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Distributions of high-density lipoprotein particle components in human follicular fluid and sera and their associations with embryo morphology parameters during IVF

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BACKGROUND: High-density lipoprotein (HDL) is the sole lipoprotein present in follicular fluid (FF). The objectives of this study were to examine HDL lipid composition and associated enzyme activities in FF and serum and to relate these levels to embryo morphology parameters in women undergoing *in vitro* fertilization (IVF). METHODS: Serum and FF were prospectively obtained from 60 women undergoing IVF. HDL lipids, apolipoprotein AI (ApoAI), paraoxonase 1 (PON1) and paraoxonase 3 (PON3) activities were determined. Bivariate analysis and ordinal logistic regression models were employed to examine the associations between biochemical measures and embryo morphology parameters [embryo cell number (ECN) and embryo fragmentation score (EFS)] as surrogate markers of oocyte health. RESULTS: All biochemical parameters were significantly ($P < 0.05$) lower in FF than serum except PON3 levels which were significantly higher. FF-HDL cholesterol (OR 0.66, 95%CI 0.46–0.96) and ApoAI (OR 0.13, 95%CI 0.03–0.97) levels were negative predictors for EFS; however, their effects were not independent and the level of one moderated the effect of the other. Limited to Day 3 embryo transfers, FF-PON1-arylesterase activity was a significant positive predictor for ECN (OR 1.09, 95%CI 1.01–1.17). CONCLUSIONS: In this pilot study, our data suggests that HDL and its component proteins within FF may play protective roles in the health of the human oocyte and subsequent early embryo development. We describe for the first time the activities of PON1 and PON3 in FF. We suspect that PON3 activity may be locally generated due to higher activities in FF compared with serum.

Keywords: high-density lipoprotein; follicular fluid; embryo cytoplasmic fragmentation; *in vitro* fertilization; paraoxonase

Introduction

HDL-cholesterol has been classically recognized as the molecule responsible for providing cholesterol as the substrate for *de novo* steroidogenesis within the ovarian follicle (Simpson *et al*., 1980; Jaspard *et al*., 1996, 1997; Azhar *et al*., 1998; Li *et al*., 2001). Yet, high-density lipoprotein (HDL) also exhibits potent anti-inflammatory and antioxidant properties that contribute to its general antiatherogenic and cytoprotective effects (Ansell *et al*., 2003; Negre-Salvayre *et al*., 2006). Determinants of HDLs antioxidant/anti-inflammatory activities include its size and density (Nobecourt *et al*., 2005), lipid composition (McPherson *et al*., 2007), the apolipoprotein moiety (ApoAI) and the presence of associated enzymes including paraoxonase 1 and 3 (PON1 and PON3) (von Eckardstein *et al*., 2005; Negre-Salvayre *et al*., 2006).

HDL is the sole lipoprotein present in follicular fluid (FF) due to the porosity of the follicle membrane which is permeable to serum proteins up to 300 kDa in size thus excluding low-density lipoprotein (LDL), very low-density lipoprotein (VLDL) and larger HDL_2s which are not expected to filter through the follicular basement membrane (Le Goff, 1994; Jaspard *et al*., 1996). HDL particles consist of a lipid monolayer and core in addition to protein components including apolipoproteins (ApoAI, AII and AIV), and lipolytic and antioxidant enzymes. Among these proteins, ApoAI and PONs appear to have the most dominant antioxidant activities (Wu *et al*., 2007).

The PON gene family in humans has three members, *PON1*, *PON2* and *PON3* (Primo-Parmo *et al*., 1996). PON1 is synthesized in the liver and carried in the blood associated

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exclusively with HDL where it is the major determinant of HDLs ability to protect LDL (Mackness *et al.*, 1991), cells (Draganov *et al.*, 2000) and lipids from oxidative and peroxidative modification (Draganov *et al.*, 2005; Teiber *et al.*, 2007). Recently, PON1 protein has been identified in FF by proteomic analysis (Angelucci *et al.*, 2006). PON1 activity is currently characterized using two substrates, paraoxon (PON activity) and/or phenyl acetate (arylesterase activity); however, these activities have not been described in FF. When PON3 protein was first discovered, it was localized to HDL and demonstrated to have similar antioxidant properties to PON1 despite the fact that it is ∼100 times lower in concentration in serum than PON1 (Reddy *et al.*, 2001; Draganov, 2007). The presence of PON3 in FF has, to our knowledge, not been previously described.

In female reproduction, evidence is accumulating on the role of reactive oxygen species, with oxidative stress (OS) in the Graafian follicle proving to be an important process in defining female reproductive potential via oocyte genetic and cytoplasmic integrity (Agarwal *et al.*, 2003). Specific antioxidant enzymes and radical scavengers are present in FF (Bisseling *et al.*, 1997; Sabatini *et al.*, 1999; Pasqualotto *et al.*, 2004) as are several markers of oxidative damage (Wiener-Megnazi *et al.*, 2004; Das *et al.*, 2006); however, the effects of these markers on oocyte quality, embryo quality and other clinical reproductive outcomes have thus far produced conflicting results.

Given the unique properties of HDL and its component proteins together with their isolated presence in FF in the absence of LDL, we sought to quantify the lipid and apolipoprotein composition of FF-HDL relative to serum obtained from patients undergoing *in vitro* fertilization (IVF) and examine possible associations with morphologic indices of embryo quality. Furthermore, the novel quantification and characterization of PON1 and PON3 activities in FF are described in the context of this prospective pilot study.

Materials and Methods

Study subjects

There were 60 patients undergoing IVF treatment at the UCSF Center for Reproductive Health recruited into this study. Subjects were enrolled prospectively prior to completion of their IVF cycle with full informed consent. No participants actively withdrew from the study after consent. The study protocol and consenting process were approved by the UCSF Committee on Human Research. Participants underwent gonadotrophin-induced ovarian stimulation per clinic protocols: (i) down-regulated luteal leuprolide acetate; (ii) microdose leuprolide acetate flare; (iii) gonadotrophin-releasing hormone (GnRH) antagonist with/without estrogen priming and (iv) demi-halt (luteal leuprolide acetate discontinued prior to gonadotrophin initiation). Transvaginal ultrasonography and serum estradiol levels were performed routinely during ovarian stimulation to assess ovarian follicle maturation. When a sufficient number of mature-sized follicles (≥16 mm diameter) developed, human chorionic gonadotrophin (5000–10 000 IU) was administered subcutaneously 36 h prior to scheduled oocyte retrieval. A blood sample was obtained from the patient 15–30 min prior to the oocyte retrieval procedure via an intravenous line placed for anesthesia and hydration purposes. FF from an individual mature 18-20 mm follicle was aspirated using a single lumen 17 gage 35 cm aspiration needle guided by transvaginal ultrasonography. After identification and removal of the cumulus mass, the FF along with serum was processed immediately. The processed samples were frozen at −80°C and stored until shipment to the OS Laboratory at the University of Buffalo. A total of 60 individually aspirated follicles from 60 patients were included in this study.

Intracytoplasmic sperm injection (ICSI) was performed in most cases based on abnormal sperm parameters from prior semen analyses. Otherwise, conventional insemination was performed. For ICSI cases, oocytes were denuded of cumulus granulosa cells prior to sperm injection. Both mature (i.e. metaphase-II) and immature oocytes were identified for all ICSI cases. All metaphase-II oocytes were fertilized, with sperm collected from the male partner on the day of oocyte collection or a frozen sperm specimen from the male partner or sperm donor, using either ICSI or conventional insemination. Zygotes were identified ∼16–18 h following insemination, by the appearance of two pronuclei (2PN) indicative of normal fertilization. All embryos were cultured in a standard commercially-available culture media (Vitrolife Series G1.3®, Kungsbacka, Sweden) for 24–48 h prior to embryo morphology evaluation. Embryo morphologic indices were determined using an inverted phase contrast Nikon Diaphot® microscope, and included individual embryo cell number (ECN) and individual embryo fragmentation score (EFS), each assessed on the day of embryo transfer for embryos generated from individually aspirated follicles. Embryo morphology assessments for all study participants were made only by experienced embryologists who were blinded to any biochemical results obtained during the study. Experienced embryologists in our laboratory undergo routine quality control assessment to minimize inter-observer variability. Similarly, the OS laboratory was blinded to any clinical outcomes data during assay analyses.

Embryo fragmentation scoring was based on the degree of fragmentation present per embryo assessed on the day of embryo transfer with the following criteria: Grade 1, 0% fragmentation; Grade 2, 1–10% fragmentation; Grade 3, 11–25% fragmentation; Grade 4, 26–50% fragmentation; and Grade 5, 51% or greater fragmentation. ECN and degree of fragmentation are both considered important clinical markers of embryo quality and viability (Puissant *et al.*, 1987; Steer *et al.*, 1992).

HDL fractionation and lipid composition analysis

Serum and FF-HDL fractions were prepared by selective precipitation methods (Gidez *et al.*, 1982), since ultracentrifugation is known to disrupt lipoprotein structure (Kunitake and Kane, 1982), and it has been suggested that HDL components may be redistributed among lipoproteins during ultracentrifugation (Cabana *et al.*, 2003). Despite the absence of non-HDL lipoproteins in FF, the precipitant was added to create the same condition as in serum. Briefly, 0.1 vol of heparin-$MnCl_2$ (40 000 USP units/ml heparin in 1.0 mol/l $MnCl_2$) solution was added to 1.0 vol of sample. After 5 min incubation at room temperature, the samples were centrifuged at 16 000 g in a microcentrifuge, and the supernatant was collected.

Total lipid analysis

ApoAI, cholesterol, phospholipids and triglycerides levels were measured using diagnostic reagent kits from Wako Diagnostics Inc. (Richmond, VA, USA) adapted to the Cobas Fara II (Hoffmann–La Roche & Co., Switzerland) automated chemistry analyzer. The analysis parameters of these assays were modified by increasing the sample size by a factor of 3–5 times to account for the lower lipid concentrations encountered in the isolated HDL preparations. All analyses were performed in duplicate and reported in mg/dl. The analytical coefficient of variation of each assay was $<5\%$.

PON1 arylesterase and PON activities

The arylesterase and PON activities of PON1 were determined exactly as recently described (Browne *et al.*, 2007). Diethyl *p*-nitrophenyl phosphate (paraoxon), 98.0%, was obtained from Chem Service (Westchester, PA, USA). PON activity was determined by the rate of formation of *p*-nitrophenol at 412 nm using 1 mmol/l paraoxon as the substrate in 50 mmol/l glycine buffer, pH 10.5, with 1.0 mmol/l $CaCl_2$. The activity was expressed as IU/l based on the molar absorptivity (18 290) of *p*-nitrophenol at 405 nm, at pH 10.5. Arylesterase activity was determined by the rate of formation of phenol at 270 nm using 4 mmol/l phenyl acetate as the substrate in 20 mM Tris–HCl, pH 8.0, with 1.0 mM $CaCl_2$. The activity, expressed as kU/l, was based on the molar absorptivity (188) of phenol at 285 nm, at pH 8.0. For all assays, water blanks were used to correct for non-enzymatic hydrolysis.

PON3 simvastatinase

PON3 is the only member of the PON family which metabolizes bulky drug substrates such as lovastatin and simvastatin (Draganov *et al.*, 2005). We have instituted and validated a method recently described by Suchocka *et al*. (2006). PON3 activity was determined by RP-HPLC monitoring of the conversion of simvastatin (SV) to β,δ-dihydroxyacid simvastatin (SVA) during incubation with serum or FF. The basal reaction mixture [940 μl of 20 mmol/l Tris–HCl (pH 7.6) containing 0.9 mmol/l $CaCl_2$ and 40 μmol/l of physostygmine] was pre-incubated for 10 min at 37°C in water-bath and then 10 μl of SV (0.5 mg/ml in methanol) was added. The reaction was initiated by adding 50 μl of serum or FF and the enzymatic reaction was stopped after 1 h by deproteinization with 1.0 ml cold acetonitrile. HPLC analysis of SV and SVA was carried out using a Shimadzu series chromatograph consisting of a LC-10AD Vp pump, SPD-10AV diode array and Sil10A autoinjector (Shimadzu Scientific Instruments, Inc., Columbia, MD, USA). SV and SVA separations were performed on a SupelcoSil C18, 5 μm, 250 mm × 4.60 mm column, at room temperature 22–25°C. Peak detection was performed at $\lambda = 239$ nm. The PON3 activity in serum or FF was expressed as the rate of formation of SVA in pmol/min/ml.

Enzyme inhibition studies

To confirm that the enzymatic activities measured were due to PON enzymes and not other hydrolytic enzymes, we performed several inhibition experiments. PONs are calcium-dependent enzymes and therefore inhibited by EDTA treatment. Inhibition by EDTA was performed by diluting samples 1:1 in buffer (50 mM Tris–HCl, 40 mmol/l EDTA, pH 7.4) and incubating for 16 h at room temperature. Buffer for the controls contained 1 mM $CaCl_2$ instead of EDTA. To rule out the possibility of cholinesterase activity in our assays, samples were inhibited with physostigmine (a cholinesterase inhibitor), phenylmethanesulfonyl fluorine (PMSF; a serine protease inhibitor) and tetraisopropyl pyrophosphoramide (iso-OMPA; a selective butrylcholinesterase inhibitor). Each inhibition was performed by adding 2 μl of the inhibitor (200 mmol/l in methanol), or methanol for controls, to samples that had been diluted 1:2 in buffer (50 mmol/l Tris–HCl, 1 mmol/l $CaCl_2$, pH 7.4) so that the final concentration of inhibitor was 2 mmol/l. Samples were then incubated at room temperature for 2 h. Aliquots of the inhibited and control samples were analyzed as described above.

Data analysis

All statistical analyses were performed using SAS version 9.1.3 with statistical significance defined as $P < 0.05$ for a two-tailed test. Non-parametric analyses were favored including Spearman rank correlation coefficients (r_{Sp}) for continuous and ordinal data; Wilcoxon rank sum tests were used for paired differences, and Mann–Whitney *U*-test, χ^2-test or Fisher's exact tests, as appropriate, were used for categorical data. Differences between the median values for sera and FF concentrations of each biochemical analyte were assessed as were correlations between serum and FF concentrations and with demographic factors.

Correlations were evaluated between concentrations for biochemical analytes and embryo morphology parameters among oocytes retrieved from the 60 sampled follicles (i.e. one per woman). These correlations, stratified by body compartment (i.e. serum or FF) were employed to select potential predictors using multivariable ordinal logistic regression, also known as proportional odds, models (McCullagh, 1980). This extension of binary logistic regression, assuming proportionality, estimates the log odds of a subject's outcome falling into the *k*th ($k = 1$, 2 and 3) outcome category or lower across $k-1$ ordered response variate categories. A more efficient capture of the ordered information inherent in an ordinal response variate, and consequently greater power and precision, may be effected than that offered by dichotomization and binary logistic regression. Biochemical correlates of embryo morphology parameters, with $P < 0.15$, were entered into a forward stepwise selection procedure (entry/exit criteria 0.05/0.10) as potential predictors for EFS (1/2/3–5; $n = 8/13/9$) and for ECN (2–4 cells/5–7 cells/8–11 cells; $n = 2/7/13$), with the latter limited to Day 3 embryo transfers. 'Unselected' potential biochemical predictors, age, BMI and patient race/ethnicity were considered as potential confounders for 'selected' models. Confounding was defined as a minimum 15% change in the magnitude for biochemical predictors following entry of the covariate into the 'selected' model (Kleinbaum *et al.*, 1998). Interaction terms were considered between 'selected' predictors and any confounders retained in the models, these were included in final models where $P < 0.10$. Multiple imputation, employing the EM algorithm, was used to impute missing values (a consequence of limited FF sample volume) during ordinal logistic regression modeling (Horton and Kleinman, 2007).

Results

The study sample

The complete demographic, treatment and treatment outcome covariates for the study population are shown in Table I. A large range for age (29–44 years) and BMI (18–34 kg/m^2) were represented among the 60 female study participants. The relatively high proportion of Asian participants (36.7%) reflects the population of the San Francisco Bay area from which the study participants were recruited. Approximately 40% of participants had undergone prior IVF procedures. Male factor (42%) was the most prevalent infertility diagnosis among study participants, followed by unexplained (22%), and diminished ovarian reserve (15%). Approximately two-thirds of study participants received a down-regulated luteal lupron stimulation protocol. ICSI was employed for 78% ($n = 47$) of subjects.

PON activity validation

To be sure that the enzyme activities measured were attributable to PONs, we performed inhibition studies in lieu of having specific PON1 or PON3 inhibitors which have yet to be identified. To conduct these inhibition studies, three FF samples

Table I. Characteristics of study participants by demographic, treatment and treatment outcomes covariates ($n = 60$).

Demographic covariates	Mean (SD)	Median	Range
Age (years)	36.5 (5.7)	37.9	28.8–44.0
BMI (kg/m^2)[a]	23.5 (3.0)	22.7	18.1–34.4
Ethnicity, *n* (%)			
Caucasian	35 (58.3)	n/a	n/a
Asian	22 (36.7)	n/a	n/a
African-American	2 (3.3)	n/a	n/a
Hispanic	1 (1.7)	n/a	n/a
Treatment covariates			
Number of prior IVF cycles[b]	1.9 (1.5)	1.0	1.0–7.0
Diagnosis, *n* (%)[c]			
Male factor	25 (41.7)	n/a	n/a
Unexplained	13 (21.7)	n/a	n/a
DOR	9 (15.0)	n/a	n/a
Donor	5 (8.3)	n/a	n/a
Tubal factor	3 (5.0)	n/a	n/a
Endometriosis	2 (3.4)	n/a	n/a
Anovulation	1 (1.7)	n/a	n/a
Recurrent SAB	1 (1.7)	n/a	n/a
Protocol, *n* (%)			
Luteal lupron	40 (66.7)	n/a	n/a
Antagonist	8 (13.3)	n/a	n/a
Demi-halt	7 (11.7)	n/a	n/a
Flare	3 (5.0)	n/a	n/a
E_2-priming antagonist	2 (3.3)	n/a	n/a

BMI, body mass index; DOR, diminished ovarian reserve; E_2, estradiol; IVF, *in vitro* fertilization; SAB, spontaneous abortion.
[a]$n = 3$ missing values.
[b]$n = 24$ (40.0%) patients with a prior history of IVF treatment.
[c]Diagnoses are mutually exclusive, $n = 1$ missing value.

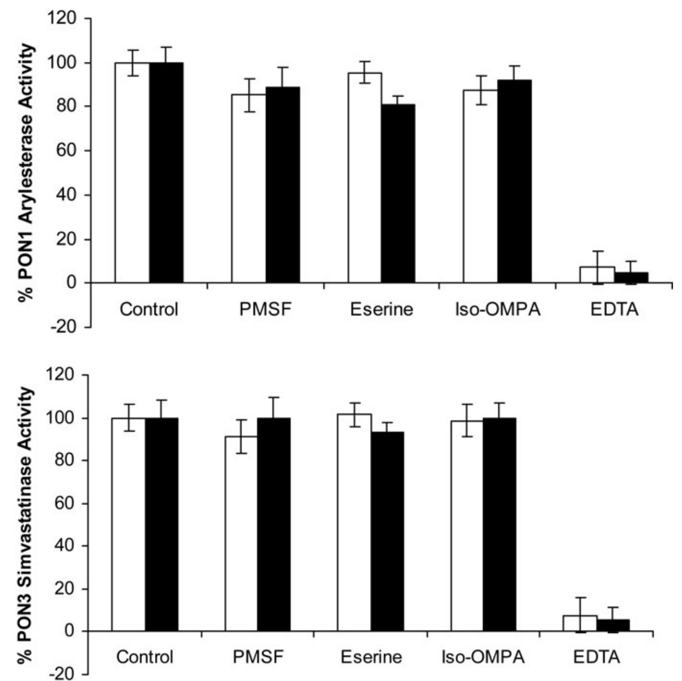

Figure 1: Inhibition of PON1 arylesterase activity and PON3 simvastatinase activity in serum (white bars) and follicular fluid (black bars) by EDTA and three enzyme inhibitors. Results are expressed as a percentage of the uninhibited, control activity (kIU/l for PON1 and pmol/min/ml for PON3).

were pooled, divided and then treated with inhibitors as described in the Methods and Materials. The results are shown in Fig. 1. Following treatment with EDTA, >90% of PON3 simvastatinase and PON1 arylesterase activities were inhibited due to calcium chelation. Alternative treatments with PMSF, eserine and iso-OMPA did not reduce the PON activities by >10% indicating that the majority of the reported activities cannot be attributed to the activities of serine proteases, cholinesterases or butrylcholinesterases, respectively. Nearly identical results were obtained for PON1 PON activities (data not shown).

Characterization of serum and FF-HDL levels and PON activities

Box and whisker plots describing the distributions for levels of HDL lipids, PON1 and PON3 enzyme activities, in serum and FF, are presented in Fig. 2. Median FF-HDL component concentrations were lower for cholesterol (20.9 versus 37.2, $P < 0.0001$, $n = 59$), phospholipids (73.1 versus 94.6, $P < 0.0001$, $n = 40$) and ApoAI (105.5 versus 157.3, $P = 0.001$, $n = 59$) when compared with serum HDL, whereas triglycerides levels were not different (7.5 versus 8.4, $P = 0.546$, $n = 39$). Except for triglycerides ($r_{Sp} = 0.09$, $P = 0.597$), levels of HDL lipids were positively correlated between FF and serum ($r_{Sp} = 0.44–0.95$, $P \leq 0.001$). Median sera activities were also greater than FF for PON1-arylesterase (154.2 versus 93.2, $P < 0.0001$, $n = 60$) and PON1-PON (186.5 versus 116.4, $P < 0.0001$, $n = 60$), and values were highly correlated between these compartments ($r_{Sp} = 0.74–0.95$, $P < 0.0001$). In contrast, median PON3-simvastatinase activity was lower in serum than in FF (10.6 versus 16.5, $P = 0.006$, $n = 58$) with an attenuated, yet statistically significant, correlation between the two compartments ($r_{Sp} = 0.36$, $P = 0.006$). With the exception of several observations for HDL-triglycerides ($n = 23$, 41.0%) and PON3 ($n = 2$, 3.4%), the observed pattern of differences between serum and FF was consistent for all study subjects.

The correlations between serum and FF-HDL components levels suggest that FF-HDL is blood-derived; however, it is significant to note that FF-HDL particle composition is proportionally different to that in serum. ApoAI is a surrogate marker of the number of HDL particles. Previous studies indicate that FF-HDL particle ApoAI is not different from blood plasma and can be useful for relative composition comparisons (Jaspard *et al*., 1997). Upon normalization of HDL components per molecule of ApoAI (i.e. HDL component concentrations divided by ApoAI concentration), it was evident that FF-HDL is 25% lower in cholesterol content ($P < 0.001$), 10% higher in phospholipids content ($P < 0.001$) and 40% higher in triglyceride content ($P < 0.01$) when compared with serum by the Wilcoxon rank sum test. These ApoAI-normalized values are reported here to be descriptive and highlight the different HDL particle composition in FF. These normalized values were not used during subsequent ordinal logistic regression analyses with clinical outcomes as measurement error and bias may be introduced as previously demonstrated for lipid normalization (Schisterman *et al*., 2005).

Substantial intercorrelations were measured among the lipid components of HDL and the levels of PON1 activities in FF, as in Table II. HDL-cholesterol, HDL phospholipids and ApoAI all correlated significantly with each other ($r_{Sp} = 0.36–0.62$,

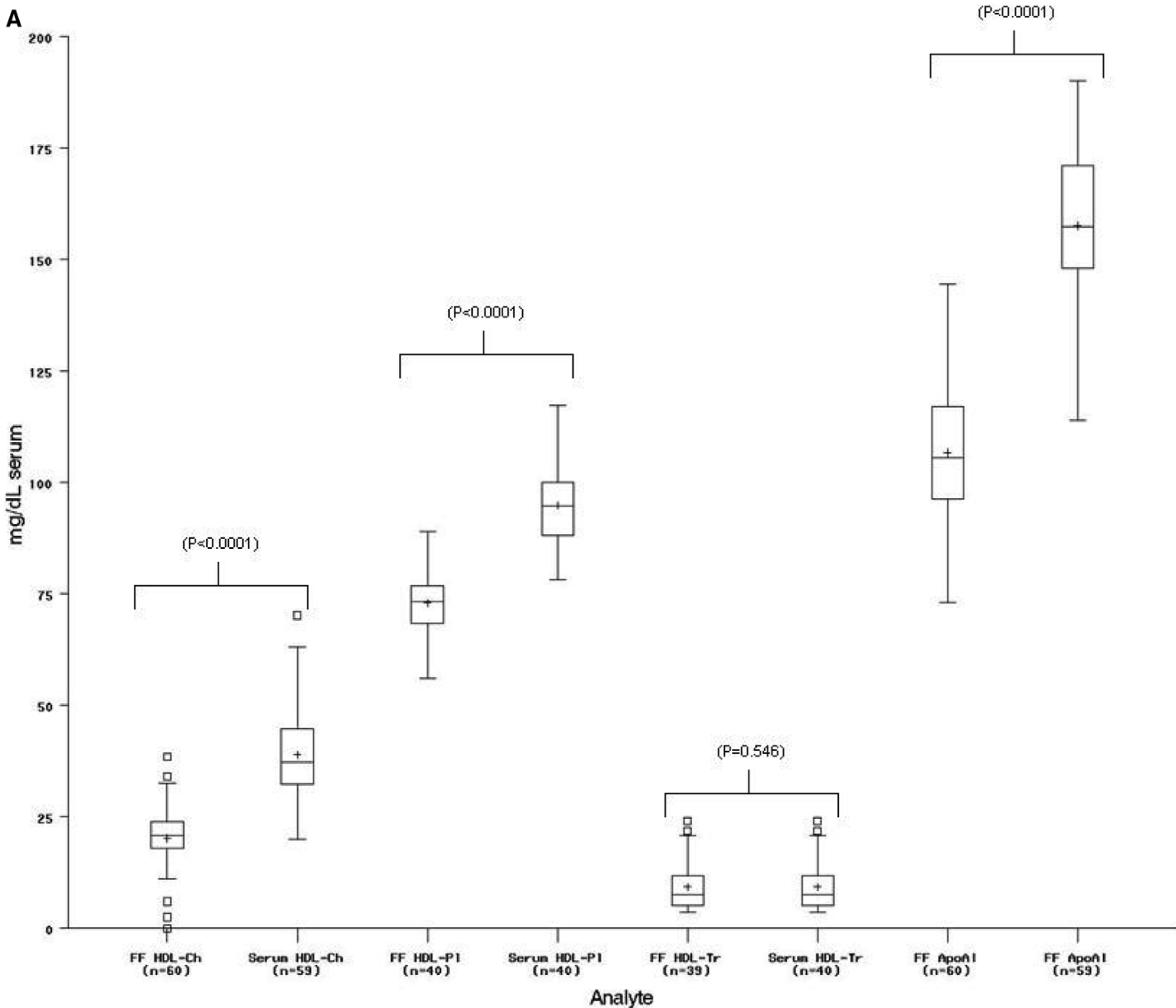

Figure 2: Box and Whisker plots describing the distributions for lipid components of fractionated HDL (**A**) and enzyme activities (**B**) from follicular fluid and serum.
HDL-Ch, high-density lipoprotein cholesterol; HDL-Pl, high-density lipoprotein phospholipids; HDL-Tr, high-density lipoprotein triglycerides; PON1-Ary, paraoxonase 1 arylesterase activity; PON1-Par, paraoxonase 1 paraoxonase activity; PON3-SIM, paraoxonase 3 simvastatinase activity.

$P \leq 0.022$), probably due to their common location on the HDL particle. PON1 arylesterase activity also correlated with these parameters albeit to a lesser degree ($r_{Sp} = 0.23$–0.36), reaching significance only in association with ApoAI ($P = 0.005$). Interestingly, a negative association was suggested between PON3 simvastatinase activity and HDL-cholesterol ($r = -0.23$, $P = 0.087$), although this correlation did not reach statistical significance. A similar pattern of correlations was measured among serum analytes (data not shown).

Correlation of HDL and PON activities with embryo morphology parameters

Metaphase-II oocytes were harvested from ~83% ($n = 50$) of individually sampled follicles. Approximately 64% ($n = 32$) of these oocytes were normally fertilized as indicated by 2PN embryos. Significant negative correlations to EFS were measured for FF-HDL cholesterol ($r_{Sp} = -0.59$, $P = 0.001$), FF ApoAI ($r_{Sp} = -0.54$, $P = 0.002$), serum-HDL cholesterol ($r_{Sp} = -0.53$, $P = 0.003$), serum ApoAI ($r_{Sp} = -0.50$, $P = 0.005$), as well as serum PON1-arylesterase activity ($r_{Sp} = -0.39$, $P = 0.032$) (Table III). Adjusting for day of embryo transfer, which was significantly associated with ECN [2-cells versus 8-cells ($P < 0.0001$) for Day 2 versus Day 3, respectively], no statistically significant correlations were measured between ECN and any FF or serum analytes. There was a positive correlation between FF-PON1-arylesterase activity and ECN, but this did not reach statistical significance ($r_{Sp} = 0.29$, $P = 0.122$). There were no statistically

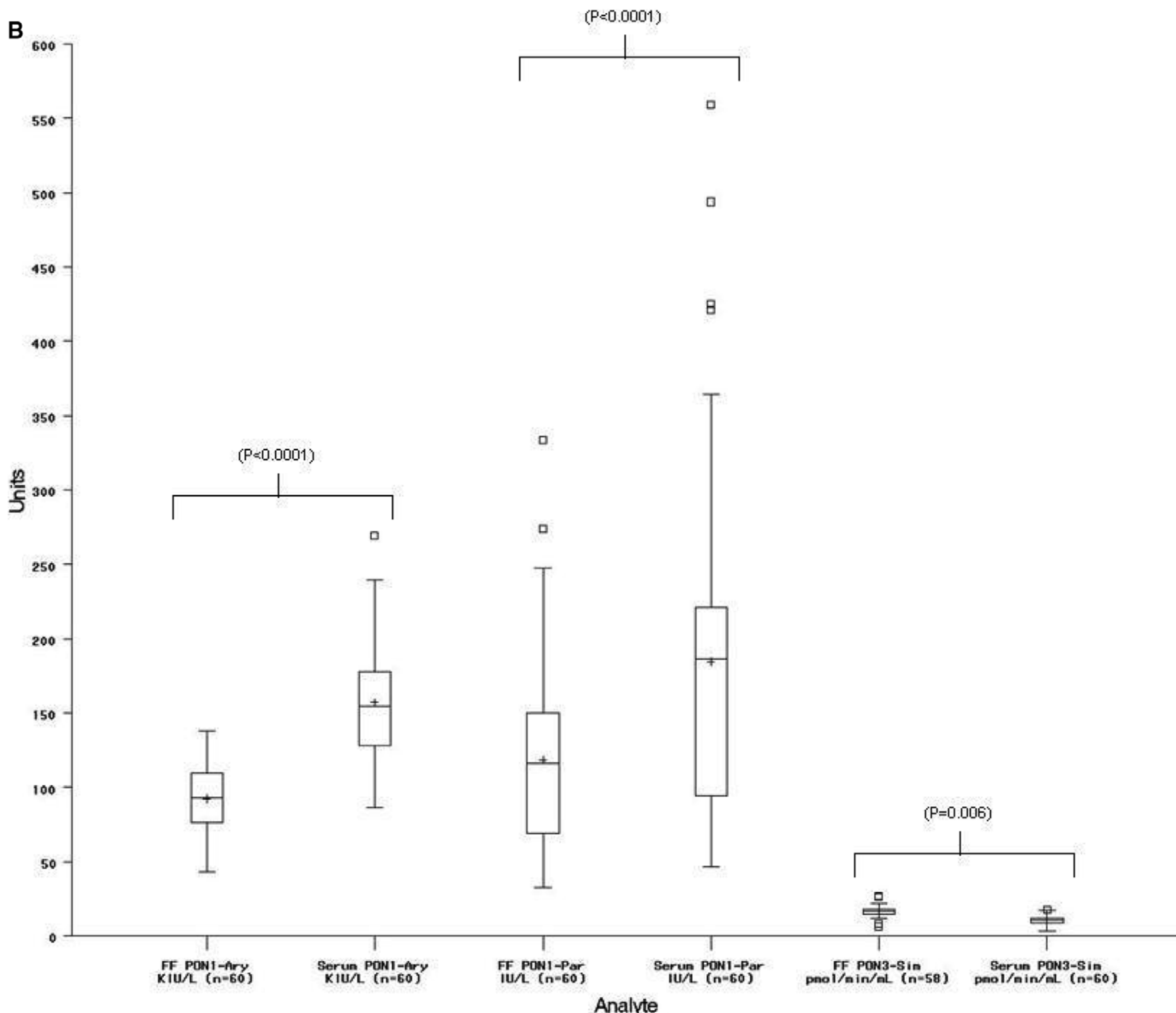

Figure 2: *Continued*

significant correlations between embryo morphology parameters and BMI or patient race/ethnicity, but a correlation of 'borderline' significance was measured for age and ECN ($r_{Sp} = -0.31$, $P = 0.088$).

Ordinal logistic regression models for embryo morphology parameters

In the ordinal logistic regression analysis shown in Table IV, both FF ApoAI (OR 0.66, 95%CI 0.46–0.96) and HDL-cholesterol

Table II. Spearman rank correlation coefficients (*P*-values) among biochemical analytes measured in 'follicular fluid' specimens.

	HDL-Ch	HDL-Pl	HDL-Tr	ApoAI	PON1-Ary	PON1-Par	PON3-Sim
HDL-Ch ($n = 60$)	1.00	**0.62 (<0.0001)**	−0.17 (0.309)	**0.47 (0.000)**	0.23 (0.074)	−0.07 (0.572)	−0.23 (0.087)
HDL-Pl ($n = 40$)	—	1.00	−0.06 (0.697)	**0.36 (0.022)**	0.28 (0.076)	−0.10 (0.550)	−0.26 (0.106)
HDL-Tr ($n = 39$)	—	—	1.00	**−0.42 (0.008)**	−0.13 (0.446)	0.15 (0.356)	−0.28 (0.094)
ApoAI ($n = 60$)	—	—	—	1.00	**0.36 (0.005)**	−0.10 (0.448)	0.14 (0.305)
PON1-Ary ($n = 60$)	—	—	—	—	1.00	**0.36 (0.005)**	−0.05 (0.702)
PON1-Par ($n = 60$)	—	—	—	—	—	1.00	−0.16 (0.222)
PON3-Sim ($n = 60$)	—	—	—	—	—	—	1.00

Correlations with $P < 0.05$ in bold typeface. ApoAI, apolipoprotein AI; HDL-Ch, high-density lipoprotein cholesterol; HDL-Pl, high-density lipoprotein phospholipids; HDL-Tr, high-density lipoprotein triglycerides; PON1-Ary, paraoxonase 1 arylesterase activity; PON1-Par, paraoxonase 1 paraoxonase activity; PON3-SIM, paraoxonase 3 simvastatinase activity.

Table III. Spearman rank correlation coefficients (P-values) between biochemical analytes and embryo quality indicators.

	EFS ($n = 30$)	ECN ($n = 31$)[a]
Follicular fluid		
HDL-Ch	**−0.59 (0.001)**	0.02 (0.896)
HDL-Pl	−0.23 (0.319)$^{n=21}$	0.14 (0.556)$^{n=21}$
HDL-Tr	0.04 (0.862)$^{n=20}$	−0.20 (0.408)$^{n=20}$
ApoAI	**−0.54 (0.002)**	0.14 (0.445)
PON1-Ary	−0.20 (0.280)	0.29 (0.122)
PON1-Par	0.15 (0.438)	0.07 (0.719)
PON3-Sim	0.20 (0.308)$^{n=29}$	−0.11 (0.585)$^{n=30}$
Serum		
HDL-Ch	**−0.53 (0.003)**	−0.11 (0.565)
HDL-Pl	−0.22 (0.336)$^{n=21}$	−0.23 (0.337)$^{n=21}$
HDL-Tr	−0.01 (0.977)$^{n=21}$	−0.12 (0.621)$^{n=21}$
ApoAI	**−0.50 (0.005)**	−0.04 (0.842)
PON1-Ary	**−0.39 (0.032)**	0.09 (0.613)
PON1-Par	0.17 (0.366)	0.04 (0.825)
PON3-Sim	−0.25 (0.192)	0.10 (0.603)

Sample sizes vary for certain analytes due to limited follicular fluid sample volume; Correlations with $P < 0.05$ in bold typeface. ApoAI, apolipoprotein AI; HDL-Ch, high-density lipoprotein cholesterol; HDL-Pl, high-density lipoprotein phospholipids; HDL-Tr, high-density lipoprotein triglycerides; PON1-Aryl, paraoxonase 1 arylesterase activity; PON1-Par, paraoxonase1 paraoxonase activity; PON3-SIM, paraoxonase 3 simvastatinase activity.
[a]Adjusted for day of embryo transfer.

(OR 0.16, 95%CI 0.03–0.97) were significant negative predictors for EFS (Table IV). Furthermore, a positive interaction term retained in this model (OR 1.02, 95%CI 1.00–1.03) suggested that the effects of FF ApoAI and HDL-cholesterol were to some extent interdependent. For example, an increase in either FF-HDL cholesterol, or in FF ApoAI, elicited a slight reduction in the ‘protective’ effect of the other on EFS. Using serum, results were similar to the FF models for EFS; however, no interaction was retained in the model, and effect estimates for ApoAI (OR 0.95, 95%CI 0.91–1.00) and HDL-cholesterol (OR 0.95, 95%CI 0.88–1.03) were substantially attenuated. In the model for ECN, limited to Day 3 embryo transfers ($n = 22$), PON1-arylesterase activity in FF was a significant positive predictor (OR 1.09, 95%CI 1.01–1.17). No ordinal logistic regression model was generated for serum measures and ECN.

Discussion

The current study was intended to generate hypotheses regarding potential clinical markers of oocyte quality, employing embryo morphology parameters, for consideration in a future investigation utilizing a larger sample size. The fundamental importance of this study is the identification of clinical markers that correlate with human embryo morphology parameters *in vitro*. Furthermore, this is the first quantification and characterization of PON1 and PON3 enzyme activities in human ovarian FF. Whereas other studies have focused on the composition of FF-HDL with respect to *de novo* steroidogenesis, HDL and its component lipids (cholesterol, phospholipids and triglycerides) and proteins (ApoAI, PON1 and PON3) have, to the best of our knowledge, never been evaluated with respect to their effects on embryo quality during IVF.

Within the scope of this preliminary work, we have noted several interesting findings with regard to HDL composition of FF and IVF embryo morphology parameters. First, HDL-cholesterol and ApoAI concentrations in both FF and serum, both indicators of HDL particle concentration, were negatively associated with Day 3 EFS. However, these effects were likely interdependent as suggested by the inclusion of a statistical interaction in the relevant ordinal logistic regression model. Second, FF-PON1-arylesterase activity was a positive predictor of Day 3 ECN. Finally, although PON3 had no correlation with embryo morphology parameters, its higher activity in FF relative to serum suggests that it may be locally generated within the follicle.

The negative association between embryo fragmentation and implantation rates with IVF has been firmly established (Giorgetti *et al*., 1995; Alikani *et al*., 1999; Ebner *et al*., 2001; Volpes *et al*., 2004; Holte *et al*., 2007). Others have described an association between embryo fragmentation and reduced blastocyst formation (Rijnders and Jansen, 1998; Hardy *et al*., 2003; Stone *et al*., 2005; della Ragione *et al*., 2007). The positive association between embryo fragmentation and programmed embryonic cell death was initially proposed by Jurisicova *et al*. (1996). Subsequent studies have confirmed this association with additional evidence for an OS-mediated etiology (Yang *et al*., 1998; Bedaiwy *et al*., 2004, 2006). Bencomo *et al*. (2006) described an association between granulosa cell apoptosis and embryo fragmentation. However, others have called into question the relationship between embryo fragmentation and cellular apoptosis (Van Blerkom *et al*., 2001, 2002; Alikani *et al*., 2005). Our preliminary findings support the hypothesis that HDL, with its component proteins including PON1, may have an important cytoprotective

Table IV. Ordinal logistic regression models for embryo quality indicators among oocytes harvested from sampled follicles.

Outcome	n	Compartment	Predictor	β (P-value)	OR	95% CI OR
Higher EFS[a]	30	FF	**ApoAI**	**−0.41 (0.032)**	**0.66**	**0.46–0.96**
			HDL-Ch	**−1.81 (0.046)**	**0.16**	**0.03–0.97**
			Interaction	0.02 (0.061)	1.02	1.00–1.03
		Serum	**ApoAI**	**−0.05 (0.045)**	**0.95**	**0.91–1.00**
			HDL-Ch	−0.05 (0.220)	0.95	0.88–1.03
Higher ECN[b]	22	FF	**PON1-Ary**	**0.08 (0.020)**	**1.09**	**1.01–1.17**

Coefficients with $P < 0.05$ in bold typeface. ApoAI, apolipoprotein AI; ECN, embryo cell number; EFS, embryo fragmentation score; FF, follicular fluid; HDL-Ch, high-density lipoprotein cholesterol; PON1-Ary, paraoxonase 1 arylesterase activity.
[a]Interaction term applies to FF ApoAI and FF-HDL-cholesterol interaction. Higher EFS is equivalent to greater embryo cytoplasmic fragmentation.
[b]Limited to embryos transferred on Day 3 following fertilization.

effect on the oocyte and its surrounding granulosa cells via mechanisms that are currently unknown.

The importance of HDL to oocyte potential is exemplified by studies in SR-BI (scavenger receptor type B class I) knockout mice (Trigatti *et al*., 1999; Miettinen *et al*., 2001; Yesilaltay *et al*., 2006). SR-BI mediates cellular uptake of HDL by binding HDL via its apolipoprotein components and regulating the intracellular movement of cholesterol. SR-BI knockout female mice were identified to be infertile as a result of embryos that uniformly arrested in early stages of development, primarily at the 1- and 2-cell stages (Trigatti *et al*., 1999). The SR-BI knockout model displays a distinctive lipoprotein abnormality characterized by abnormally-large HDL particles with a high unesterified cholesterol:total cholesterol ratio (UC:TC ratio). Restoration of fertility occurs when SR-BI$^{-/-}$ ovaries are transplanted into wild-type mice indicating that the impairment is not due to the SR-BI receptor deficient ovary, but rather the abnormal HDL particle produced by the SR-BI knockout murine liver (Miettinen *et al*., 2001). Furthermore, treatments of SR-BI$^{-/-}$ mice with probucol (to correct the UC:TC ratio) restores fertility (Miettinen *et al*., 2001), whereas partial restoration of fertility via inactivation of the ApoAI gene and reduction of total cholesterol do not normalize HDL particle size (Yesilaltay *et al*., 2006). These studies suggest the importance of HDL concentration, structure and composition in determining mammalian female reproductive potential.

Determination of the precise mechanism by which HDL may influence reproductive potential will require further studies; however, it is tempting to speculate. Potential mechanisms might include the protection against OS by the potent antioxidant composition of HDL, the regulation of steroidogenesis since HDL is the primary source of cholesterol within the follicle, or impairment of transport processes whereby HDL is unable to deliver, or remove by efflux, key compounds to/from the ovary. Alternatively, the role of HDL may be significantly related to the ovarian activity of lipolytic enzymes. Our analyses of HDL components indicate not only that the absolute levels of HDL and its components differ between FF and blood serum but also that the lipid composition of FF-HDL particles is proportionally cholesterol-poor and phospholipids-rich compared with serum-HDL particles. These results are consistent with previous findings that FF-HDL contains a higher proportion of smaller HDL_3 particles relative to larger HDL_2 particles (Le Goff, 1994; Jaspard *et al*., 1996). HDL_2s are not expected to filter through the follicular membrane and their presence in FF is thought to originate from the remodeling of smaller HDL_3 particles involving phospholipids transfer protein (PLTP) and hepatic triacylglycerol lipase. It has been shown that PLTP can convert HDL_3 particles into two populations of large (HDL_2) and small particles (pre-βHDL) (Tu *et al*., 1993) and mRNAs of PLTP have been detected in large amounts in human ovaries (Albers *et al*., 1995). Hepatic triacylglycerol lipase has been detected in ovaries and adrenal glands, with specific activity in FF (Hixenbaugh *et al*., 1989). All of these findings point to the significant remodeling of HDL in FF with a high proportion of HDL_3 particles. Furthermore, ApoAI has become increasingly important in the maintenance of a functional HDL particle with antiatherogenic properties (Wu *et al*., 2007). Thus, it is not surprising that our data would support the concept that ApoAI acts in concert with HDL-cholesterol within the HDL particle to influence embryo morphology.

In addition, we have demonstrated the presence of both PON1 and PON3 activity in human FF which, to our knowledge, has never been reported before in the literature. On the basis of the patterns of inhibition generated using several experimental conditions, it is unlikely that these activities can be attributed to enzymes other than PONs (Teiber *et al*., 2007). The data suggest an association between PON1 activity and HDL within the FF which is expected given the exclusive association of PON1 with HDL in the blood (Eckerson *et al*., 1983; Blatter Garin *et al*., 2006). This association argues for passive transfer of PON1 together with HDL through capillary mechanisms from blood into the FF compartment during folliculogenesis. Our observation that PON1-arylesterase activity was positively association with ECN, and consequently embryo cleavage, is rational given the established antioxidant properties of PON1. Thus, the associations between HDL-cholesterol, ApoAI and PON1 activity with embryo morphologic parameters suggest that the overall HDL status of the follicle is important to oocyte health.

Unlike PON1, PON3 enzymatic activity is higher in FF relative to serum and we did not observe an association between PON3 and HDL-cholesterol or ApoAI. Thus, PON3 activity appears to be independent from HDL, possibly existing in FF in a non-HDL-associated state. In mammals, PON1 is synthesized in the liver and carried exclusively in the circulation bound to HDL (Eckerson *et al*., 1983). In contrast, PON3 appears to be produced by multiple tissues, including liver, human gastrointestinal tract, mouse airway epithelium, macrophages, kidney, lung, brain and adipose tissues (Shih *et al*., 2007). Our observations suggest that the increased PON3 enzyme activity in FF may be due to *de novo* production in the human follicle with possible origination from granulosa cells.

An acknowledged limitation of this study is the small sample size. However, the number of associations found, despite this limitation, point to a potentially important role for HDL, ApoAI and PON1 activities in the overall health of the developing follicle and its resulting oocyte and embryo. Another limitation of this study involves the potential influence of sperm on early embryo development. While current literature suggests an important role for the oocyte in the etiology of early embryo cytoplasmic fragmentation, based on oocyte morphology and shared sibling oocyte studies (Serhal *et al*., 1997; Xia, 1997; Loutradis *et al*., 1999; Ebner *et al*., 2000; Salumets *et al*., 2002), a role for sperm in early embryo development has also been described (Tesarik *et al*., 2002, 2004; Tomsu *et al*., 2002; Seli and Sakkas, 2005). Tomsu *et al*. (2002) described a correlation between sperm mean head density and embryo quality that included both blastomere symmetry and degree of fragmentation in an IVF patient population. Tesarik *et al*. (2002) demonstrated consistent zygote morphology in embryos generated from the same sperm source despite different oocyte sources. However, using shared sibling oocytes,

Tesarik *et al*. (2004) found no effect of sperm DNA fragmentation using the TUNEL assay on early zygote morphology. Neither of the Tesarik *et al*. studies used embryo fragmentation during the cleavage stage as an outcome variable. Salumets *et al*. (2002) utilized a shared sibling oocyte model to demonstrate that embryo fragmentation using standard morphologic criteria similar to ours was strongly correlated with the oocyte source in 59 ovum donation cycles. This study effectively articulated the effect of intrinsic oocyte quality on embryo fragmentation in the cleavage stage embryo. This apparent dependence of early embryo morphologic observations at least in part on oocyte integrity provided the basis for using embryo morphology parameters as outcomes of interest in our study.

In summary, HDL metabolism within the follicle is undoubtedly complex, influenced by HDL composition and size, follicular vascularity, varying degrees of follicular HDL particle remodeling and variable utilization of HDL and its components by follicular cells. Within the framework of this complexity, our study points to HDL playing a potential role in the health of the human oocyte with embryo fragmentation and embryo cell number serving as surrogate markers of oocyte health. Our pilot study establishes the possibility that HDL and/or its component proteins have the potential to predict embryo fragmentation and cell number in women undergoing IVF. If validated in embryo cohort data employing a larger sample size, these biomarkers may prove to be clinically relevant for predicting patients more likely to have embryo fragmentation and cleavage problems. Except for the PON3 simvastatinase activity, the assays employed here have been adapted from commercially available reagent kits. The lipid and lipoprotein analyses are routinely available in most clinical laboratories and could be rapidly analyzed to provide timely information prior to or during the conduct of IVF treatment.

Further efforts are currently underway to examine the variability of HDL and its component proteins and lipids within FF together with the local expression of PON proteins within the follicle. We are also currently investigating other antioxidant enzymes, products of lipid peroxidation and antioxidant micronutrients to provide a comprehensive evaluation of the role of OS in the human ovarian follicle.

Beyond reproduction, the study of FF-HDL composition may have implications in the pathogenesis of disease states such as cardiovascular disease and diabetes. There is growing evidence that modification of HDL structure and composition has deleterious effects on its antioxidant and anti-inflammatory capacity rendering it actually pro-oxidant, pro-inflammatory and atherogenic (Navab, 2006; Ferretti *et al*., 2006). Identification of the specific compositional alterations responsible for these effects is difficult in systems containing LDL due to its intricate interactions with HDL. Efforts to isolate HDL by ultracentrifugation methods tend to distort its structure and composition (Kunitake and Kane, 1982; Cabana *et al*., 2003). The unique nature of FF possessing only HDL may make it an ideal matrix to study the effects of specific HDL alterations on its antioxidant and anti-inflammatory activities.

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