J Assist Reprod Genet
DOI 10.1007/s10815-016-0826-x

ASSISTED REPRODUCTION TECHNOLOGIES

Associations between follicular fluid high density lipoprotein particle components and embryo quality among in vitro fertilization patients

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Received: 1 July 2016 / Accepted: 2 October 2016

Abstract
Purpose Follicular redox balance is likely to be important for embryo quality during in vitro fertilization (IVF), and the anti-oxidative high desity lipoprotein (HDL) particle is the sole lipoprotein measured in follicular fluid (FF). Therefore, we investigated FF HDL particle components as predictors of embryo quality during IVF.
Methods Two research follicles collected from each participant were individually tracked, and 103 women having at least one developed embryo were included in the analysis. Concentrations of 15 non-cholesterol HDL particle components and 26 HDL-cholesterol (HDL-C) particle size subfractions were determined. Embryo quality was assessed for embryo cell number, embryo fragmentation, and embryo symmetry. Multivariable Poisson regression with a sandwich variance estimator was used to evaluate associations between HDL particle components and embryo quality, adjusted for covariates.
Results Higher γ-tocopherol concentration was associated with less embryo fragmentation (relative risk [RR] = 4.43; 95 % confidence interval [CI] 1.78, 11.06), and higher apolipoprotein A-1 concentration was associated with full embryo symmetry (RR = 3.92; 95 % CI 1.56, 9.90). Higher concentrations of HDL-C subfractions in the large and medium particle size ranges were associated with poorer embryo quality.
Conclusions FF HDL lipophilic micronutrients and protein components, as well as HDL-C particle size, may be important predictors of embryo quality during IVF.

Capsule FF HDL lipophilic micronutrients and protein components, as well as HDL-C particle size, may be important predictors of embryo quality during IVF.

Electronic supplementary material The online version of this article (doi:10.1007/s10815-016-0826-x) contains supplementary material, which is available to authorized users.

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Keywords Embryo cell number (ECN) · Embryo fragmentation score (EFS) · Embryo symmetry score (ESS) · Follicular fluid (FF) · High density lipoprotein (HDL) · *in vitro* fertilization (IVF)

Introduction

High density lipoprotein (HDL) transports cholesterol to the ovarian follicle, and it is the main source of cholesterol substrate for steroidogenesis in the pre-ovulatory period [1, 2]. HDL is the smallest and densest class of lipoproteins. Due to the selectivity of the follicular membrane, which permits passage only of proteins up to 300,000 Daltons, HDL is the sole lipoprotein measured in follicular fluid (FF) [3]. This selectivity excludes larger lipoprotein classes, including large HDL_2 subclass particles, low density lipoprotein (LDL), and very low density lipoprotein (VLDL) [3, 4]. Because of its anti-inflammatory and antioxidant properties [5], and presence in the FF that directly bathes a growing oocyte [6], an important

Published online: 29 November 2016

role has been suggested for HDL in the development of oocyte competence and for embryo quality [7].

HDL has anti-inflammatory and antioxidant activities [5, 8] and is generally considered as beneficial to vascular [9–11] and increasingly to reproductive health [7]. The antioxidant activity of HDL is determined in part by the lipid composition [12], the presence of apolipoprotein A-1 (ApoA-1), the activity of paraoxonases (PONs) [13, 14], and levels of lipophilic micronutrients [15]. However, HDL particle size is also an important determinant of antioxidant activity, as evidenced by more potent capabilities among smaller compared to larger size particles [16]. Structural modifications, associated with its critical role in reverse cholesterol transport [7, 17, 18], alter the antioxidant activity of HDL secondary to changes in particle size, ApoA-1 concentration or conformation [19], and PON activities [20]. Thus, the structure, size, and antioxidant activity of HDL are dynamic. We previously conducted a pilot study of HDL particle components, including lipids, ApoA-1, and paraoxonase 1 (PON1) activities measured in FF and human serum, and their associations with embryo quality on 60 women undergoing in vitro fertilization (IVF) [21]. In that study, we identified FF ApoA-1 and HDL-cholesterol (HDL-C) as predictors of embryo fragmentation, suggesting protective associations for embryo quality. In addition, FF arylesterase activity was associated with higher embryo cell number. In an expansion of that pilot study to 87 IVF patients, we detected better embryo quality in association with higher FF γ-tocopherol and β-cryptoxanthin [22]. Here, we further explored the association between HDL and embryo quality with an investigation of women undergoing IVF in a larger study sample. We measured 15 non-cholesterol HDL components in FF in addition to 26 HDL particle size subfractions and examined their associations with cleavage-stage embryo quality during IVF.

Materials and methods

Sample selection

Participant selection and recruitment was reported in detail previously [23]. Briefly, a convenience sample of 180 women undergoing IVF treatment with fresh, non-donor oocytes was enrolled at the University of California at San Francisco (UCSF), between April 10, 2010 and June 28, 2011. Through a standard IVF intake survey, comprehensive infertility and medical history data with self-reported race and cigarette smoking status were collected. There were 180 study participants each contributing two follicles for research purposes (i.e., "research follicles"); however, the current analysis includes only 103 women for whom at least one research follicle-based embryo developed. This study was conducted in accordance with the ethical standards stipulated in the 1964 Declaration of Helsinki. Informed consent was obtained prior to participation and the study protocol was approved by the UCSF Committee on Human Research.

Clinical protocol and specimen collection

Clinical and biospecimen collection protocols were described in detail elsewhere [23]. In brief, study participants underwent gonadotropin-induced controlled ovarian stimulation (COS) according to standard clinical protocols. When a sufficient number of follicles had developed to >17 mm in diameter, human chorionic gonadotropin was administered subcutaneously to precipitate ovulation. Approximately 36 h later, oocytes and FF were collected by transvaginal fine needle aspiration. Two research follicles collected from each woman, the first and largest, were not flushed in order to avoid dilution of FF analyte concentrations. Each follicle was individually evacuated into an empty 10-mL tube, the oocyte recovered for IVF, and the 3.5–5.0 mL of FF centrifuged at 1800 rpm for 10 min to pellet the residual granulosa cells. The recovered oocytes from research follicles were individually fertilized according to the usual clinical protocol and individually tracked. The supernatant FF was split into aliquots (0.6 mL) and frozen at −80 °C until the analysis. The remaining follicles were aspirated according to usual clinical procedures. No samples had evidence of trace red blood cell contamination upon visual inspection before or after centrifugation [24]. Two aliquots from each follicle were shipped to the University of Buffalo in New York State on dry ice via overnight service for biochemical analysis. A separate aliquot was shipped to LipoScience, Inc. (Chicago, IL, USA) for HDL particle size quantification.

Oocytes were fertilized by intracytoplasmic sperm injection (ICSI) or conventional IVF using fresh or frozen sperm from the male partner. The embryologist isolated oocytes from all individual "research follicles" into individual collection wells following retrieval, cultured each separately in Global media and 10 % LGPS (LifeGlobal Group, LLC, Guilford, CT, USA), then tracked until transfer. Approximately 16–18 h after insemination, fertilization was confirmed by identifying zygotes with the appearance of two pronuclei. A single embryologist who was blinded to all biochemical results assessed cleavage-stage embryo quality as: (1) embryo cell number (ECN), which is a positive predictor for IVF success and characterizes cleavage (i.e., growth) rate by the number of blastomeres counted on the day of embryo transfer [25]; (2) embryo fragmentation score (EFS), which is an inverse predictor for IVF success [26] assessed as the proportion of membrane blebs 48 h after fertilization (Grade 1, 0 % fragmentation; Grade 2, 1–10 % fragmentation; Grade 3, 11–25 % fragmentation; Grade 4, 26–50 % fragmentation; Grade 5, 51 % or greater fragmentation); and (3) embryo symmetry score (ESS), which is an positive predictor for IVF success in that greater symmetry is associated with better outcomes [27],

defined as (1) fully symmetric blastomeres in the cleaved embryo, (2) slightly asymmetric blastomeres in the cleaved embryo, and (3) highly asymmetric blastomeres in the cleaved embryo. Standardization of fragmentation and symmetry scoring has been established within our IVF laboratory. Better embryo quality was defined as ECN ≥ 6, EFS ≤ grade 2, and ESS = 1 (full symmetry), while poorer embryo quality was defined as ECN < 6, EFS > grade 2, and ESS > 1.

Analytic methods

Details of the biochemical analysis of FF HDL particle components were described elsewhere [23]. Briefly, whole FF was used to analyze ApoA-1, apolipoprotein A-2 (ApoA-2), and PON1 activities. Levels of ApoA-1 and ApoA-2 were analyzed by immunoturbidimetric methods using diagnostic kits from Kamiya Biomedical (Seattle, WA, USA) on the Cobas Fara II automated chemistry analyzer (Hoffmann-La Roche, Basel, Switzerland). Arylesterase and paraoxonase activities, which characterize PON1 activity, were also analyzed using the Cobas Fara II automated analyzer as described in detail elsewhere [28]. FF HDL fractions were prepared by selective precipitation to remove any trace amounts of apolipoprotein B containing LDLs or VLDLs [21]. The lipids comprising FF HDL particles, including phospholipids and triglycerides, were measured using diagnostic reagent kits from Sekisui Diagnostics (Lexington, MA, USA) adapted to the Cobas Fara II [23]. HDL-associated lipophilic micronutrients including retinol, α-, γ-, and δ-tocopherols, β-carotene, β-cryptoxanthin, lycopene, and lutein/zeaxanthin simultaneously using high-performance liquid chromatography (HPLC) were also measured [29]. Reported elsewhere, the interassay coefficients of variation (CVs) for FF HDL particle components ranged from a minimum of 0.6 % for arylesterase activity to a maximum of 7.2 % for β-cryptoxanthin [23].

FF HDL particle subfractions were quantified by particle size using proton nuclear magnetic resonance spectrometry (^{1}H NMR) at LipoScience, Inc. (Chicago, IL, USA) [30]. By detecting NMR signals unique to specific HDL particle sizes, these measurements generated concentrations of 26 FF HDL-C particle subfractions ranging from 7.4 to 13.5 nm diameter.

Statistical analysis

Distributions for demographic and clinical factors of 103 women who had at least one embryo developed from two "research follicles" were characterized. We linked each FF specimen to its embryo, following a "one-follicle, one-embryo" design. HDL particle components were natural log transformed to stabilize variances, and their distributions described by geometric mean and standard deviation. For multivariable analysis, modified Poisson regression models were used with FF HDL particle components as predictors and dichotomized embryo quality indicators as outcomes, defined as (1) ECN ≥ 6 or ECN < 6; (2) EFS = Grade 1–2 as "less fragmentation" and EFS ≥ Grade 3 as "more fragmentation"; and (3) ESS = 1 as "full symmetry" and ESS > 1 as "poor symmetry." Therefore, ECN ≥ 6, less fragmentation, and full symmetry are defined as positive predictors of IVF in our study. Modified Poisson regression, which employs a sandwich estimator of the variance using generalized estimating equation (GEE) [31], providing robust standard errors to accommodate clustered embryo outcomes within woman, was used [32, 33]. Confounders were identified a priori, as likely predictors of both embryo quality and FF HDL particle components, based on the literature. All models were adjusted for confounding by age (years) [34], body mass index (BMI, kg/m^2) [35, 36], race (non-Asian vs. Asian) [37, 38], and cigarette smoking (never vs. ever) [39]; age and BMI were included as continuous variables, whereas race and cigarette smoking were included as categorical variables. We restricted the analysis for EFS (n = 93) and ESS (n = 92) to women with complete case data. For ECN (n = 57), we restricted the analysis to women with complete case data and day 3 embryo transfers, to accommodate the correlation between 8-cell stage embryos and day 3 transfer.

A forward stepwise selection procedure was employed to select FF HDL particle components as predictors of embryo quality indicators in modified Poisson regression models. This approach was identified as the most appropriate compared to several other strategies commonly used to reduce large numbers of variables to a more manageable size. FF HDL particle components were first screened by regressing each on embryo quality indicators in separate modified Poisson regression models and retained only those variables with $P < 0.10$. Retained HDL particle components were subsequently entered into multivariable modified Poisson regression models predicting embryo quality in a forward stepwise fashion, adjusted for confounders. During forward stepwise selection, HDL particle components were retained or removed based on an approximation to the quasi-likelihood information criterion (QICu), which penalized additional variables added to the model in assessing model fit [40]; a smaller QICu corresponds to better fit. Interactions between HDL particle components selected as final predictors of embryo quality indicators were assessed and product terms were retained if $P < 0.10$ for the Wald test. Exponentiated regression coefficients and their 95 % confidence intervals (CIs) were obtained to provide estimates of the underlying population relative risk (RR). Statistical significance was defined as $P < 0.05$ for main effects and $P < 0.10$ for product terms using two-tailed tests and during the variable screening steps prior to the forward stepwise variable selection. Based on the exploratory nature of our work, we did not adjust the significance level to accommodate type 1 error inflation due to multiple statistical tests [41]. SAS v.9.3 (SAS Institute, Inc. Cary, NC, USA) was used for all statistical analysis.

Results

The median age and BMI of 103 women were 39 years (range 27–45) and 23.3 kg/m^2 (17.6–50.9), respectively (Table 1). Most participants were non-Asian ($n = 75$ white; $n = 2$ black; $n = 23$ Asian; $n = 3$ missing) and had never smoked cigarettes (89.9 %). The most frequent primary infertility diagnosis was "unexplained" (34.0 %), followed by "male factor" infertility (29.1 %). Most women underwent a Lupron down regulation COS protocol (LDR; $n = 54$ long luteal; $n = 13$ demi-halt). Twelve oocytes were collected on average (median) from study participants (range 1–44), with 82 % in MII-arrest (ICSI only) and 80 % normally fertilized overall (means). A median of six (range = 1–25) embryos were produced and 60 % of these were transferred on day 3.

Distributions for 15 non-cholesterol HDL particle components and 26 measured HDL-C size subfractions are shown in Table 2. The concentration of phospholipids (geometric mean ± geometric standard deviation, 71.07 ± 1.27 mg/dL) was approximately 9.2 times higher than for triglycerides (7.73 ± 1.73 mg/dL). The concentration of ApoA-1 (88.54 ± 1.29 mg/dL) was approximately 3.4 times higher than for ApoA-2 (25.95 ± 1.29 mg/dL). The concentration of α-tocopherol (3.51 ± 1.27 μg/mL) was highest among the nine HDL-

Table 1 Distribution of demographic and clinical factors for in vitro fertilization (IVF) patients

Variables	*n*	Mean (%)	SD	Min.	25th %tile	Median	75th %tile	Max.
Patients								
Age (years)	103	37.7	4.2	27	34	39	41	45
Body mass index (kg/m^2)	103	23.9	4.5	17.6	21.0	23.3	25.5	50.9
Asian[a]	23	(23.0)	–	–	–	–	–	–
Ever cigarette smoker[b]	10	(10.1)	–	–	–	–	–	–
Intracytoplasmic sperm injection	72	(69.9)	–	–	–	–	–	–
Primary infertility diagnosis								
Male factor	30	(29.1)	–	–	–	–	–	–
Unexplained[c]	35	(34.0)	–	–	–	–	–	–
Female factor: non-DOR[d]	23	(22.3)	–	–	–	–	–	–
Female factor: DOR	13	(12.6)	–	–	–	–	–	–
PGD-only	2	(1.9)	–	–	–	–	–	–
COS protocol								
Lupron down-regulated[e]	67	(65.1)	–	–	–	–	–	–
Antagonist[f]	29	(28.2)	–	–	–	–	–	–
Flare[g]	7	(6.8)	–	–	–	–	–	–
IVF endpoints								
Total oocytes collected	103	12.7	7.1	1	8	12	16	44
Proportion mature oocytes[h]	72	0.82	0.16	0.29	0.75	0.86	0.95	1
Proportion fertilized oocytes[i]	103	0.80	0.17	0.17	0.71	0.81	1.00	1
Total embryos produced	103	6.8	4.3	1	4	6	9	25
Day 3 embryo transfer	62	(60.2)	–	–	–	–	–	–

COS controlled ovarian stimulation, *DOR* diminished ovarian reserve, *Max*. maximum value, *Min*. minimum value, *PGD* preimplantation genetic diagnosis, *SD* standard deviation

[a] $n = 3$ missing values

[b] $n = 4$ missing values

[c] Includes $n = 1$ recurrent pregnancy loss

[d] $n = 8$ endometriosis, $n = 9$ tubal factor, $n = 5$ polycystic ovarian syndrome, and $n = 1$ anovulation

[e] $n = 54$ long luteal and $n = 13$ demi-halt

[f] $n = 16$ E_2 priming antagonist and $n = 13$ oral contraceptive pill antagonist

[g] $n = 1$ Clomid flare and $n = 6$ microdose flare

[h] Defined as the average proportion of oocytes recovered in metaphase-II arrest (intracytoplasmic sperm injection cases only)

[i] Defined as the average proportion of fertilized oocytes with the presence of two pronuclei and two Barr-bodies

Table 2 Distribution of high density lipoprotein (HDL) particle components measured in follicular fluid (FF) from in vitro fertilization (IVF) patients

HDL particle components	*n*	Geometric mean	Geometric SD
HDL particle components			
Phospholipids (mg/dL)	101	71.07	1.27
Triglycerides (mg/dL)	101	7.73	1.73
Arylesterase activity (kIU/L)	101	96.71	1.42
Paraoxonase activity (IU/L)	101	74.61	1.71
ApoA-1 (mg/dL)	101	88.54	1.29
ApoA-2 (mg/dL)[a]	94	25.95	1.29
δ-tocopherol (μg/mL)	102	1.04	1.07
γ-tocopherol (μg/mL)	102	1.18	1.10
α-tocopherol (μg/mL)	102	3.51	1.27
Retinol (μg/mL)	102	1.34	1.06
Lutein (μg/mL)	102	1.07	1.03
β-cryptoxanthin (μg/mL)	102	1.04	1.04
α-carotene (μg/mL)	102	1.01	1.01
β-carotene (μg/mL)	102	1.06	1.04
Lycopene (μg/mL)	102	1.06	1.02
HDL-C subfractions measured for specific particle size (μmol/L)			
LPHC_1 (13.5 nm)	102	1.02	1.03
LPHC_2 (13.0 nm)	102	1.08	1.08
LPHC_3 (12.5 nm)	102	1.04	1.06
LPHC_4 (12.0 nm)	102	1.21	1.27
LPHC_5 (11.5 nm)	102	1.07	1.14
LPHC_6 (11.0 nm)	102	1.25	1.27
LPHC_7 (10.8 nm)	102	1.34	1.40
LPHC_8 (10.6 nm)	102	1.35	1.44
LPHC_9 (10.5 nm)	102	1.76	1.60
LPHC_10 (10.0 nm)	102	2.52	1.87
LPHC_11 (9.7 nm)	102	3.47	1.70
LPHC_12 (9.4 nm)	102	2.66	1.97
LPHC_13 (9.2 nm)	102	2.81	1.58
LPHC_14 (9.0 nm)	102	2.93	1.72
LPHC_15 (8.6 nm)	102	1.21	1.18
LPHC_16 (8.5 nm)	102	2.49	1.52
LPHC_17 (8.4 nm)	102	1.35	1.35
LPHC_18 (8.3 nm)	102	1.14	1.25
LPHC_19 (8.2 nm)	102	1.22	1.35
LPHC_20 (8.1 nm)	102	1.51	1.59
LPHC_21 (8.0 nm)	102	2.06	1.49
LPHC_22 (7.9 nm)	102	2.06	1.85
LPHC_23 (7.8 nm)	102	2.07	1.62
LPHC_24 (7.6 nm)	102	6.50	1.57
LPHC_25 (7.5 nm)	102	5.48	2.14
LPHC_26 (7.4 nm)	102	2.47	1.69

ApoA-1 apolipoprotein A-1, *ApoA-2* apolipoprotein A-2, *HDL-C* high density lipoprotein-cholesterol, *SD* standard deviation

[a] Missing $n = 7$ participants with insufficient sample volume available to measure ApoA-2

associated lipophilic micronutrients measured. LPHC_24 (particle size, 7.6 nm; 6.50 ± 1.57 μmol/L) was the highest concentration measured among 26 HDL-C size subfractions, whereas the concentration was the lowest for LPHC_1 (13.5 nm; 1.02 ± 1.03 μmol/L). As illustrated in Fig. 1, geometric means > 2 μmol/L were clustered among those HDL-C size subfractions with medium and small diameters.

The results of multivariable modified Poisson regression analysis using embryo quality indicators as outcomes are presented in Table 3. For dichotomized ECN, limited to day 3 embryo transfers, six components were identified with $P < 0.10$ for bivariate associations and were entered into a forward stepwise variable selection procedure. As a result, arylesterase activity, α-tocopherol, retinol, LPHC_13 (9.2 nm), and LPHC_18 (8.3 nm) were determined as predictors of ECN. There was also evidence for an interaction between arylesterase activity and LPHC_13 (9.2 nm); however, the effect estimates were imprecise and so a product term was not included in the final modified Poisson regression model (Supplemental Table 1). Presented in Table 3, LPHC_18 (8.3 nm) was identified as a negative predictor of ECN (RR = 0.40; 95 % CI 0.16, 1.01), with marginal statistical significance.

For dichotomized EFS, four FF HDL particle components had $P < 0.10$ for bivariate associations, all of which were retained as predictors in modified Poisson regression models following entry into the forward stepwise variable selection procedure (Table 3). The final model included γ-tocopherol, LPHC_3 (12.5 nm), LPHC_9 (10.5 nm), and LPHC_17 (8.4 nm) as predictors of EFS. A positive association was detected, in which higher γ-tocopherol was associated with lower embryo fragmentation (RR = 4.43; 95 % CI 1.78, 11.06), adjusted for LPHC_3, LPHC_9, LPHC_17, age, BMI, race, and cigarette smoking. In contrast, higher LPHC_17 (8.4 nm) was associated with higher embryo fragmentation (RR = 0.69; 95 % CI 0.47, 1.01), adjusted for the covariates.

For dichotomized ESS, six FF HDL particle components had $P < 0.10$ for bivariate associations. Following entry of these six HDL particle components into a forward stepwise variable selection procedure, ApoA-1, LPHC_3 (12.5 nm), and LPHC_13 (9.2 nm) were identified as predictors of ESS in a modified Poisson regression model. Higher ApoA-1 was significantly associated with a higher likelihood for full embryo symmetry (RR = 3.92; 95 % CI 1.56, 9.90), adjusted for LPHC_3, LPHC_13, age, BMI, race, and cigarette smoking (Table 3). In contrast, higher LPHC_3 (12.5 nm; RR = 0.02; 95 % CI 2.89×10^{-4}, 0.82) and higher LPHC_13 (9.2 nm; RR = 0.56; 95 % CI 0.37, 0.86) were significantly associated with a lower likelihood for full embryo symmetry, adjusted for the covariates.

In sensitivity analyses, we excluded $n = 2$ preimplantation genetic diagnosis (PGD) patients, and then $n = 5$ polycystic ovary syndrome (PCOS) patients, repeated the models, and

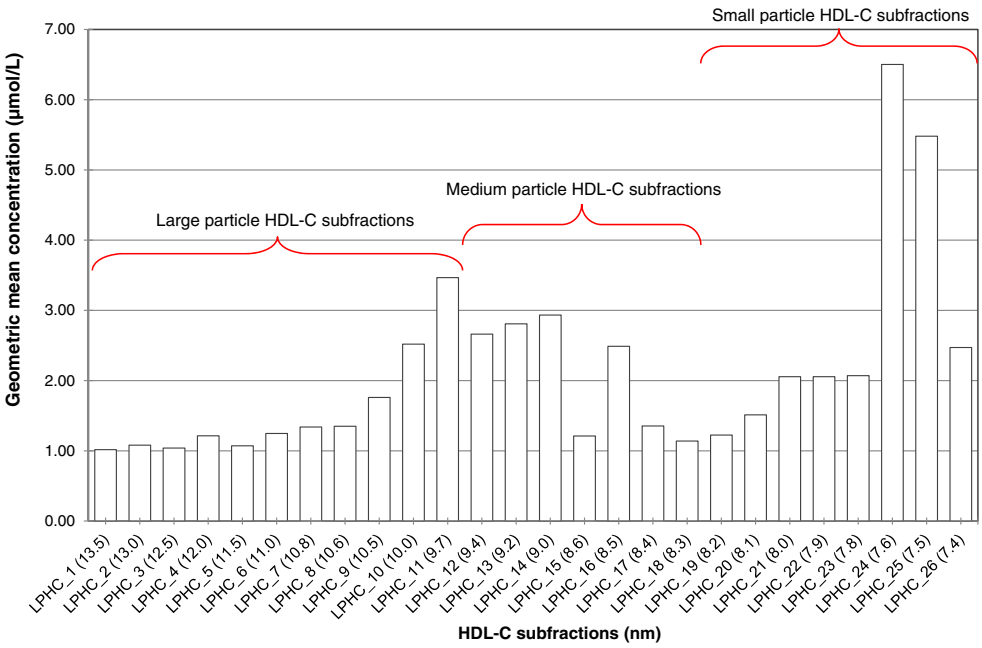

Fig. 1 Distribution of high density lipoprotein-cholesterol (HDL-C) subfractions measured by proton nuclear magnetic resonance (^{1}H NMR)

obtained similar results (data not shown). In addition, results were similar when also adjusting for potential confounding by the administered dose of gonadotropins, endometrium thickness, and total oocytes retrieved as a marker for ovarian responsiveness (data not shown).

Discussion

Using 41 FF HDL parameters measured in women undergoing IVF, we investigated associations with cleavage-stage embryo quality indicators during IVF. In our study, associations

Table 3 Modified Poisson regressions for dichotomized embryo quality indicators, using follicular fluid (FF) high density lipoprotein (HDL) particle components

FF HDL particle components (particle diameter)	*n* women (j embryo)		95 % CI		
		RR	Low	High	*P* value
ECN[a]					
Arylesterase activity	57 (77)	1.17	0.82	1.68	0.386
α-Tocopherol	–	1.21	0.53	2.78	0.654
Retinol	–	1.20	0.08	18.42	0.897
LPHC_13 (9.2 nm)	–	1.17	0.96	1.43	0.122
LPHC_18 (8.3 nm)	–	0.40	0.16	1.01	0.052
EFS					
γ-Tocopherol	93 (123)	4.43	1.78	11.06	0.001
LPHC_3 (12.5 nm)	–	0.12	0.01	1.11	0.062
LPHC_9 (10.5 nm)	–	0.93	0.75	1.14	0.469
LPHC_17 (8.4 nm)	–	0.69	0.47	1.01	0.054
ESS					
ApoA-1	92 (123)	3.92	1.56	9.90	0.004
LPHC_3 (12.5 nm)	–	0.02	2.89×10^{-4}	0.82	0.040
LPHC_13 (9.2 nm)	–	0.56	0.37	0.86	0.007

All models predicted "better" embryo quality as the outcome, adjusted for age, BMI, race, and cigarette smoking, and generalized estimating equations were used to generate robust standard errors. Only women with at least one research follicle-generated embryo were included in the analysis

ApoA-1 apolipoprotein A-1, *BMI* body mass index, *CI* confidence interval, *ECN* embryo cell number, *EFS* embryo fragmentation score, *ESS* embryo symmetry score, *RR* relative risk

[a] Limited to day 3 embryo transfers

were detected between higher FF γ-tocopherol and less embryo fragmentation and between higher ApoA-1 and full embryo symmetry. In contrast, associations were identified for higher levels of FF HDL-C subfractions in the large and medium particle size ranges with poorer embryo quality. These results suggest that antioxidant FF HDL particle components may impact embryo quality during IVF.

The presence of both oxidative stress markers [42] and antioxidant molecules and enzymes, especially lipophilic micronutrients [43, 44], has been reported in developing human ovarian follicles, implying a dynamic biochemical environment. In our recent pilot work, we measured lipophilic micronutrients in FF and demonstrated their associations with embryo quality indicators [22], supporting a potential antioxidant role in the microenvironment of developing oocytes during IVF. In our pilot study, γ-tocopherol was associated with a 66 % decrease in the cumulative odds (odds ratio (OR) = 0.34; $P = 0.035$) for a higher EFS (i.e., more fragmentation), whereas no such association was detected for ECN [22]. Among eight FF HDL-associated lipophilic micronutrients measured in our current study, higher γ-tocopherol was associated with less embryo fragmentation, consistent with our previous result. Higher α-tocopherol was also correlated to higher ECN in the current study, but the result was not statistically significant. Both α- and γ-tocopherols have antioxidant properties but with different chemical properties; human blood levels of α-tocopherol are generally higher than γ-tocopherol [45, 46]. α-Tocopherol is a potent inhibitor of oxygen-induced lipid peroxidation [47], whereas γ-tocopherol is more effective against nitric oxide-initiated peroxidative damage [48]. Although both α- and γ-tocopherols possess antioxidant properties, γ-tocopherol has demonstrated greater capacity in animal studies [49, 50], also reflected in our study in which we detected a strong association between higher FF γ-tocopherol and better embryo quality.

Humans do not synthesize lipophilic micronutrients in body, and so they must be obtained exclusively via diet. In the USA, γ-tocopherol was identified as the major form of vitamin E, mostly in association with the high consumption of soybean (76.4 %), corn, and canola oils (both 7 %) in the typical American diet [51]. Oil seeds, nuts, and cereal grains were recognized as rich sources of γ-tocopherol [52], whereas wheat-germ and sunflower oils, sunflower seeds, and almonds were rich in α-tocopherol [51]. A prior report also suggested that α-tocopherol and γ-tocopherol are differentially metabolized and excreted [53]. It is tempting to speculate that the HDL-associated lipophilic micronutrients measured in FF reflected dietary intake. Unfortunately, we did not obtain dietary information from our study participants to further investigate the dietary sources of lipophilic micronutrients measured in FF. However, given the strong association between tocopherols and embryo quality in our study, further study of associations between diet, vitamins, supplements, and FF HDL-associated lipophilic micronutrients among IVF populations is merited.

We also identified a positive association between ApoA-1 and full symmetry of the embryo, whereas no such association was detected for ECN and EFS. We previously reported an inverse association between ApoA-1 and increasing EFS (more fragmentation; OR = 0.66; 95 % CI 0.46, 0.96) [21]; both our previous and current results suggest the importance of ApoA-1 for embryo quality during IVF. ApoA-1 is a key protein component of HDL and has antioxidant properties [54], evidenced by resistance to oxidation demonstrated in both humans and animals [55]. ApoA-1 plays a crucial role in HDL remodeling by facilitating the transfer of cholesterol and phospholipids from peripheral tissues to nascent discoidal HDL and activating lecithin-cholesteryl acyl transferase (LCAT), an enzyme essential for cholesterol esterification and ultimately maturation of the HDL particle [54, 56]. Given its major role in HDL structure remodeling and antioxidant properties [54, 56], it is not surprising that we detected an association between ApoA-1 and better embryo quality, assessed by full symmetry.

The antioxidant activity of HDL is partially determined by particle size, as indicated by more potent and protective antioxidant activity in the smaller and denser HDL_3 subclass [16, 57–59]. Therefore, we measured HDL-C for 26 subfractions by particle size using ^{1}H NMR and investigated their associations with embryo quality indicators. We did not detect associations for small HDL-C particle size subfractions, which are known as potent antioxidants, and better embryo quality. However, we identified associations in which higher concentrations of the large particle size HDL-C subfraction (LPHC_3; particle size 12.5 nm) were associated with lower risks for less fragmentation and full embryo symmetry. In addition, medium range sized HDL-C subfractions (i.e., LPHC_13, LPHC_17, and LPHC_18) were associated with poorer embryo quality. Yet, only small HDL particles pass through the follicle basement membrane [3, 4] and these are more potent antioxidants than larger HDL particles [7]. This unexpected result may reflect a negative effect for larger HDL particle components formed by dynamic HDL remodeling processes within the follicle. Alternately, this result might be indicative of a compromised blood-follicle barrier, possibly associated with poorer embryo quality in some women undergoing IVF.

In our previous study, total FF HDL-C was associated with EFS interacting with ApoA-1; concurrent one unit increases in HDL-C and ApoA-1 were associated with lower odds of higher EFS (OR = 0.11; P value for the interaction = 0.061), adjusted for age, BMI, and race [21]. For example, higher HDL-C and ApoA-1 were associated with better embryo quality. Employing our current data for that same model, we also detected a lower odds of higher EFS in association with one unit rises in total FF HDL-C and ApoA-1 (OR = 0.87; P value

for the interaction = 0.031). However, using a more nuanced approach, higher levels of large and medium particle size HDL-C subfractions were associated with poorer embryo quality. Our overall results illustrate the importance of total FF HDL-C for embryo development. Yet, the inverse associations between large and medium FF HDL-C subfractions and better embryo quality illustrate an important role for HDL particle maturation within the follicle, which may have important consequences for reproduction.

Our results suggest a positive association between arylesterase activity, which characterizes PON1 activity, and ECN ≥ 6. It is consistent with our previous work, in which we detected a similarly positive association (OR = 1.09; 95 % CI 1.01–1.17) [21]. PON1 is the major determinant of HDL's antioxidant ability [60], and the results of both of our studies imply its protective role in early embryo development. In fact, arylesterase activity was strongly associated with ECN ≥ 6 (RR = 4.87; 95 % CI 1.44, 16.46), interdependently with LPHC_13 (particle size 9.2 nm); however, the effect estimates were imprecise and should thus be interpreted with caution (Supplemental Table 1). Nevertheless, altogether, our previous and current study results highlight the important impact of PON1, measured by arylesterase activity, on embryo quality during IVF.

Several other studies have examined FF composition using NMR technology and found associations between lipoproteins and oocyte/embryo outcomes [61–64]. However, these studies were limited by the absence of a "one-follicle, one-embryo" design and most were not conducted in humans. Furthermore, these studies focused only on CH_2CH_3 group signals as a biomarker for total lipoproteins and did not examine the distribution of lipoprotein particle size. The current study is therefore the first NMR examination of FF HDL particle size ever reported in humans. Use of NMR allowed us to identify large and medium sized HDL particles as predictors of poorer embryo quality, in women undergoing IVF, underscoring the importance of particle size for FF HDL biologic activity.

There are several limitations to our current results. Studies have reported changes in the levels of FF constituents with ovarian follicular growth [65, 66], but we did not measure the exact size of retrieved follicles in our study. However, to minimize the variability in follicle size across participants, and thus the impact, the two largest follicles with diameter > 17 mm were collected from each woman. During the analysis, arbitrary statistical cutoffs (i.e., $P < 0.10$) were used to identify a subset of FF HDL particle components to be used in forward stepwise variable selection procedures. These arbitrary cutoffs could have missed important FF HDL particle components associated with embryo quality that merited consideration in the multivariable models. For instance, β-cryptoxanthin was associated with higher EFS in our previous work [22] but did not qualify for inclusion in the forward stepwise modified Poisson regression model variable selection process in our current study. Conversely, we conducted numerous statistical tests, without adjusting for potential inflation of type-1 error and thus some results may in fact reflect chance. However, our aim was to maximize sensitivity to detect plausible associations for future confirmation. In addition, sperm quality is likely to be a predictor of early embryo development [67–69]; however, male data were unfortunately unavailable for this study. In our analysis, we considered day 3 embryo morphology endpoints, rather than blastocyst formation, pregnancy, or live birth, endpoints more clinically relevant to couples seeking IVF treatment. Yet, our results are a natural first step to understanding the biochemical mechanisms that underpin FF HDL particle component roles in clinical IVF outcomes.

Distributions of demographic and clinical factors and FF HDL particle components levels for $n = 103$ included in this analysis were not substantially different from our original study sample of $n = 180$ reported previously [23]. Still, including only women who had at least one embryo developed from "research follicles," regardless of embryo development from non-research follicles, may have biased the results. Our selection of research follicles was independent of measured concentrations of FF HDL particle components, and was also not based on embryo development, and therefore, any introduced bias is likely to be non-differential. However, this limitation led to a smaller sample size, especially for ECN where the multivariable analysis was further restricted to cleavage-stage embryo transfers, possibly causing imprecise effect estimates. This also limited the number of self-reported cigarette smokers in our sample, and so we collapsed "current" and "former" smokers to facilitate adjustment during the analysis; however, this may have led to misclassification. Finally, our study participants comprised of a highly selected sample recruited from women referred to IVF treatment. Therefore, our study results should be generalized to other IVF populations or non-IVF populations with caution [70].

Conclusions

In conclusion, HDL particle remodeling within the ovarian follicle is a complex and dynamic process, in which antioxidant properties appear to change with structure and size. Despite limitations, our data suggest that specific FF HDL particle components and specific FF HDL-C particle size subfractions are associated with cleavage-stage embryo quality following IVF and ICSI. More specifically, FF γ-tocopherol and ApoA-1, in addition to cholesterol metabolism characterized by large and medium HDL-C particles, may be important for embryo quality, presumably due to differential antioxidant capacities. These associations require validation in an independent group of patients. Nonetheless, a positive

association between better embryo quality and HDL-associated lipophilic micronutrients, which are mostly obtained through diet, in our data merits further investigation to elucidate the practical and clinical implications for IVF populations.

Acknowledgments The authors would like to thank Ying Wang, PhD MPH for providing critical feedback for this manuscript.

Compliance with ethical standards

Study funding Supported by the National Institutes of Health, National Institute on Aging (Grant R21 AG03957–01A2).

Conflict of interest The authors declare that they have no conflicts of interest.

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