

Chronic Stress and Reproductive Function in Female Childhood Cancer Survivors

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CHRONIC STRESS AND REPRODUCTIVE FUNCTION IN FEMALE
CHILDHOOD CANCER SURVIVORS

by

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ABSTRACT
CHRONIC STRESS AND REPRODUCTIVE FUNCTION IN FEMALE
CHILDHOOD CANCER SURVIVORS

Theresa M. Hardy, BA, BSN

Marquette University, 2018

Reproductive dysfunction is reported as a major concern for childhood cancer survivors (CCS) and is highly correlated with quality of life in this population. Few predictors of post-treatment reproductive function in CCS have been identified. CCS report high levels of psychological stress. Psychological stress activates the hypothalamic-pituitary-adrenal axis, which can disrupt reproductive function.

The purpose of this exploratory study was to explore the relationship between perceived stress, biomarkers of hypothalamic-pituitary-adrenal activity, gonadotropin levels, and anti-Müllerian hormone levels in female CCS.

This exploratory cross-sectional study included female cancer survivors (ages 16-35) treated for pediatric cancer at the Royal Hospital for Sick Children in Edinburgh, Scotland. Perceived stress was measured using the Perceived Stress Scale (PSS-10). Hypothalamic-pituitary-adrenal activity (HPA) was measured using salivary and hair cortisol levels. Ovarian function was measured using serum gonadotropin levels and serum anti-Müllerian hormone levels. Latent growth curve modeling was used to determine diurnal cortisol slope and intercept. Bayesian structural equation modeling was used to explore the relationship between perceived stress, biomarkers of HPA activity and ovarian function.

Twenty-four female (mean age 21.79 ± 5.68) CCS were included in the study. We found an inverse association between perceived stress and ovarian function and a positive association between biomarkers of HPA activity and ovarian function.

The findings from this study suggest that perceived stress is negatively associated with ovarian function and that threshold cortisol levels are required for healthy ovarian function in female childhood cancer survivors.

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Chapter 1. Introduction

Significance

An estimated one in 1000 adults under the age of 35 is a survivor of childhood cancer (Blumenfeld, 2012). Due to significant advances in the treatment of pediatric cancer, the five year survival rate now exceeds 80% in the United States (Barton et al., 2013) with similar statistics worldwide (Reinmuth et al., 2013). With an increasing number of survivors, there has been growing recognition of the potential late effects of cancer treatment (Kremer et al., 2013). Reproductive dysfunction is one such major concern for cancer survivors, and is highly correlated with quality of life in this population (Cherven, Mertens, Wasilewski-Masker, Williamson, & Meacham, 2015; Knopman, Papadopoulos, Grifo, Fino, & Noyes, 2010; Letourneau, Chan, & Rosen, 2013). Among female childhood cancer survivors (CCS), 6.3% suffer from acute ovarian failure and another 22.6% suffer from a significant reduction in ovarian function (Salih et al., 2015). Premenopausal female CCS are at risk for persistent chemotherapy-related amenorrhea and early menopause; however, not all experience these effects (Abusief, Missmer, Ginsburg, Weeks, & Partridge, 2012).

The American Society for Clinical Oncology has recommended that options for fertility preservation be discussed with cancer patients at the earliest opportunity; however, it remains challenging for healthcare providers to predict individual risk of post-treatment reproductive dysfunction because of the limited knowledge of factors associated with this late effect (Knight et al., 2015;

Letourneau et al., 2013). While the etiology of reproductive dysfunction in cancer survivors has been examined (El-Shalakany, Ali, Abdelmaksoud, Abd El-Ghany, & Hasan, 2013; Letourneau et al., 2013), few predictors of this effect of treatment have been identified (Dewailly, et al., 2014, Anderson et al., 2015). Further research is warranted to provide reliable biomarkers to help clinicians accurately identify cancer survivors at increased risk for reproductive dysfunction (Abusief et al., 2012).

In clinical practice, a woman's level of ovarian function is currently determined with measures of FSH, LH, and the presence or absence of menses. However, neither FSH nor menstrual cyclicity post-cancer treatment are reliable predictors of future fertility (Knight et al., 2015). A more reliable measure of the risk for premature ovarian failure and subsequent infertility is the size of the ovarian follicle pool (ovarian reserve). A biomarker which more closely reflects the number of remaining follicles in the ovary would have significant clinical potential.

A biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” (National Institute of Health Biomarker Definition Working Group, 2001, p. 91). A recently identified biomarker of ovarian reserve is anti-Müllerian hormone (AMH). Plasma levels of AMH reflect the continuous non-cyclic growth of small follicles, and therefore mirror the size of the remaining follicle pool (Jeppesen, Anderson, Kelsey, Christiansen, Kristensen, Jayaprakasan, Campbell, et al., 2013). Kelsey

et al. developed and validated the first model of AMH, mapping out the chronological trajectory of the hormone from conception to menopause (Kelsey, Wright, Nelson, Anderson, & Wallace, 2011). La Marca et al. went on to establish normative age-ranges to aid in the clinical interpretation of AMH values (La Marca et al., 2012).

Ovarian reserve declines naturally with age; however, recent research demonstrates that chronological age alone is not an accurate indicator of reproductive age and that other factors affect the depletion of the ovarian follicular pool over time (Dolleman et al., 2013). Identifying factors that contribute to the decline of the ovarian reserve may aid in the prevention and early detection of follicle depletion, premature ovarian insufficiency and impaired fertility. AMH is prematurely reduced in CCS (Anderson & Wallace, 2013; Charpentier et al., 2014; Miyoshi et al., 2013), and may be an early marker of significant gonadotoxicity post treatment (Brougham et al., 2012; Lie Fong et al., 2009). Several studies have demonstrated the utility of pre-treatment AMH concentrations to predict risk of post treatment ovarian dysfunction in cancer survivors (Anderson & Wallace, 2013; Lunsford, Whelan, McCormick, & McLaren, 2014). However, the predictive value of AMH in cancer survivors is limited due to incomplete knowledge of factors that influence AMH concentrations (Abusief et al., 2012; van Dorp et al., 2014). There is also limited information on how changes in AMH relate to changes in serum gonadotropin levels (e.g. follicle stimulating hormone) affecting fertility in women, and the predictive value of AMH

in assessing reproductive potential in young cancer survivors (Lunsford et al., 2014).

One of the primary regulators of reproductive function is the hypothalamic-pituitary-adrenal (HPA) axis. Precise levels of glucocorticoids are required for proper ovarian function (Whirledge & Cidlowski, 2010). At homeostatic levels, cortisol contributes to steroid biosynthesis and maintenance of gonadotropin release; at elevated levels, it suppresses gonadotropin-releasing hormone (GnRH) secretion, reduces pulsatile LH secretion, and increases rates of follicle atresia (Breen & Mellon, 2014; Kalantaridou et al., 2010; Whirledge & Cidlowski, 2010; Whirledge & Cidlowski, 2013). It is well established that psychological stress impairs reproductive function by activating the HPA axis (Louis et al., 2011; Lynch, Sundaram, Maisog, Sweeney, & Buck Louis, 2014). Stress is also associated with increased concentrations of pro-inflammatory cytokines, such as interleukin-6 (IL-6), which may reflect reduced sensitivity to the anti-inflammatory effects of cortisol (Rohleder, Aringer, & Boentert, 2012; Sribanditmongkol, Neal, Patrick, Szalacha, & McCarthy, 2014). Increased IL-6 levels diminish aromatase activity within ovarian follicles, inhibiting steroidogenesis and follicle maturation (Taghavi et al., 2014).

Cancer survivors experience high rates of psychological stress and HPA dysregulation (Oancea et al., 2014; Taylor, Absolom, Snowden, & Eiser, 2012; Zeltzer et al., 2009). As both higher rates of psychological stress and reproductive dysfunction are observed in cancer survivors, this study explored the relationship between psychological stress and biomarkers of ovarian function

in young cancer survivors. **The purpose of this study was to explore relationships between perceived stress, HPA activity, and ovarian function in a sample of female survivors of childhood cancer.**

Specific Aims

Aim 1: To describe the relationship between perceived stress and HPA activity in female childhood cancer survivors.

Aim 2: To explore the relationship between perceived stress, biomarkers of HPA activity, gonadotropin levels, and AMH in female childhood cancer survivors.

Significance to vulnerable populations

“The term ‘vulnerability’ is used to identify individuals and groups at risk of harm” (Spiers, 2000, p. 715). Thus, vulnerable individuals or groups are defined based on a real or perceived likelihood of experiencing an adverse outcome due to the nature of their circumstances. The definition of a cancer survivor starts from the time of diagnosis; accordingly, in the United States, there are more than 420,000 survivors of cancer diagnosed before the age of 21, which is approximately one in every 750 individuals (Tonorezos et al., 2015). The number of cancer survivors is growing because there have been improvements in the early detection and treatment of cancer. However, the cure is not an end to health concerns; many survivors face lifelong challenges to their health due to the late effects of cancer treatment. Over two thirds of survivors exhibit at least one chronic health condition 5 to 14 years after diagnosis (Phillips et al., 2015). At risk of numerous adverse health outcomes, cancer survivors make up a

vulnerable population, with distinct and highly variable needs based on cancer diagnosis, treatment, and duration.

CCS often do not receive adequate risk-based survivorship care as they transition from pediatric oncology to adult care. Despite the growth of survivorship clinics, a recent study by Zheng et al. (2015) found that the majority of CCS (72.2%) did not attend a clinic even at 10 years post-diagnosis. Researchers offer potential explanations for this discrepancy; CCS do not receive adequate education regarding the risks of treatment, providers lack sufficient knowledge of risks and fail to recommend proper long-term surveillance, and the transfer to adult care lacks standardization (Kremer et al., 2013; Phillips et al., 2015; Szalda et al., 2016; Zheng, Sint, Mitchell, & Kadan-Lottick, 2015).

As a response, survivorship research has emphasized the importance of developing personalized follow-up care plans based on individual risk so as to prevent chronic disease and improve quality of life in this population (Salz et al., 2015). In order to provide appropriate individualized follow-up for cancer survivors, clinicians need tools to help them to stratify risk. However, in order to develop effective risk models for the prediction of late-effects, it is necessary to have sufficient knowledge of each variable included in the models (Salz et al., 2015).

One effect of cancer treatment that is particularly concerning for cancer survivors is reproductive dysfunction. Female cancer survivors are at increased risk of premature ovarian insufficiency and fertility problems, and this effect of

treatment is associated with significant psychological distress, negatively impacting quality of life in this population (Terenziani et al., 2014). And yet, studies consistently demonstrate that cancer survivors receive inadequate fertility care, which consists of a thorough evaluation of infertility risk and counseling on options for the preservation of fertility and the treatment of infertility (Barton et al., 2013; Noyes, Knopman, Long, Coletta, & Abu-Rustum, 2011; Salih et al., 2015). Barton et al. (2013) found that compared to sibling controls, cancer survivors were approximately half as likely to be treated for infertility. Kim et al. (2016) found that survivors who received fertility counseling prior to treatment were more likely to pursue fertility care post treatment. However, the majority (73%) did not recall receiving sufficient information regarding risks of cancer treatment on future fertility or options for fertility preservation (Kim et al., 2016). One of the primary reasons for this disparity is the difficulty in quantifying risk of reproductive dysfunction after cancer treatment due to the lack of a sensitive biological marker of ovarian function (Barton et al., 2013).

This study contributes to our knowledge of AMH as a biomarker of ovarian reserve in a population at high risk for reproductive dysfunction. Existing biomarkers of reproductive function, such as FSH, may not change until infertility is advanced. AMH levels may be important in predicting risk for infertility post treatment early enough that interventions to promote fertility are likely to be successful (Gordon, Kanaoka, & Nelson, 2015).

Cancer survivors also report increased levels of perceived stress and demonstrate alterations in HPA activity. The effects of chronic stress on ovarian

reserve are poorly understood. This study therefore sought to increase the value of AMH as a biomarker of ovarian reserve by exploring the association between stress, gonadotropins, and AMH in a population of female survivors of childhood cancer. The study's findings will be used to inform future research to improve clinical decision-making based on an individual's vulnerability to the effect of cancer treatment on reproductive function.

Significance to nursing knowledge development

The late effects of cancer and survivorship care reflect 8 of the top 20 ranked priorities in oncology nursing research (LoBiondo-Wood et al., 2014). The goal of survivorship care is to individualize care to maximize quality of life. The ability to tailor treatment plans depends on the availability of accurate biomarkers that can reliably predict vulnerabilities to the late effects of cancer (Knobf et al., 2014). Cancer survivors are at increased risk of infertility and reproductive dysfunction; however the mechanism underlying this late effect is not well understood (Barton et al., 2013). Fertility is a high priority for survivors of childhood cancer and is correlated with quality of life in this population (McLaren & Bates, 2012).

Further research is needed to increase knowledge of factors contributing to reproductive dysfunction to improve prediction models for the risk of this late effect of cancer and its treatment. The knowledge gained in this study will allow patients, researchers and clinicians to collaborate in addressing the complex and multi-factorial etiology of reproductive dysfunction in female cancer survivors (Bhatia et al., 2015; Woodruff, 2013). Targeted interventions to prevent a

premature decline in ovarian functioning will decrease the prevalence of reproductive dysfunction and its associated financial and psychological burden to patients.

Chapter 2. Review of the Literature

Chapter 2 consists of two parts. Part I includes a description of life history theory and how it will be used to structure the study's main concepts and explore relationships between them. Part II will review the research on AMH as a biomarker of the ovarian reserve, its current uses in reproductive medicine, and its promising potential as a predictor of fertility post treatment in cancer survivors.

Part I: Theoretical Framework: Life History Theory

The theoretical framework used to guide this study is life history (Ellis, 2013; Whirlledge & Cidlowski, 2013). Rooted in evolutionary biology, life history theory was developed to explain variation in **life history strategies**, which are resource allocation strategies adopted by organisms to optimize survival and reproduction throughout the life course and across varying environmental conditions. The task of all organisms is essentially the same, to distribute material and energy resources to competing life functions: bodily maintenance, growth and reproduction. Because resources are limited, an organism must make **trade-offs**, prioritizing their use in one domain at the expense of other competing domains (Ellis, Figueredo, Brumbach, & Schlomer, 2009). **Life history traits**, characteristics that determine rates of bodily maintenance, reproduction, and growth, are the primary units of analysis in life history theory. Both individually and collectively, life history traits provide a window into an organism's life history strategy and the developmental continuum that results from a chain of resource allocation decisions (trade-offs). The main life history

traits are age and size at maturity, reproductive investment, reproductive lifespan, and aging (Stearns, 2000).

Influences on Life History Strategies

Life history traits are influenced by an organism's genetic characteristics and environmental conditions. These traits form the organism's life history trajectory or continuum. Based on the availability of extrinsic and intrinsic resources, the organism will coordinate tradeoffs so as to optimize its overall fitness. "A synchronized optimization of tradeoffs is therefore at the heart of what constitutes a coherent and coordinated [life history] strategy" (Ellis et al., 2009, p. 212). While life history strategies are varied, general patterns, or continua, can be observed. These continua have been described as taking either a 'slow' or 'fast' course, dependent on environmental conditions.

Ellis describes two types of environmental influence that can shape an organism's life history strategy: **environmental harshness** and **environmental unpredictability**. "Environmental harshness indexes the rates at which external factors cause disability and death at each age in a population" (Ellis et al., 2009, p. 206). While classical life history theory defines harshness as the rate of mortality in a population, Ellis expanded the concept to include non-fatal injury, disease, and other sources of *stress*, as these can also significantly impact reproductive success, and therefore the optimal allocation of resources to growth, survival and reproduction. Environmental unpredictability is defined as "the rates at which environmental harshness varies over time and space" (Ellis et al., 2009, p. 207). While environmental harshness will likely favor some life

history strategies over others (selective evolution), environmental unpredictability limits any one strategy from having an advantage over another since these conditions are largely unavoidable.

In addition to environmental influence on resource allocation decisions, individual characteristics (i.e. genetic and biological factors) impact an organism's life history strategy. As would be anticipated, this results in considerable variability, and for this reason, life history trajectories cannot be easily divided into slow and fast tracks. Instead, an organism's life history strategy is often a mixture of slow and fast traits. However, identifying general patterns that give rise to "clusters of correlated traits" (Ellis et al., 2009, p. 212) may improve prediction of future traits as well as increase understanding of current ones.

HPA axis: Regulator of Life History Strategies

Early evolutionary biologists were able to demonstrate that organisms make tradeoffs in response to changing environmental conditions and that these tradeoffs influence the life course of an organism, but they struggled to identify the biological mechanisms that allow organisms to make tradeoffs. Identifying these mechanisms is vital to understanding the sources of variation in life history strategies (Stearns, 2000). One of the primary mechanisms by which an organism responds to changing environmental conditions is the hypothalamic-pituitary-adrenal (HPA) axis (Ellis, 2013). Environmental challenges or stressors activate the HPA axis and increase glucocorticoid secretion, directing resources to vital physiological activities such as energy mobilization, cardiac output, and

cognition, and away from less vital functions. Because reproduction is energetically costly, when resources are limited, an organism is likely to prioritize survival over reproduction, delaying fertility until environmental conditions are more ideal. The HPA axis, as a primary mediator of resource allocation decisions, plays an important role in determining an organism's life history traits.

The HPA axis plays a prominent role in the regulation of the hypothalamic-pituitary-gonadal (HPG) axis through glucocorticoids, which have both supportive and suppressive effects on reproductive function. This complex and synchronized neuroendocrine cross talk between the HPA and HPG axes allows tradeoffs to occur. At homeostatic levels, glucocorticoids contribute to steroid biosynthesis and maintenance of gonadotropin release. During acute stress, endogenous glucocorticoids counteract the suppressive effects of prostaglandins on reproductive function, thus protecting gonadotropin secretion. This suggests that threshold glucocorticoid levels are necessary to maintain adequate gonadotropin release (Whirledge & Cidlowski, 2010). Elevated glucocorticoid levels suppress reproduction at multiple levels along the HPG axis. Increasing glucocorticoid levels down-regulate gonadotropin releasing hormone (GnRH) synthesis and decrease GnRH pulsatility, inhibiting the LH surge and delaying ovulation, and increasing the rate of ovarian follicle atresia (Whirledge & Cidlowski, 2010, 2013).

Strengths and Weaknesses of Life History Theory

Life History theory examines an organism's life history strategy as a dynamic process. The capacity of organisms to adapt to changing environments

and adjust life history traits to enhance overall fitness is referred to as *developmental plasticity* (Ellis & Del Giudice, 2014). Developmental plasticity is beneficial from an evolutionary perspective. It acknowledges that a single life history strategy is unlikely to afford an organism maximal fitness in every environment. The 'best' strategy for survival and reproduction varies as a function of one's environment and thus a strategy that promotes success in some environmental contexts may lead to failure in others (Ellis & Del Giudice, 2014).

Life history theory contrasts with other prevailing models of toxic stress and allostatic load in that it takes into account individual differences in developmental adaptation. Ellis and Del Giudice describe models of toxic stress as falling short in addressing the variation in life history strategies, since they operate on the assumption that chronic stress is fundamentally maladaptive. This paradigm, while compelling in the examination and prediction of chronic illness, fails to capture the ability of stress response systems to adaptively regulate development (Ellis & Del Giudice, 2014). Overall, life history theory offers an advantage over other models in its balanced perspective of stress within the context of environment and human development.

Life history theory offers an adequate framework for examining an organism's resource allocations in response to competing physiological demands (i.e. survival and reproduction) in the face of changing environmental conditions. However, life history theory does not comprehensively explain variation in human life history traits. It offers a primarily biological framework for examining phenotypic variation, as a complex interplay of biological, environmental, and

genetic influences on the human organism. Stearns, the father of life history theory, examined phenotypic variation within the context of plant and animal life. Ellis and Giudice expanded Stearns' work by applying life history principles to human biology. Their work generated a series of testable hypotheses regarding the effects of varying environmental conditions on human life history traits. Life history theory does not take into account the effect of social context and personal choice on biological processes. While human fertility is contingent upon biological, environmental and genetic factors, it cannot be fully explained by these influences.

In order to examine the effects of higher level influences such as social context and personal choice on human fertility, biological and environmental influences need to be better understood. Despite the limitation of life history theory as a biobehavioral framework, it is well suited for the current exploratory study examining associations between biobehavioral measures of stress and ovarian function. Life history theory has been successfully employed in other studies to explore variability in reproductive function and lifespan (Bleil et al., 2012).

Application of Life History Theory to Study's Main Concepts

Life history theory provides an ideal framework for exploring factors that affect life history traits such as reproductive lifespan. A description of how the main life history constructs will be conceptualized and measured in the current study follows. A complete description of measures will be presented in Chapter 3. As stated above, **the purpose of this study was to explore relationships**

between perceived stress, HPA activity, and ovarian function in a sample of female survivors of childhood cancer.

1. The life history trait that was the main outcome of interest for this study was ovarian function. As a concept, it was examined both as current and “potential” ovarian function. Current ovarian function was measured using serum gonadotropin levels (LH and FSH), serum reproductive hormones (estrogen), and menstrual cycle characteristics (regularity, age at menarche). Because an organism allocates resources for both current and predicted future environments, “potential” ovarian function was also measured using ovarian reserve (AMH). This concept is described in greater detail in the second part of this chapter.
2. The resource allocation strategy was conceptualized as HPA activity and measured using salivary cortisol and IL-6 and hair cortisol. The HPA axis is the mechanism by which an organism takes in information regarding environmental conditions and makes tradeoffs to meet current demands while also attempting to predict and prepare for future environments.
3. Environmental influences on ovarian function were conceptualized as cancer specific influences and perceived stress. Cancer specific influences included cancer diagnosis and treatment type. Stress is “a state of threatened or perceived as threatened homeostasis” (Nicolaidis, Kyratzi, Lamprokostopoulou, Chrousos, & Charmandari, 2015). Perceived stress will be measured using the Perceived Stress Scale (Cohen, Kamarck, & Mermelstein, 1983).

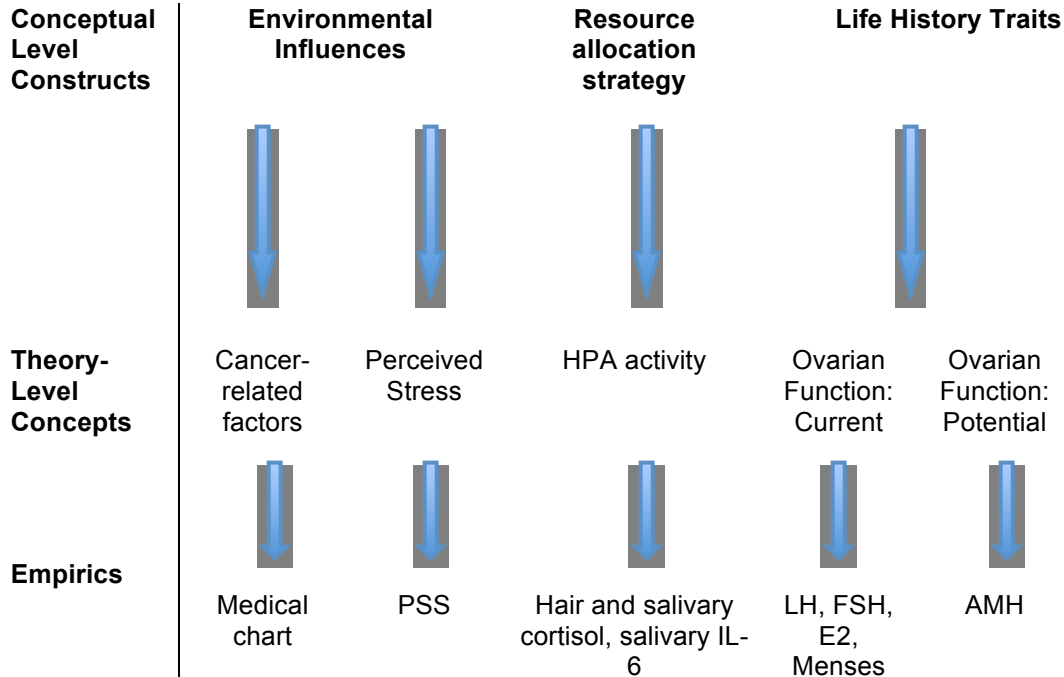


Figure 1: Conceptual-Theoretical-Empirical Model for the Study

Contribution of this study to Life History Theory

While it is well established that psychological stress interrupts normal reproductive functioning (An, Sun, Li, Zhang, & Ji, 2013; Kalantaridou et al., 2010; O'Connor et al., 2011; Whirledge & Cidlowski, 2013), the biological mechanisms underlying this effect are poorly understood (Bleil et al., 2012; Lynch, Sundaram, Buck Louis, Lum, & Pyper, 2012). In this study, we explored the influence of chronic stress on ovarian function with the goal of improving our understanding of variation in life history strategies (Stearns, 2000). The findings expand our knowledge of anti-Müllerian hormone (AMH), a biomarker of ovarian reserve. Biomarkers provide insight into life history traits, and give us a window

into the resource allocation decisions that constitute an organism's life history trajectory. This study contributes to life history theory by furthering our understanding of AMH, a biomarker of ovarian reserve. This knowledge may be used to improve the biomarker's ability to reliably predict the development of reproductive life history traits among female CCS.

Philosophical Underpinnings

A philosophical framework informs the researcher's understanding of reality (ontology), what can be known about reality and how knowledge of reality is attained (epistemology). While all scientific inquiry makes implicit claims about the nature of reality, unless these philosophical foundations are made explicit, research risks superficiality and incongruity. A solid philosophical foundation informs the conduct of research, ensuring that the right questions are asked and promoting coherence in the research aims, methodology and interpretation of the findings (Walsh & Evans, 2014).

This study was based on a post-positivist philosophical framework. Positivist epistemology is the philosophical framework that informs modern science. Positivist inquiry assumes the existence of an objective reality, which can be observed and directly perceived through the senses. In this framework, knowledge gained about reality is always true and generalizable, and truth is dependent not on belief but on knowable facts present in external reality (Clark, 1998). Positivism rejects the existence and influence of unobservable phenomena. The positivist researcher therefore is able to assume an atheoretical and unbiased perspective in the research process. While an appealing

philosophical framework for the conduct of empirical research, positivism is unrealistic in its conceptualization of uncertainty.

There is an increasing shift to the post-positivist philosophical framework in scientific inquiry. Post-positivism shares the positivistic view that metaphysical realities are outside the realm of science, but differs from positivism in that it adopts a realist perspective of science, recognizing that unobservable phenomena, aspects of reality not directly perceivable or measurable, may explain observable phenomena (Clark, 1998). Post-positivist inquiry permits theoretical explanations of reality. Thus, in addition to the positivist requirement for precision in the measurement of observable phenomena, post-positivist inquiry incorporates evidence that can be inferred about unobservable phenomena (e.g. evidence obtained through self-report, questionnaires, etc...). The post-positivist framework assumes that the “researching human, with or without instruments, must shape the research process” (Clark, 1998, p. 1246). Post-positivism not only acknowledges but also adjusts for uncertainty in the research process, allowing for greater realism in the conduct of scientific inquiry and the interpretation of findings. As a consequence, knowledge gained through post-positivistic inquiry is contextually bounded and not generalizable to all cases.

Post-positivism aligns well with the truths sought in biological nursing research (Letourneau & Allen, 1999). The post-positivist framework supports the view that truth is unknowable and that the aim of scientific inquiry is to make “warranted assertions”, which represent probabilities about human behavior

rather than universal laws governing behavior. Scientific claims are warranted when there is sufficient objective evidence about the phenomena under study, and when these claims demonstrate coherence and consistency through time and critical evaluation. In addition to permitting greater realism in the conduct of research, post-positivism also supports critical multiplism, recognizing that there is no correct or perfect method and that research questions can and should be approached through multiple perspectives.

The post-positivist framework best reflects the mode of inquiry used to conduct this study. First, the study's purpose was informed by life history theory, which provided an explanatory framework for examining the relationship between the study's main variables (stress, HPA activity, ovarian function). Second, the study was exploratory, permitting uncertainty regarding the nature of the relationship between the variables. And finally, the statistical methodology (discussed in chapter 3) selected for this study was Bayesian structural equation modeling (SEM) because it integrates uncertainty about the model parameters, appropriate for exploring the relationship between stress and ovarian function in a small sample of female cancer survivors.

Part II: Literature Review The Ovarian Reserve

The Ovarian Reserve and Anti-Müllerian Hormone

The woman's reproductive lifespan is determined by the size of the primordial follicle pool, or the ovarian reserve. The ovarian reserve is established during fetal life when germ cells populate the ovary and form primordial follicles (Dunlop & Anderson, 2014). These follicles consist of oocytes surrounded by a single layer of squamous granulosa cells, and remain in the phase of the first meiotic division until puberty. At birth, the number of primordial follicles is estimated at 1 million. This number decreases steadily during a female's lifespan and is reduced to approximately 300,000 at menarche. Throughout a female's reproductive years, follicles are recruited from the primordial pool and progress through the various stages of follicle growth. At the start of each menstrual cycle, one dominant follicle is selected for ovulation (Visser, de Jong, Laven, & Themmen, 2006).

At present, there is no way to directly assess the ovarian reserve; however, there are several biomarkers that provide an indirect measurement of its size based on the assumption that the number of growing follicles is correlated with the non-growing primordial follicle pool (Dunlop & Anderson, 2014). The association between these indirect measures and the number of non-growing follicles has been confirmed in histological studies (Hansen, Hodnett, Knowlton, & Craig, 2011; Kevenaar et al., 2006).

One biomarker that has shown considerable promise in the field of reproductive medicine is anti-Müllerian hormone (Dewailly et al., 2014). Anti-Müllerian hormone (AMH) is a dimeric glycoprotein and a member of the transforming growth factor β family of growth and differentiation factors (Dewailly et al., 2014). Initially, AMH, also known as Müllerian inhibiting substance (MIS), was studied within the context of male sexual differentiation (Dewailly et al., 2014; Visser et al., 2006). AMH is produced by the Sertoli cells of the testes, and induces regression of the Müllerian ducts, the initial clustering of embryonic cells from which the female reproductive tract develops (Visser et al., 2006). However, AMH also plays a role in ovarian follicle growth, specifically in regulating the pace of follicle recruitment and selection. AMH is expressed in granulosa cells of small growing follicles with its strongest expression in preantral and small antral follicles between 5-8mm in diameter (Jeppesen, Anderson, Kelsey, Christiansen, Kristensen, Jayaprakasan, Campbell, et al., 2013). AMH is no longer expressed by granulosa cells during FSH-dependent stages of follicle growth or by atretic follicles, degenerated follicles that do not ovulate during the menstrual cycle (Broekmans et al., 2008; Dewailly et al., 2014).

The role of AMH in follicle growth was first characterized in studies of AMH-deficient mice (Visser et al., 2007). These studies demonstrated that AMH acts as an inhibitor of primordial follicle recruitment (Durlinger et al., 1999, 2002). AMH deficient mice had more growing follicles and an increased rate of oocyte degeneration and follicle atresia (Visser et al., 2007), indicating that in the absence of AMH, primordial follicles are recruited at a faster rate, leading to a

premature depletion of the follicle pool. Another consequence of AMH absence is an increased sensitivity of growing follicles to FSH. AMH has an inhibitory effect on FSH and therefore plays an important role in the selection of the dominant follicle (Durlinger et al., 2001; Sacchi et al., 2016; Visser & Themmen, 2014; Visser, de Jong, Laven, & Themmen, 2006).

AMH: Validated Measurement and Normative Age Ranges

Until recently, FSH and inhibin B were the gold standard measures of the ovarian reserve. However, changes in FSH and inhibin B levels are not detectable in serum until there is a significant reduction in the follicle pool (Steiner, 2013), making timely detection and intervention challenging. AMH levels decline before other biomarkers of the ovarian reserve, and are therefore one of the earliest detectable changes in the process of ovarian aging (Sills, Alper, & Walsh, 2009). Several prospective longitudinal studies involving normo-ovulatory women have found that declining AMH concentrations predict with reasonable accuracy the onset of menopause (Broer et al., 2011; van Disseldorp et al., 2008). The hormone is ideal as a research and clinical measure because concentrations do not fluctuate significantly across the menstrual cycle, and are independent of FSH, LH, and E2 levels (Hehenkamp et al., 2006; La Marca et al., 2010; Shaw et al., 2011). AMH assays have been developed and serum levels correlate with the number of developing follicles in the ovary (Didier Dewailly et al., 2014).

Kelsey et al. (2011) generated the first validated model of AMH in healthy females from conception until menopause. Their findings demonstrate that AMH

rises steadily until its peak at mean age of 24.5 years, at which point it declines until menopause. The model was generated utilizing data from studies of women without a diagnosis of infertility or chronic illness. According to their model, 34% of the variation in AMH is due to age alone (Kelsey, Wright, Nelson, Anderson, & Wallace, 2011). La Marca et al. (2010) established normative AMH ranges based on age. In order for AMH values to be clinically interpreted, an established nomogram with normative age ranges is vital. La Marca et al. (2010) acknowledge the need for more studies to refine these ranges and establish standardized values for use in research and clinical/diagnostic assessment.

Current Uses of AMH in Reproductive Medicine

AMH has already demonstrated considerable clinical potential, most significantly in the field of assisted reproductive technology. Since ovarian reserve correlates with the size of the remaining follicular pool, it is thought to be associated with reproductive potential. The variable response to in vitro fertilization (IVF) treatment is thought to be related to variability in the ovarian reserve (Reijnders et al., 2016). Diminished ovarian reserve is predictive of poor response to gonadotropin therapy, therefore limiting the possibility of pregnancy (Iliodromiti, Kelsey, Wu, Anderson, & Nelson, 2014). AMH concentrations are also useful for predicting ovarian hyperstimulation syndrome, which is a potentially life-threatening condition (Dewailly et al., 2014). Currently there is insufficient evidence to base treatment decisions within assisted reproductive technology solely on a test of ovarian reserve (Iliodromiti et al., 2014). However,

tests of ovarian reserve have proven useful for tailoring treatment and pre-IVF treatment counseling (Nelson, 2013).

Since AMH regulates the pace of follicle growth by inhibiting initial recruitment from the primordial follicle pool and dominant follicle selection, it also may have potential application in understanding the pathophysiology of ovarian dysfunction (Broekmans et al., 2008). One area of particular interest is polycystic ovary syndrome (PCOS). PCOS is the most common female reproductive disorder and occurs in up to 10% of all women. The three diagnostic hallmarks of the syndrome include oligo/amenorrhea, clinical or biological signs of hyperandrogenemia, and polycystic ovaries assessed by transvaginal ultrasound (Broekmans et al., 2008). Polycystic ovarian morphology is characterized by an increased number of intermediate-sized follicles. This suggests an increased rate of initial follicle recruitment coupled with arrested follicle maturation. Several studies have demonstrated that serum AMH concentrations are markedly increased in PCOS patients, which is consistent with the increase of AMH-producing preantral and small antral follicles (Broekmans et al., 2008). Thus, AMH may serve as a serological biomarker of PCOS (Iliodromiti et al., 2013).

Factors Associated with AMH Concentrations

While ovarian reserve naturally declines with age, age alone is not predictive of ovarian age or reproductive status. The decline of ovarian reserve is primarily due to apoptotic loss of oocytes (programmed cell death) rather than ovulation (Tremellen, Kolo, Gilmore, & Lekamge, 2005). Thirty-four percent of the variation in AMH concentrations is due to age, meaning that the remaining 66%

is due to other factors (Kelsey et al., 2011). Identifying these factors may help to elucidate predictors of reproductive dysfunction and disease. Understanding factors that influence AMH levels in normo-ovulatory women may also help to establish reliable normative age ranges for use in clinical practice and research.

Several studies have explored the influence of both reproductive and lifestyle determinants of AMH concentrations (Dolleman et al., 2013; Freeman et al., 2007; La Marca et al., 2010; La Marca, Stabile, Artenisio, & Volpe, 2006; Malhotra, Bahadur, Singh, Kalaivani, & Mittal, 2013; Nelson, Stewart, Fleming, & Freeman, 2010; Steiner, Stanczyk, Patel, & Edelman, 2010; Tsepelidis et al., 2007). Oral contraceptive use is associated with lower AMH concentrations (Dolleman et al., 2013; Kristensen et al., 2012; Shaw et al., 2011). However, some studies have reported no association between oral contraceptive use and AMH concentrations (Steiner et al., 2010). Some studies have demonstrated a negative correlation between body mass index (BMI) and AMH (Freeman et al., 2007; Steiner et al., 2010), but again this association has not been consistently demonstrated (Dewailly et al., 2014; Dolleman et al., 2013). Pregnancy is associated with lower AMH concentrations (Dolleman et al., 2013; Nelson et al., 2010). Smoking is associated with lower AMH concentrations (Dafopoulos et al., 2010; Hawkins Bressler et al., 2016).

Several studies have also demonstrated an association between AMH concentrations and menstrual cycle irregularity (Dolleman et al., 2013; Kristensen et al., 2012). AMH has been studied extensively within the context of advanced reproductive technologies and various pathological conditions, but little is known

about the factors associated with variation of AMH concentrations observed in normo-ovulatory women (Dolleman et al., 2013). The first manuscript in this dissertation, entitled, “An Integrative Review of Modifiable Biobehavioral Factors Associated with Anti-Müllerian Hormone Concentrations” provides an integrative review of the biobehavioral predictors of AMH concentrations in normo-ovulatory women (Appendix A).

The relationship between AMH concentrations and reproductive outcomes is disputed in the literature. AMH is considered a useful biomarker of reproductive potential in infertile women undergoing assisted reproductive technology (Burks et al., 2015; Reijnders et al., 2016). However, its value as a predictor of natural fertility is still uncertain (Hagen, Vestergaard, et al., 2012; Steiner et al., 2011, 2017; Zarek et al., 2015). This is an important distinction as AMH concentrations are applied and interpreted in clinical practice. The diagnostic and prognostic value of AMH continues to expand, however more research is needed to examine its association with reproductive outcomes in healthy populations.

Stress and the Ovarian Reserve

This study explored the effect of chronic stress on ovarian reserve. Allsworth et al. (2001) were among the first to investigate the effect of chronic stress on the ovarian reserve. They examined whether ovarian hormone levels (FSH and estradiol [E₂]) indicative of menopausal changes were observed at an earlier age among 732 women (ages 36-44) who experienced physical or sexual violence compared with women who reported no exposure to violence. More

extreme levels of both FSH and E_2 in relation to abuse history among premenopausal women 41-45 years of age were observed, whereas little difference was seen for younger women. Allsworth et al. offer a potential explanation for the association between abuse history and ovarian function. Stress activates the HPA axis and stimulates glucocorticoid secretion, which in turn inhibits the synthesis and release of GnRH, LH, and FSH. However, the authors did not include a biomarker of stress in the study and as a result, were unable to examine this proposed biological mechanism (Allsworth, Zierler, Krieger, & Harlow, 2001).

Pal et al. (2010) expanded the work of Allsworth et al., examining associations between acute (serum cortisol) and chronic (history of abuse and/or drug use) psychosocial stress and biomarkers of ovarian reserve (FSH and Müllerian-Inhibiting-Substance (MIS), now referred to as AMH) in 89 premenopausal infertile women <42 years of age. Women were considered to have diminished ovarian reserve (DOR) if they either demonstrated early follicular phase (days 1–3) FSH levels >10 mIU/ml and/or poor ovarian response during attempts at ovarian hyperstimulation. Those with chronic stress demonstrated reduced ovarian reserve parameters: higher FSH ($p = 0.051$) and significantly lower MIS levels ($p = 0.034$), and were three times more likely to be diagnosed with DOR ($p = 0.025$). However, no association was observed between serum cortisol levels and DOR. The authors concluded that chronic, but not current stress, was associated with DOR. They proposed inappropriate HPA activation as a plausible explanation for this association (Pal, Bevilacqua, & Santoro, 2010).

Because a biomarker of chronic stress was not included in the study, they were unable to provide evidence supporting this theory.

Cancer and Reproductive Dysfunction

Cancer survivors are at increased risk of reproductive dysfunction (Barton et al., 2013). Cancer treatments cause damage to the ovaries and impair reproductive potential through accelerated follicle loss (Lie Fong et al., 2009). The proposed mechanism is apoptotic cell death in growing follicles, accelerating the movement of follicles from the primordial pool to replace those lost to atresia (Letourneau et al., 2013; Lie Fong et al., 2009). Increased follicle atresia coincides with a decrease in quantity and quality of oocytes, increasing the risk of infertility and spontaneous abortion (Knopman et al., 2010). The degree of risk of impaired fertility depends on age at cancer diagnosis (El-Shalakany et al., 2013) and the treatment regimen (Anderson & Wallace, 2013; Knopman et al., 2010).

Many chemotherapeutic agents are known to have cytotoxic effects on the ovary, and are associated with premature ovarian failure and chemotherapy-related amenorrhea (Oktem & Oktay, 2009). Chemotherapy exerts its gonadotoxic effects by activating apoptotic machinery in the oocyte and enclosing granulosa cells (Oktem & Oktay, 2009). This leads to premature depletion of the ovarian follicle pool with a subsequent decline of the ovarian reserve (McLaren & Bates, 2012). The highest risk is associated with alkylating agents (Barton et al., 2013), and with pelvic radiation (Reinmuth et al., 2013). However, the quantification of risk of infertility remains challenging for healthcare providers (Barton et al., 2013) due in large part to an incomplete understanding

of the factors contributing to long-term reproductive function in this population (Abusief et al., 2012).

AMH in Female Cancer Survivors

Reproductive potential has been identified as a factor significantly associated with quality of life in cancer survivors (Kondapalli et al., 2014). AMH has shown considerable promise in predicting post-treatment risk for infertility in female cancer survivors (Anderson, Rosendahl, Kelsey, & Cameron, 2013). Recent studies have demonstrated associations between pre- and post-treatment AMH concentrations (Anderson & Cameron, 2011; Anderson et al., 2013; Brougham et al., 2012). Lower pre-treatment AMH concentrations are predictive of long term loss of ovarian function (Anderson et al., 2013; Brougham et al., 2012).

Ovarian reserve is decreased in female survivors of childhood cancer (Charpentier et al., 2014). Several studies have examined the influence of treatment-related and biobehavioral factors on AMH concentrations post-treatment. Treatment-related factors include the therapeutic regimen (Charpentier et al., 2014), the length of exposure (El-Shalakany et al., 2013; Reinmuth et al., 2013), and the age of the patient at the time of treatment (Barton et al., 2013; El-Shalakany et al., 2013). There is also evidence to suggest that cancer pathology may contribute to prematurely reduced AMH concentrations. Van Dorp et al. found that compared to healthy age-matched controls, girls newly diagnosed with cancer presented with significantly lower serum AMH levels ($p < 0.001$) (van Dorp et al., 2014). Biobehavioral factors include obesity (van Dorp et

al., 2013) and age at menarche (El-Shalakany et al., 2013). Pre-treatment planning and the ability to provide individualized fertility prognoses depends on increased understanding of factors associated with the ovarian reserve in female childhood cancer survivors. This information can then be used to assist decision-making regarding treatment options and fertility preservation (Anderson et al., 2013; Knopman et al., 2010).

Gaps in the Literature

The study of ovarian aging as a biological process spanning many years has proven challenging. Premature ovarian aging increases risk for infertility and early menopause. In addition to reproductive dysfunction, premature menopause is associated with increased risk of osteoporosis and cardiovascular disease (Letourneau et al., 2013; Salih et al., 2015). There is great need for a reliable biomarker of the ovarian reserve to assist in the early detection of infertility. While the number of studies examining AMH as a biomarker of the ovarian reserve has increased considerably in the last fifteen years, significant gaps in the literature remain. First, the value of AMH as an age-independent predictor of fecundity has not yet been established (La Marca et al., 2010). In the context of assisted reproduction, AMH provides useful information regarding reproductive potential and ovarian responsiveness to hormonal stimulation, but its predictive accuracy of live birth is poor (Iliodromiti et al., 2014). In the context of unassisted reproduction, only a small number of studies have examined the association between AMH and fecundability (Hagen, Aksglaede, et al., 2012; Steiner et al.,

2017b; Zarek et al., 2015), and these failed to find an association between having a low ovarian reserve and a longer time to pregnancy.

Second, most of the studies that have examined the predictive value of AMH have been based on single measurements. Freeman et al. (2012) found that the rate of change in AMH levels improved the precision of estimates of time to menopause (Freeman, Sammel, Lin, Boorman, & Gracia, 2012). Longitudinal studies are needed to determine if the rate of change in AMH levels also improves prediction of fertility outcomes. Third, more studies are needed to identify the biobehavioral factors that influence serum AMH concentrations in order to account for these effects in prediction models (Dolleman et al., 2013). And finally, because AMH alone is unlikely to provide enough information to predict reproductive potential, more studies are needed to develop and test models based on multiple predictors (Venturella et al., 2015). This study contributes to the literature by improving our understanding of chronic stress on the ovarian reserve in female cancer survivors, a population at high risk of prematurely diminished ovarian reserve.

Preliminary Work

In June 2010, the author conducted a pilot study at the Hatfield Clinical Research Center at the National Institutes of Health. The study sample was obtained using an existing database that included urine cortisol levels from 36 reproductive age females with and without chronic abdominal pain (CAP). Laboratory staff assisted with the analysis of frozen serum samples for AMH concentrations using a commercially available assay (AMH GEN II ELISA,

Beckman Coulter). Women with CAP had significantly lower 5-hour urinary cortisol, a finding that is consistent with literature reporting hypocortisolemia in individuals with chronic pain (Generaal et al., 2014). Using contingency table analysis, we found that women with clinically abnormal (high or low) AMH levels had significantly lower 5-hour urinary cortisol ($p = 0.04$) compared to women with normal AMH levels (Hardy, McCarthy, Fourie, & Henderson, 2016). These findings suggest that HPA dysregulation may contribute to the variation of AMH levels warranting further exploration of this relationship.

In the summer of 2017, the author examined patterns of cardiometabolic, psychological and reproductive parameters of health in postpartum women and examined the ovarian reserve within these patterns (Hardy, Garnier-Villarreal, McCarthy, Anderson, Reynolds, unpublished manuscript) (Appendix B). Latent class analysis (LCA) was used to classify the subjects of the whole sample (heterogeneous) into smaller homogeneous groups in which members are similar to each other and different from subjects in other groups. The objective is to identify groups underlying the data, when the number of underlying groups is unknown (DiStefano, 2012; Kaplan, 2014). The LCA model included the following variables: fasting lipids (triglycerides, cholesterol, and HDL), fasting glucose, fasting cortisol, lean/obese, breastfeeding status, anxiety, depression, FSH, LH, E2, and AMH levels. The best fitting LCA model included 3 classes. For the 3 class LCA, 32.2% ($n=22$) of the sample was in Class 1, 43.4% ($n=30$) of the sample was in Class 2, and 24.3% ($n=17$) of the sample was in Class 3. We

found that women (class 3) who had lower ovarian reserve also had less favorable cardiometabolic and psychological profiles.

These findings suggest that cardiometabolic and psychological factors may contribute to the process of ovarian aging. While numerous studies have examined these factors in association with AMH (Dolleman et al., 2013; La Marca et al., 2012), few have explored patterns within and between women as a means of identifying subgroups. Accurate group classification would facilitate risk stratification and early detection in the context of ovarian aging (Pal et al., 2010). The findings from this study provided preliminary evidence to support the hypothesis that there are modifiable biobehavioral factors that contribute to variation in the process of ovarian aging.

Study Aims, Research Questions, and Hypotheses

Aim 1: To describe the relationship between perceived stress and HPA activity in female childhood cancer survivors.

Research question 1: Is perceived stress associated with HPA activity in female childhood cancer survivors?

Hypothesis: Higher levels of perceived stress will be associated with dysregulated HPA activity.

Research question 2: Is the diurnal pattern of HPA activity (salivary cortisol and IL-6) associated with long-term average cortisol secretion (hair cortisol)?

Hypothesis: Flatter diurnal patterns will be associated with lower long-term average cortisol secretion.

Aim 2: To explore relationships between perceived stress, biomarkers of HPA activity, gonadotropin levels, and AMH in female childhood cancer survivors.

Research question 1: Is perceived stress associated with ovarian function (gonadotropin and AMH levels) in female childhood cancer survivors?

Research question 2: Is dysregulated HPA activity associated with ovarian function (gonadotropin and AMH levels) in female childhood cancer survivors?

Hypothesis: Increased levels of perceived stress and dysregulated HPA activity will be associated with abnormal gonadotropin levels and diminished ovarian reserve.

Study Assumptions

This study was based on several assumptions. The first was that stress, a complex and multifaceted construct, could be measured through a combination of biological and self-report measures. It was assumed that chronic stress could be accurately measured with self-report of perceived stress levels over the past month, diurnal cortisol patterns (intercept and slope), and hair cortisol levels which reflect the average cortisol level over a month or more. Second, the study assumed that participants were reliable and trustworthy sources of information regarding their medical history and that the participants' treatment team kept accurate medical records regarding their diagnoses and treatments. Third, the study assumed that the laboratory testing of hair cortisol, serum gonadotropin levels and serum AMH levels was completed and recorded precisely and accurately.

Chapter 3. Research Design and Methods

Sample and Settings

The sample for this descriptive, cross-sectional cohort study consisted of 24 female subjects between the ages of 16 and 35 who were previously treated for cancer (including leukemia and brain tumors) at the Edinburgh Children's Cancer Centre, Royal Hospital for Sick Children (RHSC). Sample size and power estimates are challenging in an exploratory model of predictors, due to the fact that one is not aware of predictor effects and cannot put forth anticipated effect sizes.

Rules-of-thumb regarding appropriate sample sizes were used to best estimate the necessary sample size. The subject to variable (SV) ratio has been an attempt to ensure that the sample is "large enough" to minimize "parameter inflation" and improve "replicability". While numerous rules-of-thumb exist (Green, 1991), it has been shown that the power for a test of Cohen's (1988) effects sizes (medium: $f^2 = 0.15$ [$R^2 = 0.13$] to large: $f^2 = 0.35$ [$R^2 = 0.26$]) for partial correlation (regression parameters) at approximately $1 - \beta = .80$ ($\alpha = 0.05$) is achieved based on Harris' (1975) rule that $N \geq 50 + 8m$ for medium effects, where $m =$ the number of predictors. This approximates Cohen's estimates. Assuming a maximum number of predictors around six, to achieve power (0.80, $\alpha = 0.05$, two-tailed) a sampling of approximately 41 individuals for six predictors was required. Thus in order to achieve a power to detect regression effects slightly less than a large effect size, a sample of approximately 50 individuals was

needed. Due to several recruitment challenges, obtaining this target sample size was not as feasible as originally anticipated. These challenges will be discussed in detail in the results and discussion.

Cancer survivors are seen annually for follow up at the Royal Hospital for Sick Children (RHSC) (Wallace, Smith, Kelsey, Edgar, & Anderson, 2014). The inclusion criteria were: female patients treated for cancer (including leukemia and brain tumors) at the Edinburgh Children's Cancer Centre, younger than 18 years of age at cancer diagnosis and between 16-35 years of age at the time of the study visit, at least one year following the conclusion of treatment, and signed written informed consent. This age range was selected because subjects are likely to be post-pubertal and in the most fertile window of their reproductive lifespan. Cancer survivors seen for long-term follow-up at the Royal Hospital for Sick Children (RHSC) in Edinburgh, Scotland were selected because of Dr. Anderson's current research on reproductive function and fertility preservation in cancer survivors (Wallace et al., 2014). This collaboration minimized burden to subjects. In the UK, research subjects aged 16 and older are able to provide informed consent. The exclusion criteria were a positive pregnancy test, ovarian surgery in the past 6 months, hormonal therapy in the preceding 3 months (GnRH agonists, recFSH), alcohol or drug abuse/dependence according to ICD-10 criteria, and investigational drug received in the past 3 months (90 days).

Recruitment and Retention Strategies

One day a month, the RHSC medical staff hold annual review appointments for cancer survivors in long-term follow-up. This monthly clinic promotes a multi-disciplinary approach to care and encourages long-term follow-up. Recruitment letters (Appendix 2) were sent to potential study participants two weeks prior to their annual review appointment to inform them of the study and to invite them to consider participating. Subjects could either send their reply slip by mail or inform medical staff of their decision at the monthly clinic.

Research Sites

All data collection occurred at the RHSC. Laboratory analyses were conducted at the Queens Medical Research Institute (QMRI). Data analyses were conducted at Marquette University.

Instruments

Ovarian function: assessment of ovarian function included serum gonadotropin levels (luteinizing hormone (LH), follicle-stimulating hormone (FSH)) and AMH. Serum levels of these hormones are routinely measured during the annual follow-up appointment as part of standard care at NHS affiliated clinics and at regular intervals during in the parent study in Ireland. The laboratory results were retrieved from subjects' medical records. Serum AMH levels were analyzed using the Beckman Coulter AMH GEN II ELISA (Beckman Coulter Inc., Brea, CA). The AMH Gen II ELISA has a sensitivity of 0.57 pmol/l. The validated model of serum anti-Müllerian hormone by Kelsey et al. (2011) and

the nomogram with normative values for age published by LaMarca et al. (2012) will be used to interpret AMH levels (Kelsey et al., 2011a; La Marca et al., 2012).

Psychosocial stress was determined using the Perceived Stress Scale (PSS-10). Perceived stress is a concept that incorporates an understanding of stress as a “cognitively mediated emotional response to an event, not the object of the event itself” (Cohen, Kamarck, & Mermelstein, 1983). Perceived stress was measured using the Perceived Stress Scale, PSS-10, a self-report instrument which measures the degree to which situations in one’s life are appraised as stressful over the past month (Cohen, Kamarck, & Mermelstein, 1983).

Validation of the PSS-14

Cohen et al. (1983) developed the PSS to determine to what extent the respondents found their lives to be “unpredictable, uncontrollable, and overloaded”, as these had been consistently found to be central components of the experience of stress (Cohen & Williamson, 1988). The items were designed for use with community samples with at least a junior high school education. Responses to each item range from “never” to “very often” on a 5-point Likert scale and a higher score indicates greater stress (Cohen et al., 1983).

The measure was validated using two samples of college students and one sample of adults enrolled in a smoking-cessation program. Cohen et al. (1983) first calculated the mean and variance of the PSS-14 scores in each sample to determine if there were significant differences between gender and age. While mean scores were slightly higher in females than in males, the

difference was not statistically significant. They also reported that age was unrelated to scores.

Reliability of the PSS-14

Reliability of the measure was tested in each sample using Cronbach's alpha and test-retest correlations. Cronbach's alpha, a measure of internal consistency, was .84, .85, and .86 respectively. Test-retest correlations were calculated at two different intervals. In the college students, the time between the first and second administration was two days, whereas in the smoking-cessation sample, the interval was six weeks. The test-retest correlations were .85 and .55 for the two-day and the six-week interval respectively. Participants were asked to strive for accuracy rather than consistency across time. In this way, the authors demonstrated that while the test reliably examines perceived stress, test-retest correlations weaken as the time between test administration increases.

Validity of the PSS-14

Validity was examined using tests of concurrent and predictive validity. To test concurrent validity, the CSLES (College Student Life Event Scale) and the life-event scale (for the smoking-cessation sample) were used. The authors anticipated that perceived stress scores would increase with the number of stressful events reported in the life event scales (LES) and that the correlations between the scores would be even higher when the impact scores of the events were included. As predicted, there was a small to moderate correlation between LES and the PSS scores ($r = .35-.49$) and the correlations increased when

including the impact scores. While the authors considered the LES to be the best available self-report measure for testing concurrent validity, the authors recommend the possibility of using a biomarker of stress such as cortisol for future validity testing (Cohen, Kamarck, & Mermelstein, 1983).

To examine predictive validity, Cohen et al. (1983) administered a depression scale (CES-D), an anxiety scale (SADS), a physical symptom checklist (CHIPS), and monitored utilization of health services several months before and after the testing session. They predicted that the PSS would be a better predictor of depression and anxiety, physical symptomatology, and utilization of health services. As anticipated, the PSS was a better predictor of depression ($r = .65-.76$), anxiety ($r = .37-.48$), utilization of health services ($r = .11-.20$), and physical symptomatology ($r = .52-.70$) than the life events scores.

PSS-10

Cohen & Williamson (1988) tested the validity and reliability of the PSS-14 in a large sample of adults ($n = 2,387$) in the United States. The authors conducted a factor analysis using a principle components method with varimax rotation. The analysis revealed a two-factor structure accounting for 41.6% of the variance. The PSS-10, a shorter version, was derived by eliminating the four items with the lowest factor loadings. The authors conducted a second factor analysis with the PSS-10, which resulted in an improvement in both the total explained variance (48.9%) and internal reliability (alpha coefficient = .78).

To examine construct validity of the PSS-10, Cohen & Williamson (1988) examined relations between the PSS scores and other stress measures, health,

health service utilization, health behaviors, life satisfaction, and help seeking. Similar to the PSS-14, the PSS-10 was moderately correlated with the life event scale ($r = .32$). The PSS scores were weakly correlated with self-reported health behaviors, weakly or moderately correlated with self-reported physical illness, and moderately correlated with scores on the life satisfaction scale ($r = .47$). The authors conclude that the PSS-10 is as reliable and valid a measure of perceived stress as the PSS-14. The PSS-10 also had a tighter factor structure and a slightly better internal reliability. They therefore recommend the PSS-10 for use in research.

Utilization of PSS-10 in research

The PSS-10 is one of the most widely used measures of psychological stress (E.-H. Lee, 2012). It has been validated for use in numerous populations and is currently translated into 25 languages other than English (E.-H. Lee, 2012). The PSS-10 has been utilized as a measure of perceived stress to examine the association between stress and reproductive outcomes (Bleil et al., 2012; Li, Newell-Price, Jones, Ledger, & Li, 2012; Lynch, Sundaram, Maisog, Sweeney, & Buck Louis, 2014b; Schliep et al., 2015), to quantify stress levels in cancer survivors (Keir, Swartz, & Friedman, 2007), and to examine the effectiveness of interventions to reduce stress in cancer survivors (Loprinzi, Prasad, Schroeder, & Sood, 2011). For example, Loprinzi et al. (2011) assessed the effect of a SMART (Stress Management and Resiliency Training) program for increasing resiliency and decreasing stress and anxiety among breast cancer survivors. A total of twelve patients completed the intervention, and eight patients

were in the control group. No significant differences in any of the measures were noted in the control group. In the intervention group, the PSS score decreased from 22.1 ± 5.9 initially to 12.8 ± 6.6 ($p = .003$). The change in PSS score was equivalent in magnitude to other concurrent measures of anxiety and resilience. Based on previous use, the PSS-10 is an appropriate measure of stress in female cancer survivors.

HPA Activity

HPA activity was determined using measures of salivary cortisol, salivary IL-6 and hair cortisol. Analysis of salivary cortisol and IL-6 were performed in Dr. Anderson's lab under the guidance of the Queen's Medical Research Institute (QMRI) staff and in Dr. Bryan Hennessy's lab under the guidance of the Royal College of Surgeons in Ireland (RCSI) staff. Hair (1 cm) will be processed for extraction of cortisol in the laboratory of Clemens-Kirschbaum in Dresden, Germany as previously described (Kirschbaum, Tietze, Skoluda, & Dettenborn, 2009).

Salivary cortisol

Cortisol is the main glucocorticoid hormone produced in the adrenal cortex. Approximately 90% of cortisol in blood is bound to plasma proteins (corticosteroid-binding globulin and albumin), leaving only about 5-10% unbound (Kahn, Rubinow, Davis, Kling, & Post, 1988; Kirschbaum & Hellhammer, 1989). This latter fraction, which is reflected in salivary cortisol, reflects the biologically active corticosteroid that is capable of acting on target tissues (Kirschbaum &

Hellhammer, 1989). Cortisol is not secreted continuously, but rather in pulses influenced by circadian rhythmicity, evident in its well-characterized diurnal pattern with levels highest in the early morning hours and lowest around midnight (Kirschbaum & Hellhammer, 1989).

Advantages

Salivary cortisol has many advantages over prior measures used to examine HPA activity. In comparison to serum cortisol, salivary cortisol is noninvasive, providing an accurate measure of HPA activity without the risk of provoking the stress response and potentially compromising the findings (Raff, 2009). It is easy to collect, making it convenient in both the clinical and research settings, especially in cases when repeated sampling is required. Studies have reported high compliance with home collection of salivary cortisol with minimal burden to participants (Bergen et al., 2012).

Validity and Reliability

Validity of salivary cortisol has been established by examining correlations with plasma cortisol levels. The linear correlations between salivary and plasma cortisol are high, ranging from $r = 0.54$ to $r = 0.97$, with most investigators reporting correlation coefficients of $r \geq 0.90$. The lower correlations were reported in studies comparing salivary cortisol levels with total plasma cortisol levels (Kirschbaum & Hellhammer, 1989). Since salivary cortisol levels reflect unbound cortisol, correlations with total plasma cortisol levels would yield much lower coefficients (Inder, Dimeski, & Russell, 2012). Reliability is assay-dependent. A

commercially available Enzyme-Linked Immuno-Sorbent Assay (ELISA) kit was used for the quantification of cortisol in saliva (Salimetrics, Suffolk, UK KIT 1-3002). This cortisol assay kit has a lower detection limit of 7 ng/dl. The mean intra-assay coefficient is 5.7% and the mean inter-assay coefficient is 10%.

Influences on cortisol levels

Saliva flow rate and viscosity do not significantly affect salivary cortisol levels, making the measure robust in a variety of clinical and research conditions (Kirschbaum & Hellhammer, 1989). While there is considerable variation, most studies recommend participants not eat, drink, or brush their teeth prior to sample collection. There is evidence that habitual smoking increases salivary cortisol levels as well as exercising within 30 minutes of collection (Kirschbaum & Hellhammer, 1989). During pregnancy, cortisol levels are elevated due to the estrogen-induced rise in cortisol binding globulin and up-regulation of the maternal HPA axis (Jung et al., 2011). Similarly, cortisol binding globulin levels are elevated in women taking estrogen-containing oral contraceptives (Raff, 2009). Salivary cortisol is not sex-dependent, however concentrations may decline with age (Inder et al., 2012). Because cortisol is a stable molecule, saliva samples can be stored in a home refrigerator at 4-8° for up to 7 days, after which they can be frozen and stored at -20° or -80° for up to 12 months without depreciation of concentrations (Inder et al., 2012).

Use in research

Salivary cortisol has been used in numerous studies as a reliable measure of the stress response and HPA activity (An et al., 2015). Chronic stress is associated with alterations in the cortisol awakening response (Dedovic & Ngiam, 2015; Révész et al., 2013), and blunting of the morning-evening diurnal slope (Carvalho et al., 2015; Garland, Beck, Lipschitz, & Nakamura, 2014; Ho, Fong, Chan, & Chan, 2013; Schrepf et al., 2015). It has been used to measure HPA activity in cancer survivors and other populations (Du et al., 2013; Ho et al., 2013; Sephton et al., 2013).

Salivary IL-6

Interleukin-6 (IL-6) is one of the major cytokines that stimulates HPA activity in response to inflammatory stress and is thought to play a role in the mechanisms linking psychosocial stress with increased risk of chronic disease (Sjögren, Leanderson, Kristenson, & Ernerudh, 2006). Chronic stress is associated with elevated levels of pro-inflammatory cytokines, which may reflect reduced sensitivity to the inhibitory effects of cortisol (Sribanditmongkol et al., 2014).

Validity and Reliability

Unlike salivary cortisol, salivary IL-6 has not been shown to correlate with serum/plasma levels of IL-6. Salivary IL-6 is thought to reflect localized rather than systemic inflammation (Izawa, Sugaya, et al., 2013). However, Izawa et al. demonstrated elevated salivary IL-6 concentrations post-TSST (Trier Social

Stress Test) when compared to baseline concentrations ($p < .01$), indicating a close association between salivary IL-6 and psychosocial stress. They also demonstrated significant correlations between salivary IL-6 and salivary cortisol ($p < .05$) post-TSST. This association was not seen at baseline, suggesting that the correlation between the inflammatory response and HPA activity is specific to stressful conditions (Izawa, Sugaya, et al., 2013).

Izawa et al. also described the relationship between IL-6 and cortisol as dependent on proximity to and duration of the stressor. Following an acute stressor, both IL-6 and cortisol remain elevated for approximately 30-60 minutes. Cortisol then suppresses IL-6 production, changing their relationship from a positive to an inverse correlation. However, in chronic stress, hypocortisolism may occur causing salivary IL-6 to remain elevated (DeSantis et al., 2012; Nikkheslat et al., 2015). Thus, salivary IL-6 is particularly useful in helping to distinguish between acute and chronic stress when collected in conjunction with salivary cortisol (Izawa, Sugaya, et al., 2013). Salivary IL-6 has a diurnal pattern similar to that of cortisol, peaking in the morning immediately after awakening and then gradually declining into the afternoon and evening. However, unlike salivary cortisol, salivary IL-6 peaks again at midnight before going to bed (Izawa, Miki, Liu, & Ogawa, 2013).

A commercially available Enzyme-Linked Immuno-Sorbent Assay (ELISA) kit was used for the quantification of salivary IL-6 (Salimetrics, Suffolk, UK KIT 1-3602). This IL-6 assay kit has a lower detection limit of 7 ng/dl, intra-assay coefficient of 4.8% and mean inter-assay coefficient of 6.6%.

Hair cortisol

Psychosocial stress has also been associated with elevated levels of cortisol in hair (Russell et al., 2012; Wells et al., 2014). Hair has a predictable growth rate of 1 cm/month; the most proximal 1 cm segment to the scalp approximates the last month's cortisol production, the second most proximal segment the month prior to that, and so on (Russell et al., 2012). Hair cortisol provides several advantages over current measures of cortisol. It permits a retrospective analysis of cortisol secretion, allowing researchers to examine cortisol secretion before an event occurred (Stalder & Kirschbaum, 2012). Hair is also easy and non-invasive to collect; a sample of hair is cut from the vertex posterior region of the scalp as close to the skin with scissors (Raul, Cirimele, Ludes, & Kintz, 2004). Only a small amount (about 10mg) is needed to extract cortisol concentrations. Sample storage is convenient; hair cortisol can be stored at room temperature in aluminum foil for extended periods of time (Raul et al., 2004).

Protocol for hair cortisol analysis

To extract cortisol from hair, the sample is weighed and then cut into segments correlating with the time period of interest. Then, the hair is finely minced with scissors, and incubated overnight in a solvent such as methanol. The resulting solution is evaporated until the sample is dry and methanol is removed from the sample. Then the sample is reconstituted in a solution such as phosphate buffered saline (PBS). Following the extraction, an ELISA is used for

cortisol quantification (Raul et al., 2004; Russell et al., 2012; Sauvé, Koren, Walsh, Tokmakejian, & Van Uum, 2007).

Validation of hair cortisol assay

Cortisol was first measured in human hair by Raul et al. (2004) using liquid chromatography-mass spectrometry analysis and then further validated by Sauve et al. (2007) using an ELISA. The methodology and results of both studies will be summarized.

Measurement range

Raul et al. (2004) performed liquid chromatography-mass spectrometry analysis on 44 hair samples from 17 men and 27 women. Ages ranged from 2 to 90 years old (mean 36 years old). Four hair color categories were distinguished; black, brown, grey and blond (Raul et al., 2004). None of the analyzed samples' concentrations were below the limit of quantification of 5 pg/mg, defined as the "lowest reported quantitative value, within the established acceptable error of the essay" (Rifai, Gillette, & Carr, 2006). The limit of detection, defined as "the lowest value that exceeds the measurand value obtained with a blank sample" (Rifai et al., 2006), was 1 pg/mg. Linearity, which "refers to the range of measurand values over which there exists a constant relationship between observed and expected values" (Rifai et al., 2006), was between 1 and 500 pg/mg. Cortisol concentrations ranged from 5.2 to 91 pg/mg with a mean value of 19 pg/mg. Concentrations of cortisol were not different based on groups (age, natural hair color).

Sauve et al. (2007) were the first to use a modified saliva ELISA to measure cortisol concentrations in human hair (Sauvé et al., 2007). They used a sample of 39 non-obese subjects, 19 male and 20 female, to develop and validate an assay for the measurement of cortisol in human hair. For each subject, hair, urine, saliva and serum samples were collected for cortisol analysis. Sauve et al. (2007) also examined the effect of hair color (n=12) and sample location (n=14) (e.g. posterior vertex) on cortisol concentrations, as well as the difference between hair cortisol concentrations in hair that had been dyed (n=14) and hair that was untreated (n=25).

Hair cortisol levels were not normally distributed, but after log transformation, the distribution was normal. The reference range for cortisol levels in hair of healthy non-obese individuals was 17.7-153.2 pg/mg of hair with a median of 46.1 pg/mg. Hair cortisol concentrations did not differ significantly based on natural hair color. However, hair that was dyed before sample collection (n=14) had lower cortisol levels ($p < .05$) than hair that was untreated (n=25) (Sauvé et al., 2007).

Accuracy and Precision

Accuracy is the “closeness of agreement between the value of a measurement and the true concentration of the measurand in the sample” (Rifai et al., 2006). Raul et al. (2004) examined accuracy using calibration curves. To obtain calibration curves for cortisol concentrations under 20 pg/mg, the authors could not use blank hair (physiological concentrations being sometimes below 20

pg/mg). For this reason, they used melanin to obtain a “blank” hair as close as possible to human hair (Raul et al., 2004).

Sauve et al. (2007) determined accuracy by examining recovery, calculated by adding various concentrations of cortisol to the hair sample and comparing the measured amount with the added amount (Rifai et al., 2006). The mean extraction recovery of 80 ng/ml and 2 ng/ml cortisol standards (n=5) from PBS was 87.4% and 83.6% respectively. When hair samples were spiked with 100 ng/ml, 50 ng/ml and 2 ng/ml (n=6) cortisol standards, incubated and extracted, the mean extraction recoveries were 87.9%, 88.9% and 87.4%, respectively (Sauvé et al., 2007).

Precision is the “closeness of agreement between independent test results obtained under stipulated conditions” (Andreasson et al., 2015, p. 3). Raul et al. (2004) examined precision through repeatability. Intra-day repeatability (CV value) was 11% for cortisol (at 7 pg/mg). Sauve et al. examined the precision of the ELISA assay using two hair samples with an average (about 60 pg/mg) and a high cortisol level (about 600 pg/mg). The intra-assay coefficients of variation for these samples were 7.2% and 6.0%, respectively. Inter-day precision had a coefficient of variation of 10.6% for the sample of average concentration and 7.6% for the sample of high cortisol content (Sauvé et al., 2007). The mean coefficient of variation of hair cortisol levels from samples obtained from the vertex posterior of 14 individuals was 15.6%. The mean coefficient of variation for cortisol levels from hair samples taken from various parts of the head was 30.5%.

Based on these results, Sauve et al. (2007) recommend hair samples be taken from the vertex superior in order to obtain consistent results.

Validity

Sauve et al (2007) established validity of the hair cortisol ELISA assay by examining correlations between hair cortisol concentrations and concentrations of cortisol in urine, saliva, and serum. There was a correlation between hair cortisol levels and 24-hr urine cortisol ($r=0.33$; $P=0.041$) and between serum and salivary cortisol ($r=0.58$; $P=0.0016$). The strong correlation between saliva and serum cortisol can be explained because both matrices reflect short-term cortisol secretion. One explanation for the weaker correlation between hair cortisol and urinary cortisol is the difference in the time frames that are reflected by the measurements: urine represents cortisol secretion during one day, whereas hair cortisol levels represent levels during 1-2 months (Sauvé et al., 2007).

Stability reliability

Stalder et al. (2012) examined patterns of intra-individual stability in hair cortisol levels in two independent study groups. In study I, hair was collected from 45 participants at two time points one year apart from each other. In study II, 64 participants provided hair samples at three time points occurring at two-month intervals. In both studies, relevant psychosocial variables and hair-related variables were also assessed. Results of both studies consistently revealed strong test-retest associations for repeated hair cortisol measurements (r 's between 0.68 and 0.79, p 's <0.0001). The authors concluded that hair cortisol

concentrations demonstrate a considerable degree of intra-individual stability (Stalder et al., 2012).

Validity in known hypercortisolemic conditions

Several studies have demonstrated validity of hair cortisol by examining cortisol concentrations in individuals with known hypercortisolemic conditions. D'Anna-Hernandez et al. (2011) examined associations between hair and salivary cortisol throughout pregnancy and in the first three months postpartum. Cortisol levels are known to increase throughout pregnancy (D'Anna-Hernandez, Ross, Natvig, & Laudenslager, 2011). Findings demonstrated a significant effect of pregnancy phase on hair cortisol levels ($p = 0.003$), with levels higher in the third trimester relative to the first trimester ($p < 0.001$). Overall mean salivary and hair cortisol levels calculated across all time points were significantly correlated ($p = 0.047$).

Association between hair cortisol and self-report measures

Several studies have also examined the association between hair cortisol and self-report measures of stress, with variable results. Wells et al. (2014) used a pooled database from diverse community samples to examine associations between hair cortisol concentrations and self-reported measures of stress as well as mental health measures. Two centimeters of hair (reflecting the last two months) were collected from participants for analysis of cortisol. Stress was measured using the PSS-10 and the Chronic Stress Scale. To measure mental health, the following data was collected: depression and anxiety (using screening

questions taken from the DSM-IV); drug, alcohol and antidepressant use; smoking status; WHO Disability Assessment (WHO-DAS II) (to assess limitations in activities and daily functioning); and intimate partner aggression.

Hair cortisol concentrations (HCC) were significantly related to the use of antidepressants, hazardous drinking, smoking, disability and aggression by partner, after controlling for confounders. Correlations of HCC with self-reported chronic stress and perceived stress were small and both were non-significant after controlling for confounders. The authors provide several possible explanations for the lack of association between HCC and self-report measures of stress. The first explanation they offer is that HCC and self-report measures of stress such as the PSS-10 do not reflect the same time frame. Second, they offer the explanation that the relationship between hair cortisol and self-report measures of stress may not be linear. They explored alternate models, and found that HCC increased at higher levels of perceived stress but decreased at the highest levels of stress, suggesting an inverse U relationship (Wells et al., 2014).

Conclusions

The Perceived Stress Scale-10 is a valid and reliable measure of stress of an individual's perceived stress over the past month. Salivary cortisol and IL-6 are measures of current HPA activity whereas hair cortisol reflects integrated cortisol secretion over the past month. Together they can be used to measure biological and behavioral levels of stress, providing a more complete picture of the stress response. These measures were used in this study to examine the

associations between chronic stress and ovarian function in female cancer survivors.

Data Collection Schedule and Procedures

Recruitment began by sending letters of invitation to female cancer survivors in long-term follow-up, two weeks prior to their annual review appointment. Letters included a Patient Information Sheet and a reply slip (Appendix E). By returning the reply slip, patients agreed to be contacted by the research team. This manner of recruitment prevented patients from feeling any obligation to participate. After consenting to be contacted, patients were interviewed over the phone to determine eligibility to participate in the study. If eligibility requirements were met and the patient agreed to participate, a 30-minute study visit at the RHSC was scheduled at a mutually agreed upon time. The study visit was scheduled within a week of the subject's annual review visit. Written consent was obtained from subjects at the study visit. Each subject was assigned a unique study ID and data was entered into an SPSS file on an encrypted computer in a locked office.

Procedure Data Collection

Data was collected in this order: saliva, demographic and PSS-10 questionnaires, hair. Salivette collecting devices and detailed instructions for collection of samples were provided to participants at their annual review appointment. Subjects were instructed not to eat, consume caffeine or exercise for 30 minutes prior to sample collection (Schrepf et al., 2015). Subjects were

instructed to record the date/time of saliva collection and to refrigerate samples immediately following collection to preserve sample integrity. Subjects provided four timed saliva samples: the first sample was collected before going to bed on the night prior to the study visit, the second immediately upon awakening the morning of their visit before getting out of bed, and the third and fourth were collected 30 minutes after the second sample. Subjects were instructed to bring the four saliva samples to the study visit. Samples were labeled with a unique subject study ID. Saliva samples were stored at the RHSC at -20 C. Dr. Anderson's research nurse picked them up within a week of collection and brought them to the QMRI, where they were centrifuged and stored at -80 C.

During the study visit, subjects provided demographic information including age, ethnicity/race, years of formal education, marital status, employment status, number of pregnancies and live births, and income status (Appendix D). Subjects then completed the PSS-10, a 10-item questionnaire, which measures perceived stress over the past month (Cohen et al., 1983). Lastly, subjects provided a sample of hair for measurement of hair cortisol. A small sample of hair, 20mg (100-150 strands), was taken from the vertex posterior of the participant's scalp using a new pair of thinning shears (Hoffman, Karban, Benitez, Goodteacher, & Laudenslager, 2014; Van Uum et al., 2008). Hair length was recorded and proximal ends of the hair samples were marked. Hair samples were stored in aluminum foil for protection before processing (Hoffman et al., 2014) and labeled with a unique subject study ID. Wells et al. (2014) reported that 95% of 247 women consented to hair sampling conducted in

this manner. To minimize variation in sample collection, research nurses were trained on how to collect and store hair samples.

The following clinical characteristics were retrieved from subjects' medical records: age at cancer diagnosis, cancer type, treatment type and duration, age at completion of treatment, comorbidities, prescription medications, and reproductive history (age at menarche, last menstrual period, menstrual cycle regularity, and parity). Reproductive hormone levels (AMH, FSH, LH and E2), measured as part of routine care at the annual review visit, were also retrieved from subjects' medical records. This data was used to describe the sample characteristics and explore subgroup effects on associations between variables (aim 1).

Amendment to Data Collection Procedures

During data collection, it was discovered that many subjects travel a great distance to the hospital for clinic visits. Therefore, to reduce the burden of participation, subjects were given the option to receive a research packet in the mail if they were unable to return to the Royal Hospital for Sick Children for a separate study visit because of the travel distance. Since the current methods already required subjects to collect saliva samples at home, the main change was that subjects who decided to receive a research packet in the mail also self-collected a hair sample. In a study of 70 multi-ethnic breast cancer survivors, Ramirez et al. examined the feasibility of self-collection of hair samples and demonstrated that it was a reliable and non-invasive way to measure cortisol (Ramirez et al., 2017). Participants were instructed on how to collect the hair

sample after consenting to participate in the study. They were also asked to complete the PSS-10 at home. Biological samples and questionnaires were labeled with the subject's study ID so as to ensure confidentiality. No identifying information was included in or on the research packet. The research packet included prepaid return packaging and an ice pack so that subjects could return samples and questionnaires to the Royal Hospital for Sick Children for processing. Clements & Parker (1998) demonstrated that salivary cortisol concentrations of samples frozen immediately after collection and samples sent on ice were highly correlated (Clements & Parker, 1998).

Data Analysis and Interpretation

Means and standard deviations or frequency distributions, as appropriate, for all demographic and clinical variables were reported to describe the sample and reveal distributional characteristics.

Latent Growth Curve Modeling was used to examine diurnal cortisol patterns. Bayesian Structural Equation Modeling was used to explore the relationship between perceived stress and HPA activity and to explore relationships between perceived stress, biomarkers of HPA activity, gonadotropin levels, and AMH.

Latent Growth Curve Modeling

Latent Growth Curve Modeling was used to define diurnal cortisol change over time (three time points). Growth curve modeling (GCM) examines change as a trajectory and assumes that trajectories vary across participants (Barker,

Rancourt, & Jelalian, 2013). This method was selected since chronic stress is characterized by a persistent and unstable trajectory of HPA activity (Hellhammer, Wüst, & Kudielka, 2009). Change processes are determined by combining the underlying pattern of growth (slope) with the initial or baseline measurement (intercept). The intercept and slope of this trajectory were then incorporated into the structural equation model as independent predictors of ovarian function. GCM was selected instead of more commonly utilized methods of analyzing diurnal cortisol (e.g. area under the curve, cortisol awakening response) because it permits a robust examination of change in small samples.

GCM was evaluated as $y_{it} = \lambda_{int}\eta_{int} + \lambda_{slp}\eta_{slp} + e_t$, where y_{it} represents the observations for each subject i at each time t . λ_{int} represents the vector of fixed values that define the latent intercept, this is $\lambda_{int} = [1, 1, 1]$, while λ_{slp} represents the vector of fixed values that define the linear slope, this is $\lambda_{slp} = [0, 9.3, 9.8]$. This represents the average time between the first observation to the second (9.3 hours), and from the first observation to the third observation (9.8 hours). η_{int} represents the latent intercepts, while η_{slp} represents the latent slope, for which we estimate the respective mean and variance. The means represent the average starting point and average change over time, while the respective variance represents how much the subjects vary from the respective mean. Lastly, e_t represents the residual variance at each time point t , this indicates that the GCM is estimated without the assumption of homogeneity of variance over time, the residual variance is allowed to differ over time.

Bayesian analysis

In Bayesian analysis, statistical inferences are drawn from parameter values that could give rise to the observed data (Scheines, Hoijtink, & Boomsma, 1999). The term prior is used to refer to the expected parameter distribution based on theory or previous research. The posterior distribution is the parameter distribution that results from multiplying the prior distribution by the likelihood (as observed in the sample data). The posterior distribution describes the behavior of the parameters conditional on the data. Because the Bayesian framework makes inferences from the posterior distribution, it allows researchers to make direct inferences about the parameters given the observed data. The posterior distribution describes both the best estimate given the data, and the level of uncertainty about the respective parameter by the width of it, typically represented by the credible interval (Yuan & MacKinnon, 2009).

The Bayesian framework is an appropriate method for small sample sizes. Frequentist methods rely on the asymptotically correct estimation methods, basing the inference in function of the null hypothesis (Wagenmakers et al., 2008). Bayesian inference bases the inference on the observed data, which allows estimation of parameters with small samples in conditions where frequentist estimation fails (Lee & Song, 2004).

The Bayesian approach requires specification of prior distributions for each of the model parameters (Palomo, Dunson, & Bollen, 2007). The selection of the prior distribution is important because the posterior distribution is informed by the prior distribution and the likelihood of the data (Palomo et al., 2007). The

prior distribution incorporates information drawn from previous empirical studies or theory regarding the model parameters. Given the exploratory nature of this study, we first examined the model parameters using weakly informed priors. Weakly informed priors are not intended to guide the parameters, but instead provide information to delimitate the most likely data space for the parameters. Means/intercepts have a prior $\mu \sim N(0, 100)$; the standard deviations have a prior $\delta \sim \text{half-cauchy}(0, 2.5)$; the regressions have a prior $\beta \sim N(0, 50)$; and the correlations have a prior $\rho \sim U(-1, 1)$.

Strong hypotheses are tested by model comparison. A model without constraints was compared to a model with constraints in the regressions (β), constraining if the β is positive or negative (according to theory). These constraints are set by specifying the respective β prior as a truncated normal (e.g: $\beta \sim N(0, 50)T(0,)$) not allowing β to be above/below 0. Model comparison allowed us to evaluate if the model with directional constraints fit the data better than the unconstrained model. If the directional constraint model fit better, it is evidence that the β should be directional. The models were compared with Information Criteria Leave-One-Out (LOO), and Widely Applicable (WAIC) (Vehtari, Gelman, and Gabry, 2016; Gelman et al., 2013; Raftery, 1993; Kass, and Raftery, 1995). The theoretical model including the hypothesized directionality of the associations is presented in Figure 1.

Bayesian Structural Equation Modeling

Structural Equation Modeling (SEM) provides a broad framework for modeling relationships with multivariate outcomes (Palomo et al., 2007). The purpose of SEM is to test a theory by specifying a model that represents predictions about the theory's constructs measured with appropriate observed variables (Kline, 2016, p. 10). The outcome of SEM is a set of model parameters for hypothesized effects given the data. From these parameters, a set of logical implications result about the nature of the relationship or non-relationship between the variables (Kline, 2016, p. 10). While SEM typically includes an approximation of unobserved or latent constructs as well as an estimation of the structure of the relationships among these observed constructs, in this study we used a special case of SEM called path analysis. In path analysis, every variable in the model is directly measured or observed (Bryan, Schmiede, & Broaddus, 2007). While latent variables account for unreliability of measures and uncertainty in the model, they can also complicate a model, especially when the sample size is small. Path analysis was selected instead of multiple linear regression because it allowed us to test multiple relationships between variables, with multiple outcomes, conforming to the hypothesized model in this study. Establishing relationships including multiple paths and outcomes allowed us to test the presence of relationships accounting for the relationships with other variables of interest (Kline, 2015).

Bayesian SEM was selected because it facilitates greater precision in estimating the posterior distribution of the parameters for small samples.

Additionally, Bayesian SEM offers the advantage of incorporating previous knowledge about the model's parameters through the use of priors. Thus, Bayesian SEM provided an excellent framework for exploring the relationships between this study's variables, allowing for an appropriate degree of uncertainty when estimating the model's parameters (Scheines et al., 1999). We ran a series of path analysis models with salivary cortisol intercept and slope, PSS, Hair cortisol, BMI, Age, Age at diagnosis, and Age at menarche as predictors and with AMH, E2, FSH, and LH, as outcomes.

Because the aim of the study was to explore relationships between variables, the analysis was not strictly confirmatory. If the data did not fit the model specified, alternative models were tested. The purpose of exploring various models was to provide additional information about the plausible relationships between the variables of interest and make recommendations for future model testing. Data augmentation was used to account for missing data. Missing data was treated simultaneously as a parameter and data, filling any missing data points with the most likely value given the posterior distribution. At each iteration of the estimation, likely values were imputed for the missing data. These likely values were drawn from the posterior distribution estimated at that point in the iterations. In later steps of the estimation, the likely values were used as data in the path analysis, and this process continued until the model had converged (Merkle, 2011).

Data analysis was conducted in R (R Core Team, 2017). Bayesian path analysis was run with the R package *blavaan* (Merkle & Rosseel, 2016), which

estimates the model with the general Bayesian software JAGS (Plummer, 2003). Convergence of the Markov chains was determined using the potential scale reduction factor (PSRF), also known as univariate R-hat (see Gelman & Rubin, 1992). It was determined that the model converged when R-hat was lower than 1.10 for every parameter (Brooks & Gelman, 1998). The models were run with 3 chains, keeping the last 5000 iterations from each chain to build the posterior distributions. Model comparison with LOO and WAIC were done with loo R package (Vehtari, Gelman, and Gabry, 2016b); smaller values for LOO and WAIC represent a better fit.

Protection of Human Subjects

Human Subjects Involvement and Characteristics, and Design

The goal of the proposed study was to explore relationships between perceived stress, biomarkers of (hypothalamic-pituitary-adrenal) HPA activity, and ovarian function in female survivors of childhood cancer. This population was selected because female survivors of childhood cancer are at increased risk of experiencing a premature decline in ovarian function. Additionally, cancer survivors have higher levels of stress and HPA dysregulation. The association between HPA dysregulation and ovarian function in cancer survivors has not been examined. The present study sought to fill this research gap so as to better understand factors associated with this late effect of cancer treatment.

Ethics approval in Scotland

The South East Scotland Research Ethics Committee approved the study. Approval included recruitment materials and methods, consent forms, and data collection protocols. The South East Scotland Research Ethics Committee approved any changes made to the study protocol before they were implemented.

IRB Oversight

Marquette University's Institutional Review Board (IRB) reviews and approves research involving the use of human subjects under the jurisdiction of Marquette University. The IRB is charged with the responsibility of ensuring the adequacy of the research plan, to minimize risks and to maximize the potential benefits for human subjects who participate in research, based on federal and state regulations as well as institutional policies. The proposed study was approved by Marquette University's IRB.

Data Management

Data management was compliant with the most current guidelines of the Complete Health Insurance Portability and Accountability Act (HIPAA) of 1996 and in full conformance with the principles of the "Declaration of Helsinki", in accordance with the principles laid down in the Convention of the Council of Europe for the protection of human rights and dignity of the human being with regard to the application of biology and medicine.

Original patient names and hospital barcodes were de-identified by using a new, independent numbering system for all study related documentation (ie pseudo-anonymisation). A link-list was established and kept separate from other study documentation. The link-list was only accessible by the Principal Investigator (Theresa Hardy), Dr. Anderson, Dr. Wallace, and Rachel McAndrew (oncology research nurse). Theresa Hardy was granted an NHS Lothian visiting researcher passport giving her access to clinical data once participants consented to participate in the study.

Data Protection

All personal information was kept separate from the research record. Consent forms (Appendix F) were stored in locked filing cabinets in locked NHS premises at the Department of Pediatric Oncology, RHSC. Consent forms were stored on the NHS premises until the completion of the study. No identifying information will be stored on secure University computers, with all relevant files accessed only by the researchers and protected by passwords. Computer-stored data will be on password-protected computers/servers without identifying information.

Potential Risks

The risks to subjects are minimal. The time required to collect saliva samples at home as well as involvement in the study may be an inconvenience to subjects. The collection of hair samples may cause some discomfort to subjects. However, previous researchers have found taking a small hair sample

from the vertex to be very acceptable (personal communication from Dr. Jodi Ford, Ohio State University, Columbus, Ohio, July 2015). The PSS-10 is a measure of stress perceived over the past month. As subjects recall stress, they may experience some distress.

Adequacy of Protection Against Risk

Recruitment and Informed Consent

All recruitment materials and procedures were reviewed and approved by the South East Scotland Research Ethics Committee and the Marquette University IRB prior to initiating data collection. The letter of invitation (Appendix) sent to potential subjects included a description of the study, what was involved if they agreed to take part, and an explanation of risks. Written informed consent was obtained at the annual review appointment. Potential subjects had the opportunity to ask questions before providing consent. Participants were allowed to withdraw from the study at any point. Any patients identified as participating in ongoing interventional research were not recruited.

Protection Against Risk

The primary risk of the proposed study was risk of disclosure of personal data, which was minimized by the data management and security measures described above. Risk of harm associated with biological specimen collection was minimized by adhering closely to study procedures and allowing subjects to withdraw from the study at any point. NHS staff were available to treat any adverse effects due to study collection procedures, and any subject that

expressed emotional distress as a result of recalling perceived stress were referred to a member of the interdisciplinary care team.

Potential Benefits of the Proposed Research to Human Subjects

Any relevant clinical information obtained during the course of the study was made available to participants during routine care.

Importance of the Knowledge to be Gained

The findings from this study will be used to improve knowledge of factors associated with fertility and ovarian function in female survivors of childhood cancer, which may inform decision-making prior to and after treatment of cancer in young women. Reproductive potential is a major concern for cancer survivors. The knowledge gained from this study will directly contribute to quality of life for female cancer survivors. Potential risks to subjects are minimal and reasonable in relation to the importance of the knowledge that will be obtained.

Chapter 4: Results

The findings from this study are presented in the manuscript entitled, “Chronic Stress and Reproductive Function in Female Childhood Cancer Survivors” and can be found in Appendix C.

Chapter 5: Discussion, Implications, and Conclusion

In this chapter, the main findings from this dissertation will be summarized. The strengths and limitations of the study and the implications for nursing practice, education and research will be discussed.

Chronic Stress and Reproductive Function in Female Childhood Cancer Survivors

In this study, we examined the association between chronic stress and ovarian function in childhood cancer survivors. The hypothalamic-pituitary-adrenal (HPA) axis is one of the primary regulators of reproductive function (Shannon Whirledge & Cidlowski, 2013b). As high levels of psychological stress and HPA dysregulation have been observed in childhood cancer survivors (Oancea et al., 2014; Taylor et al., 2012; Zeltzer et al., 2009), we hypothesized that increased psychological stress and HPA dysregulation would be associated with lower levels of gonadotropins and anti-Müllerian hormone (AMH), a biomarker of the ovarian reserve. We found that perceived stress was negatively associated with ovarian function, and that biomarkers of HPA activity were positively associated with ovarian function in female childhood cancer survivors. The findings from this study provide preliminary evidence supporting the hypothesis that chronic stress negatively impacts ovarian function and may contribute to infertility in female childhood cancer survivors.

While many cancer survivors encounter impaired fertility after treatment, few studies have examined predictors of the ovarian reserve in childhood cancer survivors other than diagnosis and treatment-related factors. Pre-treatment

planning and the ability to provide individualized fertility prognoses depends on increased understanding of factors associated with the ovarian reserve in this population (Anderson et al., 2013; Knopman et al., 2010). The findings from this study provide the foundation for further research examining factors that contribute to risk for post-treatment reproductive dysfunction in female childhood cancer survivors.

Strengths of the study

This exploratory study has several strengths. First, this study involved interdisciplinary collaboration, bringing together expertise from oncology, reproductive endocrinology and nursing to examine factors associated with post-treatment reproductive function with the aim of improving quality of life for childhood cancer survivors (Kremer et al., 2013). Second, we used Bayesian structural equation modeling to address the study's aims, a useful approach for small sample sizes. Bayesian inference is based on the observed data, permitting estimation of parameters with small samples in conditions where frequentist estimation fails (Lee & Song, 2004). Third, while the sample size was small, the association between chronic stress, measured as both perceived stress and HPA activity, and ovarian function has not been previously examined in childhood cancer survivors. As this population is at higher risk of infertility, the findings from this study provide the foundation for further hypothesis testing.

And last, this study demonstrates the feasibility of examining factors that contribute to reproductive function in this population. Of those asked to participate in the study, all but one consented. The participants expressed

interest in contributing to research in this area. Contrary to our concern that cancer survivors might be reluctant to provide a hair sample, no one refused to participate for this reason. Hair cortisol has not been utilized as a measure of chronic stress in childhood cancer survivors; this study demonstrates that this measure may be a useful non-invasive biomarker of chronic stress in this population.

Limitations of the study

Several aspects of data collection may have impacted the study's internal validity. Clinical and demographic information was collected using self-report. To reduce the potential for recall bias, we verified information using medical records. The Perceived Stress Scale (PSS-10) also relies on subject recall. However, the PSS-10 is the most widely used measure of perceived stress and has demonstrated good to excellent reliability in varied populations (Sheldon Cohen et al., 1983). The measure has also been utilized to reliably measure stress appraisal in cancer survivors (Golden-Kreutz, Browne, Frierson, & Andersen, 2009).

As the Royal Hospital for Sick Children was far away for many patients, participants were given the choice to self-collect hair and saliva samples at home rather than returning to the hospital for a study visit. Participants were provided with a prepaid packet and an ice pack to return the samples; postal transportation of samples may have affected sample integrity. However, Clements & Parker (1998) demonstrated that salivary cortisol concentrations of samples frozen immediately after collection and samples sent on ice were highly

correlated (Clements & Parker, 1998). In addition, the amendment allowed participants to self-collect hair samples. In a study of 70 multi-ethnic breast cancer survivors, Ramirez et al. examined the feasibility of self-collection of hair samples and demonstrated that it was a reliable and non-invasive way to measure cortisol (Ramirez et al., 2017). And finally, the majority of samples had undetectable IL-6 levels, so we were unable to include this measure in the analysis. IL-6 is less stable, and sample integrity may have been affected by collection procedures (Izawa, Miki, et al., 2013).

There are also several factors that may have impacted the study's external validity. The study design was cross-sectional and relied on a convenience sample. Due to the small sample, we were unable to adjust for hormonal contraceptive use in the overall model. Hormonal contraceptive use is associated with increased production of corticotropin-binding-globulin and subsequent decreases in unbound cortisol levels (Stephens, Mahon, McCaul, & Wand, 2016). In addition, due to the wide variety of pediatric cancer diagnoses in the sample, we could not adjust for cancer diagnosis and treatment-related factors in the model. Several studies have found a significant effect of diagnosis and treatment-related factors on post-treatment ovarian function, including the cancer pathology itself (Van Dorp et al., 2014), the therapeutic regimen (Charpentier et al., 2014) and the length of exposure (El-Shalakany et al., 2013; Reinmuth et al., 2013). Another limitation is that due to the great distance that many of the participants needed to travel, we were unable to time study visits to coincide with a specific menstrual cycle phase. For this reason, we were unable

to adjust gonadotropin and reproductive hormone levels to account for menstrual cycle phase.

Integrative Review

The majority of the studies examining AMH as a biomarker of the ovarian reserve have been conducted in women diagnosed with infertility or polycystic ovary syndrome. AMH has been proposed as a biomarker of the ovarian reserve for use in reproductive medicine; in order to improve its accuracy as a reliable biomarker, more studies examining AMH in healthy samples are needed. The gaps in this area have limited the ability of AMH to reliably predict reproductive outcomes/ reproductive potential in healthy samples, thus significantly limiting the utility of the biomarker (Steiner et al., 2017a; Zarek et al., 2015). An increased understanding of factors associated with AMH concentrations in healthy women will provide greater context for the clinical evaluation and interpretation of AMH concentrations.

An integrative review was conducted with the purpose of summarizing the evidence regarding the modifiable biobehavioral factors associated with AMH concentrations in healthy reproductive age women. We found that various reproductive (contraceptive use, parity), biological (obesity, cardiometabolic) and behavioral (smoking, alcohol and caffeine consumption) factors have been examined in association with AMH concentrations. Among the studies included in the review, hormonal contraceptive use was most often examined. The majority found lower AMH levels in hormonal contraceptive users compared to non-users. Previous contraceptive use, however, was not associated with lower AMH levels

after contraceptive use was discontinued, suggesting a reversible effect. Given the current level of evidence, clinicians and researchers should be cautious when interpreting AMH levels during hormonal contraceptive use. Among the other factors examined, findings were mixed. The findings from the integrative review demonstrate that our understanding of the modifiable biobehavioral factors influencing AMH concentrations is incomplete and further research is needed.

Latent Class Analysis

In order to establish AMH as a reliable biomarker of the ovarian reserve, it is vital to explore how it behaves within the context of other health parameters. In addition to the primary study, we performed a second study exploring the feasibility of using Latent Class Analysis (LCA) to identify subgroups based on cardiometabolic (lipids, BMI, cortisol), psychological (depression, anxiety) and reproductive factors (FSH, LH, E2, AMH). We used stored postpartum blood samples of 69 women from a prospective cohort study examining mood and weight changes in pregnant women in Edinburgh, Scotland. We were able to identify three subgroups with unique class characteristics based on the factors included in the LCA, specifically focusing on AMH in our discussion of class characteristics. While the sample size was small, the findings of this exploratory study provide the foundation for further hypothesis testing regarding the effect of cardiometabolic and psychological parameters on reproductive health and fertility. In addition, it demonstrates that LCA is a feasible approach for identifying subgroups based on underlying patterns of cardiometabolic, psychological, and reproductive factors. While the findings of the study require validation in larger

cohorts, it demonstrates the potential of using LCA to examine AMH or other biomarkers in the context of other significant parameters of health, including cardiometabolic and psychological factors.

Implications for nursing practice

The findings from these studies have several important implications for oncology and woman's health nursing practice. Reproductive dysfunction is a major concern for cancer survivors, and is highly correlated with quality of life in this vulnerable population (Cherven et al., 2015; Knopman et al., 2010; Kondapalli et al., 2014; Letourneau et al., 2013). However, studies have demonstrated that cancer survivors are about half as likely to be treated for infertility as their siblings (Barton et al., 2013). While the reasons for this discrepancy are not fully understood, healthcare providers may perceive fertility treatments to be less successful in this population. Additionally, knowledge regarding factors that contribute to risk for post-treatment reproductive dysfunction is incomplete (Knight et al., 2015; Letourneau et al., 2013). The guidelines from The American Society for Clinical Oncology recommend that options for fertility preservation be discussed with cancer patients at the earliest opportunity (Knight et al., 2015; Letourneau et al., 2013). Oncology nurses play an important role in advocating for cancer survivors, helping to initiate conversations regarding future fertility as early as possible and discussing factors that may mitigate the risk for post-treatment reproductive dysfunction. The findings from this study also suggest that there may be modifiable factors such as chronic stress that contribute to risk for infertility post-treatment. Oncology

nurses can minimize the potential burden of late effects by educating patients regarding their risk and what actions can be taken prior to or after treatment to reduce this risk (McClellan et al., 2013).

This research also has important implications for woman's health nursing. Many women seek out information regarding their fertility potential. The integrative review provides nurses with an evidence-based summary of modifiable biobehavioral factors associated with AMH, a biomarker of the ovarian reserve. AMH is increasingly used in reproductive medicine as a biomarker of fertility lifespan and reproductive potential (Didier Dewailly et al., 2014). While it has demonstrated significant value in the context of assisted reproduction, its value as a predictor of natural fecundity has not been established (Hagen, Vestergaard, et al., 2012; Steiner et al., 2017a; Zarek et al., 2015). Therefore, nurses can discuss with women the current limitations in the clinical interpretation of AMH levels, given the present level of evidence.

Implications for nursing education and research

Understanding how to interpret biobehavioral data is vital to the nursing role. The studies included in this dissertation demonstrate the importance of understanding how biology and behavior interact to influence patient outcomes. Nursing education should emphasize a biobehavioral approach to health so as to prepare nurses to provide individualized care to patients, taking into account biological data while also considering the ways in which lifestyle and environment contribute to disease risk. Nursing research should continue to utilize biobehavioral methodology to address questions related to human health and

disease, integrating a depth of understanding regarding the pathophysiological mechanisms of disease with a thorough comprehension of human behavior, lifestyle and environment.

The nursing profession is tasked with health promotion and disease prevention. For this reason, nurses need an understanding of disease risk and the tools available to help identify or measure that risk (e.g. biomarkers). As a biomarker of the ovarian reserve, AMH provides a window into the process of ovarian aging. In addition to increased risk of infertility, ovarian aging is associated with increased risk of cardiovascular disease, osteoporosis and mood disturbances (F. J. Broekmans, Soules, & Fauser, 2009). Thus, the applications of AMH as a biomarker of ovarian age extend well beyond reproductive health and may provide insight into women's overall health (Pal, Bevilacqua, Zeitlian, Shu, & Santoro, 2008). Nursing education and research should continue to utilize and improve tools such as AMH to promote reproductive health and prevent the burden of infertility.

Future research

It is well established that stress disrupts reproductive function (Shannon Whirledge & Cidlowski, 2013b). The proposed explanatory mechanism is HPA dysregulation. As childhood cancer survivors are exposed to significant early life stress and are at higher risk of infertility due to the gonadotoxicity of chemotherapy, we sought to explore the relationship between stress, HPA activity and ovarian function in this population. The findings of this study provide preliminary evidence to support a negative association between perceived stress

and ovarian function in female childhood cancer survivors. Our findings also provide evidence to support the role of the HPA axis in regulating reproductive function. We found a positive association between the salivary cortisol intercept and slope and AMH as well as a positive association between hair cortisol and AMH, suggesting that threshold glucocorticoid levels are required to maintain reproductive function. These findings are consistent with other studies that have demonstrated an association between hypocortisolism and increased risk for adverse outcomes in cancer survivors (Cuneo et al., 2017).

The HPA axis mediates resource allocation decisions and plays an important role in determining an organism's life history traits. In this study, we sought to explore the association between HPA activity and the ovarian reserve in female childhood cancer survivors in order to increase our understanding of how pediatric cancer influences life history traits, specifically reproductive function. Biomarkers of HPA activity may provide valuable information regarding increased risk for infertility in this population. Additional studies with larger samples are needed to further explore this hypothesis.

More research is also needed to examine the biobehavioral factors associated with AMH as a biomarker of the ovarian reserve. Longitudinal studies are needed to examine the influence of biobehavioral exposures over time. In addition, randomized controlled trials are needed to examine the effect of lifestyle modification (i.e. stress reduction programs, smoking cessation, weight loss) and pharmacological intervention on the ovarian reserve and to determine whether an

improvement in AMH concentrations is associated with subsequent improvement in reproductive outcomes.

This dissertation examined the association between biobehavioral factors and the ovarian reserve. The mechanisms contributing to diminished ovarian reserve are complex, and in addition to biobehavioral factors, include environmental and genetic factors. Further research is needed to examine how biology, genetics and environment interact to impact reproductive lifespan and fertility. Epigenetic changes have been proposed as the link between biobehavioral exposures and reproductive disorders. Further research is needed to examine epigenetic influences on the ovarian reserve in order to obtain a better understanding of how environmental and biobehavioral exposures contribute to infertility. Findings from this research can be used develop reliable risk models incorporating environmental, lifestyle, and genetic factors for the early detection of infertility. With a better understanding of the mechanisms contributing to the ovarian reserve, nurse scientists can contribute to the development of novel therapeutic interventions for the treatment of infertility.

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APPENDIX A: MANUSCRIPT 1

An Integrative Review of Modifiable Biobehavioral Factors Associated with
Anti-Müllerian Hormone Concentrations

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Abstract

Objective: Anti-Müllerian hormone (AMH) is an emerging biomarker of ovarian reserve. There is inadequate understanding of the modifiable biobehavioral factors contributing (AMH) concentrations in normo-ovulatory women. The purpose of this integrative review was to provide a summary of the modifiable biobehavioral factors associated with AMH concentrations.

Data Sources: An electronic literature search was conducted in PubMed using the following search terms: “anti-Müllerian hormone” NOT “polycystic ovarian syndrome” NOT “cancer” NOT “endometriosis” NOT “assisted reproductive technology”.

Study Selection: Studies were included in the review if the population studied was healthy reproductive age women (ages 19+), the exposure was a *modifiable* biobehavioral factor, the outcome was serum AMH, and the biobehavioral factor was examined in 2 or more studies.

Data Extraction: For each study, the first author, year of publication, study design, sample size, age range, groups, covariates and main finding were summarized and organized by factor.

Data Synthesis: A total of 39 studies were included in the final review. Studies were published between 2007-2017. Sample sizes ranged from 20-2320. Study designs included cross-sectional, prospective cohort, retrospective cohort, and case-control. The main biobehavioral factors relating to AMH levels that emerged from the review were contraceptive use (n=14), parity (n=5), BMI (n=11),

cardiometabolic factors (n=4), smoking (n=10), alcohol consumption (n=4) and caffeine (n=3) consumption.

Conclusion: Our understanding of the modifiable biobehavioral factors influencing AMH concentrations is incomplete. Increased understanding of the biobehavioral factors associated with AMH levels will improve the quality of care for women at risk for infertility.

Introduction

Scientists continue to search for reliable and clinically useful biomarkers in the field of reproductive medicine (Palmer & Barnhart, 2013). The process of ovarian aging that eventually culminates in menopause spans approximately ten years, and there are few biomarkers that determine reproductive age. One biomarker that has received considerable attention in the field of reproductive medicine is anti-Müllerian hormone (AMH). A woman's reproductive age is determined by the size of the primordial follicle pool, referred to as the ovarian reserve. Circulating AMH levels are recognized to reliably reflect the size of the primordial follicle pool, making AMH a promising non-invasive marker of the ovarian reserve (Hansen et al., 2011).

Longitudinal studies involving normo-ovulatory women have found that serum AMH concentrations accurately predict the onset of menopause (Broer et al., 2011; van Disseldorp et al., 2008). The hormone is ideal as a research and clinical measure because, unlike follicle stimulating hormone (FSH)—another biomarker of the ovarian reserve—serum AMH concentrations remain relatively stable across the menstrual cycle, are independent of gonadotropin levels (Hehenkamp et al., 2006; La Marca et al., 2010; Shaw et al., 2011) and correlate with the number of developing follicles in the ovary (Dewailly et al., 2014). Age remains the strongest determinant of ovarian reserve, and 34% of the variation in AMH levels can be attributed to age alone (Kelsey, Wright, Nelson, Anderson, & Wallace, 2011). Although normative age ranges for AMH have been established, there is considerable variation within the same age range (Konishi, Nishihama,

lida, Yoshinaga, & Imai, 2014; La Marca et al., 2012; Lie Fong et al., 2012; Nelson, Messow, Wallace, Fleming, & McConnachie, 2011). To improve the clinical application of AMH as a reliable biomarker of the ovarian reserve, further research is needed to understand factors other than age that contribute to the observed variations in AMH levels in healthy women.

AMH has proven utility in the field of assisted reproductive medicine (Iliodromiti et al., 2014), but its value in predicting natural fecundability is less clear (Hagen, Vestergaard, et al., 2012; Steiner et al., 2017b; Zarek et al., 2015). AMH is widely used in reproductive medicine to counsel women regarding their future fertility, and many women struggling to conceive have sought out information regarding risk factors that contribute to a premature decline in AMH concentrations. While several studies have examined factors associated with AMH concentrations, the bulk of data comes from studies involving infertile women, or women diagnosed with polycystic ovary syndrome (PCOS). It is unclear if modifiable biobehavioral factors contribute to AMH concentrations in normo-ovulatory women. A greater understanding of AMH levels in the healthy female population will help to refine normative age ranges for use of this biomarker of the ovarian reserve in clinical practice and research. The purpose of this integrative review is to provide a summary of the modifiable biobehavioral factors associated with AMH concentrations.

Function of AMH

Anti-Müllerian hormone (AMH), a member of the TGF- β family, is a product of granulosa cells of pre-antral ovarian follicles. AMH plays a role in

ovarian follicle growth, specifically in regulating the pace of follicle recruitment and selection. AMH is expressed in granulosa cells of small growing follicles with its strongest expression in preantral and small antral follicles between 5-8mm in diameter (Jeppesen, Anderson, Kelsey, Christiansen, Kristensen, Jayaprakasan, Campbell, et al., 2013). AMH is no longer expressed by granulosa cells during FSH-dependent stages of follicle growth or by atretic follicles, degenerated follicles that do not ovulate during the menstrual cycle (Frank J. Broekmans et al., 2008; Didier Dewailly et al., 2014).

The role of AMH in follicle growth was first characterized in studies of AMH-deficient mice (Visser et al., 2007). These studies demonstrated that AMH acts as an inhibitor of primordial follicle recruitment (Durlinger et al., 1999, 2002). AMH-deficient mice had more growing follicles and an increased rate of oocyte degeneration and follicle atresia (Visser et al., 2007), indicating that in the absence of AMH, primordial follicles are recruited at a faster rate, leading to a premature depletion of the follicle pool. Another consequence of AMH absence is an increased sensitivity of growing follicles to FSH. AMH has an inhibitory effect on FSH and therefore plays an important role in the selection of the dominant follicle (Durlinger et al., 2001; Sacchi et al., 2016; Visser & Themmen, 2014; Visser, de Jong, Laven, & Themmen, 2006).

Methods

This review was conducted using the guidelines for integrative reviews (Whittemore & Knafl, 2005). The integrative review methodology was selected because it provides a framework for summarizing evidence from studies with

diverse methodologies. An electronic literature search was conducted in PubMed on January 17, 2018 using the following search terms: “anti-Müllerian hormone” NOT “polycystic ovarian syndrome” NOT “cancer” NOT “endometriosis” NOT “assisted reproductive technology”, using filters: females, humans, English. To focus the review, studies conducted in the pediatric/adolescent population were excluded. Titles and abstracts, if necessary, of all resulting citations were read. The full text of all potentially eligible studies was read to determine whether studies met the eligibility criteria. Reference lists of included studies were also searched in order to identify any relevant studies missed in the primary search. Studies were included if they met the following criteria:

- 1) The population studied was healthy reproductive age women. The population was considered “healthy” if they were not identified as having an infertility diagnosis or any other disease process that could potentially alter AMH levels.
- 2) The exposure was a *modifiable* biobehavioral factor, involving the interaction of biological and behavioral processes (Webster).
- 3) The outcome was serum AMH and was measured in all study participants.
- 4) The biobehavioral factor was examined in 2 or more studies.

The primary author selected and read all studies. Studies were grouped by the biobehavioral factors that emerged from the literature search. For each study, the first author, year of publication, study design, sample size, age range, groups, covariates and the main finding are summarized and organized by factor (Tables 1-7).

Results

The search strategy resulted in 408 studies. The search strategy is provided in Figure 1. Using the search criteria described above, fifty studies were selected from the primary search. After reading the full text of all 50 studies, 17 were excluded for the following reasons: the factor was only examined in one study (n=8), examined associations between AMH and non-modifiable risk factors (n=6), examined an intervention (n=1), population with illness (1), AMH examined as predictor (n=1). Six studies were added from the reference lists of included studies. A total of 39 studies were included in the final review.

Studies were published between 2007-2017. Sample sizes ranged from 20-2320. Study designs included cross-sectional, prospective cohort, retrospective cohort, and case-control. The main biobehavioral factors relating to AMH levels that emerged from the review were contraceptive use (n=14), parity (n=5), BMI (n=11), cardiometabolic factors (n=4), smoking (n=10), alcohol consumption (n=4) and caffeine (n=3) consumption.

Reproductive factors

Several studies examined whether reproductive factors were associated with a woman's ovarian reserve trajectory (Daan & Fauser, 2015). Reproductive factors that have been examined in relation to AMH levels are contraceptive use (n=14) and parity (n=5).

Contraceptive Use

Hormonal contraceptive use was examined in the greatest number of studies (n=14). Contraceptive use suppresses ovarian follicle growth by inhibiting gonadotropin secretion via exogenous administration of reproductive hormones. Ten studies found a significant inverse association between AMH levels and current hormonal contraceptive use. In a recent large retrospective cohort study, Marsh et al. (2016) examined factors associated with AMH in 1,654 African American women (23-34 years). In a multivariable model adjusting for BMI, history of abnormal menstrual bleeding, history of a thyroid condition, and history of seeking care for difficulty conceiving, current hormonal contraceptive use (27.5%) was inversely associated with AMH levels ($\beta = -0.290$, 95% CI -0.408, -0.171), $p < .0001$ (Marsh et al., 2016). Five studies were cross-sectional and examined whether there was a significant difference in AMH levels between users and non-users of oral contraceptives. In all five studies, oral contraceptive use was associated with significantly lower AMH levels (Bentzen et al., 2012; Birch Petersen et al., 2015; Dolleman et al., 2013; Kristensen et al., 2012; Shaw et al., 2011). One study was retrospective and also found a significant inverse association between oral contraceptive use and AMH levels ($p < 0.001$) (Kerkhof et al., 2010).

Two of the studies were prospective and examined AMH levels pre- and post- oral contraceptive use (Arbo, Vetori, Jimenez, Freitas, & Lemos, 2007; Kallio et al., 2013). Arbo et al. (2007) examined AMH levels pre- and one month post administration of the oral contraceptive pill (OCP) in 20 normo-ovulatory

infertile women (male factor or tubal occlusion). AMH levels were significantly reduced after pituitary suppression, with a median (inter-quartile range) of 3.02 ng/mL (1.21–6.39) before OCP and 2.22 ng/mL (0.9–3.11) after OCP, $p < 0.04$ (Arbo et al., 2007).

Kallio et al. (2013) measured AMH levels in 42 women in Finland (20–33 years) prior to contraceptive administration and at 5 and 9 weeks post administration in three groups (OCP, transdermal patch, or vaginal ring). In addition to examining the effect of hormonal contraceptive use on AMH levels, the authors examined whether the effect differed depending on route of administration. They found significant decreases in AMH levels after 9 weeks in all groups: OCP (3.88 ± 3.0 vs. 1.91 ± 1.5 ng/mL, $p < .001$), transdermal (3.86 ± 3.6 vs. 1.96 ± 1.3 ng/mL, $p < .001$), and vaginal ring (4.27 ± 3.5 vs. 2.25 ± 1.2 ng/mL, $p < 0.001$). However, they found no significant difference between the groups, demonstrating that combination (estrogen plus progestin) hormonal contraceptive use lowers AMH levels regardless of the route of administration (Kallio et al., 2013).

In the last study, van den Berg (2010) examined AMH levels in 25 Dutch women in the hormone free interval (either using the standard 21-/7- day regimen or an extended regimen) of a contraceptive cycle and two consecutive natural cycles. There was a significant increase in AMH levels between the hormone free interval of the contraceptive cycle and the consecutive natural cycles ($p = 0.005$). The authors demonstrate that AMH levels are suppressed in the hormonal contraceptive cycle and that levels increase after use of contraceptive is stopped.

In contrast, four studies found no association between AMH levels and contraceptive use. In a prospective case-control study, Deb et al. (2012) compared the AMH levels of 34 subjects who had been using the combined OCP (30 mcg of ethinylestradiol and 150 mcg of levonorgestrel) for more than a year with 36 control subjects who had not used any form of hormonal contraceptive in the last year and found that AMH levels did not differ significantly between the two groups. However, the study did not adjust for age (18-35 years), making it difficult to determine the strength of the evidence.

Contrary to the study by Kallio et al. (2013), Li et al. (2011) compared AMH levels pre- and 3-4 months post- administration of five different hormonal contraceptive types in 95 women. The five types included the (1) combined oral contraceptive (COC) (Microgynon-30®, n=23); (2) combined injectable contraceptive (CIC) (Cyclofem®, n=23); (3) progestogen-only contraceptive pill (POP) (Cerazette®, n=9); (4) progestogen-only injectable (POI) (Depo-Provera®, n=20); and (5) levonorgestrel intrauterine system (LNG-IUS) (Mirena®, n=20). There was no significant difference between pre- and post-treatment serum AMH levels in all the treatment groups ($p > .05$). However, the authors made no adjustment for age (25-50 years), and did not provide complete statistics, making it difficult to evaluate the strength of the findings (H. W. R. Li, Wong, Yeung, Ho, & Ng, 2011).

Similarly, Streuli et al. (2013) compared AMH levels in 24 women divided into three groups, (1) control group; (2) 0.02 mg of ethinylestradiol (EE) plus 0.15 mg of desogestrel (DSG) orally (Mercilon); and (3) 0.015 mg of EE and 0.12 mg

of etonogestrel, the active metabolite of DSG, through a vaginal ring (Nuvaring). They also found no significant differences in AMH levels pre- and one month post- administration of hormonal contraceptives (Streuli et al., 2008).

Finally, Kucera et al. (2016) compared AMH levels in females who had used hormonal contraceptives for a period of at least 10 years and had ceased use 1 year prior to sample collection (n=105) with females who had never used hormonal contraceptives. The median concentration of AMH in the group of long-term users of hormonal contraceptives was 2.89 ng/ml. The median concentration of AMH in the group of women who had never used contraceptives was 3.37 ng/ml. There was no statistically significant difference between the groups ($p = 0.3261$) (Kucera, Ulcova-Gallova, & Topolcan, 2016).

While there is contradictory evidence regarding the association between hormonal contraceptive use and AMH levels, the majority of the studies found an inverse association between AMH levels and hormonal contraceptive use. The findings from several small studies (Deb et al., n=34; Streuli et al., n=24) included in this review suggest that short-term administration of oral contraceptives (less than 3 months) has a limited impact on AMH levels (H. W. R. Li, Wong, et al., 2011; Streuli et al., 2008). In contrast, many studies found lower serum AMH concentrations with long-term hormonal contraceptive use, suggesting that hormonal contraceptive use suppresses follicle development (Bentzen et al., 2012). However, several studies also found that AMH levels recover after ceasing hormonal contraceptive use and that there was no significant difference in AMH levels between those who never used hormonal contraceptives and

those who previously used them, suggesting that the ovarian suppression induced by long-term hormonal contraceptive use is temporary (Dolleman et al., 2013; Kucera, Ulcova-Gallova, & Topolcan, 2016; La Marca et al., 2010; Van Den Berg et al., 2010). More research is needed to clarify the association between various characteristics of contraceptive use (duration, type, etc...) and AMH levels, as well as a more precise timeline of the return to normal AMH levels once hormonal contraceptive use is discontinued.

Parity

Several studies examined the association between parity and AMH concentrations (n=5). In a study of 294 women, Bragg et al. found that women with two ($p < 0.05$) and three or more ($p < 0.01$) children had significantly lower AMH levels than those with no children. However, they were unable to exclude women with PCOS from the study. Since PCOS is characterized by abnormally elevated AMH concentrations and subfertility, this may have skewed the results. In a sample of 420 women, Whitworth et al. (2015) found that women with the highest parity had 20% lower AMH concentrations (95% CI = -39% to 6%) than nulliparous women.

Contrary to these studies, Dolleman et al. (n = 2,320) found that higher parity was associated with higher age-specific AMH levels ($p < .02$). However, after adjusting for contraceptive use, cycle irregularity, pregnancy, and current smoking, parity was no longer significant (Dolleman et al., 2013). In a cross-sectional study of 186 women, Moini et al. (2016) found that multiparous women had significantly higher AMH levels than nulliparous women (Moini,

Hedayatshodeh, Hosseini, & Rastad, 2016). The mean AMH level was 2.53 ± 1.90 ng/ml in nulliparous women and 3.54 ± 1.42 ng/ml in women with children ($p < .0001$), suggesting that increased parity is associated with increased AMH levels. And finally, in a cross-sectional study of 277 women, La Marca et al. (2010) found no association between parity and AMH levels (La Marca et al., 2010).

Resolution of conflicting findings regarding the effects of parity on AMH requires further studies. Higher parity may result in later age at natural menopause by suppressing follicle recruitment and ovulation (during pregnancy), thus preserving the follicle pool and resulting in later menopause (Gold et al., 2001). This explanation is consistent with the findings from the studies by Dolleman et al. and Moini et al. In contrast, studies that found lower AMH levels in women with higher parity posit that this association may be explained by the costly energetic investment required for pregnancy at the expense of future reproductive potential (Bragg, Kuzawa, Ahustin, Banerjee, & McCade, 2012).

Biological factors

Obesity

Obesity inhibits reproductive function by altering the secretion of gonadotropin releasing hormone, which disrupts the ovarian follicular environment and leads to anovulation (Halawaty, ElKattan, Azab, ElGhamry, & Al-Inany, 2010; Klenov & Jungheim, 2014; Moy, Jindal, Lieman, & Buyuk, 2015; Vryonidou, Paschou, Muscogiuri, Orio, & Goulis, 2015b). Eleven studies

examined the correlation between BMI and AMH. Six observed an association (Bernardi et al., 2017; Bleil et al., 2014; Dafopoulos et al., 2010; Freeman et al., 2012; Steiner et al., 2010; Su et al., 2010) and five did not (Bentzen et al., 2013; Dolleman et al., 2013; Halawaty et al., 2010; La Marca et al., 2010; Sahmay et al., 2012).

Bernardi et al. (2017) conducted one of the largest studies examining the relationship between BMI and AMH in 1,654 African American women. At enrollment, 19.8% of participants were underweight or normal, 20.7% were overweight and 59.5% were obese. When current BMI was analyzed as a continuous variable ($15.9-79.4 \text{ kg/m}^2$), there was a significant inverse relationship with AMH levels in the age-adjusted and the multivariable linear regression model, after adjusting for current hormonal contraceptive use, history of a thyroid condition, abnormal menstrual bleeding and menstrual cycle length. When current BMI was analyzed as a categorical variable, there was also a significant association between AMH and BMI, with the strongest association observed in the group with the highest BMI. This study provides convincing evidence due to its large sample size and inclusion of participants with a wide range of BMIs.

In a prospective study of 20 women, Steiner et al. examined AMH concentrations in obese ($37.3 \pm 6.0 \text{ kg/m}^2$) and non-obese ($21.9 \pm 1.6 \text{ kg/m}^2$) women and found that AMH levels were 34% lower in the obese group (2.9 ± 2.1 vs. $4.4 \pm 1.8 \text{ ng/mL}$, $p < .05$). Similarly, in a prospective study of 122 late reproductive age women, Freeman et al. found that obese women had 65% lower mean AMH levels than non-obese women ($29.9 \pm 8.6 \text{ ng/mL}$) (geometric

mean: 0.016 ng/mL versus 0.046 ng/mL, $p < .034$). In a cross-sectional study of 947 women, Bleil et al. (2014) found that AMH decreased by 1.5% with every unit increase in BMI after adjusting for age, smoking, parity, hormonal contraception, age at menarche, psychological stress, educational attainment, and income ($p < 0.0001$) (Bleil et al., 2014). And finally, in a small comparative study ($n=36$), Su et al. (2010) found that AMH levels in obese women were 77% lower on average than in normal weight women after adjusting for age, race, smoking, and alcohol use, ($p = 0.02$).

Five studies found no association between AMH and BMI. In a study of 527 Danish women, Bentzen et al. (2013) found no significant association between BMI (median 22 kg/m²) and AMH ($p = 0.53$) after adjusting for oral contraceptive use, smoking and prenatal exposure to smoking. In a comparative study, Halawaty et al. (2010) examined differences in AMH levels between 50 women with BMI between 30-35 kg/m² and 50 age-matched controls with BMI <30 kg/m² and found no significant difference between the groups. In another comparative study, Sahmay et al. (2012) found no statistically significant difference in AMH levels between obese (>30 kg/m²) and non-obese (<30 kg/m²) participants. However, out of the 259 participants in the study, only 37 were obese. In the studies by Halawaty et al. and Sahmay et al., individuals were grouped using arbitrary BMI cut points (>30 kg/m² and <30 kg/m²). Comparing obese women to groups that include normal and overweight women is unlikely to detect differences in AMH levels, particularly given what we know regarding the disruptive effects of adipose tissue on hormone metabolism (Klenov & Jungheim,

2014). Additionally, neither of these studies adjusted for age, which has a significant effect both on AMH and BMI. The last two studies were cross-sectional. In one, the range in BMI was not reported (La Marca et al., 2012), and in the other, the mean BMI of the sample did not cover a broad range, 24.3 ± 3.9 (Dolleman et al., 2013).

There are several theories that may explain the relationship between AMH and obesity. Obesity is characterized by alterations in adipocyte-hormone secretion and insulin resistance (Crujeiras & Casanueva, 2015). Leptin, an adipokine required for activation of gonadotropin releasing hormone secretion, is present in high concentrations in obesity, and may directly inhibit gonadal functions and contribute to lower AMH levels (Crujeiras & Casanueva, 2015). Adipoectin, an adipokine that contributes to oocyte maturation and granulosa cell proliferation, is decreased in obesity and may contribute to lower AMH levels. Insulin resistance may contribute to granulosa cell dysfunction and alter AMH concentrations (Bernardi et al., 2017; Park et al., 2010). Another possibility is that AMH metabolism is altered in obese women (Freeman et al., 2007). Decreased levels of AMH in obese women may point to impaired follicular function; however, the association between AMH and BMI was not observed in all studies (Freeman et al., 2007). More research is needed to examine the association between BMI and AMH levels and to clarify the explanatory mechanisms behind this association.

Cardiometabolic

Four studies examined the association between AMH and cardiometabolic

factors (Bleil, Gregorich, McConnell, Rosen, & Cedars, 2013; Park et al., 2010; Tehrani, Erfani, Cheraghi, Tohidi, & Azizi, 2014; Zhu, Wang, Chen, Ji, & Xiong, 2017). In a study of 951 women, Bleil et al. (2013) observed that the number of cardio-metabolic risk factors was higher in women with low compared to high AMH levels and that women with low AMH levels had increased risk of high-density lipoprotein (HDL) <50 mg/dL, increased waist circumference, and hypertension. However, associations were no longer significant after controlling for BMI (Bleil, Gregorich, et al., 2013).

In a longitudinal study of 1,015 women by Tehrani et al. (2014), cholesterol and low-density lipoprotein (LDL) profiles were less favorable in women with lower age-specific AMH levels. While there were no significant differences in baseline cardiovascular risk factors, during the follow-up period, total cholesterol increased for those with the lowest age-specific AMH levels, while it decreased for those with the highest age-specific AMH levels ($p < .001$); the average decrease in LDL for those with the lowest age-specific AMH levels was less than for those with the highest age-specific AMH levels ($p < .004$) (Tehrani et al., 2014). And last, in a prospective study of 120 healthy women, Park et al. used the Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) to examine the association between insulin resistance and ovarian reserve and found an inverse association between HOMA-IR and AMH levels ($r = -0.343$, $p < .001$) (Park et al., 2010).

While numerous studies have found evidence of increased cardiovascular disease risk with advancing gynecological age (Tehrani, Behboudi-Gandevani,

Ghanbarian, & Azizi, 2014), few studies have examined potential associations between premenopausal cardiovascular risk factors and the process of ovarian aging (de Kat, Broekmans, Laven, & van der Schouw, 2015). The studies included in this review provide preliminary evidence that variability in reproductive aging may be related to cardiometabolic risk factors in premenopausal women, however more research is needed to examine this association (Maria E Bleil, Gregorich, et al., 2013).

Behavioral factors

Smoking

Toxins from smoking may have detrimental effects on the ovarian follicle pool and increase rates of follicle atresia, leading to decreased antral follicles and alterations in ovarian hormones (Schuh-Huerta et al., 2012). The association between smoking and AMH levels was examined in 10 studies. Two observed an inverse association (Dolleman et al., 2013; Plante, Cooper, Baird, & Steiner, 2010), one observed higher AMH levels in smokers vs non-smokers (Schuh-Huerta et al., 2012) and seven observed no association (Bentzen et al., 2013; Dafopoulos et al., 2010; Hawkins Bressler et al., 2016; J. Kline, Tang, & Levin, 2016; Lambert-Messerlian, Plante, Eklund, Raker, & Moore, 2016; Waylen, Jones, & Ledger, 2010).

In a cross-sectional study of 284 women, Plante et al. (2010) examined AMH levels in current smokers (defined as actively smoking within the previous two years), past smokers (defined as a history of smoking with cessation

occurring at least two years before the study), and passive smokers (defined as currently living with someone who smoked in their home). They found that current smokers, but not past smokers, had 44% lower AMH levels than the reference group (participants with neither active nor former or passive smoke exposure), $p = 0.04$. They also found that within current smokers, those who smoked 15 cigarettes or more per day tended to have lower AMH levels than those who smoked fewer than 15 cigarettes per day, but this difference was not statistically significant ($p = 0.08$). Dolleman et al. (2013) also found lower AMH levels in current smokers in comparison to never smokers ($p = 0.02$), and no association with previous smoking. When examining duration of smoking, they found an inverse association between AMH levels and 10+ pack-years ($\beta = -7.0$, $P = .003$).

Of the studies that found no association, Hawkins Bressler et al. was one of the largest with 1,654 African American participants. This study provided one of the most in depth analyses of various characteristics of smoking including age at initiation, cessation and duration. Most women (73%) never smoked regularly, 7% formerly smoked, and 19% were current smokers. Among current smokers, only 4% smoked a pack or more a day. The average age of smoking initiation was 18 (± 4) years, cessation was 25 (± 4) years, and the average duration of smoking was 6 (± 5) years, none of which were associated with AMH levels ($p > .05$) (Hawkins Bressler et al., 2016). In another study of 137 women, of which 59 (43%) smoked 8.6 ± 5.9 -pack years (range 2–20 pack years), Dafoupoulos et al. found an inverse association between smoking and AMH levels; this association

was no longer significant after adjusting for age, BMI, and reproductive hormones (Dafopoulos et al., 2010).

Waylen et al. examined the association between AMH and smoking in 335 women. Of these, 202 were non-smokers, 86 were ex-smokers and 47 were current smokers. No statistically significant difference was observed in serum AMH concentrations in women with a history of smoking compared with those that had never smoked. However, a trend towards a decrease in both previous and current smokers was observed (Waylen et al., 2010). Several other cross-sectional studies examined the association between smoking and AMH levels and also found no association (Bentzen et al., 2013; J. Kline et al., 2016; Lambert-Messerlian et al., 2016; Shaw et al., 2011). Contrary to these studies, Schuh-Huerta et al. (2012) found higher AMH levels in a sample of 947 smokers vs nonsmokers, 33.5 ± 2.4 versus 27.4 ± 1.7 pM ($p = 0.038$) and found that neither current nor previous smoking was associated with overall lower ovarian follicle counts. It is unclear why AMH levels would have been elevated in smokers in this study.

While a greater number of studies found no association between AMH and smoking, the two studies that included smoking duration in the analyses of smoking did observe an association between the duration of smoking and AMH levels. More research is needed to clarify the association between smoking and AMH levels.

Alcohol consumption

Four studies examined the association between alcohol consumption and AMH levels. In a cross-sectional study of 420 women, Whitworth et al. (2016) found lower AMH levels among women who drank alcohol (-21%, 95% CI=-36% to -3%) (Whitworth et al., 2016). In another cross-sectional study of 1654 women, Hawkins Bressler et al. (2016) found that those who binge drank twice a week or more had 21% lower AMH levels compared with those who never drank (95% CI= -41% to 7%), but found no association between drinking alcohol and AMH levels in non-binge drinkers (Hawkins Bressler et al., 2016). Hawkins Bressler et al. hypothesized that the association between binge drinking and lower AMH levels may be due to alcohol-induced alterations in hepatic catabolism of estradiol or gonadotropins and the subsequent impact on hypothalamic-pituitary axis signaling. Alternatively, they also hypothesized that repeated large-volume alcohol exposures may overwhelm the protective mechanisms of the granulosa complex and contribute to lower AMH levels (Hawkins Bressler et al., 2016). Two studies found no association between alcohol consumption and AMH levels (Dolleman et al., 2013; J. Kline et al., 2016).

Caffeine consumption

Three studies examined the association between caffeine consumption and ovarian reserve. Whitworth et al. (2016) found lower AMH levels among women who drank coffee (-19%, 95% CI -31% to -5%). Two studies of 2320

and 477 women found no association between caffeine consumption and AMH levels (Dolleman et al., 2013; J. Kline et al., 2016). In a review of epidemiologic evidence concerning the association between caffeine consumption and reproductive health, Peck et al. (2010) concluded that there was insufficient evidence to support a relationship between caffeine intake and reproductive outcomes (Peck, Leviton, & Cowan, 2010).

Discussion

This integrative review provides a summary of the evidence regarding the association between modifiable biobehavioral factors and anti-Müllerian hormone levels in reproductive age women. Various reproductive (contraceptive use and parity), biological (obesity, cardiometabolic) and behavioral (smoking, alcohol and caffeine consumption) factors have been examined in association with serum anti-Müllerian hormone concentrations.

Among the studies included, most often examined was hormonal contraceptive use (n=14). While several studies found no association, the majority (n=10) found lower AMH levels in hormonal contraceptive users compared to non-users. Previous contraceptive use, however, was not associated with lower AMH levels after contraceptive use was discontinued. These findings suggest that if contraceptive use affects serum AMH levels, the effect is reversible. Given the current level of evidence, clinicians and researchers should be cautious when interpreting AMH levels during contraceptive use; and it is important that future researchers examine how long

after discontinuing hormonal contraceptive use AMH returns to age-adjusted normal levels.

Among the biological factors examined, obesity was examined the most (n=11). However, the evidence regarding the association between BMI and AMH is contradictory. Six observed an association between increased BMI and lower AMH levels, and five found no association. There was considerable variation in the way BMI was measured and categorized. Several of the studies examined BMI as a continuous variable and several examined it as a categorical variable. The studies examining BMI as a categorical variable used different cut-points making it difficult to compare the findings (Bentzen et al., 2013; Freeman et al., 2007; Steiner et al., 2010).

There is insufficient evidence regarding the association between AMH levels and cardiometabolic risk factors; however, the studies included in this review suggest that women with lower AMH levels are more likely to have less favorable cardiometabolic profiles. And finally, the evidence regarding the effect of lifestyle factors (smoking and alcohol/caffeine consumption) is inconclusive. However, studies that observed an association between smoking and AMH levels found lower AMH levels in current smokers but no association with previous smoking, suggesting a reversible effect.

In order to facilitate direct comparison between studies and increased precision in determining effect sizes, there is a need to incorporate standardized and consistent measures of biobehavioral exposures. There was significant variation in how exposures were defined and measured (i.e. duration, quantity,

categorization, etc...). In addition, greater consistency in adjustment for covariates is needed, as this will improve our understanding of the combined effect of multiple factors as well as their total contribution to variation in the process of ovarian aging. Chronic disease risk is best explained by the synergistic effect of multiple factors, and this is likely also the case with the process of ovarian aging. The studies included in the review were all cohort, cross-sectional or case-control studies. These study designs limit the ability to detect effects and conclusions drawn are based only on associations. Longitudinal studies are needed to account for fluctuations in biobehavioral exposures over time, and to permit an examination of the mechanisms behind the associations between biobehavioral exposures and AMH levels.

The focus of this integrative review was to summarize the evidence regarding modifiable biobehavioral factors associated with AMH levels in women of reproductive age. While age at menopause is highly heritable, the rate of change in AMH levels improves the precision of estimates of time to menopause (Freeman et al., 2012). Therefore, it is helpful to also consider the evidence regarding these same biobehavioral factors and onset of menopause. In a large multi-ethnic study examining factors associated with age at natural menopause (n=14,620), Gold et al. found that ever using hormonal contraceptives was associated with later age at natural menopause (Gold et al., 2001). However, in another study that examined more specific characteristics of hormonal contraceptive use in relation to age at natural menopause, de Vries et al. found that long-term (>3 years) use of high dose oral contraceptives and long-term

(>11 years) overall oral contraceptive use were associated with an earlier age at menopause (adjusted HR = 1.12; 95% CI 1.03–1.21 and HR = 1.13; respectively). The use of lower dose oral contraceptives had no influence on menopausal age (de Vries et al., 2001). As regards parity, Gold et al. reported that higher parity was associated with later age at menopause (Gold et al., 2001).

The evidence regarding the relationship between BMI and age at menopause has been conflicting. In the study by Gold et al., in unadjusted models, women with lower BMI were more likely to be pre-menopausal, and women with higher BMI were more likely to be surgically amenorrheic (Gold et al., 2001). However, in adjusted models, BMI was not a significant predictor of age at menopause. The relationship between cardiometabolic factors and age at menopause has also proven complex to unravel. While it is widely accepted that postmenopausal status is associated with increased risk for cardiovascular disease, recent studies have demonstrated that premenopausal CVD risk factors may play a more important role in determining postmenopausal risk than previously thought (de Kat et al., 2017). It has been hypothesized that the processes of ovarian aging and cardiovascular disease risk have a synergistic relationship (de Kat, Verschuren, Eijkemans, van der Schouw, & Broekmans, 2016).

In a systematic review examining the association between smoking and age at menopause, a reduction in age at menopause was greater among current smokers than among former smokers, suggesting that the detrimental effect of smoking on ovarian follicles may be salvageable (Parente, Faerstein, Keller, &

Werneck, 2008). And in a recent systematic review and meta-analysis examining the association between alcohol consumption and age at menopause, Taneri et al. (2016) found that low and moderate alcohol consumption (more than one drink per week (RR = 0.60; 95% CI 0.49–0.75) and three or fewer drinks per week (RR = 0.75; 95% CI 0.60–0.94) were associated with later menopause onset, compared to non-drinkers. They suggest that low to moderate alcohol intake may reflect moderate lifestyle habits, which could explain the positive association between moderate intake and age at menopause (Taneri et al., 2016).

Many of the factors shown to influence AMH levels may also influence age at menopause. More research is needed to better understand the effects of these factors on AMH and whether serum AMH concentrations accurately predict the onset of menopause (Broer et al., 2011; van Disseldorp et al., 2008).

Conclusion

It is clear from this integrative review that our understanding of the modifiable biobehavioral factors influencing AMH concentrations is incomplete. Increased understanding of the biobehavioral factors associated with AMH levels will improve the quality of care for women at risk for infertility. Many reproductive age women seek evidence-based information regarding their fertility status. There is a great need for a reliable biomarker to improve early detection of infertility risk and address gaps in reproductive healthcare. Greater understanding of factors affecting AMH levels will aid in the development of evidence-based information

for improved patient education as well the development of effective interventions for the prevention and treatment of infertility.

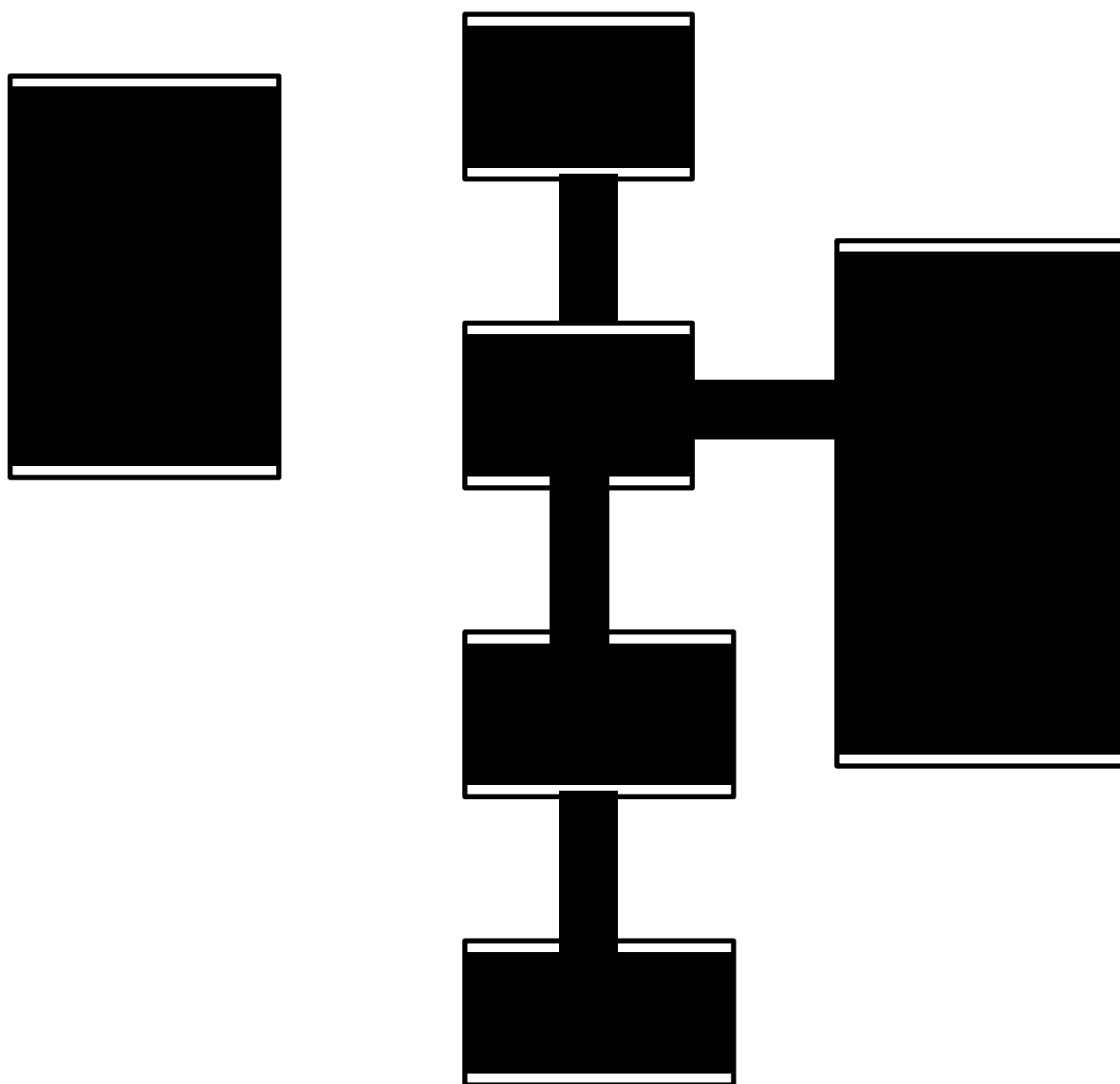


Figure 1: Search Strategy

**Table 1: Hormonal Contraceptive Use
Inverse association (n=10)**

Author et al. (date)	Design	N (age)	Race/Ethnicity	Groups	Covariates	Finding
Bentzen (2012)	CC	732 (21-41)	Denmark	228 users, 504 non-users	Age	<ul style="list-style-type: none"> AMH 29.8% lower in OCP users (p < .001, 95% CI 19.8%; 38.5%)
Birch-Peterson (2015)	CC	887 (19-46)	Denmark	244 users, 643 non-users	Age, smoking, BMI, maternal age at menopause, preterm birth, maternal smoking	<ul style="list-style-type: none"> AMH 20% lower in OCP users (p < 0.001, 95% CI 8.4%; 30.3%).
Dolleman (2013)	CC	2,320 (37.3 ± 9.2)	The Netherlands	1194 previous, 908 current, 218 never	Age, cycle regularity, OCP use, age at menarche, parity	<ul style="list-style-type: none"> Current OCP users had significantly lower AMH levels than women who had never used OCP (β = -.9; p < .0001) Previous OCP use was not associated with lower AMH levels (β = -0.3; p = .9)
Arbo (2007)	PC	20 (29.1 ± 4.1)	Brazil	Pre and post OCP use (1 month)	No	<ul style="list-style-type: none"> AMH levels were significantly reduced with OCP use Median (inter-quartile range) 3.02 ng/mL (1.21–6.39) before OCP and 2.22 ng/mL (0.9–3.11) after OCP (p < .04)
Shaw (2011)	CC	135 (41 ± 2.48)	USA	14 Users, 121 non-users	Age	<ul style="list-style-type: none"> Median AMH level in OCP users was 3.00 pmol/L (95% CI, 2.07–3.86), compared with 4.57 pmol/L (95% CI, 3.86–5.21) in nonusers (p < .001)
Kristensen (2012)	CC	256 (19-20)	Denmark	180 users, 76 non-users	No	<ul style="list-style-type: none"> Median AMH in users was 2.5 ng/mL (95% CI, 1.5–3.5), significantly lower than the median AMH level in nonusers 2.9 ng/mL (95% CI, 1.8–4.3), (p < .01)
Kallio (2013)	PC	42 (20-33)	Finland	13 OCP, 15 transdermal, 14 vaginal	No	<ul style="list-style-type: none"> Significant decreases in serum AMH levels after 9 weeks in all groups: OCP (3.88 ± 3.0 vs. 1.91 ± 1.5 ng/mL, p < .001), transdermal (3.86 ± 3.6 vs. 1.96 ±

				ring		1.3 ng/mL, $p < .001$), and vaginal ring (4.27 ± 3.5 vs. 2.25 ± 1.2 ng/mL, $p < .001$)
van den Berg (2010)	PC	25 (26.3 ± 6.2)	The Netherlands	Hormone free interval, natural cycle	Age	<ul style="list-style-type: none"> Significant increase in AMH in natural cycle vs. hormone free interval of contraceptive cycle, ($p = .005$)
Kerkhof (2010)	RC	279 (18-24)	The Netherlands	Users, non-users	Age, birth weight, fat mass, age at menarche and SES	<ul style="list-style-type: none"> OCP use inversely associated with AMH in each model ($p < 0.001$)
Marsh	RC	1654 (23-34)	USA: African American	Current users, non-users	Age, BMI	<ul style="list-style-type: none"> Current OCP use (27.5%) inversely associated with AMH levels ($\beta = -0.290$ (-0.408, -0.171), ($p < .0001$))

No association (n=4)

Author et al. (date)	Design	N (age)	Race/Ethnicity	Groups	Covariates	Finding
Deb et al. (2012)	Case-control	70 (18-35)	UK	34 Use for ≥ 1 year; 36 no use for ≥ 1 year	No	<ul style="list-style-type: none"> No significant difference between groups ($p = .44$)
Li et al. (2011)	PC	95 (26-50)	Hong Kong	Pre and 3 months post contraceptive use (5 types)	No	<ul style="list-style-type: none"> No significant difference pre and post contraceptive use in all treatment groups ($p > .05$)(statistics not provided)
Streuli (2008)	PC	24 24.1 (3.5)	Switzerland	OCP, vaginal ring, control	No	<ul style="list-style-type: none"> No significant difference between groups. Mean AMH levels for controls, 4.4 (SD ± 1.2) and 4.2 (SD ± 1.4) ng/mL for first and second cycles; for OC, 5.0 (SD ± 2.0) and 6.2 (SD ± 3.0) ng/mL; and for vaginal ring, 4.9 (SD ± 3.3) and 5.5 (SD ± 4.1) ng/mL.
Kucera (2016)	CC	149 (23-34)	Czech Republic (23-34)	Long-term users, non-users	No	<ul style="list-style-type: none"> No significant difference between groups. Median for users 2.89 ng/mL, for non-users 3.37 ng/mL

(p = .3261).

Note: Abbreviations: CC, Cross-sectional; PC, Prospective cohort; RC, Retrospective cohort; OCP, Oral Contraceptive Pill; BMI, Body Mass Index; AMH, Anti-Müllerian hormone; CI, Confidence Interval; OR, Odds Ratio; SD, Standard Deviation

Table 2: Parity**Lower AMH associated with higher parity or no association (n=3)**

Author et al. (date)	Design	N (age)	Race/Ethnicity	Groups	Covariates	Finding
Bragg (2012)	PC	294	Philippines	1, 2, 3+ children	Age, BMI, smoking status	<ul style="list-style-type: none"> The overall association with parity was significant ($F(3,286) = 4.25, p < 0.01$). Women with 2 or 3+ pregnancies had 50% of a SD decrease in AMH.
Whitworth (2015)	CC	420 (20-30)	South Africa	Nulliparous, 1, 2 children	Age, BMI, parity, education	<ul style="list-style-type: none"> Compared with nulliparous women, women with one previous birth had 21% lower AMH concentrations (95% CI -36 to -3%), and women with two or more previous births had 30% lower AMH concentrations (95% CI -46% to -10%).
La Marca (2010)	CC	277 (18-50)	The Netherlands	Nulliparous, 1+	Age	<ul style="list-style-type: none"> No significant association between parity and AMH levels.

Higher AMH associated with higher parity (n=2)

Author et al. (date)	Design	N (age)	Race/Ethnicity	Groups	Covariates	Finding
Dolleman (2013)	CC	2320 (37.3 ± 9.2)	The Netherlands	Continuous variable	Age, cycle regularity, HC use, age at menarche	<ul style="list-style-type: none"> Higher parity associated with 2 percentiles higher AMH.
Moini (2016)	CC	186 (20-35)	Iran	Nulliparous, multiparous	Age, BMI, age at menarche, cycle regularity,	<ul style="list-style-type: none"> Higher AMH in multiparous women. The mean of AMH was 2.53 ± 1.90 ng/ml in nulliparous women and

duration of menses
3.54 ± 1.42 ng/ml in women with children, which showed a significant difference between the groups (p < 0.0001).

Note: Abbreviations: CC, Cross-sectional; PC, Prospective cohort; HC, Hormonal Contraceptive; BMI, Body Mass Index; AMH, Anti-Müllerian hormone; CI, Confidence Interval; SD, Standard Deviation

Table 3: BMI Inverse association (n=6)

Author et al. (date)	Design	N (Age)	Race/Ethnicity	Groups	Covariates	Finding
Steiner (2010)	PC	20 (18-35)	USA	<25 kg/m ² (n=10), >30 kg/m ² (n=10)	No	<ul style="list-style-type: none"> AMH levels were 34% lower in the obese group (2.9 ± 2.1 vs. 4.4 ± 1.8 ng/mL, p < 0.05)
Bleil (2014)	CC	947 (25-45)	USA	Continuous variable	BMI, smoking, parity, HC, age at menarche, psychological stress, education, and income	<ul style="list-style-type: none"> With every unit increase in BMI, AMH decreased by 1.5% (p < .0001)
Dafoupoulos (2010)	PC	137 (20-49)	Greece	Continuous variable	Age, BMI, reproductive hormones	<ul style="list-style-type: none"> BMI and AMH associated without adjusting for age (r = -0.345, p < 0.05)
Freeman (2007)	PC	122 (45.8 ± 5.2)	USA	≤30 kg/m ² , >30 kg/m ² , continuous variable	BMI, age, menopausal status and race	<ul style="list-style-type: none"> Obese women had mean AMH levels that were 65% lower than AMH levels of non-obese women (geometric mean: 0.016 ng/mL versus 0.046 ng/mL; geometric mean ratio = 0.35; 95% CI 0.13, 0.92; p < .034)
Bernardi (2017)	RC	1654 (23-34)	USA: African American	<18.5kg/m ² = underweight, ≥18.5 to 24.9 kg/m ² = normal, 25 to 29.9	Current HC, history of a thyroid condition, abnormal menstrual bleeding,	<ul style="list-style-type: none"> When current BMI was analyzed as a continuous variable, there was a significant inverse relationship with AMH in the age-adjusted and the multivariable linear

kg/m²=
overweight,
≥30 kg/m²=
obese
Continuous

and
menstrual
cycle length

regression models.
When current BMI was
analyzed as a categorical
variable, the beta
coefficients for each BMI
group increased with
increasing BMI, with the
strongest association
between AMH and BMI
being observed among
those with the highest
BMI.

Su (2010)	CC	36 (40-52)	USA	<25 kg/m ² (n=18) >30 kg/m ² (n=18)	Age, BMI, race, smoking, alcohol use	<ul style="list-style-type: none"> AMH levels in obese women were 77% lower on average than in normal weight women (p = 0.02)
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No association (n=5)

Author et al. (date)	Design	N (Age)	Race/ Ethnicity	Groups	Covariates	Finding
Bentzen (2013)	CC	527 (32.7 ± 4.1)	Danish	<18 kg/m ² , 18-25 kg/m ² , >25 kg/m ² , >30 kg/m ²	HC, BMI, smoking habits and prenatal exposure to smoking	<ul style="list-style-type: none"> No significant association found between BMI and serum AMH (p = 0.53).
Dolleman (2013)	PC	2320 (37.3 ± 9.2)	The Netherlands	Continuous variables	Age, cycle regularity, HC use, age at menarche, parity	<ul style="list-style-type: none"> No significant association between BMI and AMH (β = -0.2; p = 0.16) or waist circumference and AMH (β = -0.09, p = 0.12)
La Marca (2012)	CC	416 (18-50)	Italy	Continuous variable	Age	<ul style="list-style-type: none"> AMH levels and BMI were significantly and negatively correlated (r = -0.1; p = 0.03), but not correlated after adjusting for age
Sahmay (2012)	CC	259 (27-45)	Turkey	<30 kg/m ² (n=222), >30 kg/m ² 37	No	<ul style="list-style-type: none"> AMH levels were 3.46 ± 2.79 ng/ml in non-obese participants and 3.79 ± 2.93 ng/ml in obese participants. No

Halawaty (2010)	CC	100 (40-48)	Egypt	<30 kg/m ² , 30-35 kg/m ²	No	<p>significant correlation was found between the levels of AMH and BMI ($p > 0.05$).</p> <ul style="list-style-type: none"> • There was no correlation between BMI and serum AMH.
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Note: Abbreviations: CC, Cross-sectional; PC, Prospective cohort; HC, Hormonal Contraceptive; BMI, Body Mass Index; AMH, Anti-Müllerian hormone; CI, Confidence Interval;

Table 4: Smoking Inverse association (n=2)

Author et al. (date)	Design	Sample size (Age)	Race/Ethnicity	Groups	Covariates	Finding
Dolleman (2013)	CC	2320 (37.3 ± 9.2)	The Netherlands	Current, previous, never	Age, cycle regularity, OC use, age at menarche, parity	<ul style="list-style-type: none"> • Current smoking was associated with 3.6 percentiles lower age-specific AMH values ($p = .02$) in comparison to never smokers. Significant effect of duration of smoking on AMH levels seen after 10+ pack-years ($\beta = -7.0$, $p = .003$). No significant effect of previous smoking.
Plante (2010)	CC	284 (38-50)	USA	Current, previous, passive, never	Age, BMI	<ul style="list-style-type: none"> • Current smokers, but not past smokers, had 44% lower AMH values than did the reference group (participants with neither active nor former or passive smoke exposure); $p = 0.04$.

No association (n=8)

Author et al. (date)	Design	Sample size	Race/Ethnicity	Groups	Covariates	Finding
Dafoupoulos (2010)	CC	137 (20-49)	Greece	Smokers, non-smokers	Age, BMI, reproductive hormones	<ul style="list-style-type: none"> • Pearson's correlation showed that AMH levels were negatively correlated with smoking ($r = -.807$, $p < .001$), not significant in multiple linear regression.
Bentzen	CC	527	Denmark	Current,	Age	<ul style="list-style-type: none"> • No significant effect of

(2012)		(21-41)		previous, never		current or previous smoking on AMH values ($p = .9$).
Waylen (2010)	RC	335 (24-48)	UK	Current, previous, never	Age	<ul style="list-style-type: none"> A decreasing trend was observed, but not significant: Geometric mean: Never: 1.074 (0.925–1.245), Previous: 0.955 (0.760–1.199), Current: 0.869 (0.638–1.183).
Kline (2016)	CC	477 (19-45)	USA	Current, previous, never	Age	<ul style="list-style-type: none"> Neither current nor former smoking was associated with AMH levels.
Hawkins Bressler (2016)	RC	1654 (23-34)	USA African American	Current, previous, never	Age, BMI, current HC use	<ul style="list-style-type: none"> AMH not associated with current or previous smoking, age at initiation, duration, or passive exposure ($p > .05$)
Lambert Messerlian (2016)	PC	45 (18-45)	USA	Current Not smoking	Age, BMI	<ul style="list-style-type: none"> AMH not associated with smoking
Schuh-Huerta (2012)	CC	432 (25-45)	USA	Smokers, non- smokers	Age, HC use	<ul style="list-style-type: none"> AMH was significantly higher in smokers than non-smokers AMH levels were 33.5 ± 2.4 versus 27.4 ± 1.7 pM ($p < 0.038$) in Caucasians.
Shaw (2011)	CC	135 (41 ± 2.5)	USA	Current, Previous, never	Age	<ul style="list-style-type: none"> No significant association between age and smoking status

Note: Abbreviations: CC, Cross-sectional; PC, Prospective cohort; RC, Retrospective cohort; HC, Hormonal Contraceptive; BMI, Body Mass Index; AMH, Anti-Müllerian hormone; CI, Confidence Interval

**Table 5: Cardiometabolic
Inverse association (n=4)**

Author et al. (date)	Design	Sample	Race/ Ethnicity	Groups	Covariates	Finding
Tehrani (2014)	PC	1015 (20-50)	Iran	Continuous variables	Age, BMI, menopause status	<ul style="list-style-type: none"> Total cholesterol net changes per year were higher in women with the lowest age-specific AMH but not in women with the highest age-specific AMH ($p < 0.001$). Over time, cholesterol and LDL profiles were less favorable in women with lower age-specific AMH levels.
Park (2010)	PC	120 (37.24 ± 5.48)	Korea	HOMA-IR	Age	<ul style="list-style-type: none"> There was a significant association between HOMA-IR and AMH levels, increase of HOMA-IR associated with decrease in AMH levels ($r = -0.343$, $p < .001$)
Zhu (2017)	CC	109 (23-41)	Chinese	>30, <30 years old	NA	<ul style="list-style-type: none"> In women 30 years or older, increases in triglycerides were associated with increased AMH level; however, the effect of triglycerides vanished with adjustment for testosterone level.
Bleil (2013)	CC	951 (25-45)	USA	Categorical : number of cardio-metabolic risk factors	Age, smoking, race/ethnicity, age at menarche, past use HC, and parity	<ul style="list-style-type: none"> The number of cardio-metabolic risk factors was 52.1% higher among women with low compared to high AMH levels and 46.0% higher among

women with mid compared to high AMH levels.

Note: Abbreviations: CC, Cross-sectional; PC, Prospective cohort; HC, Hormonal Contraceptive; BMI, Body Mass Index; AMH, Anti-Müllerian hormone

Table 6: Alcohol Inverse association (n=2)

Author et al. (date)	Design	N (Age)	Race/Ethnicity	Groups	Covariates	Finding
Whitworth (2016)	CC	420 (20-30)	South Africa	Yes/No Alcohol drinker	Age, BMI, parity, education	<ul style="list-style-type: none"> AMH levels were significantly lower among women who drank alcohol (-21%, 95% CI -36% to -3%).
Hawkins Bressler (2016)	RC	1654 (23-34)	USA: African American	Drinkers, non-drinkers, binge drinkers	Age, BMI, current HC	<ul style="list-style-type: none"> Those who binge drank twice a week or more had 21% lower AMH levels compared with those who never drink (95% CI, -41 to 7).

No Association (n=2)

Author et al. (date)	Design	Sample size (Age)	Race/Ethnicity	Groups	Covariates	Finding
Kline (2016)	CC	477 (19-47)	USA	Days/week drink alcohol	Age	<ul style="list-style-type: none"> No significant association between alcohol consumption and AMH levels
Dolleman (2013)	CC	2320 (37.3 ± 9.2)	The Netherlands	Daily, drinks per day	Age, cycle regularity, HC use, age at menarche, parity	<ul style="list-style-type: none"> No significant association between alcohol consumption and AMH levels ($\beta = -.4$; $p = .74$)

Note: Abbreviations: CC, Cross-sectional; RC, Retrospective cohort; HC, Hormonal Contraceptive; BMI, Body Mass Index; AMH, Anti-Müllerian hormone; CI, Confidence Interval

**Table 7: Caffeine
Inverse association (n=1)**

Author et al. (date)	Design	N (Age)	Race/Ethnicity	Groups	Covariates	Finding
Whitworth (2016)	CC	420 (20-30)	South Africa	Yes/No regular coffee consumption	Age, BMI, parity, education	<ul style="list-style-type: none"> Lower AMH levels among women who drank coffee (-19%, 95% CI -31% to -5%)

No Association (n=2)

Author et al. (date)	Design	N (Age)	Race/Ethnicity	Groups	Covariates	Finding
Kline (2016)	CC	477 (19-47)	USA	Amount of caffeine	Age	<ul style="list-style-type: none"> No significant association between caffeine consumption and AMH levels
Dolleman (2013)	CC	2320 (37.3 ± 9.2)	The Netherlands	Daily, drinks per day	Age, cycle regularity, OCP use, age at menarche, parity	<ul style="list-style-type: none"> No significant association between caffeine consumption and AMH levels

Note: Abbreviations: CC, Cross-sectional; OCP, Oral Contraceptive Pill; BMI, Body Mass Index; AMH, Anti-Müllerian hormone; CI, Confidence Interval

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APPENDIX B: MANUSCRIPT 2

Exploring the Ovarian Reserve within Health Parameters:

A Latent Class Analysis

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Abstract

The process of ovarian aging is influenced by a complex and poorly understood interplay of endocrine, metabolic and environmental factors. The purpose of this study was to explore the feasibility of using Latent Class Analysis to identify subgroups based on cardiometabolic, psychological and reproductive parameters of health and to describe patterns of anti-Müllerian hormone levels, a biomarker of the ovarian reserve, within these subgroups. Sixty-nine lean ($\text{BMI} \leq 25\text{kg/m}^2$) and severely obese ($\text{BMI} \geq 40\text{kg/m}^2$) postpartum women in Edinburgh, Scotland were included in this exploratory study. The best fitting model included 3 classes; Class 1, $n = 23$ (33.5%); Class 2; $n = 30$ (42.2%); Class 3; $n = 16$ (24.3%). Postpartum women with lower ovarian reserve had less favorable cardiometabolic and psychological profiles. Examining the ovarian reserve within distinct subgroups based on parameters of health that affect ovarian aging may facilitate risk stratification in the context of ovarian aging.

Key Words: Ovarian reserve, latent class analysis, cardiometabolic, psychological

The ovarian reserve--the number of remaining ovarian follicles--declines gradually over a women's reproductive lifespan and natural menopause occurs when the ovarian reserve is depleted (Daan & Fauser, 2015). Anti-Müllerian hormone (AMH) plays a role in ovarian follicle growth, specifically in regulating the pace of follicle recruitment and selection (Visser et al., 2007). Plasma levels of AMH reflect the continuous non-cyclic growth of small ovarian follicles, and therefore mirror the size of the remaining follicle pool (Dewailly et al., 2014; Jeppesen et al., 2013). Considerable variation in the age at natural menopause suggests that factors other than chronological age contribute to the depletion of the ovarian follicle pool over time (van Disseldorp et al., 2008).

While numerous studies have examined the effect of biobehavioral factors on AMH concentrations (Dolleman et al., 2013; La Marca et al., 2012b), few have explored patterns within and between women with proven fertility as a means of identifying subgroups. Accurate group classification would improve the clinical interpretation of serum AMH concentrations and facilitate risk stratification in the context of ovarian aging (Pal et al., 2010). As cardiovascular, metabolic, and psychological factors have each been shown to have an effect on the ovarian reserve (Balkan, Cetin, Usluogullari, Unal, & Usluogullari, 2014; Bleil et al., 2012; Bleil et al., 2012; de Kat, Broekmans, Laven, & van der Schouw, 2015), we hypothesized that latent class analysis (LCA) could be used to develop subgroup classifications using these factors and that these subgroups would provide a useful context within which to examine the complex interplay of variables involved in the process of ovarian aging.

LCA is a clustering method used to identify patterns in factor configurations and has been effectively used to examine co-occurrence patterns of risk factors in chronic disease development (Dewailly, Alebić, Duhamel, & Stojanović, 2014; Leventhal, Huh, & Dunton, 2014). Rather than providing prescriptive clinical guidelines regarding the nature of class structures, we sought to elucidate the complex process of ovarian aging by exploring AMH within the context of cardiometabolic, psychological and reproductive factors (Henry, Dymnicki, Mohatt, Allen, & Kelly, 2015; Leventhal et al., 2014). As the underlying mechanisms contributing to premature ovarian aging are not well understood, LCA may be a useful method for clarifying how co-occurrence patterns of risk factors contribute to subtle changes in ovarian functioning over time (Leventhal et al., 2014). We conducted an exploratory study with the purpose of testing the feasibility of this hypothesis in a sample of postpartum women in whom cardiovascular, metabolic, and psychological factors had been measured during pregnancy and postpartum.

Methods

Sample

This study used data from a prospective cohort study conducted in Edinburgh, Scotland from 2008-2013 examining associations between mood and weight changes in lean ($BMI \leq 25\text{kg/m}^2$) and very severely obese ($BMI \geq 40\text{kg/m}^2$) women with singleton pregnancies (Mina et al., 2015). In the parent study, cardiovascular, metabolic, and psychological measures were recorded

during pregnancy and 3 months postpartum. For this exploratory cross-sectional study, only data collected during the study visit three months postpartum were used. This time point was selected to avoid the ovarian suppression that occurs during pregnancy and to coincide with the expected recovery of AMH levels (Königer et al., 2015). Women diagnosed with gestational diabetes or polycystic ovary syndrome were excluded as these conditions are known to influence AMH levels (Iliodromiti et al., 2013; Łebkowska et al., 2016). All samples and data were collected with ethical approval (references 08/S1101/39 and 13/ES/0126), and with fully informed and written consent from all women.

Measures

Cardiometabolic risk factors included fasting lipids (total cholesterol, high density lipoprotein, triglycerides), fasting glucose, fasting morning cortisol and BMI (Mina et al., 2015). BMI was used to group women as lean ($\text{BMI} \leq 25\text{kg/m}^2$) or severely obese (SO) ($\text{BMI} \geq 40\text{kg/m}^2$).

Psychological measures included the Hospital Anxiety and Depression Scale (HADS), the State-Trait Anxiety Index (STAI) and the General Health Questionnaire (GHQ). The HADS evaluates anxiety and depression symptoms (range: 0–21) and has been reported to help in differentiating transient and enduring stress during pregnancy (Matthey & Ross-Hamid, 2012). The STAI evaluates both state and trait anxiety (range: 20–80 each), and has been previously validated in severely obese pregnant women (Gunning et al., 2010). The GHQ-12 uses binary scoring (range: 0–15) and has been shown to reliably

differentiate stress levels between pregnant and non-pregnant controls (Goldberg, 1973; van Bussel, Spitz, & Demyttenaere, 2006).

Reproductive measures included LH, FSH, estradiol (E2), AMH and breastfeeding status. Serum levels of LH, FSH, E2 and AMH were measured in stored serum samples in a single batch by electrochemiluminescence immunoassay using the Roche Elecsys assay system (West Sussex, UK) (reference numbers: 11732234122, 11775863122, 06656021190 and 06331076190 respectively). The intra-assay coefficients of variation were 6.2-6.9% for LH, 7.3-8.1% for FSH, 1.5-3.2% for E2, and 5.8-6.9% for AMH.. Breastfeeding status at both time points was assessed with a single yes/no question: 'Are you breastfeeding your baby now?'. A 'yes' answer included both exclusive and non-exclusive breastfeeding.

Data Analyses

To describe the data, means and standard deviations were calculated for clinical and demographic variables in the whole sample. A one-way ANOVA was used to explore differences in variables between lean vs. very severely obese women, and in breastfeeding vs. non-breastfeeding women. The ANOVA was conducted to control for the parent study's primary research questions and to identify meaningful differences present in the data. Bonferroni adjustment was used, dividing α of 0.05 by the number of variables ($n= 12$), to account for multiple statistical testing with only differences resulting in a p-value lower than 0.004 considered statistically significant.

AMH levels were log-transformed for parametric analysis. Anxiety and depression were analyzed using the methods outlined in Mina et al. (2015). Briefly, maternal mood outcomes were grouped into 'anxiety symptoms' and 'depression symptoms'. Anxiety symptoms were represented by Hospital Anxiety (HA from the HADS) and both the state and trait components of the STAI. Depression outcomes were represented by Hospital Depression (HD, from the HADS) and the GHQ. To avoid multiple testing and the need to include a Bonferroni correction, the z-score was calculated for each outcome and averaged z-scores were used for each symptom group in the analysis.

Latent Class Analysis (LCA) was used to classify the subjects of the whole sample (heterogeneous) into smaller homogeneous classes/groups in which members are similar to each other and differentiated from subjects in other groups using cardiometabolic, psychological and reproductive factors. The objective is to identify groups naturally occurring in the data, when the number of underlying groups is unknown (DiStefano, 2012; Kaplan, 2014). LCA assumes that the data comes from a mixture of populations that have different probability distributions and that the population consists of homogeneous subgroups, in which the groups are discrete and mutually exclusive (DiStefano, 2012). The results of LCA provide probabilities for the proportion of the population expected in each group. Groups are not defined *a priori* but are rather probabilistic (Dewailly, Alebić, Duhamel, & Stojanović, 2014); individuals are allocated to groups based on observed values in the indicator variables used to determine class membership. Individuals of the same group are similar such that their

observed values are assumed to come from the same probability distribution (Tein, Coxe, & Cham, 2013). Conditional probabilities describing the mean or likelihood of each indicator variable are provided for each class (Henry et al., 2015). LCA has demonstrated the ability to accurately determine class membership in samples as small as 50 (Henry et al., 2015). As this was an exploratory study to examine the feasibility of using LCA to identify subgroups, we used a small sample. The LCA model included the following indicator variables: age, fasting lipids (triglycerides, cholesterol, and HDL), fasting glucose, fasting cortisol, lean/obese, breastfeeding status, anxiety, depression, FSH, LH, E2, and AMH levels. All variables were continuous with the exception of lean/obese and breastfeeding status; LCA is capable of reliably identifying class structures using variables with mixed scales (DiStefano, 2012).

All analyses were conducted using R 3.3.3 (Core Team, 2017), with the package flexmix (Leisch, 2004). This software allowed us to estimate LCA with different numbers of classes and compare the fit of each number of classes to identify how many classes should be estimated. The LCA was estimated for 1 to 10 classes, comparing the models with the Bayesian Information Criterion (BIC) and Integrated Completed Likelihood Criterion (ICL) (Burnham & Anderson, 2003). These information criteria penalized the log-likelihood of the model. The BIC and ICL are used to select the model from a set of candidate models that provides the best balance of model fit, complexity and parsimony, where models with lower BIC and ICL present better fit (Burnham & Anderson, 2003). Once the number of classes was defined, we looked at the characteristics of each class

and the patterns of the variables of interest relative to the ovarian reserve. As the primary focus of this study was to establish the feasibility of using LCA to identify subgroups, we provide only a brief discussion of class characteristics.

Results

Sixty-nine lean ($n=38$) and severely obese ($n=31$) postpartum women with mean age 33.97 (SD = 4.1) years were included in the analysis. Forty-seven women were breastfeeding and 22 were not breastfeeding. Clinical and demographic data and group differences (lean/very severely obese and breastfeeding/non-breastfeeding) are summarized in Table 1. As expected, there was a significant negative correlation between age and logAMH ($r = -0.312$, $p = 0.002$).

Information criteria (BIC and ICL) and entropy were used to compare the LCA solutions from a different number of classes (Table 2). As the number of classes increases, the log-likelihood decreases, and entropy increases. The BIC and ICL decreased from 1 to 2 classes, increased minimally from 2 to 3 classes, and clearly increased from 4 or more classes. Given that the difference in information criteria between the 2 and 3 classes solution was small, we examined the parameter estimates for each of these solutions to identify which one presented results that were more theoretically fitting.

The best fitting LCA model with meaningful parameter characteristics for each class included 3 classes (Table 3). For the 3-class LCA, 33.5% ($n=23$) of the sample was in Class 1, 42.2% ($n=30$) of the sample was in Class 2, and 24.3% ($n=16$) of the sample was in Class 3. Table 4 shows the variable

characteristics for each class. For the continuous variables, the characteristic is presented as the average score of that variable within each class, for the binary variables (breastfeeding status and lean/obese), the characteristic is presented as the probability of presenting that characteristic. Comparisons between the classes by cardiometabolic, psychological and reproductive health parameters are shown in Table 3. For the 3-class solution, the entropy is 0.93. Entropy is a measure of uncertainty in the classification procedure, indicating how well the model predicts membership, with values close to 1 representing better prediction. The high entropy (0.93) for this LCA solution shows that the model accurately predicts membership. Each class is summarized briefly below:

Class 1: Highest mean AMH levels, lowest probability of breastfeeding, lowest mean cholesterol levels, lowest mean HDL levels, lowest probability of being lean, lowest mean fasting morning cortisol levels, lowest mean anxiety scores, lowest mean FSH and LH levels, and highest mean E2 levels

Class 2: Highest probability of breastfeeding, lowest mean triglyceride levels, highest mean HDL levels, highest probability of being lean, lowest mean depression score, highest mean FSH and LH levels, and lowest mean E2 levels

Class 3: Lowest mean AMH levels, highest mean triglyceride and cholesterol levels, highest mean anxiety and depression scores, and highest mean cortisol levels

Discussion

In this study, we used latent class analysis to explore possible subgroups of cardiometabolic, psychological and reproductive parameters of health in relation to ovarian reserve in a convenience sample of post-partum women. Three subgroups were identified. As expected, there was a negative correlation between age and AMH; however, age was not a strong determinant of class membership in the LCA. The subgroups identified through LCA provide insight into the complex interactions of various health related parameters in relation to ovarian reserve and contribute to our understanding of factors other than age involved in the process of ovarian aging. LCA offers a feasible approach for examining risk factors in the context of ovarian aging.

Class 1 had the highest mean AMH levels and the lowest mean cholesterol levels. Class 3 had the lowest mean AMH levels and the highest mean cholesterol and triglyceride levels. Several studies have observed the trend toward a less favorable cardiovascular risk profile in women with lower age-specific AMH levels (Bleil, Gregorich, McConnell, Rosen, & Cedars, 2013; Tehrani, Erfani, Cheraghi, Tohidi, & Azizi, 2014). Cardiovascular disease risk factors have been suggested to accelerate the process of ovarian aging by impairing vascularization of the ovaries and accelerating ovarian decline (de Kat et al., 2015). It may be that these processes occur simultaneously and share similar underlying mechanisms, such that accelerated ovarian aging increases cardiovascular disease risk and increased cardiovascular disease risk accelerates ovarian aging (de Kat et al., 2015; Kok et al., 2006).

In the present study, the probability of being lean or severely obese did not demonstrate a strong class trend in relation to AMH. Given the strong class trend of cardiovascular risk factors in relation to AMH, this was an unexpected finding since severe obesity is a well-defined risk factor for early onset cardiovascular disease. This finding may be explained by the lack of variability in cardiometabolic factors observed in the sample, irrespective of BMI. Despite their obesity, these women are still relatively young and display a narrow range of variability in cardiometabolic factors. This lack of class trend may also be explained by the use of a postpartum sample of women; breastfeeding has been shown to have a protective effect on cardiovascular health regardless of pre-conception risk factors (McClure, Catov, Ness, & Schwarz, 2012).

The suppressive effect of obesity on reproductive function is well established (Klenov & Jungheim, 2014; Nelson, Stewart, Fleming, & Freeman, 2010; Vryonidou, Paschou, Muscogiuri, Orio, & Goulis, 2015). Obesity alters the ovarian follicular environment and contributes to anovulation (Klenov & Jungheim, 2014; Moy et al., 2015). The effect of obesity on ovarian reserve is less well understood (Moy et al., 2015; Sahmay et al., 2012) with conflicting results (Malhotra et al., 2013; Moy et al., 2015; Sahmay et al., 2012; Steiner, 2013). Whether obesity also accelerates follicle loss and perhaps contributes to diminished ovarian reserve remains unclear (Klenov & Jungheim, 2014).

Class 1 had the lowest mean fasting morning cortisol levels and was the only class to have cortisol levels within the normal range. Both the mean cortisol levels in class 2 and class 3 were higher than normal. Class 2 had the highest

probability of breastfeeding. While few studies have examined hypothalamic pituitary adrenal (HPA) activity in breastfeeding, one study found that morning salivary cortisol levels were higher in women who predominantly breastfed (Ahn & Corwin, 2014). Class 3 had the highest fasting morning cortisol levels. This finding may be explained by the increased mean anxiety and depression scores as well as less favorable lipid profiles observed in this class (Veen et al., 2009). Dysregulation of the HPA axis impairs reproductive function by suppressing steroidogenesis and inhibiting gonadotropin release (Schliep et al., 2015). The effect of HPA activity on ovarian reserve is less well understood, however preliminary studies suggest associations between cortisol and abnormal AMH levels (Hardy et al., 2016).

In addition to the lowest mean AMH levels and a less favorable cardiovascular risk profile, class 3 also had the highest mean depression and anxiety scores (z-scores). Few studies have examined the association between psychological factors and biomarkers of the ovarian reserve (Bleil et al., 2012; Pal et al., 2010). Psychological disorders such as depression and anxiety are associated with impaired reproductive function (Williams, Marsh, & Rasgon, 2007) and are also associated with greater cardiovascular and metabolic disease risk (Bleil, Bromberger, et al., 2013; Lamers et al., 2012; Nikkheslat et al., 2015).

Class 2 had the highest probability of breastfeeding and of being lean, the lowest mean triglyceride levels, and the highest mean HDL levels. This is consistent with a protective effect of breastfeeding on cardiovascular health regardless of pre-conception risk factors (McClure et al., 2012). The probability of

breastfeeding did not, however, demonstrate a strong class trend in relation to AMH. Several studies have reported a suppressive effect of pregnancy on the ovarian reserve (Gerli et al., 2015; Köninger et al., 2013; Nelson et al., 2010). The effect of breastfeeding is not previously described, although AMH was not reduced in women with hyperprolactinaemia-induced amenorrhea (Li, Anderson, Yeung, Ho, & Ng, 2011). In this study, there was no significant difference in AMH concentrations between breastfeeding and non-breastfeeding postpartum women.

Consistent with this class also having the highest probability of breastfeeding, class 2 had the lowest E2 levels (McNeilly, 1993). Class 2 also had the highest gonadotropin levels, a finding that was unexpected and the basis for which is unclear. Class 1 had the highest E2 levels and the lowest FSH and LH levels. Class 1 also had the highest AMH levels. While AMH does not fluctuate significantly across the menstrual cycle, levels are at their highest in the late follicular phase, corresponding with rising estradiol levels (Wunder, Bersinger, Yared, Kretschmer, & Birkhäuser, 2008). Reproductive hormone levels were not able to be timed according to menstrual cycle phase in our postpartum sample, so these findings should be interpreted with caution.

This was an exploratory study with a small sample size. However, this was appropriate given that the study was exploratory in nature, it was the first to use latent class analysis to explore subgroups of risk profiles for ovarian reserve, and was meant to determine the feasibility of this type of analysis for this population. It is important to note that the sample consisted of a population of postpartum

women with known fertility; most studies examining factors associated with AMH concentrations have been conducted in infertile populations.

While preliminary, the findings from this study can be used as the foundation for future analyses of subgroups in relation to the ovarian reserve. The findings from this exploratory study should be replicated in larger samples to allow for independent validation, as well as in different reproductive contexts to examine how these variables behave in other populations. Current evidence suggests that the rate of change in AMH concentrations improves the accuracy of the biomarker to predict age at menopause (Freeman et al., 2012). Thus, longitudinal studies will be necessary to identify additional factors contributing to variation in the process of ovarian aging.

The process of ovarian aging spans many years, and at present, there are few clues, other than chronological age, that indicate where a woman is in her reproductive lifespan (Ottinger, 2011). In this study, we explored patterns in cardiometabolic, psychological and reproductive factors in relation to ovarian reserve and found less favorable cardiometabolic and psychological profiles in women with lower ovarian reserve. The findings from this study demonstrate that LCA is a feasible and useful approach for examining subgroups based on various parameters of health, and sheds light on factors that may contribute to variation in serum AMH concentrations, providing a window into what may be the earlier stages of ovarian aging. With validation in larger samples, this information could be used to develop reliable subgroup classifications to aid in the early detection and prevention of premature ovarian aging.

Table 1: Clinical and Demographic Data

	Overall (n = 69)	Lean (n = 38)	Obese (n = 31)	p-value	Breastfeeding (n = 47)	Not Breastfeeding (n = 22)	p-value
Age (years)	33.97 ± 4.1	34.54 ± 3.64	33.27 ± 4.56	0.203	34.6 ± 3.93	32.66 ± 4.24	0.07
Anxiety	0.04 ± 1.08	-0.2 ± 1.04	0.33 ± 1.06	0.039	-0.05 ± 1.07	0.24 ± 1.08	0.302
Depression	-0.08 ± 0.95	-0.38 ± 0.86	0.29 ± 0.94	0.003	-0.2 ± 0.91	0.17 ± 1.01	0.132
FSH (mIU/ml)	7.02 ± 3.56	7.79 ± 3.35	6.08 ± 3.64	0.046	7.4 ± 3.34	6.19 ± 3.95	0.188
LH (mIU/ml)	5.19 ± 3.93	4.9 ± 4.12	5.53 ± 3.73	0.513	5.21 ± 4.39	5.14 ± 2.82	0.952
E2 (pg/ml)	40.02 ± 38.06	31.02 ± 26.6	51.04 ± 46.71	0.029	27.9 ± 26.12	65.91 ± 46.52	<0.0001*
AMH (ng/ml)	2.79 ± 2.5	3.19 ± 2.81	2.3 ± 2.0	0.143	2.98 ± 2.57	2.4 ± 2.36	0.375
Serum cortisol (nmol/L)	855.7 ± 556.5	963.7 ± 587	742.5 ± 504.9	0.168	867.88 ± 537.53	758.96 ± 577.62	0.446
Cholesterol (mmol/L)	5.28 ± 1.47	5.57 ± 1.51	4.92 ± 1.36	0.064	5.29 ± 1.36	5.26 ± 1.71	0.937
HDL (mmol/L)	1.57 ± 0.46	1.68 ± 0.46	1.44 ± 0.43	0.027	1.62 ± 0.4	1.46 ± 0.56	0.196
Triglycerides (mmol/L)	1.12 ± 0.81	1.01 ± 0.77	1.25 ± 0.85	0.234	1.01 ± 0.79	1.34 ± 0.82	0.115

• Alpha level ≤ .0004 considered significant

Note: Anxiety and depression are z-scores. Abbreviations: AMH, Anti-Müllerian Hormone; HDL, High-Density Lipoprotein; FSH, Follicle Stimulating Hormone; LH, Luteinizing Hormone; E2, Estradiol

Table 2: Latent Class Analysis: Three-class model characteristics

	Class 1 (32.2%) (n=22)	Class 2 (43.4%) (n=30)	Class 3 (24.3%) (n=17)
AMH (ng/ml)	3.83	2.88	1.86
Age	33.25	35.01	33.08
Breastfeeding (Yes)	0.47	0.94	0.49
Triglycerides (mmol/L)	1.04	0.64	1.85
Cholesterol (mmol/L)	4.1	5.05	6.53
HDL (mmol/L)	1.22	1.77	1.56
Glucose (mmol/L)	4.73	4.48	4.61
Lean	0.29	0.73	0.51
Anxiety (z-score)	-0.18	-0.05	0.33
Depression (z-score)	0.02	-0.26	0.1
Cortisol (nmol/L)	437.15	955.91	974.67
FSH (mIU/ml)	5.1	8.13	6.99
LH (mIU/ml)	4.34	5.9	4.85
E2 (pg/ml)	74.19	22.85	37.04

Note: For the continuous variables, the characteristic is presented as the mean of that variable within each class, for the binary variables (breastfeeding status and lean/obese), the characteristic is presented as the probability of presenting that characteristic. Abbreviations: AMH, Anti-Müllerian Hormone; HDL, High-Density Lipoprotein; FSH, Follicle Stimulating Hormone; LH, Luteinizing Hormone; E2, Estradiol

Table 3: Comparisons between classes by health parameters

Class (mean AMH level)	Class 1 (3.83 ng/mL)	Class 2 (2.88 ng/mL)	Class 3 (1.86 ng/mL)
Cardiometabolic	<ul style="list-style-type: none"> • Lowest cholesterol and HDL • Lowest probability of being lean • Highest glucose • Lowest cortisol 	<ul style="list-style-type: none"> • Lowest triglycerides • Highest HDL • Highest probability of being lean • Lowest glucose 	<ul style="list-style-type: none"> • Highest triglycerides and cholesterol • Highest cortisol
Psychological	<ul style="list-style-type: none"> • Lowest anxiety 	<ul style="list-style-type: none"> • Lowest depression 	<ul style="list-style-type: none"> • Highest anxiety and depression
Reproductive	<ul style="list-style-type: none"> • Highest AMH • Lowest probability of breastfeeding • Lowest FSH and LH • Highest E2 	<ul style="list-style-type: none"> • Highest probability of breastfeeding • Highest FSH and LH • Lowest E2 	<ul style="list-style-type: none"> • Lowest AMH

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APPENDIX C: MANUSCRIPT 3

Chronic Stress and Ovarian Function in Female Childhood Cancer Survivors

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Abstract

Background: Few predictors of post-treatment reproductive function in childhood cancer survivors (CCS) have been identified. CCS report high levels of psychological stress. The association between stress and ovarian function has not been explored in this population.

Purpose: The purpose of this exploratory study was to explore the relationship between perceived stress, biomarkers of HPA activity, gonadotropin levels, and anti-Müllerian hormone (AMH) in female CCS.

Methods: Perceived stress was measured using the Perceived Stress Scale-10. HPA activity was measured using salivary and hair cortisol. Ovarian function was measured using serum gonadotropin levels and serum AMH levels. Latent Growth Modeling was used to determine diurnal cortisol slope and intercept. Bayesian Structural Equation Modeling was used to explore the relationship between perceived stress, biomarkers of HPA activity and ovarian function.

Results: Twenty-four female (mean age 21.79 ± 5.68) CCS were included in the study. We found an inverse association between perceived stress and ovarian function and a positive association between biomarkers of HPA activity and ovarian function.

Conclusion: The findings from this study provide the foundation for further research examining factors that contribute to risk for post-treatment reproductive dysfunction in female CCS.

Background

An estimated one in 1000 adults under the age of 35 is a survivor of childhood cancer (Blumenfeld, 2012). Due to significant advances in the treatment of pediatric cancer, the five year survival rate now exceeds 80% in the United States (Barton et al., 2013) with similar statistics worldwide (Reinmuth et al., 2013). With an increasing number of survivors, there has been growing recognition of the potential late effects of cancer treatment (Kremer et al., 2013). Among these, reproductive dysfunction is a major concern for cancer survivors, and is highly correlated with quality of life in this population (Cherven et al., 2015; Knopman et al., 2010; Kondapalli et al., 2014; Letourneau et al., 2013). Among female childhood cancer survivors (CCS), 6.3% suffer from acute ovarian failure and another 22.6% suffer from a significant reduction in ovarian function (Salih et al., 2015). Female CCS are at risk for persistent chemotherapy-related amenorrhea and early menopause; however, not all premenopausal CCS experience these effects (Abusief et al., 2012).

The American Society for Clinical Oncology recommends that providers discuss fertility preservation options with cancer patients at the earliest opportunity; however, it remains challenging for providers to predict individual risk of post-treatment reproductive dysfunction because of the limited knowledge of factors associated with this late effect (Knight et al., 2015; Letourneau et al., 2013). While the etiology of reproductive dysfunction in cancer survivors has been discussed (El-Shalakany, Ali, Abdelmaksoud, Abd El-Ghany, & Hasan, 2013; Letourneau et al., 2013), few predictors of this effect of treatment have

been identified (Dewailly, et al., 2014, Anderson et al., 2015). Further research is warranted to develop reliable biomarkers to help clinicians accurately identify cancer survivors at increased risk for reproductive dysfunction (Abusief et al., 2012).

In clinical practice, post-treatment ovarian function is assessed using a profile of the hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH), plus the presence or absence of menses. However, neither FSH nor menstrual cyclicity post-cancer treatment are reliable predictors of future fertility (Knight et al., 2015). Because risk of infertility related to premature ovarian failure is directly associated with the size of the ovarian follicle pool (ovarian reserve), a biomarker which more closely reflects the number of remaining follicles in the ovary would have significant clinical potential.

Anti-Müllerian hormone (AMH) is one such recently identified biomarker of ovarian reserve. Plasma levels of AMH reflect the continuous non-cyclic growth of small follicles, and therefore mirror the size of the remaining follicle pool (Jeppesen, Anderson, Kelsey, Christiansen, Kristensen, Jayaprakasan, Campbell, et al., 2013). Kelsey et al. (2011) developed and validated the first model of AMH, mapping out the chronological trajectory of the hormone from conception to menopause (Kelsey, Wright, Nelson, Anderson, & Wallace, 2011). La Marca et al. then established normative age-ranges to aid in the clinical interpretation of AMH values (La Marca et al., 2012).

Ovarian reserve declines naturally with age; however, recent research demonstrates that chronological age alone is not an accurate indicator of

reproductive age and that other factors affect the depletion of the ovarian follicle pool over time (Dolleman et al., 2013). AMH is prematurely reduced in CCS (Anderson & Wallace, 2013; Charpentier et al., 2014; Miyoshi et al., 2013), and may be an early marker of significant gonadotoxicity post treatment (Brougham et al., 2012; Lie Fong et al., 2009). Several studies have demonstrated the utility of pre-treatment AMH concentrations to predict risk of post treatment ovarian dysfunction in cancer survivors (Anderson & Wallace, 2013; Lunsford et al., 2014). However, the predictive value of AMH in cancer survivors is limited due to incomplete knowledge of factors that influence AMH concentrations (Abusief et al., 2012; Van Dorp et al., 2014).

One of the primary regulators of reproductive function is the hypothalamic-pituitary-adrenal (HPA) axis. Cancer survivors have high rates of psychological stress and hypothalamic-pituitary-adrenal (HPA) dysregulation (Oancea et al., 2014; Taylor et al., 2012; Zeltzer et al., 2009). It is well established that psychological stress impairs reproductive function by activating the HPA axis, which suppresses hypothalamic-pituitary gonadal function (Kalantaridou et al., 2010; Louis et al., 2011; Lynch, Sundaram, Maisog, Sweeney, & Buck Louis, 2014; Whirledge & Cidlowski, 2013). Increased HPA activity is associated with elevated levels of glucocorticoids, such as cortisol. Precise levels of glucocorticoids are required for proper ovarian function (Whirledge & Cidlowski, 2010). At homeostatic levels, cortisol contributes to steroid biosynthesis and maintenance of gonadotropin release; at elevated levels, it suppresses gonadotropin-releasing hormone secretion, reduces pulsatile LH secretion, and

increases rates of follicle atresia (Breen & Mellon, 2014; Whirlledge & Cidlowski, 2010; Whirlledge & Cidlowski, 2013). As both higher rates of psychological stress and reproductive dysfunction are observed in cancer survivors, this study explored the relationship between psychological stress and biomarkers of ovarian function in young cancer survivors. **The purpose of this study was to explore relationships between perceived stress, HPA activity, and ovarian function in a sample of female survivors of childhood cancer.**

Theoretical Framework

Life history theory was the framework used to guide this study (Whirlledge & Cidlowski, 2013). Rooted in evolutionary biology, life history theory was developed to explain variation in resource allocation strategies adopted by organisms to optimize survival and reproduction throughout the life course and across varying environmental conditions. The task of all organisms is essentially the same, to distribute material and energy resources to competing life functions: bodily maintenance, growth and reproduction. Life history theory posits that the allocation of biological resources is a trade-off between survival and reproduction.

Because resources are limited, an organism must make trade-offs, prioritizing their use in one domain at the expense of other competing domains (Ellis et al., 2009). Life history traits, characteristics that determine rates of bodily maintenance, reproduction, and growth, are the primary units of analysis in life history theory. Both individually and collectively, life history traits provide a window into an organism's life history strategy and the developmental continuum

that results from a chain of resource allocation decisions (trade-offs). The main life history traits are age and size at maturity, reproductive investment, reproductive lifespan, and aging (Stearns, 2000).

Early evolutionary biologists were able to demonstrate that organisms make tradeoffs in response to changing environmental conditions and that these tradeoffs influence the life course of an organism, but they struggled to identify the biological mechanisms that allow organisms to make tradeoffs. Identifying these mechanisms is vital to understanding the sources of variation in life history strategies (Stearns, 2000). One of the primary mechanisms by which an organism responds to changing environmental conditions is the hypothalamic-pituitary-adrenal (HPA) axis (Ellis, 2013). Environmental challenges or stressors activate the HPA axis and increase glucocorticoid secretion, directing resources to vital physiological activities such as energy mobilization, cardiac output, and cognition, and away from less vital functions. Because reproduction is energetically costly, when resources are limited, an organism is likely to prioritize survival over reproduction, delaying fertility until environmental conditions are more ideal. The HPA axis, as a primary mediator of resource allocation decisions, plays an important role in determining an organism's life history traits.

The HPA axis plays a prominent role in the regulation of the hypothalamic-pituitary-gonadal (HPG) axis through glucocorticoids, which have both supportive and suppressive effects on reproductive function. This complex and synchronized neuroendocrine cross talk between the HPA and HPG axes allows tradeoffs to occur (Whirledge & Cidlowski, 2010). At homeostatic levels,

glucocorticoids contribute to steroid biosynthesis and maintenance of gonadotropin release. During acute stress, endogenous glucocorticoids counteract the suppressive effects of prostaglandins on reproductive function, thus protecting gonadotropin secretion. This suggests that threshold glucocorticoid levels are necessary to maintain adequate gonadotropin release (Whirledge & Cidlowski, 2010). Elevated glucocorticoid levels suppress reproduction at multiple levels along the HPG axis. Increasing glucocorticoid levels down-regulate gonadotropin releasing hormone (GnRH) synthesis and decrease GnRH pulsatility, inhibiting the LH surge and delaying ovulation, and increasing the rate of ovarian follicle atresia (Whirledge & Cidlowski, 2010, 2013).

In this study, we hypothesized that chronic stress, operationalized as perceived stress (Perceived Stress Scale), and HPA activity, operationalized as hair cortisol and diurnal salivary cortisol patterns, would be associated with AMH, gonadotropin and reproductive hormone levels.

This study had the following aims:

Aim 1: Describe the relationship between perceived stress and biomarkers of HPA activity in female cancer survivors.

Aim 2: Explore the relationship between perceived stress, biomarkers of HPA activity, AMH and gonadotropin levels in female cancer survivors.

Methods

Sample and Settings

The sample for this exploratory cross-sectional study consisted of female childhood cancer survivors between the ages of 16 and 35 who were previously treated for cancer at the Edinburgh Children's Cancer Centre, Royal Hospital for Sick Children (RHSC). This was an exploratory study; sample size calculations are challenging in an exploratory model of potential predictors, due to the fact that one is not aware of predictor effects, and cannot put forth anticipated effect sizes.

Cancer survivors are seen annually for follow up at the Royal Hospital for Sick Children (RHSC) (Wallace et al., 2014). The inclusion criteria were: female patients treated for cancer (including leukemia and brain tumors) at the Edinburgh Children's Cancer Centre, younger than 18 years of age at cancer diagnosis and between 16-35 years of age at the time of the study visit, at least one year out from completing treatment, and signed written informed consent. In the UK, research subjects aged 16 and older are able to provide informed consent.

This inclusion age range of 16-35 was selected because subjects are likely to be post-pubertal and in the most fertile window of their reproductive lifespan. Cancer survivors seen for long-term follow-up at the Royal Hospital for Sick Children (RHSC) in Edinburgh, Scotland were selected because of Dr. Anderson's current research on reproductive function and fertility preservation in

cancer survivors (Wallace et al., 2014). This collaboration minimized burden to subjects and maximized potential findings. The exclusion criteria were a positive pregnancy test, ovarian surgery in the past 6 months, hormonal therapy in the preceding 3 months (GnRH agonists, recFSH), alcohol or drug abuse/dependence according to ICD-10 criteria, and having received an investigational drug in the past 3 months (90 days).

Instruments

Ovarian function: Assessment of ovarian function included serum gonadotropin levels (luteinizing hormone (LH), follicle-stimulating hormone (FSH)), estradiol (E2) and AMH. Serum levels of these hormones are routinely measured during the annual follow-up appointment as part of standard care at NHS affiliated clinics. The laboratory results were retrieved from participants' medical records. Serum AMH levels were analyzed using the Beckman Coulter AMH GEN II ELISA (Beckman Coulter Inc., Brea, CA). The AMH Gen II ELISA has a sensitivity of 0.57 pmol/l. The validated model of serum anti-Müllerian hormone by Kelsey et al. (2011) and the nomogram with normative values for age published by La Marca et al. (2012) was used to interpret AMH levels (Kelsey et al., 2011b; La Marca et al., 2012b).

HPA activity was determined using measures of salivary cortisol and hair cortisol. Salivary cortisol has been used to measure diurnal HPA activity in cancer survivors and other populations (Du et al., 2013; Ho et al., 2013; Sephton et al., 2013). Chronic stress is associated with alterations in the cortisol awakening response (Dedovic & Ngiam, 2015; Révész et al., 2013),

and blunting of the morning-evening diurnal slope (Garland et al., 2014; Ho et al., 2013; Schrepf et al., 2015). A commercially available Enzyme-Linked Immuno-Sorbent Assay (ELISA) kit was used for the quantification of cortisol in saliva (Salimetrics, Suffolk, UK KIT 1-3002). This cortisol assay kit has a lower detection limit of 7 ng/dl. The mean intra-assay coefficient is 5.7% and the mean inter-assay coefficient is 10%. Analysis of salivary cortisol was conducted at the Queen's Medical Research Institute in Edinburgh, Scotland under the supervision of Dr. Richard Anderson.

Chronic stress has also been associated with elevated levels of cortisol in hair (Russell et al., 2012; Wells et al., 2014a). Hair has a predictable growth rate of 1 cm/month; the most proximal 1 cm segment to the scalp approximates the last month's cortisol production, the second most proximal segment the month prior to that, and so on (Russell et al., 2012). Hair (3 cm) was processed for extraction of cortisol in the laboratory of Clemens-Kirschbaum in Dresden, Germany as previously described (Kirschbaum et al., 2009).

Perceived Stress was measured using the Perceived Stress Scale (Cohen et al., 1983). The PSS is a 10 item (PSS-10) self-report of life stress over the past 4 weeks, and is the most widely used measure of this concept. The PSS-10 was designed for use in community samples of subjects with education as low as junior high. Good to excellent reliability has been observed with the PSS-10 in varied populations (Cohen et al., 1983) with a Cronbach's alpha ranging from .78-.91 (E.-H. Lee, 2012). The measure has

been demonstrated to reliably measure stress appraisal in cancer survivors (Golden-Kreutz et al., 2009).

Data Collection

Recruitment was conducted by sending letters of invitation to female cancer survivors in long-term follow-up two weeks prior to their annual review appointment. After consenting to be contacted, patients were interviewed over the phone to determine eligibility to participate in the study. If eligibility requirements were met and the patient agreed to participate, a 30-minute study visit at the RHSC was scheduled at a mutually agreed upon time. Written consent was obtained from participants at the study visit. Each subject was assigned a unique study ID and data was entered into an SPSS file on an encrypted computer in a locked office.

Data was collected in this order: saliva, demographic and PSS-10 questionnaires, hair. Salivette collecting devices and detailed instructions for collection of samples were provided to participants at their annual review appointment. Participants were instructed not to eat, smoke, consume caffeine or exercise for 30 minutes prior to sample collection (Schrepf et al., 2015). Participants provided three timed saliva samples for the analysis of salivary cortisol: the first sample was collected before going to bed on the night prior to the study visit, the second immediately upon awakening the morning of their visit before getting out of bed, and the third was collected 30 minutes after the second sample. Participants were instructed to record the time of saliva collection on their salivette collecting devices and to keep

the saliva samples refrigerated until coming in for their study visit to preserve sample integrity. Participants brought the three saliva samples to their study visit. Samples were labeled with a unique subject study ID and stored at the RHSC at -20 C; within a week of collection, a research nurse picked them up and brought them to the QMRI, where they were centrifuged and stored at -80 C.

During the study visit, participants provided demographic information including age, ethnicity/race, level of education, marital status and employment status. Participants then completed the PSS-10 (Cohen et al., 1983). Lastly, participants provided a sample of hair for measurement of hair cortisol. A small sample of hair, 20mg (100-150 strands), was taken from the vertex posterior of the participant's scalp using a new pair of thinning shears (Hoffman et al., 2014; Van Uum et al., 2008). Hair length was recorded and proximal ends of the hair samples were marked. Hair samples were stored in aluminum foil for protection before processing (Hoffman et al., 2014) and labeled with a unique subject study ID.

During data collection, it was discovered that many subjects travel a great distance to the hospital for clinic visits. Therefore, to reduce the burden of participation, subjects were given the option to receive a research packet in the mail if they were unable to return to the RHSC for a separate study visit because of the travel distance. Since the current methods already required subjects to collect saliva samples at home, the main change was that subjects who decided to receive a research packet in the mail also self-collected a hair sample. They

were instructed on how to collect the hair sample when they consented to participate in the study. They were also asked to complete the PSS-10 at home. Biological samples and questionnaires were labeled with the subject's study ID so as to ensure confidentiality. No identifying information was included in or on the research packet. The research packet included prepaid return packaging and an ice pack so that subjects could return samples and questionnaires to the Royal Hospital for Sick Children for processing.

The following clinical characteristics were retrieved from participants' medical records: cancer diagnosis, age at cancer diagnosis, chemotherapy/radiation received (yes/no) and age at menarche. Reproductive hormone levels (AMH, FSH, LH and E2), measured as part of routine care at the annual review visit, were also retrieved from participants' medical records.

Data Analysis and Interpretation

Means and standard deviations or frequency distributions, as appropriate, for all demographic and clinical variables were reported to describe the sample and reveal distributional characteristics.

Latent Growth Curve Modeling was used to examine diurnal cortisol patterns. Bayesian Structural Equation Modeling was used to explore the relationship between perceived stress and HPA activity and to explore relationships between perceived stress, biomarkers of HPA activity, gonadotropin levels, and AMH.

Latent Growth Curve Modeling

Latent Growth Curve Modeling was used to define diurnal cortisol change over time (three time points). Growth curve modeling (GCM) examines change as a trajectory and assumes that trajectories vary across participants (Barker et al., 2013). This method was selected since chronic stress is characterized by a persistent and unstable trajectory of HPA activity (Hellhammer et al., 2009). Change processes are determined by combining the underlying pattern of growth (slope) with the initial or baseline measurement (intercept). The intercept and slope of this trajectory were then incorporated into the structural equation model as independent predictors of ovarian function. GCM was selected instead of more commonly utilized methods of analyzing diurnal cortisol (e.g. area under the curve, cortisol awakening response) because it permits a robust examination of change in small samples.

GCM was evaluated as $y_{it} = \lambda_{int}\eta_{int} + \lambda_{slp}\eta_{slp} + e_t$, where y_{it} represents the observations for each subject i at each time t . λ_{int} represents the vector of fixed values that define the latent intercept, this is $\lambda_{int} = [1, 1, 1]$, while λ_{slp} represents the vector of fixed values that define the linear slope, this is $\lambda_{slp} = [0, 9.3, 9.8]$. This represents the average time between the first observation to the second (9.3 hours), and from the first observation to the third observation (9.8 hours). η_{int} represents the latent intercepts, while η_{slp} represents the latent slope, for which we estimate the respective mean and variance. The means represent the average starting point and average change over time, while the respective variance represents how much the subjects vary from the respective mean.

Lastly, e_t represents the residual variance at each time point t , this indicates that the GCM is estimated without the assumption of homogeneity of variance over time, the residual variance is allowed to differ over time.

Bayesian analysis

In Bayesian analysis, statistical inferences are drawn from parameter values that could give rise to the observed data (Scheines et al., 1999). The term prior is used to refer to the expected parameter distribution based on theory or previous research. The posterior distribution is the parameter distribution that results from multiplying the prior distribution by the likelihood (as observed in the sample data). The posterior distribution describes the behavior of the parameters conditional on the data. Because the Bayesian framework makes inferences from the posterior distribution, it allows researchers to make direct inferences about the parameters given the observed data. The posterior distribution describes both the best estimate given the data, and the level of uncertainty about the respective parameter by the width of it, typically represented by the credible interval (Yuan & MacKinnon, 2009).

The Bayesian framework is an appropriate method for small sample sizes. Frequentist methods rely on the asymptotically correct estimation methods, basing the inference in function of the null hypothesis (Wagenmakers, Lee, Lodewyckx, & Iverson, 2008). Bayesian inference bases the inference on the observed data, which allows estimation of parameters with small samples in conditions where frequentist estimation fails (Lee & Song, 2004).

The Bayesian approach requires specification of prior distributions for each of the model parameters (Palomo et al., 2007). The selection of the prior distribution is important because the posterior distribution is informed by the prior distribution and the likelihood of the data (Palomo et al., 2007). The prior distribution incorporates information drawn from previous empirical studies or theory regarding the model parameters. Given the exploratory nature of this study, we first examined the model parameters using weakly informed priors. Weakly informed priors are not intended to guide the parameters, but instead provide information to delimitate the most likely data space for the parameters. Means/intercepts have a prior $\mu \sim N(0, 100)$; the standard deviations have a prior $\delta \sim \text{half-cauchy}(0, 2.5)$; the regressions have a prior $\beta \sim N(0, 50)$; and the correlations have a prior $\rho \sim U(-1, 1)$.

Strong hypotheses are tested by model comparison. A model without constraints was compared to a model with constraints in the regressions (β), constraining if the β is positive or negative (according to theory). These constraints are set by specifying the respective β prior as a truncated normal (e.g: $\beta \sim N(0, 50)T(0,)$) not allowing β to be above/below 0. Model comparison allowed us to evaluate if the model with directional constraints fit the data better than the unconstrained model. If the directional constraint model fit better, it is evidence that the β should be directional. The models were compared with Information Criteria Leave-One-Out (LOO), and Widely Applicable (WAIC) (Gelman et al., 2013; Kass & Raftery, 1995; Raftery, 1992; Vehtari, Gelman, &

Gabry, 2015, 2016). The theoretical model including the hypothesized directionality of the associations is presented in Figure 1.

Bayesian Structural Equation Modeling

Structural Equation Modeling (SEM) provides a broad framework for modeling relationships with multivariate outcomes (Palomo et al., 2007). The purpose of SEM is to test a theory by specifying a model that represents predictions about the theory's constructs measured with appropriate observed variables (Kline, 2015). The outcome of SEM is a set of model parameters for hypothesized effects given the data. From these parameters, a set of logical implications result about the nature of the relationship or non-relationship between the variables (Kline, 2015). While SEM typically includes an approximation of unobserved or latent constructs as well as an estimation of the structure of the relationships among these observed constructs, in this study we used a special case of SEM called path analysis. In path analysis, every variable in the model is directly measured or observed (Bryan et al., 2007). While latent variables account for unreliability of measures and uncertainty in the model, they can also complicate a model, especially when the sample size is small. Path analysis was selected instead of multiple linear regression because it allowed us to test multiple relationships between variables, with multiple outcomes, conforming to the hypothesized model in this study. Establishing relationships including multiple paths and outcomes allowed us to test the presence of relationships accounting for the relationships with other variables of interest (Kline, 2015).

Bayesian SEM was selected because it facilitates greater precision in estimating the posterior distribution of the parameters for small samples. Additionally, Bayesian SEM offers the advantage of incorporating previous knowledge about the model's parameters through the use of priors. Thus, Bayesian SEM provided an excellent framework for exploring the relationships between this study's variables, allowing for an appropriate degree of uncertainty when estimating the model's parameters (Scheines et al., 1999). We ran a series of path analysis models with salivary cortisol intercept and slope, PSS, Hair cortisol, BMI, Age, Age at diagnosis, and Age at menarche as predictors and with AMH, E2, FSH, and LH, as outcomes.

Because the aim of the study was to explore relationships between variables, the analysis was not strictly confirmatory. If the data did not fit the model specified, alternative models were tested. The purpose of exploring various models was to provide additional information about the plausible relationships between the variables of interest and make recommendations for future model testing. Data augmentation was used to account for missing data. Missing data was treated simultaneously as a parameter and data, filling any missing data points with the most likely value given the posterior distribution. At each iteration of the estimation, likely values were imputed for the missing data. These likely values were drawn from the posterior distribution estimated at that point in the iterations. In later steps of the estimation, the likely values were used as data in the path analysis, and this process continued until the model had converged (Merkle, 2011).

Data analysis was conducted in R (Core Team, 2017). Bayesian path analysis was run with the R package *blavaan* (Merkle & Rosseel, 2016) which estimates the model with the general Bayesian software JAGS (Plummer, 2003). Convergence of the Markov chains was determined using the potential scale reduction factor (PSRF), also known as univariate R-hat (Gelman & Rubin, 1992). It was determined that the model converged when R-hat was lower than 1.10 for every parameter (Brooks & Gelman, 1998). The models were run with 3 chains, keeping the last 5000 iterations from each chain to build the posterior distributions. Model comparison with LOO and WAIC were done with loo R package (Vehtari et al., 2015); smaller values for LOO and WAIC represent a better fit.

Results

Twenty-four female childhood cancer survivors (mean age 21.79 ± 5.68) participated in the study. The majority had at least a high school education (62.5%) and was unmarried (79.2%). Childhood cancer diagnoses varied considerably and included: Osteosarcoma (n=1), Rhabdomyosarcoma (n=5), Wilms Tumor (n=1), Optic Chiasmal Glioma (n=1), Non-Hodgkin's Lymphoma (n=3), Ewing's Sarcoma (n=2), Immature teratoma L ovary (n=1), B-cell lymphoma (n=1), Hodgkin's Lymphoma (n=4), Right temporal lobe pilocytic astrocytoma (n=1), Pilocytic astrocytoma of the conus (n=1), Left renal tumor (n=1), Undifferentiated sarcoma of posterior sacral region (n=1), and Acute lymphoblastic leukaemia (n=1).

Latent Growth Curve results

The three measures of cortisol were standardized in function of the first time point. For each time point, we subtracted the mean from the measure at the first time point, and divided that number by the standard deviation of the measure at the first time point. This standardization sets the metric in function of the standard deviation from cortisol at the first time point (SD = 74.67) (Figure 2). Due to the standardization, the mean of the intercepts is 0.

The GCM found that $\eta_{\text{int}} \sim N(0.00, 0.65)$, indicating that the average starting point is at the average of the first time point ($M = 105.25$ ng/dL), and the variance is $0.65 \times 74.67 = 48.53$ ng/dL, in function of the standard deviation. For the other parameter of interest, $\eta_{\text{slp}} \sim N(0.324, 0.126)$, indicating that on average, the participants' cortisol levels increased about $0.324 \times 74.67 = 24.19$ ng/dL/hour, and the variance across the sample was $0.126 \times 74.67 = 9.41$ ng/dL. The residual variances indicate that the variance increases over time; at the third time point, the variability of cortisol is greater (Table 1). We found that the salivary cortisol intercept and slope were not correlated ($r = 0.09$).

Path analysis results

The first model included weakly informative priors, which did not constrain the directionality of the regressions. The second model constrained the direction of the regressions for the outcome AMH, the third constrained the direction of the regressions for E2, the fourth model constrained the direction of the regressions for FSH, the fifth model constrained the direction of the regressions for LH, the

sixth model constrained the direction of the regressions for all the outcomes, the seventh model constrained the direction of the regressions for all the outcomes in the opposite direction to the sixth model. These models allowed us to test if the data fit better with some constraints. The seventh model allowed us to do a sensitivity test; if the original theory driven constraints are meaningful the seventh model should decrease the fit.

Table 2 shows the seven models and their fit comparison. Models 2 to 7 were compared with model 1. For model 2 and model 5, the model fit is functionally the same. For models 3, 4, and 6 the model fit improved slightly. And for model 7, the fit decreased slightly. The model comparison demonstrates that the theory driven constraints do not decrease the fit of the model, and in some cases improve it. It also indicates that the opposite direction constraints do harm the fit of the model. Although model 6 improved the fit over model 1, this change in fit was not meaningful, as its change was within the margin of the respective standard errors. Functionally, model 1 and model 6 were equivalent. We chose to keep model 1 as the model from which to interpret the results.

The results for model 1 are presented in Tables 3 to 9. We found that the predictors explained 33.2% of the variance of AMH, 41.8% of FSH, 39.2% of LH, and 0.0% of E2. Overall effects indicate that these predictors were less sensitive to E2. The standardized regression coefficients allowed us to compare the effect of different predictors for each outcome. For AMH, Age, Age at diagnosis, and Perceived Stress Scale (PSS) were the strongest predictors. As PSS increased 1 SD, AMH decreased 0.287 SD. In the case of FSH, PSS was the strongest

predictor, followed by the intercept of salivary cortisol, and age at menarche. As PSS increased 1 SD, FSH decreased 0.53 SD. For LH, we found that the 3 predictors related to cortisol (intercept, slope of salivary cortisol, and hair cortisol) and age at diagnosis were the strongest predictors. As the intercept of salivary cortisol increased by 1 SD, LH increased by 0.387 SD. In the case of E2, all the predictors were equivalent around 0.0, demonstrating that these predictors did not characterize the behavior of E2.

The correlations between predictors and the residual correlation between outcomes are presented in tables 8 and 9. The residual correlation represents the relationship between the outcomes after some variance has been explained by the model. For both sets of correlations, the variables have either a correlation functionally equal to 0, or a weak correlation ($r < 0.2$).

Discussion

Aim 1: Description of perceived stress and HPA activity in female cancer survivors.

In this small sample of female childhood cancer survivors, the mean perceived stress scale (PSS) score was 16.43 ± 6.73 ; this is higher than the mean threshold for stress in the general population, which is 13.7 for women (Cohen et al., 1983). Surviving a life-threatening illness such as pediatric cancer represents a major early life stressor (Laufer et al., 2012) and several studies have demonstrated that childhood cancer survivors report higher levels of stress than healthy controls (Brown, Madan-Swain, & Lambert, 2003; Oancea et al., 2014). Keir et al. demonstrated that long-term

cancer survivors are just as likely as cancer patients to report high levels of stress, suggesting that the time since diagnosis and treatment does not mitigate the stress effects of cancer (Keir et al., 2007).

The average salivary cortisol intercept (corresponding to the PM timepoint) was 105.25 ng/dL with a variance of 48.53 ng/dL. On average, salivary cortisol levels increased 24.19 ng/dL per hour with a variance of 9.41 ng/dL. The latent growth curve demonstrated heterogeneity of variance over time (as shown in Figure 2), with wider variability observed at the later time points. Some slopes were positive and others were flat, creating an over-dispersion at the later time points. Both higher night salivary cortisol concentrations (>100 ng/dL) and flattened diurnal cortisol slopes have been observed in studies examining HPA activity in cancer survivors, a population at risk of experiencing high levels of psychological stress (Cuneo et al., 2017; Garland, Beck, Lipschitz, & Nakamura, 2015; Schrepf et al., 2015; Sephton et al., 2013). It is hypothesized that these abnormal diurnal patterns induce systemic inflammation, which is associated with increased incidence of adverse long-term health outcomes for cancer survivors (cancer recurrence, chronic symptom experience, etc...) (Schrepf et al., 2015).

To our knowledge, no studies have yet examined hair cortisol levels in childhood cancer survivors. In our small sample of 24 CCS, we found a wide range of hair cortisol concentrations (1.08-211.70 pg/mg, mean 19.94 ± 43.23 pg/mg), a finding which is consistent with other studies that have examined hair cortisol concentrations in the general population (Russell et al., 2012; Stalder & Kirschbaum, 2012). However, the median hair cortisol

concentration in our sample was 7.01 pg/mg with 16/24 (67%) having concentrations lower than 10 pg/mg (see Figure 3). This is lower than the median concentrations reported in healthy samples (Stalder & Kirschbaum, 2012). In a meta-analysis of chronic stress and HPA activity, Miller et al. (2007) report that patterns of HPA activity in response to chronic stress vary by time since onset. When the eliciting stimulus of a chronic stressor first begins, there is an initial activation of the HPA axis, which results in elevated concentrations of cortisol. As time passes, this activity lessens, and cortisol secretion rebounds to below normal (Miller, Chen, & Zhou, 2007). This may explain the lower median hair cortisol concentrations observed in our sample of childhood cancer survivors. As both hypocortisolism and hypercortisolism are associated with adverse health outcomes, the finding of lower median hair cortisol concentrations in this sample is notable and requires further investigation (Miller et al., 2007).

Correlation between perceived stress and HPA activity in cancer survivors

In this exploratory study, perceived stress levels, salivary cortisol intercept and slope and hair cortisol concentrations were not correlated in childhood cancer survivors (Table 8). These findings are consistent with previous studies that have examined the relationship between self-reported measures of stress and measures of HPA activity (Miller et al., 2007; Stawski, Cichy, Piazza, & Almeida, 2013). Wells et al. (2014) used a pooled database from diverse community samples to examine the association between hair cortisol concentrations (HCC) and measures of perceived stress. Correlations of HCC

with self-reported stress were small and both were non-significant after controlling for confounders (Wells et al., 2014).

Correlation between biomarkers of HPA activity in cancer survivors

We also found no correlation between hair and salivary cortisol in our sample of CCS. Sauve et al (2007) examined correlations between hair cortisol and concentrations of cortisol in urine, saliva, and serum in 39 non-obese subjects. Hair cortisol and saliva cortisol were not correlated ($r = 0.306$, $p = 0.119$). One explanation for the lack of correlation between hair cortisol and salivary cortisol is the difference in time frames reflected by the measurements: salivary cortisol represents cortisol secretion at one time point during the day, whereas hair cortisol represents average cortisol secretion during 1-3 months (Sauvé et al., 2007). Since hair cortisol reflects long-term cortisol secretion, the association between hair and salivary cortisol would be expected to strengthen with an increasing number of accumulated shorter-term cortisol samples (Stalder & Kirschbaum, 2012) and this was recently confirmed in a study by Short et al.; HCC was most strongly associated with 30-day integrated cortisol production (average salivary cortisol AUC) and there were no significant associations between HCC and the 30-day summary measures using cortisol awakening response or diurnal slope (Short et al., 2016).

Aim 2: To explore the relationship between perceived stress, HPA activity and gonadotropin and reproductive hormone levels and AMH in female cancer survivors

For each outcome, the overall R^2 will be discussed as well as the highest standardized regression coefficients for that outcome.

Perceived stress, HPA activity and AMH

In this study of 24 CCS, we found that the model predictors explained 33% of the variation in AMH levels, with the highest standardized regression coefficients observed for age, age at diagnosis, perceived stress and hair cortisol (Table 4). With an increase of one standard deviation in age, age at diagnosis and perceived stress, AMH declined 5.66 pmol/L, 5.19 pmol/L, and 5.02 pmol/L respectively. The inverse association between age and AMH is well established in the literature (Kelsey et al., 2011). Barton et al. (2013) also found that older age at diagnosis increased risk of post-treatment infertility in childhood cancer survivors, but only in unadjusted models (Barton et al., 2013). The association between perceived stress, HPA activity and the ovarian reserve has not been examined in childhood cancer survivors. However, Pal et al. (2010) examined associations between acute (serum cortisol) and chronic (history of abuse and/or drug use) psychosocial stress and Müllerian-Inhibiting-Substance (MIS), now referred to as AMH, in 89 pre-menopausal infertile women. Those with chronic stress demonstrated significantly lower MIS (AMH) levels ($p = 0.034$), and were three times more likely to be diagnosed with diminished ovarian reserve ($p = 0.025$). However, no association was observed in their study between serum cortisol levels and diminished ovarian reserve. The authors concluded that chronic, but not current stress, was associated with diminished ovarian reserve. They proposed HPA dysregulation as a plausible explanation for this association;

because a biomarker of chronic stress was not included in the study, the authors were unable to provide evidence supporting this theory (Pal et al., 2010).

In this study, we found that with an increase of one standard deviation in hair cortisol concentrations, AMH increased 3.76 pmol/L. And while the standardized regression coefficients of the diurnal cortisol intercept and slope were lower, they were also positively associated with AMH levels. Taken together (positive associations between hair cortisol concentrations, diurnal cortisol slope and intercept and AMH levels), these findings suggest a positive association between cortisol concentrations and the ovarian reserve, and support the hypothesis that threshold glucocorticoid levels are necessary to sustain ovarian function (Whirledge & Cidlowski, 2013). As stated earlier, we found lower median hair cortisol concentrations in our sample than reported in healthy populations (Stalder & Kirschbaum, 2012).

Over half of the subjects in our sample exhibited flattened diurnal cortisol slopes. Lower daily cortisol output and flattened diurnal cortisol slopes have been observed in cancer survivors. Our findings are consistent with prior studies linking hypocortisolism with increased risk of experiencing the late effects of cancer treatment (Bower et al., 2005; Cuneo et al., 2017; Ho et al., 2013; Schrepf et al., 2015; Sephton et al., 2013).

Perceived stress, HPA activity and Gonadotropin levels

In this study, we found that the model predictors explained 42% of the variation in FSH levels, with the highest standardized regression coefficients observed for salivary cortisol intercept, perceived stress and age at menarche

(Table 5). The model predictors explained 39% of the variation in LH levels, with the highest standardized regression coefficients observed for salivary cortisol intercept and slope, hair cortisol and age at diagnosis (Table 6). We also found that perceived stress was inversely associated with FSH and LH levels and was not associated with E2 levels.

Because we were unable to time the collection of reproductive hormone levels according to menstrual cycle phase, we did not anticipate a strong association between perceived stress and FSH and LH levels. There are several possible explanations for the strong inverse association we observed irrespective of menstrual cycle phase. It may be that perceived stress has a significant effect on ovarian function, regardless of menstrual cycle phase.

Schliep et al. (2015) found that daily stress (measured using the Perceived Stress Scale-4) was inversely associated with E2 and LH but positively associated with FSH levels in 259 healthy women aged 18-44 years, after adjusting for age, race, percent body fat, depression score, time-varying hormones and vigorous exercise (Schliep et al., 2015). These contradictory findings regarding stress and FSH may be due to the younger age range included in our study (16-35), in which extreme levels of FSH were less likely. Another explanation may be that stress perception varies across the menstrual cycle, such that the degree to which a woman perceives her life to be stressful is in part influenced by fluctuations in gonadotropin levels (Duchesne & Pruessner, 2013).

While perceived stress was inversely associated with FSH and LH, we found that HPA activity (hair, salivary cortisol intercept and slope) was positively associated with FSH and LH levels. Similar to perceived stress, HPA activity was not associated with E2 levels. Our findings support the role of glucocorticoids in regulating the hypothalamic-pituitary-gonadal axis. In our sample, we observed lower median hair cortisol levels than those reported in healthy samples; thus, the positive association we observed suggests that threshold glucocorticoid levels are needed to sustain reproductive function (Whirledge & Cidlowski, 2013). Another explanation is that cortisol levels may fluctuate across the menstrual cycle as has been suggested by others (Kirschbaum, Kudielka, Gaab, & Schommer, 1999; Nepomnaschy et al., 2011; Stephens et al., 2016; Wolfram, Bellingrath, & Kudielka, 2011). While preliminary, the findings from our small sample support an association between perceived stress, HPA activity and gonadotropin levels in female cancer survivors. More research is needed to clarify the mechanism behind this association and to determine whether it varies depending on the temporal nature of the stressor and menstrual cycle phase.

Limitations

This exploratory study has several limitations. Due to the small sample, we were unable to adjust for hormonal contraceptive use in the overall model. Hormonal contraceptive use is associated with increased production of corticotropin-binding-globulin and subsequent decreases in unbound cortisol levels (Stephens et al., 2016). In addition, due to the wide variety of pediatric cancer diagnoses in this small sample, we could not

adjust for cancer diagnosis and treatment-related factors in the model. Several studies have found a significant effect of diagnosis and treatment-related factors on post-treatment ovarian function, including the cancer pathology itself (Van Dorp et al., 2014), the therapeutic regimen (Charpentier et al., 2014) and the length of exposure (El-Shalakany et al., 2013; Reinmuth et al., 2013). Another limitation is that due to the great distance that many of the participants needed to travel, we were unable to time study visits to coincide with a specific menstrual cycle phase. For this reason, we were unable to adjust gonadotropin and reproductive hormone levels to account for menstrual cycle phase. And last, the study design was cross-sectional and relied on a convenience sample.

Conclusions

In this exploratory study, we sought to examine the association between chronic stress and ovarian function in survivors of childhood cancer. The effects of chronic stress on the ovarian reserve are poorly understood. The findings from this study provide preliminary evidence to suggest that perceived stress is negatively associated with ovarian function, and that biomarkers of HPA activity are positively associated with ovarian function in female childhood cancer survivors. Few studies have examined predictors of the ovarian reserve in childhood cancer survivors other than diagnosis and treatment-related factors. Pre-treatment planning and the ability to provide individualized fertility prognoses depends on increased understanding of factors associated with the ovarian reserve in this

population (Anderson et al., 2013; Knopman et al., 2010). The findings from this study provide the foundation for further research examining the risk for post-treatment reproductive dysfunction in female CCS. Due to the small sample size, this study's findings require validation in larger longitudinal studies. Given that our current understanding of the ovarian reserve is limited, the findings from this exploratory study can be used to clarify relationships between variables and propose a model for future hypothesis testing in experimental research.

Table 1: Clinical and Demographic Descriptive Statistics

	N	Minimum	Maximum	Mean	SD
Age	24	17	34	21.79	5.68
Age diagnosed	24	1	16	10.38	5.22
Age at Menarche	22	10.0	15.0	12.27	1.19
BMI (kg/m²)	20	19.96	34.38	24.57	3.81
PSS Total	23	6	32	16.43	6.727
PM CORT (ng/dL)	20	18.92	262.66	105.26	74.68
AM CORT (ng/dL)	21	32.63	644.01	318.74	203.46
AM + 30 (ng/dL)	21	26.24	850.89	392.93	263.68
HAIR CORT (pg/mg)	24	1.08	211.70	19.94	43.23
FSH (U/L)	24	0.7	9.8	4.93	2.34
LH (U/L)	24	0.5	10.7	4.53	3.12
Estradiol (pmol/L)	24	50	685	197.88	175.04
AMH (pmol/L)	23	2.4	79.5	23.28	17.94

Note: Abbreviations: BMI, Body Mass Index; PSS, Perceived Stress Scale; PM CORT, Night cortisol; AM CORT, Morning cortisol; AM + 30, Morning cortisol plus 30 minutes; HAIR CORT, Hair cortisol; FSH, Follicle Stimulating Hormone; LH, Luteinizing Hormone; AMH, Anti-Müllerian Hormone

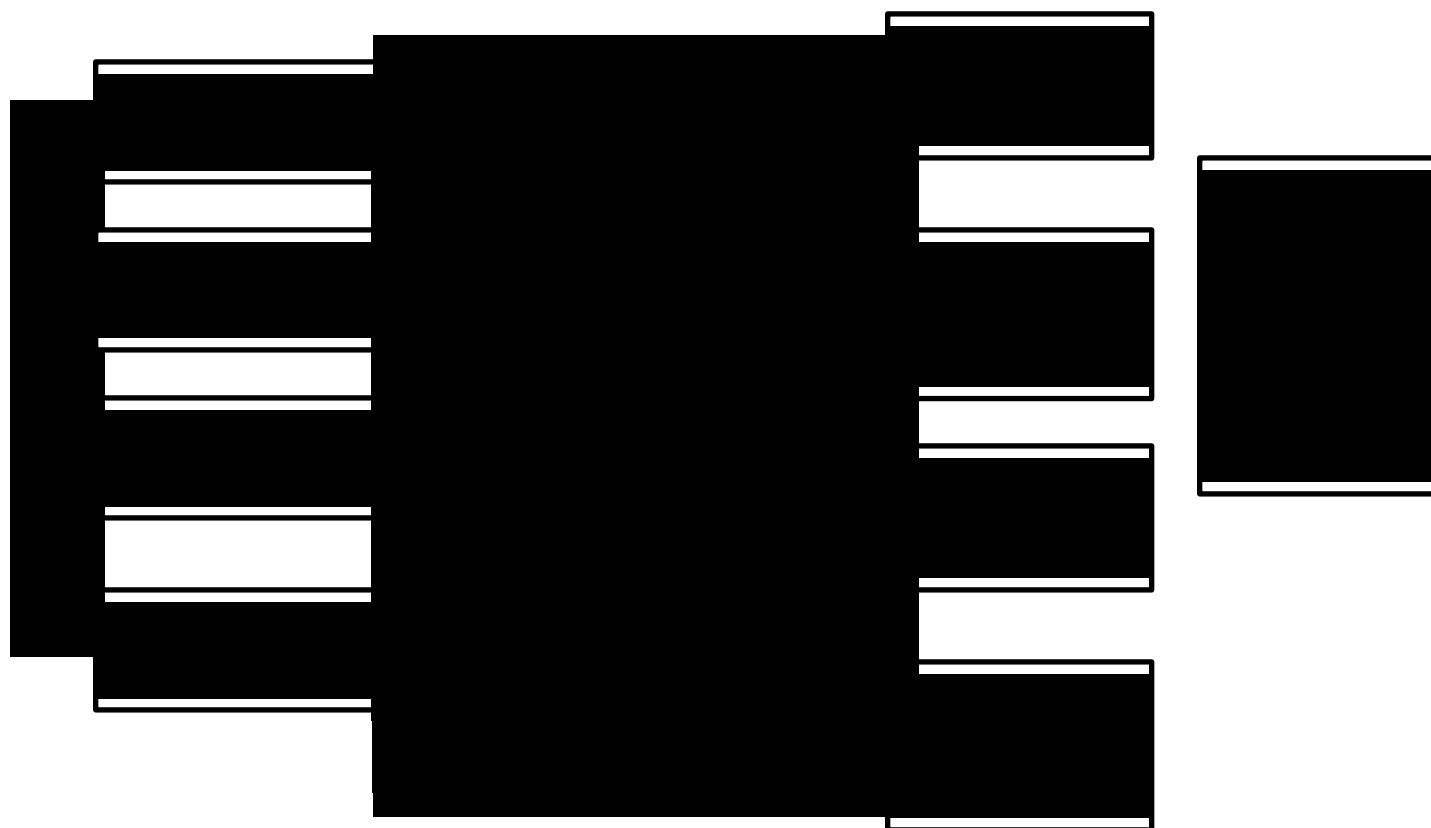


Figure 1: Bayesian Path Analysis Model

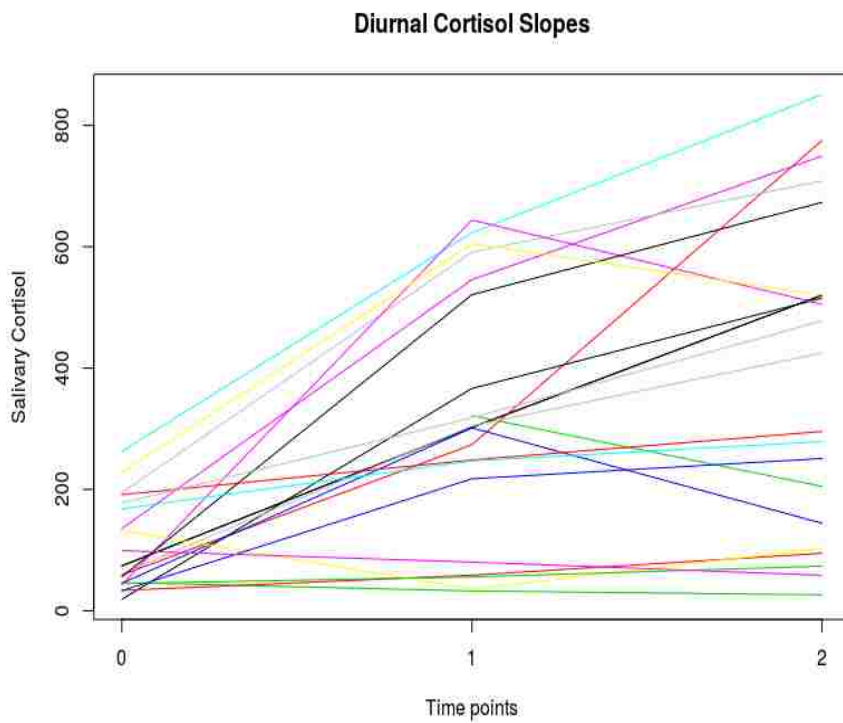


Figure 2: Graph of Diurnal Cortisol slopes including three time points

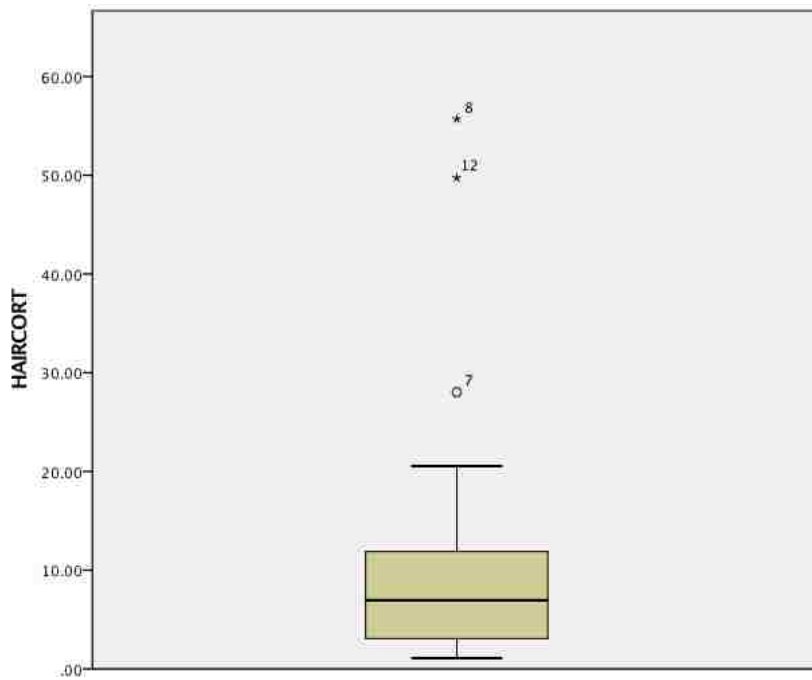


Figure 3: Boxplot of hair cortisol concentrations

Table 2: Latent Growth Curve

Parameter	Estimate	95% CI	Standardized estimate
Intercept mean	0.00	-0.45, 0.46	0.00
Intercept variance	0.65	0.09, 1.39	1.00
Slope mean	0.32	0.16, 0.49	0.91
Slope variance	0.12	0.06, 0.22	1.00
Covariance	0.03	-0.12, 0.17	0.09
Residual T1	0.482	0.00, 1.26	0.42
Residual T2	0.86	0.00, 3.42	0.07
Residual T3	4.06	0.00, 7.29	0.23

Note: Estimate: mean of the posterior distribution; CI: Credible Interval

Table 3: Path analysis model fit comparisons

	LOO (pd)	WAIC (pd)	Δ LOO (SE)	Δ WAI (SE)
Model 1	1474.0 (58.5)	1463.0 (53.1)		
Model 2	1475.3 (58.1)	1463.7 (52.3)	-0.6 (2.0)	-0.3 (1.7)
Model 3	1466.5 (55.2)	1457.3 (50.5)	3.8 (2.4)	2.9 (1.3)
Model 4	1469.0 (55.7)	1460.2 (51.3)	2.5 (2.1)	1.4 (1.7)
Model 5	1474.8 (55.3)	1464.5 (50.1)	-0.4 (3.1)	-0.7 (2.8)
Model 6	1471.5 (51.4)	1464.9 (48.1)	1.3 (4.5)	-1.0 (4.4)
Model 7	1487.1 (49.4)	1479.6 (45.6)	-6.6 (5.4)	-8.3 (5.3)

Note: LOO: leave one out information criteria; WAIC: Widely Applicable Information criteria; pd: effective number of parameters; SE: standard error; Δ : change

Bayesian Path Analysis results by outcome

Table 4: AMH

Predictor	β	95% CI	Standardized estimate
SC Intercept	4.138	-10.39, 18.81	0.133
SC Slope	5.260	-7.14, 18.28	0.082
PSS	-5.020	-10.93, 1.58	-0.287
Hair Cortisol	3.757	-2.43, 10.57	0.214
BMI	1.955	-4.65, 8.29	0.112
Age	-5.664	-12.37, 1.18	-0.312
Age at diagnosis	-5.187	-11.86, 1.86	-0.289
Age at menarche	2.996	-3.77, 9.32	0.172

Note: Abbreviations: SC Intercept, salivary cortisol intercept; SC Slope, salivary cortisol slope; PSS, Perceived Stress Scale; BMI, Body Mass Index, CI, credible interval

Table 5: FSH

Predictor	β	95% CI	Standardized estimate
SC Intercept	1.040	-6.31, 8.45	0.233
SC Slope	0.286	-5.60, 6.61	0.031
PSS	-1.332	-2.47, -0.05	-0.530
Hair Cortisol	0.381	-0.93, 1.65	0.151
BMI	0.294	-0.99, 1.54	0.117
Age	0.153	-1.22, 1.51	0.058
Age at diagnosis	0.113	-1.32, 1.52	0.044
Age at menarche	0.571	-0.77, 1.70	0.228

Note: Abbreviations: SC Intercept, salivary cortisol intercept; SC Slope, salivary cortisol slope; PSS, Perceived Stress Scale; BMI, Body Mass Index, CI, credible interval

Table 6: LH

Predictor	β	95% CI	Standardized estimate
SC Intercept	2.259	-8.73, 13.95	0.387
SC Slope	2.802	-7.85, 10.18	0.233
PSS	-0.537	-2.08, 1.04	-0.164
Hair Cortisol	0.814	-0.88, 2.87	0.247
BMI	-0.384	-2.40, 1.65	-0.117
Age	-0.430	-2.48, 1.46	-0.126
Age at diagnosis	0.857	-1.34, 2.87	0.254
Age at menarche	-0.091	-1.67, 1.55	-0.028

Note: Abbreviations: SC Intercept, salivary cortisol intercept; SC Slope, salivary cortisol slope; PSS, Perceived Stress Scale; BMI, Body Mass Index, CI, credible interval

Table 7: E2

Predictor	β	95% CI	Standardized estimate
SC Intercept	0.140	-13.51, 14.35	0.000
SC Slope	1.065	-13.37, 14.36	0.001
PSS	0.749	-12.59, 14.75	0.003
Hair Cortisol	-0.535	-14.05, 13.34	-0.002
BMI	0.589	-13.15, 13.94	0.002
Age	-0.100	-14.03, 13.36	-0.000
Age at diagnosis	-0.960	-14.56, 12.92	-0.004
Age at menarche	-1.115	-14.54, 12.86	-0.005

Note: Abbreviations: SC Intercept, salivary cortisol intercept; SC Slope, salivary cortisol slope; PSS, Perceived Stress Scale; BMI, Body Mass Index, CI, credible interval

Table 8: Correlation between predictors

	SC Int	SC Slp	PSS	Hair C	BMI	Age	Age dx	Age Mn
SC Inte	1							
SC Slp	0.045	1						
PSS	0.021	0.082	1					
Hair C	0.054	-0.035	0.006	1				
BMI	0.001	-0.067	0.081	-0.051	1			
Age	0.036	0.155	-0.027	-0.019	0.055	1		
Age dx	-0.052	0.002	-0.083	0.111	0.023	0.120	1	
Age Mn	-0.011	0.043	0.002	0.009	-0.100	0.064	0.010	1

Note: Abbreviations: SC Inte, salivary cortisol intercept; SC Slp, salivary cortisol slope; PSS, Perceived Stress Scale; Hair C, Hair cortisol; BMI, Body Mass Index; Age dx: Age at diagnosis; Age Mn, Age at menarche; CI, credible interval

Table 9: Residual correlation between outcomes

	AMH	E2	FSH	LH
AMH	1			
E2	-0.069	1		
FSH	-0.186	-0.108	1	
LH	0.173	0.192	0.059	1

Note: Abbreviations: AMH, Anti-Müllerian Hormone; E2, Estradiol; FSH, Follicle Stimulating Hormone; LH, Luteinizing Hormone

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APPENDIX D: INSTRUMENTS

Subject ID:

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Study Checklist

Please write the name of person completing and the date completed:

Item	Name of person completing	Date
Consent form		
Minimum data set		
Saliva Cortisol 1		
Saliva Cortisol 2		
Saliva Cortisol 3		
Saliva IL-6		
Hair Sample		
Demographic Sheet		
Perceived Stress Scale		

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DEMOGRAPHIC QUESTIONNAIRE
(Please check or fill in the answer)

1. What is your age? _____
2. What is the highest level of education you have completed?
 - Primary school
 - Secondary school
 - University
 - Postgraduate studies
3. What is your ethnic origin?
 - Asian
 - Indian
 - Pakistani
 - Black
 - White
 - Mixed
 - Other
 - Prefer not to say
4. What is your country of origin?

5. What is your current marital status?
 - Married
 - Living with someone
 - Divorced
 - Separated
 - Single
 - Widowed
 - Prefer not to say
6. What is your sexual orientation?
 - Heterosexual
 - Bisexual
 - Gay woman/lesbian
 - Other
 - Prefer not to say

7. What best describes your current employment status?
- Employed full time
 - Employed part time
 - Homemaker
 - Unemployed
 - Student
 - Other
8. Which of the following best describes the area you live in?
- Urban
 - Suburban
 - Rural
9. What is your current household income in GBP?
- Under £10,000
 - £10,000-£19,999
 - £20,000-£29,999
 - £30,000-£39,999
 - £40,000-£49,999
 - £50,000-£59,999
 - £60,000-£75,999
 - £76,000-£99,999
 - £100,000-£150,000
 - Above £150,000
 - Prefer not to say
10. How many people besides you live in your household?
- None
 - 1
 - 2
 - 3
 - 4 or above
11. Do you have any children?
- Yes
 - No
 - Prefer not to say
12. If you have children, how many do you have?
- N/A
 - 1
 - 2
 - 3
 - 4 or more
 - Prefer not to say

Subject ID:

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Perceived Stress Scale-10

Instructions: This set of questions is about your feelings, thoughts, and activities over the *last month*. Please give one answer for each question. Possible responses are never, almost never, sometimes, fairly often, and very often.

In the last month, how often have you...	Never	Almost never	Some- times	Fairly often	Very often
1. Been upset because of something that happened unexpectedly?	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	<input type="checkbox"/> 4
2. Felt that you were unable to control the important things in your life?	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	<input type="checkbox"/> 4
3. Felt nervous and "stressed"?	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	<input type="checkbox"/> 4
4. Felt confident about your ability to handle your personal problems?	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	<input type="checkbox"/> 4
5. Felt that things were going your way?	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	<input type="checkbox"/> 4
6. Found that you could not cope with all the things that you had to do?	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	<input type="checkbox"/> 4
7. Been able to control irritations in your life?	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	<input type="checkbox"/> 4
8. Felt that you were on top of things?	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	<input type="checkbox"/> 4
9. Been angered because of things that happened that were outside of your control?	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	<input type="checkbox"/> 4
10. Felt difficulties were piling up so high that you could not overcome them?	<input type="checkbox"/> 0	<input type="checkbox"/> 1	<input type="checkbox"/> 2	<input type="checkbox"/> 3	<input type="checkbox"/> 4

Perceived Stress Scale Scoring

PSS-10 scores are obtained by reversing the scores on the four positive items, e.g., 0=4, 1=3, 2=2, etc. and then summing across all 10 items. Items 4,5, 7, and 8 are the positively stated items.

Subject ID:

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Saliva Collection Instructions:

1. General: Please avoid sample collection within 60 minutes after eating a major meal or within 12 hours after consuming alcohol. Ten minutes before the sample is collected, please rinse your mouth thoroughly with water.
2. You will have four saliva collecting tubes. Three are for cortisol (marked with an orange sticker) and one is for IL-6 (marked with a blue sticker).
3. Saliva tubes marked with an orange sticker will also be numbered. The numbers indicate the order in which the samples should be collected:
 - a. Orange #1: This sample should be collected on the night prior to the study visit just before you go to bed.
 - b. Orange #2: This sample should be collected on the morning of the study visit immediately after waking up, before getting out of bed.
 - c. Orange #3: This sample should be collected 30 minutes after the second sample.
 - d. Blue: This sample should be collected immediately after the third sample.
4. To collect the sample, unwrap the oral cotton swab and place it underneath your tongue.
5. After collecting the samples, write your study ID, date, and time of collection on each tube.
6. Double-bag the samples in the sealable plastic bags that are provided and place them in the refrigerator until you come in for your study visit.
7. Bring the samples to the study visit and give them to the research nurse.

Subject ID:

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Saliva Sample Handling

Saliva Sample

Date collected

Time collected

Cortisol 1

D	D	M	M	Y	Y	Y	Y

H	H	M	M

Cortisol 2

D	D	M	M	Y	Y	Y	Y

H	H	M	M

Cortisol 3

D	D	M	M	Y	Y	Y	Y

H	H	M	M

IL-6

D	D	M	M	Y	Y	Y	Y

H	H	M	M

Time sample spent at 4⁰c awaiting centrifuge processing prior to freezing

--	--

Days

**Handing by Queen's Medical Research Institute
Add contact info**

Total volume of saliva sample after centrifuge

Cortisol 1

--	--

mL

Cortisol 2

--	--

mL

Cortisol 3

--	--

mL

IL-6

--	--

mL

Date and time into -80°C

D	D	M	M	Y	Y	Y	Y

H	H	M	M

Number of Freeze-thaw cycles:

--	--

Reason:.....
.....
.....

Hair collection instructions

1. Collect approximately 1cm width hair section from posterior vertex region. Place scissors as close to the scalp as possible.
2. Secure hair sample with thread provided (indicating proximal end).
3. Place hair sample in aluminum foil provided with the proximal end of the sample on the side of the foil marked "top".
4. Fold the aluminum foil to ensure sample is covered.
5. Place sample wrapped in aluminum foil in the plastic bag provided.

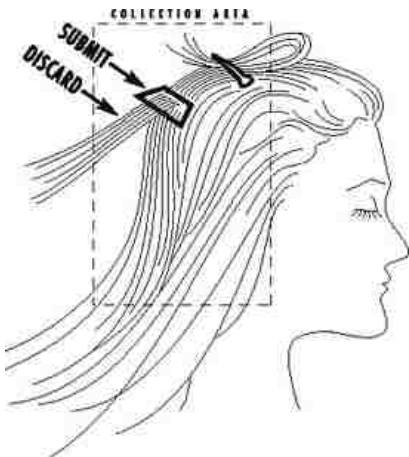


Figure 1. Hair sampling from posterior vertex region.

Subject ID:

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Hair Sample Handling

Date collected

Time collected

Hair Sample

D	D	M	M	Y	Y	Y	Y

H	H	M	M

Length of sample

--	--

 cm

Has subject ever dyed hair (yes or no)

YES / NO

If so, how recently?

Subject ID:

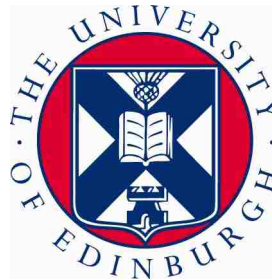
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Minimum Data Set: Please ensure the following clinical data is collected if not already available in the participant's medical records.

1. Diagnosis _____
2. Age at diagnosis _____
3. Treatment Type: _____
 - a. Chemotherapy: YES / NO
 - b. Radiotherapy: YES / NO
4. Age at completion of treatment: _____
5. Comorbidities
 - a. _____
 - b. _____
 - c. _____
 - d. _____
6. Chronic symptoms (post treatment)
 - a. _____
 - b. _____
 - c. _____
 - d. _____
7. Prescription medications
 - a. _____
 - b. _____
 - c. _____
 - d. _____
8. Age at menarche: _____
9. Average menstrual cycle length before treatment: _____
10. Average menstrual cycle length after treatment: _____
11. Date of last menstrual period: _____

D D / M M / Y Y Y Y
12. Parity: _____
13. Weight: _____
14. Height: _____
15. BP: _____

APPENDIX E: PATIENT INFORMATION SHEET



Dear _____ (name of patient),

You are receiving this letter because you may be eligible to participate in a research project examining the effect of Chronic Stress on Reproductive Function (CSRf) in female cancer survivors. Our knowledge of factors that contribute to reproductive problems in cancer survivors is incomplete. We know that stress inhibits reproductive function, and we would like to examine the extent to which it may contribute to reproductive problems in cancer survivors. In this way, if we find that stress contributes to reproductive problems, we can integrate stress reduction techniques into reproductive health care in cancer survivors.

Thank you in advance for taking the time to read through the study description. Feel free to contact us to discuss it further or request more information before making your decision. If you agree to be contacted regarding the study, please return the reply slip at the bottom of this letter, either electronically or by using the stamped envelope provided.

Yours sincerely,

Richard A Anderson
Principal Investigator

I have read the enclosed patient information sheet explaining the study, "Effect of Chronic Stress on Reproductive Function in Female Cancer Survivors."

I am interested in taking part: please contact me with further information

Please do not contact me with further information about the study

Signature: _____ Date: _____

Name:



Effect of Chronic Stress on Reproductive Function in Female Cancer Survivors
Participant Information Sheet

What is the purpose of this study?

The main purpose of this study is to explore the influence of chronic stress on reproductive function in females following treatment for childhood cancer. Reproductive function is measured by a blood test, and this is done as part of your normal care. Chronic stress will be measured by a brief questionnaire assessing perceived stress over the past month and hair and saliva samples to assess cortisol levels (a hormone released as part of the body's stress response) and IL-6 levels (released as part of the body's inflammatory response to stress).

Do I have to take part?

It is entirely your decision whether you would like to take part in this study or not. If you decide to take part, we will ask you to sign a consent form and we will ask you to keep a copy of this leaflet. If you decide not to take part, it will not affect your medical care. Even after you have decided to take part, you are free to withdraw and you need not give us a reason for withdrawal.

What will happen to me if I decide to take part?

If you decide to take part, we will schedule a brief 30-minute visit to collect study data.

Before the study visit, you will receive a saliva collection kit with detailed instructions on how to collect the saliva samples. We will ask you to collect four saliva samples in total. We will ask you to collect one sample on the night before the study visit and the remaining three on the morning before you come in for your visit. We will ask you to bring the saliva samples to the study visit.

During the visit, we will ask you some short questions about your medical history and collect a small hair sample from the back of your head. We will also ask you to fill out a brief questionnaire about your stress over the past month. The whole visit should take no more than half an hour.

No drugs or extra blood tests are involved in this study.

What do I need to do?

If you agree to participate in this study, we will arrange a suitable date for your visit.

What are the side effects of any treatment when taking part?

Collecting hair and saliva samples can be a little uncomfortable; any discomfort will be minimized as much as possible.

What are the possible disadvantages and risks of taking part?

We do not expect any disadvantages or risks from taking part in this study.

What are the possible benefits of taking part?

By taking part in this study, you may learn more about your current reproductive status and your stress levels.

What if new information becomes available?

We will continue to look at emerging studies from across the world in this field. If any new information that is relevant to this study becomes available, we will review the study in consultation with the Ethics Committee.

What if something goes wrong?

If you have a concern about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions. If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure. Details can be obtained from the hospital.

In the event that something does go wrong and you are harmed during the research study there are no special compensation arrangements. If you are harmed and this is due to someone's negligence then you may have grounds for a legal action for compensation against The University of Edinburgh but you may have to pay your legal costs. The normal National Health Service complaints mechanisms are available to you.

Will my taking part in the study be kept confidential?

With your permission, we will let your GP know that you are taking part in this study. If we find any abnormalities, we will discuss this with you and if necessary, refer you to your GP or a specialist.

We will keep your participation in this study and all related information confidential. All information that is recorded or sent to our colleagues (e.g: laboratory staff) will be labeled with a unique reference number. Your name, address and any other identifying information will be removed.

If at any point during the study you should lose the capacity to provide consent, you will be withdrawn from the study and no further data will be collected from you. Data already collected while you were able to provide consent will be retained.

What will happen to the results of the research study?

Once we have analysed all the results, we will write one or more papers, which will be submitted to medical research journals for publications. Your personal details will not be included in any of these. After completion of the study, a lay summary of the study's findings will be available upon request from the clinical team looking after you.

What will happen to my saliva and hair samples after the study is completed?

Samples will be stored for up to 5 years in the Queen's Medical Research Institute at the University of Edinburgh for use in further research studies, which would require additional ethical approval. It is possible that we may work with a commercial organization in analyzing your samples, but you will not receive compensation should the samples have commercial value.

Who is organizing and funding the research?

This study is funded by the Medical Research Council, a publicly funded UK organization.

Who has reviewed this study?

The study has been reviewed by the South East Scotland Research Ethics Committee

Contacts

Research team:

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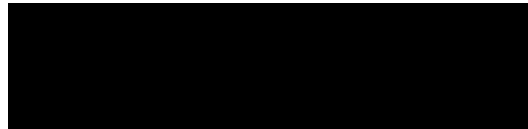
If you wish to speak to someone not involved in the study, please contact:

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Tel: 0131 2420425

NHS complaints contact:
NHS Lothian Complaints Team
2nd Floor
Waverley Gate
2-4 Waterloo Place
Edinburgh
EH1 3EG
Tel: 0131 465 5708
Email: craft@nhslothian.scot.nhs.uk

APPENDIX F: CONSENT FORM



Patient Identification Number:

Effect of Chronic Stress on Reproductive Function in Female Cancer Survivors

Please initial to confirm

1. I confirm that I have read and understand the information sheet dated 16 July 2015 (version 2) for the above study.	
2. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	
3. I understand that I will provide saliva and hair samples and answer some questions regarding my stress level over the past month as part of the research study.	
4. I understand that relevant sections of my medical notes and data collected may be looked at by individuals from the regulatory authorities and from the Sponsor(s) (NHS Lothian and the University of Edinburgh) or from the/other NHS Board(s) where it is relevant to my taking part in this research. I give permission for those individuals to have access to my records.	
5. I agree to my GP being informed of my participation in the study.	
6. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected.	
7. I consent to hair, and saliva being stored in a secure facility for use in future related ethically approved studies, which may include transfer to other academic or commercial collaborators. I understand that a unique number will identify all samples to ensure confidentiality.	

Name of Participant

Date

Signature

Name of Person taking consent
(if different from researcher)

Date

Signature

Original (x1) to be retained in site file. Copy (x1) to be included in patient notes. Copy (x1) to be retained by the participant.