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## Comprehensive profiling of prostaglandins in human ovarian follicular fluid using mass spectrometry

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### Abstract

Prostaglandins are formed by enzymatic and non-enzymatic mechanisms. They have been detected in human ovarian follicular fluid (HFF), a medium rich in growth factors and nutrients important for oocyte growth and fertility. However, the comprehensive identification of HFF prostaglandins has not been addressed. Here we use hybrid triple quadrupole time-of-flight and triple quadrupole mass spectrometers to comprehensively analyze prostaglandins in HFF. We identified PGE1, PGE2, PGF2 $\alpha$ , and other prostaglandins synthesized via prostaglandin-endoperoxide synthase (i.e. Cox) cascades. We also identified specific PGF2 $\alpha$  isomers (F2-isoprostanes) and PGF3 $\alpha$  analogs whose structures are inconsistent with Cox-dependent formation. A prospective cohort pilot study of infertility patient subtypes revealed two potential associations. F2-isoprostanes are decreased in the diminished ovarian reserve subtype and elevated PGF2 $\alpha$  may be associated with decreased live birth. Other than PGF2 $\alpha$ , only body mass index > 25 kg/m<sup>2</sup> correlated with poor *in vitro* fertilization outcome. Our studies suggest that HFF contains prostaglandins formed from at least two mechanisms, which may correlate with distinct clinical parameters.

### Keywords

prostaglandin; mass spectrometry; ovary; lipid; polyunsaturated fatty acid; fertilization; cyclooxygenase

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### Conflict of interest

The authors declare no conflicts of interest.

### Author's Contributions

BP, JKP, JWE, GWB, and MAM conceived and designed the experiments. BP, JKP, and MAM wrote the manuscript. BP, LW, AA, and RM executed the experiments. JKP and MAM assisted with data interpretation. BP, JWE, and GWB helped secure clinical samples and consent.

## 1. Introduction

Prostaglandins (PGs) are signaling molecules derived from dietary fats with clinically relevant roles in reproductive biology [1–4]. PGE<sub>2</sub>, for instance, promotes ovulation downstream of the luteinizing hormone surge [5, 6]. Excess consumption of nonsteroidal anti-inflammatory drugs, which inhibit prostaglandin-endoperoxide synthase (a.k.a. cyclooxygenase or Cox), is associated with reversible female infertility, likely due to failed ovulation [7, 8]. On the other hand, proinflammatory cytokines increase PGF<sub>2</sub> $\alpha$  associated with corpus luteum development and immune cell recruitment [9]. The mature human follicle contains mural and cumulus granulosa cells surrounding a single oocyte. During follicle development, an antrum forms that is filled with fluid containing PGs, steroids, peptide growth factors, and metabolites [10]. Human ovarian follicular fluid (HFF) is collected along with cumulus-oocyte complexes from mature follicles in patients undergoing *in vitro* fertilization (IVF), providing a window into the physiological signaling processes occurring in fertile and infertile women. Although PGs have been analyzed in HFF, most studies used radio- and enzyme-immunoassays, which lack the specificity to distinguish among the complexity of PG types and isomers known to exist today [11–13].

PGs are synthesized from the 20-carbon polyunsaturated fatty acids (PUFAs) dihomo- $\gamma$ -linolenic acid (DGLA), arachidonic acid (AA), and eicosapentaenoic acid (EPA) [1, 14, 15]. A key structural feature is the cyclopentane ring, which contains side groups that define classes. For instance, the F-series class member PGF<sub>2</sub> $\alpha$  is synthesized from AA and contains hydroxyl groups at the carbon-9 (C9) and C11 positions in the cyclopentane ring [16]. Classical PG synthesis is initiated by Cox enzymes, which convert AA into the bicyclic endoperoxide PGG<sub>2</sub> and then PGH<sub>2</sub> [17, 18]. PGD, PGE, and PGF synthases convert PGH<sub>2</sub> into bioactive forms [16, 19, 20]. PGs are also formed non-enzymatically by free radical-induced peroxidation [21, 22]. In this mechanism, reactive oxygen species (ROS) produced during oxidative stress act on C20 PUFAs in phospholipids. PGs generated by Cox or ROS can be distinguished through structural information [23–25]. Cox pathways generate free PGs with specific stereochemistry [17]. In contrast, ROS produce a broad spectrum of PG classes and isomers that are esterified to phospholipids (Fig. 1). As auto-oxidation reactions lack specificity, free radical-induced peroxidation generates 64 esterified PGF<sub>2</sub> $\alpha$  isomers alone (termed F<sub>2</sub>-isoprostanes), comprising four regioisomeric families each with 16 isomeric members. These families are called 5-F<sub>2</sub>-isoprostanes, 8-F<sub>2</sub>-isoprostanes, 12-F<sub>2</sub>-isoprostanes, and 15-F<sub>2</sub>-isoprostanes, based on position of the cyclopentane ring in the carbon chain [21, 25]. Sensitive and specific analytical methods are necessary to resolve these PG species.

There is recent evidence for a third PG metabolism pathway. The nematode *C. elegans* produces specific F<sub>1</sub>-isoprostanes, F<sub>2</sub>-isoprostanes, and F<sub>3</sub>-isoprostanes independent of Cox enzymes [26–28]. These F-series PGs are formed from DGLA, AA, and EPA precursors, respectively [27], and have an important function to attract migrating sperm to oocytes within the oviduct [27–29]. While the metabolism pathway is not well understood, insulin and TGF- $\beta$  signaling pathways regulate ovarian F-series PG metabolism [26, 28, 30]. Genetic ablation of the two *Cox* genes in mice eliminates PGI<sub>2</sub> and other classical PGs. However, specific F<sub>2</sub>-isoprostanes are still observed in wild-type and Cox null mice that are

similar to those in *C. elegans* [26]. A distinguishing feature thus far is specificity for F-series versus D-series, E-series, and I-series PGs [27]. The extent to which the metabolic process and products generated overlaps with free radical-induced peroxidation is not clear. An important distinction is that *C. elegans* PG metabolism is strictly regulated and has a function unrelated to oxidative stress.

In this study, we used a system comprising nanoscale liquid chromatography coupled to a triple-TOF 5600 instrument, a hybrid Q-time-of-flight tandem mass spectrometer (qTOF), to comprehensively analyze PGs in HFF. To correlate selected PG concentrations across patient HFF samples, we used a conventional liquid chromatography tandem mass spectrometry (LC-MS/MS) system on a triple quadrupole mass spectrometer operated in multiple reaction monitoring (MRM) mode. The most abundant PGs across all samples are PGE2 and PGF2 $\alpha$ . In addition, PGE1, PGF1 $\alpha$ , and specific F2-isoprostanes and PGF3 $\alpha$  analogs were detected. Compared to control HFF from oocyte donor and male infertility patients, HFF from patients with diminished ovarian reserve (DOR) contained significantly reduced concentration of F2-isoprostanes co-eluting with the *8-iso*-PGF2 $\alpha$  standard (also known as iPF2 $\alpha$ -III or 15-F2t-IsoP). These results document diverse PG types in HFF and suggest that multiple F-series PGs are important for female fertility.

## 2. Materials and methods

### 2.1. Chemicals

PGF2 $\alpha$ -d9, PGD2-d9, PGE2-d4, PGF1 $\alpha$ -d9, PGF2 $\alpha$ , *8-iso*-PGF2 $\alpha$ , *ent*-PGF2 $\alpha$ , *ent-8-iso*-PGF2 $\alpha$ , *ent-8-iso-15(S)*-PGF2 $\alpha$ , *15(R)*-PGF2 $\alpha$ , *8-iso-15(R)*-PGF2 $\alpha$ , *11 $\beta$* -PGF2 $\alpha$ , *8-iso-9 $\beta$* -PGF2 $\alpha$ , PGF1 $\alpha$ , PGF3 $\alpha$ , PGD2, PGE2, PGE1, PGD1, *6-keto*-PGF1 $\alpha$ , PGA1, and PGA2 standards were obtained from Cayman Chemical (Ann Arbor, MI). All HPLC solvents and reagents were purchased from Fisher Scientific Co. (Norcross, GA) and were of HPLC grade.

### 2.2 Patients and HFF collection

All patients gave consent for HFF donation for research purposes as part of a University of Alabama at Birmingham Institutional Review Board approved protocol. This work has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). Subjects were premenopausal women presenting with subfertility or as oocyte donors. No patients undergoing oocyte retrieval were excluded from this study. Pertinent patient characteristics, including laboratory values, IVF cycle laboratory values, and outcome measures were recorded. Specific variables analyzed included subject age, history of live birth, body mass index, anti-mullerian hormone level (AMH), blastocyst conversion, and IVF outcomes (no pregnancy, biochemical pregnancy, spontaneous abortion, or ongoing pregnancy). Subjects were categorized by subfertility diagnosis that led to *IVF*. A control group was comprised of oocyte donors or patient couples presenting with male factor only infertility as a diagnosis.

Transvaginal cyst puncture was performed after controlled ovarian hyperstimulation with gonadotropins (recombinant follicle stimulating hormone, human menopausal

gonadotropins). Protocols included long leuprolide, antagonist, and micro-dose leuprolide flares. HFF from the first entered follicle bilaterally was included in this study to limit potential specimen blood contamination, which may contain different PG types. The first follicles entered bilaterally were separately collected from each patient. Oocyte retrieval needles were flushed prior to follicle entry. HFF was obtained from mature sized follicles (16 mm diameter), with HFF from a total of 93 patients being collected. 42 patient samples were analyzed. Fresh HFF was stored in a  $-20^{\circ}\text{C}$  freezer within 30 minutes after oocyte retrieval, and transferred to a  $-80^{\circ}\text{C}$  freezer for further storage.  $\text{PGF}2\alpha\text{-d}9$  (1.00 ng/ml) was added to every sample as an internal standard. HFF samples were thawed in a warm bath prior to PG extraction.

### 2.3 Prostaglandin extraction

PGs were extracted using a liquid-liquid extraction method with minor modifications [31, 32]. Briefly, 2 ml of HFF (with the  $\text{PGF}2\alpha\text{-d}9$  internal standard) were mixed with 4 ml of acetone. The mixture was transferred to conical glass tubes (10 ml), mixed with hexane, and vortexed. The upper hexane soluble layer was removed and the bottom layer was mixed with 2 ml chloroform. After separation, the chloroform layer was evaporated to dryness under Nitrogen and capped immediately. The dried extract was stored in Nitrogen gas at  $-80^{\circ}\text{C}$  for a brief period (typically less than 3 days) before analysis. Butylated hydroxytoluene was used in solutions to prevent oxidation. This method produced consistent results when the same HFF sample was extracted multiple times and analyzed using LC-MS/MS operated in MRM mode (N=9). Similar results were obtained using  $\text{PGD}2\text{-d}9$ ,  $\text{PGE}2\text{-d}4$ ,  $\text{PGF}1\alpha\text{-d}9$ , and  $\text{PGA}2$  internal standards. Dried samples were resuspended in 200  $\mu\text{l}$  80% MeOH for analysis, providing 10-fold increased concentration relative to the original HFF sample.

### 2.4 Prostaglandin identification and quantification

For comprehensive PG detection, 2  $\mu\text{l}$  extracted HFF samples were loaded onto a Nano cHiPLC  $200\mu\text{m} \times 0.5\text{mm}$  ChromXP C18-CL  $3\mu\text{m}$   $120\text{\AA}$  reverse-phase trap cartridge (Eksigent, Dublin, CA) at 2 ml/min using an Eksigent autosampler. After washing the cartridge for 5 min with 0.1% formic acid in double distilled H<sub>2</sub>O, the bound lipids were flushed with a 15 minute linear (5–95%) acetonitrile gradient in 0.1% formic acid at 1000 nl/min using an Eksigent 415 NanoLC system. The column was washed with 95% acetonitrile-0.1% formic acid for 5 min and then re-equilibrated with 5% acetonitrile-0.1% formic acid for 5 min. The Sciex 5600 Triple-TOF mass spectrometer (Sciex, Toronto, Canada) was used to analyze PGs. The IonSpray voltage for positive and negative modes were  $\pm 2300$  V and the declustering potential was  $\pm 80$  V. IonSpray and curtain gases were set at 10 psi and 25 psi, respectively. The interface heater temperature was  $120^{\circ}\text{C}$ . Eluted compounds were subjected to a time-of-flight survey scan from  $m/z$  50–1000 to determine the most intense ions for MS/MS analysis. Product ion time-of-flight scans at 50 msec using a collision energy spread of 15 eV with a set collision point of 35 eV were carried out to obtain the tandem mass spectra of selected parent ions. Spectra were centroided and de-isotoped by Analyst software, version 1.6 TF (Sciex, Toronto, Canada).

Relative quantification of selected PGs was performed with liquid chromatography tandem mass spectrometry (LC-MS/MS) operated in MRM mode, following our previously

published method [26–28]. Briefly, LC-MS/MS analyses were performed using a system consisting of a Shimadzu Prominence high performance liquid chromatography column with a refrigerated auto sampler (Shimadzu Scientific Instruments, Inc., Columbia, MD) and an API 4000 (Applied Biosystems/MDS Sciex, Concord, Ontario, Canada) triple quadrupole mass spectrometer. The chromatographic separation was performed on a Synergy hydro RP-C18 column pre-equilibrated with 0.1% formic acid. The mobile phase consists of 0.1% formic acid [A] and acetonitrile containing 0.1% formic acid [B] and was pumped at a flow rate of 0.2 ml/min. The gradient started with 10% B and went up to 80% B from 0–11 min, 80–100% B from 11–14 min and returned back to 10% B at 16 min. The column effluent was introduced into the mass spectrometer using an ESI interface operating in negative ion mode. Nitrogen was used as a nebulizer and curtain gas (CUR = 10). The MRM analysis was conducted by monitoring the precursor ion to product ion transitions from  $m/z$  353/193 and 353/309 for PGF2 $\alpha$  and 8-*iso*-PGF2 $\alpha$ ,  $m/z$  355/311 for PGF1 $\alpha$ ,  $m/z$  351/191 and 351/193 for PGF3 $\alpha$ ,  $m/z$  351/189 for PGE2 and PGD2,  $m/z$  369/245 for 6-*keto*-PGF1 $\alpha$ ,  $m/z$  369/169 for thromboxane B2,  $m/z$  353/273 and 353/191 for PGE1 and PGD1,  $m/z$  333/189 for PGA2, and  $m/z$  335/273 for PGA1. The collision gas, collision energy and temperature were set at 10, –35 eV and 600°C, respectively. The LC-MS/MS system was controlled by BioAnalyst 1.4.2 software.

To estimate PG concentration, a stock solution of PG standard (1 mg/ml in 80% MeOH) was serially diluted with the same solvent to obtain 0.1, 0.5, 1.0, 10.0, and 50.0 ng/ml concentrations. The samples were analyzed by the MRM method. The standard curves exhibited excellent linearity in the range of concentration 0.1–1000 ng/ml with correlation coefficients > 0.99. Patient HFF samples were normalized to the internal control. The method exhibits excellent reproducibility and average concentration for each PG is comparable across HFF samples. Absolute PG concentrations are estimates due to potential matrix effects that can be different at each retention time [33]. Matrix effects are difficult to accurately quantify in HFF, which contains endogenous PGs. Based on analysis with PGF2 $\alpha$ -d9, PGD2-d9, PGE2-d4, PGF1 $\alpha$ -d9, and PGA2 internal standards, we estimated a 50% extraction and detection efficiency for all PGs.

## 2.5 Statistics

Mean PG levels were compared to clinical variables of interest using a student's t-test with Levene's Test for Equality of Variances. For Figure 4C, PGE2 and PGF2 $\alpha$  levels were categorized as high and low by dichotomizing levels above and below the median of the 42 samples analyzed (n=21 per cohort). High and low cohorts were compared to clinical variables of interest using chi-square testing, along with calculations for relative risk with 95% confidence interval. Analysis was performed using SPSS version 21.0, and a *P* value of <0.05 was considered to be statistically significant. Clinical pregnancy was considered positive if the patient had a normal fetus on ultrasound and a fetal heartbeat at 8 weeks or more of gestation.

### 3. Results

#### 3.1. Comprehensive PG analysis in control patients

PGF2 $\alpha$  and PGE2 have been detected in HFF previously, but the analytical methods were not specific enough to distinguish among PG isomers or comprehensively identify PG types [11–13]. To address this issue, we developed an analytical method consisting of a nano-LC separation column coupled to the triple-TOF instrument. This hybrid Q-TOF platform has accurate mass (<5 ppm), high resolution (30,000), and fast acquisition. Nano-flow chromatography (1000 nl/min) resulted in increased sensitivity and was capable of resolving isobaric and isomeric compounds, including the stereoisomers PGF2 $\alpha$ , *ent*-PGF2 $\alpha$ , *8-iso*-PGF2 $\alpha$ , and *ent-8-iso*-PGF2 $\alpha$  (Supplemental Fig. 1A).

We extracted HFF PGs from two fertile female IVF patients. As our extraction procedure removes neutral lipids, including phospholipids containing esterified PGs, we analyzed PGs in HFF as free carboxylic acids. Based on monoisotopic exact mass, retention time (RT) of commercial standards, and MS/MS spectra, we identified PGF2 $\alpha$ , PGF1 $\alpha$ , and PGE2 (Fig. 1, Supplemental Fig. 2, and Supplemental Fig. 3). PGD2, PGF3 $\alpha$ , PGA1, PGA2, and *6-keto*-PGF1 $\alpha$ , the stable metabolite of PGI2, were low or undetectable (Supplemental Fig. 3 and data not shown). Other than occasional low PGD2, we did not detect multiple PGE2 or PGF1 $\alpha$  isomers. A series of specific F2-isoprostanes and PGF3 $\alpha$  analogs were detected, as described below.

F2-isoprostanes were detected by searching for the deprotonated molecular ion  $m/z$  353.2328 [M-H]<sup>-</sup>. In both patients, extracted ion chromatograms (XIC) for  $m/z$  353.2328 showed multiple peaks ranging in RT from 18–22 minutes. 15-F2-isoprostanes, such as PGF2 $\alpha$  generate the characteristic product ion C12H17O2<sup>-</sup> with mass  $m/z$  193.122 [23]. An XIC generated from this product ion indicated several peaks from RT 18–22 minutes (Fig. 1A, B). The ion  $m/z$  353.233 at RT 20.1 was identified as PGF2 $\alpha$  (Fig. 1). The extracted ion at RT 20.8 minutes showed MS/MS product ions almost identical to PGF2 $\alpha$ , but with different RT (Fig. 1). The second patient's HFF contained a peak at RT 19.4 minutes that co-eluted with *8-iso*-PGF2 $\alpha$ , in addition to peaks at RT 20.0, 20.1, and 20.3 minutes (Supplemental Fig. 1). Thus, HFF contains PGF2 $\alpha$ , *8-iso*-PGF2 $\alpha$ , and at least one other 15-F2-isoprostane.

The MS/MS spectrum of  $m/z$  353.233 at RT 20.3 minutes showed the product ion  $m/z$  115.0402 (Supplemental Fig. 4A), which is characteristic of 5-F2-isoprostanes [21, 34]. An XIC for this product ion indicated a single prominent 5-F2-isoprostane peak in one patient (Supplemental Fig. 5) and two less abundant peaks in the other patient (not shown). In both patients, MS/MS showed product ions typical of 5-F2-isoprostanes (Supplemental Fig. 4B, C) [25, 34]. We did not detect evidence for 8-F2-isoprostanes or 12-F2-isoprostanes [21, 34] (Supplemental Fig. 5 and data not shown).

A search for the deprotonated ion  $m/z$  351.2177 revealed several ions with MS/MS spectra similar to PGF3 $\alpha$ , but different RTs. MS/MS showed the product ion  $m/z$  191.1416 and an XIC for this product ion produced several peaks (Fig. 2). The most prominent peak at RT 19.1 minutes had an MS/MS spectrum typical of PGF3 $\alpha$ , including product ions  $m/z$  333,



307, 289, 245, 193, and 191 [23, 27] (Fig. 2B). However, the presence of major ions  $m/z$  167.1067 and  $m/z$  153.0912 suggests that this lipid is similar to a PGF3 $\alpha$  analog synthesized by *C. elegans* oocytes [27]. MS/MS spectra for peaks at RT 20.32 minutes and 20.66 minutes are also consistent with these lipids being PGF3 $\alpha$  analogs (data not shown). We did not detect 5-F3-isoprostanes or 8-F3-isoprostanes containing the product ions  $m/z$  115.0402 or  $m/z$  127.0759, respectively [35]. Therefore, HFF contains a number of specific PGF3 $\alpha$  analogs.

### 3.2 Targeted analysis of selected PGs across patients

MRM on a triple quadrupole instrument is well suited for comparing relative PG concentrations across samples. In MRM mode, the parent ion to product ion mass transition is specified, providing excellent specificity and sensitivity [23, 27, 36]. We previously developed a reversed-phase LC-MS/MS method operated in MRM mode to detect and measure a wide array of PGs [27, 28, 32]. Our method is able to separate most PG isomers and stereoisomers [27].

To complement our comprehensive studies above, we selected MRM mass transitions for detecting and quantifying major PG classes (Table 1) in HFF from 42 patients, including fertile and infertile females. The most consistently detected PGs across the samples co-elute in MRM mode with PGF2 $\alpha$ , PGF1 $\alpha$ , PGE2, and PGE1 standards (Fig. 3A–D). The MRM results showed good agreement with the nano-LC qTOF method. In particular, MRM mass transition  $m/z$  353/193, which detects 15-F2-isoprostanes such as PGF2 $\alpha$ , showed three major peaks in most patients (Fig. 3A). The peak at RT 11.4 to 11.5 minutes eluted near the *8-iso*-PGF2 $\alpha$  standard, similar to analysis using nano-LC qTOF (Fig. 1 and Supplemental Fig. 1). The peak at RT 11.9 minutes eluted near the PGF2 $\alpha$  standard (Supplemental Fig. 6A). The peak at RT 12.2 minutes is also observed in *C. elegans* and *Cox-1*; *Cox-2* knockout mice [26, 27] (Supplemental Fig. 6) and may represent the unknown PGF2 $\alpha$  isomer at RT 20.8 in nano-LC qTOF (Fig. 1A and 1F). We did not focus on uncharacterized PGF3 $\alpha$  analogs because co-eluting standards are not available. The estimated concentrations of selected PGs in HFF across all samples are shown in Table 1. PGE2 and PGF2 $\alpha$  concentration varied widely in a small subset of patients, perhaps due to temporal regulation during oocyte maturation [6, 37] or an underlying inflammatory condition (see Discussion). In MRM mode, we did not consistently detect *6-keto*-PGF1 $\alpha$ , PGA1, PGA2, PGF3 $\alpha$ , or PGD2 in most patient's HFF (see Discussion).

### 3.3 Clinical characteristics

Subjects were categorized by subfertility diagnosis that led to IVF. 42 patient samples were analyzed, including 7 with endometriosis, 7 with polycystic ovary syndrome (PCOS), 7 with diminished ovarian reserve (DOR), 10 patients with unexplained infertility, and 8 fertile women undergoing IVF served as the controls. Three patients did not fall into the diagnostic categories. Endometriosis was defined by presence of endometrial implants noted during previous laparoscopy with pathologic confirmation of implants biopsied. Polycystic ovary syndrome (PCOS) was defined when the subject met at least 2 of 3 NIH Criteria for PCOS. DOR or low egg number relative to age was diagnosed with AMH <1.0 ng/mL, or FSH >10 IU/L. Unexplained infertility was diagnosed with normal ovarian reserve testing, normal

male partner semen analysis, and normal uterus and fallopian tubes on hysterosalpingogram. The control group was comprised of fertile women who were oocyte donors or presented with male factor infertility only. Patient demographics are listed in Table 2. As expected, PCOS patients had a significantly higher BMI, and patients with DOR were older and had less oocytes retrieved. We found that endometriosis and PCOS patients had a trend toward elevated PGE2 and PGF2 $\alpha$  levels, but this increase did not reach significance when compared to the control group. Age, body mass index, IVF stimulation protocol, and anti-mullerian hormone did not demonstrate a significant relationship with PG levels.

### 3.4 Clinical associations with F2-isoprostanes

F2-isoprostanes such as *8-iso*-PGF2 $\alpha$  are often linked to oxidative stress [21], but they might also have physiologic functions important for follicle development, ovulation, or fertilization [27]. To investigate free F2-isoprostanes in HFF, we measured the peak area in each HFF sample that corresponded to the *8-iso*-PGF2 $\alpha$  standard RT in MRM mode with mass transition *m/z* 353/193 (Fig. 3A). Concentration was calculated using an *8-iso*-PGF2 $\alpha$  standard curve and the internal standard. We then compared F2-isoprostane concentration in control patients ( $52 \pm 26$  pg/ml) to PCOS ( $82 \pm 28$  pg/ml) and endometriosis ( $76 \pm 64$  pg/ml) patients. Both disorders are linked to altered inflammatory state and ROS levels [38–42]. Average F2-isoprostane concentration is not statistically different in the three groups ( $P > 0.2$  using a T-test with Levene's Test for Equality of Variances). PGE2 and PGF2 $\alpha$  are 5-fold to 15-fold increased in PCOS or endometriosis samples relative to the control. These elevated values did not reach significance, however, possibly due to low sample number. Additionally, ROS damage to lipids is thought to accumulate with age [43]. F2-isoprostane concentration did not increase significantly when patients were plotted according to age (Fig. 4A). A similar trend was observed for the unidentified peak at RT 12.2 min (Fig. 3A). Therefore, increased free F2-isoprostane concentration in HFF is a poor marker for PCOS, endometriosis, and other conditions associated with oxidative stress.

When we investigated F2-isoprostane concentration in differing infertility diagnoses, the only association that reached significance was DOR patients (Fig. 4B). In these patients, the F2-isoprostane peak was significantly decreased compared to controls. As DOR patients tend to be older, this trend was opposite to that expected if this F2-isoprostane(s) is a byproduct of oxidative stress. These data raise the possibility that specific free F2-isoprostanes have a physiologic role.

### 3.5 Elevated PGF2 $\alpha$ may be associated with decreased live birth

We noticed that PGF2 $\alpha$  and PGE2 concentrations varied extensively in a subset of patients. To test whether high or low PG levels are associated with a clinical outcome, we first calculated median PGF2 $\alpha$  and PGE2 concentrations. Patients were split into groups that fell above and below the median (referred to as high and low throughout). We then compared these groups to clinical variables of interest. PGE2 was not significantly associated with a clinical outcome. High PGF2 $\alpha$  was significantly associated with a poor pregnancy outcome for the IVF cycle (negative pregnancy by HCG serum testing, biochemical pregnancy, or spontaneous abortion) when compared to low PGF2 $\alpha$  levels (Fig. 4C). Similarly, the low PGF2 $\alpha$  cohort had significantly higher live birth rate when compared to the high PGF2 $\alpha$



cohort (Fig. 4C). These results suggest that elevated PGF2 $\alpha$  concentration is detrimental to reproduction or implantation.

#### 4. Discussion

Here we use multiple liquid chromatography mass spectrometry approaches to identify and compare relative PG concentrations in HFF. Two fertile patients were analyzed using a nano-LC Q-TOF method for comprehensive PG analysis. A targeted approach using MRM was used to detect and compare relative concentrations of selected PGs in a broader population of 42 patients. We found known PGs, such as PGF2 $\alpha$  and PGE2, as well as specific F2-isoprostanes and PGF3 $\alpha$  analogs with unknown functions.

A comprehensive analysis of two control patients identified multiple 15-F2-isoprostanes and one to two 5-F2-isoprostanes. 8-F2-isoprostanes and 12-F2-isoprostanes were not detected in the two samples. MRM using mass transition  $m/z$  353/193 for 15-F2-isoprostanes detected three prominent peaks in most patients. One peak eluted with the *8-iso*-PGF2 $\alpha$  standard and a second eluted with the PGF2 $\alpha$  standard. These peaks could contain multiple PGF2 $\alpha$  isomers, as a small number of F2-isoprostanes co-elute in nano-LC and LC methods. The third peak did not co-elute with available standards. Less than half of the 64 total F2-isoprostanes are commercially available, making it difficult to develop LC methods to separate all possible isomers. MS/MS spectra among members of each regioisomer class is nearly identical. Thus, it is not currently possible to identify each F2-isoprostane and PGF3 $\alpha$  analog present in HFF. Nevertheless, it is safe to assume that HFF has specific PG composition, as opposed to a nonselective mixture. Besides PGF2 $\alpha$ , the other F2-isoprostanes and PGF3 $\alpha$  analogs that were detected are likely formed independent of Cox enzymes. Consistent with this idea, 15-F2-isoprostane chromatograms from HFF closely resemble those from Cox-deficient mice and *C. elegans* [26, 27] (Supplemental Fig. 6).

Multiple PGF3 $\alpha$  analogs were detected in nano-LC Q-TOF that did not co-elute with Cox-derived PGF3 $\alpha$ . These PGs have MS/MS spectra similar to PGF3 $\alpha$ -like lipids from *C. elegans* [27]. Their spectra do not contain abundant product ions, such as  $m/z$  115.0402 or  $m/z$  127.0759 from PGF3 $\alpha$  isomers associated with oxidative stress [34, 35]. Cox pathways specifically generate PGE2 and PGD2. Chromatograms focusing on PGE2 isomers are dominated by abundant PGE2 and occasional minor PGD2. Other isomers were not detected. Taken together, these data suggest that HFF contains PGs made by Cox pathways, as well as an alternative pathway(s).

The concentrations of free PGs in HFF are estimated (see Methods) to be in a range consistent with physiological function. While it is difficult to quantify unknown PGF2 $\alpha$  and PGF3 $\alpha$  analogs, we were able to measure peaks eluting with PGF2 $\alpha$  and *8-iso*-PGF2 $\alpha$  standards in MRM mode. In control patients, the average PGF2 $\alpha$  peak concentration is about 144 pg/ml or 406 pM. The average estimated F2-isoprostane peak (RT 11.5 min) concentration is about 52 pg/ml or 150 pM. In general, PGF2 $\alpha$  binding to its receptors occurs between 100 pM and 10 nM with reported dissociation constants in the high pM to low nM range, depending on the study [44–48]. An important mechanistic consideration comes from *C. elegans* ovaries, where numerous F-series PGs act collectively [27]. If this

signaling mechanism is valid in human ovaries, the collective concentrations of PGF1 $\alpha$ , F2-isoprostanes, and PGF3 $\alpha$  analogs could occupy many receptor sites.

It is important to note PGs that were not consistently detected in our samples. PGD1, PGD2, PGF3 $\alpha$ , PGA1, PGA2, and PGI2 were either close to or below the detection limit for most samples, suggesting that these PGs do not play an important role in the mature follicle. PGI2, in particular, was readily detectable in HFF containing blood, which could be a contaminating source. Previous studies using radio-immunoassays detected *6-keto*-PGF1 $\alpha$ , the stable metabolite of PGI2, in HFF [12, 13]. The discrepancy between these studies and ours could be due to blood contamination, poor specificity of the radioimmunoassays, or the patient population. PGE1, PGF1 $\alpha$ , and PGA1 have been shown to bind the sperm cation channel CatSper, suggesting that these PGs might have a role in fertilization [49, 50]. We detected PGE1 and PGF1 $\alpha$  in HFF, but not PGA1. However, the PG composition in the oviduct after ovulation might not reflect the composition in HFF. PGD2 has been implicated in mouse folliculogenesis [51]. Although PGD2 levels were low or undetectable in HFF from most sampled patients, it may be important early in follicle development.

Stratifying patients based on clinical diagnosis, age, and IVF outcome revealed several trends worth further investigation. Free F2-isoprostane concentration was not significantly increased with age or in conditions like PCOS and endometriosis. These findings are at odds with the conventional assumption that *8-iso*-PGF2 $\alpha$  is a marker for oxidative stress. Instead, they suggest that F2-isoprostanes and PGF3 $\alpha$  analogs in HFF may have a function(s) unrelated to stress or inflammation. Another important consideration is that we did not measure esterified PGs, which might better reflect oxidative damage.

PGF2 $\alpha$  levels above the median were associated with decreased live birth rates in our cohort undergoing IVF. PGF2 $\alpha$  concentration varied widely among a small subset of patients. Some of this variation might reflect increased synthesis in a temporally restricted fashion following gonadotropin stimulation [5, 6]. Several patients, particularly those diagnosed with endometriosis and PCOS, exhibited very high PGF2 $\alpha$  and PGE2 levels. One possibility is that these patients have an underlying inflammatory condition causing increased PGF2 $\alpha$  synthesis throughout the ovary. Indeed, elevated PGF2 $\alpha$  and PGE2 levels have been observed in endometriosis patients [39]. Excess inflammation could influence embryo implantation. Alternatively, high PGF2 $\alpha$  in HFF could indicate compromised oocyte development, leading to failed embryogenesis. Future work is necessary to determine the function of PGF2 $\alpha$  in HFF and the effects, if any, of elevated PGF2 $\alpha$  on IVF outcome.

In conclusion, HFF contains a specific composition of structurally related PGs that require sensitive analytical methods to detect. While PGE2 is important for ovulation, the roles of other PGs are not well understood. In particular, the presence of multiple F-series PGs in HFF suggests that there is more to learn about these important lipid mediators in reproduction than previously appreciated.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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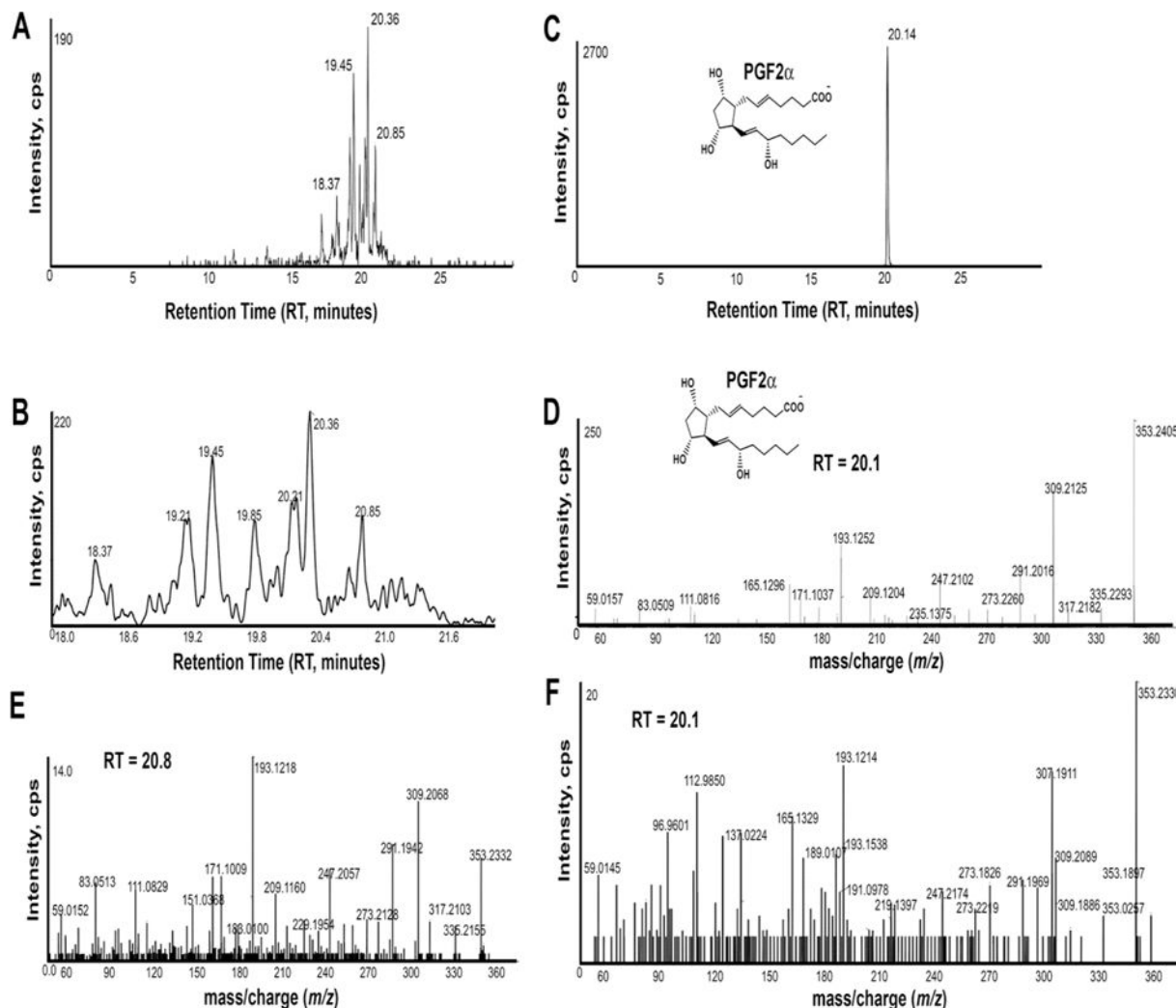
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### Highlights

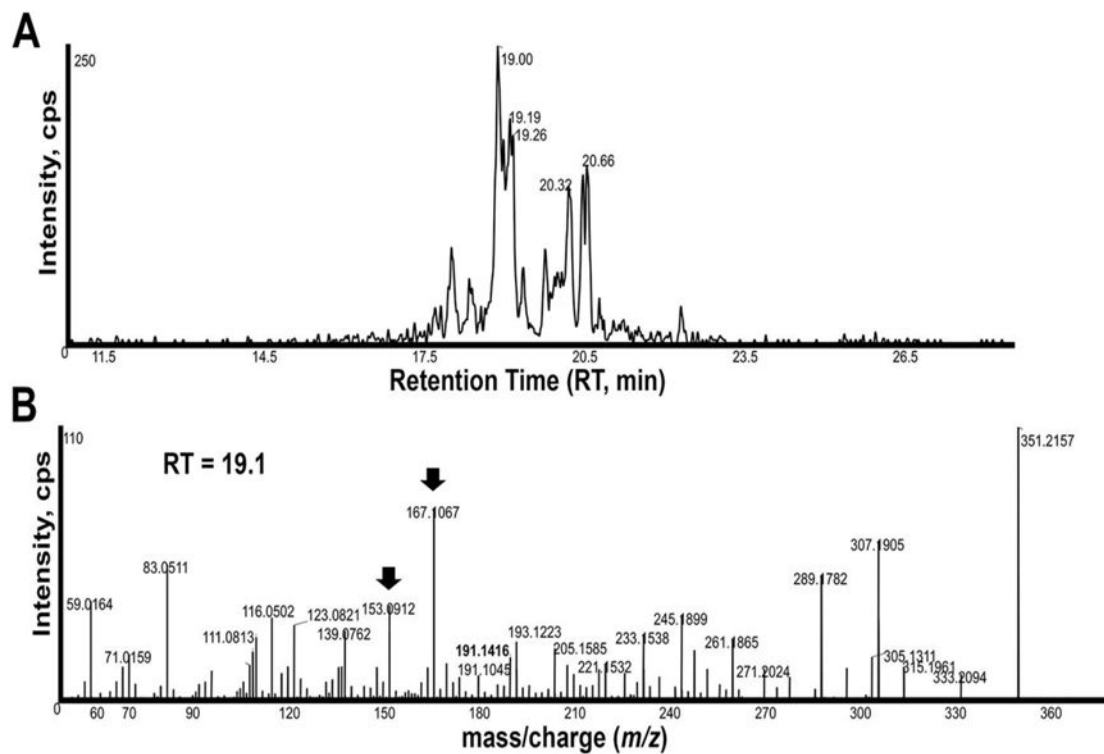
- Comprehensive profiling of prostaglandins in 42 human follicular fluid samples identified PGE1, PGE2, PGF1 $\alpha$ , and PGF2 $\alpha$
- Specific free F2-isoprostanes and PGF3 $\alpha$  analogs were detected whose structures are inconsistent with synthesis by cyclooxygenase pathways
- F2-isoprostane concentration was decreased in diminished ovarian reserve patients
- High PGF2 $\alpha$  concentration was associated with decreased live birth





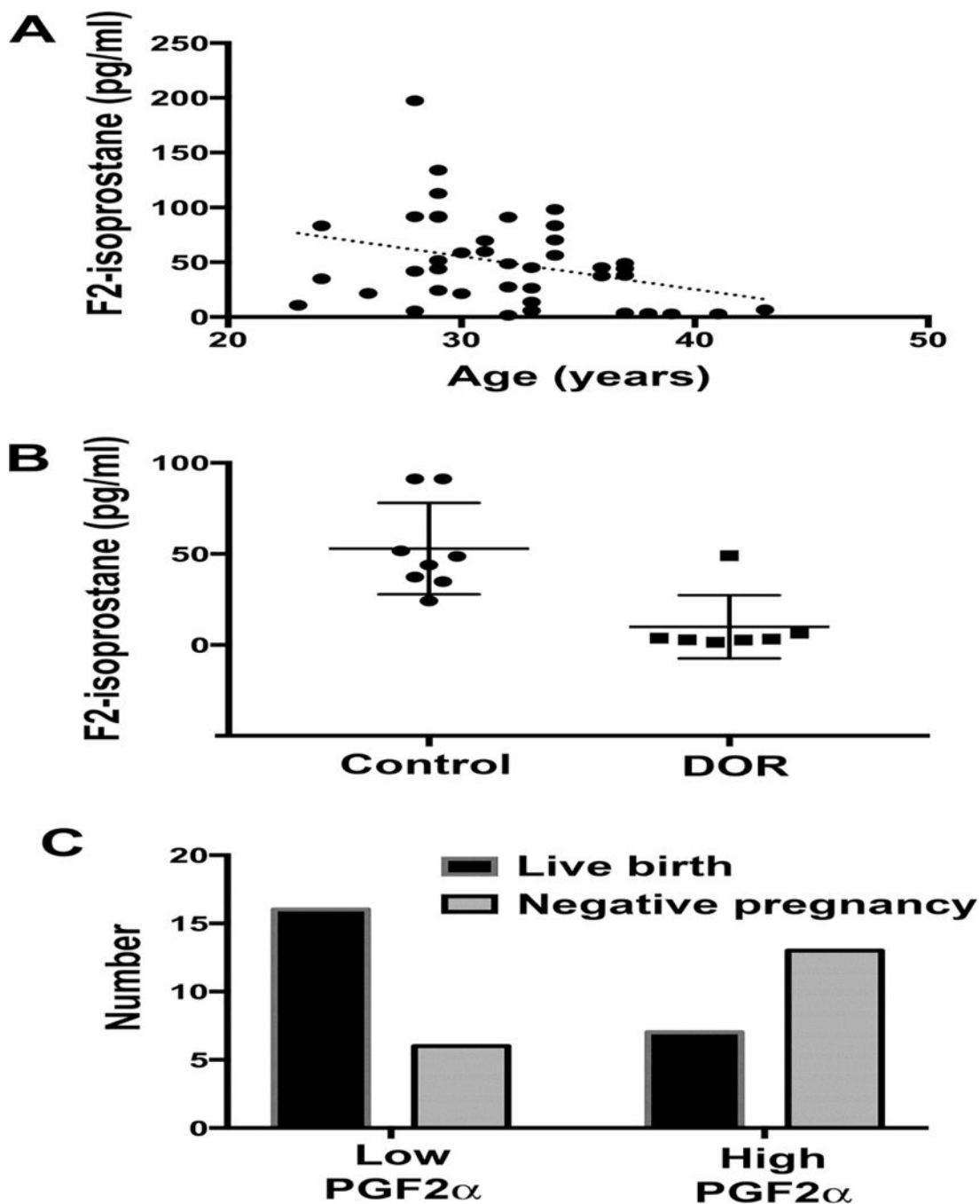
### Figure 1. PGF2 $\alpha$ isomers in HFF

An extracted ion chromatogram (XIC) generated from the product ion  $m/z$  193.122 that is found in 15-F2-isoprostanes (A). A magnified chromatogram focusing on RT 18 to 22 minutes is shown below (B). An XIC for product ion  $m/z$  193.122 shows a single peak at RT 20.14 minutes from the PGF2 $\alpha$  standard (10ng/ml) (C). MS/MS product ion spectrum of the PGF2 $\alpha$  standard (D). MS/MS product ion spectrum of  $m/z$  353.2323 at RT 20.8 minutes (E). MS/MS product ion spectrum of  $m/z$  353.232 at RT 20.2 minutes (F). This spectrum contains overlapping isobaric precursor ions, possibly a steroidal sulfate(s), as evidenced by  $m/z$  96.960 (HSO $_4^-$ ). Panels A, B, E, and F are from a single control patient. An XIC for a second control patient is shown in Supplemental Fig. 1B. Cps, counts per second.



### Figure 2. PGF3 $\alpha$ analogs in HFF

An XIC generated from the product ion  $m/z$  191.145 that is found in PGF3 $\alpha$  isomers (A). MS/MS product ion spectrum of  $m/z$  351.215 at RT 19.1 minutes in the extracted HFF sample (B). The product ions  $m/z$  333, 315, 307, 289, 245, 193, and 191 are observed in authentic PGF3 $\alpha$  [23, 27]. Standards also available online at <http://www.lipidmaps.org/data/standards/index.html>. Arrows indicate major product ions that are not observed in PGF3 $\alpha$ , but are observed in a PGF3 $\alpha$  analog from *C. elegans* [27]. Peaks at RT 20.35 minutes and 20.65 minutes have several product ions observed in PGF3 $\alpha$  spectra, including  $m/z$  333, 315, 289, 245, 193, and 191. Cps, counts per second.



**Figure 3. Representative MRM chromatograms of PG standards and HFF**

MRM using mass transition  $m/z$  353/193 detects *8-iso*-PGF2 $\alpha$  and PGF2 $\alpha$  standards (A, top). Three major peaks are observed in most HFF samples (A, bottom). Based on nano-LC qTOF analysis, these peaks may contain co-eluting PGF2 $\alpha$  isomers (see Discussion). MRM using mass transition  $m/z$  351/189 detects PGE2 (B, top) and PGD2 standards (not shown at RT = 12.6 minutes). A single major peak is observed in most HFF samples corresponding to PGE2 (B, bottom). MRM using mass transition  $m/z$  355/311 detects the PGF1 $\alpha$  (C, top) standard. A peak at RT 12.1 is observed in most HFF samples corresponding to PGF1 $\alpha$  (C,

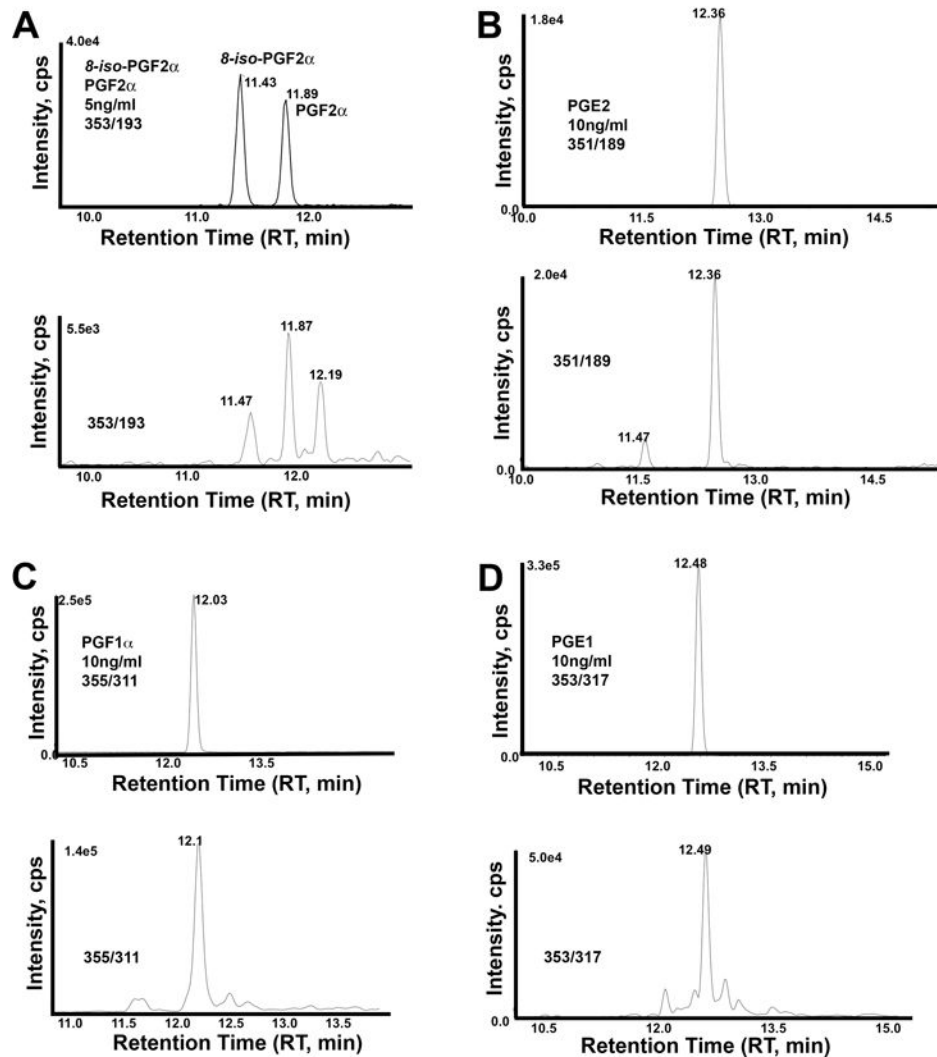
bottom). MRM using mass transition  $m/z$  353/317 detects the PGE1 (D, top) standard. A peak at RT 12.5 is observed in most HFF samples corresponding to PGE1 (C, bottom). Cps, counts per second.

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#### Figure 4. Clinical associations with 15-F2-isoprotanes

*8-iso*-PGF2 $\alpha$  concentration in HFF is plotted according to patient age (A). The dotted line represents the best fit from a linear regression model. Average *8-iso*-PGF2 $\alpha$  concentration in control patients (egg donor or male infertility) and diminished ovarian reserve (DOR) patients (B). Dots represent individual data points. Bars are standard deviation. Average *8-iso*-PGF2 $\alpha$  concentration is reduced in DOR patients compared to controls using a student's t-test ( $P < 0.05$ ). *8-iso*-PGF2 $\alpha$  concentration was calculated from the peak at RT 11.4 to 11.5 in Fig. 3A. Similar results were obtained from the unknown 15-F2-isoprostane peak at RT 12.2 in Fig. 3A. Number of live births and negative pregnancies in patients with low PGF2 $\alpha$  concentration (below the median) and high PGF2 $\alpha$  concentration (above the median) (C). Negative pregnancy was assessed by HCG serum testing and included biochemical pregnancy and spontaneous abortion.

**Table 1**

PG MRM mass transitions and average concentration across all patients.

Standard	RT (min)	MRM mass transition <i>m/z</i>	Average estimated PG concentration
PGA1	13.5	335/273	ND
PGA2	13.3	333/189	ND
PGD2	12.6	351/189	ND
PGE1	12.4	353/317	70 ± 21 pg/ml
PGE2	12.3	351/189	638 ± 235 pg/ml
<i>6-keto</i> -PGF1α	10.7	369/245	ND
PGF1α	11.9	355/311	38 ± 8 pg/ml
PGF2α	11.9	353/193	654 ± 269 pg/ml*
<i>8-iso</i> -PGF2α	11.5	353/193	50 ± 6 pg/ml*
PGF3α	11.3	351/191	ND

ND, not determined because most HFF samples had low or undetectable PG concentration. Mean ± standard error of the mean (SEM) is shown. Average estimated concentration is in picograms (pg) per milliliter (ml) of HFF, assuming a 50% extraction/detection efficiency (see Methods). HFF was concentrated 10-fold in 80% MeOH prior to analysis. RT, Retention Time in minutes.

\* this peak may contain multiple 15-F2-isoprostanes (see Discussion).



**Table 2**

Patient clinical characteristics.

	Control (n=8)	Unex (n=10)	Endo (n=7)	PCOS (n=7)	DOR (n=7)
Age	30.00	32.20	31.57	29.29	38.14*
BMI	21.99	21.66	25.23	31.29*	24.34
History of Live Birth	5/8	1/10*	3/7	0/7*	1/7
Number Oocytes Retrieved	18.13	17.20	17.71	19.43	6.71*
Total Blastocyst Number	7.25	5.60	3.00	4.86	2.43*
IVF Cycle Negative HCG	2/8	2/10	2/7	2/7	5/7
IVF Cycle Biochemical or SAB	0/8	1/10	1/7	4/7	0/7
IVF Cycle Ongoing Pregnancy	7/8	7/10	4/7	1/7	3/7

Number of patients is in parenthesis. BMI, Body Mass Index; Unex, Unexplained Infertility; Endo, Endometriosis; PCOS, Polycystic Ovary Syndrome; DOR, Diminished Ovarian Reserve; IVF, *in vitro* Fertilization; HCG, Human Chorionic Gonadotropin; SAB, Spontaneous Abortion.

\*  $P < 0.05$  comparing diagnostic groups versus control group using a student's t-test and chi-square testing for parametric and non-parametric groups, respectively.