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Xenobiotic Exposures on Women's Reproductive Health

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by

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DEDICATION

To my family – Andrew, John, Margie, and Anna Ruth. This work is a testament to the support I have received from all of you. And to my dog, Sophie, whose positivity and snuggles kept me serene.

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ABSTRACT

Environmental and chemical exposures are continually introduced into our bodies. The female reproductive cycle is complex and particularly sensitive to toxic substances. A recent rise in infertility and reproductive diseases and cancers make reproductive toxicity a public health concern. The overall purpose of this dissertation is to explore how environmental and chemical exposures impact women's reproductive health and overall wellbeing. To do this, a variety of topics related to women's reproductive health are explored, including how environmental exposures can impact reproduction, methods for studying reproductive dysfunctions, fertility preservation among cancer patients, and the overall wellbeing of female cancer patients.

The first objective, using cross-sectional data from the National Health and Nutrition Examination Survey, is to determine the association between three heavy metals (lead, cadmium, and mercury) and their mixture and infertility and long-term amenorrhea. The second objective is to review the literature of bioengineering models used by researchers in labs to better understand a variety of female reproductive diseases. The third objective is to review the literature on current oocyte preservation options in oncofertility and discuss current guidelines and practices of female fertility preservation. The fourth objective is to focus specifically on assessing knowledge, attitude, and behavior towards oncofertility among female breast cancer patients in China.

For the first objective, findings showed that higher lead blood concentrations increased the odds of infertility. Additionally, blood concentrations of lead and heavy metal mixtures were significantly higher in infertile women than pregnant women, but the concentrations of cadmium and mercury were comparable. In summary, heavy metals have endocrine disrupting effects and may increase women' risks of infertility and long-term amenorrhea. For the second objective, peer-reviewed literature demonstrated that applying bioengineering to female reproductive biology and medicine provides great potential to advance the knowledge of female fertility, genetic vulnerability, medications, environmental exposures and toxicities, aging, nutrition, and diseases. For the third objective, the literature reviewed explained that current fertility preservation options such as oocyte and embryo cryopreservation are well established for reproductive aged female cancer and infertility patients. Moreover, recent advancements in reproductive science and medicine have allowed for investigational fertility preservation options for both childhood and reproductive-age patients, including ovarian tissue cryopreservation, *in vitro* oocyte maturation, ovarian transposition, ovarian suppression, and adjuvant therapy. Finally, results of the fourth objective suggest that breast cancer patients in China view oncofertility as important, but inadequate oncofertility knowledge remains. Additionally, providers need to communicate more information to patients about how their cancer treatment will impact their fertility and what options they have for fertility preservation.

Undoubtedly, the many evident complexities of the interactions between xenobiotic exposures and women's reproductive health outcomes emphasize the need for more research. Efforts are needed to reduce endocrine disrupting exposures among women and to further develop fertility preservation approaches for cancer or infertility patients. Public health research and science-informed policies are critical to reducing adverse health outcomes, especially in the modern world where not only are environmental, occupational, and medical substances progressively introduced into the human body, but also reproductive disease and infertility rates are on the rise.

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CHAPTER 1

INTRODUCTION

As humans, we are continually subjected to a variety of substances via environmental, occupational, and medical exposures. Environmental pollution, industrial development, and agricultural processes have introduced toxic elements into the food chain, water cycle, and air.[1] Drugs also introduce chemicals into the human body.[2]

The female reproductive cycle is complex, making it difficult to study and model.[3,4] It also is particularly sensitive to toxic substances, making reproductive toxicity a public health concern.[5,6] With the incidence of infertility and reproductive diseases and cancers increasing over time,[7] more research is needed to understand the complexities of women's reproductive health.

Objectives

The overall purpose of this dissertation is to explore how environmental and chemical exposures impact women's reproductive health and overall wellbeing. To do this, this research explores a variety of topics related to women's reproductive health (Figure 1.1).

The dissertation begins with Chapter 1, an introduction to the dissertation and a summary of female reproductive biology to help the reader understand the complexities of normal female reproductive function. This introduction is followed

by Chapter 2 which examines the impacts of environmental exposures on reproductive dysfunctions in women. Specifically, this chapter determines the associations between heavy metal exposure and two reproductive dysfunctions – infertility and long-term amenorrhea - among women in the United States. Epidemiological studies have many limitations when assessing women's reproductive health. Therefore, this chapter is succeeded by Chapter 3, a review of bioengineering models used by researchers in labs to better understand a variety of female reproductive diseases. Then the dissertation dives deeper by focusing on fertility; the next piece, Chapter 4, reviews current oocyte preservation options in oncofertility and discusses current guidelines and practices of female fertility preservation. Finally in Chapter 5, the overall wellbeing of women with cancer and their reproductive health are discussed; this final section aims to understand the knowledge, attitude, and behavior towards cancer and fertility preservation among female breast cancer patients.

Hypothesis

The overall hypothesis of this dissertation is that chemical exposures adversely impact women's reproductive health and overall wellbeing. Because each chapter in this dissertation covers a specific topic, there are also sub-hypotheses for each chapter. A summary of each chapter's objective, sub-hypothesis, and focus is outlined in Table 1.1.

Women's Reproductive Biology Overview

Before reading the other chapters covered in this dissertation that dive into reproductive dysfunctions, it is important to understand the complexities of normal reproductive function.

The female reproductive system is composed of two ovaries, two fallopian tubes, a uterus, cervix, and vagina. These organs provide hormonal support and anatomical structure for embryos to undergo development, transport, implantation, placentation, and full-term pregnancy. The ovary contains various developmental stages of follicles as the basic functional unit. Each follicle consists of an oocyte (the central germ cell) and the surrounding somatic cells. There is a finite number of primordial follicles set just after birth, and these follicles remain in a quiescent state to represent ovarian reserve, a marker of female reproductive lifespan. It is believed that the ovarian follicle pool is established prior to birth and is non-renewable.[8-11] Thus, chemicals or other factors that compromise the quantity and quality of follicles and/or oocytes will result in ovarian toxicity (ovotoxicity) and infertility.[12]

The Menstrual Cycle

The endometrium, the tissue lining in the uterus, plays an essential role in women's reproductive health and fertility. In response to ovarian estrogen and progesterone exposure, the endometrium regularly undergoes cycles of proliferation, differentiation, and tissue break down (menstruation). The endometrium thickens during the monthly menstrual cycle in preparation for possible implantation of an embryo. If pregnancy fails, the upper two thirds of the

endometrium (functionalis layer) shed and regenerate on a cyclic basis (menstruation). Regular menstrual bleeding indicates continual ovarian function.[13] The menstrual cycle can be divided into two phases: 1) the follicular (proliferative) phase and 2) the luteal (secretory) phase.

Follicular Phase

The follicular phase starts at the first day of menses and lasts until ovulation. Folliculogenesis, or maturation of the ovarian follicle, begins the last few days of the previous menstrual cycle.[14] Gonadotropin-releasing hormone (GnRH), secreted by the hypothalamus, stimulates the anterior pituitary to produce luteinizing hormone (LH) and follicle-stimulating hormone (FSH).[15-17] Women are born with a set number (1-2 million) of primordial follicles known as their ovarian reserve. By puberty, approximately 400,000 to 500,000 primordial follicles remain.[18] After menarche, approximately 1,000 follicles are lost monthly, with even more after age 35. The rise in FSH from the anterior pituitary stimulates a select number of follicles (about 1,000) to start maturing.[19] Follicle diameter plays a key role in the recruitment of follicles to start the maturation process and in the selection of the dominant follicle.[20]

During folliculargenesis, follicles develop in stages, starting from primordial follicles and moving to primary, secondary, and finally antral follicles.[21] A primordial follicle contains a primary oocyte surrounded by a single layer of squamous pre-granulosa cells. FSH stimulates primordial follicles to develop into primary follicles, which consist of a primary oocyte surrounded by a single layer of cuboidal granulosa cells with FSH receptors.[17] Granulosa cells help to form the

zona pellucida and they produce estradiol.[22] Primary follicles then develop into secondary follicles, containing a primary oocyte surrounded by several layers of cuboidal granulosa cells. As the secondary follicle develops, stromal cells differentiate to form theca cells, which have LH receptors. LH stimulates theca cells to produce androgens. Androgens spread into the nearby granulosa cells which convert androgen to estrogen.[17,22] Secondary follicles develop into antral follicles where the number of granulosa cells continue to increase and begin to secrete fluid into the center of the follicle, creating an antral cavity surrounding the primary oocyte.[17]

During the first 10 days of the menstrual cycle, while follicles are still in their secondary/early antral phase and estradiol concentrations remain low, estradiol inhibits the release of LH from the anterior pituitary. Additionally, FSH is secreted primarily because of low estradiol concentrations. Consequently, as estradiol levels rise with the increasing number of granulosa cells, FSH levels fall.[17,23-25] Because granulosa cells have FSH receptors, the dominant follicle has an increased sensitivity to FSH and will continue growing as FSH levels fall. Accordingly, the other follicles in the original cohort of 1,000 follicles that aren't as mature will undergo atresia. [19] Simultaneously, estradiol stimulates endometrial growth.

Ovulation

After 10 days, estradiol levels become high enough to switch from inhibiting LH to stimulating the secretion of LH.[17,23-25] Therefore, at day 14 a sharp spike in LH occurs, triggering ovulation. The primary oocyte undergoes

meiosis I, where it produces a secondary oocyte and the first polar body.[17,26,27] The most mature antral follicle will rupture and release a secondary oocyte into the fimbriae of the Fallopian tubes.[28]

Endometrial Proliferative Phase

The endometrial cycle can be divided into three phases: the proliferative phase, the secretory phase, and menstruation. Each phase is delineated by physiologic changes controlled by estrogen and progesterone produced by the ovary.

Coinciding with folliclogenses, the proliferative phase of the endometrium occurs during the first 14 days of the cycle. Increases is estradiol help to build up the stratum functionalis layer of the endometrium, reaching its maximum thickness of around 0.5 to 5mm. Arteries provide blood flow to the endometrium and adapt with its increasing thickness. Increases in estrogen also trigger the cervix to produce watery, thin mucus, making the vagina less acidic. These endometrial and cervical changes create a less hostile environment for sperm.[23,24]

Luteal Phase

After ovulation, FSH and LH levels drop back down. The remaining dominant follicle that expelled the ovulated oocyte will transform into a corpus luteum. The corpus luteum secretes three hormones (progesterone, inhibin A, and estradiol) that maintain an environment conducive for implementation.[29] Inhibin A inhibits the secretion of FSH from the anterior pituitary.[30,31] Estradiol stimulates endometrial proliferation and primes the endometrium for implantation.

Progesterone, which is the dominant hormone produced during the luteal phase, inhibits endometrial proliferation and converts an estrogen primed endometrium into one that will be receptive to implantation.[13,14,28] If fertilization occurs, the fertilized oocyte will implant into the endometrium, and the corpus luteum will continue to function, maintaining hormone levels. If fertilization does not occur within 1 to 2 days of ovulation, the secretion of hormones from the corpus luteum will stop within 14 days after ovulation and it will degenerate into scar tissue (corpus albican).[29,32]

Endometrial Secretory Phase

The secretory phase of the endometrial cycle occurs from day 14 to 28. Progesterone prepares the corpus luteum and the endometrium for possible fertilization and implantation. The endometrium increases its vascular supply and stimulates more mucus secretions. In addition, progesterone slows the proliferation of the endometrium and works to provide more surface area within the arteries. Progesterone also decreases and thickens the cervical mucous, preventing sperm from entering since the window for fertilization has passed.[24]

Menses

Menses occurs on day 0-5 of the next menstrual cycle.[24] Menstruation is the endometrial response to the withdrawal of progesterone and estrogen as the corpus luteum dies in the absence of pregnancy. The molecular mechanisms for how hormones stimulate menses involve complex interactions between the endocrine and immune system. Key structural components of the endometrium during menses are the arterial blood and influx of leukocytes.[13,24] In women,

normal menstruation occurs every 24-38 days (averaging 28 days), lasts ≤ 8 days long (averaging 5 days), with blood loss volume being 5-80 mL.[33] The luteal phase of the cycle is relatively constant in all woman with a duration of 14 days. The variability of cycle length is usually derived from varying lengths of the follicular phase.[14]

When ovulation does not occur, the corpus luteum does not form to produce progesterone, and estrogen continues to stimulate the endometrium. This causes excessive endometrial proliferation, unpredictability, and irregular bleeding. Irregular menses can be due to a number of underlying causes, including polycystic ovary syndrome, uncontrolled diabetes mellitus, thyroid dysfunction, hyperprolactinemia, and use of antipsychotics or antiepileptics.[34]

Table 1.1: A Summary of the Chapters in this Dissertation

Chapter	Chapter Title	Objectives	Sub-Hypotheses	Summary of Methods	Focus and Flow of Chapters
2	Associations Between Heavy Metals and Female Reproductive Dysfunction	Determine the associations between blood lead, cadmium, mercury, and metal mixture levels and reproductive dysfunctions including infertility and long-term amenorrhea.	Women with higher blood heavy metal levels will be more likely to experience reproductive dysfunctions.	NHANES secondary data analysis using logistic regression	Environmental exposures on two reproductive dysfunctions in women (population-based study). ⇩
3	Bioengineering Models of Female Reproduction	Review recent advances of bioengineering models of female reproductive tissues and functions. Review current research that used bioengineering methods to study female reproductive diseases including endometriosis and gynecologic cancers.	The literature on bioengineering models of female reproduction will be relatively limited due to this emerging field.	Peer-reviewed literature review	Epidmiological studies have many limitations when assessing women's reproductive health. Therefore, in this chapter I discuss novel methods researchers use to study a variety of reproductive diseases in the lab. ⇩
4	Preserving Oocytes in Oncofertility	Review current oocyte preservation options in oncofertility and discuss current guidelines and practices of female fertility preservation.	The literature on preserving oocytes in oncofertility will be abundant and complex.	Peer-reviewed literature review	In this chapter I dive deeper into fertility. How can fertility be preserved among cancer patients? ⇩
5	Knowledge, Attitude, and Behavior Towards Oncofertility Among Female Breast Cancer Patients in China	Understand the knowledge, attitude, and behavior towards cancer and fertility preservation among female breast cancer patients in a Chinese hospital system.	Women will have limited knowledge and access to fertility preservation options and therefore, will likely not often use preservation methods.	A survey of breast cancer patients in China with basic descriptive and inferential statistical analysis	Looking further at the overall wellbeing of female cancer patients (assessing their knowledge, attitude and behavior towards fertility).

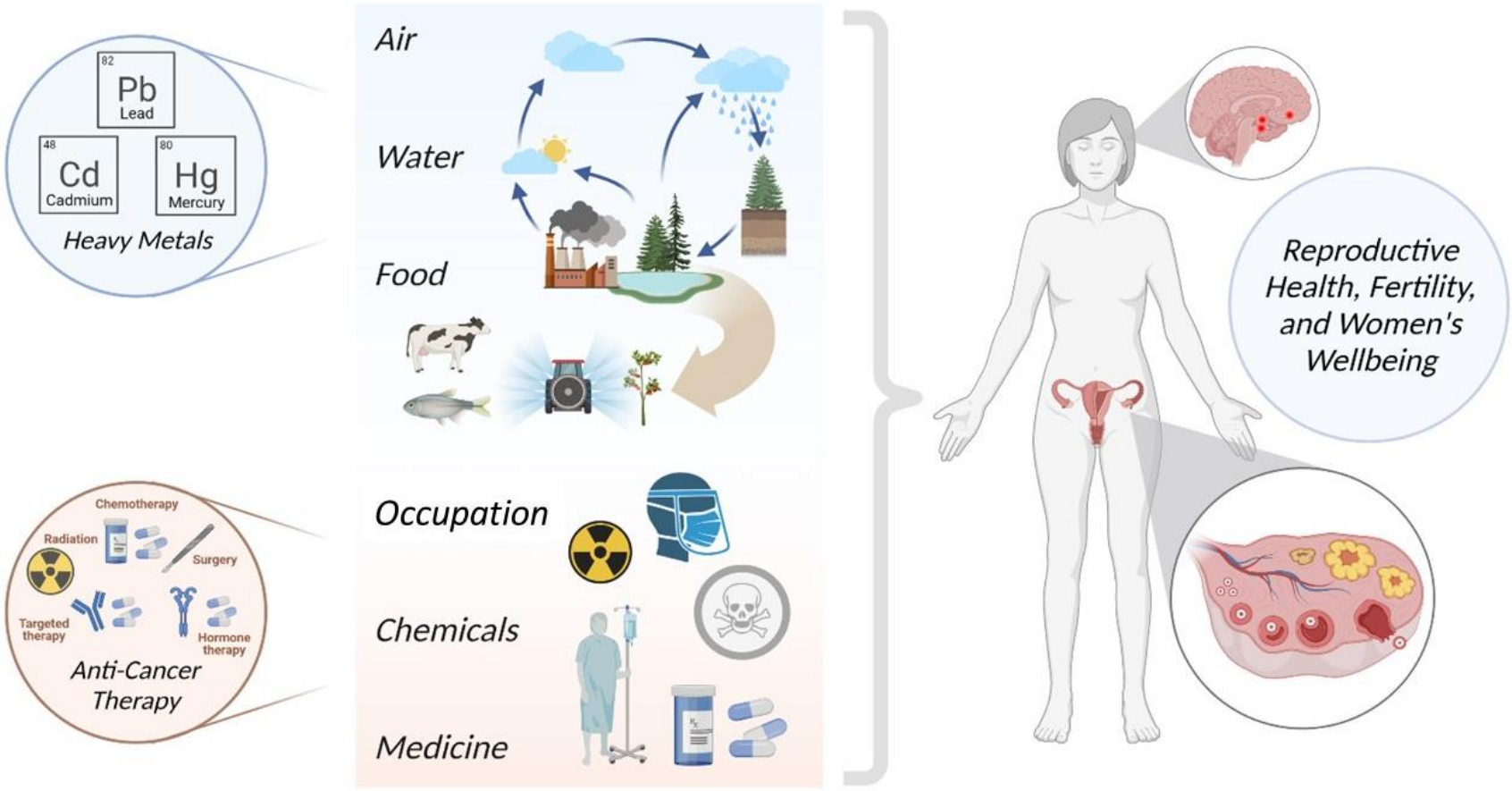

Figure 1.1: A graphical summary of this dissertation.

CHAPTER 2

ASSOCIATIONS BETWEEN EXPOSURE TO SINGLE CADMIUM, LEAD, MERCURY AND MIXTURES AND WOMEN'S INFERTILITY AND LONG-TERM AMENORRHEA[1]

[1] McClam MZ, Liu J, Fan Y, Zhan T, Zhang Q, Porter DE, Scott GI, Xiao S. 2022. Associations between exposure to single cadmium, lead, mercury and mixtures and women's infertility and long-term amenorrhea. In Review at *Journal of Clinical Endocrinology and Metabolism.*

Overview

Now that we have an understanding of women's reproductive biology and normal processes from Chapter 1, we can dive deeper into reproductive dysfunctions. Chapter 2 investigates the effects that environmental exposures can have on female reproductive dysfunction. Thus, this chapter shows results from an analysis of a national population-based study. This chapter determines the associations between heavy metal exposure and two reproductive dysfunctions – long-term amenorrhea and infertility - among women in the United States. We hypothesize for this chapter that women with higher blood heavy metal levels will be more likely to experience reproductive dysfunctions.

Abstract

Purpose

Cadmium (Cd), lead (Pb), and mercury (Hg) have been shown to exhibit endocrine disrupting properties. their effects on women's reproductive health, however, remain elusive. Here, we investigated associations between blood concentrations of single of Pb, Cd, Hg, and their mixture and infertility and long-term amenorrhea in women of reproductive age using the US National Health and Nutrition Examination Survey (NHANES) 2013-2018 cross-sectional survey.

Methods

A total of 1,990 women were included for the analysis of infertility and 1,919 women for long-term amenorrhea. The methods of log-transformation and quarterization were used to analyze blood heavy metal concentrations. Statistical differences in the covariates between the outcome groups were evaluated using

a chi-squared test for categorical variables and a t-test for continuous variables. Multiple logistic regression models were used to examine the associations.

Results

The blood concentrations of Pb and heavy metal mixtures were significantly higher in ever-infertile women than pregnant women, but the concentrations of Cd and Hg were comparable. Multiple logistic regression analysis revealed that after the full adjustment, there was a significant and dose-dependent positive association between blood Pb concentrations and women's historical infertility, a negative association between Cd and women's long-term amenorrhea, and no associations between Hg and heavy metal mixture and women's infertility or long-term amenorrhea.

Conclusions

Our study demonstrates that exposure to heavy metals exhibit differential associations with women's infertility and long-term amenorrhea.

Introduction

The female reproductive system provides hormonal control and anatomical structure to sustain a woman's menstrual cycle and fertility. Infertility is the failure of achieving clinical pregnancy after one year of unprotected intercourse, affecting up to 15% of couples worldwide.[35,36] In the US, the number of women with impaired fertility has been estimated to increase from 4.5 million in the early 1980s to about 7.7 million by 2025.[37] Although women's infertility can be caused by male factors and unexplained reasons,[38] the majority of them have recognized reproductive or neuroendocrine disorders, such as premature ovarian

insufficiency (POI),[39] oligomenorrhea or amenorrhea,[35] anovulation, [40] poor gamete quality,[41] and other reproductive diseases such as polycystic ovarian syndrome (PCOS),[42] endometriosis,[43] and hypothalamic dysfunction.[44] So far, the mechanism of women's infertility remains incompletely understood but has been attributed to both genetic factors and exposure to reproductive toxicants.[45]

Industrial development, agricultural practices, and the production and use of consumer products have introduced various toxic substances into the environment, including heavy metals that are naturally occurring metallic elements with high molecular weight and density.[46] Cadmium (Cd), lead (Pb), and mercury (Hg) are three primary heavy metals listed by the World Health Organization (WHO) under the top 10 toxicants of major public health concern.[47] The environmental contamination of heavy metals primarily stems from industrial mining, agricultural practice, and fossil fuel and waste combustion, etc.[48-51] Heavy metals persist and bioaccumulate along the food chain and in drinking water, soils, and air, making them a major source of environmental toxicants to humans.[52]

Women's reproductive health is vulnerable to environmental toxins, particularly endocrine disrupting chemicals (EDCs) that interfere with the body's normal hormone synthesis, secretion, and signaling.[53,54] Growing epidemiological and experimental research have revealed that heavy metals exert endocrine disrupting properties,[55-59] implicating the possible causative relationship between exposure to heavy metals and women's infertility and other reproductive disorders. In a cross-sectional study that compared 310 women with clinically

diagnosed infertility and 57 pregnant women in Taiwan, the blood concentrations of Pb but not Cd in infertile women were significantly higher than pregnant women.[60] Another study compared 82 infertile and 42 pregnant women in the US and found that there were positive associations between blood concentrations of Pb and Cd and women's infertility.[61] Heavy metals have also been shown to affect reproductive hormone secretion. In premenopausal women, the blood concentrations of Cd, Pb, and Hg were associated with altered means and amplitudes of follicle stimulating hormone (FSH) and luteinizing hormone (LH), two gonadotropins that regulate ovarian follicle maturation, hormone secretion, and ovulation.[62] It was also found in the same study that Pb may increase progesterone levels in the follicular phase, and both Pb and Hg cause a delay of the progesterone rise in the mid-luteal phase.[62]

Experimental research has documented that exposure to heavy metals may impact the female reproductive cycle and fertility. For example, Cd exposure in mice compromised oocyte meiotic and developmental competence by inducing oocyte oxidative stress, early apoptosis, and epigenetic modifications, which eventually resulted in decreases in litter size.[63] Pb has been found to delay vaginal opening, decrease estradiol secretion, and interfere with ovarian cyclicity in rats, suggesting the harmful effects of Pb on the ovaries or the entire hypothalamic-pituitary-gonadal (HPG) axis.[64] Heavy metals may also act as agonists or antagonists to disrupt hormone receptor-mediated signaling. All Cd, Pb, and Hg have been reported to exert estrogenic effects by binding to the estrogen receptor α and/or β, which may disrupt the expression of estrogen

target genes and the proliferation and/or differentiation of estrogen-responsive tissues such as the endometrium.[57,58] Altogether, existing epidemiological and experimental evidence suggests that exposure to heavy metals may perturb women's menstrual cycle and fertility by interfering with the homeostasis of the HPG axis, ovarian steroidogenesis, hormonal signaling, and other reproductive events. However, the majority of the epidemiological studies have small sample sizes and do not consider the complexities of the female reproductive cycle and fertility;[60-62,65] moreover, previous studies primarily focused on a single metal at a time, but women are periodically or even constantly exposed to mixtures of multiple heavy metals, which may cause cumulative effects.[60-62,65-67]

The objective of this study is to investigate associations between blood concentrations of single Pb, Cd, Hg and their mixtures and reproductive aged women's infertility in the National Health and Nutrition Examination Survey (NHANES) 2013-2018; moreover, the associations between heavy metals and women's long-term amenorrhea, a crucial contributing factor to women's infertility, was assessed. We hypothesize that women with higher blood heavy metal concentrations are more likely to experience infertility and long-term amenorrhea. We combined our robust understanding of female reproductive biology and epidemiology to create a comprehensive evaluation of the impacts of exposure to single heavy metals and their mixtures on women's reproductive health.

Materials and Methods

Study population

All data were obtained from NHANES, a nationally representative cross-sectional survey of the non-institutionalized U.S. population. NHANES is conducted by the US Centers for Disease Control and Prevention (CDC) and uses a complex multistage, probability sampling design. Since 1999, the sample design has consisted of multi-year, stratified, clustered four-stage samples, with data released in 2-year cycles. NHANES samples are drawn in four stages: (1) Primary sampling units (PSUs) (counties, clusters of tracts within counties, or combinations of neighboring counties), (2) segments within PSUs (census blocks or groupings of blocks), (3) dwelling units (DUs) (households) within segments, and (4) individuals within households. Screening is conducted at the DU level to identify individuals, based on oversampling criteria. NHANES oversamples some subgroups to increase the reliability and precision of health status indicator estimates for these particular subgroups; the population subgroups chosen for oversampling directly determine the sampling domains used to select the sample at all stages.[68] In this study, we used data from three continuous NHANES cycles, including 2013-2014, 2015-2016, and 2017-2018, where the reproductive health questionnaire addressed women's infertility and menstrual cycle. All data including sociodemographic questionnaires, physical examinations, and reproductive health questionnaires, were downloaded directly from the CDC's website.[69]

Study sample, variable descriptions, and inclusion

Among all three NHANES cycles, one-half of participants age 12+ have blood heavy metal data for the cycles of 2013-2014 and 2015-2016. All participants aged 1+ have blood heavy metal data available for the cycle of 2017-2018. The total number of participants in these three NHANES cycles was 20,113. After excluding males (n=9,934), females younger than 20 years (n=4,589), and females older than 49 years (n=2,843), there were 2,747 reproductive aged women (20-49 years) who had blood heavy metal data available. Although post-pubertal women under 20 years are also considered within reproductive age, they were not included because NHANES survey was designed to only collect reproductive data from participants 20 years of age and older. Moreover, women who had a hysterectomy (n=125) and women with missing data for the heavy metal exposures (n=136) were also excluded. Figures 2.1 and 2.2 describe the sample attrition process and amount of missingness. Participants with missing data for the questions of infertility (n=272), demographic variables (n=197), BMI (n=14), and information on the use of birth control pill and female hormones (n=4) were also excluded. Overall, a total of 1,999 women were included for comparing ever-infertile and fertile women (main group), and a total of 297 participants were included for comparing ever-infertile and pregnant women (sub-group) (Figure 2.1). For assessing long-term amenorrhea, participants with missing data for the questions of long-term amenorrhea (n=361), demographic variables (n=190), BMI (n=12), and information on the use of birth control pill and female hormone use (n=4) were also excluded. Overall, a

total of 1,919 women were included for assessing long-term amenorrhea (Figure 2.2).

Measurements of blood Pb, Cd, Hg concentrations

The blood concentrations of Pb, Cd, and Hg were measured in the whole blood using the mass spectrometry after a simple dilution sample preparation step. The full NHANES laboratory procedures can be found online.[70-72] The lower limit of detection (LLOD) of the three measured metals were: 0.07 μg/dL for Pb, 0.1 μg/dL for Cd, and 0.28 μg/dL for Hg. For analytes with analytic results below the LLOD, an imputed fill value was placed in the analyte results field. This value is LLOD divided by the square root of 2 (LLOD/sqrt [2]).

Creating a metal mixture value

Previous studies have used simple additive methods by summing all metal scores with equal weight to create a score of the metal mixture.[73,74] Here, we aimed to further fine tune this mixed metal score by using a novel method, toxic equivalency (TEQ) values that are a weighted quantity measure based on the relative toxicity potency of each chemical. TEQ values are used for reporting dioxin and dioxin-like compounds.[75] We used a similar methodology to create TEQ values for the mixture of the three heavy metals. Pb, Cd, and Hg have been shown to exhibit similar toxic mechanisms by inducing oxidative stress and endoplasmic reticulum (ER) stress,[76,77] which compromises the reduction-oxidation hemostasis and eventually results in adverse health outcomes.[78-80] ER stress has also been revealed as a key molecular mechanism in various female reproductive functions and disorders, such as ovarian injury via ER stress-

mediated apoptosis/autophagy, regulation of gestational length by the uterine ER stress, oocyte maturation, and embryo implantation.[81-84]

In the federal Tox21 program, the ER Stress Response Element β-lactamase reporter gene assay (ESRE-bla) is used to screen potential toxicants, including heavy metals.[85,86] Pb, Cd, and Hg in certain form have been shown to be 'active' in TOX21_ESRE_BLA assay, while other high-throughput assays related to oxidative stress lack the screening results for all three heavy metals in this study. Data from the assay component TOX21_ESRE_BLA_ratio were extracted from the CompTox Chemistry Dashboard for Lead(II) acetate trihydrate, Cadmium acetate dihydrate, and Mercury(II) acetate.[87] The concentration of the half-maximal activity (AC50), a common potency measure applied in pharmacological research and toxicity testing[88] was identified for each heavy metal: Lead(II) acetate trihydrate AC50 = 0.0586 μM, Cadmium acetate dihydrate AC50 = 0.0545 μM, and Mercury(II) acetate AC50 = 2.29 μM. The maximal response or efficacy of the three heavy metals are in the same order of magnitude, with that of Cd and Hg within two-fold of Pb, which is used as the reference metal to calculate the TEQ values of the other two.[89,90] Using AC50, the adjusted metal weights were 4.831e-2 for Pb, 9.565e-3 for Cd, and 1.276e-4 for Hg. The final mixed metal score was calculated using the sum of weighted blood metal concentrations as follows: Mix Metal Score = [(1*Pb Blood Metal Concentration, μg/dL*10 / 207 g/mol) + (1.0752*Cd Blood Metal Concentration, μg/L / 112.41 g/mol) + (0.0256*Hg Blood Metal Concentration, μg/L / 200.59 g/mol)] *100. The simplified formula is [(4.831e-2*Pb Blood Metal Concentration)

+ (9.565e-3*Cd Blood Metal Concentration) + (1.276e-4*Hg Blood Metal Concentration)] *100. Following TEQ approach, we refer to this as our metal mixture value of exposure throughout the paper.

Women's infertility history

The prevalence of infertility among women aged 20-49 was assessed using the question "Have you ever attempted to become pregnant over a period of at least a year without becoming pregnant?".[35] Women who responded "Yes" were considered ever-infertile. Fertile women were defined in two distinct ways: (1) fertile women or the main-group were women who answered "No" to the question of "Have you ever attempted to become pregnant over a period of at least a year without becoming pregnant?", and (2) pregnant women or the sub-group who answered "Yes" to the question "Are you pregnant now?". Infertility defined using this method represents a women's history of infertility and may not reflect their current fertility status; hence we also analyzed women's recent long-term amenorrhea in this study.

Women's recent long-term amenorrhea

Women with long-term amenorrhea were defined by those who answered "no" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)" and answered "Other" or "Don't know" to the question "What is the reason that you have not had a period in the past 12 months?". Menstruating women were defined by women who answered "Yes" to the same question. Participants who answered "Pregnancy", "Breast feeding",

and “Menopause/Change of life” to the question “What is the reason that you have not had a period in the past 12 months?” were excluded from this study. The outcome variable long-term amenorrhea defined here reflects the women’s current or recent menstrual cycle status in the past 12 months. Although menopause is defined as amenorrhea for 12 consecutive months,[91] these women did not self-report having menopause; thus, our outcome of long-term amenorrhea may reflect their most recent (last 12 months) or current fertility status.

Other Covariates

Age was included as a covariate because age is an important factor determining a woman’s menstrual cycle, menopause, and fertility. Demographic variables including race/ethnicity, education, family poverty income ratios were all included as covariates. Because this study assessed women’s reproductive capacity, which closely ties to sexual relationships, we included marital status as a covariate. We also included health insurance coverage as a covariate because health care access can impact participants’ reproductive health and fertility management.[92] Smoking status and BMI were included because they have been shown to impact women’s reproductive health.[93,94] BMI was defined by the CDC as underweight (<18.5), healthy weight (18.5 to <25), overweight (25 to <30), and obesity (30 or higher).[95] Hormonal contraception use was included because women are often prescribed hormones to regulate menstruation or prevent menstruation and unintended pregnancy. Hormonal contraception use included women who have ever taken birth control pills or used female hormones.

Additionally, when assessing infertility as an outcome, two additional covariates were included: regular menstruation and if women had seen a doctor because they were unable to be pregnant. Menstruation directly impacts women's fertility and women who see a doctor sooner for their fertility might be more likely to become pregnant in a year through assisted reproductive technology (ART) such as *in vitro* fertilization (IVF) and intrauterine insemination (IUI). We adjusted for the long-term amenorrhea when assessing for infertility because regular menstruation impacts infertility as well as blood metal concentrations. For example, the intestinal absorption of Cd, Pb, and Hg increases when the body iron stores are depleted[96] and menstruating women are more likely to have low iron stores.[97]

Statistical Analysis

For NHANES datasets, the use of sampling weights and sample design variables is recommended for all analyses because the sample design is both a clustered design and incorporates differential probabilities of selection. Statistical Analysis Software v9.4 (SAS Institute, Cary, NC) was used to perform all statistical analyses, incorporating sampling weights and non-responses while adjusting for cluster (PSUs) and strata of the complex sample design in NHANES.[98,99] Weighting was calculated using NHANES sub-sample weights and were calculated according to NHANES protocols and documentation.[100]

Descriptive statistics were calculated for both outcomes and exposures: Cd, Pb, Hg, and the mixture (Mix). Statistical differences in the covariates between the outcome groups were evaluated using a chi-squared test for

categorical variables and a t-test for continuous variables. Because blood concentrations of Pb, Cd, and Hg had skewed distributions based on normality tests, log transformed metal values were used. In addition to assessing the blood concentrations continuously, we also categorized the data into quartiles using the lowest quartile as the reference group. Multiple logistic regression analysis was used to evaluate the independent association between blood metal concentrations and metal mixture values and infertility after adjusting for above-mentioned covariates. The same approach was used to evaluate associations between blood metal concentrations and metal mixture values and long-term amenorrhea. Crude odds ratios (OR) and adjusted ORs and their corresponding 95% confidence intervals (CI) were presented. We used three models to examine associations between women's blood heavy metal concentrations and historical infertility (Table 2.3). In model 1, crude odds ratios (OR) were calculated without adjusting for any covariates. In model 2, an adjusted model was applied by including all covariates except for the other two metals not being assessed. In model 3, a fully adjusted model was run, which included all covariates including the other two metals.

Several sensitivity analyses were conducted to examine the robustness of our findings. First, we determined that there was a difference in infertility status among the 80 additional women included in the infertility group (n=1,999) compared to the long-term amenorrhea group (n=1,919). This helped us determine that there was sufficient reason to keep both outcomes (infertility and long-term amenorrhea) as separate population groups rather than taking the

smaller sample size for analysis. Second, using a chai squared test, we examined the difference in infertility status among women who may have seen a doctor and received assistance to become pregnant versus those who did not. The question of “seen a doctor because unable to become pregnant?” helped us define if an individual received medical assistance to help with her fertility or not. The purpose of this was to have additional descriptive information regarding the study population. Third, we examined the relation between long-term amenorrhea and infertility history using a chi-squared test.

Results

Exposure to heavy metals and women’s infertility

Study population

As shown in Table 2.1, a total of 238 or 12.8% of women were considered ever-infertile. These ever-infertile women were compared to two control groups: the main group of 1,761 women who self-reported being fertile and the sub-group of 59 pregnant women. Compared to fertile women, women who have been ever-infertile were more likely to be older, married, obese, smokers, and had seen a doctor because they were unable to become pregnant (all p-values < 0.05). The race/ethnicity, educational level, poverty income ratio, hormone-based contraception use, and having a period in the last 12 months were similar between ever-infertile and fertile women. Compared to pregnant women, ever-infertile women were more likely to be older, covered by health insurance, and had seen a doctor because they were unable to become pregnant (all p-values < 0.05). The distributions of race/ethnicity, education level, marital status, poverty

income ratio, BMI, smoking, use of hormonal contraception, and having a period in the last 12 months were similar between ever-infertile and pregnant women.

The question “seen a DR b/c unable to become pregnant?” enabled us to define if a woman received medical assistance to achieve pregnancy. In the main group, 166 (8.3%) women reported seeing a doctor of which 136 (6.8%) were ever-infertile compared to 30 (1.5%) who self-reported to be fertile. In the sub-group, 137 (46.1%) women reported seeing a doctor of which 136 (45.8%) were ever-infertile compared to only one woman (0.3%) who was pregnant. Women who had seen a doctor were substantially more likely to be ever-infertile in both the main group and sub-group women (*p*-value <.001).

Bivariate results and metal exposures

The median and log transformed means of blood heavy metal concentrations in women with various fertility status are summarized in Table 2.2 and illustrated in Figures 2.3 and 2.4. With respect to the main group analysis, there was no significant difference for the blood concentrations of all three single heavy metals and mixtures between ever-infertile and self-reported fertile women (Figure 2.3). In the sub-group analysis, women who have been ever-infertile had significantly higher concentrations of blood Pb and heavy metal mixture than pregnant women (Table 2.2 and Figure 2.4). The blood concentrations of Cd and Hg, however, were comparable in the main and sub-groups (Table 2.2 and Figure 2.3 and 2.4).

Multiple logistic regression analysis results

Multiple logistic regression analysis showed that after full adjustment including demographic characteristics, lifestyle factors, and two metals not being assessed (model 3), there was a positive association between blood Pb concentrations and women's ever-infertility. The continuous log transformed data of both the main-group and sub-group analyses showed that as blood Pb concentrations increased, women were more likely to be ever-infertile (OR: 1.75, 95% CI: 1.01-3.02; and OR: 3.09, 95% CI: 1.22-7.85, respectively, Table 2.3). The results of model 1 with crude OR and model 2 with adjustments of all covariates but not two metals not being assessed showed similar results, except that the crude OR of the main group analysis is insignificant (Table 2.3).

Multiple logistic regression results for the categorical data in model 3 revealed that there was no association between Pb and infertility for all quartiles of 2, 3 and 4 compared with the lowest quartile 1 in the main group analysis (ever-infertile vs. fertile). However, for the sub-group analysis (ever-infertile vs. pregnant), the blood concentrations of Pb in quartiles 3 and 4 were significantly associated with women's historical infertility (OR: 3.47, 95% CI: 1.11-10.83; and OR: 5.26, 95% CI: 1.18-23.54, respectively), and the OR from quartiles 2 to 4 exhibited a dose-dependent relationship (Table 2.3). The results of model 1 with crude OR and model 2 with adjustments of all covariates but not two metals not being assessed showed similar results (Table 2.3).

With respect to Cd and Hg summarized in Table 2.3, the results of both continuous and categorical multiple logistic regression analyses in all three

models revealed no significant associations except that the increase of blood concentrations of Hg in the quartile 3 was significantly associated with women's infertility in model 2 of the sub-group analysis (OR: 2.53, 95% CI: 0.64-11.78). Regarding the heavy metal mixture, model 3 showed no significant associations between the metal mixture and women's infertility in both the main and sub-group analyses. Contrarily, sub-group analysis in models 1 and 2 revealed that metal mixtures are positively associated with women's ever-infertility; since models 1 and 2 did not adjust for the single metals, this is likely due to the positive association found between Pb and infertility. Collectively, these results indicate that after full adjustment, exposure to Pb increases the odds of women's historical infertility; further, no associations were found between Cd, Hg, and the mixture of all three metals and women's historical infertility.

Women's historical infertility is not associated with their recent long-term amenorrhea

We next examined associations between women's historical infertility and recent long-term amenorrhea. A total of 1,918 women had complete data of both infertility and long-term amenorrhea (Table 2.4). There was no statistical correlation between women's long-term amenorrhea and historical infertility (p-value = 0.29), although the percentage of long-term amenorrhea in women who were ever-infertile (3.9%) was slightly lower than that in fertile women (5.6%) and the percentage of historical infertility in women with long-term amenorrhea (8.7%) was lower than that in menstruating women (12.2%). This negative association suggests that women's historical infertility does not reflect their recent

reproductive status. The NHANES survey asked "*Have you had at least one menstrual period in the past 12 months?*" Because the absence of a period or amenorrhea for 12 consecutive months has been suggested as an important indicator of menopause,[91] the long-term amenorrhea may reflect women's most recent reproductive and fertility status. Thus, as a secondary outcome, we chose to investigate associations between heavy metal exposure and women's recent long-term amenorrhea.

Exposure to heavy metals and women's long-term amenorrhea

Study population

As shown in Figure 2.2, a total of 1,919 participants were included to assess long-term amenorrhea after further deleting participants with missing data on long-term amenorrhea (n=361), demographic variables (n=190), BMI (n=12), and information on the use of birth control pill and female hormone use (n=4). The characteristics of these women are summarized in Table 2.5. Compared with menstruating women, women with long-term amenorrhea were more likely to be Non-Hispanic White and Non-Hispanic Black (p-value < 0.05) compared to other ethnicities. However, the distributions of age, educational level, marital status, health insurance coverage, poverty income ratio, BMI, smoking history, and hormone-based contraception use were largely similar between menstruating women and women with long-term amenorrhea (all p-values > 0.05).

Bivariate results and heavy metal exposures

The median and log transformed means of blood heavy metal concentrations are shown in Table 2.6 and illustrated in Figure 2.5. Compared

with menstruating women, women with long-term amenorrhea had comparable blood concentrations of Pb, Cd, and heavy metal mixtures but had significantly higher median blood concentrations of Hg (Figure 2.5).

Multiple logistic regression model results

Table 2.7 summarizes associations between blood heavy metal concentrations and women's long-term amenorrhea assessed by three models as we described in the fertility analysis. Multiple logistic regression analysis from continuous and categorical data showed no significant associations between blood concentrations of Pb or Hg and women's long-term amenorrhea in all three models. In the categorical multiple logistic regression analysis, after the full adjustment in model 3, there was a negative association between the blood Cd concentrations in quartiles 2 and 3 and women's long-term amenorrhea (quartile 2 OR: 0.47, 95% CI: 0.25-0.87; quartile 3 OR: 0.31, 95% CI: 0.13-0.76). Similar to model 3, the results of model 1 and 2 also showed an inverse association between the blood concentrations of Cd in quartiles 2 and 3 and long-term amenorrhea. The ORs, although still less than 1, were higher for quartile 4 than those for quartiles 2 and 3 in all three models but were not statically significant (Table 2.7). For the mixture of all three heavy metals, all three models showed insignificant associations between blood metal mixture concentrations and long-term amenorrhea.

Discussion

About 10-15% of women of reproductive age experience infertility.[101,102] Accumulating evidence reveals the endocrine disrupting effects of heavy metals,

suggesting their possible contributions to women's impaired fertility and other reproductive disorders. Here, we performed a cross-sectional analysis of NHANES 2013-2018 to investigate associations between exposure to single Cd, Pb, Hg and mixtures and women's infertility and long-term amenorrhea. Our results show that (1) the blood concentrations of Pb and heavy metal mixtures were significantly higher in ever-infertile women than pregnant women, but the concentrations of Cd and Hg were comparable; (2) exposure to Pb is positively associated with women's historical infertility; and (3) the increase of blood concentrations of Cd is inversely related to women's recent long-term amenorrhea.

Comparisons of blood heavy metal levels between this study and guidelines from federal or other organizations.

So far, there are no recognized biological functions of Pb, Cd, and Hg for human health. The typical blood levels of Pb in adults is less than 1 μg/dL, and 5 μg/dL is designated as the elevated blood lead level in adults by the US CDC.[103] This is also the level for required medical removal in the workplace if occupational exposures exist for women who are pregnant or are trying to be pregnant due possible reproductive and developmental adversities.[104] In our study, 81.5% of women had blood Pb levels < 1 μg/dL, 17.9% had levels at 1-5 μg/dL, and 11 women (0.55%) had levels > 5 μg/dL. The blood levels of Cd are usually < 5 μg/L, with most in the range of 0.5-2 μg/L; Blood Cd levels of 50 μg/L or more have been shown to cause acute toxicities.[105,106] The women's blood concentrations of Cd in our study ranged from 0.07 - 5.14 μg/L, with only one

woman having blood Cd levels > 5 µg/L and 97.2% had levels < 2 µg/L. The blood concentrations of Hg are usually < 10 µg/L. Significant exposure is defined when the concentration is > 50 µg/L if exposure is due to alkyl Hg, or > 200 µg/L if exposure is due to Hg(2+).[107] In our study, women's blood Hg concentrations ranged from 0.2 - 26.87 µg/L, with 99.1% of them having Hg levels <10 µg/L and 18 women (0.9%) having blood Hg levels >10 µg/L. Altogether, the percentages of women that exceeded typical or normal levels of blood heavy metals were 18.5% for Pb, 0.05% for Cd, and 0.9% for Hg. Observed elevated blood heavy metal levels, particularly for Pb, pose a threat to women's reproductive health and fertility, highlighting an urgent unmet need to prevent and reduce heavy metal exposure.

Impacts of heavy metal exposure and women's fertility and menstrual cycle

The impacts of heavy metal exposure on women's fertility and menstrual outcomes remain elusive. Consistent to our data, a cross-sectional study in Taiwan from Lei et al. and another cross-sectional analysis by Lee et al. using NHANES 2013-2016 found that the blood concentrations of Pb in ever-infertile women were significantly higher than pregnant woman and this association was dose-dependent.[60,61] Similar to Lee et al., we also found a positive association between the log transformed Pb concentrations and women's infertility, but we found a negative association between Cd and long-term amenorrhea after quarterization. We also discovered similar results to another NHANES 2013-2016 analysis that found no associations between Hg and infertility.[108]

The absorption, distribution, metabolism, and excretion (ADME) of metals, particularly Cd, depend on nutritional status. The intestinal absorption of Cd increases when the body iron stores are depleted.[96] In addition, women typically have higher levels of Cd than men because women are more susceptible to having low iron stores due to the monthly menstruation.[97,109] It has also been found that people with vegan/vegetarian diets often have low iron, while concurrently these people on vegan/vegetarian diets tend to have higher blood levels of Cd.[110] These results suggest that although we did not anticipate Cd being protective against women's long-term amenorrhea, it is possible that women who have normal menstruation and thus the metal transporters in the GI track are more upregulated than amenorrhea women tend to have higher blood levels of Cd, resulting in a negative association in our analysis. Therefore, future research is necessary to consider associations between Cd levels, dietary patterns, iron levels, and amenorrhea.

So far, evidence regarding the effects of heavy metal exposure on women's reproduction is limited and inconsistent; however, the rationale behind our observed associations can be explained by previous *in vitro* and *in vivo* studies.[57,111-115] With respect to Pb, results from experimental research suggest that Pb may impact female fertility through various mechanisms, including disrupting menstrual cycle, altering hormone levels, and impairing fetal development.[116,117] It was also found in mice that Pb accumulates in the ovary and disrupts folliculogenesis, decreases ovarian reserve, and increases follicle

atresia,[113,115,118,119] suggesting that all these Pb-induced reproductive toxicities may contribute to women's historical infertility observed in our NHANES analysis.

Animal studies found that Cd may adversely impact female reproduction.[119] For example, Cd has been shown to decrease the number of growing follicles,[119-121] induce follicle atresia,[119,122] alter follicular cell structure,[119,123,124] decrease ovarian reserve,[119,125,126] reduce FSH and LH levels,[119,127] and increase ovarian cycle length.[114,119] Additionally, Cd has also been found to affect follicle maturation, induce luteoolysis,[119,128] and thicken endometrium.[57,119] All these results suggest that exposure to Cd may impair women's fertility. However, results obtained from epidemiological studies have been conflicting. Several cohort studies investigating associations between exposure to Cd and women's fertility had conflicting results including no associations[129] or even reduced fecundity.[130] In contrast, Cd has also been found to disrupt reproductive hormone secretion.[112,131] A study from Lee et al. discovered an inverse relationship between blood concentrations of Cd and Anti-Mullerian hormone (AMH) – a peptide hormone secreted from growing follicles and is commonly used as a biomarker of ovarian reserve, suggesting that exposure to Cd may increase women's infertility risk by diminishing ovarian reserve.[132] Collectively, as we study the role of nutrition status on the toxicokinetics of Cd, it is essential to integrate both experimental and epidemiological evidence and include all possible confounding factors to determine the effects of Cd on women's reproductive health and fertility.

Experimental evidence reveals that Hg accumulates in the ovaries and impacts female reproduction[111,119,133] by interfering with the secretion patterns of gonadotropins of LH and FSH, altering ovarian cyclicity, and inducing follicular cell apoptosis and follicle atresia.[119,134-136] Although some other studies reported that Hg is associated with female infertility, the evidence to support this is limited and inconclusive.[111,119,137,138] Thus, evidence is inadequate to draw meaningful conclusions about how Hg impacts female reproductive outcomes, underscoring the need for additional research.

Heavy metal mixtures on women's fertility in epidemiological and experimental studies

Previous studies have examined heavy metals and individual reproductive outcomes without examining the complexities of reproductive cycles and the interactions of these exposures. Both epidemiological and experimental literature is lacking for assessing the mixture of heavy metals on women's reproductive outcomes. Previous studies assessing other health outcomes have used the simple concentration additive method or other statistical methods[139] for combining metals.[73,74] Here, we integrated multiple heavy metal concentrations by considering each individual metal's toxicity related to the ER stress, a key mediator of the adverse outcome pathway in female reproduction.[83,140] EPA's framework for metal risk assessment outlines that some metals act additively while others are antagonistic or synergistic when they are present together.[141] These interactions occur during absorption, excretion, or sequestration.[141] However, the exact fate and joint effects of Pb, Cd, and Hg together in women

has yet to be determined; additionally, metal mixtures in women can be dependent on other factors that are different across individuals, making it hard to quantify.

The link between women's infertility and long-term amenorrhea

The menstrual cycle, or periodic vaginal bleeding due to the shedding of uterine endometrium, is regulated by the cyclic changes of reproductive hormones, including both gonadotropins from the pituitary and sex hormones from the ovaries.[142] Pathological amenorrhea that are not caused by pregnancy, lactation, or menopause occurs in 3 – 4% of women in the US.[143,144] In our study, we found no association between women's ever-infertility and their recent long-term amenorrhea, suggesting that women's recent menstrual cycle status does not reflect their fertility history. It is also likely that amenorrhea is only one of many complex contributing factors towards women's fertility success. For example, although up to 25% of infertile women have disturbed menstrual cycle such as amenorrhea,[35,145] infertility can also be attributed to sperm defects from the male partner and other unexplained reasons.[38] The underlying mechanism of women's amenorrhea remains poorly understood and has been attributed to both genetic and environmental factors.[146,147] In addition to causing infertility, amenorrhea can have additional health consequences. For example, continual anovulation for two to three years increases the risk of developing endometrial cancer,[34] suggesting that long-term amenorrhea is a risk factor of other female reproductive disorders.

Advantages and limitations

This study overcomes several limitations in previous papers using NHANES database to investigate associations between heavy metals and women's infertility.[61,108] First, both of these studies only included participants from two NHANES cycles (2013-2014 and 2015-2016), whereas we further added the cycle of 2017-2018. Second, the study from Lee et al. only included infertile women up to age 39 and compared them to pregnant women, which resulted in a smaller sample size of n=124.[61] Here, in addition to women of 20-39 years, we also included women of 40-49 years, because these women may have experienced infertility before and are also within reproductive age; moreover, we defined the fertile women in two ways, including self-reported fertile women and pregnant women. Third, our study accounts for additional covariates related to reproduction that are essential for understanding infertility, such as hormonal contraception use, menstruation patterns, and possible help from a doctor for fertility issues; further, our study also examined the difference in infertility status among women who may have seen a doctor and gotten assistance to become pregnant vs. those who did not. Fourth, we chose to define fertile women in two sub-groups due to some limitations in the NHANES survey questions. The main-group or women who self-reported to be fertile or ever-infertile could include women who have never 'tried' to become pregnant. Therefore, it is possible to include several misclassifications. Thus, we additionally looked at a sub-group of current pregnant women. There were ten women who were pregnant but also answered "yes" to the question of "Have you ever attempted to become pregnant

over a period of at least a year without becoming pregnant?" We chose to include these ten women in the ever-infertile group because they reported having had issues with their fertility in the past. Indeed, five of those ten women responded that they had previously seen a doctor because they were unable to become pregnant, indicating that these women likely have received ART such as IVF to become pregnant. Lastly, this study takes a unique approach to assessing the reproductive toxicity of the mixture of heavy metals using weighted TEQ values.

Our study has several limitations due to NAHNES study design, the complexities of assessing female reproduction and reproductive toxicities of heavy metals. First, there are limitations due to NHANES study design. NHANES is a cross-sectional study, therefore casual and temporal relationships cannot be confirmed. Cross-sectional data are also prone to survival bias. Although women aged 15-19 are still considered of reproductive age, we did not include them because the study design did not collect exposure or outcome information in this age group. Additionally, NHANES did not collect information on some reproductive diseases that also play a role in infertility, such as endometriosis and PCOS. For example, about 30 to 50% of women with endometriosis are infertile and PCOS is a leading cause of infertility.[148,149] Moreover, the NHANES questionnaire only collected historical use of birth control and female hormones. Second, the reproductive health outcomes are measured using a self-reported questionnaire. Although self-reported information is useful, various definitions may affect the prevalence of a measured outcome. With the information collected, we did our best to define the outcomes (ever-infertile, fertile, pregnant,

long-term amenorrhea, menstruating). However, there are limitations for the definitions we used. For example, amenorrhea is defined as the absence of menstruation for at least a 90-day period.[150] However, NHANES only collects information on absence of menstruation for the past 12 months.[91] Although menopause can also be defined by one year of no menses, we chose to name our variable long-term amenorrhea because these women self-reported not having menopause. Thus, women categorized with long-term amenorrhea may have suspected early menopause or POF. Third, male factors account for approximately 40-50% of all cases of infertility.[151] The NHANES questionnaire only addressed females. Therefore, male infertility factors were not considered. However, the relationship between male reproduction and heavy metals has been well studied, while female reproductive function is lacking.

Understanding what blood metal concentrations represent is also worth discussing. A single measurement of blood metal concentration may not reflect long-term exposure though some studies suggested that under steady state conditions a single measurement of blood metal level seems to be acceptable as it can reflect body metal burden of long-term exposure.[152] Our study assumes women's blood metal concentrations during the time of the examination were the same as when they experienced infertility or long-term amenorrhea. However, by study design, there is no way of knowing temporality. Although the biological half-lives for heavy metals in the human body are long, the half-lives in blood specifically can be shorter and vary (Hg = 50 days,[153] Cd = 3-4 months for the fast component and 7-16 years for the slow component,[154-156] Pb = 1-2

months[157]). Blood metal concentrations are used to represent both recent and chronic exposures.[158,159] However, it is important to note that the acute exposures can modify blood metal concentrations. For example, eating fish right before the examination could markedly elevate blood Hg concentrations, however, someone who has been chronically exposed to Hg, maintains high concentrations in their blood even after exposure has ended.[160,161] This same concept could be applied to Cd blood concentrations with a participant who smoked before the examination. Our study assumes that the individual's behavior prior to the examination is consistent to their daily behaviors. Additionally, those with chronic past exposure are often underestimated when assessing blood levels because metals like Pb can be stored in the bone. Therefore, individuals can have a high body burden of Pb but still appear to have normal Pb concentrations in the blood.[162] Lastly, we used the ER stress, an important contributing factors of female reproductive dysfunctions, to calculate the mixture score of heavy metals because the Tox21 program has the screening results of all three metals available. However, it is possible that heavy metals may compromise female reproduction through other mechanisms such as DNA damage, oxidative stress, and epigenetic modification. Thus, an optimized calculation method of heavy meatal mixtures is highly desired.

Conclusion

In summary, the results of our studies using NHANES 2013-2018 reveal that there are significant percentages of women having blood heavy metal levels exceeding typical or normal levels. Moreover, the blood concentrations of single

Pb and heavy metal mixtures are associated with an increase of women's historical infertility. This study highlights the threat of heavy metal exposure on women's reproductive health and fertility as well as an urgent unmet need to prevent and reduce heavy metal exposure.

Table 2.1: Women's Characteristics for Studying Associations Between Blood Heavy Metal Concentrations and Historical Infertility

Characteristics	**Main group sample (ever-infertile vs fertile)**				**Sub-group sample (ever-infertile vs pregnant)**			
	Total Sample N (%)	**Ever-infertile[1] N (%)**	**Fertile[2] N (%)**	***p*-Value[4]**	**Total Sample N (%)**	**Ever-infertile[1] N (%)**	**Pregnant[3] N (%)**	***p*-Value[4]**
Total Subjects	1999	238 (12.8)	1761 (87.2)		297	238 (81.6)	59 (18.4)	
Age, *mean* ± *SE* (years)	34 ± 0.23	37 ± 0.79	33 ± 0.22	<.001	35 ± 0.71	37 ± 0.70	27 ± 0.58	<.001
Race/Ethnicity				0.85				0.26
Hispanic	518 (18.2)	62 (17.9)	456 (18.2)		78 (18.5)	62 (17.9)	16 (21.4)	
Non-Hispanic White	697 (58.4)	90 (60.5)	607 (58.1)		108 (57.8)	90 (60.5)	18 (45.8)	
Non-Hispanic Black	426 (13.2)	47 (13.0)	379 (13.2)		61 (14.4)	47 (13.0)	14 (20.4)	
Other Race Including Multi-Racial	358 (10.2)	39 (8.6)	319 (10.5)		50 (9.3)	39 (8.6)	11 (12.4)	
Education Level				0.53				0.73
Less than High School	297 (10.8)	36 (11.7)	261 (10.7)		46 (15.5)	36 (11.7)	10 (14.1)	
High School	397 (19.6)	50 (22.1)	347 (19.2)		62 (20.9)	50 (22.1)	12 (17.1)	
More than High School	1305 (69.6)	152 (66.3)	1153 (70.1)		189 (63.6)	152 (66.3)	37 (68.8)	
Marital Status				<.001				0.59
Married / Living with Partner	1173 (61.3)	175 (78.1)	998 (58.9)		223 (78.6)	175 (78.1)	48 (80.6)	
Divorced / Widowed / Separated	243 (10.3)	29 (8.8)	214 (10.5)		31 (8.0)	29 (8.8)	2 (4.2)	
Never Married	583 (28.4)	34 (13.0)	549 (30.6)		43 (13.4)	34 (13.0)	9 (15.2)	
Poverty Income Ratio, *mean* ± *SE*	2.770 ± 0.07	2.95 ± 0.14	2.75 ± 0.070	0.15	2.90 ± 0.14	2.95 ± 0.14	2.72 ± 0.23	0.52
Covered by Health Insurance				0.33				0.03
Yes	1601 (83.0)	187 (80.7)	1414 (83.3)		239 (82.6)	187 (80.7)	52 (91.0)	
No	398 (17.0)	51 (19.3)	347 (16.7)		58 (17.4)	51 (19.3)	7 (9.0)	
Body Mass Index (kg/m**2)				0.007				0.16

Underweight (<18.5)	39 (2.0)	4 (1.2)	35 (2.1)		5 (1.2)	4 (1.2)	1 (1.4)	
Normal Weight (18.5-24.9)	615 (32.1)	64 (27.8)	551 (32.7)		78 (26.2)	64 (27.8)	14 (19.2)	
Overweight (25.0-29.9)	471 (24.9)	38 (18.5)	433 (25.9)		53 (21.1)	38 (18.5)	15 (32.4)	
Obesity (>30)	874 (50.0)	132 (52.5)	742 (39.3)		161 (51.5)	132 (52.5)	29 (47.0)	
Ever Smoked				0.028				0.50
Yes	613 (33.0)	88 (40.5)	525 (31.9)		109 (39.3)	88 (40.5)	21 (34.1)	
No	1386 (67.0)	150 (59.5)	1236 (68.1)		188 (60.7)	150 (59.5)	38 (65.9)	
Ever taken hormone-based contraception?				0.85				0.15
Yes	1389 (75.7)	170 (76.3)	1219 (75.6)		204 (74.3)	170 (76.3)	34 (65.1)	
No	610 (24.3)	68 (23.7)	542 (24.4)		93 (25.7)	68 (23.7)	25 (34.9)	
At least one period in past 12 months				0.73				n/a
Yes	1815 (89.8)	222 (89.0)	1593 (89.9)		281 (91.0)	222 (89.0)	59 (100)	
No	183 (10.2)	16 (11.0)	167 (10.1)		16 (9.0)	16 (11.0)	0 (0)	
Seen a DR b/c unable to become pregnant?				<.001				<.001
Yes	166 (9.3)	136 (60.3)	30 (1.8)		137 (49.5)	136 (60.3)	1 (1.9)	
No	1833 (90.7)	102 (39.7)	1731 (98.2)		160 (50.5)	102 (39.7)	58 (98.1)	

Values for continuous variables are mean +/- SD.
Values for categorical variables are n (unweighted sample counts) and % (weighted sample percentages to account for NHANES survey design).
[1] 'Ever-infertile' if subject responded 'yes' to the following question: "Have you ever attempted to become pregnant over a period of at least a year without becoming pregnant?"
[2] 'Fertile' if answered "No" to the following question: "Have you ever attempted to become pregnant over a period of at least a year without becoming pregnant?"
[3] 'Pregnant' if women answered "Yes" to the question "Are you pregnant now?"
[4] *p*-Value for categorical variables comes from a chi-squared test, which determines if there is a significant difference between demographics in ever infertile vs. fertile or pregnant. *p*-values for continuous variables comes from a t-test to determine if there is a significant difference between the means of ever infertile vs. fertile or pregnant.

Table 2.2: Medians and Log Transformed Means of Blood Heavy Metal Concentrations and Heavy Metal Mixture Scores in Ever-infertile or Fertile/Pregnant Women

Metal	Main group sample (ever-infertile vs fertile n=1999)				Sub-group sample (ever-infertile vs pregnant n=297)			
	Total Sample	Ever-infertile[1]	Fertile[2]	*p*-Value[4]	Total Sample -	Ever-infertile[1]	Pregnant[3]	*p*-Value[4]
Lead, *median, IQR* (ug / dL)	0.53 (0.34 - 0.78)	0.56 (0.42 - 0.79)	0.53 (0.38 - 0.78)	0.19	0.54 (0.36 - 0.74)	0.56 (0.42 - 0.79)	0.36 (0.26 - 0.53)	0.001
Log Transformed Lead, *Mean, SE*	-0.57 ± 0.02	-0.49 ± 0.04	-0.58 ± 0.03	0.11	-0.58 ± 0.05	-0.49 ± 0.04	-0.99 ± 0.06	<.001
Cadmium, *median, IQR* (ug / L)	0.25 (0.16 - 0.44)	0.26 (0.15 - 0.47)	0.25 (0.60 - 0.44)	0.68	0.25 (0.14 - 0.44)	0.26 (0.15 - 0.47)	0.19 (0.11 - 0.35)	0.21
Log Transformed Cadmium, *Mean, SE*	-1.25 ± 0.03	-1.24 ± 0.07	-1.25 ± 0.03	0.91	-1.30 ± 0.07	-1.24 ± 0.07	-1.54 ± 0.10	0.07
Mercury, *median, IQR* (ug / L)	0.61 (0.33 - 1.26)	0.60 (0.37 - 1.15)	0.61 (0.32 - 1.27)	0.72	0.59 (0.35 - 1.16)	0.60 (0.37 - 1.15)	0.55 (0.26 - 1.14)	0.051
Log Transformed Mercury, *Mean, SE*	-0.37 ± 0.03	-0.36 ± 0.08	-0.37 ± 0.03	0.89	-0.40 ± 0.07	-0.36 ± 0.08	-0.58 ± 0.12	0.21
Mixed Metal, *median, IQR*	3.01 (2.12 – 4.39)	3.10 (2.27 – 4.31)	2.10 (2.98 – 4.39)	0.19	2.94 (2.03 – 4.23)	3.10 (2.27 – 4.31)	1.39 (2.04 – 2.79)	0.001
Log Transformed Mix, *Mean, SE*	1.14 ± 0.02	1.21 ± 0.04	1.13 ± 0.02	0.15	1.12 ± 0.04	1.21 ± 0.04	0.73 ± 0.06	<.001

Blood metal distributions were skewed. Therefore, we presented the median and IQR (25th and 75th percentile) and the mean of the Log Transformed blood heavy metal levels. These results are weighted to account for NHANES survey design.

[1] 'Ever-infertile' if subject responded 'yes' to the following question: "Have you ever attempted to become pregnant over a period of at least a year without becoming pregnant?"

[2] 'Fertile' if answered "No" to the following question: "Have you ever attempted to become pregnant over a period of at least a year without becoming pregnant?"

[3] 'Pregnant' if women answered "Yes" to the question "Are you pregnant now?"

[4] *p*-values represent a t-test to determine if there is a significant difference between the means of ever-infertile vs. fertile or pregnant.

Table 2.3: Associations Between Blood Heavy Metal Concentrations and Heavy Metal Mixture Scores and Women's Infertility

Characteristics	Total N (%)[1]	Main group sample (ever infertile[2] vs fertile[3] n=1999) Ever-infertile[2] n (%) or Mean (SD) [1]	Crude OR (95% CI) Model 1	*Adj* OR (95% CI) Model 2	*Fully Adj* OR (95% CI) Model 3	Sub-group sample (ever-infertile[2] vs pregnant[4] n=297) Ever-infertile[2] n (%) or Mean (SD) [1]	Crude OR (95% CI) Model 1	*Adj* OR (95% CI) Model 2	*Fully Adj* OR (95% CI) Model 3
LEAD									
Log Transformed, Mean (SD)	-0.52 (0.62)	-0.48 (0.61)	1.28 (0.97 - 1.68)	1.69 (1.01 - 2.85)*	1.75 (1.02 - 3.02)*	-0.48 (0.61)	5.02 (2.73 - 9.23)*	5.19 (2.14 - 12.59)*	3.09 (1.22 - 7.85)*
Lead quartiles, n (%)									
Q1 (Ref) (≤0.40)	488 (24.41%)	52 (2.6%)	Ref	Ref	Ref	52 (17.51%)	Ref	Ref	Ref
Q2 (0.41 – 0.56)	492 (24.61%)	56 (2.8%)	1.15 (0.70 - 1.89)	1.37 (0.67 - 2.80)	1.38 (0.68 - 2.79)	56 (18.86%)	3.23 (1.18 - 8.86)*	2.88 (0.61 - 13.55)	2.52 (0.53 - 12.09)
Q3 (0.57 – 0.86)	513 (25.66%)	70 (3.5%)	1.52 (1.00 - 2.29)*	1.60 (0.82 - 3.10)	1.61 (0.83 - 3.09)	70 (23.57%)	5.32 (2.20 - 12.88)*	5.60 (1.67 - 18.73)*	3.47 (1.11 - 10.83)*
Q4 (>0.86)	506 (25.31)	60 (3.0%)	1.30 (0.80 - 2.11)	1.71 (0.76 - 3.85)	1.72 (0.75 - 3.95)	60 (20.2%)	6.71 (2.85 - 15.81)*	12.62 (2.48 - 64.21)*	5.26 (1.18 - 23.54)*
CADMIUM									
Log Transformed, Mean (SD)	-1.16 (0.84)	-1.13 (0.84)	1.01 (0.81 - 1.26)	1.01 (0.68 - 1.52)	0.94 (0.63 - 1.41)	-1.13 (0.84)	1.51 (0.94 - 2.43)	2.29 (0.87 - 6.04)	1.90 (0.64 - 5.63)
Cadmium quartiles, n (%)									
Q1 (Ref) (≤0.18)	469 (23.46%)	52 (2.6%)	Ref	Ref	Ref	52 (17.51%)	Ref	Ref	Ref
Q2 (0.19 – 0.29)	509 (25.46%)	67 (3.35%)	1.08 (0.66 - 1.77)	0.65 (0.34 - 1.25)	0.67 (0.35 - 1.27)	67 (22.56%)	2.06 (0.81 - 5.27)	0.82 (0.16 - 4.05)	0.58 (0.10 - 3.41)
Q3 (0.30-0.51)	513 (25.66%)	53 (2.65%)	0.87 (0.57 - 1.32)	0.71 (0.38 - 1.33)	0.70 (0.37 - 1.31)	53 (17.85%)	1.83 (0.76 - 4.41)	0.38 (0.05 - 2.83)	0.37 (0.04 - 3.20)

Q4 (>0.51)	508 (25.41%)	66 (3.3%)	1.07 (0.64 - 1.81)	0.95 (0.41 - 2.18)	0.83 (0.36 - 1.94)	66 (22.22%)	2.19 (0.80 - 6.02)	1.33 (0.09 - 19.70)	0.61 (0.04 - 9.94)
MERCURY									
Log Transformed, Mean (SD)	-0.29 (0.99)	-0.28 (0.96)	1.01 (0.85 - 1.21)	1.07 (0.80 - 1.44)	1.02 (0.76 - 1.36)	-0.28 (0.96)	1.35 (0.84 - 2.17)	1.37 (0.71 - 2.67)	1.38 (0.76 - 2.51)
Mercury quartiles, n (%)									
Q1 (Ref) (≤0.34)	491 (24.56%)	46 (2.3%)	Ref	Ref	Ref	46 (15.49%)	Ref	Ref	Ref
Q2 (0.34 – 0.67)	508 (25.41%)	72 (3.6%)	1.69 (0.96 - 2.97)	1.70 (0.76 - 3.81)	1.59 (0.70 - 3.61)	72 (24.24%)	2.54 (0.90 - 7.17)	2.26 (0.47 - 10.90)	1.48 (0.43 - 5.03)
Q3 (0.68-1.38)	493 (24.66%)	63 (3.15%)	1.43 (0.82 - 2.49)	1.54 (0.75 - 3.18)	1.49 (0.72 - 3.08)	63 (21.21%)	1.91 (0.62 – 5.90)	2.53 (0.54 - 11.78)*	2.51 (0.60 - 10.59)
Q4 (>1.38)	507 (25.36%)	57 (2.85%)	1.20 (0.66 - 2.17)	0.58 (3.66 - 3.11)	1.26 (0.51 - 3.11)	57 (19.19%)	1.90 (0.61 – 5.94)	2.01 (0.40 - 10.20)	1.67 (0.33 - 8.43)
MIX									
Log Transformed, Mean (SD)	1.19 (0.60)	1.22 (0.61)	1.26 (0.94 - 1.69)	1.69 (0.95 -2.99)	1.00 (0.46 - 2.19)	1.22 (0.61)	4.60 (2.42 - 8.76)*	6.21 (2.24 - 17.20) *	1.30 (0.09 - 18.13)
Mix quartiles, n (%)									
Q1 (Ref) (≤2.24)	499 (24.96%)	53 (2.65%)	Ref	Ref	Ref	53 (17.85%)	Ref	Ref	Ref
Q2 (2.24-3.14)	500 (25.01%)	65 (3.25%)	1.34 (0.91 - 1.99)	1.58 (0.86 - 2.91)	1.47 (0.80 - 2.71)	65 (21.89%)	2.66 (1.33 - 5.32) *	1.86 (0.53 - 6.47)	0.87 (0.18 - 4.30)
Q3 (3.14-4.74)	500 (25.01%)	59 (2.95%)	1.39 (0.90 - 2.15)	1.31 (0.65 - 2.63)	1.12 (0.56 - 2.25)	59 (19.87%)	9.62 (2.74 - 33.80) *	15.87 (3.11 - 80.91) *	6.92 (0.72 - 66.79)
Q4 (>4.74)	500 (25.01%)	61 (3.05%)	1.29 (0.82 - 2.05)	2.02 (0.89 - 4.75)	1.17 (0.44 - 3.10)	61 (20.54%)	4.80 (1.77 - 13.03) *	13.22 (2.39 - 73.17) *	0.48 (0.01 - 16.32)

* Statistically significant and corresponding *p*-value <0.05

[1] Values are unweighted sample counts and percentages.

[2] 'Ever infertile' if subject responded 'yes' to the following question: "Have you ever attempted to become pregnant over a period of at least a year without becoming pregnant?"

[3] 'Fertile' if answered "No" to the following question: "Have you ever attempted to become pregnant over a period of at least a year without becoming pregnant?"

[4] 'Pregnant' if women answered "Yes" to the question "Are you pregnant now?"

Table 2.4: Association Between Women's Infertility and Recent Long-term Amenorrhea

	Amenorrhea	**Menstruating**	**Total**	**% of women with amenorrhea by infertility status**	***p*-value**
Infertile	9	222	231	3.9%	0.29
Fertile	94	1593	1687	5.6%	
Total	103	1815	1918		
% of women who are infertile by menstrual status	8.7%	12.2%			

Table 2.5: Women's Characteristics for Studying Associations Between Blood Heavy Metal Concentrations and Long-term Amenorrhea

Characteristics	**Total Sample N (%)**	**Long-term amenorrhea[1] N (%)**	**Menstruating[2] N (%)**	***p*-Value[3]**
Total Subjects	1919	103 (6.4)	1816 (93.6)	
Age, *mean* ± *SE* (years)	34 ± 0.2	35 ± 0.1	33 ± 0.2	0.24
Race/Ethnicity				0.03
Hispanic	501 (18.4)	23 (11.9)	478 (18.8)	
Non-Hispanic White	664 (58.0)	47 (68.5)	617 (57.3)	
Non-Hispanic Black	409 (13.4)	26 (14.1)	383 (13.3)	
Other Race Including Multi-Racial	345 (10.3)	7 (5.6)	338 (10.6)	
Education Level				0.65
Less than High School	285 (10.7)	16 (11.8)	269 (10.6)	
High School	382 (19.9)	27 (23.0)	355 (19.6)	
More than High School	1252 (69.4)	60 (65.3)	1192 (69.7)	
Marital Status				0.77
Married / Living with Partner	1117 (60.8)	57 (63.5)	1060 (60.6)	
Divorced / Widowed / Separated	228 (10.1)	13 (10.8)	215 (10.1)	
Never Married	574 (29.1)	33 (25.7)	541 (29.3)	
Covered by Health Insurance				0.47
Yes	1533 (82.5)	88 (85.7)	1445 (82.3)	
No	386 (17.5)	15 (14.3)	371 (17.7)	
Poverty Income Ratio, *mean* ± *SE*	2.75 ± 0.065	2.59 ± 0.189	2.77 ± 0.067	0.39
Body Mass Index (kg/m**2)				0.88
Underweight (<18.5)	38 (2.1)	1 (1.2)	37 (2.1)	
Normal Weight (18.5-24.9)	591 (31.8)	34 (31.9)	557 (31.8)	
Overweight (25.0-29.9)	451 (25.0)	21 (22.6)	430 (25.2)	

Obesity (>30)	839 (41.1)	47 (44.4)	792 (40.9)	
Ever Smoked				0.51
Yes	580 (32.6)	34 (35.9)	546 (32.3)	
No	1339 (67.4)	69 (64.1)	1270 (67.7)	
Ever taken hormone-based contraception?				0.38
Yes	1328 (75.4)	73 (71.3)	1255 (75.7)	
No	591 (24.6)	30 (28.7)	561 (24.3)	

Values for continuous variables are mean +/- the Standard Error of the Mean.
Values for categorical variables are n (unweighted sample counts) and % (weighted sample percentages to account for NHANES survey design). If precents do not equal 100% it is due to rounding.
[1] 'Long-term amenorrhea' answered "no" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)" AND answered "Other" or "Don't know" to the question "What is the reason that you have not had a period in the past 12 months?"
[2] 'Menstruating' if answered "Yes" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)"
[3] P-Value for categorical variables comes from a chi-squared test, which determines if there is a significant difference between demographics in long-term amenorrhea vs. menstruating women. P-values for continuous variables comes from a t test to determine if there is a significant difference between the means of long-term amenorrhea vs. menstruating.

Table 2.6: Medians and Log Transformed Means of Blood Heavy Metal Concentrations and Heavy Metal Mixture Scores in Women with Normal Menstruation and Long-term Amenorrhea

Metal	Total Sample	Long-term amenorrhea[1]	Menstruating[2]	*p*-Value[3]
Lead, *median, IQR* (ug / dL)	0.53 (0.38 - 0.78)	0.52 (0.35 - 0.71)	0.53 (0.38 - 0.78)	0.72
Log Transformed Lead, *Mean, SE*	-0.58 ± 0.02	-0.62 ± 0.09	-0.57 ± 0.02	0.60
Cadmium, *median, IQR* (ug / L)	0.25 (0.16 - 0.44)	0.23 (0.12 - 0.41)	0.26 (0.16 - 0.44)	0.99
Log Transformed Cadmium, *Mean, SE*	-1.25 ± 0.03	-1.37 ± 0.11	-1.25 ± 0.03	0.33
Mercury, *median, IQR* (ug / L)	0.61 (0.33 - 1.26)	0.65 (0.37 - 1.01)	0.61 (0.32 - 1.29)	0.004
Log Transformed Mercury, *Mean, SE*	-0.37 ± 0.03	-0.38 ± 0.06	-0.37 ± 0.03	0.84
Mixed Metal, *median, IQR*	2.97 (2.10 – 4.34)	2.91 (1.84 – 3.82)	2.97 (2.10 – 4.37)	0.74
Log Transformed Mix, *Mean, SE*	1.13 ± 0.02	1.08 ± 0.08	1.13 ± 0.02	0.57

Blood metal distributions were skewed. Therefore, we presented the median and IQR (25th and 75th percentile) and the mean of the Log Transformed blood metal levels. These results are weighted to account for NHANES survey design.

[1] 'Long-term amenorrhea' answered "no" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)" AND answered "Other" or "Don't know" to the question "What is the reason that you have not had a period in the past 12 months?"

[2] 'Menstruating' if answered "Yes" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)"

[3] P-Values represent a t test to determine if there is a significant difference between the means of long-term amenorrhea vs. menstruating.

Table 2.7: Associations Between Blood Heavy Metal Concentrations and Heavy Metal Mixture Scores and Women's Long-term Amenorrhea

Characteristics	Total N (%)[1]	Full sample (Long-term amenorrhea[1] vs Menstruating[2] n=1919) Long-term amenorrhea n (%) or Mean (SD)	Crude OR (95% CI) Model 1	*Adj* OR (95% CI) Model 2	*Fully Adj* OR (95% CI) Model 3
LEAD					
Log Transformed, Mean (SD)	-0.53 (0.62)	-0.54 (0.56)	0.88 (0.53 - 1.46)	0.89 (0.53 - 1.50)	0.93 (0.54 - 1.59)
Quartiles, n (%)					
Q1 (≤0.39)	460 (23.97%)	22 (1.15%)	Ref	Ref	Ref
Q2 (0.40 - 0.55)	475 (24.75%)	27 (1.41%)	0.99 (0.41 - 2.35)	1.02 (0.42 - 2.50)	1.04 (0.42 - 2.54)
Q3 (0.56 - 0.83)	504 (26.26%)	32 (1.67%)	1.03 (0.46 - 2.32)	1.03 (0.46 - 2.32)	1.06 (0.46 - 2.42)
Q4 (>0.83)	480 (25.01%)	22 (1.15%)	0.71 (0.31 - 1.61)	0.72 (0.31 - 1.68)	0.76 (0.31 - 1.85)
CADMIUM					
Log Transformed, Mean (SD)	-1.16 (0.83)	-1.27 (0.94)	0.84 (0.58 - 1.22)	0.73 (0.48 - 1.09)	0.72 (0.49 - 1.08)
Quartiles, n (%)					
Q1 (≤0.18)	455 (23.71%)	38 (1.98%)	Ref	Ref	Ref
Q2 (0.18 - 0.28)	490 (25.53%)	23 (1.20%)	0.49 (0.26 - 0.91)*	0.47 (0.25 - 0.87)*	0.47 (0.25 - 0.87)*
Q3 (0.29 - 0.51)	488 (25.43%)	16 (0.83%)	0.36 (0.16 - 0.82)*	0.31 (0.13 - 0.75)*	0.31 (0.13 - 0.76)*
Q4 (>0.51)	486 (25.33%)	26 (1.35%)	0.69 (0.34 - 1.38)	0.52 (0.23 - 1.20)	0.53 (0.24 - 1.21)
MERCURY					
Log Transformed, Mean (SD)	-0.29 (0.98)	-0.38 (-1.61)	0.98 (0.84 - 1.15)	1.10 (0.92 - 1.32)	1.11 (0.91 - 1.36)
Quartiles, n (%)					
Q1 (≤0.34)	471 (24.54%)	25 (1.30%)	Ref	Ref	Ref
Q2 (0.35 – 0.68)	485 (25.27%)	30 (1.56%)	1.60 (0.82 - 3.13)	1.68 (0.83 - 3.42)	1.70 (0.84 - 3.44)
Q3 (0.69 – 1.39)	480 (25.01%)	27 (1.41%)	1.60 (0.81 - 3.19)	1.87 (0.92 - 3.82)	1.87 (0.92 - 3.77)
Q4 (>1.39)	483 (25.17%)	21 (1.09%)	0.91 (0.53 - 1.56)	1.18 (0.65 - 2.14)	1.19 (0.63 - 2.25)
MIX					
Log Transformed, Mean (SD)	1.18 (0.60)	1.17 (0.56)	0.86 (0.51 -1.46)	0.86 (0.50 - 1.47)	0.82 (0.35 -1.93)
Quartiles, n (%)					

Q1 (≤2.21)	479 (24.96%)	24 (1.25%)	Ref	Ref	Ref
Q2 (2.21 – 3.10)	480 (25.01%)	25 (1.30%)	0.71 (0.31 - 1.63)	0.71 (0.30 - 1.67)	0.74 (0.29 -1.85)
Q3 (3.10 –4.66)	480 (25.01%)	27 (1.41%)	0.86 (0.39 – 1.90)	0.86 (0.39 - 1.92)	0.93 (0.37 - 2.34)
Q4 (>4.66)	480 (25.01%)	27 (1.41%)	0.83 (0.40 - 1.74)	0.85 (0.39 - 1.84)	1.04 (0.42 - 2.58)

*Statistically significant and corresponding *p*-value <0.05

Values are unweighted sample counts and percentages.

[1] 'Long-term amenorrhea' answered "no" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)" AND answered "Other" or "Don't know" to the question "What is the reason that you have not had a period in the past 12 months?"

[2] 'Menstruating' if answered "Yes" to the question "Have you had at least one menstrual period in the past 12 months? (Please do not include bleedings caused by medical conditions, hormone therapy, or surgeries.)"

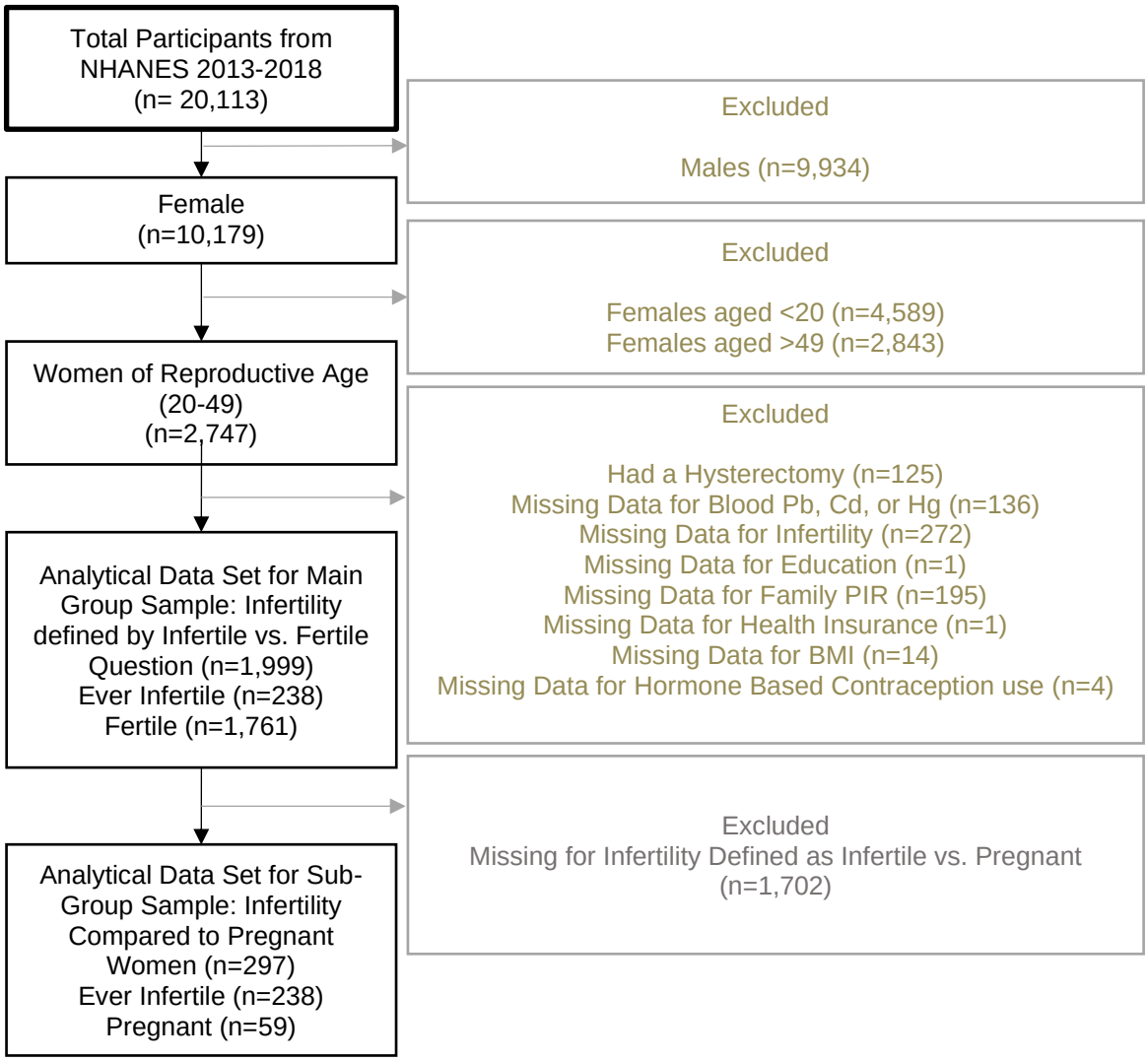

Figure 2.1: Schematic diagram depicting the process of inclusion of women from NHANES 2013–2018 for investigating associations between blood heavy metal concentrations and women's fertility.

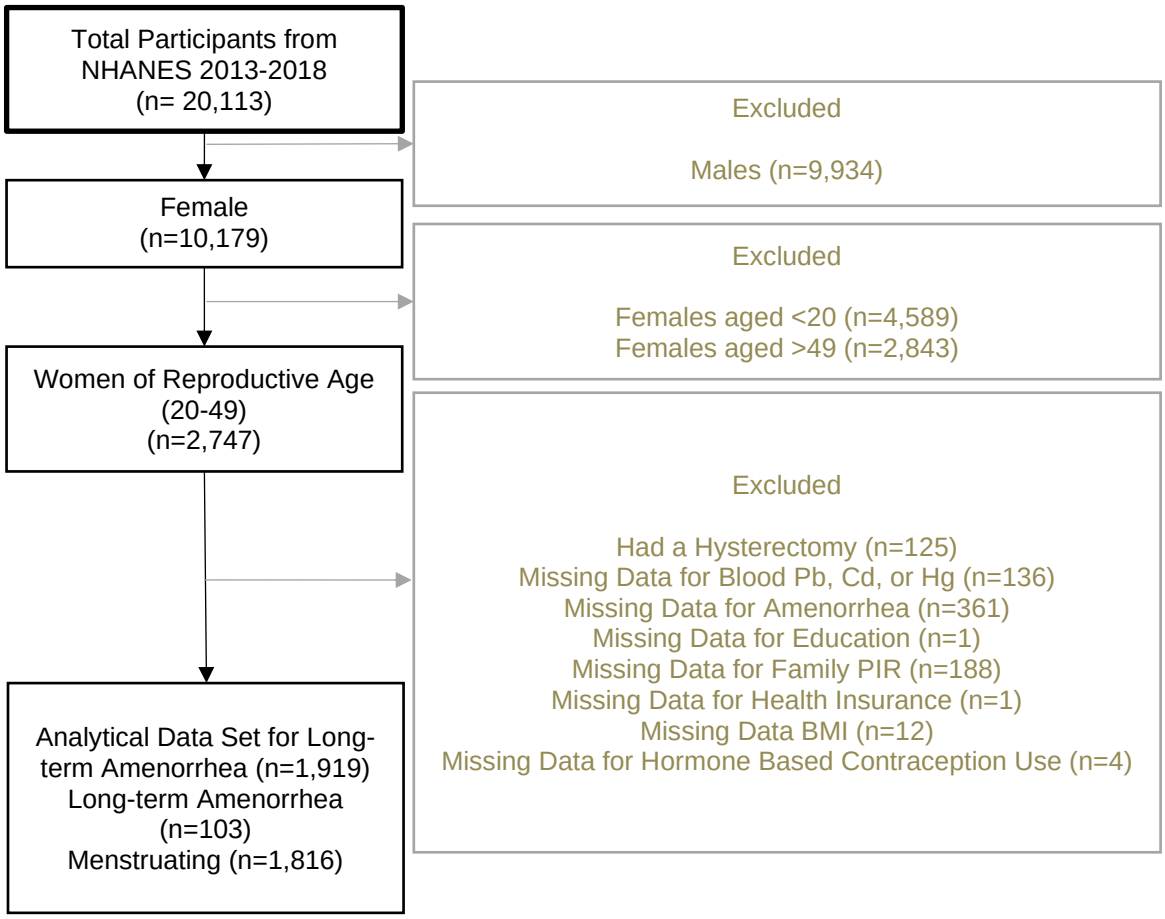

Figure 2.2: Schematic diagram depicting the process of inclusion of women from NHANES 2013–2018 for investigating associations between blood heavy metal concentrations and women's long-term amenorrhea.

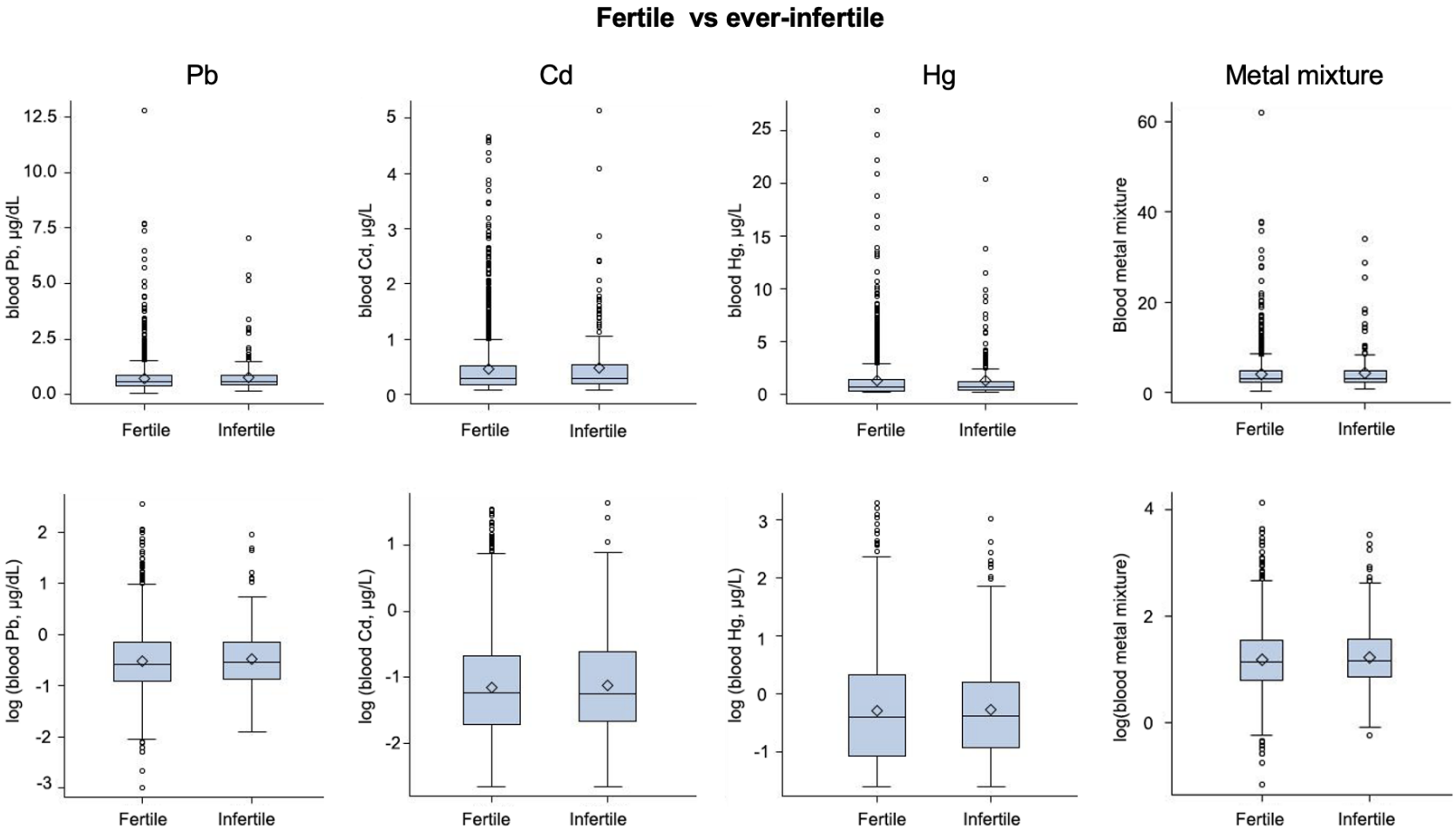

Figure 2.3: The original and log-transformed blood heavy metal concentrations and heavy metal mixture scores in women for the main-group (ever-infertile and fertile) comparison. Each box plot includes the lower (25%) and upper (75%) quartile, median (string), and mean (diamond dot). These results are un-weighted.

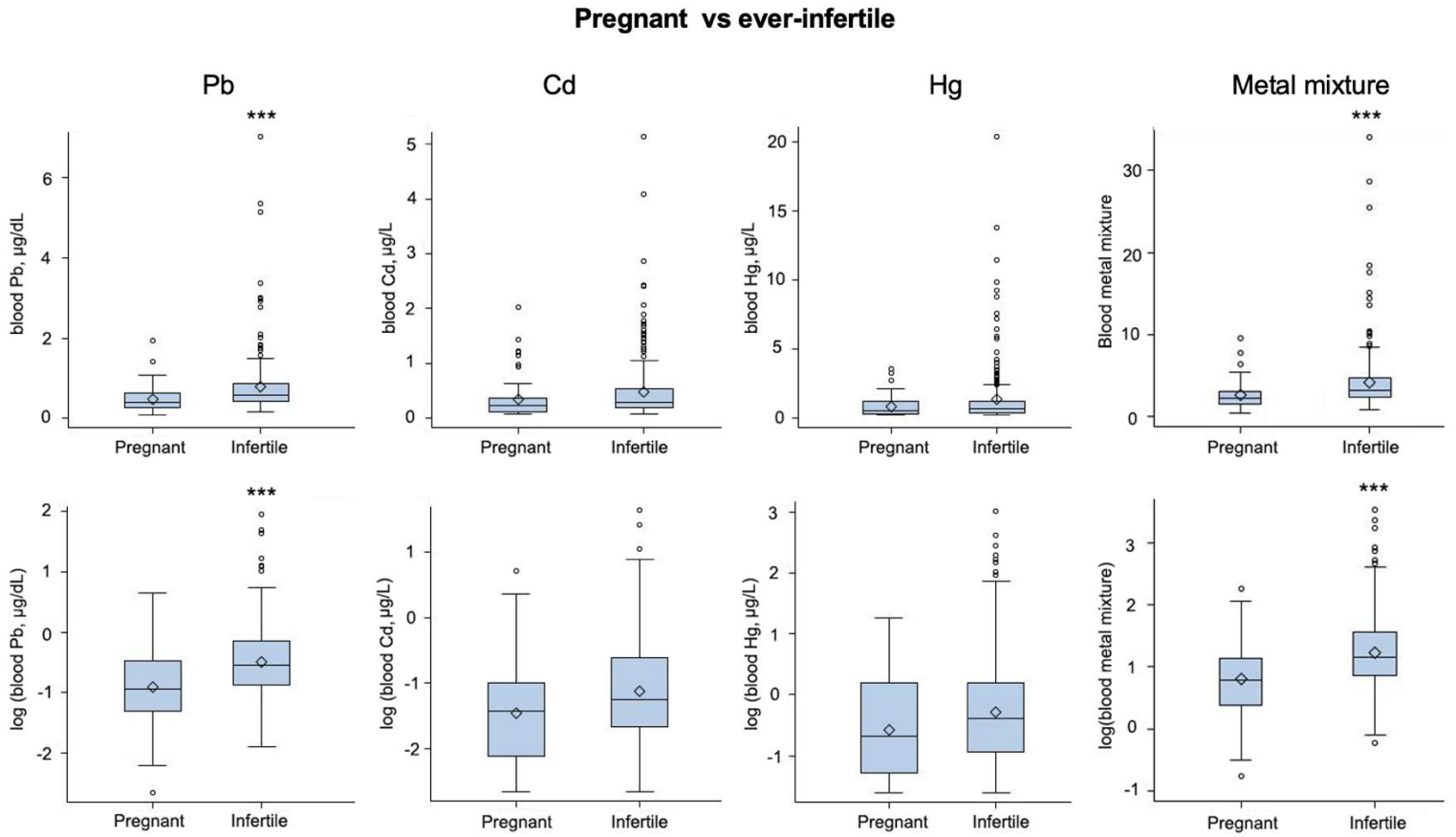

Figure 2.4: The blood heavy metal distributions among the sub-group (pregnant and ever-infertile) samples. These results are un-weighted. The original and log-transformed blood heavy metal concentrations and heavy metal mixture scores in women for the sub-group (pregnant and fertile) comparison. Each box plot includes the lower (25%) and upper (75%) quartile, median (string), and mean (diamond dot). These results are un-weighted. ***p<0.001.

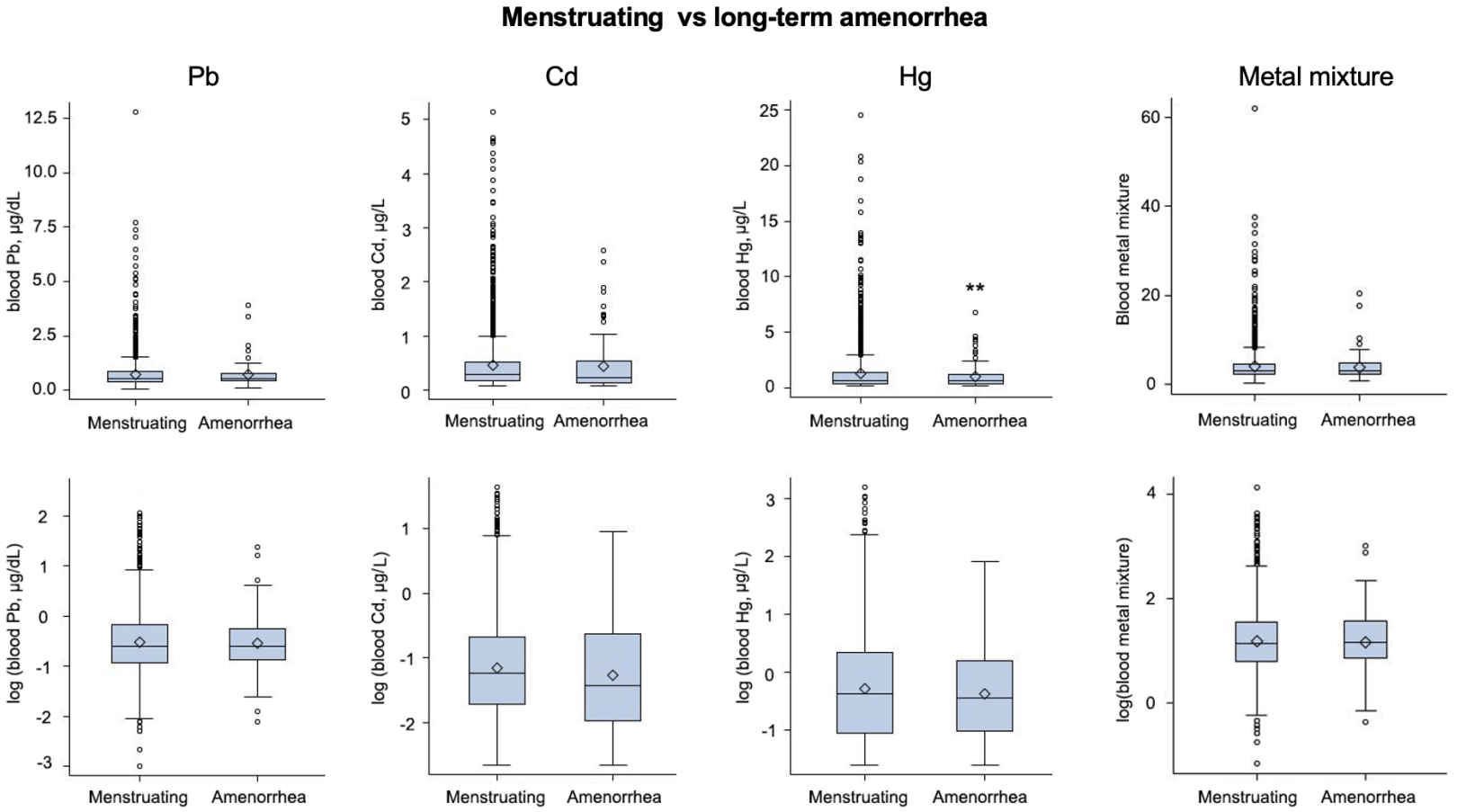

Figure 2.5: The original and log-transformed blood heavy metal concentrations and heavy metal mixture scores in women with normal menstruation and long-term amenorrhea. Each box plot includes the lower (25%) and upper (75%) quartile, median (string), and mean (diamond dot). These results are un-weighted.

CHAPTER 3

BIOENGINEERING MODELS OF FEMALE REPRODUCTION[2]

[2] Zubizarreta ME, Xiao S. Bioengineering models of female reproduction. *Biodes Manuf.* 2020;3(3):237-251. doi:10.1007/s42242-020-00082-8
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Overview

Chapter 1 gave us the background knowledge to fully understand the female reproductive system. In Chapter 2 we learned about a population-based study or epidemiological study assessing environmental exposures and reproductive dysfunction. However, epidemiological studies have many limitations when assessing women's reproductive health. Therefore, Chapter 3 discusses novel methods researchers use to study a variety of reproductive diseases in the lab. This chapter presents a literature review of recent advances and current research that uses bioengineering methods to study female reproductive diseases, including endometriosis and gynecologic cancers. We hypothesize for this chapter that the literature on bioengineering models of female reproduction will be relatively limited due to this emerging field.

Abstract

The female reproductive system consists of the ovaries, the female gonads, and the reproductive tract organs of the fallopian tubes, uterus, cervix, and vagina. It functions to provide hormonal support and anatomical structure for the production of new offspring. A number of endogenous and exogenous factors can impact female reproductive health and fertility, including genetic vulnerability, medications, environmental exposures, age, nutrition, and diseases. To date, due to the ethical concerns of using human subjects in biomedical research, the majority of studies use *in vivo* animal models and 2D cell/tissue culture models to study female reproduction. However, the complexity and species difference of the female reproductive system in humans make it difficult to compare to those of

animals. Moreover, the monolayered cells cultured on flat plastics or glass lose their 3D architecture as well as the physical and/or biochemical contacts with other cells *in vivo*. Further, all reproductive organs do not work alone but interconnect with each other and also with non-reproductive organs to support female reproductive, endocrine, and systemic health. These facts suggest that there is an urgent and unmet need to develop representative, effective, and efficient *in vitro* models for studying human female reproduction. The prodigious advancements of bioengineering (e.g., biomaterials, 3D printing, and organ-on-a-chip) allow us to study female reproduction in an entirely new way. Here, we review recent advances that use bioengineering methods to study female reproduction, including the bioengineering models of the ovary, fallopian tube, uterus, embryo implantation, placenta, and reproductive disease.

Introduction

The female reproductive system is composed of the female gonads, the ovaries, and the female reproductive tract, which includes the fallopian tubes (termed oviducts in non-primate species), uterus, uterine cervix, and vagina (Figure 3.1). It functions to provide hormonal support and anatomical structure for the production of new offspring. In addition to fertility, it is also important for women's systemic health because hormones secreted from the ovaries contribute to the general health of their endocrine, cardiovascular, skeletal, and immune systems.[163]

Abnormal female reproduction is caused by a number of factors including genetic vulnerability, medical treatments, environmental exposures, age,

nutrition, and diseases. In the USA, about 16.2% of married women within reproductive age (15-49 years old) have impaired fecundity, 8.8% of them are diagnosed as infertile, and 12.7% of them have received fertility treatments such as *in vitro* fertilization (IVF).[164] Moreover, female reproductive diseases such as endometriosis and polycystic ovary syndrome (PCOS) are becoming more and more prevalent.[165] Furthermore, gynecologic cancers do not only affect women's fertility but also threaten their lives. For example, ovarian cancer is the fifth leading cause of cancer death among women and cervical cancer is the fourth most frequent female cancer disease.[166] The female reproductive system is also one of the major off-targets of clinical drugs. Both chemotherapy and irradiation have been demonstrated to exhibit highly detrimental effects on the ovaries and increase childhood and young adult female cancer patients' risks of ovarian failure, early menopause, and infertility.[167-169] Our recent studies also suggested that doxorubicin (DOX), a commonly used chemotherapeutic chemical, permanently altered the uterine response to estrogen, an ovarian steroid hormone, indicating that anticancer agents can also directly target the uterus to impair female reproductive health and fertility.[170] In addition to pharmaceutical compounds, increasing evidence suggests that the environmental chemicals, particularly those identified as endocrine disrupting chemicals (EDCs) such as the bisphenols, phthalates, and flame retardants, can also cause female reproductive toxicities.

Thus far, due to the ethical concerns of using human subjects to study female reproduction, particularly when women are pregnant, the majority of the

explorations for female reproductive physiology, pathology, and toxicology have been through *in vivo* animal models and two-dimensional (2D) cell or tissue culture models. However, the complexity and species difference of the female reproductive system in humans make it difficult to compare to those of animals. For example, the average ovarian cycle is 4-5 days for rodents but 28 days for humans, and the average gestation period is 20-23 days for rodents but 40 weeks for humans. Moreover, *in vivo* animal models are time consuming and costly, and it is unethical to sacrifice a large number of animals for human benefit. Regarding 2D culture models, the cells cultured on flat plastics or glass lose their 3D architecture as well as the physical or biochemical contacts with other cells *in vivo*. For instance, ovarian cell lines, including both somatic cells and oocytes, have been cultured *in vitro.*[171-173] However, these individual cell lines lack the 3D cell/tissue architecture and also the bidirectional communications between somatic cells and their enclosed oocytes,[174-178] which are required for supporting normal ovarian development and functions.[179-183] These facts indicate that there is an urgent and unmet need to develop representative and effective models for studying human female reproduction.

Bioengineering aims to use the principles and technologies of engineering to solve problems in biology and medicine. Over the past decades, the prodigious advancements of bioengineering (e.g., biomaterials, 3D printing, and microfluidics) allow us to study the female reproductive system and reproductive diseases in an entirely new way. Here, we review recent advances of bioengineering models of female reproductive tissues and functions, which

provide us with encouraging research models to study the female reproductive science and medicine. We first discuss the research related to bioengineering and the ovaries, the primary female reproductive organs, which is followed by the downstream female reproductive tract including the fallopian tube, uterus, and placenta. Further, we reviewed current research that used bioengineering methods to study female reproductive diseases including endometriosis and gynecologic cancers.

Bioengineering models of ovaries

The ovary is the female gonad and contains various developmental stages of follicles as the functional units (Figure 3.2A). Each follicle consists of a central germ cell, the oocyte, and the surrounding somatic cells. It is believed that the ovarian follicle pool is established prior to birth and is non-renewable.[10,11] Thus, the diseases, medications, environmental exposures or other factors that compromise the quantity and quality of follicles and/or oocytes will increase women's risks of premature ovarian insufficiency (POI), hormonal imbalance, and infertility.[12] Growing ovarian tissues or follicles *in vitro* has a long history because it provides valuable research models and significant applications in female reproductive science and medicine, including understanding the basic ovarian biology, preserving and restoring women's fertility and endocrine functions after cancer therapy, and ovarian toxicity screening. However, when follicles are cultured on flat plastics or glass, the connections between the oocyte and somatic cells will be crushed and follicles will lose their 3D architecture and die. Here, we review the existing bioengineering methods that have been applied to

grow ovarian tissues or follicles in 3D, including the static ovarian tissue/ follicle culture models using hydrogel encapsulation, decellularized ECM, and 3D bioprinted scaffold, as well as the dynamic culture models using microfluidic systems.

Hydrogel encapsulation

Hydrogel encapsulation has been increasingly used and becomes a promising bioengineering method to support 3D *in vitro* cell or tissue culture. The liquid precursor solutions of hydrogels can become solid hydrogels after crosslinking with curing agents, which is termed gelation. The gelated hydrogels are water swellable and the formed hydrogels exhibit tissue-like elastic properties as well as allow for the diffusion of nutrients, wastes, and oxygen for the encapsulated cells or tissues. These attributes make hydrogels ideal scaffolds to mimic the native extracellular matrices (ECMs), provide tunable stiffness similar to the cellular environment *in vivo*, and maintain the 3D architecture of encapsulated cells or tissues.[184,185]

Several research groups have used hydrogels (e.g. alginate, collagen, and fibrin) to grow ovarian tissues or follicles *in vitro* and obtained remarkable advancements. A good example is that in 2003, Dr. Woodruff and her team started to use alginate hydrogel, a polysaccharide derived from brown algae, to encapsulate and culture mouse ovarian follicles, which is termed encapsulated *in vitro* follicle growth (eIVFG).[186] Their results showed that the 3D architecture of follicular complex was well-maintained during eIVFG, the granulosa cells proliferated, and the oocytes grew in volume and obtained structural

characteristics of mature oocytes such as the developed zona pellucida, presence of gap junctions between granulosa cells and oocytes, and meiosis resumption after human chorionic gonadotropin (hCG) stimulation.[186] Their following studies further demonstrated that eIVFG recapitulates all key events of mouse folliculogenesis and oogenesis *in vivo*, including the follicle development from preantral to antral stage, differentiation of mural granulosa cells and cumulus cells, development of the theca cell layer, ovarian hormone synthesis and secretion, oocyte maturation and ovulation, and luteinization (Figure 3.2B). Moreover, the ovulated oocytes from eIVFG were able to be fertilized after IVF and produced live birth after embryo transfer. [187,188] In addition to supporting mouse ovarian follicles, eIVFG has also been successful in other species including rat, dog, sheep, non-human primate, and human. In Appendix B (Table B.1), we summarized previous works that used hydrogel encapsulation method to grow ovarian tissues or follicles *in vitro*, including species, hydrogels, ovarian tissue types, and achieved reproductive outcomes.

In addition to supporting follicle development and oocyte maturation processes *in vitro*, eIVFG is also a valuable research model to explore key ovarian biology questions that have been challenging using *in vivo* models. For example, by encapsulating and culturing multiple primary mouse follicles in a single alginate bead, it was found that follicles communicated with each other to develop and mature.[189] Although the underlying mechanism is not fully understood, the conditioned culture medium from eIVFG provides a valuable resource to identify the interfollicular factors, which may include miRNAs,

exosomes, bioactive lipids, or other active peptide or protein factors. Another example to study ovarian biology using eIVFG is that the ovarian physical environment determines follicle fate. It has been demonstrated that changing the rigidity or stiffness of alginate hydrogel impacted follicle and oocyte reproductive outcomes. Specifically, decreasing the stiffness of alginate hydrogels enhanced granulosa cell proliferation, estradiol secretion, and oocyte maturation. In contrast, the rigid matrix environment induced higher production of androgen as well as compromised oocyte developmental competence.[190,191] These results are consistent with the phenotypes of aging or cystic ovaries, which have more fibrotic ovarian stroma tissues, compromised follicle/oocyte quality, and high androgen production. Such results suggest that the ovarian physical environment plays an essential role in determining the quality of follicles and oocytes. These research findings have also been translated from mouse to human. For example, since human preantral follicles require a much longer time to develop to the antral stage for maturation, we developed a two-step human follicle culture method that provided a more rigid growth environment during the preantral stage and then a permissive environment after follicles grow to antral stage. Using this two-step and dynamic follicle culture regimen, we were able to, for the first time, generate human MII oocytes using eVIFG.[192] In summary, these studies indicate that the hydrogel encapsulation method is a robust bioengineering model to grow and mature ovarian follicles *in vitro*, which can be used for multiple reproductive biology and medicine applications.

Decellularized ECM scaffold

Decellularization is a process of using the physical, chemical, or enzymatic methods to remove cellular components and only preserve the intact ECM scaffold of the original tissue.[193] ECM does not only provide a natural physical environment for the cells, but also plays essential biochemical roles in regulating a number of cellular functions, such as the cell adhesion, migration, proliferation, and differentiation.[194] Following injecting the decellularized ECM scaffold with targeting cells (e.g., stem cells, primary cells, or cell lines), the recellularized ECM scaffold has been increasingly researched for regenerative medicine and organ transplantation.

Making an artificial ovary using the decellularization method has been studied as a potential fertility preservation option for young female cancer patients. Compared to the conventional ovarian tissue transplantation, the decellularized ECM scaffold allows for the malignant cell-free recellularization and transplantation, eliminating the risk of reintroduction of cancer cells to cancer survivors. Laronda *et al.* used to seed primary murine ovarian cells into the decellularized bovine ECM scaffolds to reconstruct mouse ovaries. They demonstrated that the recellularized ECM scaffolds were able to produce estradiol *in vitro* and also induced puberty in ovariectomized mice following renal graft.[195] Moreover, the same research group further used bovine ovary ECM powders to fabricate ovarian tissue pieces, which were termed ovarian tissue papers (OTPs).[196] The OTPs were used to reconstitute human ovaries by co-culturing with human ovarian cortical strip. Their results showed that the

reconstituted human ovaries supported follicle viability and hormone secretion. However, whether the reconstituted human ovaries can support more advanced folliculogenesis and oogenesis requires further investigations. More profoundly, Oktay *et al.* transplanted human decellularized ECM scaffolds seeded with ovarian cortical tissues to two patients who had POI after cancer treatments.[197,198] Results showed that the ECM scaffolds supported vascularization in the grafted ovarian tissues and follicles secreted estradiol and developed to the antral stage in both patients. Moreover, both patients had pregnancy following IVF and embryo transfer and one of them successfully delivered a healthy baby. Recently, another research group also used the decellularized human ovary ECM scaffold and isolated human preantral follicles to reconstruct human ovaries.[199] After the reconstructed human ovaries were subcutaneously grafted to the immunodeficient mice for 3 weeks, about 40% of preantral follicles developed to antral stage, indicating that the decellularized ovarian scaffold provides a promising environment to support follicle growth and development. Taken together, these studies demonstrate that decellularized ECM scaffolds provide a promising method to reconstitute ovaries and also ovarian functions for restoring female fertility and endocrine functions.

3D printed scaffold

3D printing is novel emerging tissue engineering method that has remarkable potential in regenerative medicine and tissue transplantation. Compared to the decellularized ECM scaffold that depends on natural tissue structurality, 3D printing uses biomaterials to fabricate tissue scaffolds, which

allows for precise control of the scaffold shape, size, geometry, porosity, and other physical and biochemical properties to meet the specific research and medical needs. Researchers have used various biomaterials and 3D printing approaches to fabricate live tissues and organs, such as the heart,[200] blood vessel,[201] aortic valve,[202] skin,[203] and bone and cartilage,[204] etc.

With respect to the 3D printed female reproductive tissues, one good example is that Laronda *et al* created bioprosthetic ovaries using the 3D printed microporous gelatin scaffolds.[205] It was found that the pore geometry of the printed scaffolds influenced the seeded follicle reproductive outcomes. The 30° and 60° with underlying struts better supported follicle development and survival than the 90° without underlying struts. After *in vivo* transplantation using ovariectomized female mice, the reconstructed ovaries became highly vascularized and animals fully restored their ovarian functions and delivered live pups by natural mating. In addition to gelatin hydrogel, other researchers also used the mixture of poly (epsilon caprolactone) (PCL), a biodegradable polyester, and gelatin for scaffold fabrication to reduce the hydrophobicity as well as improve the biocompatibility of the 3D printed scaffold.[206,207] Results revealed that the unique presence of fibers in the middle of scaffold macropores promoted the adhesion, infiltration, and growth of seeded porcine ovarian follicles. Another emerging tissue engineering method based on 3D printing is bioprinting. Different from the conventional 3D printing using biomaterials only, bioprinting refers to the use of the mixture of both biomaterials and encapsulated cells, termed bioink, to print tissue constructs.[208] The bioink can be stabilized through crosslinking during

or immediately after bioprinting to generate the desired size, shape and architecture of engineered tissues or organs. In summary, both the 3D printing and bioprinting indicate a significant potential to reconstruct and build artificial, functional, and implantable human ovaries that allow prepubertal girls and young adult women to restore their fertility and endocrine functions.

Microfluidic system

The bioengineering models described above are considered as static models. Microfluidics is another emerging bioengineering method that can provide cells or tissues with a dynamic culture environment. It is defined as the application of a simplified 3D cell/tissue culture under the fluidic flow in a micron-sized channel to yield a functional tissue unit. This can recapitulate the entire organ-level of functionalities and responses. The functional unit together with the microfluidic system is also called 'organ-on-a-chip'.[209-211] Compared to static 2D or conventional 3D cultures, the microfluidic culture provides the benefits of dynamic and desired oxygen and nutrient delivery, prompt waste removal, and mechanical input by controlling the volume or rate of fluidic flow. Another unique feature of the microfluidic system is the interconnection of multiple types of tissues together to build an organ system-on-a-chip or even an entire body-on-a-chip, which will be discussed in another section of this review.

With respect to the ovary, we collaborated with bioengineers and created a microfluidic platform to culture both individual follicles and ovarian explants, which is shown in Figure 3.3A. Our results showed that the microfluidic culture of mouse primary and early secondary follicles completely recapitulated follicle

development and oocyte maturation as eIVFG does. More profoundly, the follicles cultured in the microfluidic environment further promoted follicle survival as well as the production of both steroid and peptide hormones, producing a human 28-day menstrual cycle-like hormone profile.[212] Furthermore, the microfluidic platform also supported a long-term (28 day) culture of mouse ovarian explants that contain all developmental stages of immature follicles, which has been challenging due to the insufficient oxygen and nutrient diffusion in static cultures (Figure 3.3B). In addition to mouse ovarian tissues, the microfluidic culture has also been applied to large mammalian species. Nagashima *et al.* used the microfluidic system to culture both individual follicles and ovarian cortex tissues isolated from domestic cats and dogs.[213] The dynamic fluidic flow significantly promoted the growth of both primordial follicles and preantral growing follicles, indicating a promising exploration toward the development and maturation of ovarian tissues or follicles of large mammalian species. These results gained from rodent and large mammalian species using microfluidic technology provide encouraging clue to create a human-ovary-on-a-chip.

Bioengineering models of fallopian tubes

The fallopian tubes are paired tubes with one end of the tube adjacent to the ovary and the other end extended to the uterus (Figure 3.1). They provide space and a biological environment to support several essential events during early pregnancy, including fertilization, embryo development from a fertilized oocyte to morula stage, and transport of developing embryos to the uterus.[214]

The fallopian tube consists of five segments: fimbriae (opening to the ovary), infundibulum, ampulla, isthmus, and uterotubal junction (Figure. 3.1). There are three main layers of the fallopian tube: epithelium, stroma, and inner circular and outer longitudinal layers of the smooth muscle. The epithelium layer of the fallopian tubes contains both epithelial cells and cilia. The coordination of cilia beating and smooth muscle contraction controls the uptake of ovulated oocytes and transport of developing embryos to the uterus. Nowadays, although the artificial reproductive technologies (ARTs) allow fertilization and early embryogenesis to fully complete *in vitro* without the fallopian tube, it is increasingly believed that the hormones, growth factors, and other components in the fallopian tubes have important roles in supporting fertilization and embryo development and transport.[215,216] Below, we reviewed previous studies that used bioengineering methods to recapitulate fallopian tubes or oviducts and their associated functions *in vitro*, which is particularly essential for improving the IVF success of humans, agricultural animals, and some endangered species. Compared to the ovaries, since there are limited studies focusing on the bioengineering models of fallopian tubes and the other downstream reproductive organs and diseases, we did not separate different model types as we did for the ovaries.

Human fallopian tube fimbriae have been encapsulated in 0.5% alginate hydrogel and cultured *in vitro* for 7 days. [217] Compared to a control group without alginate encapsulation, the alginate matrix retained the 3D architecture of fimbriae tissue and morphologically normal epithelial and stromal compartments.

Further, unlike the loss of ciliated epithelia cultured in 2D, the alginate encapsulation retained both ciliated and secretory features of fallopian tube epithelium. Furthermore, we and others also used transwell inserts and a microfluidic platform to co-culture human fallopian tube epithelium and murine ovarian follicles.[212,218] It was found that the fallopian tube cilia beating and secretion of oviduct-specific glycoprotein (OVGP1) were regulated by the dynamic ovarian secretion of estradiol. Moreover, the co-culture of fallopian tube-ovary also prolonged the secretion of progesterone of the formed CL. These results illustrate the crosstalk between the ovaries and fallopian tubes. Additionally, another microfluidic platform has also been designed to build a bovine oviduct-on-a-chip, which maintained the polarization and differentiation of bovine oviductal epithelial cells for 6 weeks.[194,219] Further, the oviduct-on-a-chip improved bovine IVF outcomes such as preventing poly-sperm fertilization and parthenogenic activation and producing *in vivo* like-zygote transcriptome and epigenome, compared to static bovine IVF system. These studies indicate that the bioengineered fallopian tube or oviduct has significant potential to provide a more physiological IVF and embryo culture system.

Bioengineering models of uterus, embryo implantation, and beyond

The uterus is where the developing embryo or fetus resides during pregnancy. Histologically, the uterus consists of three main layers: luminal epithelium (LE), stroma, and myometrium (Figure. 3.4A). The uterine endometrium forms the uterine cavity and includes both LE and stroma layers. In mammals, the thickness and components of uterine endometrium change with

the menstrual cycle. After fertilization, the uterus stops its transition to the next cycle and prepares for embryo implantation, a process by which a competent embryo implants into the receptive uterus. Upon embryo implantation, the uterine LE cells at the embryo attachment site undergo apoptosis to allow for further penetration of the implanting embryo, and the stromal cells differentiate into secretory decidual cells to provide a nutritive and immune-privileged matrix to support embryo and placenta development. The transition of uterus from a non-receptive to receptive status is highly regulated by the coordination of ovarian hormones of estradiol and progesterone.[220] It has been estimated that 75% of pregnancy loss is caused by implantation failure and is not clinically recognized.[221,222] Therefore, understanding the mechanisms of uterine receptivity and embryo implantation is critical for reproductive biology and medicine.

Thus far, due to the complexity of both embryo and uterus as well as their elaborate interactions during implantation, the gold standard for studying embryo implantation relies on *in vivo* animal models, in particular of mice. However, generating early pregnant animals is time and effort consuming and costly and the species difference also underscores the translation of research findings from mouse to human. The traditional 2D culture of human uterine cell lines (e.g. ECC-1 and Ishikawa cells) has been used to study uterine biology *in vitro*. However, these monolayer cells cultured in 2D miss the 3D tissue-specific architecture as well as the interconnection of different cell types. In recent years, uterine explants or bioengineered uterine tissues have been used for recapitulating human uterine functions *in vitro*. One example is that Cook *et al.*

co-cultured human uterine epithelial cells on top of the polyethylene glycol (PEG) hydrogels encapsulated with uterine stromal cells.[223] Their results showed that the bioengineered uterine endometrium formed *in vivo*-like tissue architecture and also displayed hormone-mediated differentiation such as the secretion of prolactin and IGFBP-1 (insulin like growth factor binding protein 1), two decidualization markers. Moreover, we and others have combined the microfluidics and decellularized ECM scaffold methods to engineer human uterine endometrium (Figure 3.4B).[212,224] Specifically, we repopulated decellularized human uterine endometrium scaffolds with primary human uterine epithelial and stroma cells and demonstrated that the seeded endometrial cells remain viable for a 28-day microfluidic culture period. These cells also expressed endometrial cell markers of estrogen receptor (ER) and progesterone receptor (PR), secreted uterine decidualization markers of prolactin and IGFBP-1, and responded to the upstream ovarian hormones. Another research group designed a polydimethylsiloxane (PDMS)-based microfluidic device that allowed for the co-culture of primary human endometrial stromal cells and endothelial cells isolated from umbilical vein or uterine blood vessels.[225,226] Their results revealed that the co-culture system successfully differentiated the stroma cells into decidual cells and the decidualization was also significantly enhanced by the co-cultured endothelial cells, suggesting that the uterine vascularization plays an essential role in human stroma decidualization during peri-implantation period.

In addition to the uterus, a competent embryo is another critical factor to secure successful embryo implantation. Because of the extremely limited

resource of human embryos and ethical considerations of human embryo research, using human pluripotent stem cells (hESCs) to develop embryo-like organoids has significant potential to study human embryo implantation.[227] For instance, Zheng *et al.* designed a microfluidic device which created a 3D environment and supported the growth and differentiation of loaded hESCs into embryonic-sac-like-structures.[228] The formed embryo-like organoids recapitulated many key events of embryos during the peri-implantation period, such as the anteriorization, posteriorization, and initial germ cell layer differentiation, indicating a valuable model to study embryo implantation *in vitro*. However, whether the induced 'embryos' are able to attach to uterus lining for implantation and post-implantation differentiation requires further studies. On the other hand, although embryos can be cultured and cryopreserved up until the prezygote, 8 cell, or blastocyst stage,[229] there remains no *in vitro* models to support embryo or fetus development after this. Some research has investigated post-implantation embryo or fetal development through engineering an artificial uterus that is able to closely reproduce the environment of the womb. A good example is that a device has been constructed for a fetus of a lamb and incorporated an umbilical cord structure with an amniotic fluid like system.[230] This device included a pumpless arterial–venous circuit, a closed fluid environment with continuous fluid exchange, and umbilical vascular access. Their results demonstrated that the system kept the fetus alive for four weeks without organ failure.

Bioengineering models of placenta and maternal-fetal interface

The placenta is another essential female reproductive organ inside the uterus during pregnancy. The primary function of the placenta is to serve as the interface to separate the maternal and fetal circulation and also mediate the exchange of oxygen, nutrients, and fetal waste. The exchange of these substances is through a multilayered membranous structure which is often called blood placenta barrier (BPB, Figure 3.5A). The BPB is composed of trophoblasts (syncytiotrophoblasts and cytotrophoblasts), basal lamina, and fetal capillary endothelium. The abnormal placenta development and functions are associated with a number of pregnancy complications, such as the preeclampsia, fetal growth restriction, miscarriage, and still birth.[231]

Because the ethical considerations prevent researchers from using human placenta during pregnancy and the placenta tissues collected after birth cannot fully represent the placenta functions during gestation, there is a long history of research to develop *in vitro* human placenta models. For example, previous studies developed *in vitro* trophoblast cell culture on transwells and *ex vivo* placental perfusion systems to study the placenta physiology and pathology (Figure. 3.5B).[232-234] However, these models cannot completely recapitulate the complex structure of the multilayered BPB as well as the transport and metabolism of both endogenous and exogenous substances across the placenta. The recent advances of microfluidic or organ-on-a-chip technologies allow for the bioengineering of human placenta in a more complex and representative manner. Blundell *et al.* created a microfluidic device to reproduce the trophoblast-

endothelial interface by culturing human trophoblast BeWo cell line and human primary villous endothelial cells appositely using a semipermeable ECM membrane under flow conditions, which was called placenta-on-a-chip (Figure. 3.5C).[235] This microfluidic culture system maintained the long-term viability of placental cells, which produced highly confluent monolayers on both sides of the placental membrane. More importantly, the bioengineered placenta barrier allowed for a more physiologically relevant glucose transport from the maternal to the fetal side compared to the other two *in vitro* models made of a bare membrane and a monolayer of BeWo cells without the co-cultured endothelial cells. Additionally, this microfluidic system was also used to culture another two types of placental cells, human trophoblast JEG-E cells line and human primary umbilical vein endothelial cells, to engineer a placenta-on-a-chip, receiving similar results.[236] The same research group also used the created placenta-on-a-chip to study how placenta regulates the maternal-to-fetal transfer of clinical drugs that are commonly used during pregnancy.[237] They demonstrated that the bioengineered placenta maintained the barrier integrity of BPB and also prevented the passage of heparin, a widely used medication for the treatment of deep vein thrombosis and pulmonary embolism during pregnancy. Additionally, the bioengineered BPB also expressed functional breast cancer resistance protein (BCRP), an important efflux drug transporter in the apical membrane of trophoblast cells in human placenta. In summary, these results suggest that the microfluidic platform allows for the bioengineer of human placenta-on-a-chip,

which can recapitulate both *in vivo* placenta barrier structure and transporter functions.

In addition to the 'barrier' functions, another critical role of the placenta is to provide the developing fetus with oxygen and also accept carbon dioxide from the fetus, letting the fetus 'breathe'. Preterm neonates that suffer from respiratory distress syndrome require support from ventilators, which can lead to long term complications. Another microfluidic system has been created and combined with a lung support device to improve oxygenation status in preterm neonates.[238] The creation of this artificial placenta type microfluidic oxygenator allows for gas permeable membranes that work through arterio-venous pressure difference. This oxygenates the blood through exposure directly to the air without any pumping. The device has the ability to support 30% of the oxygen needs of a preterm neonate. Reasons for premature birth are complex, but engineered artificial placenta could help us create better outcomes for premature neonates.

Bioengineering models of female reproductive diseases

Although engineered tissues, organs and organ system models have been applied for a variety of research, studies regarding bioengineering and female reproductive diseases remain limited. Several studies have been published using microfluidics and scaffold engineering to understand endometriosis[239,240] and reproductive cancers,[241,242] though none to study polycystic ovary syndrome (PCOS).

Bioengineering and endometriosis

Endometriosis occurs when endometrial tissues are outside of the uterine cavity. It is one of the most common gynecological diseases in women of reproductive age worldwide.[243] An *in vitro* model was used to investigate the cell interactions between endometrial stromal cells (ESCs) and human peritoneal mesothelial cells (HPMCs) that are similar to endometriosis conditions.[239] Microfluidic channels and cover slips were used to observe interactions between ESCs and HPMCs, mimicking the physiological processes of peritoneal endometriosis. Control HPMCs resisted introduction of ESCs from both control and endometriotic individuals. However, HPMCs from endometriotic individuals were unable to resist ESCs from both normal and endometriotic individuals. This study developed an adaptable and simple *in vitro* method for real-time monitoring of interactions between ESCs and HPMCs. This approach can be used for demonstrating interactions among three or more types of cells and for investigating organ development of other diseases. The data suggest that endometriosis is related not only to the condition of endometrial cells, but also to the locations where there is the disease. Another study used the microfluidic system to investigate the drug pathologies and biomarkers for those with endometriosis vs. those without.[240] Authors aimed to evaluate an *in vitro* model that was designed to look at inflammation and could be applied to endometriosis tissues. Results showed that the developed droplet-based microfluidic platform allowed for the observation of hundreds of protease enzyme activity reactions for

hours, creating physiologically relevant differences in controls and those with endometriosis.[240]

Bioengineering and female reproductive cancers

Traditional cell cultures fail to repeat the natural tumor microenvironment. Recently, functional 3D *in vitro* models have been investigated and engineered for studying cervical cancers.[241] Normal epithelial and immortalized cervical epithelial carcinoma cell lines were used to mimic 3D artificial normal cervical and cervical cancerous tissues. Human skin cells were used as a scaffold for both models. Results indicated that the created 3D *in vitro* cervical cancer model showed stratified epithelial layers and expressed the same types and patterns of differentiation marker proteins as seen in corresponding *in vivo* tissue in either normal cervical or cervical cancerous tissues. Additionally, other 3D microfluidic systems have been investigated to mimic ovarian cancer environments.[217,242,244] For example, a microfluidic platform of the peritoneum was constructed to mimic ovarian cancer spheroids in the peritoneal cavity with mesothelial cells under hydrodynamic conditions. The interactions between cancer cells and mesothelial cells were analyzed. This model can help future researchers understand mechanisms of metastatic progression and assist with therapeutic development.[242] High-grade serous carcinoma (HGSC), which is the most common type of ovarian cancer, originates in epithelial cells in the fallopian tube. Several studies use oviductal epithelia cultured in a dynamic microfluidic chip to create an *in vitro* model that recapitulated human carcinoma.[245,246] These *in vitro* models can allow for the study of biomarkers for early detection of cancer and for

improved therapeutic treatments. Theory shows that cortical inclusion cysts (CICs) in the ovary play a role in HGSC progression. Other studies use human samples to engineer an *in vitro* model that mimics the size, shape, and extracellular matrix properties of CICs.[247,248] Taken together, these engineered platforms can provide new knowledge on basic gynecologic cancer biology and pathology and for potential drug screening and development.

Co-culture of reproductive and non-reproductive organs in a microfluidic setting

One unique feature of the female reproductive system is that no reproductive organ functions alone. For example, the ovarian follicle development and ovulation are highly regulated by the pituitary hormones of follicle stimulating hormone (FSH) and luteinizing hormone (LH). The ovarian hormones of estradiol and progesterone control the downstream reproductive tract organs. As we described above, we have used the transwell insert or the microfluidic system to co-culture ovarian tissues and fallopian tube epithelium and demonstrated the crosstalk between these two female reproductive organs.[212,218] Furthermore, we collaborated with bioengineers and created a novel microfluidic device that can interconnect five different organs in one platform, which is termed 'EVATAR' (Figure. 3.6).[212] The five organs we cultured using EVATAR were human liver spheroids, mouse ovarian explants, human fallopian tube epithelium, human endometrium, and human cervix tissues. The EVATAR interconnected these reproductive and non-reproductive tissues through embedded microfluidic channels and the universal culture medium. The results generated from this bioengineered female reproductive system-on-a-chip

indicated that the dynamic fluidic flow maintained the viability of all culture tissues for 28 days and the downstream reproductive tract tissues responded to the upstream ovarian hormones. Additionally, compared to ovarian tissue culture alone, the co-culture system also produced a more physiologically relevant human 28-day menstrual cycle hormone profiles.

Another important application of the microfluidic system is to introduce liver metabolism for drug screening or toxicity testing. In our previous works using EVATAR,[212] the liver spheroids remained viable and secreted albumin over the entire 28 days culture period with other co-cultured female reproductive tissues; however, further studies are necessary to determine whether the co-cultured liver spheroids were able to metabolize the hormone and other secreted factors from reproductive tissues and whether the reproductive tissues can influence the metabolic activities of co-cultured liver tissues. A number of clinical drugs or environmental chemicals require liver metabolism to exhibit therapeutic or toxic effects. For example, cyclophosphamide, a widely used chemotherapeutic drug, needs to be metabolized to phosphoramide mustard to exhibit both anti-cancer effects and ovarian toxicities. Di(2-ethylhexyl)phthalate (DEHP), a plasticizer and well-identified EDC, only exhibits female reproductive toxicities when it is metabolized to the Mono-(2-ethylhexyl) phthalate (MEHP).[249] The co-culture of liver and female reproductive tissues in a microfluidic setting will allow us to introduce pharmacokinetics or toxicokinetics of the tested compounds, providing a more complex and representative *in vitro* models.

Conclusion, challenge, and future direction

There are limited ways to study female reproduction because of the complex interactions among cells, organs, hormones, and organ systems. Recent advances that use bioengineering methods to study female reproduction allow for a better understanding of the ovary, fallopian tube, uterus, embryo implantation, placenta, and reproductive disease. Bioengineering methods have been applied to study basic female reproductive biology, reproductive medicine, and toxicity screening. Hydrogel encapsulation, decellularized ECM scaffolds, 3D printing, microfluidic platform and other engineered advances indicate significant potentials to reconstruct and build artificial, functional, and implantable reproductive organs that may allow prepubertal girls and young adult women to restore their fertility and endocrine functions. Additionally, advances in *in vitro* systems like artificial wombs can help build better outcomes for preterm neonates. Furthermore, these engineered platforms can provide new knowledge on basic gynecologic cancer biology and pathology and for potential drug screening and development. While remarkable advancements have been achieved, most of these bioengineering models copy female reproduction at organotypic or cellular levels. Therefore, more in-depth analyses are required to investigate whether these bioengineering methods can recapitulate female reproduction at the molecular, genetic, and epigenetic levels, etc. For example, it is not well understood that whether the reconstructed ovaries using decellularized or 3D printed ECM scaffolds completely support the acquisition of oocyte transcriptome profiling to ensure its meiotic and developmental

competence and whether the engineered oocyte or embryos fully preserve *in vivo* epigenetic reprogramming signatures. Compared to static culture environment, the microfluidic platform significantly promotes reproductive cell proliferation and differentiation. However, different microfluidic designs have been used and the precise microfluidic settings, such as the fluid flow pattern, flow rate, and shear stress, and whether these settings depend on cell/tissue types have not been well determined. Further, the safety assessment of applied biomaterials and fabrication methods also needs to be considered when bioengineering female reproductive organs and function. In conclusion, although many endeavors are required in future studies, the intersection of bioengineering and female reproductive biology and medicine provides great potential to advance the knowledge of female fertility, genetic vulnerability, medications, environmental exposures and toxicities, aging, nutrition, and diseases.

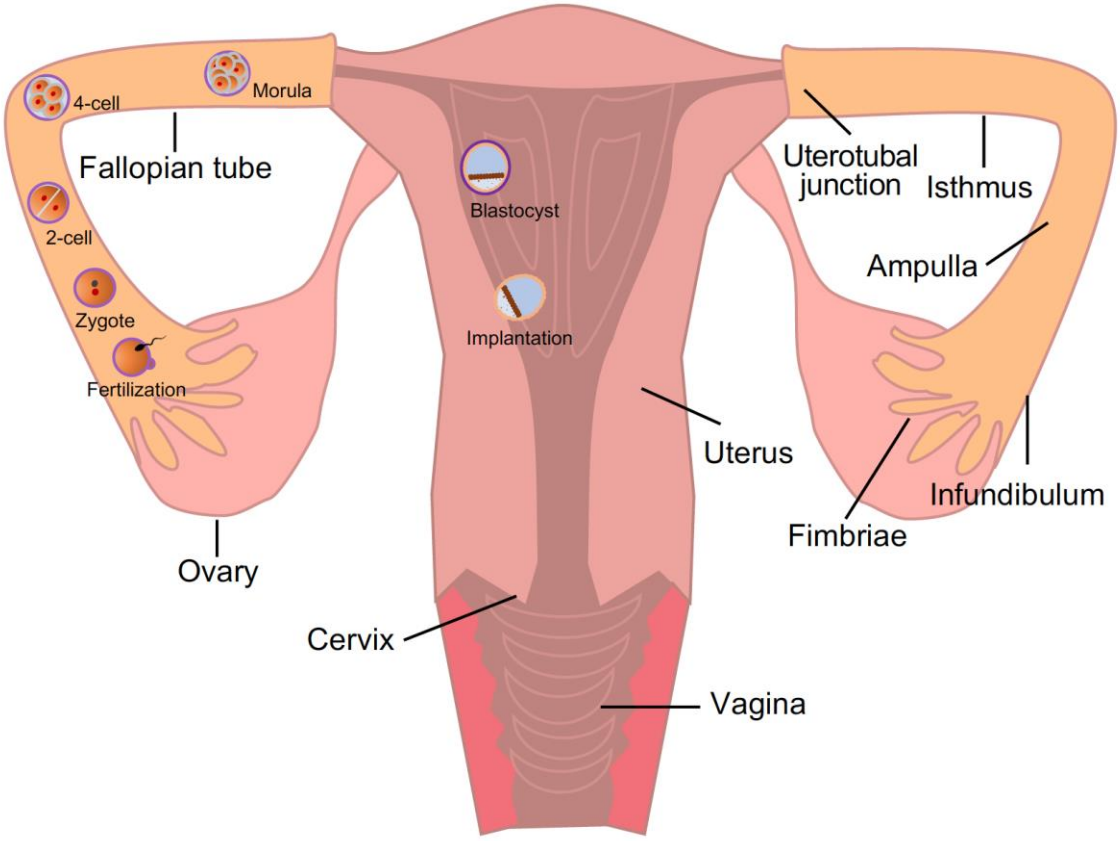

Figure 3.1: The anatomy of the human female reproductive system and early pregnant events. The female reproductive system consists of the ovaries, fallopian tubes, uterus, cervix, and vagina. Following fertilization in the fallopian tube, the zygote undergoes 5–6 miotic cell division to form the morula, a 16-cell embryo. Simultaneously, the developing embryo continues to be projected toward the uterus in the fallopian tube and reaches the uterus approximately 3 days after fertilization. Once inside the uterus, the morula continues to divide and produce an approximately 100-cell embryo, which is called the blastocyst. The blastocyst consists of two major structures: the mass of cells inside of the blastocyst is called the inner cell mass (ICM) which will become the embryo; and the cells that form the outer shell of the blastocyst are trophoblasts, which will develop into the chorionic sac and fetal portion of the placenta. The trophoblasts of blastocyst then secrete enzymes to degrade the zona pellucida, which is referred to as hatching. At about day 7 after fertilization, the hatched blastocyst adheres to the uterine endometrium for embryo implantation, which is complete at about day 10 after fertilization.

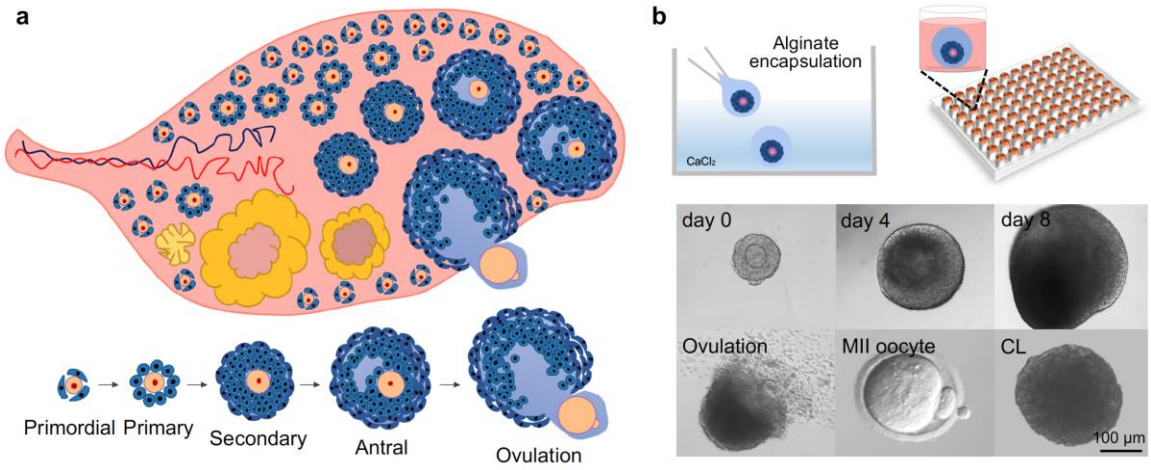

Figure 3.2: The ovary anatomy, folliculogenesis, oogenesis, and encapsulate *in vitro* follicle growth (eIVFG). (**A**) The earliest stage of ovarian follicles is termed primordial follicles, which remain quiescent to represent ovarian reserve. After birth, the primordial follicles are activated and grow to the primary, secondary, and antral stages for ovulation until menopause, which is termed folliculogenesis. In parallel to folliculogenesis, the oocytes increase in size and store mRNAs and proteins to gain meiotic and developmental competence, which is termed oogenesis. The ovulation is characterized by the rupture of antral follicles and release of oocytes into the fallopian tube. During ovulation, the oocytes resume meiosis and progress to metaphase II (MII) stage. After ovulation, the remaining granulosa cells and theca cells differentiate into luteal cells, form corpus luteum (CL), and secrete progesterone. (**B**) The eIVFG maintains the 3D architecture of ovarian follicles and supports mouse follicle development from multilayered secondary stage to antral stage for maturation, ovulation, oocyte meiotic division, and luteinization.

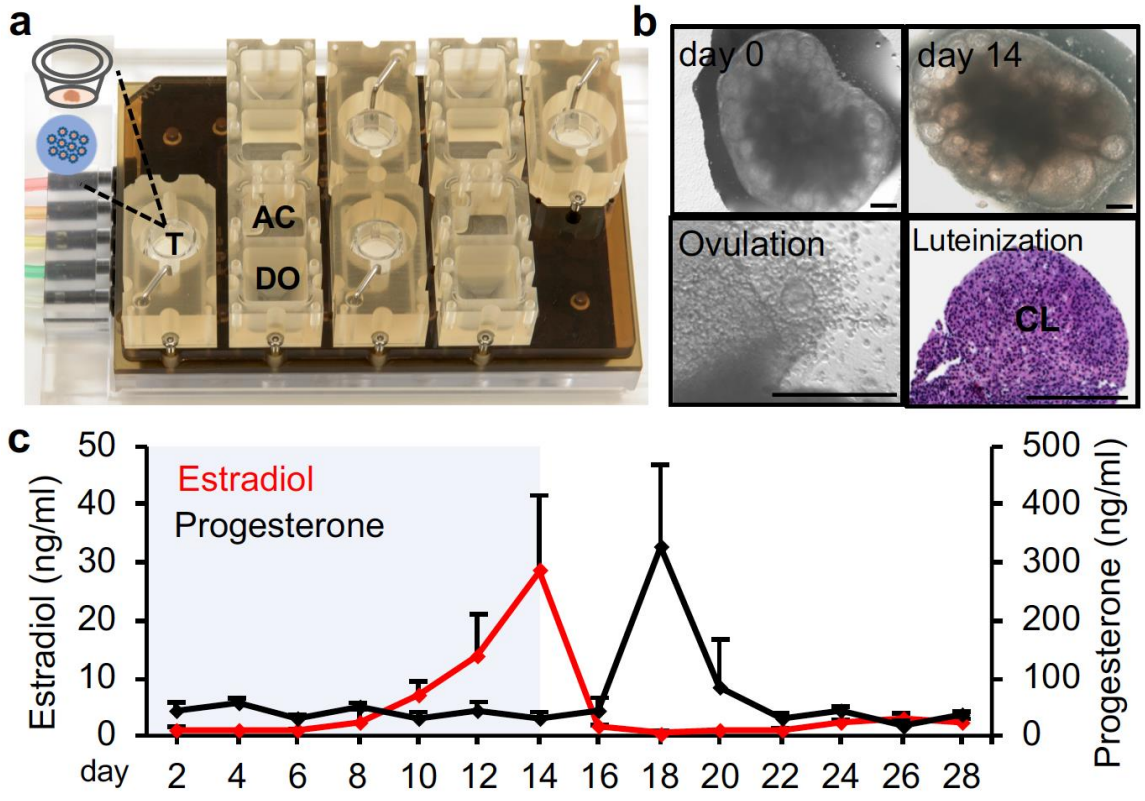

Figure 3.3: The ovary-on-a-chip. (**A**) Each microfluidic platform consists of 4 replicates of a fluidic circuit and each replicate has 3 connected modules: inlet media donor module (DO), tissue culture module (T), and outlet media acceptor module (AC). The microfluidic system can introduce a unidirectional transport fresh medium at a controlled flow rate from the DO to the T, and then remove the conditioned media and secreted hormones and other factors from the T to the AC. (**B**) The ovary-on-a-chip supported long-term culture of mouse ovarian explants, which supported follicle development, oocyte maturation, ovulation, and luteinization. CL: corpus luteum. Scale bar: 300 μm. (**C**) The ovary-on-a-chip produced a 28-day menstrual cycle-like hormone secretion profile including both follicular phase and luteal phase.

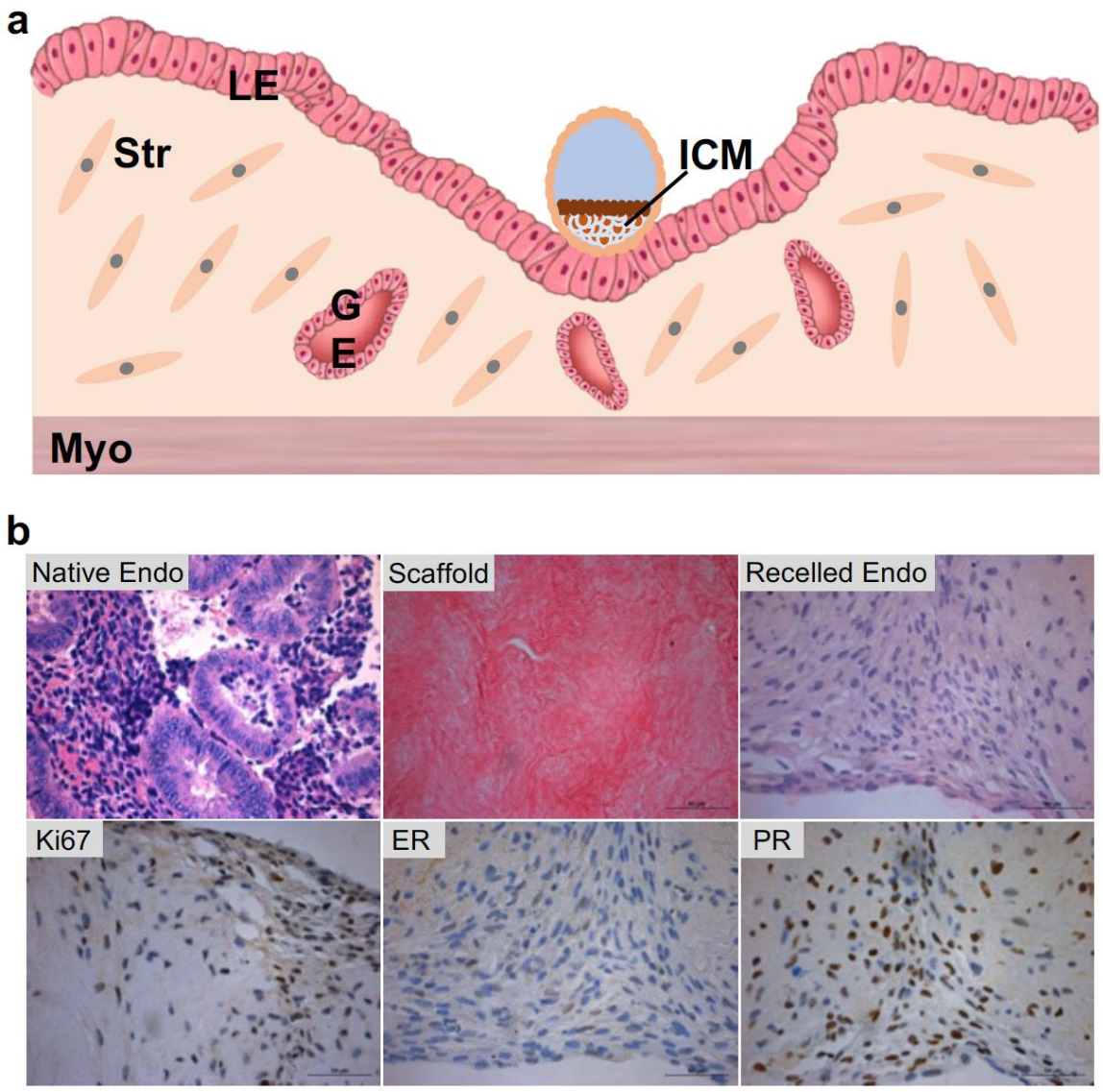

Figure 3.4: Uterine anatomy, embryo implantation, and bioengineering model of human uterine endometrium. (**A**) Uterine anatomy and embryo implantation. The blastocyst initiates the implantation process through embryo apposition, adhesion, and penetration to the uterine luminal epithelium (LE). The LE cells around the attachment site will start apoptosis upon blastocyst attachment, and help the blastocyst penetrate the LE layer into the stroma. ICM: inner cell mass. Str: stroma cells. GE: glandular epithelium. Myo: myometrium. Modified based reference[250]. (**B**) Bioengineering model of human uterine endometrium. Human endometrium tissue before and after the decellularization, after recellularization, and immunohistochemistry staining of Ki67, estrogen receptor (ER), and progesterone receptor (PR) on day 14 (28 days in microfluidic culture) in EVATAR.

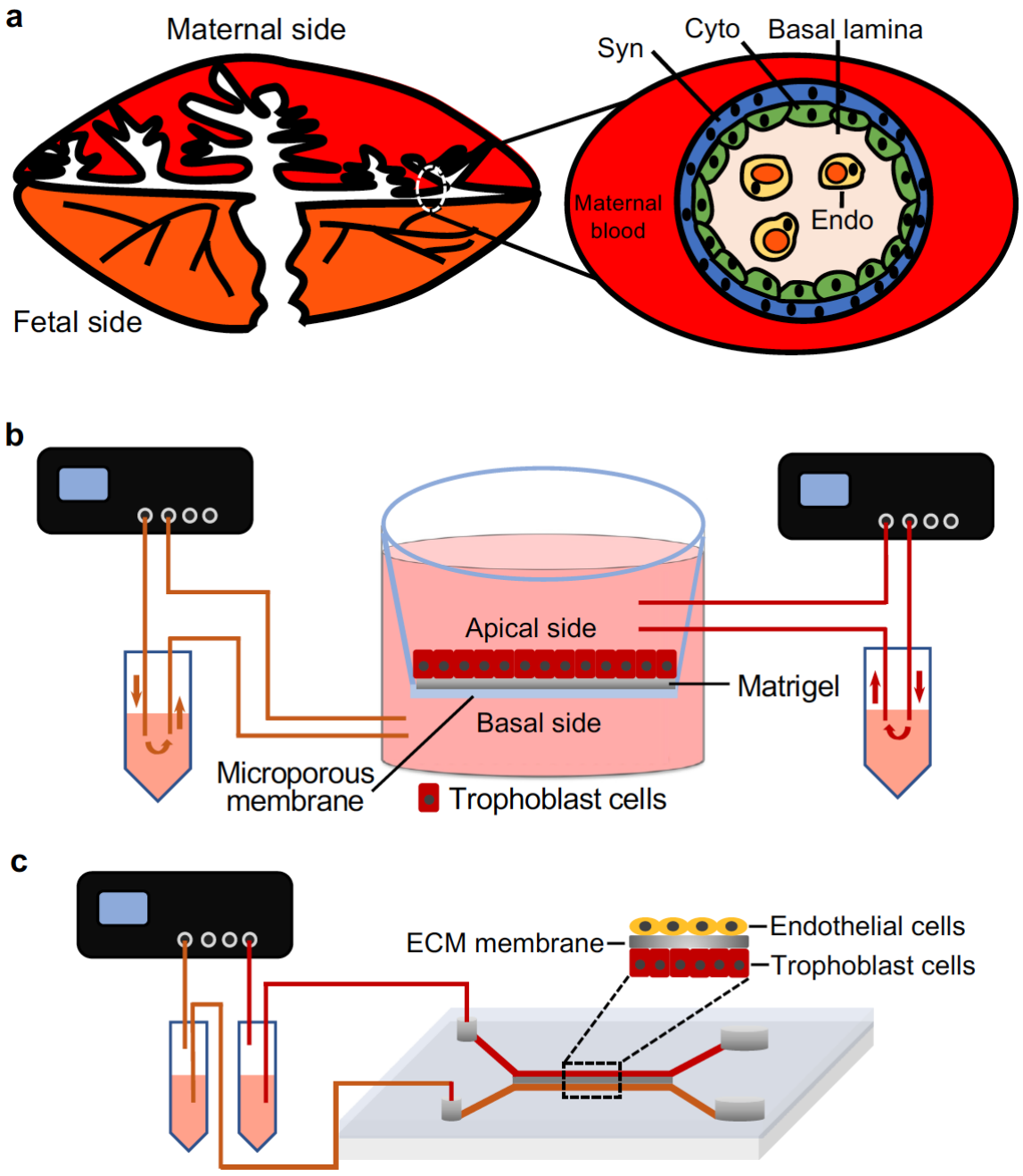

Figure 3.5: The blood-placenta-barrier and placenta-on-a-chip. (**A**) The anatomy of blood-placenta-barrier (BPB) that consists of trophoblasts (syncytiotrophoblasts, Syn, and cytotrophoblasts, Cyto), basal lamina, and fetal capillary endothelium (Endo). (**B**) The illustration of perfusion *ex-vivo* placenta model with the fetal reservoir on the left side and maternal reservoir on the right side. (**C**) The illustration of placenta-on-a-chip based on microfluidic technology, which consists of the upper villous endothelial cells (maternal side), middle semipermeable membrane, and lower trophoblast cells (fetal side).

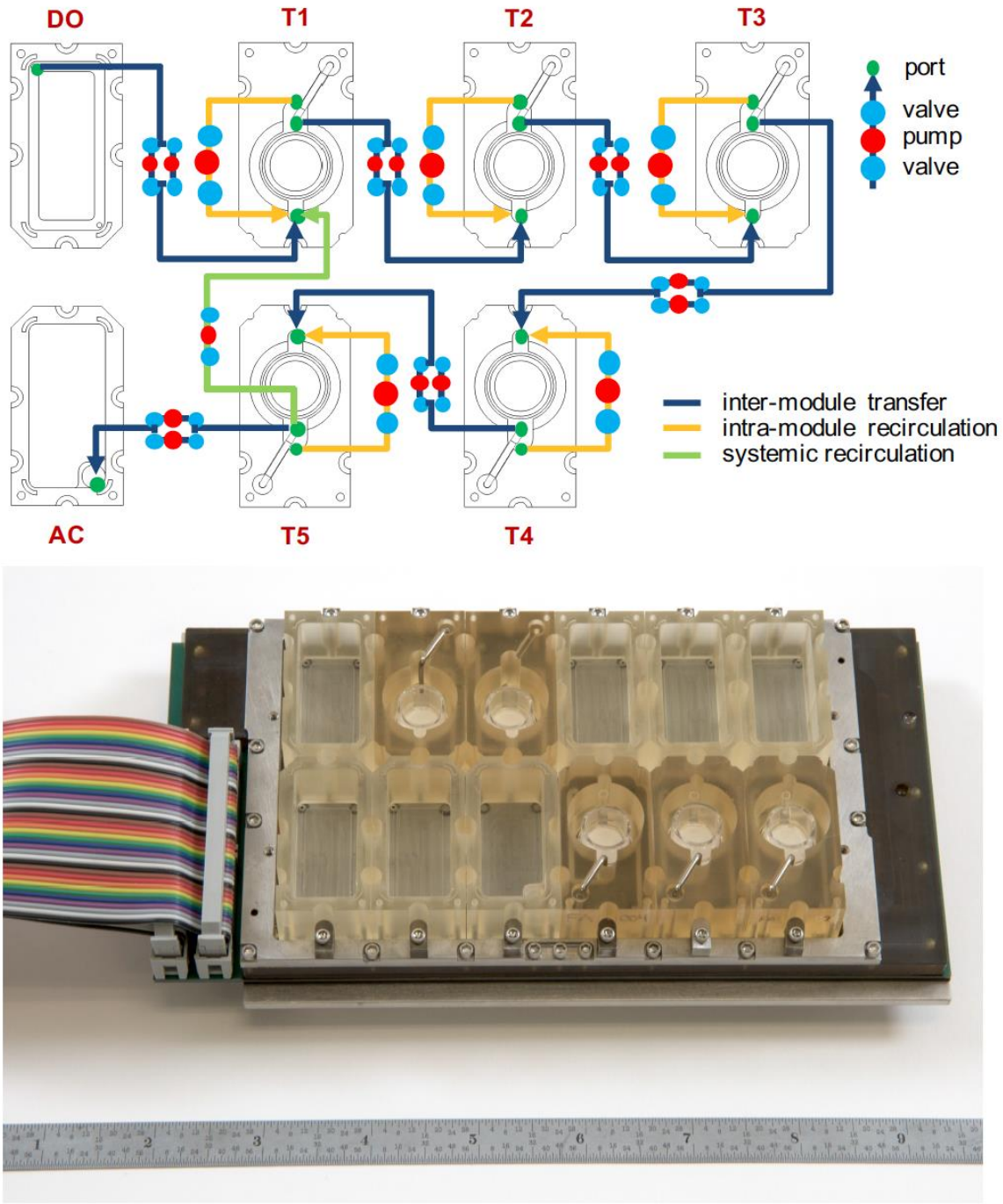

Figure 3.6: The female reproductive system-on-a-chip. The microfluidic platform of 'EVATAR' that can interconnect different tissues/organs to make a female reproductive system-on-a-chip.[212] The system was designed based on pneumatic actuation technology. The interconnection between different modules was accomplished by embedding electromagnetically actuated micro-pumps and microfluidic channels. Each module allowed for recirculation within each module (orange), ensuring that the system was well mixed and enabling homogenous

exposure of cultured tissues to factors within the media. Additionally, the fluidic path design also allowed for whole-system recirculation (blue), which enabled a well-mixed system within and across all tissue/organ modules. DO: donor module, T: tissue module, AC: acceptor module.

CHAPTER 4

PRESERVING OOCYTES IN ONCOFERTILITY[3]

[3] McClam M, Xiao S. Preserving Oocytes in Oncofertility†. *Biol Reprod.* 2022;106(2):328-337. doi:10.1093/biolre/ioac008

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Overview

In Chapter 1 we outlined how the female reproductive system works. Chapter 2 gave us a look at how environmental exposures can impact reproductive dysfunction. Chapter 3 then discussed novel methods researchers use to study reproductive dysfunction. This chapter, Chapter 4, dives deeper into fertility and specifically answers the question of how fertility can be preserved among cancer patients. This chapter reviews current literature on oocyte preservation options in oncofertility and discusses current guidelines and practices of female fertility preservation. We hypothesize in this chapter that the literature on preserving oocytes in oncofertility will be abundant and complex.

Abstract

The prodigious rise of cancer survival rates enables many cancer survivors to live long lives. Therefore, the side effects of cancer treatments as well as the long-term quality of life after cancer have become more relevant. Ovarian toxicity is a major off-target effect of anticancer agents for childhood and young adult female cancer patients. Both chemotherapy and irradiation have been demonstrated to damage the ovary and increase the risks of premature ovarian failure (POF), early menopause, ovarian endocrine disorders, and sub- or infertility. Oncofertility is an emerging and multidisciplinary research and medical field that focuses on providing cancer patients with fertility preservation options. Oocyte quality and quantity are one of the most important factors to determine women’s fertility success; therefore, preserving oocytes is paramount for maintaining the ability of young female cancer patients' reproduction after

their recovery. This review summarizes peer-reviewed literature on current oocyte preservation options in oncofertility. We describe in-depth oocyte and embryo cryopreservation, ovarian suppression, ovarian tissue cryopreservation, *in vitro* maturation, ovarian transposition, and adjuvant therapy. Further, we discuss current guidelines and practices of female fertility preservation that cover preserving oocytes.

Introduction

Each year there are approximately 210,000 newly diagnosed cancer cases in the USA in women who are before or within reproductive age (0- 49 years); this number increases to more than 3 million women per year worldwide.[251] The remarkable advances of early cancer diagnoses and treatment methods make the overall cancer survival rate greatly increased,[252] and several types of cancers, such as leukemia and breast cancer, can be nearly or completely cured.[253] These improvements allow many young female cancer survivors to live long and productive lives, leading to an increasing awareness regarding the side effects of cancer treatments as well as the long-term quality of life after cancer.[169]

Ovarian toxicity is a major side effect of cancer therapy in young female cancer patients. Both chemotherapy and irradiation have been shown to be toxic to the ovary and to heighten women's risks of premature ovarian failure (POF), early menopause, ovarian endocrine disorders, and infertility.[254,255] To find a balanced solution between "saving life" and "saving fertility," there have been numerous efforts devoted toward preserving patients' fertility before, during, and

after cancer therapy since the 1990s.[252] In 2006, a new discipline termed oncofertility was developed by Dr. Teresa Woodruff and her team,[256] which is dedicated to preserving childhood, adolescent, and young adult-aged cancer patients' fertility and associated reproductive and endocrine functions.[257-259]

As the female gonad, an ovary contains hundreds of thousands of follicles at various stages (Figure 4.1). Primordial follicles are at the earliest stage, characterized by a central oocyte surrounded by a single layer of flattened granulosa cells. There is a finite number of primordial follicles set at birth. These primordial follicles remain in a quiescent state for months or years, representing the ovarian reserve which is a marker of female fertility potential or reproductive lifespan.[8-11] Dormant primordial follicles are activated in waves of cohorts and develop into primary follicles. Activation occurs particularly after puberty when a woman has a fully developed hypothalamus-pituitary-gonad (HPG) axis. Primary follicles further grow into secondary follicles which then develop into the early antral and antral stages for maturation. Follicle development occurs from birth until menopause when the pool of primordial follicles is depleted. Folliculogenesis refers to the development process starting from the activation of the quiescent primordial follicles to the emergence of the preovulatory follicles ready for ovulating a fertilizable oocyte (Figure 4.1). This is a lengthy process estimated to take about 50 days in rodents and 200 days in humans to complete.[260,261] In parallel to folliculogenesis, the follicle-enclosed oocytes grow in size and develop to become meiotically and developmentally competent for ovulation, fertilization, and embryogenesis, which is termed oogenesis. The folliculogenesis and

oogenesis require orchestrated bidirectional communications between somatic cells and oocytes, so the damage to either one or both cell types can lead to the atresia of an entire follicle.[179-183]

It is well accepted that the number of primordial follicles and follicle-enclosed oocytes are established prior to birth and are non-renewable.[8-11] Thus, chemicals or other factors that compromise the quantity and quality of follicles and/or oocytes will result in ovarian toxicity, reproductive and endocrine disorders, and infertility.[12] Most of the clinically used anti-cancer agents act by inducing DNA damage in highly-proliferating cancer cells, which consequently results in cancer cell death.[262] Not surprisingly, the mitotically-active granulosa cells in growing follicles are potential targets of cancer treatments. Although the underlying mechanism is largely unknown, dormant primordial follicles, particularly for oocytes, are also sensitive to DNA damaging agents.[229] So far, multiple chemotherapeutic chemicals have been found to cause ovarian atrophy, a reduction of the primordial follicle reserve, DNA abnormalities or damage, and stromal fibrosis.[263-266] Moreover, radiotherapy, particularly for pelvic radiation, has been reported to induce POF.[267,268] In Figure 4.1, we briefly summarized the molecular control of folliculogenesis and the ovarian toxic effects and mechanisms of anti-cancer agents; this information has been thoroughly reviewed in previous articles.[26,254,269,270] Although various factors including cancer treatment strategies, age, or reproductive diseases impact fertility in young female cancer patients, the success of fertilization and developmental fate of the embryo/fetus are predominantly determined by the quantity and quality of the

oocyte.[271] Therefore, preserving oocytes before, during, or after cancer is critically important. This review article summarizes peer-reviewed literatures on current oocyte preservation options in oncofertility.

Oocyte Preservation Options in Oncofertility

There are multiple oocyte preservation methods for young female cancer patients, each of which has different considerations specific to the needs and condition of the patients.[264] It is not always feasible or desirable to go through fertility preservation options at the time of cancer diagnosis due to the cost, stress, or risks of delaying cancer treatments. In addition, not all cancer treatments bear the same toxicity to the ovary. Although anti-cancer agents generally kill cancer cells by inducing DNA damage, the precise mechanisms vary.[272] It is thus expected that different cancer treatments will exhibit different cytotoxicities to non-tumorous tissues such as the ovary. For example, cyclophosphamide, one of the most widely used alkylating anticancer agents, has been shown to primarily damage growing follicles. Consequently, this activates dormant primordial follicles and exhausts or even depletes the ovarian reserve to induce POF and infertility.[273,274] In contrast, cisplatin and irradiation directly promote the apoptosis of oocytes in primordial follicles, leading to POF.[275-278] These results indicate that there are mechanistic differences in the induction of ovarian damages in response to different anti-cancer agents, thus requiring different fertility preservation methods, including preserving the quality and quantity of oocytes in oncofertility. Currently, the most effective and first-line fertility preservation strategies for cancer patients are oocyte and embryo

cryopreservation, following ovarian hyperstimulation. Recent advancements in reproductive biology and medicine have allowed for other fertility preservation options for both childhood and/or young adult female cancer patients, including ovarian tissue cryopreservation, *in vitro* follicle or oocyte maturation, ovarian transposition, ovarian suppression, and adjuvant therapy. These fertility preservation options are summarized in Figure 4.2 and the following sections explain these vital technologies and their roles in preserving oocytes in oncofertility in detail.

Efficacy of Oocyte and Embryo Cryopreservation

Cryopreservation of the oocyte or embryo is an important component of assisted reproductive technology (ART) for female cancer patients. While embryo and oocyte cryopreservation are routine treatments for infertility patients, these methods are also key fertility preservation methods for female cancer patients in oncofertility and, therefore, are discussed in this review. Depending on the cancer diagnosis and treatment plan, some women can delay treatment and go through ovarian stimulation to collect oocytes from preovulatory follicles and freeze them. Standard hormonal stimulation for oocyte retrieval and cryopreservation usually requires 12-14 days.[229] The timing of these stimulation procedures has decreased in recent years and is no longer dependent on the phase of the menstrual cycle.[279] Oocyte cryopreservation is a valuable technique for women who do not have a male partner because there is no need for a sperm source. Since the first live birth from oocyte cryopreservation in 1986,[280] many advancements have been made to increase live birth success rates and to

incorporate these methods and technologies into IVF clinics worldwide. Embryo cryopreservation is a good option for women who have a male partner. Similar to the cryopreservation of oocytes, embryo cryopreservation also requires hormonal stimulation of the ovaries to collect fertilizable oocytes. After oocyte collection, regular IVF or intracytoplasmic sperm injection (ICSI) is performed and embryos are cryopreserved at the 8-cell, morula, or blastocyst stage.[229]

Oocytes or embryos can be cryopreserved for years at extremely low temperatures using slow freezing or vitrification methods.[281] Slow freezing cools the oocytes or embryos at a much slower rate than vitrification. Vitrification, which is less time consuming, requires a higher cooling rate but more cryoprotectants.[282] Previously, controlled slow freezing methods were commonly used and multiple studies have demonstrated the success of this technique, which has been summarized in previous reviews.[283,284] Recently, vitrification has been reported to have excellent clinical outcomes and higher success rates.[285] Compared to slow freezing, vitrification is also simple and cost-effective, requiring less procedural time.[284] Studies have reported successful clinical outcomes with oocyte cryopreservation. In a randomized control trial, embryo implantation rates from cryopreserved oocytes were the same as those undergoing IVF with freshly harvested oocytes.[286] The American Society for Reproductive Medicine (ASRM) and Society for Assisted Reproductive Technology (SART) practice guidelines approximate that the survival rate of oocytes after vitrification and thawing is about 90% - 97%; the fertilization rate is 71% - 79%; the implantation rate is 17% - 41%; and their estimation for clinical

pregnancy rate per thawed oocyte is 4.5% - 12% (clinical pregnancy rate is defined by the presence of a fetal heartbeat at 6-7 weeks of pregnancy).[287] These high rates are why vitrification is a first-line solution for preserving women's fertility, including young female cancer patients in oncofertility.

Effects of Cryopreservation on Oocyte Transcriptome and Epigenome

During oogenesis, the growing oocyte transcribes and stores large quantities of mRNAs that will be translated to corresponding proteins in a timely manner, some of which are crucial for fertilization, male genome processing, early embryo development, and zygotic genome activation.[288] These important maternal transcripts in oocytes are defined as maternal-effect genes/factors.[288] Oocyte quality is highly dependent on these stored maternal-effect factors. However, research describing how oocyte cryopreservation influences the gamete transcriptome is still lacking. A recent study published in 2020 demonstrated that embryos produced from cryopreserved oocytes displayed 200 up-regulated and 105 down-regulated genes compared to freshly harvested oocytes, and these differentially expressed genes were associated with mitochondrial, protein, fatty acid/lipid, and cell cycle regulation and function.[289]

Epigenetic mechanisms play a fundamental role in healthy oogenesis, fertilization, and embryogenesis. Research suggests that vitrification can impact the patterns of some epigenetic processes including DNA methylation and histone acetylation.[290] For example, vitrification of murine embryos has been reported to cause irregular methylation of the H19 and IGF2 genes.[290,291] H19 is a gene that has a role in the negative regulation of body weight and cell

proliferation[292]; IGF2 (insulin-like growth factor 2) is a critical growth factor expressed in tissues and is important during pregnancy for promoting fetal and placental growth as well as transferring nutrients from the placenta to the fetus.[293] Additionally, vitrification was found to decrease expression of oocyte-specific DNA methyltransferase-1 (DNMT1o), an enzyme that is synthesized and stored in the cytoplasm of the oocyte and is used after fertilization to maintain methylation patterns on imprinted genes.[290,294] Global DNA hypo-methylation levels were also found after slow freezing in bovine oocytes.[290,295] Epigenetic changes during gamete formation and early embryo development can change gene expression, having negative impacts on the embryo.[290,296] Previous studies and review articles have also shown an increased risk of imprinting disorders in children conceived using ART.[297-300] Several animal studies indicate that oocyte vitrification can lead to the loss of DNA methylation of imprinted genes.[301] However, human studies remain inconclusive and require further investigations.[302,303]

Effects of Cryopreservation on Oocyte Mitochondria

High amounts (~100,000 per mature human oocyte) of mitochondria are found in oocytes.[304] Mitochondria provide energy for oocyte maturation, fertilization, and embryo formation via oxidative phosphorylation and play a crucial role in the aging process of the oocyte.[305] The proper function and distribution of mitochondria in oocytes play essential roles in the process of oocyte maturation and early embryo development[306]; hence, the quality of the mitochondria in the oocyte determines the quality of the oocyte. Animal studies

suggest that when oocytes are cryopreserved, the freezing process can cause ultrastructural changes to the oocyte and changes to the mitochondria.[307,308] For example, one study observed that about half of vitrified oocytes contained atypical, small and slender mitochondria-smooth endoplasmic reticulum aggregates as well as a non-homogeneous microvillar pattern in 30% of the vitrified oocytes.[307] Another study also observed disorganization of mitochondria-smooth endoplasmic reticulum aggregates and a decreased complement of microvilli and cortical granules in frozen-thawed oocytes.[308] Vitrification can also affect mitochondrial function and distribution in oocytes by reducing the percentage and distribution of polarized mitochondria.[309] Moreover, the morphology and function of mitochondria were shown to be damaged during vitrification.[310] However, it is worth noting that the literature remains inconclusive because other studies indicate no observed mitochondrial differences between fresh and cryopreserved oocytes.[311,312]

Special Considerations for Oocyte or Embryo Cryopreservation in Oncofertility

Several aspects should be taken into consideration when thinking about oocyte or embryo cryopreservation as fertility preservation methods for cancer patients. Although the females undergoing oocyte or embryo cryopreservation may be young, they must be post pubertal. This poses an issue for pediatric female cancer patients who have no meiotically and developmentally mature oocyte available. In addition, hormonal stimulation to retrieve mature oocytes takes about 2-5 weeks. Not all women are able to delay cancer treatment that

long. Further, certain types of malignancies are hormone sensitive, requiring individual considerations when performing hormonal stimulation for oocyte collection. For example, breast cancer is the most common malignancy diagnosed during reproductive years and treatment usually consists of gonadotoxic agents.[229] Traditional ovarian stimulation regimens used to harvest oocytes increase circulating estrogen levels and may cause the hormone-dependent breast cancer to progress faster. However, altering these stimulation protocols to aromatase inhibitor-based stimulation can reduce the risk because studies have shown no increased cancer recurrence in aromatase inhibitor ovarian stimulation.[279]

Clinical Outcomes after Oocyte Cryopreservation

Despite the numerous reports demonstrating successful reproductive outcomes, oocyte or embryo cryopreservation still raises several concerns – in particular, the fear of alterations in meiotic spindle integrity, creating chromosomal abnormalities in babies born.[313] However, recent studies looking at the perinatal outcomes have provided support that abnormality rates in naturally conceived babies are not any different than those born from oocyte or embryo cryopreservation methods. In an analysis of 58 published reports that included 609 live born babies from cryopreserved oocytes, there were no differences in the rates of congenital abnormalities, compared to naturally conceived infants.[314] The study concluded that with the collection of increasing live born data, oocyte cryopreservation may become more conventional as a fertility preservation option, particularly for women diagnosed with cancer.[314] A randomized control

trial found that there was no difference in the rates of embryonic aneuploidy among women undergoing IVF with cryopreserved oocytes compared to their own fresh oocytes.[286] Another study of 200 live births following oocyte cryopreservation reported that the mean birth weight and incidence of congenital abnormalities were similar to those born from the regular IVF or spontaneous conceptions.[315]

For the long-term outcomes of IVF babies, there have been concerns of a higher cancer risk among IVF babies compared to natural birth babies. For instance, in a recent cohort study of 275,686 IVF children and 2,266,847 naturally conceived children, the overall cancer rate (per 1,000,000 children) of IVF children was about 17 percent higher than for non-IVF children.[316] Another study differentiated between IVF children who were born from oocytes/embryos with and without cryopreservation. With a total of 12.2 million person-years of follow-up, the incidence rate of childhood cancer was 17.5 per 100,000 for children born to fertile women and 44.4 per 100,000 for children born after the use of frozen embryo transfer.[317] Because oocyte and embryo cryopreservation are relatively new treatments for fertility preservation, the second generational or transgenerational health outcomes have not been investigated yet.

Ovarian Suppression to Enhance Fertility Preservation

Ovarian suppression is a treatment that aims to suppress the recruitment of immature follicles to develop and reach maturation. There is conflicting research for using gonadotropin releasing hormone agonist (GnRHa) for ovarian suppression as a fertility preservation method. However, it is recognized that this

option may be offered for cancer patients facing hormone sensitive malignancies such as breast cancer and endometrial cancer.[279,318,319] GnRHa's lower both gonadotropins and sex hormone levels by binding to the GnRH receptors in pituitary gonadotropin-producing cells, which causes a decline in luteinizing hormone (LH) and follicle stimulating hormone (FSH).[320] The mechanism and rationale for using GnRHa's to protect fertility during chemotherapy is not completely clear but animal models indicate that ovarian toxicity is higher if chemotherapy is administered while the ovaries are active.[320] A meta-analysis[321] that included five randomized clinical trials among breast cancer patients[322-327] revealed that women who received GnRHa for ovarian suppression while on chemotherapy treatment were less likely to experience ovarian failure and had higher rates of resumed menses.[321] The meta-analysis included a total of 873 breast cancer patients; 436 were randomly assigned to receive GnRHa and 437 were assigned to the control group (received cytotoxic therapy only, no GnRHa). Approximately 14% (51/363) of the GnRHa patients developed POF, as compared with 31.9% (111/359) of the patients in the control group. Moreover, their analysis showed that treatment with GnRHa and a younger age at cancer diagnosis were significantly associated with reduced risk of developing chemotherapy-induced ovarian failure.[321] The three largest trials (totaling 726 patients) also reported post-treatment pregnancies. In the GnRHa groups, 10.3% (37/359) women had at least one post-treatment pregnancy compared to 5.5% (20/367) in the control groups. This indicates that ovarian suppression is at least an effective option to preserve ovarian functions.[321] In addition, manipulating the

HPG axis, directly suppressing primordial follicle activation, is another way to preserve oocytes. For example, anti-müllerian hormone (AMH, also termed müllerian inhibiting substance, MIS) can suppress primordial follicle activation, maintaining the ovarian reserve during cancer therapy.[328] This will be further discussed in the section about adjuvant therapy below. Although ovarian suppression can enhance fertility preservation in both clinical and experimental studies, understanding whether the oocyte quality is completely preserved requires further evaluations.[322,324,325]

Assessing Ovarian Tissue Cryopreservation on Oocyte Quality

Ovarian tissue cryopreservation involves freezing ovarian tissue, which primarily contains early stage follicles. The ovarian cortex, the outer layer of the ovary, is collected laproscopocially. Once patients have undergone and finished their cancer treatments, the frozen ovarian tissue is thawed and can be used for transplant back into the ovary. This method of fertility preservation can be used in pediatric pre-pubertal female cancer patients.[329] Thus far, there have been many successful cases of fertility restoration and live births after ovarian tissue cryopreservation and transplantation.[330-335] Ovarian tissue cryopreservation that is used for transplantation does not require ovarian stimulation and can be performed immediately.[336] This is also the only option available for childhood female cancer patients who have not reached sexual maturity. It also has the potential to restore cancer survivors' ovarian endocrine functions. In January of 2020, the most recent Practice Committee Opinion of ASRM indicated that ovarian tissue banking is now an acceptable fertility preservation technique and

is no longer considered experimental.[337] However, further research needs to be conducted to determine if this is a safe and effective method for all cancer types.[279]

The research to date demonstrates that ovarian tissue cryopreservation methods preserve the molecular integrity of ovarian tissues, particularly the quality of follicles and oocytes. Previous studies found that cryopreservation protocols did not affect the incidence of apoptosis in human ovarian tissues,[338-340] but it has been shown to affect the expression of some pro- or anti-apoptotic genes in ovarian or follicular cells.[339,341] However, the specific cell types with altered apoptosis pathway were not determined. In our recently published works, we used a closed vitrification method to cryopreserve individual mouse immature follicles.[342,343] Using a 3D encapsulated *in vitro* follicle growth (eIVFG) method, we demonstrated that after warming, vitrified follicles had normal follicle and oocyte reproductive outcomes compared to freshly harvested follicles during eIVFG. Moreover, oocytes from vitrified follicles had comparable follicular cell transcriptomic profiles and expression of oocyte-specific genes, including Gdf9, Bmp15, Zp1, Zp2, and Zp3.[342,343] Although our studies focused on the cryopreservation of individual follicles but not more complex ovarian tissues, these results suggest that vitrification may be able to preserve oocyte quality. However, more systemic analyses on the oocyte developmental competence and epigenome are required in future studies.

In Vitro Maturation (IVM) and *In Vitro* Follicle Growth (IVFG) to Preserve Oocytes

IVM and IVFG are two procedures that can be used to preserve oocytes in oncofertility. IVM involves surgically retrieving women's immature oocytes from growing antral follicles and completing nuclear and cytoplasmic maturation outside the body; IVFG involves collecting women's ovarian tissues or individual immature follicles for *in vitro* culture and maturation of follicles and follicle-enclosed oocytes.[336] Once the oocytes reach maturity *in vitro*, they can be fertilized using traditional IVF or ICSI and the embryos are cultured *in vitro*. IVM and IVFG require no or minimal hormonal stimulation prior to oocyte retrieval. Thus, this makes them a faster fertility preservation option for cancer patients dealing with aggressive and time sensitive malignancies as well as allowing women with hormone sensitive cancers to avoid issues related to hormone stimulation. Beside cryopreserving ovarian tissues, IVM and IVFG are additional fertility preservation options for prepubertal girls who have no mature gamete available. Furthermore, since the oocytes will completely or partially mature *in vitro*, there is no risk of reintroducing malignant cells back to cancer survivors. Regarding the detailed methods of IVM or IVFG, please refer to another two articles in this special issue entitled "Oocyte Quality Following *In Vitro* Follicle Development" and "Present State and Future Outlook for the Application of *In Vitro* Oocyte Maturation (IVM) in Human Infertility Treatment" and a few recently published review articles.[344,345]

Ovarian Transposition as a Surgical Procedure to Preserve Fertility

Ovarian transposition or oophoropexy is a procedure that moves the ovaries out of the field of radiation by separating one or both ovaries and fallopian tubes from the uterus and attaching them to the wall of the abdomen away from where radiation will be targeted. However, this method is not always successful and risks the ovaries and embedded oocytes migrating back into the field of radiation.[279]

Adjuvant Therapy as a Method to Prevent Infertility Among Cancer Patients

Fertility preserving adjuvants work by modifying the impact of chemotherapy drugs through blocking of certain molecular pathways. For example, in terms of exposure to anti-cancer agents that primarily damage growing follicles and cause primordial follicle overactivation and exhaustion, several animal studies have found that the overactivation and exhaustion of primordial follicles was primarily through activating the PTEN/PI3K/AKT signaling pathway in the oocytes of primordial follicles.[346,347] Thus, after treating mice with agents that can suppress primordial overactivation, such as the immunomodulator AS101 and AMH, the overactivation of primordial follicles and POF can be prevented.[273,274,328,347]

With respect to anti-cancer agents that directly target primordial follicles, follicle atresia is the major mechanism to result in POF. Compared to granulosa cells, the oocytes of primordial follicles are extremely sensitive to genotoxic insults through prompt detection and repair of DNA damage or apoptosis to ensure the genomic integrity of female germline.[348,349] Even a very low level of

DNA damage has been found to be sufficient to activate the oogenic DNA damage response (DDR) pathway, which initiates oocyte apoptosis and then the entire primordial follicle atresia and POF.[350] Based on this scenario, previous animal studies have demonstrated that blocking the activation of key components of the DDR pathway in primordial follicle oocytes, such as the checkpoint kinase 2 (CHEK2), p63, and PUMA, can prevent cancer treatment-induced POF and infertility in mice.[275-278,351,352] Although the use of adjuvants to preserve fertility is hopeful, the risk of simultaneously reducing the efficacy of the chemotherapy drug is a significant concern. In addition, whether the quality of primordial follicles, including both granulosa cells and oocytes, is well preserved following the long-term process of folliculogenesis and oogenesis is still not completely understood. A recent study from Stringer et al. reported that when the γ-irradiation-induced mouse primordial follicle oocyte apoptosis was blocked, oocytes were able to repair DNA damage through homologous recombination repair. Furthermore, this DNA damage repair is sufficient to preserve animal fertility and allow for the birth of healthy offspring with genetic integrity.[352] Although these results are exciting, there remains a need for more in-depth research and clinical cases before this method is defined as an appropriate approach for fertility preservation in oncofertility.[254]

Current Guidelines and Practices of Female Fertility Preservation

Receiving specific fertility preservation counseling and/or preservation treatment helps cancer patients with their stress or coping, reduces their long-term regret or disappointment concerning fertility, and improves their quality of

life.[353-355] The American Society of Clinical Oncology (ASCO), the ASRM, and the European Society for Medical Oncology (ESMO) guidelines state that oncologists are responsible for addressing fertility preservation options with all reproductive aged women prior to any cancer therapy and that the conversation be documented.[279,318,319,356] For pediatric patients, informing the patient and/or the family of all options, benefits, and risks and referring them to a fertility specialist should be implemented.[357] ASCO and ASRM consider oocyte and embryo cryopreservation as the gold standard.[279,318,319] In January of 2020, the Practice Committee of ASRM published an opinion and indicated that ovarian tissue banking is an acceptable fertility preservation technique and is no longer considered experimental.[337] ASCO acknowledges that when proven fertility preservation methods are not feasible, ovarian suppression with hormones is a good option but should not be used in place of established fertility preservation methods.[319] However, ASCO states that the research is conflicting for using gonadotropin releasing hormone analogs (GnRHa) for ovarian suppression and other fertility preservation methods should be offered before considering this treatment. In addition, this method should only be offered in a research setting.[279,318] ASCO guidelines also state that providers should inform patients that these other options, including ovarian transposition and ovarian suppression, do not have enough evidence to be dependable options for fertility preservation.[279,319] ASRM guidelines indicate that concern about the wellbeing of children born via fertility treatments is not a sufficient reason to deny patients facing gonadotoxic treatments.[279,318]

Although it is highly recommended that cancer patients receiving gonadotoxic treatment should be informed of infertility risk and fertility preservation options, fertility preservation consultation, referral, and tissue/gamete cryopreservation services have yet to be integrated into the medical field or required by the regulations in many countries worldwide. Many cancer patients do not preserve their fertility before receiving gonadotoxic cancer treatments. For example, a previous retrospective study reported that of all the 918 surveyed cancer survivors who had potential reproductive toxic cancer treatments, 61% of them were counseled by an oncologist about their infertility risk, but only 5% of them visited a fertility specialist and 4% of them ultimately chose to preserve their fertility.[354,358] Similarly, another study collected ovarian tissues from 44 young female cancer patients who chose to cryopreserve their ovaries through the National Physician Cooperative (NPC) of the Oncofertility Consortium. However, only 50% (22) of them had not already undergone chemotherapy and/or radiation prior to ovarian tissue removal [192]. In three of our recent studies, we found that both reproductive endocrinologists and oncologist play important roles in counseling and referring cancer patients for pursuing fertility preservation in oncofertility.[359-361]

Conclusion

With the remarkable advances of early cancer diagnosis and cancer treatment methods, many reproductive-aged and childhood female cancer survivors can live long lives. Efficient and effective fertility preservation options are important to ensure long-term quality of life after cancer. Preserving oocytes

for young female cancer patients is fundamental for maintaining the ability to reproduce. As oogenesis relies on bi-directional communications between the oocytes and somatic cells, preserving the viability and functionality of an entire follicle or even the whole ovary is also critical. Current fertility preservation options that are well established for reproductive aged female cancer and infertility patients include oocyte and embryo cryopreservation. Furthermore, recent advancements in reproductive science and medicine have allowed for investigational fertility preservation options for both childhood and reproductive-age patients, including ovarian tissue cryopreservation, *in vitro* oocyte maturation, ovarian transposition, ovarian suppression, and adjuvant therapy.

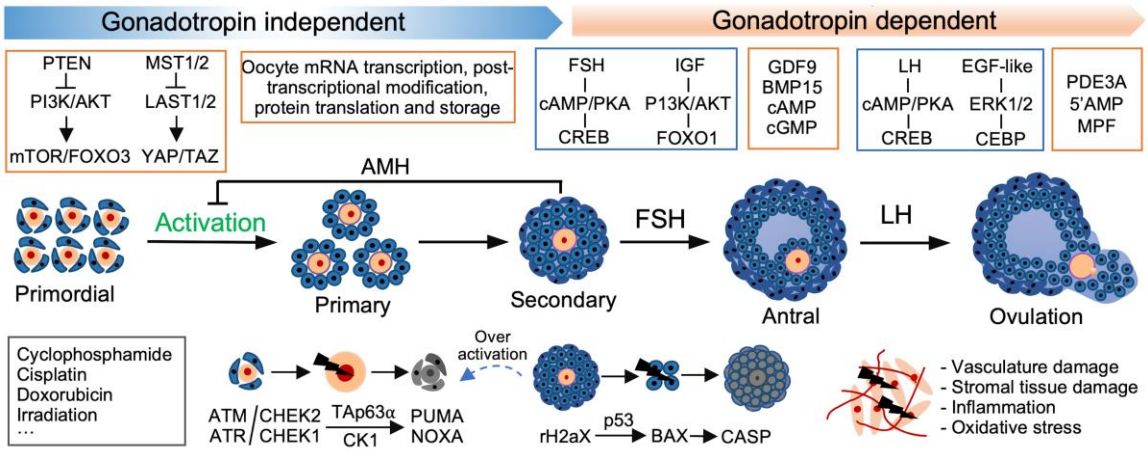

Figure 4.1: Summary of the molecular control of folliculogenesis and oogenesis (top) as well as the ovarian toxic effects and mechanisms of anti-cancer agents (bottom). Orange squares: molecular events in oocytes. Blue squares: molecular events in granulosa cells. Black lightning bolts: anti-cancer agent-induced DNA damage or other cellular stress. Follicles with gray cells: follicle atresia. Red lines: ovarian blood vessels. Orange spindle cells: ovarian stromal cells. PTEN: Phosphatase and tensin homolog; PI3K: Phosphoinositide 3-kinase; AKT: protein kinase B; mTOR: Mammalian target of rapamycin; FOXO3: Forkhead box O3; MST1/2: Macrophage stimulating1/2; LAST1/2: Large tumor suppressor kinase1/2; YAP: Yes1 associated transcriptional regulator; TAZ: transcriptional coactivator with PDZ-binding motif; AMH: Anti-müllerian hormone; FSH: Follicle stimulating hormone, cAMP: Cyclic adenosine monophosphate; PKA: Protein kinase A; IGF: Insulin-like growth factor; FOXO1/3: Forkhead box O1/3; GDF9: Growth differentiation factor 9; BMP15: Bone morphogenetic protein 15; cGMP: Cyclic guanosine monophosphate; LH: Luteinizing hormone; CREB: cAMP response element-binding protein; EGF: Epidermal growth factor; ERK1/2: Extracellular signal-regulated protein kinase 1/2; CEBP: CCAAT Enhancer Binding Protein; PDE3A: Phosphodiesterase 3A; 5'AMP: 5' adenosine monophosphate; MPF: Maturation promoting factor; ATM: Ataxia telangiectasia mutated; ATR: Ataxia telangiectasia and Rad3 related; CHEK1/2: Checkpoint kinase 1/2; TAPp63α: Tumor protein p63 α; CK1: Casein kinase 1; PUMA: p53 upregulated modulator of apoptosis; NOXA: Phorbol-12-Myristate-13-Acetate-Induced Protein 1; rH2AX: r-H2A.X Variant Histone; p53: Tumor protein p53; BAX: BCL2 associated X; CASP: Caspase.

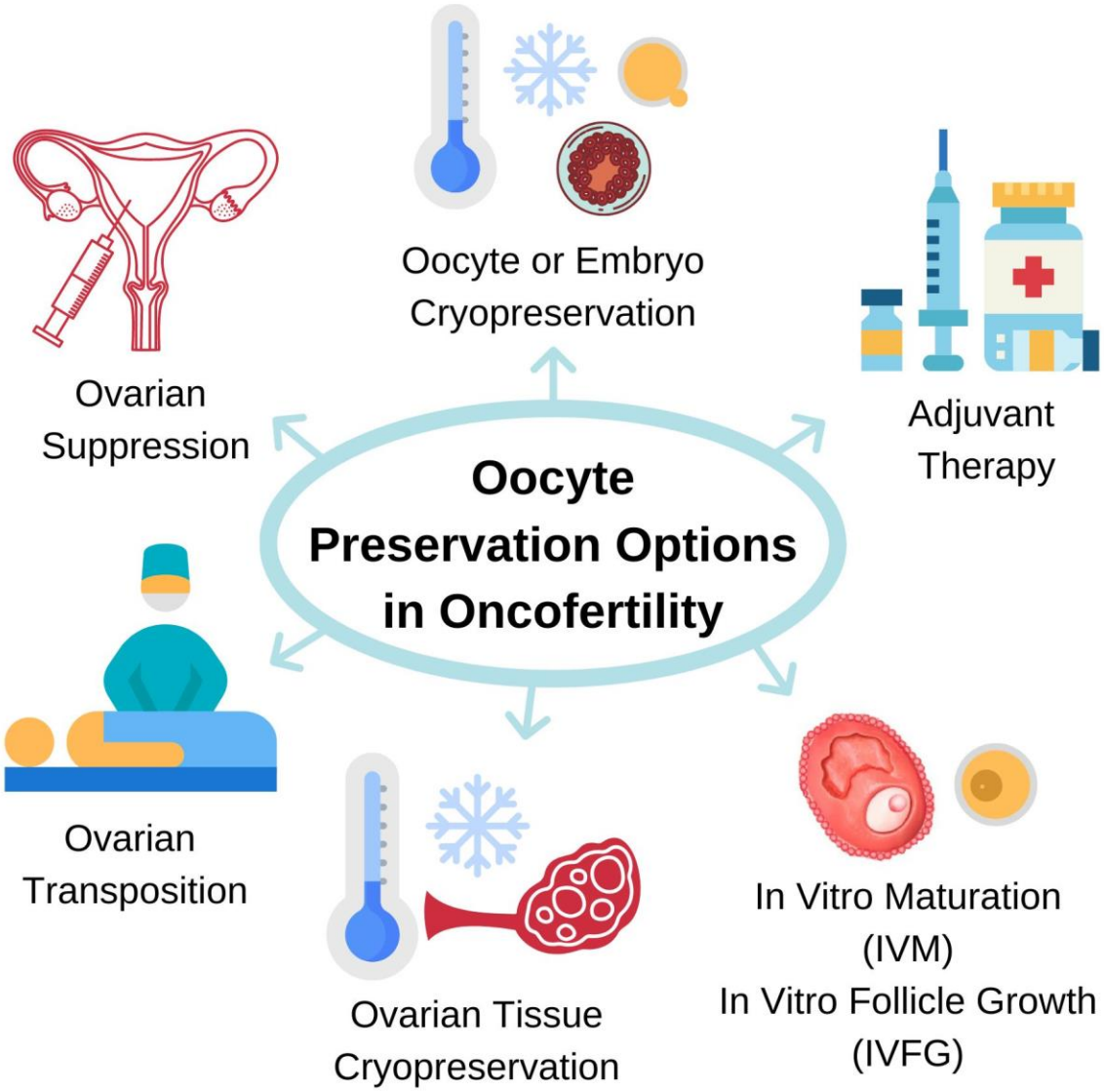

Figure 4.2: Oocyte preservation options in oncofertility. The summary of oocyte preservation methods in oncofertility, including oocyte or embryo cryopreservation, adjuvant therapy, *in vitro* maturation (IVM) or *in vitro* follicle growth (IVFG), ovarian tissue cryopreservation, ovarian transposition, and ovarian suppression.

CHAPTER 5

KNOWLEDGE, ATTITUDE, AND BEHAVIOR TOWARDS ONCOFERTILITY AMONG FEMALE BREAST CANCER PATIENTS IN CHINA[4]

[4] McClam MZ, Yan R, Su Y, Xiao S, Zhang X. 2022. To be submitted.

Overview

Chapter 1 gave us information on the female reproductive system. Chapter 2 assessed how environmental exposures can impact reproduction. Chapter 3 then discussed ways to study reproductive dysfunctions. Chapter 4, dives deeper into fertility preservation among cancer patients. This chapter, Chapter 5, considers the overall wellbeing of female cancer patients by assessing their knowledge, attitude and behavior towards fertility. This chapter shows results from a survey conducted among female breast cancer patients in a Chinese hospital system to understand their knowledge, attitude, and behavior towards oncofertility. We hypothesize for this chapter that women will have limited knowledge and access to fertility preservation options and therefore, will likely not often use preservation methods.

Abstract

Increased cancer survival rates and advances in cancer treatments have allowed many adolescent and young adult-aged cancer patients to live long lives after having cancer. Cancer treatment-induced reproductive toxicities and infertility is important for cancer patients to understand so they can make informed decisions about their reproductive health. This study aimed to assess the knowledge, attitude, and behavior towards oncofertility and fertility preservation among female breast cancer patients in Sichuan, China. We created an online questionnaire survey to examine 113 Chineese breast cancer patients' demographics, knowledge, attitude, experience, and behavior regarding their cancer and fertility preservation. Results showed that there is an inadequate

oncofertility knowledge among surveyed breast cancer patients. On average, patients answered only half (49%) of the questions assessing their knowledge of oncofertility correctly. Although most breast cancer patients reported not proceeding with fertility preservation, they view oncofertility as important and said there remains a lack of communication about oncofertility from providers to patients. Patients expressed a need for more information on how cancer treatment impacts fertility and what options there are for fertility preservation. Our study demonstrates that there is a need to improve patients' oncofertility knowledge in China as well as increase communication between oncologists, fertility specialists, and their patients.

Background

Oncofertility refers to an emerging field in reproductive science and medicine that is devoted to protecting and preserving pediatric, adolescent, and young adult cancer patients' fertility and other reproductive and endocrine functions.[256,362] Over the past few decades, significant advances in early cancer diagnostic and detection technologies as well as anti-cancer treatment methods enable an increasing number of young cancer patients to live long lives after having cancer.[363,364] There is a rising concern regarding the side effects of anti-cancer therapeutics, including reproductive toxicities and infertility. In women, both chemotherapeutic chemical compounds and radiotherapy have been documented to impair the ovaries, the female gonad, and heighten young female cancer patients' risks of premature ovarian insufficiency (POI), early menopause,

infertility, and other ovarian endocrine disorders.[365] Therefore, young female cancer patients tend to seek fertility preservation before cancer therapy.

Preserving the fertility of young female cancer patients has become standard and includes methods like cryopreservation of oocytes or embryos for women of reproductive age and cryopreservation of ovarian tissues and *in vitro* maturation of oocytes for prepubertal and adolescent girls.[366,367] Various fertility preservation guidelines have been published by professional societies including the American Society of Clinical Oncology (ASCO),[368] the American Society for Reproductive Medicine (ASRM),[367] the European Society for Medical Oncology,[369,370] the Oncofertility Consortium (OC),[258,371] the International Society for Fertility Preservation (ISFP),[372] the National Comprehensive Cancer Network (NCCN),[373] the American Academy of Pediatrics (AAP),[374] the Association of Pediatric Hematology/Oncology Nurses (APHON),[375] and the Japan Society of Clinical Oncology (JSCO).[376] These guidelines all recommend that young female cancer patients should be informed about the gonadotoxic risk of anti-cancer agents, with timely referrals to reproductive specialists, fertility preservation options, and follow up. Although the concept of fertility preservation and available options have been increasing recognized, determining which one to use especially on cancer patients can be a far more complex decision due to patients' age, marital status, and cancer location, type, and stage. Moreover, there exists a lack of knowledge around fertility preservation in general, low uptake from providers for referring cancer patients to fertility specialists, and inadequate

communications and interactions between patients, oncologists and fertility specialists.[377]

In China, fertility preservation in young cancer patients continues to be under-applied because oncofertility practice has been slow to be incorporated in medical fields, clinical regulations, and in guidelines/laws. The Chinese Fertility Preservation Society was initiated in 2017 to promote the practice of fertility preservation.[378] However, ferto-protective strategies commonly used in the west are not well utilized in China due to the lack of funding, services, or timely referrals.[359,379] For example, embryo and oocyte cryopreservation is an established fertility preservation service but ovarian tissue cryopreservation remain limited in research settings [380] In addition, surrogacy and oocyte donation are not accessible by law in China.[381]

Breast cancer in women surpassed lung cancer in 2020 as the leading cause of global cancer incidence, with approximately 2.3 million new cases per year and is the fifth leading cause of cancer mortality worldwide, accounting for 24.5% of all malignancies in women.[382] In 2020, China accounted for 24% of newly diagnosed cases and 30% of cancer-related deaths worldwide. Among women, breast cancer is the most frequent type of cancer in China and incidence continues to increase.[383]

Sichuan, a province in southwest China with about 83 million people, accounts for 5.93% of the national population.[384] The province covers an area of 486,000 square kilometers, of which 74.2% is mountainous areas. Sichuan province also has the largest Yi minority community, the second largest Tibetan

community and the only Qiang community. There is 56.73% (47 million people) of the population living in urban areas and 43.27% (36 million people) or more in rural areas.[385] According to the China National Health Database, the incidence of breast cancer in Sichuan province in 2019 was 38.3 per 100,000 people.[386] Women who live in urban areas of Sichuan are more likely to have breast cancer (new cases in 2018 was 6,239 or 5.37%) compared to women who live in townships or rural areas (new cases in 2018 was 5,440 or 4.09%).[387] In 2018, breast cancer became the second most common cancer among women in Sichuan with 11,917 cases.

The prestigious advances of cancer survival rates and long lives after cancer have shifted the goal of cancer therapy from “solely saving lives” toward “saving both lives and post-cancer life quality” for both professional physicians and cancer patients. Though explaining potential sterility can be overwhelming for newly diagnosed patients and their families, studies advise that failure to do so can significantly impact patient’s life quality.[388] Previous surveys among medical providers in China show that there are significant knowledge gaps about oncofertility, suggesting an urgent unmet need to establish an interdisciplinary fertility preservation education and service delivery system.[389,390] However, literature assessing the knowledge and uptake of fertility preservation among cancer patients is still lacking. Thus, the objective of this study is to assess the knowledge, attitude, and behavior towards oncofertility and fertility preservation among female breast cancer patients in Sichuan, China.

Methods

To understand the knowledge, attitude, and behavior of female breast cancer patients in Sichuan, China regarding oncofertility and fertility preservation, we conducted an online survey (Appendix C) in November 2021 amongst young female breast cancer patients in a Chinese hospital, the Sichuan Provincial People's Hospital. Sichuan Provincial People's Hospital is located in Chengdu, the capital city of Sichuan Province. It was founded in 1941 and ranked 57th in the comprehensive ranking of Hospitals in China by Fudan University in 2020, ranking second in Sichuan Province.[391]

The survey was initially created in English and then translated into Chinese by research team members at Sichuan Academy of Medical Sciences – Sichuan Provincial People's Hospital (SAMSPH), University of South Carolina, and Rutgers University. Breast cancer patients were recruited by providers in the Departments of OBGYN, Oncology, and Plastic Surgery using a purposive sampling framework. The target population for the survey included female breast cancer patients over 15 years old. The online survey link was distributed via WeChat, a Chinese social media platform, and administered by Questionnaire Star, an online survey tool. The survey and data collection protocol were approved by the Institutional Review Boards (IRB) at SAMSPH and at the University of South Carolina.

The survey, totaling 45 items, asked questions about their knowledge and attitude towards oncofertility as well as their behavior with fertility preservation. The questionnaire consisted of four domains, respectively: 1) general information

about the person and their cancer, 2) their knowledge of oncoferility, 3) their attitude towards fertility preservation, and 4) their behavior around fertility preservation. Questions ranged from multiple choice, true/false, strongly agree to strongly disagree, and open-ended response text boxes. We adapted questions from three of our previous studies assessing oncologists' knowledge, attitude, and practice.[359-361]

A total of 113 breast cancer patients responded to the survey. Data was exported into Excel and translated into English for analysis. Data was analyzed using the Statistical Analysis Software SAS.[392] Basic descriptive and inferential statistics were be calculated for survey responses, by question. The answers for the oncofertility knowledge questions included two choices, true and false. If participants correctly answered an oncofertility knowledge question, 1 point was added to his/her total oncofertility knowledge score. To assess if knowledge scores varied by demographic groups, we used Pearson's correlation and analysis of variance (ANOVA) tests. Additionally, participants were categorized into two age groups consisting of those under age 40 and those 40 years and older. Behavior questions were assessed by age group using Pearson chi-square test or Fisher's exact test when one or more of the cells had an expected frequency of less than five. All open-ended questions were analyzed using an inductive approach to identify key themes.

Results

Sample Characteristics

Characteristics of the study population and information about their breast cancer are outlined in Table 5.1. The mean age of all surveyed participants was 44 years with 25 being the minimum and 68 the maximum. The majority of participants were of Han ethnic group (96%), had lower than a high school degree (40%), and were married (88%). All patients reported having at least one child, with the mean being two and maximum, four. Patients had varying stages of breast cancer with the majority being stage two (32.7%). The mean age for cancer diagnoses was 40 years old, ranging from 1 to 68 years. Most participants were covered by the social health insurance at the time of diagnosis (47.8%). All patients were receiving some type if not multiple types of cancer treatments with chemotherapy (73.5%), surgery (53.1%), and irradiation (33.6%) being the most reported.

Attitude Towards Oncofertility

The majority of surveyed participants reported having no plans or wanting to become pregnant after cancer (88.5%) (Table 5.2). Family opinions (82.3%), pressure from the society to become pregnant in the future (54%), and cancer treatment survival rates (54%) were all seen as important factors that patients consider when determining their fertility options. Additionally, More than half of patients were uncertain if medical insurance would cover the cost of fertility preservation (54.9%). Furthermore, patients felt it is necessary for oncologists to be trained on the reproductive and fertility outcomes related to cancer treatment

(81.4%), necessary for oncologists to talk to cancer patients about fertility preservation (92%), and necessary to provide psychological counseling to cancer patients who have a risk of infertility before or during cancer treatment (91%). When patients were asked if they had sufficient information about fertility preservation, most said they were uncertain (48.7%) or that they did not (36.3%). Conversely, about half responded that they thought everyone could get enough information about fertility preservation (50.4%) and the other half responded that they thought everyone could not get enough information about fertility preservation (47.8%).

Knowledge of Oncoferility

Breast cancer patients' knowledge of oncofertility was basic and limited. Most patients reported they had heard that cancer therapy such as chemotherapy and irradiation can impair their ovaries and uterus and increase the risk of premature ovarian failure (POF), infertility, or premature menopause (63.7%) (Table 5.3). However, patients' knowledge of China's laws regarding fertility preservation were limited. For example, more than half of patients said they did not know that the Chinese law does not require women to be married to freeze oocytes or ovarian tissue but does require marriage to freeze embryos (56%) and about half were uncertain whether Chinese law allows young cancer patients who are infertile due to cancer treatment to choose surrogacy (50.4%).

When patients were asked if they knew the concept of fertility preservation, 62% said they did not know it at all (n=70), some said they had heard of it (n=35, 31%), several said they knew some about it (n=6, 5.3%), and a

couple reported being very familiar with it (n=2, 1.8%). However, when patients were asked specifically if they know any methods of fertility preservation such as egg cryopreservation or embryo cryopreservation, most reported they knew some (n=59, 52.2%) or did not know (n=48, 42.5%), and only 5.31% reported be familiar (n=6). Of the six people who were familiar, five people reported being familiar with egg cryopreservation and one person with embryo cryopreservation.

At the end of the survey, all surveyed breast cancer patients were asked a series of true/false questions to assess their knowledge regarding oncofertility (Table 5.4). On average, patients answered only half (49%) of the knowledge questions correctly. On a scale of 0-7 with 0 meaning no questions were answered correctly and 7 meaning all questions were answered correctly. Patients' mean score was a 3.44 with the minimum score of 1 and maximum score of 6. The results of univariate analyses showed that age, ethnicity, education level, marital status, and number of children had no significant impacts on patients' oncofertility knowledge scores (all *p*-values >0.05). We also assessed knowledge by age group. The two age groups assessed consisted of participants under age 40 (n=32) and those 40 years or older (n=81). Overall, mean knowledge scores significantly differed by age group (<40 years mean=3.8, SD=1.03; $\geq$40 years mean=3.3, SD=1.24; *p*-value=0.04). Only two individual items were significantly different by age group; A higher percentage of those aged less than 40 years answered correctly for the items "The use of fertility preservation methods increases the risk of future cancer recurrence" (*p*-value =0.046) and "Freezing eggs and freezing embryos have the same chance

of success for future pregnancies" (*p*-value=0.02) compared to those aged 40 or older (Table 5.4).

Behavior Around Fertility Preservation

Breast cancer patients reported that most had never inquired their oncologist about the potential damage to fertility caused by cancer treatment (72.6%) but that they felt comfortable discussing fertility issues with their doctor (71.7%) (Table 5.5). Furthermore, almost half of surveyed participants reported never being asked about their plans for future pregnancies or being informed of the risk of infertility by their oncologist (46%). Additionally, most patients reported that they were not referred by a doctor or nurse to an expert in fertility preservation (86.7%) and were also uncertain if their hospital provides fertility preservation services (66.4%). The majority of respondents said they had not received fertility preservation consultations for a variety of physicians including gynecologists and oncologists (85.8%) or were provided psychological counseling about fertility concerns from cancer treatment (77%). Ultimately, only five (4.4%) survey participants reported that they had or were going to proceed with fertility preservation.

Of the few (n=3) patients who received fertility preservation consultation from a variety of physicians, including gynecologists and oncologists, all said they sought out consultation to help them make an informed discission and one also said to help avoid any future regrets. All three patients were uncertain how long it was between cancer diagnosis and when they received fertility preservation consultation. Two out of the three patients said they were able to

see a fertility preservation specialist before starting they cancer treatment. Two of the patients were unsure what types of fertility preservation options were offered to them while the third recalled egg cryopreservation, sperm cryopreservation, embryo cryopreservation, and ovarian tissue cryopreservation. These three patients ultimately said they did not proceed with fertility preservation and therefore were ineligible to answer the next few questions about the order in which their treatment and consultations were received. However, five other patients recorded that they did proceed with fertility preservation. Of the five patients who proceeded with fertility preservation, the order in which their treatment and consultations were received varied (Table 5.5). Three out of five patients received fertility preservation consultation first.

When patients were asked what their reason was for not proceeding with fertility preservation, most said it was due to concerns about delaying cancer treatment (n=45, 39.8%). However, other reasons were also reported including that patients did not know about fertility preservation prior to tumor treatment (n=38, 33.6%), fertility preservation costs (n=28, 24.8%), and mental and physical demands (n=28, 24.8%). Additionally, patients also reported they did not proceed with fertility preservation for other reasons (n=38, 33.6%), some of which were specified as not having a plan when they got cancer, already having children and no desire to have more, or feeling like they were too old to have more children. Most patients said they did not have any regrets about not proceeding with fertility preservation (67.3%), while some were uncertain (20.4%), and a few did have regrets (8.0%) (Table 5.6).

We also compared responses for the behavior questions displayed in Table 5.6 by age group. We found that most responses did not significantly differ by age group. However, responses to the questions “Have you ever asked your oncologist about the potential damage to fertility and fertility preservation caused by cancer treatment?” (*p*-value 0.01), “Have you been asked about your plans for future pregnancies and informed of the risk of infertility by oncologists?” (*p*-value 0.02), and “Do you feel uncomfortable discussing fertility issues with your doctor?” (*p*-value 0.03), differed significantly by age group.

When patients were asked what factors were most important and influential for their final decision regarding fertility preservation, most answered family (51%, n=58), doctors’ recommendation or approval (38%, n=43), access to resources including medial, social, and budget (29%, n=33), and available services (13%, n=15). Several patients also expressed that the effect of their cancer-treatment or risk of delaying treatment (n=3) and their age (n=2) were important factors for their decision regarding fertility preservation.

Perceived Opportunities for Improvement in Oncofertility suggested by surveyed patients

Patients described that they needed access to more medically accurate information regarding fertility preservation (39%, n=45) as well as access to resources (27%, n=31) and services (31%, n=35). Participants explained that it would be nice to “know the pros and cons of fertility preservation,” and “provide more information about what happens to fertility after cancer treatment.” Another patient suggested using “multiple channels to enable patients to acquire more

knowledge.” Several participants noted that medical treatment, doctors, and complex medical institutions focusing on oncofertility are still lacking. One patient suggested “popularizing the work” could be a solution to getting more professionals in the field while another said that “the allocation of resources” at the system level could use improvement. Additionally, patients mentioned a need for “psychological counseling,” “creating a positive and harmonious atmosphere,” and doctors with good attitudes.

When patients were asked for suggestions to improve fertility counselling for cancer patients in the future, participants explained that information regarding oncofertility should become more publicly available and assessible, doctors should give patients more information and options prior to starting anti-cancer treatments, and science should inform policy and decision making (e.g. one patient suggested legalizing surrogacy). One participant explained “every woman has the right to choose whether or not to have children” and another said “I hope that the future of medicine will be more advanced so that the cancer patients no longer suffer and our dreams of motherhood will not be affected.” Moreover, patients expressed the need for more fertility preservation options to be covered by health insurance or free. One patient explained, “it is also necessary for doctors in hospitals or gynecology departments to establish a better platform to provide free consultation on gynecological issues for women.” Additionally, a few patients reconized the importance of science by saying “the popularization of science in this field is urgently needed.”

Discussion

Overall, there remains a need for more communication between oncologists and reproductive specialists, to improve patients' oncofertility knowledge in China. Our results show that breast cancer patients feel strongly that they need more information about fertility preservation options prior to receiving cancer treatment so that they can make informed decisions. Although most of the patients surveyed did not have a plan or want to become pregnant after cancer, they still felt oncologists should be trained on the reproductive and fertility outcomes related to cancer treatment, speak to their patients about this, and provide psychological counselling for patients. Our results also demonstrate the complexities of decision making when it comes to oncofertility. Patients consider family, society, budget, and efficacy of cancer treatments when deciding about fertility preservation. However, it is clear that patients need more information on types of fertility preservation, success rates, how fertility preservation impacts cancer, and Chinese laws to make informed decisions. Overall, there remains a lack of knowledge of oncofertility among the Chinese breast cancer patients we surveyed. Moreover, more communication regarding oncofertility is needed between providers and patients.

Most previous studies assessing knowledge, attitude, and behavior towards oncofertility have been among providers and professionals rather than among patients.[360,390,393-395] A previous study we conducted found that there is inadequate knowledge of oncofertility among Chinese oncologists.[390] In that study, we found that only 11.8% of surveyed oncologists often referred their

patients for fertility preservation, while 66.3% and 21.9% of them have referred once or never, respectively; thus, demonstrating an urgent unmet need to improve oncologists' oncofertility knowledge, attitude, and practice in China as well as remove the communication barrier between oncologists and fertility specialists.

Our study has several limitations. First, the mean age of our study participants was 43 years and all patients had already had at least one child. This may make those surveyed less likely to be concerned about having children or preserving their fertility. This could explain why only five participants indicated they proceeded with fertility preservation. Logically it would make sense that the five patients who recorded proceeding with fertility preservation should have also selected that they received fertility preservation consultation from a variety of physicians, including gynecologists and oncologists. It could be that these patients only got information from one provider rather than a variety or that they didn’t understand the question. Four out of the five patients that reported receiving fertility preservation were unsure what type they received. It is possible these patients received Gonadotropin-releasing hormone analogs (GnRHa) as we did not have that as a response item they could pick in the survey. Future studies may want to consider increasing sample size to get a more diverse sample. Second, selection bias may exist because all breast cancer patients who participated in the survey were recruited from the same hospital. In future studies, we can explore and use more representative survey sampling methods to avoid this bias.

In summary, our results suggest that patients' view oncofertility as important and that providers should communicate more information to patients about how cancer treatment impacts fertility and what options there are for fertility preservation. Our study shows there is inadequate oncofertility knowledge among breast cancer patients in China.

Table 5.1: Characteristics of the Study Population

	n (%)[1] or Mean (SD)
Age	43 (6.3)
Ethnicity	
Han	108 (96%)
Zang	4 (3.5%)
Prefer not to say	1 (0.9%)
Education	
< high school degree	45 (39.8%)
High school degree	30 (26.6%)
Bachelor's degree	37 (32.7%)
Master's degree	1 (0.9%)
Marital Status	
Single	1 (0.9%)
Married	99 (87.6%)
Divorced	11 (9.7%)
Widowed	2 (1.8%)
Number of Children	2 (0.6)
Stage of Cancer	
Stage 0 (carcinoma in situ)	6 (5.3%)
Stage 1	24 (21.2%)
Stage 2	37 (32.7%)
Stage 3	20 (17.7%)
Stage 4	7 (6.2%)
Stage 5	19 (16.8%)
Age when diagnosed with cancer	40 (8.1)
Type of heath insurance when diagnosed with cancer	
No insurance	3 (2.7%)
Rural cooperative medical insurance	25 (22.1%)
Social insurance	54 (47.8%)
Social insurance and commercial insurance	25 (22.1%)
Others (for example, commercial insurance only)	6 (5.3%)
Type of cancer treatments the patient is receiving	
None	0 (0%)
Surgery	60 (53.1%)
Radiation therapy	38 (33.6%)
Chemotherapy	83 (73.5%)
Immunotherapy to treat cancer	13 (11.5%)
Others[2]	18 (15.9%)
Does the patient have family or friends who have had cancer	
Yes	4 (3.5%)
No	109 (96.5%)

[1] If precents don't equal 100% it is because of rounding.
[2] Others included endocrine therapy, targeted therapy, and traditional Chinese medicine

Table 5.2: Survey Questions and Responses Regarding Patients' Attitude Towards Oncofertility

Survey Question	Response		
	Yes n (%)	No n (%)	Uncertain n (%)
Do you plan to or want to be pregnant after cancer?	10 (8.9%)	100 (88.5%)	3 (2.7%)
Do you take family members' opinions into account when deciding your family planning?	93 (82.3%)	11 (9.7%)	9 (8.0%)
Do you feel pressure from society to get pregnant in the future?	61 (54.0%)	39 (34.5%)	13 (11.5%)
Do you think medical insurance will cover the cost of fertility preservation?	17 (15.0%)	34 (30.1%)	62 (54.9%)
Would you like to choose the lower infertility-damage cancer treatment regimen even if it has a lower survival rate?	15 (13.3%)	61 (54.0%)	37 (32.7%)
Do you think it is necessary for oncologists to be trained on the reproductive and fertility outcomes related to cancer treatment?	92 (81.4%)	3 (2.7%)	18 (15.9%)
Do you think it is necessary for oncologists to talk to cancer patients about fertility preservation?	104 (92.0%)	9 (8.0%)	
Is it necessary to provide psychological counseling to cancer patients who have a risk of infertility before or during cancer treatment?	103 (91.1%)	2 (1.8%)	8 (7.1%)
Do you feel like you have sufficient information about fertility preservation?	17 (15.0%)	41 (36.3%)	55 (48.7%)
I think everyone can get enough information about fertility preservation	57 (50.4%)	54 (47.8%)	2 (1.8%)

Table 5.3: Survey Questions and Responses Regarding Patients' General Understanding of Oncofertility and Chinese Law

Survey Question	Response		
	Yes n (%)	No n (%)	Uncertain n (%)
Have you heard that cancer therapy such as chemotherapy and radiation can impair your ovary and uterus function and increase the risk of premature ovarian failure, infertility, or premature menopause?	72 (63.7%)	18 (15.9%)	23 (20.4%)
Do you know that the Chinese law stipulates that young women including young women with cancer, do not need to be married to freeze eggs or ovarian tissue, but women must be married to freeze embryos?	15 (13.3%)	64 (56.6%)	34 (30.1%)
Do you think Chinese law allows young cancer patients who are infertile due to cancer treatment to choose surrogacy?	16 (14.2%)	40 (35.4%)	57 (50.4%)

Table 5.4: Survey Questions and Responses Regarding Patients' Knowledge of Oncofertility

Survey Question	**Response** **Correct Answer**	**Total Sample** **Answered Correctly n (%)**	**< 40 years old** **Answered Correctly n (%)**	**≥40 years old** **Answered Correctly n (%)**	***p*-value**
All fertility preservation methods have consistent success rates in preserving fertility	False	66 (58.4%)	23 (71.9%)	43 (53.1%)	0.07
Fertility preservation methods are the same before and after cancer treatment	False	87 (77.0%)	28 (87.5%)	59 (72.8%)	0.10
The use of fertility preservation methods increases the risk of future cancer recurrence	False	76 (67.3%)	26 (81.3%)	50 (61.7%)	0.046
Freezing eggs and freezing embryos have the same chance of success for future pregnancies	False	44 (38.9%)	18 (56.3%)	26 (32.1%)	0.02
A woman who freezes her eggs will be able to use them whenever she needs them in the future	True	75 (66.4%)	17 (53.1%)	58 (71.6%)	0.06
Freezing eggs or embryos can guarantee future pregnancies	False	12 (10.6%)	4 (12.5%)	8 (9.9%)	0.74
Chemotherapy increases the risk of birth defects in children	False	29 (25.7%)	6 (18.8%)	23 (28.4%)	0.29
TOTAL Mean (SD) Knowledge Score		3.44 (1.20)	3.81 (1.03)	3.30 (1.24)	0.04

Table 5.5: The Order and Timing of Which Patients Received their Fertility Preservation Counseling, Treatment, and Cancer Treatment.

Service	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Fertility Preservation Consultation					
Order Received	Last	.	First	First	First
Time from cancer diagnosis to service	>3weeks	.	1-2days	3-5days	1-2days
Fertility Preservation Treatment					
Order Received	Second	.	Last	Last	Second
Time from cancer diagnosis to service	>3weeks	.	1-2days	>3weeks	Unsure
Cancer Treatment					
Order Received	First	First	Second	Second	Last
Time from cancer diagnosis to service	>3weeks	1-2days	1-2days	1week	Unsure
Final Treatment Received					
	Ovarian tissue cryopreservation	Unsure	Unsure	Unsure	Unsure

. is missing data

Table 5.6: Survey Questions and Responses Regarding Patients' Behavior Towards Oncofertility

Survey Question	Response in total sample			Participants Age <40			Participants Aged 40 and over			
	Yes n (%)	No n (%)	Uncertain n (%)	Yes n (%)	No n (%)	Uncertain n (%)	Yes n (%)	No n (%)	Uncertain n (%)	*p*-value
Have you ever asked your oncologist about the potential damage to fertility and fertility preservation caused by cancer treatment?	31 (27.4%)	82 (72.6%)	n/a	14 (43.8%)	18 (56.5%)	n/a	17 (21.0%)	64 (79.0%)	n/a	0.02
Have you been asked about your plans for future pregnancies and informed of the risk of infertility by oncologists?	46 (40.7%)	52 (46.0%)	15 (13.3%)	19 (59.4%)	12 (37.5%)	1 (3.13%)	27 (33.3%)	40 (49.4%)	14 (17.3%)	0.02
Were you provided psychological counseling about fertility concern from cancer treatment?	12 (10.6%)	87 (77.0%)	14 (12.4%)	4 (12.5%)	25 (78.1%)	3 (9.4%)	8 (9.9%)	62 (76.5%)	11 (13.6%)	0.82
Do you feel uncomfortable discussing fertility issues with your doctor?	8 (7.1%)	81 (71.7%)	24 (21.2%)	2 (6.25%	28 (87.5%)	2 (6.25%)	6 (7.4%)	53 (65.4%)	22 (27.2%)	0.04
Have you been referred by a doctor or nurse to an expert in fertility preservation?	5 (4.4%)	98 (86.7%)	10 (8.9%)	0 (0%)	31 (96.9%)	1 (3.1%)	5 (6.2%)	67 (82.7%	9 (11.1%)	0.17
Does your hospital provide fertility preservation services for female cancer patients?	29 (25.7%)	9 (8.0%)	75 (66.4%)	7 (21.9%)	3 (9.4%)	22 (68.8%)	22 (27.2%)	6 (7.4%)	53 (65.4%)	0.82
Have you received fertility preservation consultations from a variety of physicians, including gynecologists and oncologists?	3 (2.7%)	97 (85.8%)	13 (11.5%)	1 (3.1%)	26 (81.3%)	5 (15.6%)	2 (2.5%)	71 (87.7%)	8 (9.9%)	0.61

Did you proceed or are you proceeding with fertility preservation?	5 (4.4%)	108 (95.6%)	n/a	3 (9.4%)	29 (90.6%)	n/a	2 (2.5%)	79 (97.5%)	n/a	0.14
Do you have any regrets about not proceeding with fertility preservation?	9 (8.0%)	76 (67.3%)	23 (20.4%)	3 (9.4%)	19 (59.4%)	7 (21.9%)	6 (7.4%)	57 (70.4%)	16 (19.8%)	0.36

If percentages don't equal 100% it is because of rounding.

CHAPTER 6

CONCLUSIONS

This work aims to explore how environmental and chemical exposures impact women's reproductive health and overall wellbeing. To do this, we explore a variety of topics related to women's reproductive health including how environmental exposures can impact reproduction, methods for studying reproductive dysfunctions, fertility preservation among cancer patients, and the overall wellbeing of female cancer patients. With recent rises in infertility and reproductive diseases and cancers, reproductive toxicity is a public health concern. The scope of this work begins with an introduction and summary of female reproductive biology.

Following an understanding of normal reproductive function, Chapter 2 investigates the associations between blood concentrations of single of Pb, Cd, Hg, and their mixture and infertility and long-term amenorrhea in women of reproductive age in the United States from the National Health and Nutrition Examination Survey (NHANES) 2013-2018 cross-sectional survey. Overall, this study demonstrates that heavy metals may exhibit endocrine disrupting effects and heighten women' risks of infertility, and exposure to different heavy metals may cause differential female reproductive disorders. Future research is needed to confirm the roles of heavy metal exposure in women's reproductive disorders as well as the mechanisms involved.

Chapter 3 presents a literature review of recent advances and current research that uses bioengineering methods to study female reproductive diseases, including endometriosis and gynecologic cancers. Although many endeavors are required in future studies, the intersection of bioengineering and female reproductive biology and medicine provides great potential to advance the knowledge of female fertility, genetic vulnerability, medications, environmental exposures and toxicities, aging, nutrition, and diseases.

Another literature review is displayed in Chapter 4 to understand oocyte preservation options in oncofertility and discusses current guidelines and practices of female fertility preservation. Current fertility preservation options that are well established for reproductive aged female cancer and infertility patients include oocyte and embryo cryopreservation. Furthermore, recent advancements in reproductive science and medicine have allowed for investigational fertility preservation options for both childhood and reproductive-age patients, including ovarian tissue cryopreservation, *in vitro* oocyte maturation, ovarian transposition, ovarian suppression, and adjuvant therapy. Taken together, efficient and effective fertility preservation options are important to ensure long-term quality of life after cancer.

Lastly, Chapter 5 considers the overall wellbeing of female breast cancer patients in Sichuan, China by assessing their knowledge, attitude and behavior towards oncofertility via a questionnaire. In summary, results from the survey suggest that patients' view oncofertility as important and that providers should communicate more information to patients about how cancer treatment impacts

fertility and what options there are for fertility preservation. This study also displayed inadequate oncofertility knowledge among breast cancer patients in China.

In conclusion, findings from this research will advance the scientific understanding of women's reproductive health. Moreover, environmental pollution from anthropogenic factors and the distribution of toxic substances are a major public health concern. Advancing the scientific knowledge about the link between endocrine disrupting chemicals and women's reproductive health may play an essential role in the mitigation of adverse reproductive health outcomes.

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APPENDIX B

SUPPLIMENTAL TABLES

Table B.1: A Review of Previous Works that Used Hydrogels to Bioengineer Ovarian Functions

Species	Hydrogel	Ovarian tissue	Major reproductive outcomes	Year
Mouse	Collagen	Two-layered secondary follicles	Collagen-gel matrix maintained follicle 3D architecture and integrity and supported follicle development	1989[1]
Human	Agar	Preantral follicles	Human preantral follicles developed to antral stage	1989[2]
Hamster	Agar	Preantral follicles	FSH promoted follicle development and steroid hormone secretion	1996[3]
Mouse	Collagen	Multilayered secondary follicles	Collagen maintained follicle integrity and supported follicle development	1999[4]
Human	Collagen	Primary or early secondary follicles	Collagen maintained follicle integrity and follicles increased in size during 24 hours' culture	1999[5]
Sheep	Agar	Primary and secondary follicles	Secondary follicles exhibited better follicle reproductive outcomes than primary follicles	2000[6]
Human	Collagen	Preantral follicles	3D collagen culture supported human follicle growth	2001[7]
Pig	Collagen	Secondary follicles	Follicles developed to antral stage	2002[8]
Mouse	Alginate	Two-layered and multilayered secondary follicles	Follicles developed to antral stage and oocytes underwent meiosis	2003[9]
Mouse	Collagen	Early secondary follicles	3D ovarian follicle culture using collagen hydrogel promoted oocyte growth	2003[10]
Mouse	Alginate-collagen I matrix	Two-layered and multilayered secondary follicles	FSH (5 - 25 mIU/mL) does-dependently supported follicle development and FSH > 25 mIU/mL compromised follicle development	2005[11]
Mouse	Agar	Early secondary follicles	Agar hydrogel membrane enhanced follicle and oocyte reproductive outcomes	2005[12]

Mouse	Alginate	Multilayered secondary follicles	Follicle developed to antral stages and produced MII oocytes that can be fertilized and produced live birth	2006[13]
Mouse	Alginate	Two-layered secondary follicles	Decreasing matrix stiffness enhanced follicle development	2007[14]
Mouse	Matrigel	Early secondary follicles	Follicles developed to antral stage	2007[15]
Human	Alginate	Pre-antral follicles	Follicles from frozen–thawed tissues survived and developed	2009[16]
Mouse	Alginate	Multilayered secondary follicles	Follicle microenvironment controlled antral formation and steroidogenesis	2009[17]
Mouse	Alginate	Cryopreserved preantral follicles or ovaries	Ovary and follicle morphology are similar to fresh tissue; follicle development to antral stage and oocyte meiosis resumption	2009[18]
Rhesus monkeys	Alginate	Secondary follicles	Follicles survived and developed for 30 days and secreted steroid hormones	2009[19]
Bovine	Collagen	Primary follicles	3D collagen hydrogel culture supported bovine follicle development to antral stage	2009[20]
Human	Alginate	Secondary follicles	Follicles were steroidogenically active, developed to antral stage, and contained healthy oocytes	2009[21]
Mouse	FA (fibrin-alginate matrix)	Whole ovaries and secondary follicles	FA hydrogel produced higher MII rate and more oocytes formed two-cell embryos	2010[22]
Rat	Alginate	Preantral follicles	Enhanced follicle and oocyte reproductive outcomes using solid-surface vitrification method compared to open-pulled straws vitrification and slow-rate freezing:	2010[23]
Macaque	Alginate	Secondary follicles	Alginate encapsulation supported macaque follicle growth and follicles from young adult animals produced more healthy oocytes	2010[24]
Mouse	Alginate	Secondary follicles	Difference of genes expression between in vivo follicles and in vitro follicle culture	2011[25]
Mouse	Fibrin-alginate interpenetrating network (FA-IPN)	Secondary follicles	Follicle developed and matured, ovulating MII oocytes; follicles degraded fibrin component of the FA-IPN during the in vitro culture period; the dynamic mechanical environment mimics the natural ovarian environment	2011[26]
Dog	Alginate	Preantral follicles	Follicles retained structural integrity, grew in size and were hormonally active	2011[27]
Mouse	PEG hydrogel	Multi-layered secondary follicles	The network formation of hydrogel was improved and supported follicle development	2011[28]

Rhesus monkey	Alginate	Multi-layered secondary follicles	Alginate encapsulation supported follicle development to antral stage and produced MII oocytes	2011[29]
Baboon	Fibrin-alginate-matrigel matrix (FAM)	Preantral follicles	FAM supported follicle growth to antral stage and generated MII oocytes	2011[30]
Mouse	Alginate	Co-culture of primary/early secondary follicles with embryonic fibroblasts (MEFs)	Co-culture group developed antral cavities, resulting in 72% - 80% GVBD and 41% - 69% MII oocyte.	2012[31]
Mouse	Hyaluronan (HA) or ECM-HA hydrogel	Secondary follicles	ECM-HA produced better follicle reproductive and oocyte outcome compared to HA only	2012[32]
Rhesus monkey	Alginate	Ovarian cortex	More rigid alginate encapsulation maintained primordial survival	2012[33]
Human	Alginate	Primordial/primary follicles	The use of DMSO ethylene glycol (EG) as cryoprotectants to cryopreserve follicles using slow-freezing method; DMSO group showed better follicle reproductive outcomes than EG	2013[34]
Macaque	Fibrin-alginate	Primary and secondary follicles	Fibrin further promoted follicle development, MII oocytes were produced and developed to morula stage after IVF	2013[35]
Mouse	Alginate	Primary follicles	Multiple primary follicle culture supported follicle development and oocyte maturation	2013[36]
Mouse	Alginate	Ovarian surface epithelium (OSE)	Supplement of insulin and IGF-I induced OSE hyperplasia and proliferation and decreased follicular integrity through upregulation of the PI3-kinase pathway	2013[37]
Human	Alginate	Primordial follicles	More atretic follicles in samples cultured within alginate than PEG- fibrinogen hydrogels;	2013[38]
Mouse	Alginate	Primary and early secondary follicles	Co-culture with mouse embryonic fibroblasts (MEF) enhanced follicle reproductive outcomes	2013[39]
Mouse	Collagen	Primary and early secondary follicles	A multiple-step follicle culture based on collagen hydrogels produced oocytes that were able to generate live birth	2013[40]
Caprines	Alginate	Preantral follicles	0.25% alginate produced the best follicle and oocyte reproductive outcomes	2014[41]

Human	Alginate	Primordial follicles; human ovarian cortex	Human ovarian tissue could be kept at 4 °C for up to 24 h while still maintaining follicle viability; primordial follicles isolated from ovarian tissue did not survive in vitro; encapsulation of ovarian cortical pieces supported the survival, activation,and growth to primary stage	2014[42]
Mouse	Alginate	Early secondary follicles	Improved growth and survival of early secondary follicles cultured in a hypoxic environment	2014[43]
Mouse	Alginate	Primordial follicles	The transplanted follicles encapsulated within alginate were able to mature and restore ovarian function in ovariectomized mice	2014[44]
Mouse	Alginate	Primordial, primary and secondary follicles	Ascorbic acid enhanced primary follicle survival	2014[45]
Hyline chickens	Alginate	Primordial follicles	Follicular recovery and survival were different among different enzymes and methods used; Alginate encapsulation produced the maximal follicle survival rate	2015[46]
Mouse	Matrigel	14-day ovarian pieces	Matrigel supported follicle development oocyte maturation; activin A enhanced follicle culture success; oocytes were competent to generate live birth	2015[47]
Mouse	Alginate	Multi-layered secondary follicles	Follicles size as a non-invasive marker to determine oocyte quality	2015[48]
Mouse, human	Alginate	Multi-layered secondary follicles	eIVFG of human follicles supported an entire ovarian cycle	2015[49]
Human	Alginate	Multilayer secondary follicles	Human follicle development oocyte meiotic maturation using a two-step culture method	2015[50]
Mouse	Fibrin-alginate inetwork	Two-layered secondary follicles	Fibrin degradation positively correlated with *in vitro* folliculogenesis; doxorubicin exhibited ovarian toxicities	2015[51]
Mouse	Poly(ethylene) glycol (PEG)-hydrogel	Early secondary and preantral follicles	Hydrogel stiffness impacted follicle and oocyte reproductive outcomes	2015[52]
Goats	Alginate, fibrin–alginate (FA)	Multilayered secondary follicles	FA enhanced follicle and oocyte reproductive outcomes	2016[53]
Rat	Collagen	Secondary follicle	3% collagen produced the best follicle development and survival outcomes	2016[54]
Human	Alginate	Pre-antral follicles	Follicle development and similar secretion levels of estradiol and testosterone on chip compared to culture dish.	2017[55]

Mouse	Alginate	Multi-layered secondary follicles	Encapsulated in vitro follicle growth determined the ovarian toxicity of doxorubicin	2017[56]
Deer mouse	Collagen core in alginate shell	Preantral follicle	Preantral follicles developed to the antral stage, releasing cumulus - oocyte complex	2017[57]
Bovine	Alginate	Preantral follicles	No differences were observed between follicle viability and morphology between short-term 2D and 3D culture systems	2018[58]
Mouse	Alginate	Primary follicles	The differential synergism and correlation of transcription factors activity and secretome during folliculogenesis within group culture were analyzed.	2018[59]
Mouse	Fibrin alginate interpenetrating network (FA-IPN)	Secondary follicles	Follicle exposure to mixtures of lindane and 7,12-dimenthylbenz(a)anthracene (DMBA) affected follicle survival, diameter and oocyte MII percentage	2018[60]
Mouse	Poly(ethylene) glycol (PEG)-hydrogel	Multilayered secondary follicles	PEG hydrogel supported co-culture of ovarian stromal cells and follicles and further enhanced follicle development	2018[61]
Dog	fibrinogen-alginate	Preantral, early antral and antral follicles	FSH improved follicle growth and antral cavity expansion; activin increased oocyte diameter and improved nuclear integrity	2019[62]
Rat	Alginate	Preantral follicle	differential survival, growth, and maturation of rat oocytes and hormone levels between 3D and 2D culture; using this model to evaluate the effect of bisphenol-A (BPA) on female reproductive toxicity	2019[63]
Mouse	Alginate	Preantral follicles	Chitosan hydrogels better supported follicle reproductive outcomes compared to alginate encapsulation	2020[64]
Mouse	Alginate	Preantral follicles	1% alginate compromised oocyte quality compared to 0.5% alginate and co-culture of follicles with interstitial cells increased follicle and oocyte reproductive outcomes	2020[65]
Mouse	Alginate	Secondary follicles	Follicles cultured in extracellular matrix-derived soft hydrogel enhanced follicle and oocyte reproductive outcomes	2020[66]
Mouse	Alginate	Multilayered secondary follicles	Vitrification allowed for a high-content ovarian follicle biobank for ovarian toxicity screening	2020[67]

*These papers represent the majority but not all of the previous works done in the field of using hydrogel for in vitro follicle growth. Our apologies to colleagues not listed here due to journal space constraints.

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APPENDIX C

ONCOFERTILITY SURVEY

Dear patient,

The Sichuan Academy of Medical Sciences – Sichuan Provincial People's Hospital (SAMSPH) is collaborating with Rutgers University and the University of South Carolina, hoping to learn about your perception, attitude and behavior regarding the preservation of fertility in young female cancer patients. If you decide to participate, you will be asked to fill out a brief survey that will take about 15 minutes to complete.

In particular, you will be asked questions about your attitude and knowledge regarding fertility preservation. You may feel uncomfortable answering some of the questions. You do not have to answer any questions that you do not wish to answer.

Participation is confidential. Study information will be kept in a secure location at The Sichuan Academy of Medical Sciences. The results of the study may be published or presented at professional meetings, but your identity will not be revealed.

We will be happy to answer any questions you have about the study. Thank you for your consideration. If you would like to participate, please email Shuo Xiao at sx106@pharmacy.rutgers.edu

With kind regards,
Shuo Xiao
sx106@pharmacy.rutgers.edu

1. What is your age?

2. What is your Ethnicity?

3. What is your highest level of completed education?
   A. ≤High school degree
   B. High school degree

C. Bachelor's degree
D. Master's degree
E. Doctoral degree

4. What is your marital status?
A. Unmarried
B. Married
C. Divorce
D. Widowed

5. How many children do you have?
A 0.
B 1.
C 2.
D ≥3

6. What type of cancer do you have?

_____

7. What stage is your cancer?
0（carcinoma in situ）
I
II
III
IV

8. How old were you when you were diagnosed with cancer?

_____

9. Did you have health insurance when you were diagnosed with cancer? If yes, what kind?
A. None
B. The new rural cooperative medical insurance
C. Social insurance
D. Commercial insurance and social insurance
E. Other cases（e.g., Only have commercial insurance)

10. What cancer treatments have you received or are currently receiving?
    A. Surgery
    B. Radiation therapy
    C. Drug therapy（including Chemotherapy）
    D. Immunotherapy to Treat Cancer
    E. Other cases

11. Do you plan to or want to be pregnant after being diagnosed with cancer?
    Yes
    No
    Uncertain

12. Do you feel pressure from other family members to get pregnant in the future?
    Yes
    No
    Uncertain

13. Do you feel pressure from society to get pregnant in the future?
    Yes
    No
    Uncertain

14. Have you heard that cancer therapy such as chemotherapy and radiation can impair your ovary and uterus function and may increase the risk of infertility or early menopause?
    Yes
    No
    Uncertain

15. Have you heard about fertility preservation?
    A. Familiar
    B. Know some
    C. Know a little
    D. Not known

16. Do you know any methods for fertility preservation?
    A. Know some
    B. Know a little
    C. Not known

[*if yes*] What kind do you know?

_____

17. Do you think medical insurance can reimburse the cost of fertility preservation?

Yes
No
Uncertain

18. If given the option, I would choose the lower infertility-damage cancer treatment regimen even if it has a lower survival rate.

Yes
No
Uncertain

19. Do you know that Chinese law stipulates young women, including young women with cancer, must be married before they can accept the fertility preservation methods of frozen eggs or frozen embryos?

Yes
No
Uncertain

20. Do you think that Chinese law allows young cancer patients who are infertile due to cancer treatment to choose surrogacy?

Yes
No
Uncertain

21. Do you think it is necessary for oncologists to be trained on the reproductive and fertility outcomes related to cancer treatment?

Yes
No
Uncertain

22. Do you think it is necessary for oncologists to inform patients about the damage that cancer treatment may cause to fertility before cancer treatment?

Yes
No
Uncertain

23. Is it important to provide psychological counseling to cancer patients who have a risk of infertility before or during cancer treatment?

Yes
No
Uncertain

24. Have you ever asked the oncologist about the possible damage to fertility and the preservation of fertility from cancer treatment?

Yes
No

25. Have oncologists asked about your future reproductive plans and told you that cancer treatment may have side effects on fertility?

Yes
No
Uncertain

26. Have you received psychological counseling about cancer treatment affecting fertility?

Yes
No
Uncertain

27. I feel uncomfortable discussing infertility issues with my doctor(s）

Yes
No
Uncertain

28. My doctor or nurse referred me to a fertility specialist.

Yes
No
Uncertain

29. Does your hospital provide fertility preservation services for female cancer patients or do you have to go to another facility?

Yes
No
Uncertain

30. Have you received consultations on fertility preservation provided by doctors such as obstetricians and gynecologists and oncologists?

Yes
No
Uncertain

31. Have you received fertility preservation consultation?

Yes
No

a. [*if yes*] What was your reason for seeking fertility preservation consultation

A. Knowing the options
B. Making an informed decision
C. Hope to be a mother
D. Avoid future regrets

b. [*if yes*] Time from cancer diagnosis to fertility preservation consultation

A. 1-2 days
B. 3-5 days
C. 1 week
D. 2 weeks
E. >3 weeks
F. unsure

c. [*if yes*] I was able to see a fertility preservation specialist before I started cancer treatment.

Yes
No

d. [*if yes*] What types of fertility preservation were offered to you?

A. Egg cryopreservation
B. Sperm cryopreservation
C. Embryo cryopreservation
D. Ovarian tissue cryopreservation
E. Testicular tissue cryopreservation
F. Surrogacy
G. Unsure

32. Did you proceed with fertility preservation?
    Yes
    No
    Uncertain

a. [*if yes*] What is the timeline between fertility preservation consultation, fertility preservation treatment, and cancer treatment? How long did each go through? (The investigator needs to record the timeline in detail and fill in the corresponding duration)
    A. 1-2 days
    B. 3-5 days
    C. 1 week
    D. 2 weeks
    E. >3 weeks
    F. Unsure

b. [*if yes*] What type of fertility preservation will you be using?
    A. Egg cryopreservation
    B. Sperm cryopreservation
    C. Embryo cryopreservation
    D. Ovarian tissue cryopreservation
    E. Testicular tissue cryopreservation
    F. Surrogacy
    G. I don’t know

c. [*if no*] What was your reason for not proceeding with fertility preservation?
    A. Don't know that fertility can be preserved before cancer treatment
    B. Worried about delays in cancer treatment
    C. Does not meet the physical and mental conditions of fertility preservation treatment
    D. The cost of fertility preservation is too high

d. [*if no*] Do you have any regrets about not proceeding with fertility preservation?
    Yes
    No
    Uncertain

33. Do you feel like you have sufficient information about fertility preservation?
    Yes
    No

34. I feel that fertility preservation educational materials are available and accessible to everyone

- Strongly Agree
- Agree
- Undecided
- Disagree
- Strongly Disagree

35. All fertility preservation treatments have similar success rates at achieving pregnancy

- True
- False

36. Fertility preservation methods are the same before and after cancer treatment

- True
- False

37. Women who utilize fertility preservation methods increase their risk of cancer recurrence in the future

- True
- False

38. Egg freezing and embryo freezing have the same chances of future pregnancy

- True
- False

39. A woman who freezes her eggs will have access to them whenever she is ready to use them in the future

- True
- False

40. Future pregnancy is guaranteed with frozen eggs and embryos

- True
- False

41. The risk of birth defects in future children increases with chemotherapy

treatments

True

False

42. Who has supported you the most in your decisions about your fertility? (e.g., family, specific doctors, resources, services)

43. What information, services, or resources on fertility do you really need, but not have?

44. Do you have any suggestions for how to improve fertility counselling for cancer patients in the future?

45. What else would you like to share about your experience with fertility and cancer?