Running Title: Preconceptional diet and embryo development

The effect of a six week 'Mediterranean' dietary intervention consisting of a supplement drink containing omega-3 fatty acids and vitamin D together with the use of olive oil and olive oil based spreads on *in vitro* human embryo development: The 'PREPARE*' double blinded randomized controlled trial.

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* (PREconception dietary suPplements in Assisted REproduction)

Capsule: Couples randomized to the nutrient rich diet had higher blood levels of EPA, DHA and vitamin D, and their embryos showed altered morphokinetic markers of development.

ABSTRACT

Objective: To study the impact of increased dietary intake of omega-3 fatty acids, vitamin D and olive oil for six weeks prior to IVF or IVF-ICSI on morphokinetic markers of early embryo development.

Design: A double blinded randomised controlled trial.

Setting: Academic IVF unit in the UK.

Patients: 111 couples undergoing IVF or IVF-ICSI were recruited.

Interventions: 55 couples received the six week study intervention of a daily supplement drink enriched with omega-3 fatty acids and vitamin D plus additional olive oil and olive oil based spread and 56 couples received the control intervention.

Main outcome measures: The primary endpoint for the study was the time taken for completion of the second cell cycle after fertilization (CC2). Secondary endpoints included time to complete the third and fourth cell cycles (CC3 and CC4), the synchrony of the second and third cell cycles (S2 and S3), the day 3 and day 5 Known Implantation Data Scores (KIDScores).

Results: There was no difference in CC2 between the two groups ($p=0.707$). However, CC4 was accelerated in the study group compared to the control group ($p<0.001$) and a significantly shortened S3 ($p=0.031$) as well as an increase in KIDScore on day 3 ($p=0.05$) were observed; indicating improved embryo quality in the study group.

Conclusions: This study demonstrates that a short period of dietary supplementation alters the rate of embryo cleavage. Further research is required to investigate the mechanisms which regulate this effect, and whether the impact on embryo development translates into improved clinical outcomes.

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Key words: Diet, omega-3, IVF, morphokinetic markers, embryo development

INTRODUCTION

A growing body of evidence from prospective cohort observational studies indicates that the peri-conceptional nutritional status of both men and women impacts on early fetal development and perinatal and long term health of the offspring (1). In recent years, a number of observational studies have indicated that variations in preconceptional diet may impact early embryo development. Particular interest has been afforded to the possible effects of a Mediterranean diet (2, 3). An observational study demonstrated an increased chance that embryos would form a blastocyst when they were from women who reported consuming higher quantities of fruit and fish and a decreased chance of blastocyst formation in those consuming more red meat or who were on a weight loss diet (4). Furthermore, a prospective observational study reported that a 'Mediterranean' diet high in vegetable oils, fish, vegetables and legumes and low in carbohydrate-rich snacks was positively associated with red blood cell folate and vitamin B6 in blood and follicular fluid and with a 40% reported increase in the probability of achieving a pregnancy (5). A more recent cohort study demonstrated a significantly increased clinical pregnancy rate and almost double the live birth rate in couples consuming a more Mediterranean diet (according to the validated MedDietScore) compared to those who did not (6). The PREDIMED study (7) investigated the effect of the Mediterranean diet on cardiovascular disease and offered specific dietary advice and nuts or extra-virgin olive oil (intervention groups) or guidance on decreasing fat intake (control group). A significant difference in mortality between those adhering to the Mediterranean diet and those in the low fat control group was demonstrated; however, the trial has recently been criticised due to the complexities of ensuring that a specified diet is followed in a randomised trial (8).

Acknowledging the difficulties of significantly altering overall diet in a study setting, the PREconception dietary suPplements in Assisted REproduction (PREPARE) trial was developed to examine the importance of key components of the Mediterranean diet including olive oil, omega-3 fatty acids (FAs) from seafood and vitamin D. The effect of increased dietary intake of the omega-3 FAs eicosapentaenoic acid (EPA) and

docosahexaenoic acid (DHA) on markers of embryo morphology is unclear, with some studies demonstrating a benefit (9) and others showing no advantage (10). It is recognised that FAs are required by the developing embryo as a source of energy (11) and for synthesis of newly forming cell membranes (12). There is also some evidence that increased levels of omega-3 FAs in the cell membrane may increase the gap junction capacity of the morula.(13).

The importance of vitamin D as a determinant of embryo development is unclear, although it has also been implicated as a key factor in fertilisation, affecting sperm-egg binding and the activity of acrosine which digests the zona pellucida (14). However, despite evidence from a recent prospective cross sectional study demonstrating a significantly decreased pregnancy rate in women with a serum level below 20 ng/mL (15) and the inclusion of vitamin D in many conception multivitamin preparations, increased levels in follicular fluid vitamin D have been associated with poorer oocyte (16) and embryo quality (17).

While a growing body of literature suggest that preconceptional exposure to key components of the Mediterranean diet may affect gamete and embryo development, the reported data are largely observational and there remains a lack of intervention studies designed to clarify the validity of this association. The PREPARE trial (18) was a prospective, double blinded randomized controlled trial investigating whether a drink high in omega-3 FAs (both EPA and DHA) and the recommended dose of vitamin D in combination with increased intake of olive oil taken by both the man and the woman for 6 weeks prior to IVF altered morphokinetic markers of early embryo development.

MATERIALS AND METHODS

The full protocol for the PREPARE trial has been previously published (18).

Recruitment

Full ethical approval (13/SC/0544) was granted from South Central (Oxford A) Research Ethics Committee (NRES) via the Integrated Research Application System (IRAS) and from the Research and Development Department (O+G0211) at University Hospital Southampton. Following approvals, couples awaiting IVF treatment who met the study inclusion criteria were invited to provide written consent to participate. These criteria were female age between 18 and 41 years, a body mass index (BMI) between 18 and 32 kg/m^2, and the use of partner sperm. Exclusion criteria included more than two previous unsuccessful IVF cycles; low ovarian reserve indicated by an anti-Mullerian hormone (AMH) level of less than 2 pmol/L (0.28 ng/mL) (Beckman AMH Gen II, Glasgow); any medical contraindication to IVF or IVF-ICSI treatment or to the specific dietary intervention; previously diagnosed diabetes; the use of prescribed medication or herbal remedies other than simple analgesia; or eating fatty fish (as defined by the UK Food Standards Agency) more than once a week. Those who were eligible and wished to participate were randomized to the study or control intervention groups.

The intervention

The study group received olive oil for cooking, an olive oil based spread, and a daily supplement drink enriched with EPA (800 mg), DHA (1200 mg) and vitamin D (10 μg). The dose of EPA+DHA provided is consistent with doses used in other trials, can be obtained from foods (fatty fish), and is much greater than can be obtained from over the counter fish oil supplements. The dose of vitamin D used is in accordance with current UK recommendations for intake by pregnant women. The control group received sunflower seed oil for cooking, a sunflower seed oil based spread, and a daily supplement drink without EPA, DHA or Vitamin D. For the purpose of blinding, the drinks were provided by Smartfish (Oslo, Norway) in identical

unmarked containers. The cooking oils and spreads were supermarket purchased and repackaged into identical unmarked containers.

The dietary intervention was to be taken for a minimum of six weeks immediately preceding an IVF treatment cycle. The duration of the intervention was determined by two considerations: long enough to impact on gamete maturation but not so long as to represent a burden or delay to treatment that would discourage participation in the study and compliance with the interventions. Support for this duration was 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 (19) 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 (20, 21).

Power calculation and sample size

Recently, morphokinetic analysis of human embryos has shown the duration of key development phases to be correlated to standard conventional morphological criteria, but to be more highly predictive of implantation potential (22, 23). In particular, the time taken to reach specific pre-implantation developmental milestones has been shown to be predictive of embryo viability. A key morphokinetic marker of embryo viability has been shown to be the length of the second cell cycle (CC2) (Figure 1). A shortened CC2 has previously been associated with an increased likelihood that the embryo will develop to a blastocyst (24). In addition, embryos with a CC2 shorter than 11.9 hours (pooled SD 2.25 hours, but not normally distributed) have been reported to have an implantation rate of 35% compared to 28% when this key developmental step took longer than 11.9 hours (22). Moreover, in a study correlating morphokinetic parameters with static morphology scoring, a CC2 less than 11.9 hours was shown to correlate with an overall increase in embryo score of 0.5 points on a 5 point scale (22). This is similar in magnitude to the impact on embryo morphology reported in a previous observational study, where a diet rich in omega-3 FAs was shown to be associated with altered embryo morphology markers when assessed on day 3 post

143 fertilization (9). Given the evidence that CC2 may constitute a functional marker of embryo development,
144 the primary end point of this study was the difference in mean CC2 score of embryos generated by IVF
145 after exposure of the couple to the study versus control diet. Based on the work by Meseguer et al.(22), a
146 12% absolute difference (1.4 hours) in mean CC2, or effect size of 0.670 was considered to represent a
147 developmentally significant effect. In order to show this with power ≥ 80% at $p < 0.05$, a non-parametric
148 comparison (Wilcoxon test) indicated a requirement of 46 couples per group in the analysis. To allow for
149 drop outs and failure to produce sufficient viable embryos, a further 20% were recruited. This required the
150 randomization of a minimum of 55 couples per group (110 in total).

151 ***Baseline assessment***

152 In order to address other possible factors determining embryo development, study participants were
153 invited to complete a baseline preconception questionnaire establishing their characteristics and lifestyle
154 including their age, ethnicity, education and occupation, exercise levels, alcohol and caffeine consumption,
155 time spent outside and sun cream use. Their BMI was measured and they completed the short
156 Southampton Food Frequency Questionnaire (FFQ) (25) to assess the prudency of their diet. This
157 comprised of asking each individual about the frequency with which they consumed 20 food items (that
158 best characterized a prudent dietary pattern from a 100 item, interviewer administered, food frequency
159 questionnaire used during the Southampton Women's Survey (25)). The more positive the score, the more
160 prudent (or healthier) the described diet. Samples of blood were collected from the participants at the
161 time of recruitment. Serum was used to measure vitamin D concentrations while red blood cells (RBCs)
162 were used to measure FAs (see below).

163 ***Randomization***

164 After collecting the baseline data, participating couples were randomized to one of the two intervention
165 groups and were provided with a 6 week supply of the respective intervention components of drinks, oil
166 and spread in unmarked containers. 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; neither the couples nor the research or clinical teams knew which arm of the study a couple had been assigned to. Unblinding was only performed once all couples had completed the dietary intervention and the annotation of all embryos had been performed.

Compliance

Compliance was monitored by weekly communication (either phone calls or email) by the research team.

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 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 gonadotrophin releasing hormone agonist (GnRH agonist). IVF alone or ICSI was performed where clinically indicated. On the day of oocyte retrieval (after approximately six weeks of dietary study intervention or control), the initial lifestyle questionnaire was re-administered and a further blood sample was collected from the participants.

Embryo culture

The embryos were cultured in sequential G-series culture media (Vitrolife, Sweden) in a validated time-lapse incubator (EmbryoScope D, Vitrolife AS, Denmark) in 6% CO_2, 5% O_2 and at 37°C, according to the standard laboratory protocol. During incubation, seven focal plane images were taken every 10 minutes, generating forty two plane focal images every hour, and these were analysed according to morphological and morphokinetic markers (26). The morphokinetic markers of 750 embryos (356 in the study group and 394 in the control group) were annotated by embryologists blind to the study group and the endpoint parameters calculated. The morphokinetic markers were measured from the time of insemination or the start of the injection for ICSI. Standard time points were annotated and calculated (including the time for

the second (CC2), third (CC3) and fourth (CC4) cell cycles and the synchrony of the second (S2) and third (S3) cell cycles). These morphokinetic markers were then used to calculate the day 3 and day 5 KIDScores (known implantation data scores), in accordance with published (27) and unpublished (28) algorithms, respectively. The embryos were then transferred, cryopreserved or destroyed according to the clinic's standard operating procedures. The embryo or embryos with the highest morphological score based on validated criteria (Gardner's) (29) were transferred; embryos were cryopreserved if they were grade 3 or higher with an a or b grade trophectoderm and inner cell mass as graded on the Gardner score (30).

Primary and secondary end points

As previously stated, the primary endpoint for the study was one of a series of validated morphokinetic parameters of healthy embryo development, namely the time taken for completion of the second cell cycle after fertilization (CC2). Secondary endpoints included blood measurements of EPA, DHA and Vitamin D and additional validated parameters of embryo development such as time to complete the third and fourth cell cycles (CC3 and CC4), the synchrony of the second and third cell cycles (S2 and S3), associated with increased chance of development to a blastocyst (24, 31, 32), implantation (22, 24, 33, 34) and clinical pregnancy (24). In addition, the day 3 and day 5 known implantation data scores (KIDScores) were calculated and pregnancy data were analysed. Other studies have examined fertilisation rates as the primary outcome; however, utilising morphokinetic markers and algorithms enabled the PREPARE trial to study the effect of the intervention on the development of the embryo.

Analysis of fatty acids in red blood cells

The fatty acid content 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 (35).

Analysis of serum vitamin D concentration

Serum vitamin D concentrations were determined by Liquid Chromatography/Tandem Mass Spectrometry (Waters, Milford, MA, USA). The pathology laboratory, University Hospital Southampton NHS Foundation Trust, that undertook the analysis, is a member of the Vitamin D EQA scheme (DEQAS).

Statistical analysis

Results are reported as mean ± standard deviation unless otherwise stated. Differences between the sociodemographic characteristics of participants in the two groups were analysed using ANOVA; characteristics that were not normally distributed and were scalar were adjusted by log transforming and then included in the ANOVA; this enabled a more powerful statistical analysis than utilising non-parametric tests.

ANOVA was also used to compare the levels of FAs and vitamin D in the blood of the participants in the two groups. Results that were not normally distributed were log transformed and then included in the analysis.

A mixed effect model was used to analyse the effect of the intervention on the morphokinetic markers of embryo development. A random effect was fitted for each couple and a fixed effect for treatment and the methodology (i.e. IVF or IVF-ICSI) used to inseminate the embryo. Treatment effects are summarised by the regression coefficient and its standard error. The intra-class correlation coefficient measures the within to between couple variation.

RESULTS

Demographic data

One hundred and eleven couples were recruited between February 2014 and November 2015 (Figure 2). Of these, 102 completed the trial and had at least one embryo analysed, 4 became pregnant prior to their treatment; 2 had cycles which were converted to intrauterine insemination (IUI) following ovarian stimulation; 1 was a recruiting error and 2 had health issues leading to withdrawal.

238 More than 90% of study participants were of white Caucasian ethnicity, representative of the local ethnic
239 profile. The mean age (± SD) of the participants in the trial was 33.4 years (± 4.2) for the women and 36.0
240 years (± 5.5) for the men. There was no significant difference in BMI, prudent diet score, quantity of
241 alcohol and caffeine consumed and number of hours of exercise per week between those randomized to
242 the study group and the control group (Table 1). Monitoring of these markers showed no evidence that
243 participants instituted other lifestyle changes during the study period.

244 At the trial commencement, 25 women (23%) and 61 men (55%) reported not taking any supplements. 52
245 women (47%) and 32 men (29%) were taking a multivitamin supplement (specific to either conception or
246 pregnancy), and 6% of women and 11% of men stated they were taking an omega-3 supplement (either in
247 conjunction with a multivitamin or on its own). All omega-3 supplements were of a sufficiently low dose
248 (less than 200 mg of EPA and DHA) to render these patients eligible for inclusion in the trial.

249 ***Compliance***

250 62% of women and 50% of men reported full compliance with their allocated intervention. There was no
251 statistical difference in compliance between the study group and the control group in either the women
252 (p=0.799) or the men (p=0.089). The median compliance to the drinks was 100.0% (LQ 97.2, UQ 100.0) for
253 women and 99.3% (LQ 95.3, UQ 100.0) for men.

254 ***Red blood cell fatty acids and serum vitamin D***

255 There were no significant differences in the pre-intervention levels of any of the 19 different fatty acids
256 measured in RBCs between the study and control groups in either the women or men (data for EPA and
257 DHA shown in Table1).

258 Among both women and men, both EPA and DHA increased in the study group. There was an increase in
259 EPA of 2.30% (2.80, 3.21) in women and 2.51% (2.30, 2.74) in men and an increase in DHA of 2.80% (2.57,

3.03) in women and 2.42% (2.12, 2.71) in men in the study group (all p<0.001). No significant increase was observed in the control group except a 0.82% (0.87, 0.97) increase in EPA in women (p=0.002).

At study entry, 7% of men and 4% of women had a total serum vitamin D concentration of less than 25 nmol/L, indicative of deficiency. A further 20% of men and 19% of women had borderline deficiency (concentrations between 25 and 50 nmol/L). As expected, the season of recruitment affected the vitamin D concentration measured; men and women who were recruited in the summer (77.75 nmol/L ± 25.01 and 82.13 nmol/L ± 26.06) had higher mean total concentrations compared to those recruited in the winter (49.07 nmol/L ± 22.51 and 49.47 nmol/L ± 18.42, p<0.001 and p=0.001, respectively).

At the end of the treatment period, men and women in the study group had higher serum total vitamin D concentrations than those in the control group (Geometric mean ± SD; in women: 154.63 nmol/L ± 1.56 vs. 68.50 nmol/L ± 1.51, p<0.001; In men: 137.31 nmol/L ± 1.54 vs. 66.57 nmol/L ± 1.49, p<0.001).

Analysis of embryo development

There was no difference between the study group and the control group in the number of oocytes retrieved (median 10.00 (LQ 6.00, UQ 15.75), median 11.00 (LQ 7.50, UQ 18.50), p=0.500) or in the number of normally fertilized embryos obtained (median 6.00 (LQ 2.00, UQ 9.00), median 6.00 (LQ 3.50, UQ 12.00), p=0.299).

Seven hundred and fifty embryos were analysed (356 in the study group and 394 in the control group). Of these, 742 embryos cleaved to the two cell stage (351/356, 98.6% vs. 391/394, 99.2%, p=0.392), 719 cleaved to the four cell stage (344/356, 96.6% vs. 375/394, 95.2%, p=0.319) and 610 to the eight cell stage (295/356, 82.9% vs. 315/394, 79.9%, p=0.306). Furthermore, 487 embryos formed a blastocyst (231/356, 64.9% vs. 256/394, 65.0%, p=0.980).

Table 2 shows the median and quartile values of the morphokinetic markers for the study and control groups. The markers are expressed in standardised form and the treatment effects are shown in Table 2.

283 CC2 times were 0.04 standard deviations shorter in the study group (95% CI -0.16 to 0.24, p=0.71) than the
284 control group, but the two groups were not significantly different. There were statistically significant
285 reductions in CC4 and S3 times (p<0.001 and p=0.02, respectively) and an increase in KIDScore on day 3
286 (p=0.05) in the study group compared to the control group (Table 2). There was no significant difference
287 observed in the amount of fragmentation, blastomere eveness or multinucleation on day 3 between the
288 two groups. No difference between groups was observed in the time it took the embryos to form a morula
289 (tM), start blastulation (tSB), form a blastocyst (tB), or form an expanded blastocyst (tEB) or a hatching
290 blastocyst (tHB) (Table 2). There was also no difference between the average number of blastocysts
291 formed (median 4.00 (LQ 0.00, UQ 6.00) vs. median 3.00 (LQ 0.00, UQ 9.00), p=0.619) or the average
292 number of blastocysts suitable for cryopreservation (median 3.00 (LQ 0.00, UQ 4.00) vs. median 2.00 (LQ
293 0.00, UQ 5.00), p=0.823) between the study group and the control group.

294

295 ***Pregnancy rates***

296 The study was not adequately powered to look at pregnancy rates and no difference was observed
297 between the two groups; either in the first cycle following the trial (30/53 in the study group and 28/49 in
298 the control group, p=0.956) or per embryo transferred (fresh and frozen) to date (169 embryos; with a
299 pregnancy rate of 41/78 in the study group and 55/91 in the control group, p=0.303). Furthermore, there
300 was no difference in live birth rates: 42% (22/53) in the study group and 33% (16/49) in the control group
301 following the first cycle (p=0.355). Live births per embryo transferred to date have been 27/78 in the study
302 group and 28/91 in the control group (p=0.595).

303

DISCUSSION

To our knowledge, this is the first randomized controlled trial using IVF as a model to study the impact of a preconceptional dietary intervention on markers of pre-implantation human embryo development. The dietary intervention did not reveal a significant effect on CC2, but the effect on other key morphokinetic markers indicated a potentially positive influence of the study intervention on the developing embryo. Specifically, CC4 and S3 were shortened in embryos derived from couples who had taken a six week intervention of high dose of omega-3 FAs and the recommended vitamin D intake along with olive oil. The observed impact of the dietary intervention on CC4 and S3, as opposed to the earlier morphokinetic markers (including CC2), may reflect a cumulative amplification of the effect over time. A difference in the day 3 KIDScore was also observed in the study group, demonstrating an overall improvement in the morphokinetic markers of embryo quality. Interestingly, a difference in time to blastocyst formation was not observed which might suggest that blastocysts with a slower CC4 have fewer cells in the trophectoderm and inner cell mass; this needs further research but was beyond the scope of this trial. Furthermore, this study did not enable us to determine whether it was an effect of the dietary intervention on the female or male gametes or an additive effect that resulted in the change in embryo development. While the focus of this study was the impact of a dietary supplement on markers of embryo development rather than clinical outcomes of IVF, a considerable body of evidence from observational studies indicates that the reported findings may have implications for clinical practice. A shortened CC4 has been shown to be positively predictive of continuing development to the blastocyst stage, and of achieving a clinical pregnancy (24). Similarly, a relationship has been demonstrated between a shortened S3 and the chance that the embryo will develop to the blastocyst stage (24). It has therefore been postulated that a diet rich in omega-3 FAs, vitamin D and olive oil may increase pregnancy rates in couples undergoing ART. This notion is supported by an observational study reporting that higher serum omega-3 FAs in women undergoing IVF were associated with an increased chance of clinical pregnancy and live birth (10).

328 Furthermore, a recent publication examining protein intake and fertility treatment outcome demonstrated
329 a positive correlation between intake of fish (the main dietary source of bioactive omega-3 FAs) and live
330 birth rate (36).
331 Vitamin D levels have been implicated as a factor affecting endometrial receptivity (37). However, to date
332 benefit of vitamin D in ART patients has been demonstrated in couples who were depleted prior to starting
333 the supplements (3). In the PREPARE trial, less than 5% of the women were depleted at recruitment,
334 compared to cited levels of between 35 and 45% (38). The lack of deficiency may mean it is possible that
335 the full effect of the vitamin D supplementation was not elicited; due to the small numbers of deficient
336 patients it was not possible to do a sub analysis. A previous observational study reported an associaton
337 between serum vitamin D levels during ovarian stimulation in women who were not deficient and a higher
338 fertilisation rate. However no correlation with clinical pregnancy or live birth rate was observed (39). Olive
339 oil was included in the trial because it represents a key element of the Mediterranean diet. A recent study
340 showed that when taken with a high fish diet in the periconceptional period , olive oil accelerated embryo
341 development between six and eleven weeks gestation (40).
342 It should also be recognised that the trial design meant that the individual components of the study
343 intervention resulting in the alteration of the morphokinetic markers could not be determined; it is
344 possible that the benefit seen might be due to either omega-3 FAs, vitamin D or olive oil individually.
345 Additional randomized interventional studies are required to confirm or refute the proposed benefits of
346 omega-3 FAs, vitamin D and olive oil, independently or synergistically, for improving fertility outcomes, but
347 the current work indicates that even a relatively short and thus well tolerated intervention may have
348 beneficial effects.
349 This study also demonstrated the feasibility of recruiting couples who are trying to conceive to randomized
350 controlled nutritional intervention trials. Compliance was high and biochemical markers of omega-3 FA

status and vitamin D in blood indicated significant enrichment in the both men and women in the study group, consistent with good compliance.

Some limitations of this trial should be noted. The duration of the intervention was six weeks, which might have limited the effect on embryo development as it was shorter than the reported duration of oocyte and sperm maturation, considered to take around 3 months (41) and 72 days (42), respectively. However, although human data are lacking, the latter phases of gamete maturation have been shown to be sensitive to environmental factors (43) and as outlined previously the duration of the intervention was supported by studies in mice that have demonstrated a remarkable impact of a very short term preconceptional dietary intervention (3.5 days) on in-uteri growth trajectories and even behaviour development (44). Moreover, RBC levels of omega-3 FAs and serum vitamin D were increased by six weeks in this study, showing that the trial intervention was efficacious in altering the *in vivo* nutritional milieu.

The FA profile of the women prior to the intervention was similar to that reported in a previous fish oil supplementation study in pregnancy (45). This suggests that the RBC fatty acid profile of the studied women is representative of the wider female population of this age. However, it should be noted that the study population was predominantly white Caucasian and care should be taken when extrapolating the evidence to those from other ethnic backgrounds.

CONCLUSIONS

In conclusion, this study provides further confirmation that preconceptional nutritional status can impact embryo development. Whilst the dietary intervention did not result in an alteration in the CC2 rate, omega-3 FAs and vitamin D in the blood were increased and a demonstrable impact on the development of the pre-implantation embryo in the CC4 and S3 rate was observed. Further intervention studies of sufficient power are required to determine the optimal duration of a preconceptional dietary intervention containing omega-3 FAs, vitamin D and olive oil that might impact clinical outcomes.

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Author's roles

PCC and NSM conceived the study. AJK, MM, FDH, PCC and NSM designed the experiments. AJK, SJW and HLF performed the experiments. AJK, PL, MM and CO analysed the data. All authors were involved in the preparation of the manuscript. The corresponding author attests that all listed authors meet authorship criteria and that no others meeting the criteria have been omitted.

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Conflict of interest

MM is a consultant to Vitrolife AB, Sweden. PCC is an advisor to Smartfish, DSM, BASF AS, Danone/Nutricia Research, Friesland Campina, Cargill and Fresenius-Kabi. NM has received research grants and fees from Merck Serono, Ferring, IBSA, Schering Plough, Gedeon Richter, Anecova, ArtPRED, Abbott, Vivoplex. None of the other authors has any conflict of interest to declare in relation to this manuscript.

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499 **FIGURE LEGENDS**

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501 **FIGURE 1: Development of the embryo from the point of fertilization of the oocyte to morula formation**

502 (timings taken from averages for the general population (46)) (Images taken from PREPARE patient 157).

503 **FIGURE 2: CONSORT diagram for the trial.**

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