The fatty acid composition of human follicular fluid is altered by a six-week dietary intervention that includes marine omega-3 fatty acids

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Capsule: The fatty acid composition of human follicular fluid was altered by a six-week dietary intervention.

ABSTRACT

INTRODUCTION

The fatty acid composition of human follicular fluid is important for oocyte development and for pregnancy following *in vitro* fertilisation (IVF). This study investigated whether a dietary intervention that included an increase in marine omega-3 fatty acids, olive oil and vitamin D alters the fatty acid composition of human follicular fluid. The association of lifestyle factors with follicular fluid fatty acid composition was also investigated.

METHODS

55 couples awaiting IVF were randomized to receive the six-week treatment intervention of olive oil for cooking, an olive oil-based spread, and a daily supplement drink enriched with vitamin D and the marine omega-3 fatty acids docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) and 56 couples were randomized to receive placebo equivalents. Dietary questionnaires were completed and samples of blood were taken before and after the intervention. Follicular fluid was collected at oocyte retrieval and the fatty acid profile assessed using gas chromatography.

RESULTS

In the control group, individual fatty acids in red blood cells and follicular fluid were significantly correlated. Furthermore, a healthier diet was associated with a lower percentage of follicular fluid arachidonic acid. The follicular fluid of women in the treatment group contained significantly higher amounts of EPA and DHA compared to the control group, whilst the omega-6 fatty acids linoleic, γ-linolenic, dihomo-γ-linolenic and arachidonic were lower.

CONCLUSION

This is the first report of a dietary intervention altering the fatty acid composition of follicular fluid in humans. Further research is required to determine whether this intervention improves oocyte quality.

TRIAL REGISTRY: ISRCTN50956936

Key words: Diet, omega-3, DHA, EPA, vitamin D, IVF, follicular fluid

LIST OF ABBREVIATIONS

AMH	anti-Mullerian hormone
BMI	body mass index
DHA	docosahexaenoic acid
EPA	eicosapentaenoic acid
FAMEs	fatty acid methyl esters
FFQ	food frequency questionnaire
GnRH	gonadotrophin releasing hormone
hCG	human chorionic gonadotrophin
IVF	*in vitro* fertilisation
IVF-ICSI	*in vitro* fertilisation-intracytoplasmic sperm injection
PCOS	polycystic ovary syndrome
RBC	red blood cell

INTRODUCTION

Approximately one third of subfertility is unexplained and it is possible that a proportion of these cases may be due to an impact of the follicular fluid milieu on oocyte quality. Previous studies have identified a number of lifestyle and clinical factors associated with the composition of the follicular fluid. Lower follicular fluid antioxidant enzyme levels have been reported in older compared with younger women (Carbone *et al.* 2003) and increased follicular fluid levels of stearic acid have been observed in women with a higher body mass index (BMI) (Mirabi *et al.* 2017). Furthermore, follicular fluid from obese women with polycystic ovary syndrome (PCOS) was reported to have higher palmitoleic and oleic acids than that from non-obese women with PCOS and from normal weight healthy controls (Niu *et al.* 2014). The effect of other lifestyle factors, such as diet, on follicular fluid composition has not been well investigated.

The importance of lipid metabolism in the process of oocyte maturation is becoming increasingly well recognised. Fatty acids are required as an energy source (utilised by β-oxidation following the luteinising hormone surge) in order to support oocyte maturation and early embryo development. In both mice and cows, inhibition of β-oxidation decreased oocyte quality and subsequent blastocyst formation (Ferguson & Leese 2006; Dunning *et al.* 2010). In addition to being an energy source, fatty acids are required for the biosynthesis of cell membranes of the rapidly dividing early embryo (O'Gorman *et al.* 2013); and play a vital role in the process of embryo cavitation (Watson 1992; Berger & Wood 2004). Human embryos that developed beyond the four cell stage contained higher levels of linoleic and oleic acids and lower levels of saturated fatty acids compared to those that arrested (Haggarty *et al.* 2006).

In humans, linoleic acid in follicular fluid has been shown to be positively correlated with oocyte fertilisation rates as opposed to arachidonic acid which had a negative correlation; in the same study the ratio of omega-6 to omega-3 fatty acids was lower in the follicular fluid of women who became pregnant following assisted reproductive treatment (Shaaker *et al.* 2012). This suggests that omega-3 fatty acids might play an important role in achieving pregnancy. Another study examining the fatty acid content of human follicular fluid, demonstrated that higher levels of oleic, palmitic, linoleic and stearic acids were associated with a lower percentage of cumulus oocyte complexes with favourable morphology (Jungheim *et al.* 2011). In addition, palmitic, linoleic and stearic acids were shown to be higher in the follicular fluid of women who did not achieve a pregnancy following in vitro fertilisation-intracytoplasmic sperm injection (IVF-ICSI) (Mirabi *et al.* 2017), possibly due to the disruption of oocyte development (Marei *et al.* 2010) or the decreased ability of these oocytes to form pronuclei after IVF-ICSI (Ciepiela *et al.* 2015).

An intravenous emulsion of soybean oil, which contains both linoleic acid and the plant omega-3 fatty acid alpha-linolenic acid, has been used to attempt to improve IVF success rates in women with recurrent implantation failure. However, a recent systematic review concluded that, when studies with a high potential for bias were excluded, there is no significant benefit of this therapy (Zhou *et al.* 2020).

Dietary supplementation with trace elements has been shown to alter the nutritional milieu of human follicular fluid (Özkaya *et al.* 2011). The ability to alter the fatty acid content of follicular fluid by diet has been demonstrated in bovine studies (Childs *et al.* 2008) but to date, no intervention trials with fatty acids have been conducted in humans that report on follicular fluid composition.

The aim of this research was to determine the fatty acid composition of human follicular fluid in a small cohort of IVF patients, to examine demographic and lifestyle factors which may affect the composition, and to test whether this composition was different after a short intervention that provided DHA, EPA and vitamin D, olive oil and olive oil-based spread.

METHODS

This research was conducted as part of the PREconception dietary suPplements in Assisted REproduction (PREPARE) trial. The full protocol for the PREPARE trial has been published (Kermack *et al.* 2014), as has the effect of the intervention on embryo quality, the primary outcome (Kermack *et al.* 2020). The trial is registered as ISRCTN50956936.

Recruitment

After obtaining ethical approval (13/SC/0544) to perform the study, couples awaiting IVF treatment were screened for eligibility. Inclusion criteria were females aged between 18 and 41 years, BMI between 18 and 32 kg/m^2, and the use of partner sperm. Potential participants were excluded from participating if they consumed oily fish more than once a week (as defined by the UK Food Standards Agency); if they had any contraindications to IVF or IVF-ICSI or to the supplements provided; if they had had more than two previous unsuccessful IVF cycles or low ovarian reserve indicated by an anti-Mullerian hormone (AMH) level of less than 2 pmol/L (Beckman AMH Gen II, Glasgow); or they were using prescribed medications or herbal remedies other than simple analgesia. Written consent was taken from those who were eligible and wished to participate and they were then randomized to the treatment or control groups.

The intervention

The treatment group received a six week supply of olive oil for cooking, an olive oil-based spread, and a daily supplement drink enriched with marine omega-3 fatty acids (EPA (800 mg) and DHA (1200 mg)) and vitamin D (10 micrograms), compared to the control group who received sunflower seed oil for cooking, a sunflower seed oil-based spread, and a daily supplement drink without EPA, DHA or vitamin D. All oils, spreads and drinks were provided in identical unmarked containers, with the couple's unique identifiable number for reference. Olive oil and the olive oil-based spread were used to limit intake of omega 6 fatty acids in the treatment group. The EPA and DHA dose provided is a significant increment (at least 10-times) above habitual daily intakes and is achievable through diet (e.g. from one serving of oily fish such as salmon a day); this dose is consistent with doses used in many other trials of marine omega-3 fatty acids in different settings and is much greater than can be obtained from over the counter fish oil supplements. It was not deemed ethical to give a higher dose of marine omega-3 fatty acids than could be achieved by diet to couples trying to conceive as potential adverse effects were unknown. The dose of vitamin D used is in accordance with current UK recommendations for intake by pregnant women.

The duration of the intervention was a minimum of six weeks preceding an IVF treatment cycle. This duration would not delay couples access to IVF treatment which would discourage participation. Support for this duration is provided by evidence that some lipid pools would have achieved maximal, or near-maximal, changes in EPA and DHA content within the 6 week period (Browning *et al.* 2012) and from rodent studies demonstrating that dietary manipulations within a very short window around the time of implantation can have profound effects on early development (Fleming *et al.* 2011; Watkins *et al.* 2008).

Baseline assessment

A preconception questionnaire was completed prior to commencing the interventions by all participants. This ascertained participants characteristics and lifestyle factors for example age, ethnicity, occupation, exercise levels, and alcohol and caffeine consumption. The couples' diets were assessed using the short Southampton Food Frequency Questionnaire (FFQ) (Crozier *et al.* 2010) to assess the prudency of their diet and their BMI was measured.

Samples of blood were collected from the participants at the time of recruitment and red blood cell (RBC) fatty acid composition was determined.

Randomization

Following this initial data collection, participating couples were randomized to one of the two intervention groups and were provided with a six-week supply of the drinks, oil and spread. Permuted block randomization was used with blocks of varying size and allocation concealment, stratification was performed at randomisation for planned mode of fertilisation (IVF or IVF-ICSI). The trial was double blinded and unblinding was only performed once all couples had completed the dietary intervention and analysis was complete.

Compliance

Compliance was monitored by the research team using weekly phone calls or email, depending on participant preference.

IVF cycle

Women embarking on the study underwent ovarian stimulation according to the standard protocols employed by the IVF unit using gonadotrophins and co-treatment with a gonadotrophin releasing hormone agonist (GnRH agonist) or GnRH antagonist to prevent premature luteinisation. Oocyte retrieval was performed 36 hours following triggering of final oocyte maturation by a single

subcutaneous dose of human chorionic gonadotrophin (hCG) or GnRH agonist. Follicular fluid was collected during the oocyte retrieval. Once the oocytes within it had been removed, the first 5 ml collected was placed into a conical centrifuge tube and centrifuged at 1500 r.p.m. (400 g) for 5 minutes. The supernatant was then removed by pipette and placed into 1 ml aliquots (three or four per patient). On the day of oocyte retrieval (after approximately six weeks of dietary study intervention or control), the initial lifestyle questionnaire was repeated and a further blood sample was collected from the participants.

Analysis of fatty acids in red blood cells

The fatty acid composition of RBCs was measured using gas chromatography, allowing the separation and identification of nineteen fatty acids including EPA and DHA. The protocol used was as described elsewhere (Fisk *et al.* 2014).

Follicular fluid analysis

Fatty acid analysis was performed on the follicular fluid. Total lipid was extracted into chloroform: methanol (2:1, vol/vol). The lipid extract was heated to 50°C for 2 hours with 2% methanol in sulphuric acid to produce fatty acid methyl esters (FAMEs). FAMEs were separated and identified by gas chromatography performed according to conditions described elsewhere (Fisk *et al.* 2014). FAMEs were identified by comparison of run times with those of authentic standards. Fatty acid concentrations are expressed as weight % of the total fatty acids present.

Statistical analysis

Statistical analysis was performed using SPSS Statistics 21 (IBM, Armonk, NY, USA). Results are reported as mean ± standard deviation unless otherwise stated. Correlations were examined between the proportions of the specific fatty acids in the RBCs and follicular fluid, using Pearson's correlation (R) for normally distributed, continuous data and Spearman's rho (R_s) for non-normally distributed data. Comparisons were made between the proportions of the fatty acids in the follicular fluid between the placebo and treatment groups using ANOVA (any non-normally distributed data were log transformed prior to the analysis). In all cases a value for p of less than 0.05 was considered significant.

RESULTS

Characteristics of women in the two groups

The two groups did not differ according to age (mean age ~33 yr), BMI (mean BMI ~25 kg/m^2), prudent diet score, alcohol consumption, caffeine consumption, or amount of exercise undertaken (Kermack *et al.* 2020).

Fatty acid composition of human follicular fluid

21 fatty acids were identified in the follicular fluid of 49 women in the control group in order to ascertain a normal follicular fluid profile in this population (Figure 1). Fatty acids are expressed as weight percentage of the total fatty acids present. Palmitic acid was found at the highest levels (28.50% ± 1.33), followed by linoleic acid (23.12% ± 2.86), oleic acid (17.36% ± 1.93) and stearic acid (12.01% ± 0.91). Arachidonic acid, EPA and DHA were present at 7.65% ± 1.35, 0.58% ± 1.65 and 2.45% ± 1.35, respectively.

Red blood cell fatty acids

There were no significant differences in the pre-intervention levels of any of the 19 different fatty acids measured in RBCs between the treatment and control groups. Both EPA and DHA increased in the study group (mean increase of 2.30% (95% CI: 2.80, 3.21) and 2.80% (95% CI: 2.57, 3.03) respectively, both $p<0.001$). In the control group, an increase in EPA was also observed but this was smaller (mean 0.82% (95% CI: 0.87, 0.97), $p=0.002$), but there was no change in DHA.

Correlations between fatty acids in RBCs and follicular fluid

In the women in the control group, positive correlations were observed between the following fatty acids in RBCs taken at the time of oocyte retrieval and in follicular fluid: myristic acid ($R=0.307$, $p=0.036$), palmitic acid ($R=0.464$, $p=0.001$), palmitoleic acid ($R_s=0.468$, $p=0.001$), stearic acid ($R=0.377$, $p=0.009$), oleic acid ($R_s=0.298$, $p=0.042$), vaccenic acid ($R_s=0.619$, $p<0.001$), linoleic acid ($R=0.785$, $p<0.001$), gamma linolenic acid ($R_s=0.544$, $p<0.001$), alpha linolenic acid ($R_s=0.326$, $p=0.025$), eicosadienoic acid ($R=0.395$, $p=0.006$), dihomo gamma linoleic acid ($R=0.565$, $p<0.001$), arachidonic acid ($R=0.685$, $p<0.001$), EPA ($R_s=0.406$, $p=0.005$) and DHA ($R_s=0.538$, $p<0.001$).

Associations of follicular fluid fatty acids with demographic and lifestyle factors

Associations of follicular fluid fatty acids with demographic and lifestyle factors were explored in women in the control group. There were no associations between follicular fluid fatty acids and consumption of coffee or amount of habitual exercise. A negative correlation was observed between alcohol intake and follicular fluid palmitic acid (R=-0.313, p=0.029). A higher prudent diet score (and hence a healthier diet) was associated with a lower percentage of arachidonic acid in follicular fluid from women in the control group (R=-0.367, p=0.010) (Figure 2). There were no other significant associations between any individual fatty acids and prudent diet score.

BMI was correlated with palmitoleic acid (R_s=0.286, p=0.047), vaccenic acid (R_s=-0.315, p=0.028), gamma linolenic acid (R_s=0.571, p<0.001), dihomo-gamma linolenic acid (R=0.441, p=0.002), and eicosatetraenoic acid (R=0.418, p=0.003).

Seven fatty acids were significantly correlated with the woman's age; negatively for palmitoleic acid (R_s=-0.315, p=0.028); oleic acid (R_s=-0.355, p=0.012), dihomo-gamma linolenic acid (R=-0.297, p=0.038), and lignoceric acid (R_s=-0.312, p=0.029), and positively for alpha linolenic acid (R_s=0.298, p=0.038), EPA (R_s=0.496, p<0.001) and DHA (R_s=0.484, p<0.001).

Differences in follicular fluid fatty acid profile following an intervention providing EPA and DHA

A five-fold higher follicular fluid EPA and a two-fold higher DHA were observed in the treatment group compared to the control group (Table 1). Furthermore, higher percentages of gondoic acid (p<0.001), eicosatetraenoic acid (p=0.034), and docosapentaenoic acid (p<0.001) were observed in the treatment group (Table 1). Lower percentages of linoleic acid (p<0.001), gamma linolenic acid (p<0.001), arachidic acid (p=0.001), eicosadienoic acid (p<0.001), dihomo gamma linolenic acid (p<0.001), arachidonic acid (p<0.001), and lignoceric acid (p<0.001) were observed in the treatment group compared to the control group (Table 1).

Discussion

We report the fatty acid composition of follicular fluid from 102 women in the UK undergoing IVF or IVF-ICSI. The most common fatty acids present in follicular fluid were palmitic, linoleic, oleic and stearic, which agrees with previous reports (Dunning *et al.* 2014; Leroy *et al.* 2005; Valckx *et al.* 2014). In the control group there were significant correlations between percentages of 14 fatty acids in follicular fluid and in RBCs; such correlations have been reported previously (Hauschka *et al.* 2009). The novelty of the current study is that percentages of the omega-3 fatty acids EPA, DPA and DHA were higher in follicular fluid from women who had received the drink containing EPA and DHA than in the control group while percentages of the omega-6 fatty acids linoleic and arachidonic were lower. This has not previously been demonstrated, although intervention with EPA and DHA is known to increase the levels of those fatty acids in blood plasma/serum, blood cells and many tissues (Calder, 2018). We previously reported RBC fatty acids in these women; RBC EPA and DHA increased in the treatment group (Kermack *et al.* 2020). We did not report that RBC DPA also increased (by 0.21% (95% CI 0.10 – 0.31%), $p<0.001$). It is likely that DPA is higher in RBC and follicular fluid in the treatment group because EPA is converted in the body to DPA.

We studied the effect of increased intake of EPA and DHA because these fatty acids have been linked to increased chance of achieving pregnancy (Chiu *et al.* 2018) and to improved pregnancy outcomes (Chiu *et al.* 2018; Nassan *et al.* 2018). In addition, we reported previously that morphokinetic markers of embryo quality (length and synchrony of the fourth cell cycle) were better in the treatment group than in the control group (Kermack *et al.* 2020).

EPA and DHA levels were higher in the follicular fluid of the treatment group, whereas linoleic acid and arachidonic acid were lower. Linoleic acid is an omega 6 PUFA and animal studies have suggested that increased linoleic acid inhibits cumulus cell expansion and development of the mature oocyte (Marei *et al.* 2010). Furthermore, in human studies, associations have been made between linoleic acid and more cumulus oocyte complexes with unfavourable morphology (Jungheim *et al.* 2011) and decreased success following IVF-ICSI (Mirabi *et al.* 2017). Using the "one follicle – one retrieved oocyte – one resulting embryo" investigational model, it was observed that higher arachidonic and linoleic acids and their derivatives resulted in oocytes that did not develop two pronuclei and degenerated following ICSI (Ciepiela *et al.* 2015). Hence the lower linoleic and arachidonic acids in the treatment group could be beneficial to oocyte quality, early embryo development and success from IVF. Interestingly, we identified that a healthier diet was also associated with lower follicular fluid arachidonic acid. Thus, both a generally healthy diet and a higher intake of marine omega-3 fatty acids seem to determine lower follicular fluid arachidonic acid levels and this might contribute to the effects

of a heathy diet on pregnancy success (Chen *et al.* 2016). Further research is needed to examine the effect of the follicular fluid fatty acid composition on oocyte quality, which was beyond the scope of this project.

In addition to the specific association of linoleic acid with the cumulus oocyte complex morphology (Jungheim *et al.* 2011), a lower ratio of omega-6 to omega-3 fatty acids in follicular fluid has been associated with a greater likelihood of achieving a pregnancy with assisted reproductive treatment (Shaaker *et al.* 2012). Furthermore, lower omega-6 fatty acids in the follicular fluid has been associated with improved folliculogenesis and IVF performance in bovine studies (Moallem *et al.* 2013). Thus, both the lower omega-6 fatty acids and higher EPA and DHA in follicular fluid following the dietary intervention could be clinically relevant, and may relate to the improved embryo quality already reported (Kermack *et al.* 2020).

Within the control group, follicular fluid palmitoleic acid was positively associated with BMI. Outcomes from IVF are poorer in obese women (Kudesia *et al.* 2018) and whether higher palmitoleic acid in follicular fluid and elsewhere has a role in this should be explored. There are many reports that blood and adipose tissue palmitoleic acid are higher in obese than normal weight individuals (de Souza *et al.* 2018). Furthermore, Valckx *et al*. report fatty acids in different lipid fractions within follicular fluid from normal weight, overweight and obese women. They find no differences in fatty acids in cholesteryl esters or triglycerides and few differences in phospholipids among the different body weight groups (Valckx *et al.* 2014). Within non-esterified fatty acids, they find increasing follicular fluid palmitoleic acid concentration going from normal weight to overweight and from overweight to obese women. The significance of higher follicular fluid palmitoleic acid with increasing BMI is not clear but higher blood or adipose tissue palmitoleic acid is linked with poor metabolic outcomes (de Souza *et al.* 2018). We identified associations between follicular fluid fatty acids and age. There were positive associations for the omega-3 fatty acids alpha-linolenic acid, EPA and DHA. Using a large cohort of New Zealand women (n = approx. 1300) aged 15 to 65+ years, Crowe *et al*. identified that concentrations of EPA and DHA in blood serum lipids increased with age (Crowe *et al.* 2008). Our findings of positive association of follicular fluid omega-3 fatty acids with age, agree with previous description of such associations for EPA, DPA and DHA (Ruiz-Sanz *et al.* 2019). Higher omega-3 fatty acids with age may relate to dietary or metabolic differences. There were no associations between follicular fluid fatty acids and consumption of coffee or amount of habitual exercise. However, there were inverse associations between alcohol consumption and palmitic acid and also between a prudent diet score and arachidonic acid. The latter association may be explained by lower intake of animal-

derived foods especially red meat in those women with a higher prudent diet score, since animal-derived foods are the main sources of dietary arachidonic acid

Strengths of this study include its randomised controlled trial design, its novelty and the high retention and compliance of the women to the interventions, as reported previously (Kermack *et al.* 2020). Most previous studies of fatty acid composition of human follicular fluid have had smaller sample sizes than here and there are no studies in humans reporting on the effect of an intervention to increase or decrease intake of specific fatty acids. One limitation of the study is that we measured fatty acids within the follicular fluid as a whole; the fatty acids are mainly carried esterified in different complex lipids (phospholipids, cholesteryl esters and phospholipids) and these have different fatty acid compositions (Valckx *et al.* 2014). It would be interesting to know how each of these lipid fractions is affected by the treatment intervention. Also, we did not measure the fatty acid content or composition of the oocytes themselves; previous research in cattle has demonstrated selective uptake of saturated fatty acids as compared with polyunsaturated fatty acids by the cumulus-oocyte complex (Fouladi-Nashta *et al.* 2009; Adamiak *et al.* 2006).

In conclusion, increased dietary intake of marine omega-3 fatty acids is reflected in an altered fatty acid profile of human follicular fluid with increased amounts of EPA and DHA. The effects of this may be clinically relevant but require further investigation in order that evidence-based dietary advice can be given to women undergoing *in vitro* fertilisation. An observational study utilising the "one follicle – one resulting oocyte – one resulting embryo" model to examine the levels of omega-3 fatty acids and resulting embryo quality would lead to more robust evidence to inform clinical dietary advice.

DECLARATIONS

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Authorship

NSM and PCC conceived the study. AJK, FDH, NSM and PCC designed the experiments. AJK, SJW and HLF performed the experiments. AJK analysed the data. AJK and PCC drafted the manuscript. All authors provided input into the manuscript.

Conflict of Interest

PCC is an advisor to Smartfish, DSM, BASF AS, Cargill and Fresenius-Kabi. NSM has received fees and research grants a number of pharmaceutical companies, but none directly related to this study. None of the other authors has any conflict of interest to declare in relation to this manuscript.

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Figure 1: a. Fatty acid profile of follicular fluid from women in the control group of the PREPARE trial. b. Graph to demonstrate % of total fatty acids when < 2%.

Data are mean ± SD.

Figure 2: Correlation between prudent diet score and percentage of arachidonic acid in follicular fluid

Table 1: Fatty acid composition of follicular fluid in the treatment and control groups at the end of the 6 week intervention.