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# Biomarkers of preconception stress and the incidence of pregnancy loss

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**STUDY QUESTION:** Are biomarkers of preconception stress associated with pregnancy loss?

**SUMMARY ANSWER:** Preconception stress, as measured by basal salivary cortisol and alpha-amylase concentrations, is not associated with pregnancy loss.

**WHAT IS KNOWN ALREADY:** Many studies, most of which have been retrospective, have identified an association between stressful life events and perceived stress and miscarriage.

**STUDY DESIGN, SIZE, DURATION:** A prospective pregnancy study with preconception enrollment was conducted between 2005 and 2009. Among the 344 women who became pregnant during the Longitudinal Investigation of Fertility and the Environment (LIFE) study, 337 (98%) had salivary biomarker data for analysis.

**PARTICIPANTS/MATERIALS, SETTING, METHODS:** Couples planning pregnancy were followed for up to 12 months as they tried to become pregnant and through pregnancy if it occurred. Participating women collected a basal saliva sample on the morning following enrollment and a second on the morning following their next menses to measure cortisol and alpha-amylase, biomarkers of stress. Women used home pregnancy tests on the day of expected menses. A pregnancy loss was defined as a negative pregnancy test following a positive pregnancy test, the onset of menses, or for pregnancies that survived to clinical recognition, recognition of the loss by a healthcare provider.

**MAIN RESULTS AND THE ROLE OF CHANCE:** Among the 337 couples, the median age of female and male partners was 29 and 31 years, respectively. Most of the women were non-Hispanic white (83%) and highly educated. There were 97 pregnancy losses reported among the 337 pregnancies. The median gestational age at loss was 6 weeks 5 days with only two losses occurring in the second trimester. Using Cox proportional hazards models, we found no clear pattern of association between two preconceptional biomarkers of stress (salivary cortisol and alpha-amylase concentrations) modeled both continuously or in tertiles and incident pregnancy loss after adjustment for confounders.

**LIMITATIONS REASONS FOR CAUTION:** Our prior work suggests that women enrolled in the LIFE Study had lower stress levels than women in the general population. Owing to concerns regarding participant burden, we were unable to collect serial saliva measurements, which would have allowed us to examine the association between stress in early pregnancy and pregnancy loss. Further, with regard to the measurement of perceived stress, the Cohen's Perceived Stress Scale was only administered at baseline. While every attempt was made to ensure diversity in the cohort, non-Hispanic white women were over-represented, therefore it is possible that the results might not be generalizable to all women.

**WIDER IMPLICATIONS OF THE FINDINGS:** In one of the largest studies in the USA to prospectively capture data on the incidence of early pregnancy loss, we found no clear association between two biomarkers of preconception stress (measured in saliva) and pregnancy loss.

**STUDY FUNDING/COMPETING INTEREST(S):** This study was supported by the Intramural Research Program of the Eunice Kennedy Shriver National Institute of Child Health and Human Development (contracts #N01-HD-3–3355, N01-HD-3-3356, N01-HD-3358). There are no conflicts of interest to declare.

**TRIAL REGISTRATION NUMBER:** Not applicable.

**Key words:** miscarriage / fecundity / stress / cortisol / alpha-amylase / pregnancy / spontaneous abortion / biomarkers

# Introduction

Early work suggests that 31–33% of pregnancies are lost following identification with hCG testing (Wilcox *et al.*, 1988; Zinaman *et al.*, 1996; Wang *et al.*, 2003). Recently, we reported the incidence of pregnancy loss to be 28% among a group of couples trying to conceive and using highly sensitive digital home pregnancy tests (Buck Louis *et al.*, 2016). While about half of pregnancy losses are thought to be due to aneuploidy (Alberman and Creasy, 1977; Goddijn and Leschot, 2000), and there are other known etiologies (e.g. endocrine, autoimmune and thrombotic abnormalities), some of the causes of miscarriage remain unexplained (Regan and Rai, 2000). In an attempt to identify additional causes of unexplained miscarriage, investigators have spent a lot of time over the years examining various environmental risk factors, including lifestyle factors such as smoking, alcohol consumption and caffeine intake. One area that has received considerable attention over time is the role of stress in pregnancy loss.

While a number of studies have examined the association between stress and pregnancy loss, nearly all of them have examined the frequency of stressors themselves (i.e. life events scales) or perceived stress. There is only one study of which we are aware that included a physiologic measure of stress (i.e. urinary cortisol) (Nepomnaschy *et al.*, 2006). The study of pregnancy loss is methodologically difficult in that it requires that women who are at risk of pregnancy (irrespective of pregnancy intentions) are followed over time with timed hCG testing in each cycle because most pregnancy losses that do occur happen early and are often mistaken as a late menses. As a result, many of the studies that have been conducted to date have used a case–control approach that is subject to incomplete ascertainment of the outcome and recall bias, with women experiencing a loss being more likely to report exposures (Savitz *et al.*, 2008).

Despite the variations in study design and exposure ascertainment, most studies have reported an association between stress and miscarriage (Fenster *et al.*, 1995; Neugebauer *et al.*, 1996; Schenker *et al.*, 1997; Bashour and Abdul, 2001; Nepomnaschy *et al.*, 2006; Arck *et al.*, 2008; Clark *et al.*, 2005), while several did not (Klebanoff *et al.*, 1990; Milad *et al.*, 1998). Previously, we reported that women with high levels of a stress biomarker, salivary alpha-amylase (sAA), in the preconception period experienced a 29% decrease in fecundity or an increased time-to-pregnancy compared to women with lower levels of the biomarker, which translated into a more than 2-fold increased risk of infertility (Lynch *et al.*, 2014). This finding was consistent with our other work that reported lower day-specific probabilities of conception among women with high levels of sAA (Louis *et al.*, 2011) The goal of this analysis was to assess whether higher levels of stress biomarkers (i.e. sAA and cortisol) measured before conception were also associated with an increased risk of pregnancy loss in our population.

# Materials and Methods

## Population and eligibility

The data for this study were collected as part of the Longitudinal Investigation of Fertility and the Environment (LIFE) study that took place in 2005–2009 (Buck Louis *et al.*, 2011). In brief, we enrolled 501 couples in the states of MI and TX, USA, who were discontinuing contraception with the intent of getting pregnant. The primary aim of the study was to examine the association between the environment, broadly defined, and human fecundity and fertility. Eligibility criteria included: non-pregnant females aged 18–40 years; married or in a committed relationship; male partner age 18+ years; self-reported menstrual cycle length of 21–42 days (to comply with fertility monitor specifications); ability to communicate in English or Spanish; no use of hormonal birth control injections in the prior 12 months (because of the uncertainty surrounding return to fertility in this group); woman and her partner had never been told by a healthcare provider that they could not get pregnant without medical help; and actively trying to get pregnant and off contraception for ≤2 months at study entry. Enrolled couples were followed for up to 12 months as they attempted to get pregnant and through to pregnancy if it occurred.

## Data collection

The study team conducted home visits with enrolled couples to provide training and collect data. Separate questionnaires were completed by each partner of the couple to collect demographic, health history, reproductive history and lifestyle information (e.g. stress, smoking and alcohol use). Couples were trained in the use of study journals and other data collection elements, such as the digital home pregnancy tests and the fertility monitor provided by the study (Clearblue® fertility monitor, SPD Swiss Precision Diagnostics GMBH, Bedford, UK). Each partner completed a daily journal while trying to conceive and up until 8 weeks of pregnancy. Monthly pregnancy journals were completed by women thereafter. The protocol was reviewed and approved by the institutional review boards at each participating institution. Written informed consent was obtained from all participants.

## Exposure assessment

Physiologic stress was assessed via the measurement of salivary cortisol and sAA concentrations. Female partners collected a first morning (basal) saliva specimen using a Salivette® collection device (Sarstedt, Nümbrecht, Germany) on the morning following enrollment and then again on the morning following their first study-observed menses. Both cortisol and sAA exhibit a diurnal pattern. Cortisol levels peak in the morning and fall slowly throughout the day, whereas sAA falls quickly within 60 min of awakening and rises slowly throughout the day (Weitzman *et al.*, 1971; Nater *et al.*, 2007). Women were asked to collect the specimen immediately upon awakening before eating, drinking, smoking or brushing their teeth. Samples were returned to study staff via prepaid overnight shipping and samples were stored at −20°C until analyzed.

Saliva specimens were analyzed by one of the leading laboratories in the area of salivary biomarker research (Salimetrics, LLC, State College, PA, USA). Cortisol (μg/dl) was measured using a highly sensitive enzyme immunoassay (Raff *et al.*, 2003). The lower limit of sensitivity of the assay was <0.007 μg/dl. sAA (U/ml) was quantified using a commercially available (Salimetrics, State College, PA, USA) kinetic reaction assay (Granger *et al.*, 2007). The lower limit of sensitivity of assay was 0.4 (U/ml). Complete details regarding the assays and quality control procedures can be found elsewhere (Salimetrics 2016a, 2016b).

## Outcome assessment

Pregnancies were detected using ClearBlue digital home pregnancy tests that are sensitive for 25 mIU/ml hCG (SPD Swiss Precision Diagnostics GMBH, Bedford, UK) (Johnson *et al.*, 2015). Pregnancy losses were identified in one of three ways depending upon gestational age: a positive home pregnancy test followed by a negative test; a positive home pregnancy test followed by self-report of a pregnancy loss on the study's Pregnancy Loss Information Card, which collected information regarding the loss, including the method of detection (e.g. bleeding indicative of miscarriage, healthcare provider noted no heartbeat) and provided some bereavement information. In the first situation, the date of the loss was taken to be the date of the first negative pregnancy test following a positive test. While ovarian cysts, menopause and some rare medical conditions can lead to a false positive pregnancy test, there is no reason to expect those conditions to result in a positive and then a negative test 1 week later (i.e. result in misclassification of pregnancy loss). In the second instance, the date of the loss was considered to be the day the woman recognized it. For those recognized by clinical detection, the date of the loss was the day it was noted by a healthcare provider.

## Covariate information

Covariates were chosen by review of the literature and examination of a Directed Acyclic Graph (DAG) (Greenland *et al.*, 1999). Covariates that were considered included: female and male ages (years), female race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, other), past history of loss conditional on gravidity (nulliparous, gravid with no prior history of loss, gravid with prior history), female BMI (categorized according to National Heart Lung and Blood Institute, National Institutes of Health, USA standards as underweight, healthy, overweight, and obese), female educational attainment (high school or less, some college and college graduate), household income (<$50 000, $50 000–99 999, $100 000+), health insurance status, and smoking, vitamin use, alcohol use, and caffeinated beverage consumption both before and during pregnancy. Vitamin use was defined as the percentage of daily vitamins taken over the periconception window. So, for a woman who reported taking vitamins 5 out of 7 days, the percentage of expected vitamins would be 71%. For lifestyle factors, we modeled use consistent with current recommendations: smoking (none versus any), alcohol (none versus any) and for caffeinated beverages (<2 versus 2+ average drinks per day). For covariates with missing data, multiple imputation was used.

## Statistical analysis

The analysis was limited to pregnant woman with saliva biomarker data available. To examine the relation between preconceptional stress and pregnancy loss using stress biomarkers, we averaged the two preconception (morning after enrollment and the morning after their first menses in the study) cortisol and alpha-amylase concentrations for analysis, given their lack of variability (Lynch *et al.*, 2014). We looked at the biomarkers modeled two ways: continuously; and by dividing the distributions of salivary cortisol and alpha-amylase into tertiles based on the distribution among pregnant women (i.e. the ones at risk of pregnancy loss).

As in our prior work, we used the fetuses-at-risk approach to examine time to loss (measured by post-conception gestational age in days) as competing risks data with miscarriage as the primary event of interest, with the other outcome (live birth) as a competing event (Buck Louis *et al.*, 2016; Sapra *et al.*, 2016). Date of conception was estimated using the ClearBlue Easy Fertility Monitor, which has been shown to detect the LH surge with 99% accuracy (Behre *et al.*, 2000). Loss to follow up of pregnancies was addressed using right censoring of the competing risks data. That is, women contributed time until they were lost to follow-up or withdrew, at which point they were censored.

Using the estimates from the above models, we assessed the association of the main exposure of interest in each instance on the cumulative incidence function for miscarriage. This was implemented using PROC PHREG in SAS (SAS Institute Inc, Cary, NC, USA).

# Results

Among the 344 women who became pregnant with a singleton during the LIFE Study, 337 had available data for both stress biomarkers and were included in the current analysis. Among these 337 women, the median age was 29 (range: 19–40) years, and their partners' median age was 31 (range: 21–50) years. Most pregnant women were non-Hispanic white (84%), with 9% of women being Hispanic and 2% of women being non-Hispanic black. Pregnant women were well-educated with 83% having at least a college degree.

There were 97 pregnancy losses recorded during the LIFE Study. Among those losses, 42 (43%) occurred before 6 weeks of gestation, 53 (55%) happened later in the first trimester, and two (2%) were second trimester losses (<21 weeks). As shown in Table I, females who experienced a pregnancy loss were more likely than women without losses to be 35 years of age or older (19 versus 10%) and to have drunk two or more caffeinated beverages per day both before and during pregnancy (32 versus 19% before pregnancy and 17 versus 9% during pregnancy).

Table II presents measures of stress by pregnancy outcome and reflects the absence of significant differences between groups. The unadjusted and adjusted associations between biomarkers of preconception stress and pregnancy loss are presented in Table III. Biomarkers exhibited skewness, and as such we log-transformed both variables when modeled continuously. The associations between stress and pregnancy loss showed no meaningful pattern, even after adjustment for female age, race, health insurance status, periconceptional smoking, periconceptional alcohol consumption, periconceptional caffeinated beverage consumption and periconceptional prenatal/multivitamin adherence. Most of the point estimates for preconception stress, as measured by salivary cortisol, were below one, whereas estimates for alpha-amylase hovered around one. None of the associations were statistically significant.

# Discussion

In a contemporary cohort of couples trying to get pregnant, we found no evidence that preconception stress, as measured by salivary cortisol and alpha amylase, is associated with pregnancy loss (Buck Louis *et al.*, 2016). In our prior work, we reported that preconception stress as measured by salivary alpha amylase was associated with a longer time to pregnancy, which translated into a 2-fold increased risk of

**Table I Demographic and lifestyle characteristics of participants in the LIFE study by pregnancy outcome ($n = 337$).**

| | Live birth, $n = 216$ | Pregnancy loss, $n = 97$ | Lost to follow-up while pregnant, $n = 24$ |
|---|---|---|---|
| Female age (years)* | | | |
| ≤29 | 115 (53%) | 48 (50%) | 18 (75%) |
| 30–34 | 80 (37%) | 31 (32%) | 3 (13%) |
| ≥35 | 21 (10%) | 18 (19%) | 3 (13%) |
| Mean (SD) | 29.7 (4) | 30.3 (4) | 29.3 (4) |
| Male age (years) | | | |
| ≤29 | 84 (39%) | 28 (29%) | 9 (38%) |
| 30–34 | 82 (38%) | 40 (41%) | 9 (38%) |
| ≥35 | 50 (23%) | 29 (30%) | 6 (25%) |
| Mean (SD) | 31.4 (5) | 32.3 (5) | 31.0 (4) |
| Race/ethnicity | | | |
| White, non-Hispanic | 176 (82%) | 81 (84%) | 23 (96%) |
| Black, non-Hispanic | 3 (1%) | 3 (3%) | 0 (0%) |
| Hispanic | 19 (9%) | 7 (7%) | 1 (4%.) |
| Other, non-Hispanic | 16 (7%) | 5 (5%) | 0 (0%) |
| Missing data | 2 (1%) | 1 (1%) | 0 (0%) |
| Education | | | |
| ≤High school | 8 (4%.) | 6 (6%) | 0 (0%) |
| Some/college graduate | 208 (96%) | 91 (94%) | 24 (100%) |
| Health insurance | | | |
| No | 6 (3%) | 6 (6%) | 3 (12.5%) |
| Yes | 210 (97%) | 91 (94%) | 21 (87.5%) |
| Household income | | | |
| <$50 000 | 27 (13%) | 12 (12%) | 3 (13%) |
| $50 000–$99 999 | 96 (44%) | 53 (55%) | 14 (58%) |
| ≥$100 000 | 93 (43%) | 32 (33%) | 7 (29%) |
| Employed | | | |
| No | 42 (19%) | 22 (23%) | 4 (17%) |
| Yes | 174 (81%) | 75 (77%) | 20 (83%) |
| BMI (kg/m$^2$) | | | |
| Under/healthy ≤24.9 | 110 (51%) | 43 (44%) | 15 (63%) |
| Overweight 25.0–29.9 | 59 (27%) | 23 (24%) | 4 (17%) |
| Obese ≥30 | 47 (22%) | 31 (32%) | 5 (21%) |
| Mean (SD) | 26.6 (7) | 27.8 (7) | 26.9 (7.3) |
| Prior history of pregnancy loss | | | |
| Nulligravid | 86 (40%) | 37 (38%) | 10 (42%) |
| Gravid, with no prior history of loss | 16 (7%) | 7 (7%) | 4 (17%) |
| Gravid, with prior history of loss | 112 (52%) | 52 (54%) | 10 (42%) |
| Missing data | 2 (1%) | 1 (1%) | 0 (0%) |
| Prenatal/multivitamin adherence** | 0.76 (0.3) | 0.63 (0.3) | 0.71 (0.3) |
| Smoking status prior to pregnancy | | | |
| Non-smoker | 202 (94%) | 85 (88%) | 22 (92%) |
| Smoker | 14 (6%) | 12 (12%) | 1 (4%) |
| Missing data | 0 (0%) | 0 (0%) | 1 (4%) |
| Smoking status during pregnancy | | | |
| Non-smoker | 202 (93%) | 85 (88%) | 20 (83%) |
| Smoker | 4 (2%) | 6 (6%) | 0 (0%) |
| Missing data | 10 (5%) | 6 (6%) | 4 (17%) |

*Continued*

**Table I** *Continued*

| | Live birth, $n = 216$ | Pregnancy loss, $n = 97$ | Lost to follow-up while pregnant, $n = 24$ |
|---|---|---|---|
| Average alcohol consumption prior to pregnancy | | | |
| <1 drinks/day | 68 (32%) | 28 (29%) | 8 (33%) |
| ≥1 drinks/day | 148 (68%) | 69 (71%) | 15 (63%) |
| Missing data | 0 (0%) | 0 (0%) | 1 (4%) |
| Average alcohol consumption during pregnancy | | | |
| <1 drinks/day | 202 (93%) | 77 (79%) | 19 (79%) |
| ≥1 drinks/day | 4 (2%) | 14 (15%) | 1 (4%) |
| Missing data | 10 (5%) | 6 (6%) | 4 (17%) |
| Average caffeinated beverage consumption prior to pregnancy* | | | |
| <2 drinks/day | 175 (81%) | 66 (68%) | 21 (88%) |
| ≥2 drinks/day | 41 (19%) | 31 (32%) | 2 (8%) |
| Missing data | 0 (0%) | 0 (0%) | 1 (4%) |
| Average caffeinated beverage consumption during pregnancy* | | | |
| <2 drinks/day | 185 (86%) | 75 (77%) | 20 (83%) |
| ≥2 drinks/day | 20 (9%) | 16 (17%) | 0 (0%) |
| Missing data | 11 (5%) | 6 (6%) | 4 (17%) |

LIFE, Longitudinal Investigation of Fertility and the Environment.
*Note*: All female variables except where noted. Data are *n* (%) unless stated otherwise.
*$P < 0.05$ from chi-square tests for categorical variables and ANOVA for means.
**Mean (SD) percentage of expected (one/day) prenatal/multivitamins consumed since enrollment.

**Table II Preconception stress biomarkers and pregnancy loss among pregnant women ($n = 337$).**

| | Live birth, $n = 216$ | Pregnancy loss, $n = 97$ | Lost to follow-up while pregnant, $n = 24$ |
|---|---|---|---|
| Time to pregnancy (cycles)* | 2.0 (1.0, 4.0) | 2.0 (1.0, 4.0) | 2.5 (1.0, 4.5) |
| Cortisol (μg/dl)* | 0.39 (0.30, 0.50) | 0.34 (0.28, 0.50) | 0.33 (0.27, 0.42) |
| Cortisol tertile (μg/dl) | | | |
| Lowest (0.02–0.30) | 66 (31%) | 36 (37%) | 10 (42%) |
| Middle (0.30–0.44) | 71 (33%) | 34 (36%) | 9 (38%) |
| Highest (0.45–7.25) | 79 (37%) | 27 (28%) | 5 (21%) |
| Alpha-amylase (U/ml)* | 14.53 (7.37, 26.07) | 14.59 (8.76, 26.74) | 15.87 (11.21, 31.9) |
| Alpha-amylase tertile (U/ml) | | | |
| Lowest (0.40–10.03) | 75 (35%) | 32 (33%) | 5 (21%) |
| Middle (10.04–21.06) | 65 (30%) | 38 (39%) | 11 (46%) |
| Highest (21.06–360.64) | 76 (35%) | 27 (28%) | 8 (33%) |
| Four-item Cohen's perceived stress scale† | 3.31 (2.3) | 3.79 (2.7) | 4.21 (3.0) |

Salivary biomarker levels reflect the mean of the first and second sample.
*Median (interquartile range).
†Mean (SD).

infertility (Lynch *et al.*, 2014). There are several possible explanations why we did not see a similar association with pregnancy loss. Perhaps there is indeed no association between preconceptional levels of stress, as measured by these biomarkers, and pregnancy loss. While we were unable to collect serial saliva measurements due to concerns regarding participant burden, there remains the possibility that the critical window for exposure to stress is in early pregnancy rather than preconceptionally. It remains possible that participants' stress levels simply did not reach the threshold required to adversely impact a pregnancy. As noted in a prior publication involving this cohort, we found no association between the salivary biomarkers and related self-reported measures, including perceived stress (Lynch *et al.*, 2012). Additionally, the correlation between salivary cortisol and alpha-amylase is low, which has been reported by others and is not

**Table III Association between average preconception stress biomarker level and the hazard of pregnancy loss ($n = 337$).**

| | Hazard ratio | 95% CI | Adjusted hazard ratio† | 95% CI |
|---|---|---|---|---|
| Alpha-amylase* | 1.01 | [0.80, 1.27] | 1.08 | [0.84, 1.39] |
| Lowest | Referent | – | Referent | – |
| Middle | 1.16 | [0.73, 1.86] | 1.00 | [0.61, 1.63] |
| Highest | 0.90 | [0.54, 1.49] | 1.01 | [0.59, 1.72] |
| Cortisol* | 0.58 | [0.13, 2.56] | 0.60 | [0.14, 2.53] |
| Lowest | Referent | – | Referent | – |
| Middle | 0.88 | [0.55, 1.41] | 1.04 | [0.64, 1.70] |
| Highest | 0.68 | [0.42, 1.12] | 0.72 | [0.43, 1.19] |

*Modeled as a continuous variable then in tertiles (separate models). Multiple imputation was used for covariates with missing values.
†Adjusted for female age, race, health insurance status, periconceptional smoking, periconceptional alcohol consumption, periconceptional caffeinated beverage consumption and periconceptional prenatal/multivitamin adherence.

surprising as the measures are biomarkers of different components of the stress system (i.e. acute versus chronic stress) (Chatterton *et al.*, 1997; Lynch *et al.*, 2014).

Prior work has pointed to the need to study the association between stress and pregnancy loss among women who are followed in very early pregnancy. This is for several reasons. First, most of the pregnancy losses that do occur happen within the first month after conception before most pregnant women present for routine prenatal care. Second, prospective studies suggest that developing embryos are most vulnerable to stress during the periods of implantation and placentation (Hjollund *et al.*, 2000; Nepomnaschy *et al.*, 2004). Indeed, women with Cushing's Disease, a disease involving high levels of circulating cortisol, have difficulty maintaining a pregnancy (Lindsay and Nieman, 2005). Stress can shorten a woman's luteal phase resulting in suboptimal progesterone levels, which are incompatible with implantation (Hatch *et al.*, 1999).

This is the first study of which we are aware to examine the association between biomarkers of preconception stress pathways and early pregnancy loss; therefore, our work is not directly comparable with prior studies, which have been conflicting and plagued with methodologic limitations. A case–control study reported a statistically significantly higher number of severe life events among women who experienced a miscarriage compared to those who did not (O'Hare and Creed, 1995). Neugeberger *et al.* (1996) reported that woman in their study who experienced at least one negative life event were more than twice as likely to experience a chromosomally normal miscarriage. A study of female resident physicians (presumably under psychosocial stress) and the wives of male resident physicians (believed to be under less stress) found no differences in miscarriage between the two groups (Klebanoff *et al.*, 1990).

A major strength of this study is the cohort design in which women who were trying to conceive were followed with serial hCG monitoring over time, permitting an unbiased ascertainment of pregnancy and pregnancy loss, most notably early pregnancy loss. Levels of stress were measured objectively with biomarkers and all exposure data were collected prior to the study outcome. Given the nature of our data, we believe that the analytic approach that we used was appropriate. However, to examine if our findings were robust to model misspecification, we also examined the association between the stress biomarkers and pregnancy loss using logistic regression and the results remained consistent in terms of directionality and magnitude.

Our study, however, is not without limitations. First, it would have been ideal to have detailed self-reported perceived stress data in early pregnancy, but the four-item Cohen's Perceived Stress Scale was administered only at baseline. The period of recall for that scale is the past month. Therefore, while half of the women in our study had achieved pregnancy by the second cycle of trying, it is possible that it did not accurately reflect stress in early pregnancy, particularly for those with a longer time to pregnancy. Further, as discussed in detail in our publication involving this cohort, the women in the LIFE Study had lower levels of stress biomarkers and perceived stress than the general population, likely because the study itself was very time intensive (and so stressed individuals were less likely to enroll) (Lynch *et al.*, 2014). In addition, this kind of work can only be done with couples who are planning pregnancy. Moreover, while every attempt was made to ensure racial and ethnic diversity in the cohort, non-Hispanic white women were overrepresented. Therefore, it is possible that the results might not be generalizable to all women.

While we could not collect serial saliva measurements out of concern for participant burden, it would have been helpful to have measurements at additional time points so that we could have examined the potential for critical windows of exposure to stress as they relate to pregnancy loss. Further, while it has been reported that a single basal saliva measurement is sufficient to capture stress-related changes in cortisol, more recent data suggest that collecting multiple saliva samples per day to measure the cortisol awakening response is preferable (Yehuda *et al.*, 2003; Stalder *et al.*, 2016). Finally, while we found no association between preconception stress and pregnancy loss it is possible that the role of stress in pregnancy loss is small and as such we were underpowered to detect the association despite the fact that our study is one of the largest in the USA to date to capture incident data on early pregnancy loss.

While the association between stress and pregnancy loss remains unclear, we found no association between preconception stress, as measured by salivary biomarkers, and pregnancy loss. Future work that incorporates serial measurements of stress biomarkers over time would go a long way to clarify what role, if any, that stress might play in pregnancy loss.

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## Authors' roles

C.D.L. assisted in the design of the study, conceptualized the article, and wrote first draft and took the lead on subsequent edits. R.S. designed and implemented the analytic plan and provided substantive edits on the article. G.M.B.L. designed the study and provided substantive edits on the analytic plan and article.

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## Conflict of interest

The authors have no conflicts of interest to declare in relation to this article.

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