SECONDHAND TOBACCO SMOKE EXPOSURE AND ITS EFFECTS AMONG WOMEN UNDERGOING IN VITRO FERTILIZATION

by

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ABSTRACT

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Infertility and early pregnancy loss are prevalent in the United States and worldwide, and a large proportion of non-tobacco users are exposed to secondhand tobacco smoke (STS). While the effects of active smoking on these endpoints have been well-documented, studies on STS exposure remain limited. In one of the only previous studies of early pregnancy that utilized exposure biomarkers of STS exposure, no relationship was observed between urinary cotinine and *in vitro* fertilization (IVF) outcomes. However, we hypothesize that urine may not be the most relevant sample media within which to measure biomarkers for a study of early pregnancy, and that the lack of an association may have been due to a large degree exposure measurement error. In the first chapter of this dissertation we report a weak rank-order relationship (Spearman r <0.2) and poor exposure category agreement between cotinine concentrations measured in urine and the

potentially more biologically-relevant follicular fluid (FF) among 255 women undergoing IVF. We observed fair reliability (ICC = 0.42-0.52) in FF cotinine concentrations from 415 women undergoing multiple IVF cycles. In the second chapter, cotinine was measured in FF collected during 3,270 IVF cycles from 1,909 nonsmoking women to investigate the relationship between STS exposure and failed embryo implantation. We observed a significant increase in the risk of implantation failure among women exposed to STS compared to those who were unexposed (Odds Ratio [OR] = 1.52; 95% confidence interval [CI] = 1.20-1.92; Risk Ratio [RR] = 1.17; 95% CI = 1.10-1.25). We also found a significant decrease in the odds for a livebirth among STS-exposed women (OR = 0.75; 95% CI = 0.57-0.99; RR = 0.81; 95% CI = 0.66-0.99). In the third chapter, we explored the relationship between STS exposure and serum levels of prolactin and TSH measured at baseline among 337 of the women in the study. STS exposure was associated with a significant increase in circulating prolactin (p = 0.03) but was not associated with serum TSH. The findings reported here are likely of great public health significance due to continued widespread STS exposure, the rapidly increasing number of couples utilizing IVF, and the potential relationship between elevated prolactin concentrations and a range of potential downstream adverse health effects. In addition, FF cotinine concentrations, if available, may be desired as a biomarker of low-level tobacco smoke exposure over urinary cotinine in studies of early reproduction. Finally, due to only moderate temporal reliability, cotinine measured in FF samples collected at each IVF cycle may be needed to most accurately represent STS exposure in studies utilizing outcome data from multiple cycles.

CHAPTER I

Introduction

Infertility and early pregnancy loss are prevalent in the US and worldwide. The CDC reported that, in 2002, 7.4% of all married women 15-44 years of age were infertile (i.e. not surgically sterile, have not used contraceptives in the past 12 months, and have not become pregnant; Chandra et al., 2005). 11.8% of these women had impaired fecundity (i.e. the ability to become pregnant and carry a child full term), an increase of about 2% from the levels seen in 1988 and 1995. The World Health Organization estimates that infertility affects up to 15% of reproductive-aged couples worldwide, and in some countries, more than 30% of women aged 25–49 suffer from secondary infertility, the failure to conceive after the first pregnancy (Cui, 2010).

The causes of infertility and adverse pregnancy outcomes remain poorly understood. Female infertility may result from anovulation, oligoovulation, failed fallopian transport of the oocyte, failed fertilization, or from the loss of a fertilized embryo before implantation. It has been estimated that only 50-60% of all conceptions advance beyond 20 weeks of gestation (Wilcox et al., 1988) and up to 75% of lost pregnancies are the result of blastocyst implantation failure which are never clinically recognized as pregnancies (Norwitz et al., 2001). Thus, these early losses often manifest clinically as

female infertility. The ability of a blastocyst to implant in the uterine wall may be associated with uterine receptivity, oocyte quality, or delayed implantation, though oocyte quality is likely the most important factor (Norwitz et al., 2001). Much of the difficulty in studying the causes of these adverse outcomes lies in our limited ability to observe the early stages in the reproductive process in the general population.

While active smoking has been known to be harmful to pregnancy for years, there is growing concern about adverse reproductive health effects resulting from secondhand tobacco smoke (STS) exposure. Exposure to STS, sometimes referred to as environmental tobacco smoke, is dangerous and prevalent worldwide. STS is a mixture of more than 4000 chemicals, over 60 of which are known or suspected carcinogens or reproductive toxicants (Lindbohm et al., 2002). For example, STS contains carbon monoxide, cadmium, lead, benzene, nicotine, formaldehyde, and radioactive polonium-210, to name just a few.

The majority of STS is in the form of sidestream smoke, which is generated from the burning end of a lighted cigarette when not actively smoked. Mainstream smoke is the smoke inhaled by individuals who are actively smoking. Thus, the remainder of STS is exhaled mainstream smoke. Mainstream and sidestream smoke both contain thousands of chemicals, many of which are harmful to humans. However, the proportion of these compounds differs between mainstream and sidestream smoke because each type of smoke is produced at different temperatures and oxygen concentrations. For example, sidestream smoke contains higher concentrations of combustion products formed by

amination and nitrosation than mainstream smoke (Woodward & al-Delaimy, 1999). Sidestream smoke also contains more carbon monoxide and, therefore, less carbon dioxide than mainstream smoke.

Exposure to STS frequently occurs at home, in automobiles, and, in states with less stringent anti-smoking laws, at work and in other public places such as bars and restaurants. Despite a significant reduction in the proportion of the general population exposed to STS, exposure remains prevalent. According to a recent report, during 2007-2008, approximately 88 million nonsmokers in the US aged three and older were exposed to STS based on an objective exposure measure (Centers for Disease Control and Prevention, 2010). An older study estimated that 87.9% of non-tobacco users in the US were exposed to STS (Pirkle et al., 1996). Yet, the same study found that only 33% of all women in the US reported that they were exposed to STS, indicating that many are likely unaware of, or underreport, their exposure. Due to the persistence of widespread STS exposure, even minor associations between exposure and fertility and pregnancy outcomes may have a significant impact on public health.

STS exposure can be estimated through self-report, environmental monitoring, or measuring the constituents of tobacco smoke or their metabolites in biological matrices (i.e. biomarkers of STS exposure). Some biomarkers used in the past, however, are not specific to tobacco smoke exposure, such as exhaled carbon monoxide and polycyclic aromatic hydrocarbon metabolite and thiocyanate measures in urine, blood, or saliva (Benowitz et al., 2009a). Cotinine, the major proximate metabolite of nicotine, is specific

to tobacco smoke and is the most widely used biomarker of exposure because of its sensitivity and relatively long half-life (~17 hours) in body fluids compared to nicotine (~2 hours; Benowitz, 1999; Benowitz et al., 1983). Cotinine can be measured in urine, serum, saliva, hair, nails, and other biological samples, and its measurement reduces errors and biases inherent to self-reported exposure.

Nicotine is rapidly absorbed into the blood stream once tobacco smoke reaches the alveoli. For example, nicotine reaches the brain in 10–20 seconds after a puff of a cigarette (Benowitz et al., 2009a). Once in the bloodstream, nicotine is distributed widely to body tissues. The liver, kidney, spleen, and lungs seem to have the highest affinity, and adipose tissue the lowest, for this chemical based on human autopsy samples from smokers (Benowitz et al., 2009a). Nicotine accumulates in some tissues due to the increased presence of nicotinic cholinergic receptors (i.e. ligand-gated ion channels in the plasma membrane) and in other tissues due to passive ion trapping (Perry et al., 1999; Breese et al., 1997; Lindell et al., 1996).

The liver is responsible for nicotine metabolism, and six primary metabolites have been identified (Benowitz et al., 2009a). From a quantitative standpoint, cotinine is the most important of these in mammals. Approximately 75% of nicotine is metabolized into cotinine in humans. The first step of this transformation is primarily mediated by the CYP2A6 enzyme, which produces the intermediate nicotine-Δ1'(5')-iminium ion. This intermediate is then catalyzed by cytoplasmic aldehyde oxidase to form cotinine (Benowitz et al., 2009a).

Despite the large proportion of nicotine that is metabolized into cotinine in humans, only 10-15% of nicotine that is absorbed by smokers is excreted in urine as unchanged cotinine (Benowitz et al., 1994). This is a minor route of elimination because, after glomerular filtration, cotinine is extensively reabsorbed into the blood stream, likely due to the absence of cotinine-protein binding. Unexcreted cotinine is further metabolized into a number of other chemicals, each with varying rates of renal clearance (Benowitz et al., 2009a). Of these, *trans*-3'-hydroxycotinine is the main metabolite. This, along with one other cotinine metabolite, cotinine glucuronide, accounts for 40-60% of the nicotine dose that is excreted in urine (Benowitz et al., 1994; Byrd et al., 1992). Because such a small proportion of absorbed nicotine is excreted in urine as unchanged cotinine, the measurement of cotinine in other matrices (e.g. serum), although potentially more invasive, may provide more accurate biomarkers of tobacco smoke exposure than cotinine measures in urine.

The increasing use of assisted reproductive technologies (ART), particularly *in vitro* fertilization (IVF), has improved our ability to study contributors to infertility and early pregnancy loss by allowing the observation of early and discrete stages in the reproduction process. Follicular fluid (FF), the fluid surrounding the preovulatory oocyte, is routinely collected during ART but is seldom used despite its superior biological relevance in studies of early pregnancy as a matrix within which to measure markers of exposure to STS or other environmental agents. Cotinine levels in FF reflect

a developing oocyte's direct exposure to constituents of tobacco smoke (i.e. it is a measure of dose at the target tissue).

FF provides a very important microenvironment in which the oocyte matures and granulosa cells differentiate (Fahiminiya & Grard, 2010; Fortune et al., 2004). Though some of its functions remain unknown, FF likely facilitates the oocyte's escape from the follicle and its transport to the oviduct (Edwards, 1974). It may also provide a favorable environment around the oocyte for fertilization (Hong et al., 1993). Chemicals in FF can interact directly with the cumulus-oocyte complex, and chemicals present in FF, the oocyte, and/or the preimplantation blastocyst may lead to adverse effects on fertilization and implantation (Fabro, 1978).

FF is composed of constituents of blood plasma that cross the blood-follicle barrier as well as secretions from granulosa and thecal cells (Fortune, 1994). Urine, on the other hand, is composed of water and the byproducts of cellular metabolism, including urea, chloride, sodium and creatinine. Since the ovarian follicle does not have a direct blood supply, in order for cotinine to enter FF it must diffuse through interstitial fluid or be transported through thecal and granulosa cells which surround the antrum and oocyte (Fabro, 1978).

Gap junctions (nonspecific pores between cells) are likely involved in the passive transport of cotinine into FF. These junctions can pass molecules between cells up to 1000 Da in molecular mass (Weber et al., 2004). Cotinine has a molecular mass of only

176.2 Da, enabling it to move relatively freely through gap junctions. Thus, cotinine concentrations in urine are measures of plasma cotinine not reabsorbed during glomerular filtration; while cotinine measures in FF represent plasma cotinine that diffused through the blood-follicle barrier and largely depend upon concentration gradients between the blood, interstitial fluid, and cells surrounding the oocyte.

Cotinine was first measured in FF from smokers in 1989 (Weiss & Eckert, 1989), and in 1996, Zenzes et al. were the first to detect cotinine in the FF of passive smokers (i.e. STS exposed nonsmokers). Cadmium, another constituent of tobacco smoke, has also been measured in the FF of both active and passive smokers (Zenzes et al., 1995). These findings suggest that tobacco smoke exposure may allow toxic compounds to interact directly with cells of the follicle and the developing oocyte. Zenzes et al. (1996) reported that FF cotinine concentrations followed a dose-dependent pattern when comparing self-reported active and passive smokers demonstrating that FF cotinine could be a feasible and relevant biomarker of exposure in an epidemiological study among women undergoing ART treatments.

In addition to the findings of Zenzes et al., a prospective cohort study in Chile reported a strong, positive correlation ($r = 0.95$) between serum and FF cotinine levels among women who had recently actively smoked (Fuentes et al., 2010). This concurs with an earlier report that no significant gradient exists between cotinine in blood serum and FF (Paszkowski, 1998), indicating that cotinine easily crosses the blood/follicle barrier.

However, both of these studies included a large proportion of active smokers, which may have strengthened the observed cotinine correlation between matrices.

Only one study has examined inter-fluid cotinine agreement among those exposed to low levels of tobacco smoke. After dosing subjects with nicotine intended to simulate STS exposure, Benowitz et al. (2009b) reported a strong correlation ($R^2 = 0.84$) between cotinine in blood plasma and creatinine-adjusted urine. No previous studies have assessed the agreement between urinary and FF cotinine or FF and plasma cotinine among passively exposed women.

In addition, little is known about the ability of cotinine to estimate long-term STS exposure. Since the half-life of cotinine in body fluids is approximately 17 hours, it likely only reflects exposure over the past 3-4 days (Benowitz et al., 2009a). This depends, however, on intra-individual variability in STS exposure and cotinine concentrations over time. A study examining within-subject variation of urinary cotinine in young children with smoking parents, reported that a single urine sample yielded highly accurate estimates of recent exposure only (i.e. 2-3 days) and up to 12 urine samples may be needed to obtain similar precision in estimates of STS exposure over a 4-13 month period (Matt et al., 2007). To our knowledge, no studies to date have examined the intra-individual variability of FF cotinine over time.

Between-person variability of FF cotinine is also important to consider. High between-subject variation in FF cotinine within smoking groups and within women who reported

smoking the same number of cigarettes was previously demonstrated (Zenzes et al., 1996). This may reflect differing toxicant metabolism, distribution, and excretion between individuals (Swan et al., 2005) or may simply serve as further evidence of the limitations in the precision of self-reported exposure.

The effects of active smoking on fertility and early pregnancy are very well established and have been recently reviewed (Cooper & Moley, 2008). Less is known, however, about the relationship between STS exposure and reproductive health, and most research is focused on birth outcomes (e.g. measures of fetal growth, preterm birth) as opposed to early pregnancy (Lindbohm et al., 2002). In addition, in much of the literature, self-report is the only means of estimating STS exposure (Florescu et al., 2009), likely leading to exposure misclassification and biased risk estimates.

A recent study relying on self-reported STS exposure among approximately 4,800 women found that those exposed to STS had greater difficulty becoming pregnant and experienced increased fetal loss compared to those unexposed (Peppone et al., 2009). Self-reported exposure to STS has been linked to many other adverse reproductive outcomes, including altered menstrual function (Chen et al., 2005; Chen et al., 2000), early menopause (Cooper et al., 1995; Everson et al., 1986), delayed conception (Hull et al., 2000), early pregnancy loss (Venners et al., 2004), first-trimester fetal loss (Ahlborg & Bodin, 1991), spontaneous abortion (Windham et al., 1992), reduced mean birthweight (Ward et al., 2007), early preterm birth (gestational age < 35 weeks; Fantuzzi et al., 2007), and preterm birth (gestational age < 37 weeks; Goel et al., 2004). A study among

225 women undergoing IVF or ICSI (intra cytoplasmic sperm injection) found significant decreases in implantation and pregnancy rates among both active and passive smokers compared to non-smokers (Neal et al., 2005). The decreases in pregnancy and implantation rates were strikingly similar between active and passive smokers suggesting that exposure to STS may be just as detrimental to fertility and early pregnancy as active smoking. However, these findings were also based on self-reported exposure.

Several studies have used biomarkers to assess birth outcomes associated with maternal STS exposure, and these have been recently reviewed (Leonardi-Bee et al., 2008). However, as far as we are aware, only three studies exist that used a biomarker of exposure to assess fertility or early pregnancy outcomes among STS exposed women, and their results have been inconsistent. One such study used FF cotinine as a measure of STS exposure but found no significant difference in fertilization or pregnancy rates between active, passive, and non-smokers in a small cohort of IVF patients (n = 197) (Sterzik et al., 1996). On the other hand, a case-control study in Sweden found increased odds of spontaneous abortion in STS exposed women versus unexposed women based on plasma cotinine levels (George et al., 2006).

In a study conducted using creatinine-adjusted urinary cotinine to estimate exposure, Meeker et al. (2007a) found no association between female STS exposure and failed fertilization, failed implantation or spontaneous abortion, nor was there evidence of a dose-response relationship. A follow-up study with 2,162 ART patients, however, established suggestive evidence for an increased risk of failed implantation from current,

self-reported STS exposure (Meeker et al., 2007b). Overall, studies of the effects of STS on fertility and early pregnancy have had differing, but suggestive results underscoring the need for additional research using biologically relevant markers of exposure.

The present work leveraged data collected in a previous study of predictors of IVF success (Cramer et al., 2003). The studies by Meeker et al. (2007a & 2007b) referenced above also relied on data from this cohort. Because of the null findings by Meeker et al. (2007a) when relying on urinary cotinine to estimate STS exposure and their subsequent positive findings when relying on self-reported exposure (Meeker et al., 2007b), the present work reexamined the relationship between STS exposure (this time estimated through FF cotinine) and early pregnancy difficulties among these women. We hypothesized that urinary cotinine concentrations agree poorly with FF cotinine concentrations among STS exposed subjects and that the null findings of Meeker et al. (2007a) may have been due to a high degree of exposure misclassification. We also hypothesized that early pregnancy (e.g. implantation) is adversely affected by STS exposure and that using biomarkers of exposure more proximate to the outcome of interest (e.g. FF cotinine) will enable us to more clearly observe the relationship between exposure and some of these early pregnancy outcomes.

Little is known about the potential mechanisms involved in infertility and early pregnancy loss due to tobacco smoke exposure. Prolactin (PRL) and thyroid stimulating hormone (TSH) may play mechanistic roles because both have important functions during early reproduction. Decreases in both maternal and fetal circulating TSH levels

have been observed among women who actively smoke during pregnancy (Shields et al., 2009; McDonald et al., 2008). STS exposure also disrupts the thyroid (Carrillo et al., 2009; Soldin et al., 2009; Flouris et al., 2008).

Tobacco smoke exposure is associated with changes in PRL concentrations, as well; though studies have had differing results. One study found a significant increase in PRL concentrations among men who were active smokers compared to nonsmokers (Xue et al., 2010). Two other studies reported increases and decreases, respectively, in PRL concentrations among animals exposed to tobacco smoke (Ng et al., 2006; Muraki et al., 1979). To our knowledge no human studies to date have examined the relationship between STS exposure and PRL concentrations.

The present work begins with an evaluation of the agreement between cotinine measures in FF and urine among STS-exposed women and the intra-subject variability of FF cotinine measures over time to determine how well a single FF cotinine measure estimates longer-term STS exposure. An examination of the relationship between implantation failure and IVF success (i.e. odds of a successful livebirth) and STS exposure is also presented. Finally, the relationship between STS exposure and circulating levels of TSH and PRL was examined.

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CHAPTER II

Cotinine Concentrations in Follicular Fluid as a Measure of Secondhand Tobacco Smoke Exposure in Women Undergoing *In Vitro* Fertilization: Inter-matrix Comparisons with Urine and Temporal Variability

Abstract

We examined the relationship between cotinine measures in follicular fluid (FF) and urine to inform our exposure assessment strategy for an ongoing epidemiological study of secondhand tobacco smoke (STS) exposure and early pregnancy loss. Among subjects undergoing *in vitro* fertilization (IVF), we compared cotinine levels in paired urine and FF samples from the same women and examined FF cotinine levels over time. We found a weak rank-order relationship (Spearman r <0.2) and poor agreement for classifying nonsmoking individuals as exposed to STS (sensitivity = 0.29-0.71; specificity = 0.35-0.72) between cotinine concentrations in FF and urine. We observed fair reliability (ICC = 0.42-0.52) in FF cotinine concentrations from women undergoing multiple IVF cycles. If available, FF cotinine concentrations may be desired as a biomarker of low-level tobacco smoke exposure over urinary cotinine in studies of early reproduction. Collection of multiple FF samples for cotinine analysis may be needed to accurately represent long-term STS exposure.

Introduction

Active smoking is associated with adverse effects on fertility and early pregnancy (Cooper and Moley, 2008). Recent studies have also suggested that secondhand tobacco smoke (STS) exposure has deleterious effects on early reproduction (Neal *et al.*, 2005; Peppone *et al.*, 2009), though these studies rely on self-reported exposure. Conversely, an earlier study using a biomarker of tobacco smoke exposure found no difference in fertilization or pregnancy rates between active, passive and nonsmokers (Sterzik *et al.*, 1996).

It is currently unclear which method of STS exposure assessment is most appropriate in studies of early pregnancy. Self-reported exposure is commonly used due to its convenience and economy, but is subject to recall bias and misclassification errors (George *et al.*, 2006). Objective markers of tobacco smoke exposure, such as nicotine and its metabolites, are also frequently measured in biological samples. Approximately 75% of nicotine is converted to cotinine in humans. Cotinine is a widely accepted biomarker because of its specificity and relatively long half-life in body fluids (~16 hours) compared to nicotine (~2 hours; Benowitz *et al.*, 2009a). However, a single measure of cotinine in fluids such as blood or urine may be limited because it reflects only recent exposure (3-4 days; Benowitz *et al.*, 2009a; Matt *et al.*, 2007).

Assisted reproduction technologies (ART) provide the opportunity to study the effects of environmental insults on stages of early reproduction otherwise not observable (e.g. fertilization, implantation). ART also facilitate the collection of follicular fluid (FF),

which surrounds the preovulatory oocyte. FF is composed of constituents of blood plasma that cross the blood-follicle barrier as well as secretions from granulosa and thecal cells (Fortune, 1994). FF provides a very important microenvironment in which the oocyte matures and granulosa cells differentiate (Fahiminiya and Gerard, 2010; Fortune *et al.*, 2004).

Since the ovarian follicle has no direct blood supply, in order for cotinine and other chemicals to enter FF they must diffuse through interstitial fluid and/or be transported through thecal and granulosa cells which surround the antrum and oocyte (Fabro, 1978). Gap junctions (nonspecific pores between cells) can transport molecules up to 1 kDa in molecular mass and, since cotinine has a molecular mass of only 176.2 Da, are likely involved in the passive transport of cotinine into FF (Weber *et al.*, 2004). Once within the FF, cotinine and other chemicals can interact directly with the cumulus-oocyte complex and may contribute to adverse effects on fertilization and blastocyst implantation (Fabro, 1978). Thus, cotinine measured in FF, as opposed to other media (e.g. urine, serum), may be a more appropriate biomarker of tobacco smoke exposure in studies of early pregnancy because it reflects a maturing oocyte's direct exposure to the constituents of tobacco smoke.

Cotinine was first measured in FF in smokers in 1989 (Weiss and Eckert, 1989), and in 1996, Zenzes *et al.* detected cotinine in the FF of passive smokers (i.e. those exposed to STS). Shortly thereafter, Zenzes and Reed (1998) demonstrated the ability of FF cotinine to discriminate between active, passive and nonsmokers.

Using urinary cotinine concentrations to estimate exposure in a large cohort of women undergoing *in vitro* fertilization (IVF), we recently reported no increased risk of failed implantation or spontaneous abortion among exposed nonsmokers compared to those unexposed (Meeker *et al.*, 2007). However, few studies have examined whether urinary cotinine is strongly related to more biologically relevant markers of STS exposure in studies of early pregnancy (e.g. cotinine in FF). Cotinine concentrations in urine are measures of plasma cotinine not reabsorbed during glomerular filtration; while cotinine concentrations in FF represent plasma cotinine that diffused through the blood-follicle barrier and largely depend upon concentration gradients between the blood, interstitial fluid, and cells surrounding the oocyte. In addition to these biological differences, relying on biomarkers more remote to the target area (e.g. gametes) may increase exposure measurement error and misclassification, which tend to bias effect estimates toward the null (Armstrong, 1998). Thus, we hypothesized poor agreement between cotinine concentrations in follicular fluid and urine, which may partially explain the lack of association between exposure and pregnancy outcomes in our previous report.

In the present study we compared cotinine levels in paired urine and FF samples from the same women. We also examined FF cotinine levels over time among subjects undergoing multiple IVF treatment cycles to determine if repeated exposure measures would be needed in epidemiology studies of STS exposure and IVF outcomes. These efforts were intended to inform our exposure assessment strategy for a large, ongoing epidemiological study of STS exposure and early pregnancy loss among ART patients. As far as we are

aware, this is the first study to examine the relationship between cotinine concentrations in FF and urine and the intra-individual variability of FF cotinine concentrations over time.

Methods

Study Population

For the present study, 415 subjects were randomly selected from among those who underwent oocyte retrieval (i.e. for whom a FF sample was taken) in a larger study previously described (Meeker *et al.*, 2007). Briefly, in the larger study, couples undergoing IVF or intracytoplasmic sperm injection (ICSI) between 1994 and 2003 were recruited through three Boston-area clinics to study predictors of IVF success. Protocols were approved by the Human Research Committees at Brigham and Women's Hospital, the Harvard School of Public Health, and the University of Michigan. Approximately 65% of couples approached agreed to participate in the study. Couples excluded from the study were those who underwent gamete intra-fallopian transfer (GIFT) or were gestational carriers, as well as those who required donor oocytes or donor semen. After exclusions, 2,350 couples who underwent from one to six IVF/ICSI treatment cycles were enrolled in the parent study.

Exposure Assessment

A self-administered questionnaire was used to obtain information from each subject on medical history and lifestyle factors such as: demographics, ages of both male and female partner, medical and reproductive history, smoking history, and duration of

infertility. Self-reported STS exposure at home or at work was also obtained as well as information on male partner's present smoking status.

Physicians and technicians were asked to retain the FF from study patients during egg retrieval for each cycle. FF was aspirated from follicles using a 16-gauge needle attached to a 100 mm Hg pump-operated aspirator (Rocket, Branford, CT) under constant suction. To ensure that FF was not contaminated with other fluids, samples were collected during the first puncture of the oocyte retrieval from the largest follicle visualized on ultrasound before using any flushing medium and only consisted of fluid from one follicle. The FF was then transferred to a sterile Petri dish where oocytes were scanned for and removed. The fluid, normally discarded at this point, was placed into a 15 mL conical tube and centrifuged for 15 minutes at low speed (200*g*). The supernatant was placed into a clean storage tube, labeled, refrigerated, and transferred to the Brigham and Women's Hospital laboratory within 12 hours. At the laboratory, the specimens were aliquoted into 2 mL specimens and frozen at -80 degrees centigrade. FF was analyzed for cotinine using a quantitative enzyme-linked immunosorbent assay (ELISA; BioQuant, Inc., San Diego, CA). This single-step, competitive test uses spectrometric measurement to determine cotinine in body fluids. It has a lower reporting limit of 0.3 ng/mL and inter- and intra-assay variations of 4 and 6%, respectively.

A first-morning, void urine sample was collected from participants in only the first half of the parent study (1994-1998). These samples were available for 255 of the 415 subjects in the present sub-study. Urine samples were collected in a sterile, wide-

mouthed, 1-liter plastic container, aliquoted in the laboratory, and frozen at -80 degrees centigrade. Samples were later analyzed for cotinine via competitive radioimmunoassay techniques described elsewhere (Langone *et al.*, 1973, Van Vunakis *et al.*, 1993). The procedure had a lower reporting limit of 0.1 ng/mL and inter- and intra-assay variations of 5%. After analysis, urinary cotinine concentrations were adjusted by creatinine (Cr) to account for individual differences in hydration and urine output (Boeniger *et al.*, 1993). Before we compared FF and urinary cotinine concentrations, we matched each subject's urine sample to the IVF/ICSI treatment cycle that occurred nearest in time to its collection. Urine samples were matched to the initial cycle for 97% of the subjects in the present study.

Statistical analysis

Data analysis was performed using SAS software (version 9.2; SAS Institute Inc., Cary, NC). Quantified cotinine concentrations below the limit of detection (LOD) were kept as the reported value. Unquantified concentrations were assigned a value of one half of the LOD. We compared cotinine levels in urine and FF among the 255 subjects from whom both types of samples were collected. In preliminary analyses we tabulated cotinine distributions in both FF and urine, stratified by self-reported smoking status. Self-reported active smokers (n = 13) were excluded to calculate Spearman correlation coefficients between concentrations of cotinine measured in paired FF and urine samples. We also conducted this correlation analysis after excluding smokers based on a published cutpoint or threshold (Fuentes *et al.*, 2010). To determine whether the time between urine

and FF sample collection affected the strength of cotinine correlation, we examined coefficients after stratifying subjects by the duration between sample collections.

We assessed the ability of a single urinary cotinine concentration to correctly classify an individual's STS exposure status by calculating its percent agreement with the individual's exposure classification based on the cotinine concentration in a paired FF sample. For this analysis we considered FF cotinine to represent a woman's "true" or "gold standard" exposure because it reflects a developing oocyte's direct exposure to the constituents of tobacco smoke. Also, FF samples were collected during IVF treatment at oocyte retrieval, while urine samples were often collected before oocyte retrieval. Thus, in studies of female fertility and early pregnancy, we hypothesize that FF cotinine represents a more biologically and, in our case, temporally relevant biomarker of STS exposure.

Among self-reported nonsmoking subjects in this agreement analysis, we considered those in the highest cotinine tertile as exposed. For comparison, we also used a published cutpoint to classify exposure. Following Zielinska-Danch *et al.* (2007), we categorized unexposed nonsmokers as those below an unadjusted urinary cotinine concentration of 50 ng/mL. To the best of our knowledge, no STS exposure cutpoints exist for cotinine in Cr-adjusted urine or FF. Thus, we extended 50 ng/mL in unadjusted urine to our data distributions to establish cutpoints for cotinine in Cr-adjusted urine and in FF. We first determined the percentile in our unadjusted urinary cotinine distribution that corresponded to 50 ng/mL, which was the 42nd percentile. We then matched this percent

rank to the 42nd percent values in our FF cotinine and our Cr-adjusted urinary cotinine distributions, which were 1.11 ng cotinine/ mL FF and 66 ng cotinine/ g creatinine, respectively. These values were also used as STS exposure cutpoints for subsequent analyses. After nonsmoking participants were categorized as exposed or unexposed based cotinine concentrations in each sample media, sensitivity, specificity, and positive and negative predictive values were calculated and compared between classification methods (i.e. when using highest tertile or published cutpoints). Percent agreement was also assessed after stratifying cotinine concentrations by time between urine and FF sample collections.

Since women in the parent study underwent up to six IVF/ICSI cycles, we also examined the temporal variability (i.e. changes over time) in FF cotinine concentrations to assess how well a single FF cotinine measure may represent one's average exposure to STS over longer periods of time. Among all 415 subjects selected for the present study, we stratified FF cotinine levels by study year and examined cotinine distributions over time.

To assess the between- and within-person variability in FF cotinine concentrations, we calculated the intraclass correlation coefficient (ICC) and 95% confidence intervals for ln-transformed FF cotinine concentrations using SAS PROC MIXED (Hankinson *et al.*, 1995). ICC is a measure of the reliability of repeat measures over time and is defined as the ratio of between-subject variance to total variance. Values range from zero to one indicating poor and excellent reliability, respectively (Rosner, 2000). The ICC was

calculated for differing smoking groups for comparison (e.g. when including or excluding active smokers).

ICC is an indicator of reliability for continuous measures, though it does not quantify the degree of misclassification that may occur as subjects are categorized into exposure groups. Thus, we calculated the percent agreement of exposure categorization from a single cotinine measure to the category in which women were assigned when considering all their repeated FF cotinine measures (Mahalingaiah *et al.*, 2008; Meeker *et al.*, 2005). The geometric mean FF cotinine concentration for each subject was defined as their "true" exposure. As described earlier, a concentration of 1.11 ng cotinine/ mL FF was used to categorize women as exposed or unexposed to STS. Thus, we determined the percent agreement between each cycle and the subject's "true" exposure. Sensitivity, specificity, and positive and negative predictive values were evaluated and compared between smoking groups.

Results

The 415 subjects selected for the present study had FF collected from one to six IVF/ICSI treatment cycles, with a mean of 1.8 cycles per women. The number of women who underwent 1, 2, 3, 4, 5, and 6 cycles, were, respectively, 209, 109, 63, 24, 7, and 3. A single urine sample was available for 255 subjects recruited between 1994 and 1998 (i.e. in the first half of the parent study). Detectable cotinine concentrations were measured in 100% of urine samples and 82% of FF samples.

Large differences ($p < 0.0001$) were observed between the cotinine distributions of self-reported active smokers and nonsmokers and are presented in Table 2.1. No significant differences were found between the cotinine distributions of nonsmokers who reported they were exposed to STS and nonsmokers who did not report STS exposure. Cotinine concentrations were consistently higher in urine than in FF.

No clear relationship between urinary and FF cotinine concentrations was observed visually (Figure 2.1). Several self-reported nonsmokers had very high levels of cotinine in either FF or urine or both, which may reflect active smokers who inaccurately reported their smoking status. Overall, a weak correlation (Spearman $r < 0.2$) was observed between cotinine concentrations in these two media and is presented in Table 2.2. Because of the short biological half-life of cotinine in body fluids, we hypothesized that the correlation between cotinine in urine and FF would strengthen as the time between collections of the two samples decreased. For subjects whose urine and FF samples were collected more than 15 days apart, correlation coefficients declined somewhat compared to all samples, as expected. Likewise, correlations were slightly strengthened among samples collected within 15 days of each other compared to all samples. However, as subjects were grouped by shorter time intervals between sample collections, we observed a decreasing trend in the strength of cotinine correlation. When a published cutpoint was used to distinguish active from passive smokers, coefficients decreased compared to coefficients calculated among self-reported nonsmokers. This is may be due to the presence of active smokers who misreported their smoking status. Thus, the lower half of the table is likely representing the true correlation of cotinine concentrations in urine and

FF samples among nonsmokers. Similar results were seen when calculating Pearson correlation coefficients using cotinine concentrations transformed by the natural logarithm (results not shown).

To determine the extent of exposure misclassification based on categories of urinary cotinine as a biomarker of STS exposure, we calculated its percent agreement with FF cotinine categories (Table 2.3). Agreement between cotinine in urine and FF was poor, and results were consistent among various data stratification approaches (e.g. when using different exposure cutpoints or Cr-adjusted urine). Sensitivity and positive predictive values did strengthen when we classified exposure by a published cutpoint versus the tertile cutpoint, but specificity and negative predictive values decreased proportionally, negating any overall improvement in percent agreement. Finally, urine and FF samples collected closer together in time did not have better agreement than those collected farther apart.

To assess the reliability of FF cotinine concentrations, we calculated intraclass correlation coefficients (ICC) and their 95% confidence intervals (Table 2.4). Good reproducibility (ICC = 0.67) was demonstrated when including all participants; though, as expected, when active smokers were excluded, coefficients were reduced for self-reported nonsmokers (ICC = 0.52) and “true” nonsmokers (ICC = 0.42). We also assessed the ability of a single FF cotinine measure to correctly categorize women as exposed to STS when compared to their exposure categorization that considered multiple FF cotinine measures. Among 97 women undergoing three or more cycles, FF cotinine agreement

was good (Table 2.5). Results were consistent when active smokers were omitted and when exposure status was defined by a published cutpoint versus self-report.

Finally, since the parent study spanned 10 years we hypothesized that there would be a decline in FF cotinine concentrations over time due to increased tobacco awareness and regulation in the US. However, no temporal trend was observed (results not shown).

Discussion

In this study, we compared cotinine concentrations in FF and urine samples and examined the temporal variability of FF cotinine measures. Among nonsmoking IVF patients, we found a weak relationship between cotinine levels in paired FF and urine samples. FF cotinine was a more temporally relevant biomarker in the present study because FF samples were collected during ART treatment, while urine samples were often taken before treatment. Since FF is also likely a more biologically relevant sample matrix when assessing STS exposure's effects on early female reproduction, our results provide evidence that measuring cotinine in FF may be a more appropriate biomarker of low-level tobacco smoke exposure in studies of fertility and early pregnancy.

Cotinine in FF and serum correlated highly with one another ($R^2 = 0.95$ and $R^2 = 0.89$) in two previous reports (Fuentes *et al.*, 2010; Paszkowski, 1998). This inter-fluid cotinine agreement was much stronger than what we observed in the present study, though active smokers were included in both previous studies, likely strengthening the observed correlations. As far as we are aware, only one other study has examined inter-fluid

cotinine agreement exclusively among nonsmokers. After dosing subjects with nicotine levels intended to simulate STS exposure, Benowitz *et al.* (2009b) recently reported a strong relationship ($R^2 = 0.84$) between cotinine in blood plasma and Cr-adjusted urine. This reported correlation is also much stronger than what was observed in the present study, but it is difficult to compare results from this study and ours since the relationship between cotinine in blood plasma and FF among nonsmokers is unknown. Paszkowski *et al.* (1998) found no significant gradient between cotinine in serum and FF, indicating that cotinine easily crosses the blood-follicle barrier; however, 22% of the subjects in that study were self-reported active smokers. Thus, this finding may be influenced by cotinine-saturated biological compartments within those who actively smoke.

To the best of our knowledge, the present study is also the first to examine intra-individual variability in FF cotinine concentrations. We found moderate to high within-subject variability in FF cotinine concentrations when assessing concentrations as a continuous variable. Our ICC analyses showed fair to poor reproducibility in FF cotinine levels over time, demonstrating the need for multiple FF samples to accurately predict long-term STS exposure. On the other hand, when using FF cotinine concentrations to categorize women as exposed or unexposed, we found a high level of agreement in sensitivity analyses. Thus, if broad exposure categories are used as opposed to continuous FF cotinine concentrations, there may be a smaller degree of exposure misclassification when using a single FF sample to estimate exposure over a longer period of time. However, in our analysis sensitivity may have been overestimated because we used subjects' geometric mean cotinine concentrations as their "gold standard" exposure

measure. In other words, the predicted measurements, cotinine measures from a single FF sample, are not independent of the standard against which they are being compared. The lack of independence between these two variables may have increased the observed levels of agreement.

While somewhat low, the ICCs are higher here than what has been seen for other non-persistent chemicals such as phthalates and bisphenol-A among other studies of infertile couples (Hauser *et al.*, 2004; Mahalingaiah *et al.*, 2008). This may indicate that people are more consistently and repeatedly exposed to STS compared to these other contaminants, perhaps because exposure to the other ubiquitous contaminants is likely to be more multi-source, multi-route, and multi-pathway compared to STS exposure.

The variability of cotinine concentrations over time has not been extensively studied in any sample media. In a study examining temporal patterns in urinary cotinine among children whose parents smoked, Matt *et al.* (2007) reported that over a 7–13 month period, within-subject variability of urinary cotinine levels was 10–20 times higher than would be expected based on measurement error alone. They also reported that a single urine sample yielded highly accurate estimates of recent exposure only (i.e. 2-3 days) and up to 12 urine samples may be needed to obtain similar precision in estimates of STS exposure over a 4-13 month period. In their study, ICCs for cotinine measured in single urine samples collected four days apart ranged from 0.81 and 0.93. For cotinine measured in samples collected 138 days or more apart, ICCs ranged from 0.65 to 0.71. Similarly, in the present study, our subjects' FF samples were collected from one month to a few years

apart. We found ICCs of 0.52 and 0.42 for cotinine measured in single FF samples among self-reported nonsmokers and "true" nonsmokers, respectively. We expected ICCs from the present study to be lower than what Matt *et al.* reported, since all subjects in that study were children whose parents smoked. Matt *et al.* also reported that ICCs for averaged cotinine measures from multiple samples (between 6 and 15) were much higher than for single samples, at times exceeding 0.93 for time intervals of 138 to 403 days. These findings demonstrate that within-subject cotinine variability increases with time. In other words, a single measure's accuracy as an estimate of exposure decreases as time increase. Thus, collecting and conducting cotinine analysis on multiple biological samples over time should improve long-term STS exposure estimate accuracy.

Findings from Matt *et al.* may represent true variability in exposure to STS over time. However, high between-subject variation in FF cotinine within smoking groups and within women reporting smoking the same number of cigarettes has also been demonstrated (Zenzes *et al.*, 1996), potentially suggesting variation in toxicant metabolism, distribution, and excretion between individuals due to genetic differences (Swan *et al.*, 2005). If so, using a more biologically-relevant exposure biomarker, such as FF, may be vital for minimizing exposure measurement error in epidemiological studies. Alternatively, the results of Zenzes *et al.* (1996) may serve as further evidence of the inaccuracy of self-reported exposure since self-report was used to create smoking groups in that study.

There are several potential alternate explanations for our findings. Some of the cotinine disagreement we observed between matrices may be the result of using different analytical methods to measure cotinine in urine and FF. However, we expect that this would only account for a small proportion of the observed disagreement because the precision for both methods was good and our sample size was large. Since cotinine levels generally reflect exposure to tobacco smoke within the past 3-4 days (Benowitz *et al.*, 2009a), the time between urine and FF sample collections could also partially explain this lack of agreement. We stratified our correlation and percent agreement analyses, however, by the time between sample collections. In doing so we did not observe an improvement in percent agreement or correlation between samples collected within three days of each other verses all samples. Additionally, relying solely on self-report can lead to exposure misclassification. Thus, for comparison, we used an objective cotinine cutpoint or threshold to determine exposure status. Our results did not change when exposure classification was based upon cutpoints. The use of a first-morning void urine sample may also partially explain the observed disagreement. Other markers of exposure, such as cotinine measured in a 24-hour urine sample, may have been helpful. Unfortunately, such markers were not available in the present study.

Another explanation for our findings may be the difference in storage times of the urine and FF samples. FF samples were frozen/stored for much longer than urine samples before cotinine measurement. The stability of cotinine in FF has not been tested, though the results of a study examining the stability of urinary cotinine and creatinine concentrations suggest that these analytes retain their ability to discriminate between

smokers and nonsmokers for at least 10 years of storage at -20 degrees centigrade (Riboli *et al.*, 1995). Also, our findings of weaker within-subject cotinine relationships between FF and urine than between repeat measures from FF collected over time argue that the lack of correlation between urine and FF is true and that cotinine degradation from long-term storage of FF did not occur.

In conclusion, our results indicate that FF cotinine may be an improved biomarker of exposure to STS compared to urinary cotinine in studies of early pregnancy and that cotinine measures from multiple FF samples may be necessary to accurately represent long-term STS exposure. In the future, we plan to measure cotinine in multiple FF samples from a larger number of women to reevaluate relationships between STS exposure and early pregnancy outcomes.

Tables and Figures

Table 2.1: Distributions of cotinine in follicular fluid and urine and time between sample collections by self-reported smoking status among 255 women undergoing their first[a] *in vitro* fertilization treatment cycle

	Geometric	Selected percentiles				
	Mean	Min	25th	50th	75th	95th	Max
Follicular fluid cotinine (ng/mL)[b]							
unexposed nonsmokers[c]	1.00	0.05	0.32	1.49	2.37	3.97	69.8
exposed nonsmokers[d]	1.17	0.15	0.15	1.64	2.54	118	331
active smokers[e]	72.6	3.27	40.9	100	219	369	382
Unadjusted urinary cotinine (ng/mL)							
unexposed nonsmokers	60.0	4.00	37.1	58.9	88.1	210	11190
exposed nonsmokers	75.5	13.2	36.9	63.6	107	1768	7421
active smokers	1397	46.2	982	1267	4819	10996	15000
Creatinine-adjusted cotinine (ng/g creatinine)							
unexposed nonsmokers	85.6	9.71	49.3	74.1	127	362	86078
exposed nonsmokers	109	19.9	49.7	87.5	207	2731	5377
active smokers	2114	70.0	2178	3249	5737	8217	9389
Time between urine and follicular fluid sample collections (days)							
all nonsmokers[f]	15.9	1.00	6.00	15.0	38.8	76.9	1108

[a] A cycle other than the first was used for seven subjects because that cycle occurred nearer in time to the collection of the subject's urine sample

[b] Unquantified follicular fluid cotinine concentrations were given a value of one half the limit of detection (LOD). Quantified values below the LOD (< 0.3 ng/mL) were kept as the reported value

[c] N = 211; [d] N = 31; [e] N = 13

[f] Both unexposed and exposed nonsmokers; N = 242

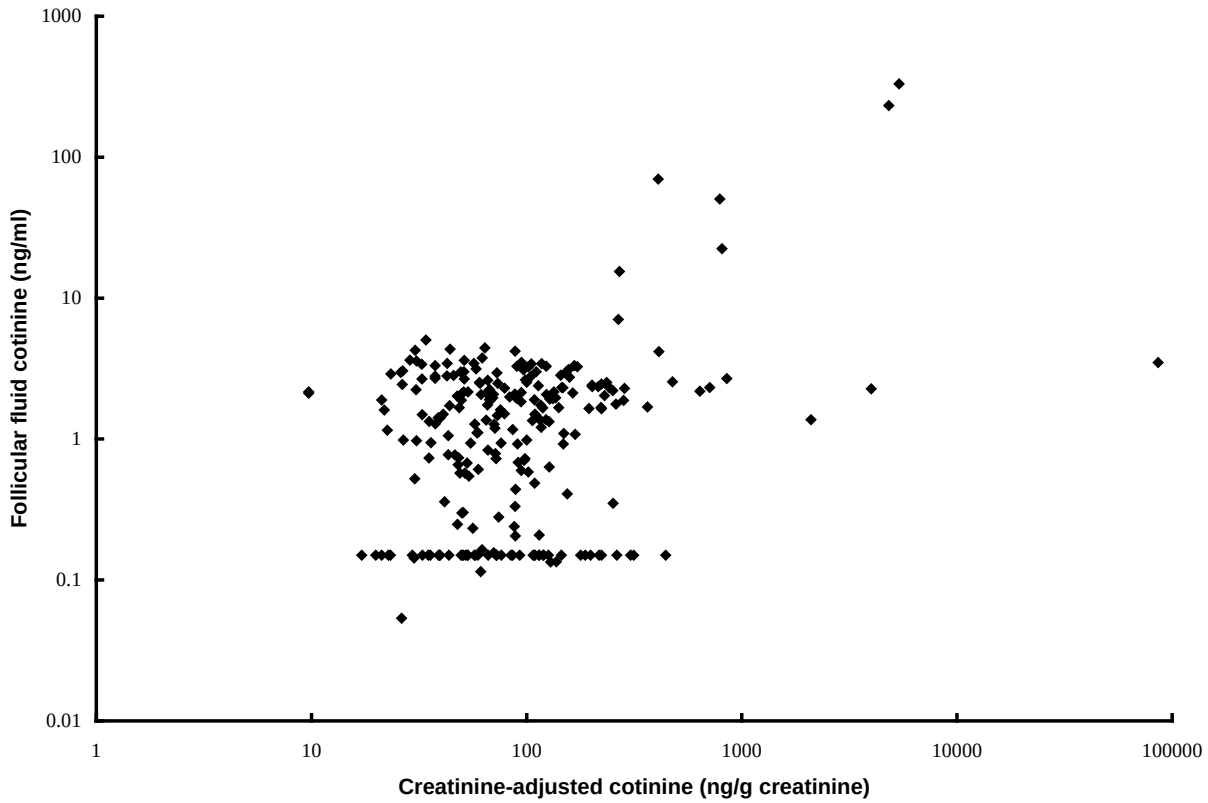

Figure 2.1: Urinary cotinine versus follicular fluid cotinine in paired samples from the same women (N = 242 self-reported nonsmokers)

Table 2.2: Spearman correlation coefficients between cotinine measured in follicular fluid and urine samples among nonsmoking *in vitro* fertilization patients

	N	Unadjusted urine	Creatinine-adjusted urine
Nonsmokers determined by self-report			
all samples	242	0.13[a]	0.15[a]
> 15 days apart	122	0.06	0.13
≤ 15 days apart	120	0.20[a]	0.16
≤ 7 days apart	82	0.14	0.11
≤ 3 days apart	17	0.12	-0.08
Nonsmokers determined by published cutoff [b]			
all samples	238	0.07	0.08
> 15 days apart	120	0.01	0.08
≤ 15 days apart	118	0.12	0.07
≤ 7 days apart	80	0.08	0.04
≤ 3 days apart	16	-0.06	-0.29

[a] p-value ≤ 0.05

[b] Those participants with a follicular fluid cotinine concentration below 10 ng/mL, indicating they are not active smokers following Fuentes *et al.* (2010)

Table 2.3: Percent agreement of secondhand tobacco smoke exposure classification between cotinine in follicular fluid and urine among 242 self-reported nonsmokers using two different methods to group exposures

	Number exposed	Percent exposed	Unadjusted urine				Creatinine-adjusted urine			
			Sens[a]	Spec[b]	Ppv[c]	Npv[d]	Sens	Spec	Ppv	Npv
Highest tertile[e]										
All samples	81	0.33	0.38	0.69	0.38	0.69	0.43	0.71	0.43	0.71
> 15 days apart	35	0.29	0.40	0.69	0.35	0.74	0.49	0.71	0.40	0.77
≤ 15 days apart	46	0.38	0.37	0.68	0.41	0.64	0.39	0.72	0.46	0.66
≤ 7 days apart	34	0.41	0.41	0.69	0.48	0.62	0.35	0.69	0.44	0.60
≤ 3 days apart	7	0.41	0.43	0.70	0.50	0.64	0.29	0.60	0.33	0.55
Published cutoff[f]										
All samples	141	0.58	0.62	0.47	0.62	0.47	0.62	0.48	0.62	0.48
> 15 days apart	67	0.56	0.55	0.49	0.58	0.46	0.64	0.42	0.58	0.48
≤ 15 days apart	74	0.61	0.68	0.44	0.65	0.47	0.61	0.54	0.67	0.47
≤ 7days apart	53	0.65	0.72	0.35	0.67	0.40	0.57	0.52	0.68	0.40
≤ 3 days apart	12	0.71	0.58	0.40	0.70	0.29	0.58	0.40	0.70	0.29

[a] Sensitivity; [b] Specificity; [c] Positive predictive value; [d] Negative predictive value

[e] Highest cotinine tertile, indicating those exposed to secondhand tobacco smoke

[f] 50 ng cotinine/mL unadjusted urine, indicating those exposed to secondhand tobacco smoke following Zielinska-Danch *et al.* (2007); in our dataset, this concentration corresponds to 1.11 ng cotinine/ mL follicular fluid and 66 ng cotinine/ g creatinine based on percent rank

Table 2.4: Intraclass correlation coefficients (ICC) for cotinine levels in repeated follicular fluid samples

	No. of women	No. of cycles	ICC	95% CI
All subjects	415	765	0.67	0.61-0.73
Self-reported nonsmokers	388	718	0.52	0.44-0.59
"True" nonsmokers[a]	379	696	0.42	0.34-0.52

[a] Geometric mean below 10 ng cotinine/ mL follicular fluid, indicating participants who are not active smokers following Fuentes *et al.* (2010)

Table 2.5: Percent agreement of secondhand tobacco smoke exposure classification when considering only a single cotinine measure versus multiple cotinine measures in follicular fluid among women undergoing three or more *in vitro* fertilization treatment cycles

	No. of women	No. exposed[a]	No. of cycles	No. exposed[b]	Sens[c]	Spec[d]	Ppv[e]	Npv[f]
All cycles	97	53	338	208	0.91	0.72	0.79	0.87
Self-reported nonsmkrs[g]	91	46	318	188	0.90	0.71	0.76	0.88
"True" nonsmokers[h]	86	42	302	173	0.89	0.72	0.75	0.88

[a] Participants with a geometric mean cotinine concentration greater than 1.11 ng/mL follicular fluid, indicating nonsmokers exposed to secondhand tobacco smoke following Zielinska-Danch *et al.* (2007)

[b] Participants with cotinine concentrations greater than 1.11 ng/mL follicular fluid measured during a single cycle

[c] Sensitivity; [d] Specificity; [e] Positive predictive value; [f] Negative predictive value

[g] One cycle from one subject was omitted in this analysis because it occurred during self-reported active smoking; the subject's geometric mean was recalculated after omission

[h] Participants with a geometric mean cotinine concentration less than 10 ng/mL follicular fluid, indicating those who are not active smokers following Fuentes *et al.* (2010)

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CHAPTER III

Secondhand Tobacco Smoke Exposure is associated with Increased Risk of Failed Implantation and Reduced IVF Success

Abstract

Infertility and early pregnancy loss are common in the US and worldwide, as is exposure to secondhand tobacco smoke (STS). Previous research has suggested a relationship between STS exposure and early pregnancy loss, but studies have been limited by small study sizes and/or imprecise methods for exposure estimation. *In vitro* fertilization (IVF) allows for the collection of follicular fluid (FF), the fluid surrounding the preovulatory oocyte, which may be a more biologically relevant sample media than urine or serum in studies of early reproduction. We measured cotinine in FF collected during 3,270 IVF treatment cycles from 1,909 nonsmoking women between 1994 and 2003 to examine the relationship between STS exposure and implantation failure. In adjusted models, we found a significant increase in the risk of implantation failure among women exposed to STS compared to those unexposed (Odds Ratio [OR] = 1.52; 95% confidence interval [CI] = 1.20-1.92; Risk Ratio [RR] = 1.17; 95% CI = 1.10-1.25). We also found a significant decrease in the odds for a livebirth among STS-exposed women (OR = 0.75; 95% CI = 0.57-0.99; RR = 0.81; 95% CI = 0.66-0.99). Female STS exposure, estimated through the measurement of cotinine in FF, is associated with an increased risk of implantation failure and reduced odds of a livebirth.

Introduction

Infertility and early pregnancy loss (e.g. spontaneous abortion) are prevalent in the US and worldwide (Chandra *et al.*, 2005; Norwitz *et al.*, 2001), and there is growing concern about adverse reproductive health effects resulting from secondhand tobacco smoke (STS) exposure. Since STS exposure is widespread, even minor associations between exposure and fertility or pregnancy outcomes may have a significant impact on public health.

Self-reported female STS exposure was associated with decreased implantation and pregnancy rates among 225 women undergoing *in vitro* fertilization (IVF) or intracytoplasmic sperm injection (ICSI; Neal *et al.*, 2005), though this method of exposure assessment may lead to exposure misclassification and biased risk estimates. We recently conducted a study using an objective biomarker (creatinine-adjusted urinary cotinine) to estimate female STS exposure in 921 women undergoing IVF but found no association between exposure and failed fertilization, failed implantation or spontaneous abortion (Meeker *et al.*, 2007a). A case-control study in Sweden did, however, find an increased odds of spontaneous abortion in STS exposed women versus unexposed women based on plasma cotinine levels (George *et al.*, 2006).

The increasing use of assisted reproductive technologies, particularly IVF, has improved our ability to study contributors to infertility and early pregnancy loss by allowing the observation of early and discrete stages in the reproduction process. Follicular fluid (FF), the fluid surrounding the preovulatory oocyte, is routinely collected during IVF treatment

but is seldom used despite its superior biological relevance as a measure of exposure to STS or other environmental agents. Cotinine levels in FF reflect a developing oocyte's direct exposure to constituents of tobacco smoke (i.e. it is a measure of dose at the target tissue). To our knowledge, the only study to rely on FF cotinine to assess the relationship between STS exposure and early reproduction found no significant difference in fertilization or pregnancy rates between active, passive, and non-smokers in a small cohort of IVF patients (n = 197, 26 of whom were categorized as being exposed to STS; Sterzik *et al.*, 1996).

Overall, studies of the effects of STS on fertility and early pregnancy have had differing, but suggestive results underscoring the need for additional research. The present study was designed to examine the relationship between female STS exposure and failed implantation using cotinine measured in FF as a biomarker of exposure among a large cohort of women undergoing IVF.

Methods

Study population

Participants in the present study were couples undergoing IVF treatment between August 1994 and June 2003 at one of three Boston-area clinics. Elements of the original study have been described previously (Meeker *et al.*, 2007a; Meeker *et al.*, 2007b). Protocols were approved by the Human Research Committees at all participating institutions. Approximately 65% of couples approached agreed to participate in the study. Couples excluded from the study were those who underwent gamete intra-fallopian transfer

(GIFT) or were gestational carriers, as well as those who required donor oocytes or donor semen. Couples in which the woman self-reported active smoking were also excluded. In addition, cycles that failed or were discontinued prior to embryo transfer were excluded from the present analysis. After these exclusions, there were 1,909 couples, with a total of 3,270 treatment cycles, enrolled in the present study. Participants underwent from one to six treatment cycles. A self-administered questionnaire was used to obtain information from each couple on medical history and lifestyle factors such as: demographics, medical and reproductive history, smoking history, duration of infertility, and STS exposure status.

Treatment Outcomes

All IVF treatment and outcome variables were abstracted from the clinic record. When at least one embryo was transferred but human chorionic gonadotropin (hCG) levels never reached 5.0 mIU/ml, the cycle outcome was defined as a failed implantation. A chemical pregnancy was defined by a measurement of luteal hCG of 5.0 mIU/ml or greater with no further evidence (e.g. gestational sac, fetal heartbeat) of a continued pregnancy. Clinical pregnancy was determined by ultrasound visualization of a gestational sac or a fetal heartbeat. Outcomes among clinically recognized pregnancies included an ectopic pregnancy (gestation outside of the uterus), a molar pregnancy (placental formation with no fetus), a spontaneous abortion (fetal demise before 20 weeks of gestation), stillbirth (fetal demise at or beyond 20 weeks gestation), or livebirth of at least one infant.

Exposure assessment

Physicians and technicians were asked to retain the FF from study participants during egg retrieval for each cycle. FF was aspirated from follicles using a 16-gauge needle and constant suction from a Rocket pump apparatus. Fluid was collected from the largest visible follicle before using any flushing medium and then transferred to a sterile Petri dish. Oocytes were scanned for and removed. The fluid, normally discarded at this point, was placed into a 15 ml conical tube and centrifuged for 15 minutes. The supernatant was placed into a clean storage tube, labeled, refrigerated, and transferred to the Brigham and Women's Hospital laboratory within 12 hours. At the laboratory, the specimens were aliquoted into 2 ml specimens and frozen at -80 degrees centigrade. FF was analyzed for cotinine using a quantitative enzyme-linked immunosorbent assay (ELISA; BioQuant, Inc., San Diego, CA). This single-step, competitive test uses spectrometric measurement to determine cotinine in body fluids. It has a lower reporting limit of 0.3 ng/ml and inter- and intra-assay variations of 4 and 6%, respectively.

Statistical analysis

Data analysis was performed using SAS software (version 9.2; SAS Institute Inc., Cary, NC). Quantified cotinine concentrations below the limit of detection (LOD) were kept as the reported value. Unquantified cotinine concentrations were assigned a value of one half of the LOD. Although self-reported smokers were omitted from the study, smokers who may have misreported their smoking status were identified and excluded based on concentrations of cotinine in FF. Treatment cycles were considered to be from an active

smoker if the cycle yielded a FF cotinine concentration of ≥10 ng/ml (n = 81 cycles), following Fuentes *et al*. (2010).

To the best of our knowledge, no STS exposure cutpoint exists for cotinine in FF. Thus, we extended to our data a cutpoint for unadjusted urinary cotinine of 50 ng/ml (Zielińska-Danch *et al.*, 2007). In a previous analysis among the current cohort, FF and urine samples were collected from 255 participants and analyzed for cotinine (unpublished data). We determined the percentile in our unadjusted urinary cotinine distribution that corresponded to 50 ng/ml, which was the 42nd percentile. We then matched this percent rank to the 42nd percent value in our FF cotinine distribution to establish a STS exposure cutpoint, which was 1.11 ng cotinine/ ml FF. Cycles from STS exposed nonsmokers were defined as those that yielded FF cotinine concentrations of <10 ng/ml and >1.11 ng/ml (n = 386 cycles). Treatment cycles from unexposed nonsmokers were defined by FF cotinine concentrations ≤1.11 ng/ml (n = 2,803 cycles).

After excluding the treatment cycles of active smokers based on FF cotinine concentration, preliminary exploratory analyses were performed to evaluate variable distributions and to assess bivariate relationships among key covariates. Variables considered as potential confounders were female age, body mass index (BMI), ethnicity, primary infertility diagnosis, site of treatment, year of treatment, months spent trying to get pregnant, whether the woman had experienced a previous livebirth, ampules of gonadotropins, down-regulation protocol, use of ICSI, use of assisted hatching, number of embryos transferred, and day of embryo transfer. Bivariate relationships between each

covariate and the exposure and outcome variables were examined to identify covariates to include in the multivariate models. Covariates included in the final models were considered to be biologically or clinically important in models in which they were not statistically significant (Hosmer & Lemeshow, 1989). The same covariates were included in each model to maintain consistency.

The relationship between STS exposure and implantation failure was initially modeled using only data from subjects' first treatment cycles to maintain consistency with previous studies that have been conducted on this topic, followed by analysis of data from all cycles to further improve statistical power. Conditional analyses were performed in that only the subset of subjects that had not experienced a failure up to that point were included. Thus, all results presented are among cycles that proceeded to embryo transfer. When considering only subjects' first treatment cycles, conditional logistic regression was used to model the association between STS exposure and implantation failure. Generalized estimating equations (GEE) were used when considering all treatment cycles. GEE in their simplest form are an extension of logistic regression and are a method of analyzing correlated data (e.g. longitudinal data) that otherwise could be modeled as a generalized linear model (Liang & Zeger, 1986).

As a potentially more clinically relevant measure of effect we also calculated the odds of a livebirth in relation to STS exposure for both first cycle-only data and when considering all the data. When analyzing the multi-cycle data for livebirth outcomes we first used discrete survival analysis, which was carried out by using a logistic regression model and

adjusting for cycle number (Cox & Oakes, 1984). Discrete survival analysis censors on the outcome (i.e. a woman can only have the event once), and thus was not used to analyze implantation failure since a woman may experience multiple implantation failures across cycles. We also modeled the multi-cycle livebirth data using GEE for comparison.

Since odds ratios for common outcomes (≥ 10%) tend to overestimate the relative risk (McNutt *et al*., 2003), odds ratios and risk ratios were calculated and compared for both implantation failure and successful livebirth outcomes. Thus, both log-binomial and logistic regression models were used to compute effect estimates. Like logistic regression, the log-binomial model is used for the analysis of a dichotomous outcome and models the probability of that outcome (McNutt *et al.*, 2003). Both modeling approaches also assume the error terms have a binomial distribution. These two approaches differ in that logistic regression uses the logit function as the link between the independent variables and the probability of the outcome. Using the logit function yields an odds ratio. In the log-binomial model the log link is used, yielding a risk ratio.

Results

Demographic data for the women in this study are presented in Table 3.1. Participants had a mean (SD) age of 35.3 (4.3) years and were predominantly white (90%). Most reported that they had never actively smoked (69%). Male factor and tubal inflammation/occlusion were the most common causes of infertility, accounting for 33% and 20% of primary infertility diagnoses, respectively. The cause of infertility remained

unexplained for 18% of couples. Table 3.2 presents the treatment outcomes for couples in the study. Just over one-half (53%) of couples experienced a failed implantation in their first IVF cycle and 32% of initial treatment cycles resulted in a livebirth.

The relationship between STS exposure and implantation failure is presented in Table 3.3. In crude and adjusted models, we observed a significant increase in the risk of failed implantation among women exposed to STS versus those unexposed when considering only each subject's first treatment cycle (adjusted OR = 1.59; 95% CI = 1.17-2.17; adjusted RR = 1.17; 95% CI = 1.07-1.28) as well as when considering all cycles (adjusted OR = 1. 52; 95% CI = 1.20-1.92; RR = 1.17; 95% CI = 1.10-1.25).

We also observed a relationship between STS exposure and IVF treatment success (i.e. livebirth; see Table 3.4). In adjusted models, STS exposure was associated with a suggestive decline in the odds of a livebirth when considering only each subject's first treatment cycle (OR = 0.71; 95% CI = 0.50-1.02). When considering all cycles, there was a statistically significant reduction in the odds of a successful IVF cycle in relation to STS exposure (OR = 0.75; 95% CI = 0.57-0.99; RR = 0.81; 95% CI = 0.66-0.99).

When comparing odds ratios and risk ratios in our results, odds ratios yielded stronger effected estimates than risk ratios in all analyses performed (i.e. odds ratios were always further from the null). Effect estimates were not sensitive to the cotinine cutpoint chosen to define the STS exposed group. For example, similar results were obtained when using

the median FF cotinine concentration to define STS exposed or unexposed nonsmoking women (not shown).

Discussion

The primary aim of the present study was to determine the association between female STS exposure and implantation failure among couples undergoing IVF. In models adjusted for potential confounders, we found an increased risk of failed implantation among women exposed to STS versus those who were unexposed based on cotinine concentrations measured in FF. As a secondary aim, we also examined the relationship between STS exposure and the odds of a successful livebirth as a potentially more clinically-relevant effect measure. In our adjusted analysis, women exposed to STS were less likely to have a successful livebirth compared to those who were unexposed.

We examined the relationship between STS exposure and implantation failure in this population in two previous studies that utilized different exposure measurement methods from the present study. In an analysis among 921 women who had urine samples available for cotinine measurement, we found that creatinine-adjusted cotinine levels in urine were associated with a slight decrease in first-cycle implantation rates among nonsmoking women (11.1% in the lowest cotinine quintile versus 8.2% in the highest quintile; P = 0.13; (Meeker *et al.*, 2007a). However, in a multivariate analysis, creatinine-adjusted cotinine levels were not associated with failed implantation. Shortly thereafter, in a much larger follow-up study among all non-smoking participants in the study, we found a suggestive association between self-reported STS exposure and failed

implantation (Meeker *et al.*, 2007b). We have improved upon our earlier work with our current findings since we believe cotinine in FF is a more biologically relevant exposure measure, as opposed to self-report and urinary concentrations, as it more likely reflects the extent to which the oocyte was directly exposed to the constituents of tobacco smoke during its late development.

We found that, in all analyses for both implantation failure and successful livebirth, the odds ratio was farther from the null than the risk ratio. Odds ratios for common outcomes (≥ 10%) tend to overestimate the relative risk (McNutt *et al.*, 2003). Since implantation failure (53%) and successful livebirth (32%) were common outcomes in our study, risk ratios are likely a more accurate effect estimate than odds ratios. However, both odds ratios and risk ratios were statistically significant in adjusted models for both implantation failure and successful livebirth outcomes, which adds confidence that our results were robust to the type of model used.

Several other studies have explored the relationship between STS exposure and infertility or early pregnancy loss. A Canadian study among 255 women undergoing IVF or ICSI examined differences in implantation and pregnancy rates between smoking groups: those exposed to sidestream (SS) smoke (defined in this study as those who live with a partner that actively smokes), those exposed to mainstream (MS) smoke, the smoke inhaled by the smoker, and nonsmokers (NS; Neal *et al.*, 2005). The authors reported that embryo quality was similar between the three groups; however, consistent with our findings, there was a significant difference in implantation rates (MS = 12.0%, SS =

12.6%, and NS = 25%; $P < 0.01$) and pregnancy rates (MS = 19.4%, SS = 20.0%, and NS = 48.3%; $P < 0.001$) per embryo transfer between groups. Limitations of that study included a small sample size, lack of adjustment for confounding variables, and reliance on self-reported exposure. However, despite those limitations, the similarity in results for implantation failure and successful livebirths between that study and the present study suggest that STS exposure may be detrimental to early pregnancy.

Similar to the present study, Sterzik *et al.* (1996) utilized cotinine levels in FF to examine the effects of STS exposure on fertility and pregnancy among an IVF cohort. They reported no change in pregnancy rates between active, passive, and nonsmokers. Though not statistically significant, a decrease in fertilization rates was seen among passively exposed subjects (58%) compared to nonsmoking subjects (68%). This study's small sample size (n = 197; 26 passive smokers) and resultant lack of statistical power may partially explain its null findings. Further, the FF cotinine cutpoints used by Sterzik *et al.* (1996) were much higher (nonsmokers ≤20 ng/ml; passive smoker >20 ng/ml and ≤50 ng/ml; active smokers >50 ng/ml) than what was used in the present study (nonsmokers ≤1.11 ng/ml; passive smokers >1.11ng/ml and <10 ng/ml; active smokers ≥10 ng/ml). In other words, participants with FF cotinine concentrations as high as 20 ng/ml were considered nonsmokers in the Sterzik study, but concentrations of that magnitude are more likely to reflect those who actively smoke (Fuentes *et al.*, 2010).

A more recent study investigated associations between paternal smoking and pregnancy loss measured via daily urinary hCG assays among 526 nonsmoking Chinese female

textile workers (Venners *et al.*, 2004). Increased odds of early pregnancy loss was reported among women whose husbands smoked more than 20 cigarettes per day. The results of that study may reflect either effects related to female STS exposure or sperm damage associated with active smoking in males (Calogero *et al.*, 2009), or a combination of both. Another study of fertile women found that the risk of experiencing delayed conception for at least six months was significantly elevated among women who reported STS exposure (Hull *et al.*, 2000). The risk estimate for STS exposure was similar in magnitude to that found for women who actively smoked in the study.

Studies of the mechanisms of early pregnancy difficulties associated with STS exposure are fairly limited. Oxidative stress and DNA damage are plausible mechanisms due to the carcinogenic, mutagenic, and otherwise toxic constituents of STS. Human studies have found that increased FF cotinine levels were associated with a significant increase in follicular lipid peroxidation intensity (Paszkowski *et al.*, 2002) and an increased risk of DNA damage in granulose-lutein cells (Zenzes *et al.*, 1998). Decreased ovarian function and decreased number and quality of oocytes among smokers versus nonsmokers have also been reported (Van Voorhis *et al.*, 1996; Zenzes *et al.*, 1995). An early animal study of the effects of cadmium (a component of tobacco smoke) on reproduction reported that exposure resulted in an increased proportion of oocytes and embryos with chromosomal abnormalities and a decline in the number of oocytes reaching metaphase II (Watanabe *et al.*, 1979). Cadmium may also contribute to placental necrosis, slow trophoblastic development, and suppressed steroid biosynthesis and transfer of nutrient metals across

the placenta; all of which may contribute to implantation failure and early pregnancy loss (Thompson & Bannigan, 2008).

Because the present study only included couples undergoing IVF, the generalizability of our findings may be limited. Demographic characteristics of an IVF cohort are likely different from the general population. For example, IVF patients tend to be of a higher socioeconomic status due to the cost of treatment, and smoking rates and STS exposure may vary by socioeconomic status. If socioeconomic groups respond differently to STS exposure this could limit our generalizability. Also, infertile couples' gametes may be more sensitive to STS exposure. Another reason our results may have limited generalizability is because the IVF treatment process does not represent what occurs in natural pregnancy. For instance, fertilization occurs in a laboratory and only the best embryos are selected for transfer. In other words, our results would only be generalizable to similar populations if these conditions are associated with a differential response to STS exposure. However, there is no evidence to date that these factors are associated with differential sensitivity to STS exposure. In addition, implantation failure is not observable in other study designs conducted among the general population which typically rely on estimates of time-to-pregnancy.

Despite some potential limitations in generalizability, the present study has several strengths. To the best of our knowledge, it is the largest study to date on the effects of STS exposure, estimated through an objective biomarker, on fertility or early pregnancy among IVF patients. Thus, we may have been able to observe associations between

exposure and outcome that similar, smaller studies would be underpowered to detect. As mentioned above, the use of an IVF cohort also offers the advantage of being able to monitor stages of early reproduction that are otherwise unobservable in the general population. We were also able to leverage longitudinal data from couples who underwent multiple treatment cycles, which resulted in a more thorough analysis than previous studies that relied on only data from IVF patients' first treatment cycles.

Another strength lies in our use of FF cotinine to estimate STS exposure, including the use of FF cotinine concentrations measured at multiple points in women who underwent multiple IVF cycles. We hypothesize that cotinine in FF may be a more biologically relevant marker of STS exposure versus cotinine in urine or serum because it represents the developing oocytes' direct exposure to the constituents of tobacco smoke. When comparing FF cotinine concentrations with cotinine in urine from the same nonsmoking participants from our previous analysis (Meeker *et al.*, 2007a) we found that the measures were weakly correlated with one another (unpublished data). Self-reported STS exposure was also poorly predictive of FF cotinine concentrations in this cohort, possibly because many people may be unaware of or underreport their exposure. Thus, the present study may have been less susceptible to exposure misclassification compared to studies relying on urinary cotinine or other markers of STS exposure. Finally, since the study was conducted among IVF patients, it involved a motivated study group which likely resulted in a higher participation rate (65%) and, therefore, potentially less selection bias, than what would be achieved in a similarly invasive study among the general population.

In conclusion, we found a significant increase in the risk of implantation failure following IVF among women exposed to STS compared to those who were unexposed based on cotinine concentrations measured in the FF of the women at oocyte retrieval from repeated treatment cycles. We also observed a significant decrease in the odds of achieving a successful livebirth among STS exposed women. These findings are likely of great public health significance due to continued widespread STS exposure worldwide.

Tables

Table 3.1: Study demographics for 1909 self-reported nonsmoking women undergoing IVF who proceeded to embryo transfer

Female age at first cycle, mean (SD)	35.3 (4.3)
Race, n (%)[a]	
White	1,716 (89.9)
Non-white	191 (10.0)
Smoking status (self-report), n (%)	
Never smoker	1319 (69.1)
Ex-smoker	590 (30.9)
Primary infertility diagnosis, n (%)[b]	
Male factor	637 (33.4)
Ovulatory	230 (12.0)
Endometriosis	245 (12.8)
Tubal inflammation/occlusion	386 (20.2)
Cervical/uterine	66 (3.5)
Unexplained	342 (17.9)
Year of first cycle treatment, n (%)	
1994	5 (0.3)
1995	319 (16.7)
1996	340 (17.8)
1997	212 (11.1)
1998	6 (0.3)
1999	178 (9.3)
2000	195 (10.2)
2001	284 (14.9)
2002	267 (14.0)
2003	103 (5.4)

[a] Information on race was missing for two subjects.
[b] Information on primary infertility diagnosis was missing for three subjects.

Table 3.2: Outcome of IVF treatment cycles for 1909 self-reported nonsmoking women who proceeded to embryo transfer

Reason for failure	First cycles, n (%)	All cycles, n (%)
Failure of implantation (i.e., never achieved chemical pregnancy)	1013 (53.1)	1812 (55.4)
Failure of development		
Chemical pregnancy but never achieved clinical pregnancy	153 (8.0)	271 (8.3)
Clinical pregnancy was molar	1 (0.1)	1 (0.0)
Clinical pregnancy was ectopic	24 (1.3)	43 (1.3)
Clinical pregnancy was therapeutically aborted	2 (0.1)	3 (0.1)
Clinical pregnancy was spontaneously aborted	104 (5.4)	197 (6.0)
Fetus was stillborn	6 (0.3)	10 (0.3)
Successful livebirth	606 (31.7)	933 (28.5)
Total	1909 (100)	3270 (100)

Table 3.3: Odds ratios and risk ratios with 95% confidence intervals (CI) for implantation failure associated with female secondhand tobacco smoke exposure based on cotinine concentrations in follicular fluid

	Model	
	Crude	Adjusted[a]
First cycle only[b]		
OR, 95% CI (p-value)	2.23, 1.66-3.00 (<0.0001)	1.59, 1.17-2.17 (0.004)
RR	1.37, 1.25-1.51 (<0.0001)	1.17, 1.07-1.28 (0.0005)
All cycles[c]		
OR	1.93, 1.54-2.42 (<0.0001)	1.52, 1.20-1.92 (0.0005)
RR	1.31, 1.21-1.41 (<0.0001)	1.17, 1.10-1.25 (<0.0001)

[a] Adjusted for age, BMI, year of treatment, and down regulation protocol
[b] Female secondhand tobacco smoke exposure was present during 224 initial treatment cycles
[c] Female secondhand tobacco smoke exposure was present during 386 total treatment cycles

Table 3.4: Odds ratios and risk ratios with 95% confidence intervals (CI) for successful livebirths associated with female secondhand tobacco smoke exposure based on cotinine concentrations in follicular fluid

	Model	
	Crude	Adjusted[a]
First cycle only[b]		
OR[c], 95% CI (p-value)	0.48, 0.35-0.68 (<0.0001)	0.71, 0.50-1.02 (0.06)
All cycles[d]		
OR[e]	0.57, 0.44-0.74 (<0.0001)	0.76, 0.58-0.99 (0.045)
All cycles[d]		
OR[f]	0.57, 0.44-0.73 (<0.0001)	0.75, 0.57-0.99 (0.04)
RR[f]	0.66, 0.54-0.80 (<0.0001)	0.81, 0.66-0.99 (0.04)

[a] Adjusted for age, BMI, year of treatment, and down regulation protocol
[b] Female secondhand tobacco smoke exposure was present during 224 initial treatment cycles
[c] Calculated using logistic regression
[d] Female secondhand tobacco smoke exposure was present during 386 total treatment cycles
[e] Calculated using discrete survival analysis
[f] Calculated using a generalized estimating equation

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CHAPTER IV

Secondhand Tobacco Smoke Exposure is Associated with Serum Levels of Prolactin but not TSH among Women Seeking *In Vitro* Fertilization Treatment

Abstract

Prolactin (PRL) and thyroid stimulating hormone (TSH) serve important roles in the reproductive and other systems. Active smoking is associated with changes in PRL and TSH secretion, but the relationship between secondhand tobacco smoke (STS) exposure and these hormones is unclear. We measured serum PRL and TSH as well as cotinine in follicular fluid to estimate STS exposure among 337 women undergoing *in vitro* fertilization treatment. Our results demonstrated a significant increase in PRL concentrations ($p = 0.03$) among STS-exposed nonsmokers compared to unexposed nonsmokers. We did not observe a significant difference in TSH concentration ($p > 0.4$) among those exposed to STS compared to those who were unexposed. Future studies are needed to confirm our results, identify biological mechanisms involved, and better understand the potential clinical and public health implications.

Introduction

Prolactin (PRL) and thyroid stimulating hormone (TSH; also known as thyrotropin) are important reproductive hormones. PRL is secreted by the anterior pituitary and was originally identified by its ability to stimulate mammary gland development and lactation. We now know that it is involved in over 300 separate actions in various vertebrates, including effects on reproduction, growth and development, metabolism, water and electrolyte balance, brain and behavior, and immunoregulation (Bole-Feysot et al., 1998). The largest group of actions for PRL pertains to reproductive processes.

TSH is also secreted by the anterior pituitary, and it stimulates the thyroid gland to produce and secrete thyroid hormones. TSH is regulated via negative feedback from thyroid hormones. Normal thyroid function is an important component of reproductive health. In females, thyroid dysfunction has been linked to menstrual disturbances, reduced fertility, spontaneous abortion and various late-pregnancy outcomes, including preterm birth and low birth weight (Krassas et al., 2010). Proper thyroid function is important to many other processes, as well, including energy balance, metabolism, and other functions in the nervous, cardiovascular, and pulmonary systems.

Studies have examined the effects of active smoking on TSH and thyroid function. McDonald et al. (2008) found that women who smoke during pregnancy had significantly lower TSH levels than nonsmokers. Triiodothyronine (T3) was not measured in that study, but free thyroxine (T4) concentrations did not differ between exposure groups,

neither did cord blood TSH concentration from infants born of smokers compared to infants of nonsmokers (McDonald et al., 2008).

Shields et al. (2009) later confirmed some of these findings. They also observed lower TSH concentrations in serum among pregnant smokers compared to nonsmokers and no significant difference in free T4 concentrations between exposure groups; though they did find significantly higher median free T3 concentrations among smoking mothers as well as significantly lower cord serum TSH concentrations in babies born to smoking mothers compared to those whose mothers were nonsmokers.

Active smoking is also associated with changes in PRL concentrations; though studies have had differing results. One study found a significant increase in PRL concentrations among men who were active smokers compared to nonsmokers (Xue et al., 2010). Two other studies reported increases and decreases, respectively, in PRL concentrations among animals exposed to tobacco smoke (Ng et al., 2006; Muraki et al., 1979).

Data is limited on the effects of STS exposure on TSH and PRL concentrations. Several studies have shown that exposure can disrupt the thyroid (Carrillo et al., 2009; Soldin et al., 2009; Flouris et al., 2008), but to our knowledge no studies to date have examined the relationship between STS exposure and PRL concentrations. Thus, the present study is intended to increase our understanding of the relationship between STS exposure and circulating TSH and PRL. We hypothesized that STS exposure is associated with increased serum levels of PRL and decreased serum TSH.

Methods

Study population

Subjects for the present study are a subset of a larger, ongoing study examining predictors of IVF success, including STS exposure, and have been previously described (Meeker et al., 2007; Cramer et al., 2003). Briefly, in the larger study, couples undergoing IVF or intracytoplasmic sperm injection (ICSI) between 1994-1998 (study 1) and 1999-2003 (study 2) were recruited through three Boston-area clinics to study predictors of IVF success. Protocols were approved by the Human Research Committees at Brigham and Women's Hospital, the Harvard School of Public Health, and the University of Michigan. Approximately 65% of couples approached agreed to participate in the study. Couples excluded from the study were those who were gestational carriers or who underwent gamete intra-fallopian transfer (GIFT), as well as those who required donor oocytes or donor semen. After exclusions, 2,350 couples who underwent from one to six IVF/ICSI treatment cycles were enrolled in the parent study. A self-administered questionnaire was used to obtain information from each subject on medical history and lifestyle factors such as: demographics, ages of both male and female partner, medical and reproductive history, smoking history, and duration of infertility. Information on IVF treatment and outcome was abstracted from clinical records. 337 patients for whom a blood sample was analyzed for PRL and TSH and for whom first-treatment-cycle follicular fluid (FF) was analyzed for cotinine were included in the present analysis.

Hormone measurement

When possible, a basal blood sample was collected from study participants. This sample was taken sometime during days one through five of the menstrual cycle and designated as the "true baseline." When a blood sample timed with the menses could not be collected, a sample was collected before IVF treatment began and was designated the "initial" specimen. Samples were aliquoted and stored at -80 degrees Centigrade. PRL and TSH were measured in archived serum samples using the AxSYM Immunoassay system (Abbott Diagnostics, Chicago, IL), which was described previously (Cramer et al., 2003). Briefly, the tests for PRL and TSH are solid-phase double antibody enzyme immunoassays employing microparticle enzyme immunoassay (MEIA) technology. For PRL, the limit of detection (LOD) was 0.6 ng/ml and assay performance was monitored using three quality control sera (Abbott Diagnostics). The coefficients of variation (CV) for PRL in the three control sera were 8.3, 6.8, and 4.8%. TSH was analyzed using the MEIA technology (Ultrasensitive hTSH II). TSH levels were quantified as µIU/ml based on assay calibrators standardized using the World Health Organization TSH 80/558. The LOD was 0.03 µIU/ml. TSH assay performance was also monitored using three quality control sera and the CV were 7.1, 6.2, and 7.4%.

Cotinine measurement

Physicians and technicians were asked to retain FF during egg retrieval for each IVF cycle. FF was aspirated from follicles using a 16-gauge needle and constant suction from a Rocket pump apparatus. Fluid was collected from the largest visible follicle before using any flushing medium and then transferred to a sterile Petri dish. Oocytes were

scanned for and removed. The fluid, normally discarded at this point, was placed into a 15 ml conical tube and centrifuged for 15 minutes. The supernatant was placed into a clean storage tube, labeled, refrigerated, and transferred to the Brigham and Women's Hospital laboratory within 12 hours. At the laboratory, the specimens were aliquoted into 2 ml specimens and frozen at -80 degrees centigrade. FF was analyzed for cotinine using a quantitative enzyme-linked immunosorbent assay (ELISA; BioQuant, Inc., San Diego, CA). This single-step, competitive test uses spectrometric measurement to determine cotinine in body fluids. It has a lower reporting limit of 0.3 ng/ml and inter- and intra-assay variations of four and six percent, respectively.

Statistical Methods

Data analysis was performed using SAS software (version 9.2; SAS Institute Inc., Cary, NC). Quantified cotinine concentrations below the LOD were kept as the reported value. Unquantified cotinine concentrations were assigned a value of one half of the LOD. All TSH and PRL levels were quantified and those below the LOD were also kept as the reported value. Since serum samples were collected before any treatment, we matched subject's hormone concentrations to the cotinine concentration measured in first-treatment-cycle FF samples.

Methods for categorizing tobacco smoke exposure have been previously described (Benedict et al., 2011). Briefly, we considered active smokers to be those participants with FF cotinine concentrations greater than or equal to 10 ng/ml, following Fuentes et al. (2010). Passive smokers, or STS-exposed nonsmokers, were those with FF cotinine

concentrations less than 10 ng/ml and greater than 1.11 ng/ml. This value was extended to our data from a urinary cotinine cutpoint established by Zielinska-Danch et al. (2007). We defined unexposed nonsmokers as those with cotinine concentrations less than or equal to 1.11 ng/ml. For comparison we also defined exposure based on the LOD of the cotinine assay. In this approach to classifying exposure, participants whose cotinine concentration was less than or equal to 0.15 ng/ml (½ the LOD) were considered unexposed. Those whose cotinine concentrations were greater than 0.15 ng/ml and less than 10 ng/ml were classified as exposed nonsmokers. Like the former approach, those with FF cotinine concentrations greater than or equal to 10 ng/ml were considered active smokers.

In preliminary analyses, Spearman correlation coefficients were calculated between FF cotinine concentrations, TSH, and PRL. Due to a large proportion of non-detect cotinine measurements, the distributions of TSH and PRL were examined and stratified by smoking status based on both published cotinine cutpoints and the LOD, for comparison. Analysis of variance (ANOVA) was performed to determine if hormone levels differed between smoking groups.

The relationships between exposure and outcome variables and key covariates were examined. PRL and TSH concentrations were transformed by the natural logarithm. Multiple linear regression was then used to examine the relationship between hormone concentrations and STS exposure categories (i.e. STS-exposed vs. unexposed). Regression was first performed when using published cotinine cutpoints to classify

exposure, followed by using the cotinine assay LOD, for comparison. Covariates considered for inclusion in adjusted models were age, BMI, primary infertility diagnosis, date that the treatment cycle commenced, study number, timing of blood sample collection, and ethnicity. Covariates were included in the final models depending on biological and statistical considerations (Hosmer & Lemeshow, 1989). For example, PRL levels changed significantly with fertility diagnoses among study participants (Table 3). Also, moderately elevated TSH concentrations are frequently found in obese humans (Reinehr, 2010), hyperprolactinemia is associated with weight gain and obesity (Shibli-Rahhal & Schlechte, 2009), and STS exposure may be associated with BMI (Braun et al., 2010; Kwok et al., 2010). Thus, BMI was included in adjusted models. We also included participant age and year of treatment in our final models since the study spanned 10 years (US population STS exposure levels have declined over time) and since PRL levels in women decrease steadily with age (Vekemans & Robyn, 1975).

Due to the large number of non-detect cotinine concentrations in FF, we also examined the relationship between hormone levels and self-reported exposure. Self-reported exposure was obtained through the questionnaire. Participants were asked about their smoking behavior and STS exposure at home or at work and were considered exposed if they reported any STS exposure. The same covariates were included in both sets of regression models (i.e. when categorizing exposure based on FF cotinine and self-report) to maintain consistency.

Results

Among 337 participants, 32 percent had first cycle cotinine concentrations above the LOD Hormone concentrations were quantified in all women. In preliminary analyses, we found a statistically suggestive difference ($p = 0.05$) in PRL concentrations between active smokers, STS-exposed nonsmokers and unexposed nonsmokers based on FF cotinine concentrations (Table 4.1). A student's t-test clarified that this difference in PRL levels was greatest between STS-exposed and unexposed nonsmokers ($p = 0.05$).

Table 4.2 displays results from our initial correlation analysis. There was a significant, positive correlation ($r \geq 0.23$; $p \leq 0.04$) between cotinine and PRL concentrations among those exposed to STS when either exposure classification method is employed (i.e. when exposures are grouped by either published cutpoints or the LOD). No significant correlation was observed between TSH and cotinine among all participants, among non-smoking participants only, or among STS-exposed non-smokers. TSH and PRL levels were significantly and positively correlated with one another ($r = 0.23$; $p < 0.0001$).

In regression models adjusted for covariates, we observed a significant increase in PRL levels ($p = 0.03$) among STS-exposed nonsmokers compared to unexposed nonsmokers based on published FF cotinine cutpoints (Table 4.4). However, only a suggestive difference in PRL ($p = 0.10$) was observed between exposure groups when using the cotinine assay LOD to categorize exposure. No difference in TSH levels was observed between groups ($p > 0.4$).

Because of the large proportion of non-detect FF cotinine concentrations and because cotinine concentrations measured at a given time may only reflect recent exposure to tobacco smoke, we sought to test the observed increase in PRL levels among STS-exposed nonsmokers through self-reported STS exposure. Using self-report to categorize exposure, we found a suggestive increase ($p = 0.09$) in PRL among those who reported exposure to STS compared to women who didn't report exposure (Table 4.5). As with the analysis using FF cotinine concentrations, no significant change in TSH was observed when using self-report to categorize STS exposure.

Discussion

The aim of the present study was to explore the relationship between STS exposure and serum PRL and TSH concentrations in women who participated in a study on predictors of IVF success. As far as we are aware, this is the first study examining the effects of STS exposure on circulating PRL levels in humans. In adjusted models, we observed a significant increase in PRL concentrations among women exposed to STS compared to those unexposed based on cotinine concentrations measured in FF. The relationship between STS exposure and PRL was stronger when using a published cutpoint compared to using the cotinine analytical method LOD to categorize exposure. To further assess the results of these analyses, we conducted a sub-analysis using self-report to categorized exposure. There was a statistically suggestive increase in circulating PRL levels among subjects who self-reported STS exposure, but the association was weaker compared to the use of cotinine concentrations in FF to categorize exposure. The stronger relationships observed when using published cotinine cutpoints, compared to the cotinine method LOD

or self-reported exposure, suggests it was the exposure categorization approach associated with the least amount of misclassification.

Similar to the findings of the present study, Xue et al. (2010) recently reported that tobacco smoke exposure, measured through plasma nicotine, was correlated with increased PRL levels ($r = 0.53$; $p < 0.05$). However, that study was conducted among men who were active smokers. Conversely, in another human study, Mello et al. (2001) reported that smoking mothers have reduced breast milk production and shorter lactation periods, findings which suggest that exposure to tobacco smoke may reduce PRL levels.

Animal studies on tobacco smoke and PRL have had conflicting results, as well. Decreased PRL levels were observed in female rats exposed to tobacco smoke from one to four cigarettes over a 90-minute period (Andersson et al., 1988). A study of the effects of tobacco smoke on gestational hormone levels among pregnant mice, however, found a suggestive increase ($p = 0.07$) in PRL levels among mice exposed to mainstream cigarette smoke compared to those that were unexposed (Ng et al., 2006).

Tobacco smoke-induced changes in PRL levels may depend on species and/or gender (Shaw & al'Absi, 2010; Andersson et al., 1988), which could explain some of the conflicting results in the literature. In addition, endocrine responses to tobacco smoke exposure may differ depending on the duration, frequency and magnitude of exposure. Fuxe et al. (1989) reported that the initial effects of nicotine are characterized by a marked hypersecretion of PRL (which rapidly disappears) and that PRL secretion is

inhibited with acute, intermittent nicotine treatment or exposure to cigarette smoke. This reduced PRL secretion due to tobacco smoke exposure is found mainly in chronic, habitual smokers.

The rapid disappearance of the acute stimulatory effects of nicotine may be due to a desensitization of central nicotinic cholinergic receptors from higher-level exposure during active smoking. Evidence also indicates that the inhibitory effects of nicotine on PRL secretion are produced through an activation of dopamine neurons by nicotinic receptors (Fuxe et al., 1989). In other words, chronic and/or high-level nicotine exposure may induce the release of dopamine, which inhibits PRL secretion. On the other hand, STS exposure may not be sufficient to desensitize nicotinic cholinergic receptors and/or activate dopamine neurons, which could result in elevated PRL concentrations. It should also be noted that one or several of the many constituents of tobacco smoke other than nicotine may be responsible for the observed relationship between exposure and changes in PRL secretion.

The findings of increased PRL concentrations among those exposed to STS versus those unexposed may have implications for a range of conditions. For example, hyperprolactinemia may be a cause of infertility in women with endometriosis (Gregoriou et al., 1999). In addition, epidemiology studies recently reviewed by Bernichtein et al. (2010) indicate that high levels of circulating PRL may be a risk factor for breast cancer. Data from two large prospective case-control studies (Nurses' Health Studies; NHS and NHS II) demonstrated a 40% increase in breast cancer risk for pre-

menopausal women with PRL concentrations in the highest versus the lowest quartile of normal range (*p trend* = 0.05; Bernichtein et al., 2010). A 30% increase in breast cancer risk was observed among post-menopausal women (*p trend* = 0.01). There is also increasing evidence that locally-produced PRL (i.e. PRL expressed by human tissues other than the anterior pituitary, such as the mammary glands, the prostate, the skin, the brain and adipocytes) is associated with breast and prostate tumor growth (Bernichtein et al., 2010; Ben-Jonathan et al., 1996).

Studies have linked STS exposure with breast cancer, but there may not be sufficient evidence to infer a causal relationship. At least 21 studies have investigated the association between STS exposure and breast cancer risk among lifetime nonsmokers and these were recently reviewed (Centers for Disease Control and Prevention, 2006). Results of these studies varied, but considered collectively in a meta-analysis, breast cancer risk in lifetime nonsmokers was significantly associated with STS exposure. After stratification by menopausal status the association only remained significant among premenopausal women. Thus, it is possible that an association between PRL hypersecretion and STS exposure may increase breast cancer risk. If so, our results showing increased PRL in relation to STS exposure may provide important information on biological mechanisms in the discussion of previous reports of STS exposure and increased risk of breast cancer.

In the present study we did not observe associations between exposure to tobacco smoke and serum TSH levels. No difference in TSH levels was found between exposure groups

based on FF cotinine concentrations or based on self-reported exposure. Another study among 237 women aged 18-44 years, however, found a significant decrease ($p < 0.05$) in TSH concentrations among passive smokers compared to nonsmokers defined by serum cotinine (Soldin et al., 2009). The same study also reported a significant decrease ($p < 0.01$) in TSH levels in active smokers compared to nonsmokers, but no significant difference in TSH levels between active and passive smokers.

Because participants for the present study were IVF patients, the generalizability of our findings may be limited. For example, elevated levels of circulating PRL may be a cause of infertility (Wang et al., 2009). Thus, IVF patients may tend to have higher levels of circulating PRL than the general population, regardless of STS exposure. Also, demographic characteristics of an IVF cohort are likely different from the general population. If PRL is associated with socioeconomic status, for example, this could limit our generalizability. Another potential limitation of the present study is a relatively small sample size. Future studies with a larger number of participants exposed to STS may be needed to support our findings.

A strength of the present study was the use of a precise biomarker to estimate STS exposure. However, our choice of biomarker may have influenced our results. FF cotinine data was accessible from a larger study among these women on implantation failure and IVF success. Cotinine concentrations in FF represent the fraction of plasma cotinine that diffused through the blood-follicle barrier and largely depend on concentrations gradients between the blood plasma, interstitial fluid and cells surrounding

the oocytes (Benedict et al., 2011). Thus, future research could explore the relationship between PRL secretion and STS exposure based on markers of exposure (e.g. serum cotinine) that may be more biologically relevant. However, based on two previous reports, cotinine in serum and FF is highly correlated ($R^2 = 0.95$ and $R^2 = 0.89$; Fuentes et al., 2010; Paszkowski, 1998). Thus, a high degree of tobacco smoke exposure measurement error in the present study was not likely. In addition, the strength of the relationship between PRL and STS exposure based on published cutpoints ($p = 0.03$) was stronger than the relationships observed when using the LOD or self-report to classify exposure ($p = 0.10$ and 0.09, respectively), which suggests that using the cutpoints may have resulted in less exposure misclassification. Moreover, the suggestive increase we observed in PRL concentrations among self-reported STS exposed women compared to those reporting no STS exposure further supports our conclusions.

In conclusion, we did not observe a significant difference in TSH concentration among those exposed to STS compared to those who were unexposed. However, our results indicate that STS exposure is associated with increased circulating PRL. This finding could have large public health significance due to the range of downstream adverse health effects potentially related to altered PRL levels. Future studies are needed to confirm our results, identify biological mechanisms involved, and better define the potential clinical and public health implications.

Tables

Table 4.1: Distributions of serum thyrotropin (TSH) and prolactin (PRL) concentrations by smoking group for women undergoing IVF treatment

		TSH (uIU/ml)								PRL (ng/ml)							
Smoking Group	N	Geometric mean	Min	25th	50th	75th	95th	Max	*p* value[1]	Geometric mean	Min	25th	50th	75th	95th	Max	*p* value
Overall	337	1.72	0.012	1.27	1.74	2.42	4.14	22.3		14.0	0.56	9.88	13.4	20.5	33.7	66.1	
Smokers[2]	23	1.47	0.41	1.03	1.38	1.91	3.70	3.85		11.9	4.76	8.87	11.8	14.8	23.5	46.4	
Exposure based on published cutpoints									0.42								0.05[7]
Exposed[3]	69	1.75	0.39	1.32	1.74	2.42	3.88	7.04		15.9	3.87	10.7	16.6	23.7	40.7	54.7	
Unexposed[4]	245	1.75	0.012	1.28	1.79	2.43	4.17	22.3		13.7	0.56	9.88	13.2	19.5	32.7	66.1	
Exposure based on the limit of detection									0.28								0.12
Exposed[5]	84	1.81	0.39	1.34	1.82	2.43	3.98	7.04		15.5	2.85	10.6	15.3	23.6	38	54.7	
Unexposed[6]	230	1.72	0.012	1.25	1.74	2.43	4.29	22.3		13.7	0.56	9.88	13.2	19.4	33	66.1	

[1] From ANOVA (denotes whether there is significant difference in TSH or PRL concentrations between active smokers, STS-exposed nonsmokers, and unexposed nonsmokers)
[2] Follicular fluid (FF) cotinine concentration greater than or equal to 10 ng/ml, following Fuentes et al. (2008)
[3] Secondhand-tobacco-smoke-exposed nonsmokers; FF cotinine concentration greater than or equal to 1.11 ng/ml and less than 10 ng/ml
[4] Unexposed nonsmokers; FF cotinine concentration less than 1.11 ng/ml following Benedict et al. (2011)
[5] FF cotinine concentration greater than ½ the limit of detection (0.15 ng/ml) and less than 10 ng/ml
[6] FF cotinine concentration less than or equal to ½ the limit of detection
[7] Pooled t-test *p*-value for difference in PRL concentrations between exposed and unexposed nonsmokers was 0.05 with equal variance (folded F *p*-value = 0.77)

Table 4.2: Spearman correlation coefficients between follicular fluid cotinine and serum thyrotropin (TSH) or serum prolactin (PRL) for women undergoing IVF treatment

	N	TSH (uIU/ml)	PRL (ng/ml)
Overall	337		
Correlation		-0.050	0.026
p value		0.36	0.64
Smokers[1]	23		
Correlation		-0.35	-0.13
p value		0.11	0.56
Exposure based on published cutpoints			
Exposed NS[2]	69		
Correlation		-0.078	0.25
p value		0.52	0.04
Unexposed NS[3]	245		
Correlation		0.018	-0.032
p value		0.78	0.62
Exposure based on the limit of detection			
Exposed NS[4]	84		
Correlation		-0.13	0.23
p value		0.20	0.02
Unexposed NS[5]	230		
Correlation		-0.070	-0.054
p value		0.31	0.43

[1] Follicular fluid (FF) cotinine concentration greater than or equal to 10 ng/ml following Fuentes et al. (2008)
[2] Exposed nonsmokers; FF cotinine concentration greater than or equal to 1.11 ng/ml and less than 10 ng/ml
[3] Unexposed nonsmokers; FF cotinine concentration less than 1.11 ng/ml following Benedict et al. (2011)
[4] FF cotinine concentration greater than ½ the limit of detection (0.15 ng/ml) and less than 10 ng/ml
[5] FF cotinine concentration less than or equal to ½ the limit of detection

Table 4.3: TSH and prolactin (PRL) distributions stratified by key covariates for 337 women undergoing IVF treatment

	N (%)	Smokers[1], N (%)	STS Exposed[2], N (%)	Unexposed [3], N (%)	TSH (uIU/ml), Median (25^{th}, 75^{th})	PRL (ng/ml), Median (25^{th}, 75^{th})
Fertility diagnosis						*p* value = 0.02 [4]
Unexplained	48 (14)	3 (13)	7 (10)	38 (16)	1.63 (1.14, 2.37)	10.8 (8.87, 16.6)
Male factor	108 (32)	4 (17)	25 (36)	79 (32)	2.01 (1.40, 2.60)	14.8 (10.6, 21.5)
Ovulatory	41 (12)	2 (9)	8 (12)	31 (13)	1.44 (1.03, 2.04)	12.0 (9.88, 16.5)
Endometriosis	43 (13)	0 (0)	10 (14)	33 (13)	1.79 (1.17, 2.39)	13.7 (10.6, 24.0)
Tubal	87 (26)	14 (61)	15 (22)	58 (24)	1.69 (1.30, 2.42)	13.9 (9.88, 21.7)
Cervical/uterine	10 (3)	0 (0)	4 (6)	6 (2)	1.42 (1.25, 1.78)	9.38 (8.32, 23.7)
Study						
1	200 (59)	15 (65)	51 (74)	134 (55)	1.81 (1.31, 2.43)	13.4 (9.93, 20.4)
2	137 (41)	8 (35)	18 (26)	111 (45)	1.62 (1.21, 2.29)	13.4 (9.48, 21.1)
Sample time						
True baseline	144 (43)	13 (57)	23 (33)	108 (44)	1.61 (1.13, 2.19)	12.9 (9.80, 18.4)
Initial	193 (57)	10 (43)	46 (67)	137 (56)	1.86 (1.37, 2.51)	13.7 (9.92, 21.6)
Race						
White	316 (94)	21 (91)	65 (94)	230 (94)	1.75 (1.27, 2.42)	13.2 (9.76, 19.8)
Non-white	21 (6)	2 (9)	4(6)	15 (6)	1.72 (1.22, 2.20)	15. 8 (12.2, 23.4)
Total						
N (%)	337	23 (7)	69 (20)	245 (73)		

[1] Active smokers; follicular fluid (FF) cotinine concentration greater than or equal to 10 ng/ml, following Fuentes et al. (2008)
[2] Secondhand-tobacco-smoke-exposed nonsmokers; FF cotinine concentration greater than or equal to 1.11 ng/ml and less than 10 ng/ml
[3] Unexposed nonsmokers; FF cotinine concentration less than 1.11 ng/ml following Benedict et al. (2011)
[4] Change in ln-PRL levels with fertility diagnosis based on ANOVA (no significant change in TSH or PRL was observed between any of the other covariates groups)

Table 4.4: Adjusted[1] regression coefficients and 95% confidence intervals for change in ln-serum hormone concentrations associated with secondhand tobacco smoke exposure[2] among 314 nonsmoking women undergoing IVF treatment

	Published cutpoint, n_{exp} = 69			Limit of detection, n_{exp} = 84		
	parameter estimate	95%CI	p-value	parameter estimate	95%CI	p-value
TSH	-0.013	-0.18, 0.16	0.88	0.057	-0.097, 0.21	0.46
Prolactin	0.16	0.013, 0.32	0.03	0.12	-0.023, 0.25	0.10

[1] Adjusted for age, BMI, primary fertility diagnosis and year of IVF treatment
[2] Exposure groups defined by cotinine concentrations in follicular fluid
n_{exp} = Number exposed to secondhand tobacco smoke

Table 4.5: Adjusted[1] regression coefficients and 95% confidence intervals for change in ln-serum hormone concentrations associated with self-reported secondhand tobacco smoke exposure[2] among 314 nonsmoking women undergoing IVF treatment

	Parameter estimate	95% CI	p-value
TSH	-0.14	-0.35, 0.072	0.20
Prolactin	0.16	-0.026, 0.35	0.09

[1] Adjusted for age, BMI, primary fertility diagnosis and year of IVF treatment
[2] Number reporting secondhand tobacco smoke exposure = 40

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CHAPTER V

Conclusion

The present work used a large cohort of IVF patients and cotinine concentrations measured in follicular fluid (FF) to study the effects of female secondhand tobacco smoke (STS) exposure on early pregnancy and endocrine function. Our first aim was to determine the agreement between cotinine measures in FF and urine among STS exposed women and the intra-individual variability of FF cotinine measures over time among women who underwent multiple IVF treatment cycles. We hypothesized poor agreement between cotinine concentrations in FF and urine and high with-person FF cotinine variability over time. Our second aim was to examine the relationship between female STS exposure estimated through FF cotinine and implantation failure and overall IVF success (i.e. odds of a livebirth). We hypothesized that STS exposure was associated with an increased risk of implantation failure and reduced odds of achieving a successful livebirth. Our final aim was to study the effects of STS exposure on prolactin (PRL) and thyroids stimulating hormone (TSH) concentrations. We hypothesized an increase in circulating PRL and a decrease in circulating TSH among STS-exposed women compared to those who were unexposed.

In the first portion of this work, we compared cotinine concentrations in FF and urine samples and examined the variability of FF cotinine measures over time. Among

nonsmoking IVF patients, we found a weak relationship between cotinine levels in paired FF and urine samples. FF cotinine was a more temporally relevant biomarker than urinary cotinine in the present study because FF samples were collected during ART treatment, while urine samples were often collected before treatment. Since FF is also likely a more biologically relevant sample matrix when assessing STS exposure's effects on female reproduction related to oocyte development, fertilization, and early pregnancy, our results provide evidence that measuring cotinine in FF may be a more appropriate biomarker of low-level tobacco smoke exposure in studies of fertility and early pregnancy.

To our knowledge, this is the first study to compare cotinine concentrations in FF and urine samples, and only one other study has compared inter-fluid cotinine concentrations exclusively among nonsmokers. After dosing subjects with nicotine levels intended to simulate STS exposure, Benowitz et al. (2009) reported a strong correlation ($R^2 = 0.84$) between cotinine in blood plasma and creatinine-adjusted urine. This correlation is much stronger than what we observed. However, since the quantitative relationship between cotinine in blood plasma and FF among nonsmokers is unknown, it is difficult to compare results from this study and our own.

Studies which included active smokers have found a strong correlation ($R^2 \geq 0.89$) between cotinine in FF and serum (Fuentes et al., 2010; Paszkowski, 1998). However, agreement between cotinine in FF and serum among nonsmokers may not be nearly as strong, since cotinine's diffusion into FF is dependent upon concentration gradients

between the blood, FF and interstitial fluids. Among smokers, gradients will likely be strong, pushing cotinine downgradient out of the blood and into FF and other tissues. Future studies should examine the relationship between cotinine in FF and serum concentrations among STS-exposed nonsmokers in order to further validate FF cotinine as a biomarker of STS exposure and to further elucidate cotinine's distribution to various tissues in the body when concentrations are low.

Since the half-life of cotinine in body fluids is relatively short, a single cotinine measure may only reflect exposure over the past 2-3 days. Thus, the first chapter of this dissertation also presents an examination of within-subject variability in FF cotinine concentrations. This was done to assess the reliability of FF cotinine concentrations (i.e. to determine how many samples may be needed to estimate longer-term STS exposure). We found moderate to high within-subject variability in FF cotinine concentrations when assessing concentrations as a continuous variable. Our ICC analyses showed fair to poor reproducibility in FF cotinine levels over time, demonstrating the need for multiple FF samples to accurately predict long-term STS exposure.

On the other hand, when using FF cotinine concentrations to categorize women as exposed or unexposed, we found a high level of agreement in sensitivity analyses. Thus, if broad exposure categories are used as opposed to continuous FF cotinine concentrations, there may be a smaller degree of exposure misclassification when using a single FF sample to estimate exposure over a longer period of time. However, in our analysis, sensitivity may have been overestimated because we used subjects' geometric

mean cotinine concentrations as their "gold standard" exposure measure. In other words, the predicted measurements, cotinine measures from a single FF sample, are not independent of the standard against which they are being compared. The lack of independence between these two variables may have increased the observed levels of agreement when using cotinine to categorize exposure. Similar to the agreement analysis between FF and urinary cotinine, this is also the first study of its kind. In other words, it is the first study to assess the temporal variability of FF cotinine concentrations in repeat samples over time. Additionally, this chapter proposes a STS exposure cutpoint for cotinine concentrations in FF (1.11 ng/ml). No cutpoint of this type had been published previously.

Another study examined the temporal variability of cotinine in urine samples from children whose parents smoked (Matt et al., 2007). That study found that within-subject cotinine variability increases with time. In other words, a single measure's accuracy as an estimate of exposure decreases as time increase. Thus, in future work, collecting and conducting cotinine analysis on multiple biological samples over time should improve the accuracy of long-term STS exposure estimates.

Chapter 2 presents the main study in this body of work, which is an analysis of the effects of female STS exposure on embryo implantation and overall IVF success. We found an increased risk of failed implantation among women exposed to STS versus those who were unexposed based on cotinine concentrations measured in FF. As a secondary aim, we also examined the relationship between STS exposure and the odds of a successful

livebirth as a potentially more clinically-relevant effect measure. In our adjusted analysis, women exposed to STS were less likely to have a successful livebirth compared to those who were unexposed.

The relationship between STS exposure and implantation failure in this population was examined in two previous studies that utilized different exposure measurement methods from the present study. An analysis among 921 women who had urine samples available for cotinine measurement found that creatinine-adjusted cotinine levels in urine were associated with a slight decrease in first-cycle implantation rates among nonsmoking women (11.1% in the lowest cotinine quintile versus 8.2% in the highest quintile; p = 0.13; Meeker et al., 2007a). However, in a multivariate analysis, creatinine-adjusted cotinine levels were not associated with failed implantation. Shortly thereafter, a much larger follow-up study among all non-smoking participants in the study found a suggestive association between self-reported STS exposure and failed implantation (Meeker et al., 2007b).

Since we observed poor agreement between cotinine concentrations in urine and FF (presented in Chapter 1), we believe that urinary cotinine may not accurately represent STS exposure in studies of early pregnancy. Thus, the null implantation failure findings based on creatinine-adjusted cotinine concentrations presented by Meeker et al. (2007a) may have been due to exposure measurement error and exposure misclassification, since these tend to bias effect estimates toward the null.

Cotinine measured in FF reflects the extent to which the oocyte was directly exposed to the constituents of tobacco smoke during its late development. Thus, FF cotinine is a more biologically relevant exposure measure compared to self-report and urinary cotinine concentrations. Therefore, we believe we have improved upon the earlier work by Meeker et al. and that our current findings more likely represent the true relationship between STS and implantation failure.

The results from a study among 255 women undergoing IVF or ICSI agree with our present findings. Neal et al. (2005) reported significant declines in implantation and pregnancy rates among STS-exposed women compared to unexposed women based on self-reported exposure. An association between STS exposure and other early pregnancy difficulties has been demonstrated in several studies. A study of fertile women found that the risk of experiencing delayed conception for at least six months was significantly elevated among women who reported STS exposure (Hull et al., 2000). Peppone et al. (2009) reported that STS exposure was associated with fetal losses and reduced fecundity (the ability to become pregnant and carry a child full-term).

A recent meta-analysis reported that STS exposure significantly increased the risk of stillbirth (pooled odds ratio [OR] = 1.23; 95% confidence interval [CI] = 1.09-1.38) and congenital malformation (pooled OR = 1.13; 95% CI = 1.01-1.26), based on four and seven studies, respectively (Leonardi-Bee et al., 2011). The same study, however, found no evidence of a statistically significant increase in the risk of spontaneous abortion among those exposed to STS compared to those unexposed (pooled OR = 1.17; 95% CI =

0.88-1.54) based on a pooled analysis of six studies. In the present study, we did not have enough data to examine other pregnancy endpoint of interest (e.g. failed fertilization, spontaneous abortion). Thus, because FF cotinine may be an improved biomarker of low-level tobacco smoke exposure, future studies using cotinine in FF, or, depending on study design, cotinine in other biologically relevant and available matrices, should be conducted to determine if STS exposure also adversely affects some of these other early pregnancy endpoints.

Future work should also seek to determine if early-life exposure to STS increases the risk of reproductive difficulties in adulthood. Jensen et al. (2006) found that women who were exposed to maternal active smoking *in utero* are more likely to experience reduced fecundity later in life. Declined semen quality has also been observed in studies among men who were exposed to active maternal smoking *in utero* (Jensen et al., 2005; Storgaard et al., 2003).

At least three studies have reported an increased risk of pregnancy difficulties based on childhood STS exposure. Peppone et al. (2009) reported that women whose parents smoked had more difficulty becoming pregnant and were more likely to experience fetal losses. Another study found that exposure to STS from parents as a child was associated with a significant increase in the risk of spontaneous abortion later in life (Meeker et al., 2007a). A follow-up study with a much larger cohort found a dose-dependent increase in odds of spontaneous abortion when 0, 1, or 2 parents smoked (Meeker et al., 2007b).

Exposure assessment strategies for studies such as these, however, are prone to errors, since self-report is often the only feasible means of estimating childhood exposures. Thus, long-term (e.g. >20 years) longitudinal studies may be required to obtain objective measures of exposure (e.g. biomarkers) to STS during childhood. Future studies should also examine the latent effects of STS exposure during pregnancy on the offspring. For example, long-term studies could be designed to, perhaps, measure maternal exposure to STS while pregnant via cotinine; then, determine if there is a relationship between those maternal STS exposures and the offspring's reproductive health later in life.

There is some evidence that STS exposure can be just as detrimental to reproductive health as active smoking. A study of delayed conception reported similar risk estimates among STS-exposed women and women who actively smoke (Hull et al., 2000). Also, Neal et al. (2005) reported very similar declines in implantation and pregnancy rates between women exposed to STS and women who were active smokers. More studies are needed, however, to elucidate the difference in risk of adverse pregnancy outcomes between active smokers and STS-exposed nonsmokers.

In the final portion of the present work, we examined the relationship between STS exposure and prolactin (PRL) and thyroid stimulating hormone (TSH) concentrations. Other studies have evaluated this relationship among active smokers, but, to the best of our knowledge, this is the first study to evaluate changes in PRL among STS-exposed nonsmokers. We observed a significant increase in PRL concentrations among women exposed to STS compared to those unexposed. There is a fair amount of disagreement

among the findings of studies on this topic, and it is unclear whether exposure to tobacco smoke increases or decreases PRL concentrations. A study among men who were active smokers found a positive correlation between plasma nicotine and PRL levels (Xue et al., 2010). Another study, however, reported that smoking mothers had shorter lactation periods and reduced breast milk production, indicating that PRL levels were likely reduced among these active smokers (Mello et al., 2001). Meanwhile, an increase in PRL levels was observed among pregnant mice exposed to tobacco smoke compared to those unexposed (Ng et al., 2006). Conversely, among rats, nicotine exposure is associated with decreased PRL levels (Andersson et al., 1985; Muraki et al., 1979).

These differences in study results may be due to differing physiological responses (e.g. PRL secretion) to tobacco smoke exposure between males and females and between species (Shaw & al'Absi, 2010; Andersson et al. 1988). Further, pregnancy may change the way women respond to environmental insults. There may also be a non-linear dose-response "curve" for tobacco smoke exposure. In other words, STS exposure, as we observed in the present study, may truly be associated with an increase in PRL concentrations, while active smoking may result in decreased PRL concentrations. Many factors play a role in PRL secretion, and studies are needed to determine which are likely affected by tobacco smoke exposure. Studies should also explore the possible differential response to tobacco smoke between males and females and pregnant and non-pregnant women. Studies should seek to delineate the PRL-tobacco smoke dose-response curve, as well, to determine if PRL secretion changes between active smokers and those who are exposed to STS.

Our study of the relationship between PRL and TSH concentration and STS exposure was exploratory in nature and more studies are needed to clarify the present findings as well as the findings of others. Serum cotinine, as opposed to FF cotinine, may be a more biologically relevant marker of STS exposure in studies of endocrine changes. For the present study, PRL and TSH concentrations were measured in serum, while cotinine was measured in FF. FF cotinine was used to estimate STS exposure simply because it was available as a result of our primary aim in Chapter 2 and serum cotinine was not. In addition, since PRL may be a risk factor for adverse health effects including certain types of cancers (e.g. breast, prostate), future studies on the relationship between STS exposure and PRL secretion may help to elucidate the mechanisms behind the carcinogenic potential of STS.

To the best of our knowledge, this is the largest study to date on the effects of STS exposure, estimated through an objective biomarker, on fertility or early pregnancy. Thus, we may have been able to observe associations between exposure and outcome that similar, smaller studies would be underpowered to detect. We were also able to leverage longitudinal data from couples who underwent multiple IVF treatment cycles, which resulted in a more thorough analysis than previous studies that relied on only data from IVF patients' first treatment cycles.

The use of IVF patients for the present work, however, may have limited the generalizability of our findings. For example, demographic characteristics of an IVF

cohort are likely different from the general population and infertile couples' gametes may have an increased sensitivity to STS exposure. In addition, the IVF treatment process does not represent what occurs in natural pregnancy. In other words, our results may only apply to the general population if these conditions are not associated with a differential response to STS exposure. However, there is no evidence to date that these factors are associated with differential sensitivity to STS exposure. Thus, we feel our results are likely generalizable to women conceiving naturally.

On the other hand, using an IVF cohort has provided several advantages. IVF patients provide a willing and motivated study group. The IVF treatment process also allows researchers to observe and study early pregnancy endpoints, such as implantation, that are otherwise unobservable in the general population. IVF also facilitates the invasive collection of FF, which is likely a more biologically relevant marker of STS exposure versus cotinine in urine or serum in studies of early pregnancy because it represents the developing oocytes' direct exposure to the constituents of tobacco smoke. In other words, it provides a measure of dose at the target tissue.

Most research on STS exposure and early reproduction has relied on self-reported exposure and a few studies have relied on other biomarkers of STS exposure (e.g. urinary cotinine). Thus, the present study may have been less susceptible to exposure misclassification compared to studies relying on self-report or other markers of STS exposure. Only one other study has measured cotinine in FF to examine the effects of STS exposure on early pregnancy. This study found no significant difference in

fertilization or pregnancy rates between active, passive, and non-smokers in a small cohort of IVF patients (n = 197, 26 of whom were categorized as being exposed to STS; Sterzik et al., 1996). Because of the large sample size of the present study, we may have been able to observe an association that this earlier study was underpowered to detect.

Future studies should examine the relationship between cotinine concentrations in FF and serum among STS-exposed nonsmokers in order to further validate FF cotinine as a biomarker of low-level tobacco smoke exposure. Measuring other biomarkers of STS exposure may also be beneficial in future work. A study evaluating analytical methods of urinary nicotine and its metabolites, reported that the half-life of trans-3'-hydroxycotinine was approximately 63 hours (compared to 20 hours for cotinine) and that the assay used had a limit of detection low enough to be used in studies of STS exposure (Matsuki et al., 2008). Because of trans-3'-hydroxycotinine's longer half-life and because it is specific to nicotine exposure, it may be a preferred biomarker of long-term STS exposure. The utility of this biomarker, however, and its agreement with cotinine should be further explored. In the meantime, to improve the accuracy of long-term STS exposure estimates, multiple biological samples should be collected over time and analyzed for cotinine.

Because FF cotinine may be an improved biomarker of low-level tobacco smoke exposure in studies of early reproduction, future studies using cotinine in FF should be conducted to determine if STS exposure also adversely affects other early pregnancy endpoints that were not evaluated in the present study (e.g. fertilization, spontaneous

abortion). Early-life exposures could also be measured to determine if STS increases the risk of reproductive difficulties in adulthood. And more studies are needed to elucidate the difference in risk of adverse pregnancy outcomes between active smokers and STS-exposed nonsmokers.

Future work should also explore the possible differential response to tobacco smoke between males and females and pregnant and non-pregnant women. Further, studies should seek to delineate the PRL-tobacco smoke dose-response curve, as well, to determine if PRL secretion changes between active smokers and those who are exposed to STS. Since many factors play a role in PRL secretion, studies are needed to determine which are likely affected by tobacco smoke exposure. In addition, since PRL may be a risk factor for adverse health effects including certain types of cancers, future studies on the relationship between STS exposure and PRL secretion may help to elucidate the mechanisms behind the carcinogenic potential of STS.

In conclusion, our results indicate that STS exposure, estimated through FF cotinine, is associated with an increased risk of implantation failure and decreased odds of achieving a successful livebirth among women undergoing IVF treatment. Increased PRL concentrations are also associated with STS exposure. These findings are likely of great public health significance due to continued widespread STS exposure worldwide, the commonness of couples experiencing problems conceiving and the rapidly increasing number of couples utilizing IVF, and the potential relationship between elevated PRL concentrations and certain types of cancer. In addition, FF cotinine concentrations, if

available, may be desired as a biomarker of low-level tobacco smoke exposure over urinary cotinine in studies of early reproduction. Collection of multiple FF samples for cotinine analysis may be needed to accurately represent long-term STS exposure.

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