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BACKGROUND. Several lines of evidence suggest that male embryos may have greater vulnerability than female embryos to disordered inflammation; therefore, antiinflammatory drugs, such as low-dose aspirin (LDA), may alter the sex ratio. Here, we assessed the effect of LDA on male live birth and male offspring, incorporating pregnancy losses ($n = 56$) via genetic assessment, as part of a parallel-design, block-randomized, placebo-controlled trial of preconception LDA.

METHODS. Participants (615 treated with LDA, 613 treated with placebo) ranged in age from 18 to 40 years of age, with 1 to 2 prior pregnancy losses. We estimated the intention-to-treat (ITT) risk ratio (RR) and 95% CI and assessed interaction with baseline high-sensitivity C-reactive protein (hsCRP) serum concentration — a marker of systemic inflammation.

RESULTS. Among the 1,078 women who completed follow-up (535 treated with LDA, 543 treated with placebo), the male live birth ITT RR equaled 1.31 (95% CI: 1.07–1.59). With increasing tertile of hsCRP, the proportion of males at birth decreased in the placebo group, and the effect of LDA on male live birth increased (first tertile: 48% male in LDA vs. 52% in placebo, ITT RR = 0.97, 95% CI: [...])

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Sex ratio following preconception low-dose aspirin in women with prior pregnancy loss

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METHODS. Participants (615 treated with LDA, 613 treated with placebo) ranged in age from 18 to 40 years of age, with 1 to 2 prior pregnancy losses. We estimated the intention-to-treat (ITT) risk ratio (RR) and 95% CI and assessed interaction with baseline high-sensitivity C-reactive protein (hsCRP) serum concentration – a marker of systemic inflammation.

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CONCLUSION. Initiation of LDA prior to conception restored numbers of male live births and pregnancy with male offspring among women with 1 to 2 prior pregnancy losses. Moreover, our data suggest that LDA modulates inflammation that would otherwise reduce the conception or survival of male embryos.

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Introduction

The ratio of male to female newborn infants has trended downward subtly but significantly over decades in North America (1, 2), Europe (3, 4), and Japan (5). More dramatic decreases in this ratio have been associated with parental exposure to environmental hazards, such as smoking (6), dioxin (7), methylmercury (8), and earthquakes (9, 10), suggesting that parental exposure to toxic chemicals and stress is particularly hazardous to male conception or survival. One potential pathway to altered sex ratios is maternal inflammation, which exhibits sex-dependent embryonic effects in bovines (11) and mice (12). In humans, a decidual proteotoxic stress response prevents implantation of nonviable

embryos (13), and increased endometrial inflammation is associated with recurrent pregnancy loss (13, 14). Given the influence of inflammation on implantation, low-dose aspirin (LDA), an antiinflammatory drug, may improve implantation through reduction of inflammation. Indeed, some small trials noted that LDA increased the clinical pregnancy rate among women undergoing in vitro fertilization, although results were mixed (15–19). Recently, the Effects of Aspirin in Gestation and Reproduction (EAGeR) trial reported that preconception LDA treatment increased the probability of becoming pregnant, but did not prevent pregnancy loss, among women who were attempting pregnancy without fertility treatments and had a history of 1 pregnancy loss in the previous 12 months (20). LDA may also alter the sex ratio of pregnancies among women with a history of pregnancy loss, given the sex-dependent embryonic effects of maternal inflammation.

Conflict of interest: The authors have declared that no conflict of interest exists.

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Table 1. Effect of LDA on the probability of male live birth among 1,086 women and mother-offspring pairs who completed follow-up (EAGeR trial, US, 2007–2012^A)

	N ^C	Among all participants (n = 1,086) ^A		Among pregnancies ^B (n = 783) ^A		Among live births (n = 601) ^A	
		RR (95% CI)	RD (95% CI) ^D	RR (95% CI) ^E	RD (95% CI) ^F	RR (95% CI) ^G	RD (95% CI) ^H
Overall							
LDA	164	1.31 (1.07–1.59)	7.18 (1.92–12.4)	1.22 (1.01–1.48)	7.33 (0.57–14.1)	1.24 (1.04–1.47)	10.0 (1.93–18.2)
Placebo	128	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)
Stratified by tertile of preconception hsCRP at randomization							
Tertile 1: 0.15–0.71 mg/l							
LDA	49	0.97 (0.70–1.35)	–0.90 (–10.4–8.54)	0.93 (0.68–1.28)	–2.56 (–14.3–9.19)	0.89 (0.66–1.20)	–5.61 (–20.0–8.76)
Placebo	50	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)
Tertile 2: 0.72–2.08 mg/l							
LDA	57	1.36 (0.98–1.90)	9.07 (–0.48–18.6)	1.24 (0.91–1.70)	8.67 (–3.41–20.8)	1.33 (1.00–1.77)	14.26 (0.51–28.0)
Placebo	45	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)
Tertile 3: 2.09–62.7 mg/l							
LDA	48	1.70 (1.13–2.57)	11.5 (2.87–20.2)	1.64 (1.08–2.46)	14.5 (2.99–25.9)	1.62 (1.09–2.40)	19.0 (4.34–33.4)
Placebo	29	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)
P interaction ^I		0.03	0.05	0.04	0.04	0.04	0.05
Stratified by sex of previous live-born children							
Nulliparous women							
LDA	64	1.46 (1.04–2.04)	8.45 (1.07–15.8)	1.33 (0.96–1.83)	9.04 (–1.07–19.1)	1.28 (0.95–1.73)	10.8 (–1.95–23.6)
Placebo	46	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)
Parous women, no son							
LDA	43	1.19 (0.81–1.74)	5.43 (–6.62–17.5)	1.17 (0.81–1.68)	6.13 (–7.91–20.2)	1.13 (0.81–1.57)	6.11 (–10.25–22.5)
Placebo	31	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)
Parous women, with son							
LDA	56	1.21 (0.88–1.66)	4.90 (–3.84–15.4)	1.15 (0.86–1.55)	5.72 (–6.03–17.5)	1.22 (0.94–1.60)	10.3 (–3.14–23.8)
Placebo	51	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)

^AThe unit of analysis was the participant and, in the case of pregnancy, the mother-offspring pair. Thus, 8 twin gestations each contributed two observations to the analysis, with accounting for correlated data using the generalized estimating equations of log-binomial regression. ^BPregnancies that were detected by urine hCG test. ^CNumber of live-born males. ^DRD per 100 participants. ^ERR and 95% CI were calculated out of pregnancies, with stabilized inverse probability weighting to adjust for the selection of pregnant participants. ^FRD per 100 embryos and 95% CI were calculated out of embryos identified in pregnancies, with stabilized inverse probability weighting to adjust for the selection of pregnancies. ^GRR and 95% CI were calculated out of live-born infants, with stabilized inverse probability weighting to adjust for the selection of pregnancies and live births. ^HRD per 100 live-born infants and 95% CI were calculated out of live-born infants, with stabilized inverse probability weighting to adjust for the selection of pregnancies and live births.

^IP values were calculated from the χ^2 test of the regression coefficient for a cross-product term that modeled tertile of hsCRP and LDA assignment.

Among women with 1 to 2 prior losses, we evaluated the effect of daily preconception LDA treatment versus placebo on the secondary sex ratio, calculated as the proportion of males at birth, and on the sex ratio at implantation (males to females among human chorionic gonadotropin-detected [hCG-detected] pregnancies), by using genetic data that were collected systematically.

Results

The majority of the 1,228 women randomized to the LDA treatment group (615 women) and to the placebo group (613 women) had education beyond high school and an annual household income of \geq \$75,000 (ref. 20 and Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI82357DS1). The LDA and placebo groups were on average very similar with respect to demographic characteristics and reproductive history, including race (mixed European descent 94% LDA vs. 96% placebo), employment status (employed, 73% LDA vs. 72% placebo), nulliparity (46% LDA vs. 47% placebo), and number of prior pregnancy losses (1 prior loss, 69% LDA vs. 66% placebo) (20).

Sex ratio at birth. Among the 1,078 women who completed the trial (535 in the LDA group, 543 in the placebo group), in the intention-to-treat (ITT) analysis, women assigned to LDA treatment were more likely to give birth to a live-born male than women assigned to the placebo (31% LDA vs. 23% placebo, risk ratio [RR] = 1.31, 95% CI: 1.07–1.59; risk difference [RD] per 100 women = 7.18, 95% CI: 1.92–12.4; Table 1). This was also true when the population was restricted to women who became pregnant (live-born males among pregnancies, 44% LDA vs. 37% placebo, RR = 1.22, 95% CI: 1.01–1.48) and to women who had a live birth (live-born male among participants with a live birth, 53% LDA vs. 44% placebo, RR = 1.24, 95% CI: 1.04–1.47). The probability of having a live-born female infant was similar in the LDA and placebo groups (among all participants who completed the trial, 24% LDA vs. 26% placebo, RR = 0.93, 95% CI: 0.76–1.12).

Sex ratio at implantation. In agreement with the analysis of male live births, LDA treatment increased the probability of pregnancy with male offspring among all participants (37% LDA vs. 28% placebo, RR = 1.30, 95% CI: 1.08–1.56; Table 2) and

Table 2. Effect of LDA on the probability of a pregnancy with male offspring among 960 women and mother-offspring pairs, with either a pregnancy with offspring sex known or completion of follow-up without a pregnancy (EAGeR trial, US, 2007–2012^A)

	N ^c	Among all women (n = 960) ^A		Among pregnancies ^B (n = 657) ^A	
		RR (95% CI)	RD (95% CI) ^D	RR (95% CI) ^E	RD (95% CI) ^F
Overall					
LDA	175	1.30 (1.08–1.56)	8.52 (2.59–14.5)	1.19 (1.01–1.41)	8.18 (0.40–16.0)
Placebo	138	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)
Stratified by tertile of preconception hsCRP at randomization					
hsCRP 0.15–0.71 mg/l					
LDA	55	1.00 (0.74–1.34)	–0.18 (–10.9–10.6)	0.95 (0.73–1.24)	–2.61 (–16.0–10.8)
Placebo	56	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)
hsCRP 0.72–2.05 mg/l					
LDA	60	1.46 (1.07–2.00)	13.2 (2.52–23.8)	1.32 (1.00–1.75)	13.6 (0.19–27.0)
Placebo	47	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)
hsCRP 2.06–62.7 mg/l					
LDA	50	1.58 (1.07–2.33)	12.9 (2.09–21.6)	1.44 (0.98–2.12)	14.0 (0.0–28.0)
Placebo	31	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)
P interaction ^G		0.06	0.09	0.07	0.10
Stratified by sex of previous live-born children					
Nulliparous women					
LDA	68	1.36 (1.00–1.85)	8.57 (0.16–17.0)	1.30 (0.96–1.77)	10.38 (–1.46–22.2)
Placebo	53	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)
Parous women, no son					
LDA	46	1.12 (0.79–1.59)	4.51 (–8.86–17.9)	1.11 (0.78–1.57)	4.59 (–11.0–20.2)
Placebo	34	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)
Parous women with sons					
LDA	61	1.34 (0.99–1.81)	10.2 (–0.23–20.7)	1.19 (0.90–1.57)	8.38 (–4.75–21.5)
Placebo	51	1.00 (reference)	0.00 (reference)	1.00 (reference)	0.00 (reference)

^AThe unit of analysis was the participant and, in the case of pregnancy, the mother-offspring pair. Thus, 8 twin gestations each contributed two observations to the analysis, with accounting for correlated data using the generalized estimating equations extension of the log-binomial regression model. This analysis excluded 126 women with a pregnancy in which offspring sex was undetermined. ^BPregnancies that were detected by urine hCG test. ^CNumber of male embryos. ^DRD per 100 women. ^ERR and 95% CI were calculated out of embryos identified in pregnancies, with stabilized inverse probability weighting adjusted for the selection of pregnancies. ^FRD per 100 embryos in confirmed pregnancies, with stabilized inverse probability weighting adjusted for the selection of pregnancies. ^GP values were calculated from the χ^2 test of the regression coefficient for a cross-product term that modeled tertile of hsCRP and LDA assignment.

among pregnancies (RR = 1.19, 95% CI: 1.01–1.41). In the simple quantitative bias analysis that evaluated bias from maternal cell contamination (MCC), the ITT RR for pregnancy with male offspring was 1.29 and the RD was 8.03 per 100 embryos, similar to the estimates from the primary analysis (RR = 1.30, RD = 8.52). In the sensitivity analysis that imputed outcome data for pregnancy losses with undetermined sex, the RRs for pregnancy with male offspring among pregnancies ranged from 1.13 to 1.18 ($P = 0.007$ – 0.047) (Figure 1) in data sets with plausible values for percentage male offspring out of missing data in each treatment group (44%–56%). LDA treatment was not associated with male offspring among 56 pregnancy losses with sex determined (Table 3). This was also true after restricting to aneuploid and euploid pregnancy losses (Table 3).

Stratified analyses. In the analysis stratified by serum concentration of preconception high-sensitivity C-reactive protein (hsCRP) at randomization, there was no effect of LDA treatment on male live births in the first tertile of hsCRP. However, LDA's effect increased with increasing tertile (first tertile: RR = 0.97, 95% CI: 0.70–1.35; second tertile: RR = 1.36, 95% CI: 0.98–1.90; third

tertile: RR = 1.70, 95% CI: 1.13–2.57; P interaction = 0.03; Table 1). The effect of LDA treatment on pregnancy incidence with male offspring was also stronger with increasing tertile of hsCRP (first tertile: RR = 1.00, 95% CI: 0.74–1.34; second tertile: RR = 1.46, 95% CI: 1.07–2.00; third tertile: RR = 1.58, 95% CI: 1.07–2.33; P interaction = 0.06; Table 2). In the placebo group, the proportion of males among live births by tertile of hsCRP was 52%, 43%, and 35% in the first, second, and third tertiles, respectively. In the LDA group, the respective values were 48%, 57%, and 52%. Thus, hsCRP was inversely associated with having a live-born male in the placebo group (third tertile vs. first tertile, RR = 0.56, 95% CI: 0.38–0.84, P trend = 0.006) but not in the LDA group (RR = 0.99, 95% CI: 0.70–1.39, P trend = 0.66), after adjusting for maternal age, smoking, income, race, and marital status. LDA was not associated with female live births in any hsCRP tertile, and hsCRP was not associated with female live births (Table 4).

Stratified by parity and previous son, the ITT RR of a live-born male was 1.46 (95% CI: 1.04–2.04) among nulliparous women, 1.19 (95% CI: 0.81–1.74) among parous women with no sons, and 1.21 (95% CI: 0.88–1.66) among parous women with ≥ 1 son (Table 1).

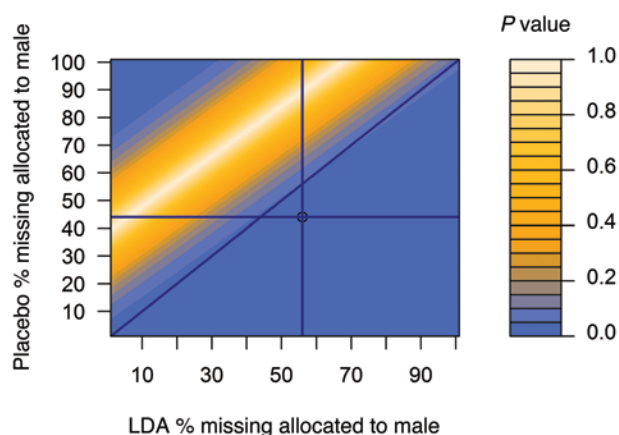


Figure 1. LDA and pregnancy with male offspring among 775 women with a pregnancy detected by a urine hCG test. Colors depict the *P* values from the χ^2 test of independence between LDA and male offspring, calculated in each of the 10,201 data sets containing imputed outcomes for 126 women who had a pregnancy with undetermined offspring sex. Each data set represents one possible scenario for the percentage male among missing data in the LDA and placebo groups, respectively. The central triangle comprises the data sets that met assumptions for plausible values of percentage male in the respective groups, i.e., 44%–56%, with the percentage male in LDA group greater than or equal to that of the placebo group. The analysis excluded 453 women who did not become pregnant. Each woman contributed one observation, with inverse probability weighting to adjust for selection of pregnant women.

Discussion

Among women with 1 to 2 prior pregnancy losses, women who were randomized to daily LDA treatment while attempting pregnancy were more likely to have a live-born male, translating to an increased sex ratio at birth. We observed an interaction between LDA treatment and preconception hsCRP measured at randomization, a putative marker of the maternal inflammatory milieu. Higher maternal inflammation was associated with a reduced sex ratio among women taking placebo but not LDA, suggesting that LDA restored the number of male offspring in women with higher levels of inflammation. These effects were similar after restricting to pregnancies, demonstrating an effect operating on the embryos at risk, and after restricting to live births, demonstrating an effect estimate that followed the convention of the sex ratio literature (1, 2). Using genetic data from pregnancy losses, we found that there was a higher probability of having a pregnancy with male offspring—sex ratio at implantation—in the LDA group and that this association was not meaningfully biased by pregnancy losses with unknown offspring sex. The LDA and placebo groups were similar with respect to live-born females among all women randomized and males among the pregnancy losses.

Collectively, our results suggest that inflammation reduces conception or survival of male embryos and that LDA initiated

prior to conception restores the number of males to a conventional level, most likely by modulating inflammation. LDA was not associated with the overall risk of pregnancy loss or with offspring sex among pregnancy losses, suggesting that its actions to enhance the conception or survival of viable male embryos were complete soon after implantation. It is uncertain whether LDA had an effect before implantation, as spontaneous conceptions cannot be detected prior to this point.

In this population of women with 1 to 2 prior documented pregnancy losses, the low proportion of males at birth in the placebo group (44%) may be related to a disordered inflammatory milieu that is harmful for male conception or survival. This population is well-suited for studying this hypothesis, since the trial excluded women with diagnosed medical disorders, including polycystic ovarian syndrome, antiphospholipid syndrome, and others that reflect other causes of pregnancy loss (exclusion criteria were described previously, ref. 21). Because the proportion of males at birth was low only among women in the placebo group who had high levels of baseline inflammation, we suspect that inflammation may be hazardous to the conception or survival of male embryos. This is in agreement with *in vivo* animal studies (11, 12).

Emerging evidence suggests that maternal inflammation may have sexually dimorphic effects on preimplantation embryos. Pre-

Table 3. LDA in relation to male offspring among 56 pregnancy losses with sex determined by genetic analysis (EAGeR trial, US, 2007–2012^a)

		All pregnancy losses ^b		Euploid losses ^c		Aneuploid losses ^c	
		LDA	Placebo	LDA	Placebo	LDA	Placebo
Original	Boy	11	10	2	7	9	2
	Girl	20	15	10	7	10	8
	Total	31	25	12	14	19	10
<i>P</i> value			0.73		0.11		0.23
Revised ^d	Boy	14	12	3	8	11	3
	Girl	17	13	5	4	12	9
	Total	31	25	8	12	23	12
<i>P</i> value			1.0		0.36		0.28

^aThe unit of analysis was the mother-offspring pair (*n* = 54). Pregnancy losses included two twin gestations: one male-male pair and one female-female pair. Genetic analysis was not performed on one phenotypic male, gestational age 15 weeks. This observation was included only in the analysis of all pregnancy losses. ^bTwo-sided *P* values were calculated from χ^2 test of independence. ^cTwo-sided *P* values were calculated from Fisher's exact test. ^dDistributions of offspring sex and aneuploid status were revised according to the results of an external validation study.

Table 4. Female live birth in relation to treatment assignment and hsCRP at randomization among 1,046 women and mother-offspring pairs (EAGeR trial, US, 2007–2012^A)

	N ^B	RR (95% CI)
LDA	142	0.93 (0.76–1.12)
Placebo	158	1.00 (reference)
Stratified by tertile of hsCRP at randomization		
hsCRP tertile 1: 0.15–0.71 mg/l		
LDA	52	1.12 (0.79–1.58)
Placebo	46	1.00 (reference)
hsCRP tertile 2: 0.72–2.08 mg/l		
LDA	43	0.79 (0.56–1.10)
Placebo	59	1.00 (reference)
hsCRP tertile 3: 2.09–62.7 mg/l		
LDA	47	0.91 (0.65–1.28)
Placebo	53	1.00 (reference)
hsCRP tertile 1: 0.15–0.71 mg/l ^C	98	1.00 (reference)
hsCRP tertile 2: 0.72–2.08 mg/l ^C	102	1.04 (0.82–1.32)
hsCRP tertile 3: 2.09–62.7 mg/l ^C	100	1.02 (0.80–1.29)
Stratified by treatment assignment		
LDA		
hsCRP tertile 1: 0.15–0.71 mg/l ^C	52	1.00 (reference)
hsCRP tertile 2: 0.72–2.08 mg/l ^C	43	0.86 (0.61–1.22)
hsCRP tertile 3: 2.09–62.7 mg/l ^C	47	0.92 (0.66–1.29)
Placebo		
hsCRP tertile 1: 0.15–0.71 mg/l ^C	46	1.00 (reference)
hsCRP tertile 2: 0.72–2.08 mg/l ^C	59	1.22 (0.88–1.70)
hsCRP tertile 3: 2.09–62.7 mg/l ^C	53	1.13 (0.80–1.59)

^AThis analysis included data from 1,039 participants who had hsCRP measured at randomization and completed follow-up. In the case of twin gestation, the embryo was the unit of analysis, with accounting for correlated data by using the generalized estimating equations extension of log-binomial regression. ^BNumber of live-born females. ^CModel adjusted for maternal age, smoking, income, race, and marital status.

implantation embryos exhibit sexually dimorphic physiology (22, 23). In bovines, maternally derived colony-stimulating factor 2 cytokine decreased length and interferon-tau secretion of female embryos but increased length and interferon-tau secretion of male embryos (11). Colony-stimulating factor 2 also caused sex-dependent changes in the embryonic transcriptome and methylome (11), indicating that maternal inflammation may exert broader, sexually dimorphic effects. In mice, heat stress resulted in lower survival and higher hydrogen peroxide production among male preimplantation embryos relative to those of female preimplantation embryos (12). This was explained by the lower expression by male embryos of the X-linked gene, glucose 6-phosphate dehydrogenase, which contributes to controlling free radicals (12).

LDA may have aided the implantation of viable male embryos by modulating inflammation in the decidua among individuals with a tendency toward overactive inflammation (14). Decidualized endometrial stromal cells respond to a poor-quality embryo with profound downregulation of the *HSPA8* gene to induce a proteotoxic stress response (24). Embryonic metabolism may influence signaling, as poor-quality embryos are more active

metabolically due to greater demands for cell repair (25, 26). The male embryo in particular may have elicited this over-active response due to its potentially greater metabolic activity (22, 23), thereby presenting more opportunity for “rescue” through LDA’s antiinflammatory actions compared with female embryos. As the science of maternal-fetal recognition is evolving, there is uncertainty around the precise biological mechanisms that may have produced our results.

The concept of a biological mechanism linked with both pregnancy loss and reduced sex ratio has some precedent in the literature. To our knowledge, there are two other broad categories of exposure that increase inflammation; these categories are associated both with increased risk of pregnancy loss and with reduced sex ratio at birth. While these mechanisms may not be acting in our study, their existence enhances the plausibility of an analogous mechanism that could have produced a reduced sex ratio among women with 1 to 2 prior pregnancy losses in the placebo group. First, maternal immunization against male-specific histocompatibility antigens (27) was hypothesized to explain a sex ratio of 0.76 among 213 births to Danish women following a diagnosis of unexplained secondary recurrent (≥ 3) pregnancy loss (27). This mechanism also increases inflammation. Second, parental exposure to certain environmental toxins — including lead (28), methylmercury (29), and high exposure to pesticides (30) — has been linked to both pregnancy loss and reduced sex ratio (31), which is a plausible mechanism for lower sex ratio among women with prior pregnancy loss, perhaps by inducing inflammation. In sum, prior studies have implicated inflammatory mechanisms in their findings of reduced sex ratio and pregnancy loss, and, by analogy, the lower sex ratio among women in the overall placebo group observed here can be regarded as potentially valid.

This study has several limitations to consider. It is possible — as it is in any study — that the differences observed were the result of a type I error. The observed sex ratio among the placebo group overall was lower than expected in the general population, while among the LDA group it was as expected. Thus, replication of our findings among a similar population with 1 to 2 prior pregnancy losses is needed to confirm our findings. Additionally, selection bias may have produced a reduced sex ratio among women in the placebo group if women who withdrew early from the placebo group were more likely to have a male live birth. This is unlikely since early withdrawal was fairly low and balanced between the groups (13% in the LDA group, 11% in the placebo group). Spontaneous fertilization is unobservable, and so effects on implantation are indistinguishable from effects on fertilization. In addition, MCC was not ruled out in 15 of 17 euploid female results. However, a sensitivity analysis concluded that this produced negligible bias in the RD and RR of pregnancy with male offspring. Our results should be applicable to women similar to those in the study population: 18 to 40 years of age, with 1 to 2 prior pregnancy losses and no major medical problems or history of infertility (21).

Our findings, that we believe to be novel, suggest that maternal inflammation may be hazardous to male conception or embryonic survival and that LDA may restore the proportion of male embryos to a normal level by modulating inflammation. These findings implicate fundamental inflammatory processes, which may affect the sex ratio of populations. However, the observed effect would

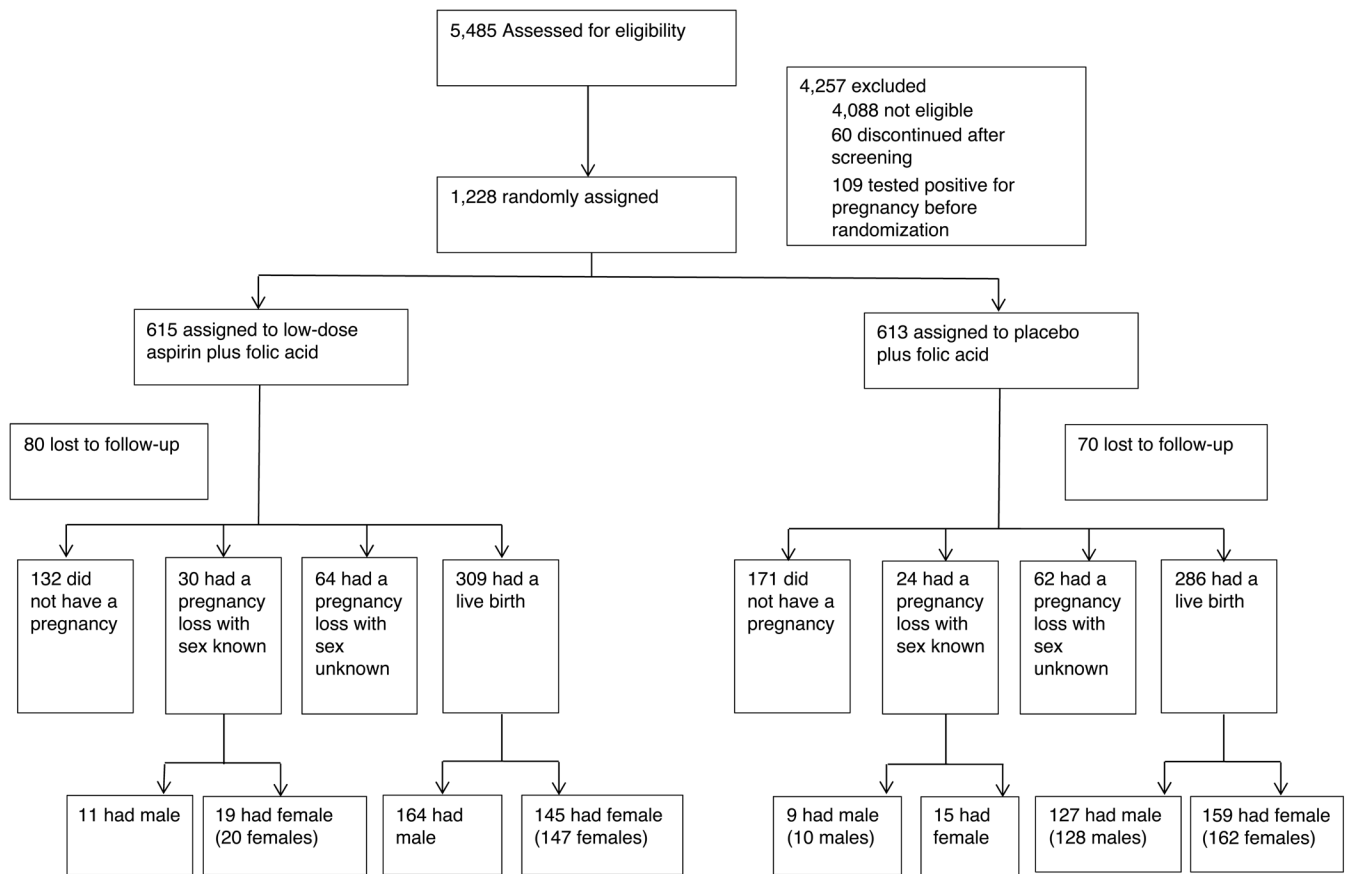


Figure 2. EAGeR trial flow diagram. The total numbers of male and female offspring are shown in parentheses, reflecting 8 twin gestations.

make little difference on an individual level, and LDA is not recommended for increasing the probability of having a son. We hope that our data will prompt further study of the relationships among inflammation, pregnancy loss, and sex ratio, ultimately leading to better reproductive outcomes.

Methods

The EAGeR trial was a block-randomized, double-blind, placebo-controlled, parallel-design trial of daily preconception 81 mg aspirin to prevent pregnancy loss (ClinicalTrials.gov NCT00467363) (21). It was conducted at 4 clinical sites in the US from 2007 to 2012 (Salt Lake City, Utah; Scranton, Pennsylvania; Denver, Colorado; and Buffalo, New York).

Participants were 18–40 years old; had regular menstrual cycles, 21 to 42 days in length; and were trying to conceive without fertility treatments. The original eligibility criteria specified 1 documented pregnancy loss that occurred at <20 weeks gestation in the previous 12 months and ≤1 live birth. Expanded eligibility criteria were implemented to increase enrollment, allowing women to enroll if they had 1 to 2 documented pregnancy losses at any time in the past that may have occurred before or after 20 weeks gestation and ≤2 live births.

Women were randomized in a 1:1 ratio to receive 81 mg aspirin daily or identical placebo (Figure 2). An automatic computer-generated randomization algorithm developed by the Data Coordinating Center used a permuted block design, with blocks of 6 or 8 in random order, defined by study center and eligibility criteria. At the random-

ization visit, which coincided with menstrual cycle days 2 to 4, participants provided a blood specimen, and preconception hsCRP serum concentration — a marker of systemic inflammation — was quantified using the Roche COBAS 6000 Chemistry Analyzer (Roche Diagnostics; limit of detection = 0.15 mg/l).

For up to 6 menstrual cycles, while attempting pregnancy, participants used study-provided fertility monitors (ClearBlue, Swiss Precision Diagnostics GmbH) and urine hCG pregnancy tests (QuickVue, Quidel Corporation) at home and at end-cycle clinic visits. Those with a positive pregnancy test returned for an ultrasound at 6 to 7 weeks gestation to determine an ultrasound-confirmed pregnancy (clinical pregnancy). If there was no visible gestational sac, the participant was diagnosed with a peri-implantation loss and continued with pre-pregnancy follow-up for the remainder of the 6 cycles. Participants who became pregnant continued with monthly study visits throughout pregnancy and study medication through gestational week 36. Study completion occurred as planned in 2012.

Adverse effects are detailed in the Supplemental Methods.

Ascertainment of biological sex. The sex of live-born infants was ascertained by medical chart abstraction. In the event of a loss of a clinical pregnancy, products of conception were collected when possible for genetic testing. At the 6- to 7-week ultrasound visit, participants were asked to contact study staff as soon as possible if a pregnancy loss was diagnosed in a clinical setting and to collect a specimen with study-issued equipment if a pregnancy loss occurred at home. When possible, an immediate attempt was made to assess fetal or placental

karyotype or chromosomal microarray. Specimens were refrigerated for up to 24 hours and then frozen at -80°C , with some additional frozen specimens subjected to chromosomal microarray. Genetic testing was performed on 84 of 127 clinical pregnancy losses (including 2 twin gestations): 55 tests determined sex and 29 tests had no results due to testing failure ($n = 5$) or indeterminate result ($n = 24$). One 15-week phenotypic male had no genetic analysis.

Statistics. The ideal method to estimate an effect of LDA on embryonic survival that operates differentially by sex would assess the association with male offspring at various stages of the reproductive process. Our data analysis aimed to approximate this ideal, given the constraints on feasibility, by including the sex of pregnancy losses determined by genetic testing, when available, and by conducting a sensitivity analysis that imputed outcome data for pregnancy losses with no determination of offspring sex.

The primary analysis used an ITT approach, comparing male live births among participants with complete follow-up in treatment and placebo groups by calculating the RR, RD, and corresponding 95% CI. The purpose was to estimate the effect of LDA on carrying and giving birth to a live-born male among all women offered treatment. We also estimated the effect of LDA on female live births to clarify whether it was male or female survival that produced a change in the sex ratio. Because the ITT analysis included women who did not become pregnant, we conducted secondary analyses that were restricted to pregnancies detected by urine hCG testing and to live births. As restrictions to pregnancies and live births might break the randomization, we adjusted for the selection through the use of stabilized inverse probability weights (32) in a weighted log-binomial regression model with robust variance estimation. The unit of analysis was the woman and, in the case of pregnancy, the offspring. Thus, 8 twin gestations contributed 2 observations each, and we estimated the RR and RD with robust standard errors by using PROC GENMOD in SAS version 9.4 (SAS Institute). For all statistical tests, a P value of less than 0.05 was considered significant.

The analysis that evaluated the relation of LDA with having a pregnancy with male offspring (sex ratio at implantation) assessed the effect of LDA on a pregnancy with a male embryo that implanted and was detected by a urine hCG test, whether or not it survived to live birth. An ITT analysis as well as a secondary analysis that restricted to pregnancies counted women as having the event of interest if they had either a live-born male or a pregnancy loss with male sex determined. The unit of analysis was the woman and, in the case of pregnancy, the offspring.

Sensitivity analyses. Female karyotypes of first-trimester losses may be inaccurate due to MCC (33), and 15 of 17 euploid female karyotype results did not exclude possible MCC. A simple quantitative bias analysis (34) that was informed by an external validation study (33) evaluated bias from outcome misclassification to the estimated effect of LDA on pregnancy with male offspring.

Furthermore, the analysis of pregnancy with male offspring excluded 126 offspring with undetermined sex (55 peri-implantation losses and 71 clinical pregnancy losses). To assess potential bias from missing data, we first imputed the offspring sex of these pregnancy losses under every possible scenario of the percentage male of missing data (0%–100%) in each treatment group. Then, for each scenario, we calculated the P value from the χ^2 test of the association of LDA and male offspring if the sex was determined in all pregnancies (Figure 1). We restricted our discussion to results from data sets that met our

assumptions for plausible values of percentage male in the treatment and placebo groups (44%–56%, the central triangular area of Figure 1): (a) the percentage male out of missing data in each treatment group could vary from the observed value among pregnancies in the placebo group to the observed value among pregnancies in the treatment group; and (b) the percentage male of missing data in the treatment group was greater than or equal to that of the placebo group. The median gestational age of these pregnancy losses was 8 weeks (interquartile range: 7–10 weeks, range: 2–20 weeks). Our range of plausible values for percentage male is compatible with the finding that a majority of pregnancy losses are female during the period from implantation through the first half of the second trimester (35). The observed values for percentage of male and female pregnancy losses were corrected for outcome misclassification due to MCC (33).

Stratified analyses. To further test whether LDA may affect sex ratio by modulating inflammation, we stratified analyses by preconception hsCRP serum concentration. Participants with hsCRP concentration measured ($n = 1,039$) were categorized according to tertiles of preconception serum hsCRP (first tertile: 0.15–0.71 mg/l; second tertile: 0.72–2.08 mg/l; third tertile: 2.09–62.7 mg/l). The presence of statistical interaction was tested with the Wald χ^2 test of the coefficient of one cross-product variable that modeled treatment assignment and hsCRP tertile median. Furthermore, we estimated the RR and 95% CI of male live birth in the highest-versus-lowest tertile of preconception hsCRP among women assigned to placebo and among women assigned to LDA and calculated the P value for linear trend in each group by using the Wald χ^2 test of the coefficient of a linear variable for hsCRP tertile median. Finally, we stratified the analysis of LDA and male live birth by previous live-born children (nulliparous, parous with no sons, parous with ≥ 1 son) in order to assess whether LDA may affect the sex ratio by modulating an aberrant maternal immune response to H-Y antigens (27).

The study size for this clinical trial was based on the enrollment goal of 1,600 participants in order to detect a 10% absolute difference in the EAGeR trial's primary endpoint, live birth rate, with 80% power and a 5% type I error rate. The sample-size calculation assumed a live birth rate of 40% over 6 cycles in the placebo arm and loss to follow-up of 20%. However, actual performance was better than the assumptions, with live birth rate of 53% in the placebo arm and loss to follow-up of 12%.

Study approval. The study protocol was approved by the Intermountain Healthcare Institutional Review Board (Salt Lake City, Utah, USA), The Wright Center for Graduate Medical Education Institutional Review Board (Scranton, Pennsylvania, USA), the University at Buffalo Health Sciences Institutional Review Board (Buffalo, New York, USA), and the Colorado Multiple Institutional Review Board (Aurora, Colorado, USA). Participants provided written informed consent prior to study enrollment.

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