same age (3-4 years).

4.4 Conclusions

To conclude, this thesis was among the first (to my knowledge) to measure telomere length in 385, healthy, 3- to 4-year-old children from Southwestern Ontario and study the effects of variables like maternal depression and exposure to stress on their telomere length. A summary of results from my study are presented in Table 4.1. Telomere length was successfully and reliably measured using monochrome multiplex quantitative polymerase chain reaction method (MMqPCR). Apart from conducting bivariate analyses for studying the individual effects of variables on telomere length in children, multivariate analyses helped in confirming the results and associations of telomere length with different family and child-specific variables in children of this cohort.

In all, I found no significant associations found between exposure to chronic stress and maternal depression and telomere length in this cohort. The key factors here are the age and health of these children. Children of such a young age can very well perceive stress, but it does not lead to a decline in their telomere length (with respect to unaffected children). The negative results are
a good indicator of children’s cellular state (in terms of telomere length), that is not affected during early stressful environment. Children of such a young age, if affected at the molecular level, may lead to several health concerns in the future. Children recruited in this study were free from any illness and disorder and were completely healthy. This plays a large contribution in their telomere length as well. A disease or disorder might lead to shorter telomeres in children.

Increase in levels of cortisol (obtained after performing a stress task) predicted telomere length in the multiple regression analysis. A positive correlation was found between cortisol levels and telomere length measured in these children. However, not many studies measure this variable, but one of the most recent studies, shows a negative association between cortisol levels and telomere length in healthy, young daughters (10-14 years) with depressed mothers (Gotlib et al., 2014). Further, the sample size of my study is larger (n=384) as compared to Gotlib’s study (n=97). The results obtained in my study suggest that there might be a mechanism protecting such young children from telomere erosion. However, further studies are required to confirm these results.

Other variables like age and sex of children did not show any significant effects on the telomere length of these children. In all, more studies are required in young children to measure the effects of environmental stressors and adversities on their cellular state, particularly in terms of their telomere length as telomere damage can lead to a number of diseases and age-related illnesses in the future.
Table 4.1: Sample characteristics, telomere length data and the outcomes of this study

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Variable assessed</th>
<th>Sample size</th>
<th>Mean telomere length in children</th>
<th>Telomere length range</th>
<th>Results/ Conclusions</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Maternal depression (MDD)/ No maternal depression (Control)</td>
<td>97 (MDD)/ 270 (C)</td>
<td>2.31± (MDD)/ 2.50± (C)</td>
<td>0.10-9.89 (MDD)</td>
<td>No significant correlation found between maternal depression and TL in children</td>
</tr>
<tr>
<td>2.</td>
<td>Chronic family stress</td>
<td>380</td>
<td>2.43±0.10</td>
<td>0.04-9.89</td>
<td>No significant correlation found between chronic family stress and TL in children</td>
</tr>
<tr>
<td>3.</td>
<td>Child sex (Boys/Girls)</td>
<td>186 (boys)/ 197 (girls)</td>
<td>2.36±0.15(boys)/ 2.49±0.13(girls)</td>
<td>0.08-9.89 (boys)/ 0.04-9.89 (girls)</td>
<td>TL of boys was not significantly different from girls</td>
</tr>
</tbody>
</table>
Table 4.2: Previous studies on telomere length in subjects with/exposed to major depressive disorder

<table>
<thead>
<tr>
<th>Authors/Reference</th>
<th>Psychiatric disorder</th>
<th>Sample type and size (S/C)</th>
<th>Mean Age (yrs) (S/C)</th>
<th>Method used</th>
<th>Mean Telomere length (S/C)</th>
<th>Telomere length Range (S/C)</th>
<th>Results</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gotlib et al., 2014</td>
<td>Healthy daughters either having mothers with MDD/without MDD (control)</td>
<td>Saliva 50/47</td>
<td>10-14</td>
<td>qPCR</td>
<td>1.754/1.524 (T/S)</td>
<td>N/A</td>
<td>Shorter telomeres in daughters of depressed mothers.</td>
<td>Small samples size.</td>
</tr>
<tr>
<td>Wojcicki et al., 2015</td>
<td>Children with maternal clinical depression</td>
<td>Dried blood spots 203 (total)</td>
<td>Birth-5 years (4-6 weeks, 6, 12 months, 3, 4 and 5 years)</td>
<td>qPCR</td>
<td>7828.67±137.9/7792.08±76.00 bp (4-6 weeks) 8082.31±162.93/7770.15±79.62 bp (6 months) 7571.43±137.08/7794.28±6.15 bp (3 years) 7926.05±192.77/7785.83±73.89 bp (4 years)</td>
<td>N/A</td>
<td>No association between child TL and exposure to maternal depression. Significant association found only at 3 years of age.</td>
<td></td>
</tr>
<tr>
<td>Simon et al., 2006</td>
<td>Chronic MDD/BD with or without anxiety (SCID-DSM-IV) /</td>
<td>Blood leukocytes 44/44</td>
<td>51/51</td>
<td>Southern Blot</td>
<td>6.98/7.64 (kb)</td>
<td>MDD-5.61-8.73 Control-5.69-10.30</td>
<td>Shorter LTL in mood disorders</td>
<td>Analysis not conducted on individual MDD group.</td>
</tr>
<tr>
<td>Study Authors &amp; Year</td>
<td>Group Description</td>
<td>Methodology</td>
<td>MDD Score</td>
<td>Control Score</td>
<td>Method</td>
<td>LTL Score</td>
<td>LTL Difference</td>
<td>Notes</td>
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<tr>
<td>Lung et al., 2007</td>
<td>MDD (SCID-DSM-IV)/Control (undocumented method)</td>
<td>Peripheral blood (WBC) 253/411</td>
<td>44/45</td>
<td>Southern Blot</td>
<td>8.17/9.13 (kbp)</td>
<td>N/A</td>
<td>Shorter LTL in MDD</td>
<td>Duration/number of episodes of illness unknown</td>
</tr>
<tr>
<td>Hartmann et al., 2010</td>
<td>MDD (DSM-IV)/Control (undocumented method)</td>
<td>54/20</td>
<td>49/49</td>
<td>Southern Blot</td>
<td>7.20/7.55 (kb)</td>
<td></td>
<td>Shorter LTL in MDD</td>
<td></td>
</tr>
<tr>
<td>Hoen et al., 2011</td>
<td>MDD (CDIS-IV-DSM-IV) with stable coronary heart disease/control without MDD but had stable CHD</td>
<td>Peripheral blood leukocytes 206/746</td>
<td>62/68</td>
<td>qPCR</td>
<td>0.86/0.90 (T/S)</td>
<td>N/A</td>
<td>Significant short LTL in MDD</td>
<td>Mainly older men, with CHD. Association of TL may have been due to CHD more than depression</td>
</tr>
<tr>
<td>Wolkowitz et al., 2011</td>
<td>MDD not medicated outpatients (SCID-DSM-IV)</td>
<td>Blood 18/17</td>
<td>37/37</td>
<td>qPCR</td>
<td>5101±425/5141±282 (bp)</td>
<td>N/A</td>
<td>No difference in LTL across all MDD subjects. Shorter LTL was observed in more chronic MDD cases.</td>
<td>Small sample size.</td>
</tr>
<tr>
<td>Authors</td>
<td>Diagnosis Conditions</td>
<td>Sample Type</td>
<td>Sample Size</td>
<td>Method</td>
<td>LTL Measurement</td>
<td>Findings</td>
<td></td>
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<tr>
<td>Wikgren et al., 2012</td>
<td>MDD (severe depression phenotype) by DIGS/FIGS-DSM-IV, 90% had melancholia</td>
<td>Blood leukocytes</td>
<td>60/59</td>
<td>qPCR</td>
<td>5261/5538 (bp)</td>
<td>N/A</td>
<td>Shorter LTL in MDD</td>
<td></td>
</tr>
<tr>
<td>Teyssier et al., 2012</td>
<td>MDD (SCID-DSM-IV and MINI)</td>
<td>17/16</td>
<td>40/38</td>
<td>qPCR</td>
<td>13.42/13.60 (Mean Ct)</td>
<td>N/A</td>
<td>No significant difference in LTL</td>
<td></td>
</tr>
<tr>
<td>Garcia-Rizo et al. (2013)</td>
<td>MDD (SCID-DSM-IV)</td>
<td>Blood leukocytes</td>
<td>Unknown for the subset for TL. In the entire study 31/28</td>
<td>Fluorimetric assay</td>
<td>89.0/103.7 (telomere content)</td>
<td>N/A</td>
<td>Lower telomere content in MDD as compared to controls.</td>
<td></td>
</tr>
</tbody>
</table>

Small sample, all females, all Caucasian, most were relatively recent onset of depressive disorder.
<table>
<thead>
<tr>
<th>Study</th>
<th>Design</th>
<th>Sample Size</th>
<th>LTL Measurement</th>
<th>Method</th>
<th>qPCR Results</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Verhoeven et al., 2014</td>
<td>Netherlands Study of Depression and Anxiety (NESDA)</td>
<td>Blood leukocytes 1095 (current MDD)+802 (remitted MDD)/510 controls</td>
<td>41 (current MDD)/44 (remitter MDD)/41 (control)</td>
<td>qPCR</td>
<td>1.11 (current MDD)/1.09 (remitted MDD)/1.15 (controls) (T/S)</td>
<td>Shorter LTL in current and remitted MDD as compared to controls</td>
</tr>
<tr>
<td>Needham et al., 2014</td>
<td>MDD (Composite International Diagnostic inventory)</td>
<td>Whole blood 75 MDD/966 controls</td>
<td>30.3/29.2</td>
<td>qPCR</td>
<td>1.21/1.14 (T/S)</td>
<td>No group effect on LTL.</td>
</tr>
</tbody>
</table>
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## Appendices

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<th>5</th>
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</thead>
<tbody>
<tr>
<td>1. Child age</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>2. Child sex</td>
<td>.067</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Maternal depression</td>
<td>-.006</td>
<td>-.038</td>
<td>.075</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>4. Chronic family stress</td>
<td>-.067</td>
<td>.040</td>
<td>.123</td>
<td>.244**</td>
<td>--</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. AUC&lt;sub&gt;g&lt;/sub&gt;</td>
<td>-.046</td>
<td>-.001</td>
<td>.126*</td>
<td>.064</td>
<td>.165**</td>
<td>--</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. AUC&lt;sub&gt;i&lt;/sub&gt;</td>
<td>-.038</td>
<td>.024</td>
<td>.016</td>
<td>-.026</td>
<td>.109*</td>
<td>.769**</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>7. Telomere length</td>
<td>.038</td>
<td>.079</td>
<td>.117*</td>
<td>-.053</td>
<td>.042</td>
<td>.159*</td>
<td>.186*</td>
<td>--</td>
</tr>
</tbody>
</table>

Note: AUC<sub>g</sub> = area under the curve ground, AUC<sub>i</sub> = area under the curve increase.

*p<0.05; **p<0.01.
ADITI THAKUR
CURRICULUM VITAE

EDUCATION

2014-2016  M.Sc. Candidate, Department of Biology, University of Western Ontario, Canada
2011-2013  M.Sc. in Biotechnology, Amity University, India
2008-2011  B.Sc. in Life Sciences, Lucknow University, India

RESEARCH EXPERIENCE

2014-Present  Graduate student, Department of Biology, University of Western Ontario, Canada
2014(April-July)  Internship, MRD Lifesciences, India
2013(Feb-May)  Internship, Department of Biology, Mc Master University, Canada
2012(Mar-May)  Internship, Biotech Park, India

ACADEMIC HONOURS AND AWARDS

2014-Present  Western Graduate Research Scholarship, The University of Western Ontario, Canada
2014-Present  Graduate Teaching Assistantship (GTA), The University of Western Ontario, Canada
2011-2013  Bronze in Academics (M.Sc. In Biotechnology), Amity University, India

RELATED WORK EXPERIENCE

2014-2016  Teaching Assistant, Department of Biology, The University of Western Ontario, Canada