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# Shadows Cast Before Birth: In Utero and Perinatal Origins of Premature Ovarian Insufficiency—A Multi-Generational Cohort Study

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## Abstract

Background: Premature ovarian insufficiency (POI) affects 1%-3.7% of women under 40, causing infertility and long-term health morbidities. While genetic, autoimmune, and iatrogenic factors are well-established, emerging evidence suggests that ovarian reserve, established entirely during fetal life, may be profoundly shaped by intrauterine exposures. This study investigates whether maternal nutritional status, metabolic dysfunction, and environmental toxin exposure during pregnancy predict reduced ovarian reserve in female offspring. Methods: This prospective multi-generational cohort study enrolled 1,248 mother-daughter dyads from Fatima Memorial Hospital, Lahore, Pakistan (2008-2012). Maternal data included dietary diversity scores (MDD-W), serum micronutrients, heavy metal profiles (lead, cadmium, mercury), glycaemic indices, and obstetric records. Daughters were followed through age 14-18 years, when ovarian reserve was assessed using serum anti-Müllerian hormone (AMH) and antral follicle count (AFC). Multivariate regression and structural equation modelling were applied. Results: Of 1,248 enrolled dyads, 1,024 (82.1%) completed follow-up. Daughters of mothers in the lowest MDD-W quartile had significantly lower AMH (1.42 vs. 2.87 ng/mL; $p < 0.001$). Maternal gestational diabetes was associated with 34.7% reduction in daughter's AFC ($\beta = -2.3$, $p < 0.001$). Blood lead > 5 μg/dL predicted AMH reduction of 0.84 ng/mL ($p < 0.001$). Intrauterine growth restriction mediated 38.2% of the undernutrition-AMH pathway. Cumulative exposures produced additive effects, with triple-exposed daughters showing 1.96 ng/mL lower AMH. Conclusions: Maternal nutritional deficiency, environmental toxins, and metabolic dysfunction during pregnancy represent modifiable determinants of daughter's ovarian reserve. These findings support fetal programming of POI and argue for antenatal health optimization and targeted screening in high-risk pregnancies.

**Keywords**

Premature ovarian insufficiency, Fetal programming, Ovarian reserve, Developmental origins of health and disease, Anti-Müllerian hormone, Environmental exposures, Maternal nutrition, Multi-generational cohort

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## 1. Introduction

Premature ovarian insufficiency (POI), previously designated as premature ovarian failure, represents one of the most significant causes of female infertility and premature estrogen deficiency. The European Society of Human Reproduction and Embryology (ESHRE) defines POI as the loss of normal ovarian function before the age of 40 years, characterized by oligo/amenorrhoea for at least four months in combination with elevated follicle-stimulating hormone (FSH) levels greater than 25 IU/L on two occasions, measured more than four weeks apart [1]. Its prevalence ranges from 1% to 3.7% globally, though data from South Asian populations remain substantially underrepresented in the scientific literature [2]. The condition is not merely a reproductive concern; the consequent hypoestrogenism carries profound implications across cardiovascular, skeletal, neurological, and psychosocial domains that collectively contribute to a measurable reduction in quality of life and longevity [3].

The human ovarian reserve—the total cohort of primordial follicles available for maturation throughout a woman's reproductive life—is established entirely during the fetal period, peaking at approximately six to seven million germ cells around gestational weeks 16-20 and declining through atresia to roughly one to two million by birth [4]. No new follicles are generated postnatally. This biological reality situates the intrauterine environment as a uniquely sensitive and irrevocable determinant of lifelong reproductive potential. If conditions in utero compromise oogonial proliferation, meiotic progression, or folliculogenesis, the resulting follicular deficit is permanent and may manifest clinically as diminished ovarian reserve (DOR) or frank POI decades later [5].

The developmental origins of health and disease (DOHaD) framework, introduced conceptually by Barker and colleagues in the late 1980s and subsequently expanded through landmark longitudinal studies, has established compelling mechanistic evidence that suboptimal fetal environments—characterized by nutrient deprivation, hormonal imbalance, oxidative stress, or toxicant exposure—program the structure and function of multiple organ systems in ways that confer risk for chronic disease in adult life [6,7]. Remarkably, the ovary, as an organ entirely reliant upon its antenatal endowment, represents a compelling candidate for DOHaD-mediated vulnerability, yet this axis of inquiry has remained substantially understudied, particularly within low- and middle-income country (LMIC) populations where both nutritional insufficiency and environmental toxicant burden are disproportionately prevalent [8].

Recent advances in reproductive epidemiology have begun to illuminate the profound intergenerational consequences of maternal exposures during pregnancy. Richardson and colleagues [9] demonstrated that maternal undernutrition during the Dutch Hunger Winter of 1944-1945 was associated with altered reproductive outcomes in offspring conceived during that period, providing some of the earliest human evidence for nutritional programming of reproductive function. More contemporary work by Anand-Ivell et al. [10] demonstrated that maternal environmental exposures, including smoking and phthalate load, during pregnancy are associated with altered amniotic insulin-like factor 3 (INSL3) levels in male offspring, suggesting that environmental toxicants can specifically target gonadal development across sexes. However, systematic investigation of multiple concurrent exposures—nutritional, metabolic, and environmental—has remained limited by the logistical challenges of prospective multi-decade follow-up [11].

Pakistan presents a particularly salient context for this investigation. National nutrition surveys consistently document high rates of iron, folate, zinc, and vitamin D deficiency among women of reproductive age, with approximately 41% of pregnant women experiencing anemia and 62% demonstrating vitamin D insufficiency [12]. Industrial urbanization has precipitated significant heavy metal contamination in peri-urban communities, particularly around Lahore's industrial corridors where tanneries, battery recycling facilities, and informal electronic waste processing operations release substantial quantities of lead and cadmium (Cd) into the environment [13]. Simultaneously, the country bears a rapidly escalating burden of gestational diabetes mellitus, driven by rising obesity prevalence and dietary transition; current estimates suggest that 16%-20% of pregnancies in urban Pakistani centers are complicated by gestational diabetes [14]. Each of these factors has been individually implicated—though never comprehensively interrogated in combination—as a potential modulator of fetal ovarian development [15].

The present study was therefore designed to prospectively examine, over a follow-up period of 14 to 18 years, whether measurable maternal exposures during pregnancy—nutritional, metabolic, and environmental—independently or synergistically predict reduced ovarian reserve in female offspring. To our knowledge, this constitutes the first multi-generational cohort study from Pakistan, and among the few globally, to systematically integrate maternal environmental toxicology, dietary assessment, and offspring ovarian ultrasonography within a single longitudinal framework. The findings hold potential to reshape our understanding of POI pathogenesis and to inform preventive strategies targeting the earliest stages of human development [16].

## 2. Background and Theoretical Framework

### 2.1 Fetal Folliculogenesis and Its Vulnerability Windows

Primordial germ cells (PGCs) migrate from the yolk sac to the gonadal ridge by gestational week five, where they differentiate into oogonia under the influence of NOBOX, FIGLA, FOXL2, and BMP15 signalling cascades [17]. Between weeks 8 and 20, oogonia undergo mitotic amplification, followed by entry into prophase I of meiosis and encapsulation by pregranulosa cells to form primordial follicles. This window of active folliculogenesis represents the most sensitive period for exogenous disruption [18].

The molecular choreography of human ovarian development involves precisely timed expression of thousands of genes, many of which are epigenetically regulated and susceptible to environmental modulation [19]. Recent single-cell RNA sequencing studies of human fetal ovaries have revealed remarkable heterogeneity in germ cell populations during development, with distinct transcriptional signatures characterizing mitotic, meiotic, and quiescent states [20]. These findings underscore the complexity of ovarian development and the multiple points at which disruption might occur.

Nutrient availability during this phase is critical: folate is essential for DNA methylation and meiotic progression; zinc acts as a co-factor for multiple folliculogenic enzymes including DNA polymerase and superoxide dismutase; and iron mediates mitochondrial biogenesis within the oocyte, which must support the entire subsequent developmental trajectory of the embryo [21]. Experimental murine models have demonstrated that maternal folate restriction during the equivalent of the second trimester reduces offspring primordial follicle number by up to 40%, accompanied by accelerated follicular atresia via upregulation of the Bcl-2/BAX apoptotic pathway [22]. These animal data provide mechanistic plausibility for the human observations that form the basis of the present study.

Furthermore, emerging evidence suggests that the ovarian reserve may be influenced not only by the number of follicles initially formed but also by the rate of follicular activation and atresia during childhood and adolescence [23]. The mTOR signalling pathway, which integrates nutritional and growth factor signals, plays a central role in regulating the transition of primordial follicles into the growing pool [24]. Dysregulation of this pathway by intrauterine exposures could theoretically alter the delicate balance between follicle preservation and activation, with long-term consequences for reproductive lifespan [25].

### 2.2 Environmental Endocrine Disruption and Gonadotoxicity

Heavy metals—particularly lead (Pb), Cd, and mercury (Hg)—are well-established ovarian gonadotoxins [26]. Cd mimics the biological activity of zinc and competes at zinc-dependent enzymatic sites critical for folliculogenesis, including those involved in DNA synthesis and repair. In human studies, higher urinary Cd concentrations have been associated with reduced serum AMH and earlier menopause, with effect sizes comparable to those observed for cigarette smoking [27]. Lead exposure has been linked to dysregulation of the hypothalamic-pituitary-ovarian (HPO) axis, inhibiting GnRH pulsatility and reducing gonadotropin-dependent follicular recruitment [28]. Importantly, maternal heavy metal burden crosses the placenta with high efficiency; fetal blood concentrations of lead typically approximate 80%-90% of maternal blood levels, and the fetal blood-brain barrier provides incomplete protection against neuroendocrine disruption [29].

The mechanisms by which heavy metals exert their gonadotoxic effects are multifactorial. Oxidative stress appears to play a central role, with metals catalyzing the formation of reactive oxygen species that damage cellular membranes, proteins, and DNA [30]. Mitochondrial dysfunction is particularly relevant to oocytes, which contain the highest mitochondrial DNA content of any cell type and rely on mitochondrial function for energy production and calcium homeostasis [31]. Lead has been shown to impair mitochondrial respiration and induce permeability transition pore opening, triggering apoptosis in granulosa cells [32].

Emerging evidence also implicates epigenetic mechanisms in metal-induced reproductive toxicity. Chronic lead exposure has been associated with altered DNA methylation patterns in genes involved in ovarian function, including those encoding hormone receptors and steroidogenic enzymes [33]. These epigenetic modifications could potentially persist across cell divisions, contributing to long-term alterations in ovarian function even after the initial exposure has ceased [34].

### 2.3 Gestational Diabetes and Ovarian Organogenesis

Hyperglycaemia during sensitive windows of ovarian development generates an intrauterine milieu of chronic oxidative stress, advanced glycation end-product accumulation, and insulin-like growth factor (IGF-1) dysregulation—conditions collectively inimical to granulosa cell survival and primordial follicle formation [35]. Animal data from streptozotocin-induced diabetic rat models reveal significantly smaller ovarian follicular pools in female offspring compared to controls, mediated in part through FOXO3a-dependent mechanisms that accelerate primordial follicle activation [36]. The extrapolation of these observations to women with gestational diabetes, though biologically plausible, had not been confirmed in large-scale longitudinal human studies prior to the present investigation.

Recent work has further elucidated the molecular pathways linking maternal hyperglycaemia to offspring ovarian development. Hyperglycaemia induces endoplasmic reticulum stress in fetal oogonia, activating the unfolded protein response and promoting apoptosis through CHOP-dependent mechanisms [37]. Additionally, elevated glucose levels increase flux through the hexosamine biosynthesis pathway, leading to O-GlcNAcylation of key transcription factors involved in ovarian development, potentially altering their transcriptional activity [38].

The timing of hyperglycaemic exposure may be critical in determining its effects on the developing ovary. Gestational diabetes typically manifests in the late second or early third trimester, coinciding with the latter stages of folliculogenesis when primordial follicles are being established [39]. This timing may be particularly detrimental because it affects not only oogonial proliferation but also the initial stages of follicle assembly and survival [40].

### 2.4 The DOHaD Framework and Reproductive Aging

The DOHaD paradigm has evolved substantially since its initial formulation, incorporating concepts of developmental plasticity, predictive adaptive responses, and intergenerational transmission of risk [41]. According to this framework, organisms facing adverse intrauterine conditions make adaptive adjustments in their development that prioritize short-term survival at the expense of long-term health [42]. The ovary, as an organ whose primary function is required only decades after birth, may be particularly susceptible to such trade-offs.

Epidemiological studies have provided indirect support for this hypothesis. Women born with low birth weight, a marker of adverse intrauterine conditions, have been shown to experience earlier menopause and reduced fecundability in some [43], though not all [44], studies. However, birth weight is a crude proxy for the complex intrauterine environment, and more precise characterization of specific exposures is needed to establish causal relationships [45]. The present study addresses this gap by prospectively measuring multiple dimensions of the intrauterine environment and relating them to directly assessed ovarian reserve in offspring.

## 3. Materials and Methods

### 3.1 Study Design and Setting

This was a prospective multi-generational cohort study conducted at Fatima Memorial Hospital (FMH), a tertiary care centre in Lahore, Pakistan. The institution serves a diverse patient population drawn from both urban and peri-urban communities, providing a broad representation of socioeconomic and environmental exposures relevant to the research questions. Written informed consent was obtained from all participants. The study was conducted in accordance with the Declaration of Helsinki (2013 revision) and registered with the Pakistan National Health Research Registry (NHRC-2008-LHR-0112).

### 3.2 Participant Recruitment and Enrolment

Pregnant women were enrolled consecutively between January 2008 and December 2012 during their first antenatal visit (8-12 weeks gestation). Inclusion criteria required singleton pregnancy, maternal age 18-38 years, and residence within a defined 30-kilometre catchment area around the hospital to facilitate long-term follow-up. Women with pre-existing ovarian pathology, known chromosomal abnormalities, twin pregnancies, or severe comorbidities precluding standard antenatal follow-up were excluded. Of 1,412 women initially screened, 1,248 mother-daughter dyads were enrolled following confirmation of viable female singleton gestation at the anatomy scan (18-22 weeks). The sample size was calculated to detect a 0.3 ng/mL difference in AMH between exposure groups with 80% power at $\alpha = 0.05$, assuming 20% attrition over the follow-up period.

### 3.3 Maternal Exposure Assessment

Dietary diversity was assessed at enrolment and again at 28 weeks using the validated Minimum Dietary Diversity for Women (MDD-W) tool, a 24-hour dietary recall instrument scoring consumption across ten food groups (FAO/FHI360, 2016) [46]. This tool has been extensively validated in LMIC settings and correlates well with micronutrient adequacy. Serum micronutrient analyses—folate, ferritin, zinc, 25-hydroxyvitamin D, and vitamin B12—were performed at 12 and 28 weeks using standardized laboratory assays at a CAP-accredited laboratory. Quality control procedures included internal and external proficiency testing.

Whole blood lead, urine Cd (normalized to creatinine), and hair Hg were measured at 16 weeks gestation using inductively coupled plasma mass spectrometry (ICP-MS) at a certified reference laboratory. Detection limits were 0.5 μg/dL for lead, 0.05 μg/L for Cd, and 0.01 μg/g for Hg. Samples below the detection limit were assigned a value of half the detection limit for statistical analysis. Rigorous contamination control procedures were followed, including the use of metal-free collection tubes and thorough participant instruction to avoid environmental contamination.

Gestational diabetes mellitus was diagnosed at 24-28 weeks using the IADPSG one-step criteria (fasting plasma glucose $\geq$5.1 mmol/L, or one-hour $\geq$ 10.0 mmol/L, or two-hour $\geq$8.5 mmol/L following a 75g OGTT) [47]. Thyroid function (TSH, free T4) was measured at enrolment using electrochemiluminescence immunoassays. Obstetric outcomes including birth weight, gestational age at delivery, APGAR scores, and presence of IUGR (defined as birth weight below the 10th percentile for gestational age using Fenton growth charts) were extracted from hospital records by trained research staff.

### 3.4 Offspring Follow-Up and Ovarian Reserve Assessment

Daughter participants were followed through scheduled clinical visits at 6 months, 2, 5, 10, and 14-18 years of age. A dedicated tracking system, including telephone reminders, home visits, and coordination with local schools and community health workers, was implemented to minimize attrition. At the final follow-up visit (conducted 2022-2026), daughters aged 14-18 years underwent the following ovarian reserve assessments:

(1) Fasting serum AMH measured using the Elecsys AMH Plus immunoassay (Roche Diagnostics), which demonstrates high interlaboratory reproducibility and has been validated for use in adolescent populations [48]. Samples were analyzed in duplicate, and the mean value was used for analysis.

(2) Transabdominal ultrasonographic antral follicle count (AFC) counting bilateral antral follicles of 2-10 mm diameter, performed by a single trained sonologist blinded to maternal exposure data using a standardized protocol. For participants with adequate acoustic windows, transvaginal ultrasound was offered to improve visualization where clinically appropriate; this was performed only in post-menarchal participants who provided separate written assent (with written parental/guardian consent), and only when the participant and guardian explicitly agreed after counselling by a trained female research staff member. Age-appropriate care protocols were followed throughout, including the option to have a female chaperone present. All scans were performed in the early follicular phase (days 2-5 of the menstrual cycle) when possible; for oligomenorrheic participants, scans were scheduled without regard to menstrual timing.

Pubertal staging was assessed using Tanner criteria by a trained female research assistant. Daughters with irregular menstruation (oligomenorrhoea or secondary amenorrhoea) at follow-up underwent additional FSH and estradiol measurement to screen for subclinical POI. None of the daughters received hormonal contraception or other medications known to suppress ovarian reserve at the time of assessment.

### 3.5 Statistical Analysis

Continuous variables were summarized using means and standard deviations or medians and interquartile ranges, as appropriate following Shapiro-Wilk normality testing. Categorical variables were expressed as frequencies and percentages. AMH values were log-transformed for regression analyses given their right-skewed distribution, with results back-transformed for presentation.

Multivariate linear regression models were constructed to examine associations between each maternal exposure and offspring AMH and AFC, adjusting for potential confounders including maternal age, parity, socioeconomic status (assessed by the multidimensional poverty index), mode of delivery, gestational age at birth, and daughter's age and Tanner stage at follow-up. Model building proceeded in a stepwise fashion, with covariates retained if they changed the exposure coefficient by > 10% or were significant at $p < 0.10$. Effect modification by birth weight and gestational age was explored through interaction terms and stratified analyses.

Structural equation modelling (SEM) was performed using AMOS version 24.0 (IBM Corp.) to test mediation pathways and assess the cumulative exposure model. The SEM approach allowed simultaneous estimation of direct and indirect effects while accounting for measurement error and correlations among exposures. Model fit was assessed using the comparative fit index (CFI), Tucker-Lewis index (TLI), and root mean square error of approximation (RMSEA), with values > 0.95 for CFI/TLI and < 0.06 for RMSEA indicating good fit. Mediation analysis used the bootstrap method with 5,000 resamples to generate bias-corrected confidence intervals.

Multiple imputation by chained equations was performed for missing covariate data (present in < 5% of cases), assuming data were missing at random. Sensitivity analyses using complete-case analysis yielded similar results, supporting the robustness of the findings. Statistical significance was set at $p < 0.05$ (two-tailed). All analyses were performed using SPSS version 27.0 and R version 4.2.1.

## 4. Results

### 4.1 Cohort Characteristics

Of the 1,248 enrolled mother-daughter dyads, 1,024 (82.1%) completed the full 14-18-year follow-up with valid ovarian reserve data. Attrition was attributable primarily to migration out of the catchment area (n = 143), loss of contact (n = 52), daughter's refusal of ultrasonographic assessment (n = 19), and laboratory processing failure (n = 10). Maternal and daughter baseline characteristics of completers versus non-completers did not differ significantly on key demographic variables ($\chi^2$ analysis, $p > 0.05$ for all), minimizing the risk of informative attrition bias.

Table 1 summarizes the maternal sociodemographic and clinical characteristics of the final analytic sample. Mean maternal age at enrolment was 26.4 ± 4.7 years. The majority of participants (67.3%) belonged to lower-middle socioeconomic strata. GDM was diagnosed in 18.4% of enrolled women—a prevalence consistent with regional epidemiological estimates.

**Table 1.** Maternal sociodemographic and clinical characteristics (n = 1,024).

| Characteristic | n (%) | Mean ± SD / Median (IQR) |
|---|---|---|
| Maternal age at enrolment (years) | 1,024 (100) | 26.4 ± 4.7 |
| Primigravida | 431 (42.1) | — |
| Gestational diabetes mellitus | 188 (18.4) | — |
| Pre-existing hypertension | 74 (7.2) | — |
| Thyroid dysfunction | 121 (11.8) | — |
| Low dietary diversity (MDD-W < 5) | 389 (38.0) | — |
| Serum ferritin (μg/L) | — | 18.4 (10.2-30.6) |
| 25-OH Vitamin D (nmol/L) | — | 34.7 ± 18.3 |
| Blood lead level > 5 μg/dL | 316 (30.9) | 4.1 (2.2-7.8)* |
| Urinary Cd (μg/g creatinine) | — | 0.9 (0.4-2.1) |
| IUGR in index pregnancy | 163 (15.9) | — |

Note: *Median (IQR) for continuous lead levels presented; categorical threshold shown separately. MDD-W: Minimum dietary diversity-women; IUGR: Intrauterine growth restriction; Thyroid dysfunction defined as TSH > 4.5 mIU/L or current thyroid medication use at enrolment. Note that 30.9% (n = 316) of mothers had blood lead concentrations exceeding the WHO reference level of 5 μg/dL, reflecting the substantial environmental lead burden in the study population. Thyroid dysfunction was present in 11.8% (n = 121), predominantly subclinical hypothyroidism.

### 4.2 Daughter Ovarian Reserve at Follow-Up

The mean age of daughters at ovarian reserve assessment was 16.2 ± 1.3 years (range 14.1-18.7 years). Mean serum AMH in the cohort was 2.31 ± 1.07 ng/mL and mean AFC was 14.8 ± 5.6 follicles. These values are consistent with published normative data for adolescents, though at the lower end of the range reported in European populations [49]. Thirty-four daughters (3.3%) demonstrated markedly DOR, defined by AMH below the fifth percentile for age (< 0.7 ng/mL) with corroborating FSH elevation (> 10 IU/L); these individuals are classified as high risk for future POI and warrant long-term follow-up. An additional 72 (7.0%) had AMH in the diminished reserve range (0.7-1.1 ng/mL), suggesting early-stage compromise. It is emphasized that formal ESHRE criteria for POI (FSH > 25 IU/L on two occasions with menstrual disturbance) were not met in these adolescents, and these findings should be interpreted as early markers of DOR rather than as diagnoses of POI. The prevalence of markedly DOR in this cohort is approximately twice that reported in comparable European studies [50], consistent with the hypothesis that adverse intrauterine exposures in this population contribute to reduced ovarian reserve.

Pubertal development was within normal limits for the majority of participants, with 93% having achieved menarche by the time of assessment. Mean age at menarche was 12.8 ± 1.2 years, comparable to national averages. Tanner stage was significantly associated with AMH levels ($\beta = 0.18$ per stage, $p = 0.02$), underscoring the importance of adjusting for pubertal status in all analyses.

### 4.3 Association Between Maternal Dietary Diversity and Offspring Ovarian Reserve

After full covariate adjustment, maternal dietary diversity score (MDD-W) was positively and independently associated with daughter AMH ($\beta = 0.23$, 95% CI: 0.17-0.29; $p < 0.001$) and AFC ($\beta = 1.4$, 95% CI: 0.9-1.9; $p < 0.001$). The relationship was monotonic across the range of MDD-W scores, with no evidence of threshold effects. Daughters born to mothers in the lowest MDD-W quartile (score ≤ 3) had a mean AMH of 1.42 ng/mL (95% CI: 1.28-1.56), compared to 2.87 ng/mL (95% CI: 2.71-3.03) in the highest quartile (score ≥ 8), representing a 50.5% difference ($p < 0.001$). This effect size is clinically meaningful, equivalent to approximately 10-15 years of age-related AMH decline [51].

Serum folate and zinc were the micronutrients most strongly correlated with AMH in mediation analyses (standardized regression coefficient $\beta = 0.18$ and 0.16 respectively; $p < 0.01$ for both). When both folate and zinc were in the lowest tertile, the combined effect on AMH was greater than the sum of individual effects (interaction term $p = 0.04$), suggesting synergistic action. Vitamin D insufficiency (< 50 nmol/L) was associated with modest AMH reduction ($\beta = -0.22$, $p = 0.012$), consistent with emerging evidence linking vitamin D to ovarian function [52].

### 4.4 Gestational Diabetes and Offspring Ovarian Reserve

Daughters of mothers with GDM had a mean AMH of 1.74 ng/mL (95% CI: 1.58-1.90) compared to 2.52 ng/mL (95% CI: 2.44-2.60) in the non-GDM group (difference = -0.78 ng/mL; 95% CI: -1.03 to -0.53; $p < 0.001$). In multivariate regression, GDM remained independently associated with a 34.7% reduction in daughter's AFC ($\beta = -2.3$, 95% CI: -3.1 to -1.5; $p < 0.001$) after adjustment for maternal BMI, gestational weight gain, and offspring birth weight.

The association was dose-dependent with respect to maternal glycaemia. Women with fasting glucose in the highest tertile (> 5.3 mmol/L) showed the greatest AMH attenuation in offspring ($\beta = -0.92$, 95% CI: -1.24 to -0.60; $p < 0.001$), while those in the middle tertile (5.1-5.3 mmol/L) had intermediate effects (β = -0.41, 95% CI: -0.68 to -0.14; p = 0.003). This dose-response relationship strengthens causal inference and suggests that even modest elevations in fasting glucose below the diagnostic threshold for GDM may influence fetal ovarian development [53].

Notably, the GDM effect was not explained by offspring birth weight or macrosomia. Including these variables in the model attenuated the GDM coefficient by only 8%, indicating that the ovarian effects are likely mediated through direct metabolic programming rather than through altered fetal growth trajectories. Sensitivity analyses excluding women with pre-existing diabetes or those requiring insulin therapy yielded similar results.

### 4.5 Heavy Metal Exposure and Ovarian Reserve

Maternal blood lead above 5 μg/dL was associated with an adjusted AMH reduction of 0.84 ng/mL in daughters (95% CI: -1.12 to -0.56; $p < 0.001$). The relationship was linear across the observed range of lead concentrations, with each 1 μg/dL increment in maternal blood lead associated with a 0.16 ng/mL decrease in daughter AMH (95% CI: -0.21 to -0.11; $p < 0.001$). This finding is particularly concerning given that 30.9% of mothers exceeded the 5 μg/dL threshold, and 8.2% had levels > 10 μg/dL, the former occupational exposure limit.

For Cd, each 1 μg/g creatinine increment in maternal urinary Cd was associated with a 0.31 ng/mL decrease in daughter AMH (95% CI: -0.47 to -0.15; $p < 0.001$). Hg showed a directionally consistent but non-significant negative association (β = -0.18, p = 0.09), possibly due to the lower prevalence of elevated Hg exposure in this population compared to coastal communities where fish consumption is higher [54].

As illustrated in Table 2, the heavy metal effects were stronger in daughters born with IUGR, consistent with cumulative fetal stress augmenting gonadotoxic effects. For lead, the AMH reduction associated with levels > 5 μg/dL was -0.96 ng/mL (95% CI: -1.31 to -0.61) in IUGR-affected dyads compared to -0.71 ng/mL (95% CI: -0.98 to -0.44) in those without IUGR, though the interaction term did not reach statistical significance (p = 0.12).

**Table 2.** Multivariate regression–maternal predictors of daughter's AMH (ng/mL).

| Predictor Variable | β | 95% CI | p-value | $R^2$ |
|---|---|---|---|---|
| Dietary Diversity (MDD-W score) | +0.23 | 0.17 to 0.29 | < 0.001 | .11 |
| Gestational Diabetes (vs. none) | -0.78 | -1.03 to -0.53 | < 0.001 | .09 |
| Blood Lead > 5 μg/dL | -0.84 | -1.12 to -0.56 | < 0.001 | .08 |
| Urinary Cd (per 1 μg/g Cr) | -0.31 | -0.47 to -0.15 | < 0.001 | .05 |
| Serum Folate (per 10 nmol/L) | +0.18 | 0.09 to 0.27 | 0.001 | .04 |
| Serum Zinc (per 10 μmol/L) | +0.16 | 0.07 to 0.25 | 0.004 | .03 |
| IUGR (present vs. absent) | -0.61 | -0.84 to -0.38 | < 0.001 | .07 |
| Vitamin D < 50 nmol/L | -0.22 | -0.39 to -0.05 | 0.012 | .02 |

Note: All models adjusted for maternal age, parity, socioeconomic status, gestational age at delivery, and daughter's age and Tanner stage at assessment. MDD-W = Minimum Dietary Diversity-Women; Cr = creatinine; IUGR = intrauterine growth restriction. $R^2$ values represent semi-partial (incremental) variance explained by each predictor beyond covariates; baseline model $R^2$ (covariates only) = 0.14.

### 4.6 Cumulative Exposure Model and SEM

SEM analysis confirmed IUGR as a significant partial mediator of the relationship between maternal undernutrition and offspring AMH (indirect effect: β = -0.24; 95% bootstrap CI: -0.33 to -0.15), accounting for 38.2% of the total effect. The direct effect of undernutrition remained significant (β = -0.39, $p < 0.001$), suggesting that additional mechanisms beyond growth restriction contribute to ovarian programming.

When all three primary risk exposures co-occurred–dietary diversity score below 4, blood lead above 5 μg/dL, and GDM–daughters experienced a cumulative AMH reduction of 1.96 ng/mL compared to unexposed controls (95% CI: -2.41 to -1.51; $p < 0.001$). This additive effect was statistically greater than the sum of individual exposure coefficients (test for interaction p = 0.03), suggesting possible biological synergy. The proportion of daughters with AMH < 0.7 ng/mL increased from 1.2% in the unexposed group to 11.8% in the triple-exposed group ($p < 0.001$), underscoring the clinical significance of cumulative risk.

The final SEM model demonstrated excellent fit (CFI = 0.97, TLI = 0.95, RMSEA = 0.04), supporting the hypothesized causal structure. Sensitivity analyses excluding influential cases and using alternative mediation methods (e.g., causal mediation analysis with inverse probability weighting) yielded consistent results.

## 5. Discussion

This multi-generational cohort study, spanning 14-18 years of follow-up and involving over 1,000 mother-daughter pairs, provides substantive prospective evidence that the intrauterine environment—shaped by maternal nutritional status, metabolic function, and environmental toxicant burden—exerts a measurable and enduring influence on daughter's ovarian reserve. The findings advance the DOHaD paradigm specifically into the domain of ovarian biology and carry both scientific and public health significance.

### 5.1 Nutritional Programming of Ovarian Reserve

The association between maternal dietary diversity and offspring AMH is, perhaps, the most clinically actionable finding. Dietary diversity is a modifiable exposure; the MDD-W tool is a validated, low-resource instrument deployable in primary care settings. Our observation that daughters born to women with dietary diversity scores in the lowest quartile had AMH levels approximately 50% lower than their counterparts from the highest quartile is striking, and comparable in magnitude to the effect of carrying a BRCA1 mutation on ovarian reserve [55]. It bears emphasis that AMH in this adolescent age group reflects the growing follicle pool and serves as an early marker of ovarian reserve rather than a definitive measure of future reproductive outcomes; these differences should be interpreted as altered ovarian reserve parameters in early life, with long-term follow-up required to determine whether they translate into earlier menopause, reduced fecundity, or clinical POI. While the causal architecture is complex—poor dietary diversity co-segregates with poverty, food insecurity, and micronutrient deficiency—the specificity of the folate and zinc associations in our mediation models points to biologically plausible mechanistic intermediaries.

Folate's role in one-carbon metabolism and epigenetic programming of the germ line has been well characterized in rodent models; the present data suggest this pathway may extend meaningfully to human fetal ovarian development [22]. Folate is essential for the synthesis of S-adenosylmethionine, the universal methyl donor for DNA methylation, and for the production of nucleotides required for rapid cell division during oogonial proliferation [56]. Zinc, similarly, is a critical cofactor for over 300 enzymes, including those involved in DNA synthesis, repair, and protection against oxidative damage [57]. The observed synergy between low folate and low zinc is biologically plausible, as both nutrients converge on common pathways of cell proliferation and epigenetic regulation.

### 5.2 Metabolic Programming Through Gestational Diabetes

The GDM-offspring AMH association deserves particular attention given Pakistan's escalating prevalence of gestational metabolic dysfunction. The 34.7% reduction in AFC observed in daughters of GDM mothers, robust to adjustment for maternal BMI and offspring birth weight, suggests that intrauterine hyperglycaemia operates through mechanisms beyond macrosomia or adiposity programming alone. Oxidative stress-mediated apoptosis of granulosa cells during the critical window of folliculogenesis—a pathway demonstrated experimentally by Guo and colleagues [36]—represents the most parsimonious biological explanation consistent with our findings.

Recent experimental work has further elucidated potential mechanisms. Pan and colleagues [37] demonstrated that exposure of cultured fetal ovaries to high glucose concentrations activates the unfolded protein response and induces apoptosis through CHOP-dependent pathways. These effects were attenuated by antioxidant treatment, supporting a causal role for oxidative stress. In a non-human primate model, maternal hyperglycaemia was associated with altered DNA methylation patterns in offspring ovarian tissue, particularly at genes involved in mitochondrial function and insulin signalling [58]. These epigenetic changes persisted into adulthood and were associated with reduced ovarian reserve, providing a plausible mechanism for the long-term effects observed in our study.

The dose-response relationship between maternal fasting glucose and offspring AMH is particularly noteworthy. It suggests that even modest elevations in glucose below the diagnostic threshold for GDM may influence fetal ovarian development, a finding with significant implications for clinical practice. Current GDM screening and treatment thresholds are based primarily on risks of macrosomia and perinatal complications [47]; our findings suggest that lower thresholds might be appropriate for preventing long-term reproductive consequences in offspring.

### 5.3 Environmental Gonadotoxicity and Intergenerational Justice

The heavy metal data from this cohort are particularly noteworthy given the environmental burden faced by many communities in Pakistan's urban-industrial belt. Lahore's peri-urban areas encompass active tanneries, battery recycling facilities, and informal electronic waste processing, all of which are known sources of lead and Cd contamination [13]. Our finding that blood lead concentrations exceeding 5 μg/dL—a threshold now deemed hazardous at any level by the WHO—are associated with nearly 0.84 ng/mL lower AMH in daughters underscores the reproductive inter-generational toll of unregulated industrial pollution.

The mechanisms by which lead affects ovarian development are increasingly well understood. Lead disrupts calcium signalling in granulosa cells, impairing gap junction communication and reducing the transfer of nutrients and regulatory signals to developing oocytes [59]. It also interferes with steroidogenesis by inhibiting cytochrome P450 enzymes, potentially altering the hormonal milieu required for normal follicular development [60]. At the epigenetic level, lead exposure has been associated with altered DNA methylation at imprinted genes, which play critical roles in embryonic and placental development [61].

These findings raise important questions of environmental justice. The individual-level associations between maternal blood lead and offspring AMH observed in this cohort are robust and biologically plausible; however, attributing population-level causality requires consideration of unmeasured community-level factors, including overall pollution burden, poverty concentration, and access to healthcare, which were not fully captured in this study. The communities most heavily exposed to industrial pollutants are typically those with the fewest resources to mitigate their effects or to advocate for regulatory protections [62]. Our data suggest that the health consequences of this inequity may extend across generations; addressing these disparities will require coordinated action across multiple sectors, including environmental regulation, urban planning, and public health surveillance. It should also be acknowledged that blood lead was measured at a single time point (16 weeks gestation), which may not capture the full range of fetal exposure across the vulnerable window of folliculogenesis; cumulative exposure estimates derived from serial measurements would provide a more complete picture in future studies.

### 5.4 Mediation Through Intrauterine Growth Restriction

The SEM results illuminate IUGR as a critical biological mediator linking maternal undernutrition to reduced follicular endowment. This is conceptually important: IUGR may represent the phenotypic expression of a broader fetal nutrient-deficient state, within which the ovary—as a relatively metabolically active but non-vital organ—is selectively deprived of the substrates required for folliculogenesis. Growth-restricted fetuses prioritize perfusion to the brain and heart through haemodynamic redistribution, potentially at the expense of splanchnic and gonadal blood flow [63]. Reduced gonadal blood flow during critical windows of follicular formation may constitute the proximal physiological mechanism linking maternal nutritional deprivation to diminished lifetime follicular endowment.

The finding that IUGR mediates only 38% of the undernutrition effect indicates that additional mechanisms are operative. Direct effects of nutrient deficiencies on oogonial proliferation and meiosis, independent of overall fetal growth, likely contribute. Epigenetic programming of ovarian genes by nutritional signals may also play a role, as suggested by animal studies showing altered DNA methylation in the ovaries of offspring born to undernourished mothers [64]. These epigenetic changes can persist across cell divisions and potentially across generations, contributing to the intergenerational transmission of reproductive risk.

### 5.5 Clinical and Public Health Implications

The cumulative exposure model demonstrating additive—and potentially synergistic—effects of multiple risk factors has important implications for risk stratification and intervention. Rather than focusing on single exposures in isolation, clinicians and public health practitioners should consider the total burden of developmental risk when identifying individuals who might benefit from enhanced surveillance or preventive interventions [65]. In our study, daughters with triple exposure (poor nutrition, elevated lead, and GDM) had AMH levels approaching the threshold for DOR, despite being only 14-18 years old. These AMH differences should be regarded as early markers of altered ovarian reserve rather than as evidence of established POI; whether they predict clinically significant outcomes such as earlier menopause or impaired fertility requires confirmation through long-term follow-up of this cohort. Nonetheless, these individuals may represent a high-priority group for ongoing monitoring, fertility counselling as they approach their reproductive years, and, where appropriate, consideration of fertility preservation options once adulthood is reached.

From a public health perspective, our findings argue for a fundamental repositioning of POI prevention strategies. Rather than focusing exclusively on identifying and managing POI once it has manifested, health systems should invest in interventions targeting the earliest stages of development. Preconceptional and antenatal nutritional supplementation programs, environmental remediation efforts to reduce heavy metal exposure, and improved screening and management of GDM could yield substantial dividends for the reproductive health of future generations [66]. The cost-effectiveness of such investments is likely to be favorable given the substantial personal and societal costs of POI, including infertility, premature estrogen deficiency, and their associated morbidities [67].

### 5.6 Strengths and Limitations

The principal strength of this study lies in its prospective, multi-generational design with comprehensive exposure assessment during pregnancy and long-term follow-up through adolescence. This design minimizes recall bias, enables examination of temporality, and allows for the assessment of multiple exposures simultaneously. The use of directly measured ovarian reserve parameters (AMH and AFC) rather than proxy outcomes such as age at menopause represents another important strength, as these measures provide a more direct assessment of follicular endowment during the reproductive years [68].

The inclusion of a population from a LMIC context is both a strength and a limitation. It provides novel data from an understudied population with high levels of relevant exposures, enhancing the generalizability of DOHaD findings beyond high-income settings. However, it also raises questions about the applicability of findings to populations with different exposure profiles and genetic backgrounds.

Several limitations merit acknowledgement. First, daughters in this cohort were assessed at a single point during mid-adolescence; ovarian reserve is known to decline with age, and a proportion of girls who appeared to have adequate reserve at age 14-18 may develop POI in their twenties or thirties. Long-term follow-up into adulthood is essential to determine whether the observed differences in AMH translate into differences in age at menopause and reproductive success. Second, AMH measurement in adolescents may be confounded by pubertal stage and ongoing HPO axis maturation, a potential source of measurement imprecision addressed only partially by Tanner staging [69]. Third, dietary recall data are inherently subject to recall and social desirability bias, though the use of a validated instrument and repeated assessments mitigates this concern. Fourth, while the cohort is drawn from a large referral centre in Lahore, generalizability to rural Pakistani populations, other South Asian contexts, and high-income country settings requires caution; the specific exposure profile of Lahore's peri-urban industrial belt may not be representative of other populations, and replication in geographically and ethnically diverse cohorts is needed before conclusions can be broadly applied. Fifth, we cannot exclude the possibility of residual confounding by several categories of unmeasured factors. Genetic predisposition represents an important gap: variants in genes such as FMR1, BMP15, FOXL2, and NOBOX are known to influence ovarian reserve, and maternal reproductive history (e.g., age at menopause, history of POI) was not systematically collected as a proxy for heritable risk. Future analyses should incorporate maternal reproductive history and, where feasible, explore stored biospecimens for epigenetic and genetic characterisation. Paternal factors—including paternal age, smoking, and occupational exposures that may affect offspring via epigenetic mechanisms—were not captured in this dataset and warrant inclusion in future studies. Postnatal environmental factors, including the daughter's own nutrition, physical activity, body composition, and environmental exposures from birth through adolescence, may also independently influence ovarian reserve and could not be fully characterised in this study; while our focus was intentionally on intrauterine determinants, postnatal influences represent an important complementary axis of inquiry. Sixth, heavy metal exposure was measured at a single time point (16 weeks gestation); blood lead reflects primarily recent exposure and a fraction of bone stores rather than cumulative fetal burden across the full vulnerable window of folliculogenesis. This likely results in underestimation of true exposure and attenuation of observed effect sizes. Future studies should incorporate serial measurements across trimesters or use biomarkers of cumulative exposure [70].

Despite these limitations, the multi-generational cohort design—combining rigorous prospective maternal data collection with blinded offspring assessment—provides a level of evidence substantially higher than retrospective or cross-sectional approaches. The breadth of exposure data collected antenatally, spanning dietary, metabolic, hormonal, and toxicological domains, enabled simultaneous examination and disentanglement of multiple candidate determinants within a single analytical framework.

## 6. Conclusions

The data from this prospective multi-generational cohort study establish, with a degree of rigour not previously achieved in the literature, that maternal health behaviours and environmental exposures during pregnancy cast measurable shadows over a daughter's ovarian reserve more than a decade later. POI—an understudied cause of reproductive morbidity in low- and middle-income countries—may in a meaningful proportion of cases have its origins not in the postnatal or reproductive life of the woman who suffers it, but in the womb she inhabited.

These findings argue for a fundamental repositioning of POI prevention strategies. Rather than focusing exclusively on identifying and managing POI once it has manifested, clinicians and health policymakers should consider whether earlier interventions—dietary diversification programs for pregnant women, reduction of occupational and environmental heavy metal exposures, gestational diabetes prevention and management, and IUGR surveillance—might confer lasting protective benefits for the reproductive futures of the daughters being carried. The integration of these considerations into routine antenatal care represents a paradigm shift with the potential to improve reproductive health across generations.

A practical clinical implication emerging from this work is the potential role of targeted ovarian reserve screening in daughters born to mothers with documented high-risk pregnancies—specifically those complicated by severe undernutrition, heavy metal toxicant exposure, GDM with poor glycaemic control, or IUGR. Identifying DOR early in adolescence would allow for timely counselling about future fertility, consideration of fertility preservation options, and proactive monitoring for the non-reproductive sequelae of hypoestrogenism. While the optimal timing and frequency of such screening remain to be established, our findings suggest that adolescence represents an appropriate window for initial risk stratification.

Future research should pursue long-term follow-up of the present cohort into the third and fourth decades of daughter participants' lives to determine whether the observed differences in adolescent AMH—which represent early markers of altered ovarian reserve rather than confirmed clinical outcomes—translate into differences in age at menopause, time to conception, fertility outcomes, and long-term health. This follow-up is essential before the current findings can be used to guide individual clinical decision-making. Examination of epigenetic mechanisms linking in utero exposures to follicular atresia, using stored maternal and offspring biospecimens, could provide mechanistic insights and identify potential biomarkers of exposure and effect. Finally, randomized controlled trials of preconceptional nutritional supplementation and environmental remediation programmes are needed to establish whether modifying these exposures can improve offspring ovarian reserve. The reproductive health of future generations may depend, in ways we are only beginning to appreciate, on decisions and exposures that their mothers experience long before the first cry of birth.

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## Ethics Statement

This study was conducted in accordance with the Declaration of Helsinki (2013 revision). Written informed consent was obtained from all participants and their guardians (where applicable). The study was registered with the Pakistan National Health Research Registry (NHRC-2008-LHR-0112).

## Data Availability Statement

De-identified participant data are available from the corresponding author upon reasonable request, subject to data sharing agreement.

## Conflicts of Interest

The author declares no conflict of interest.

## Generative AI Statement

The author declares that no Gen AI was used in the creation of this manuscript.

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