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ORIGINAL ARTICLE *Infertility*

Trimethylamine-N-oxide is present in human follicular fluid and is a negative predictor of embryo quality

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STUDY QUESTION: Are levels of trimethylamine-N-oxide (TMAO) in human follicular fluid (FF) related to IVF outcomes?

SUMMARY ANSWER: Higher levels of TMAO are a negative predictor of oocyte fertilization and embryo quality.

WHAT IS KNOWN ALREADY: TMAO is a metabolic product of dietary choline and L-carnitine produced via subsequent enzymatic modifications by the intestinal microbiota and hepatocytes. TMAO promotes inflammatory and oxidative stress pathways and has been characterized as a causative biomarker for the development of cardiometabolic disease.

STUDY DESIGN, SIZE, DURATION: For the present cross-sectional study, samples (FF and plasma) from 431 modified natural cycle (MNC)-IVF cycles of 132 patients were collected prospectively between October 2014 and March 2018 in a single academic medical center.

PARTICIPANTS/MATERIALS, SETTING, METHODS: TMAO and its precursors (choline, L-carnitine and gamma-butyrobetaine) were measured by ultra-high-performance liquid chromatography/mass spectrometry in (i) matched FF and plasma from 63 MNC-IVF cycles, in order to compare metabolite levels in the two matrices and (ii) FF from 232 MNC-IVF cycles in which only one oocyte was retrieved at follicular puncture. The association between metabolite levels and oocyte fertilization, embryo fragmentation percentage, embryo quality and the occurrence of pregnancy was analyzed using multilevel generalized estimating equations with adjustment for patient and cycle characteristics.

MAIN RESULTS AND THE ROLE OF CHANCE: The level of choline was higher in FF as compared to matched plasma ($P < 0.001$). Conversely, the levels of TMAO and gamma-butyrobetaine were lower in FF as compared to plasma ($P = 0.001$ and $P = 0.075$, respectively). For all metabolites, there was a positive correlation between FF and plasma levels. Finally, levels of TMAO and its gut-derived precursor gamma-butyrobetaine were lower in FF from oocytes that underwent normal fertilization (TMAO: odds ratio [OR] 0.66 [0.49–0.90], $P = 0.008$ per 1.0-µmol/L increase; gamma-butyrobetaine: OR 0.77 [0.60–1.00], $P = 0.047$ per 0.1-µmol/L increase) and developed into top-quality embryos (TMAO: OR 0.56 [0.42–0.76], $P < 0.001$ per 1.0-µmol/L increase; gamma-butyrobetaine: OR 0.79 [0.62–1.00], $P = 0.050$ per 0.1-µmol/L increase) than in FF from oocytes of suboptimal development.

LIMITATIONS, REASONS FOR CAUTION: The individual contributions of diet, gut bacteria and liver to the metabolite pools have not been quantified in this analysis.

WIDER IMPLICATIONS OF THE FINDINGS: More research on the contribution of diet and the effect of gut bacteria on FF TMAO is warranted. Since TMAO integrates diet, microbiota and genetic setup of the person, our results indicate potential important clinical implications for its use as biomarker for lifestyle interventions to improve fertility.

STUDY FUNDING/COMPETING INTEREST(S): No external funding was received for this project. The Department of Obstetrics and Gynecology of the University Medical Center Groningen received an unrestricted educational grant of Ferring Pharmaceutical BV, the Netherlands. The authors have no other conflicts of interest.

TRIAL REGISTRATION NUMBER: Netherlands Trial Register number NTR4409.

Key words: fertility / TMAO / microbiota / intestine / liver / gamma-butyrobetaine / L-carnitine / choline / oocyte

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Introduction

Patients attending infertility clinics often display features of an unhealthy lifestyle frequently manifesting as overweight and obesity (Broughton and Moley, 2017). In addition to lower fecundity, obesity has been associated with decreased rates of implantation, pregnancy and live birth in assisted reproduction (Jungheim and Moley, 2010; Bellver *et al.*, 2013; Kawwass *et al.*, 2016). One of the ways in which excessive nutrition influences reproductive outcomes is through a decrease in oocyte quality. Mice with diet-induced obesity had an increased number of apoptotic follicles, a decreased number and size of mature oocytes and displayed impaired mitochondrial function as compared to controls (Jungheim *et al.*, 2010; Grindler and Moley, 2013). Moreover, in human studies, oocytes from overweight and obese women were more frequently of lower quality and of reduced number and displayed phenotypic and metabolic abnormalities (Wittemer *et al.*, 2000; Marquard *et al.*, 2011; Leary *et al.*, 2015). In summary, changes in nutritional status impact fertility by inducing complex modifications in oocyte and embryo developmental potential, which are likely mediated by alterations in ovarian follicular conditions. During oocyte maturation, alterations in the oocyte's natural environment, the follicular fluid (FF), are known to impact oocyte development and maturation (Sutton *et al.*, 2003). Consequently, changes in FF composition have been related to decreased oocyte quality in obese women. Although women with higher BMI have been shown to have higher FF triglyceride, insulin and glucose levels than women with a normal BMI (compositional changes associated with decreased oocyte quality in animal models) (Valckx *et al.*, 2012; Yang *et al.*, 2012), in general such differences in FF composition do not seem to be directly related to BMI (Valckx *et al.*, 2012). Rather, metabolic and hormonal changes (e.g. alterations in steroid hormones and leptin levels, development of insulin resistance and excess free fatty acids) occurring as complications of an increased BMI seem to mediate the impact of obesity on female fertility (Broughton and Moley, 2017; Hallajzadeh *et al.*, 2018).

One of the components of lifestyle that might be involved in obesity-mediated subfertility is nutrition. However, thus far the picture of how diet impacts fertility is inconclusive, reflected by the fact that no nutritional guidelines have been established for sub/infertility. One of the underlying reasons could be that existing studies have focused on the pure composition of food, leaving out the diverse potential metabolic modifications that can occur by the intestinal microbiota or by enzyme systems of the host (Lynch and Pedersen, 2016).

The gut microbiota is an important regulator of health and disease, with major involvements in energy homeostasis, whole-body metabolism, obesity and onset of chronic disease, such as atherosclerosis (Vinje *et al.*, 2014; Lynch and Pedersen, 2016). Recently, two gut-derived metabolites, indole-3-propionic acid and shikimic acid, have been identified by untargeted metabolomics to be differentially abundant in FF from obese versus normal weight women (Ruebel *et al.*, 2019).

A relevant recent example of a metabolite integrating diet, microbiota and host metabolism is trimethylamine-N-oxide (TMAO) (Fig. 1). Dietary choline (present in, for e.g. dairy products, fish) and L-carnitine (present in, for e.g. red meat) are converted by the gut microbiota into trimethylamine (TMA) (Koeth *et al.*, 2013; Koeth *et al.*, 2014). TMA is a gas that is further metabolized by hepatic flavin monooxygenases (FMO) 1 and FMO3 into TMAO. TMAO is involved in a number of processes that influence cell and whole-body metabolism, such as coagulation, inflammation and sterol metabolism (Schugar and Brown, 2015; Zhu *et al.*, 2016; Chen *et al.*, 2017). Importantly, there is an increasing body of evidence that TMAO is a causative biomarker for the development of atherosclerosis, diabetes and overweight (Bogiatzi *et al.*, 2018; Heianza *et al.*, 2018; Zheng *et al.*, 2019; Zhuang *et al.*, 2019). Studies on TMAO from the cardiovascular field lend strong support to the idea that nutrition modulates the risk of metabolic syndrome through the effect of bacteria-derived metabolites. Despite the evidence for a relationship between nutrition, metabolic syndrome and infertility, the influence of gut metabolites on fertility has been scarcely studied. Therefore, in the present study, we investigated the relationship between FF TMAO and its precursors with oocyte and embryo quality in modified natural cycle (MNC)-IVF. In contrast to classic hyperstimulation IVF, in MNC-IVF only one oocyte is retrieved at follicular puncture, which makes it possible to clearly link FF composition to oocyte, embryo and pregnancy outcomes.

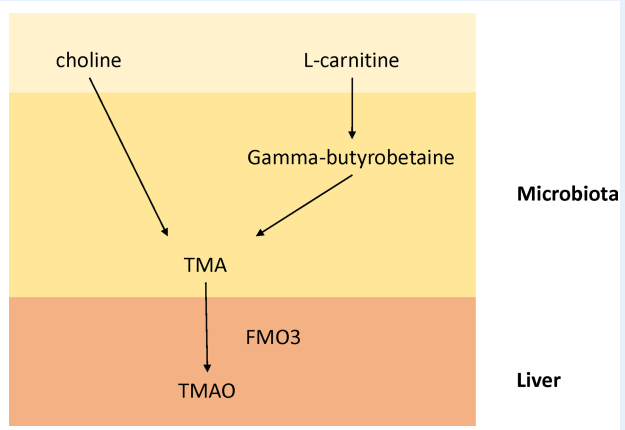

Figure 1 Schematic drawing of trimethylamine N-oxide synthesis. TMA, trimethylamine; TMAO; trimethylamine N-oxide; FMO, flavin-containing monooxygenase.

Materials and Methods

Sample and data collection

FF and plasma were prospectively collected from women enrolled in a large observational cohort study at the University Medical Center Groningen on the relationship between women's nutrition, serum and FF composition, and the outcomes of MNC-IVF (Netherlands Trial Register number NTR4409, first patient enrolled in September 2014). Material collected between October 2014 and March 2018 from women attending the fertility clinic for a maximum of six MNC-IVF cycles was used for the present study. Each study participant was assigned a non-traceable code under which the materials, cycle characteristics and outcomes of the fertility treatments were stored.

Fasted blood samples and FF were collected on the day of oocyte retrieval and stored at −80°C. After collection of the oocyte, FF was centrifuged for 20 min at 300*g* and the supernatant was stored at −80°C until needed for analysis. FF with any red color as sign of blood

contamination was discarded. For the present study, only samples from cycles in which one dominant follicle developed and one oocyte was retrieved were used.

Ethical approval

Ethical approval was obtained from the local Medical Ethics Committee (METc 2014/007, number NL47569.042.13) prior to start of the clinical study and signed consent was obtained from all study participants.

MNC-*in vitro* fertilization procedure and outcomes

MNC-IVF was performed following a standard protocol as previously described (Nagy *et al.*, 2015). In short, from cycle days 6–8 onwards, growth of the dominant follicle was followed by vaginal ultrasound and serum hormone levels (estradiol and luteinizing hormone) were measured regularly. Once the diameter of the dominant follicle reached 14 mm, self-administered, subcutaneous daily injections of 0.25 mg GnRH antagonist cetrorelix (Cetrotide®, Merck BV, The Netherlands) and of 150 IU recombinant FSH (r-FSH, Follitropin-alpha: Gonal-F®, Merck BV, The Netherlands) were started. Cetrorelix was administered up to and including the day of ovulation triggering and r-FSH up to the day of ovulation triggering. Ovulation was triggered by administration of 10 000 IU hCG (Pregnyl®, Organon, The Netherlands) when the size of the dominant follicle reached 18 mm and/or serum estradiol levels were higher than 0.8 nmol/L. Approximately 34 h later, the oocyte was retrieved by ultrasound-guided transvaginal follicle aspiration without either sedation or local anesthesia, with a single-lumen aspiration needle and without flushing of the follicle. The oocyte was inseminated either by incubation with culture medium containing spermatozoa or by intracytoplasmatic sperm injection (ICSI) (within 6 h of follicular puncture). On the morning of Day 1 after insemination/ICSI, the number of pronuclei was assessed. On Day 2 after insemination/ICSI, the cleavage, number of blastomeres, percentage of fragmentation and the presence of multinucleated blastomeres (MNBs) were assessed. Normal fertilization was defined as the presence of zero, one or two pronuclei on Day 1 after insemination/ICSI and the occurrence of cell cleavage on Day 2 after insemination/ICSI. Fertilization was considered abnormal if three pronuclei were present on the day after insemination/ICSI or if the embryo failed to cleave on Day 2 after insemination/ICSI, irrespective of the number of pronuclei. Top-quality embryos were defined as the presence of two pronuclei on Day 1 after insemination/ICSI and presence of four cells, absence of MNBs and less than 20% fragmentation on Day 2 after insemination/ICSI. Embryos that displayed abnormal fertilization or more than 40% fragmentation were discarded. Embryo transfer was performed on Day 2 after follicle puncture. For luteal support, 1500 IU hCG was administered on Days 5, 8 and 11 after oocyte retrieval. The occurrence of pregnancy was defined by a positive serum hCG test at 2 weeks after embryo transfer.

Measurements of TMAO and its precursors

In FF and plasma samples, TMAO and its precursors (choline, L-carnitine and gamma-butyrobetaine) were analyzed by ultra-high-performance liquid chromatography in combination with isotope dilution tandem mass spectrometry (UPLC-MS/MS) as previously published (Brandsma *et al.*, 2019).

Statistical analysis

Levels of TMAO and its precursors were expressed as mean ± standard deviation (for normally distributed variables) or median [interquartile range (IQR)] (for not normally distributed variables). Levels in matched FF and plasma were compared by paired sample testing. The relationship between l-carnitine, choline, gamma-butyrobetaine and TMAO in plasma and matched FF, as well as between diet-derived (L-carnitine and choline) and gut-derived metabolites (gamma-butyrobetaine and TMAO), was expressed as Pearson correlation coefficient (r, for normally distributed variables) or Spearman's rank correlation coefficient (r_s, for not normally distributed variables). Since FF samples from several cycles corresponding to the same patient were used, multilevel generalized estimating equation (GEE) analysis was used to study the relationship between measurements of TMAO and its precursors and the outcomes of MNC-IVF (Zeger and Liang, 1986). Confounders known from literature to affect embryo quality and pregnancy (i.e. maternal age, BMI and smoking) were included in the adjusted models. Additionally, cycle characteristics that were related to MNC-IVF outcomes were also included in the adjusted model. In order to avoid overfitting of the model, predictors with the highest P value were removed one by one until a model with 10 cases per predictor was obtained. Results of GEE analysis were presented as odds ratio (OR) [confidence interval (CI)] for the change in either 1 µmol/L (for choline, L-carnitine and TMAO) or 0.1 µmol/L (for gamma-butyrobetaine, since values are below 1 µmol/L). A P value lower than 0.05 was considered significant, except for the selection of possible confounders where a cut-off of 0.15 was used. SPSS version 23 (SPSS, Inc., Chicago, IL, USA) was used for data analysis.

Results

Study population characteristics

Between October 2014 and March 2018, FF and plasma were collected from 431 MNC-IVF cycles of 132 patients attending the fertility clinic of the UMCG (Fig. 2). FF samples with macroscopic blood contamination were discarded ($n = 68$). FF from cycles where either more than one follicle was punctured or more than one oocyte was retrieved were likewise discarded ($n = 14$). Finally, a small number of samples were lost during preparation for storage ($n = 5$). For the present analysis, cycles in which no oocyte was retrieved were excluded ($n = 107$). Further cases were excluded due to cycle number exceeding the standard maximum of 6 ($n = 1$) and due to the inability to track samples back in storage ($n = 4$). Finally, 232 cycles corresponding to 111 patients were selected for the present study. Cycle characteristics are detailed in Table I. In 143 cycles, embryo transfer took place, which resulted in 42 positive pregnancy tests.

Metabolites in plasma compared to FF

Firstly, we determined whether TMAO and its precursors (Fig. 1) are present in FF and how their levels compare to those in plasma (Supplementary Fig. S1). TMAO and its precursors were measured

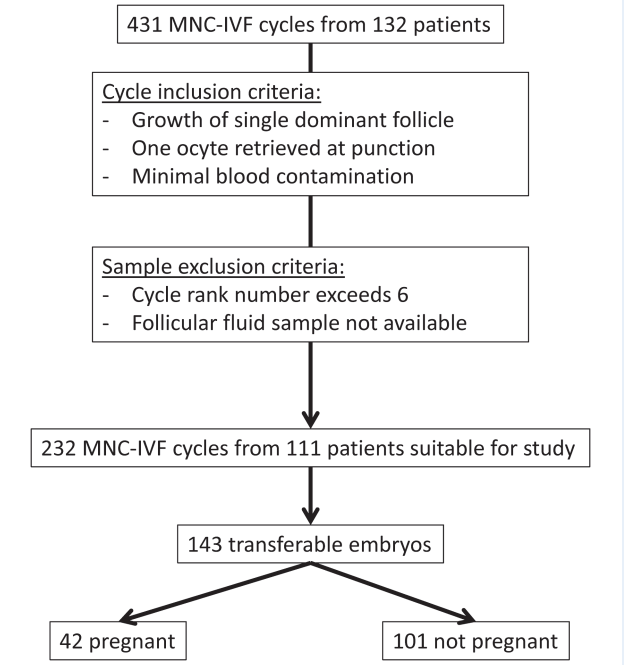

Figure 2 Flow diagram of cycle selection.

Table I Cycle characteristics ($n = 232$) corresponding to 111 subjects.

	Total group
Age (years)	31.4 ± 3.50
BMI (kg/m^2)	23.1 [20.92–25.69]
Smoking	
Yes	20 (9%)
Stopped before cycle	68 (29%)
No	144 (62%)
Alcohol consumption	
Yes	124 (53%)
No	108 (47%)
Duration of subfertility (months)[a]	36.5 [22.34–50.27]
Indication	
Male factor	160 (69%)
Tubal factor	31 (13%)
Unexplained	41 (18%)
Fertility treatment	
ICSI	195 (84%)
IVF	37 (16%)

Of note, materials from several cycles corresponding to the same patients were used.
[a]Values missing for six cycles corresponding to four patients.

and compared in FF and matched plasma from first cycle MNC-IVF procedures from 63 women (patient characteristics are described in Supplementary Table SI). The level of choline was significantly higher in FF as compared to matched plasma (FF: 25.4 [20.51–29.09] μmol/L versus plasma: 6.4 [5.56–7.32] μmol/L, $P < 0.001$). Conversely, the level of gamma-butyrobetaine was significantly lower in FF as compared to plasma (FF: 0.6 [0.51–0.66] μmol/L versus plasma: 0.7 [0.45–0.84] μmol/L, $P = 0.001$), and a similar though not significant trend was observed for TMAO (FF: 2.0 [1.57–2.93] μmol/L versus plasma: 2.1 [1.67–3.02] μmol/L, $P = 0.075$). There was no significant difference in L-carnitine levels between FF and plasma (FF 29.8 ± 7.39 μmol/L versus plasma 30.6 ± 6.58 μmol/L, $P = 0.175$). Finally, there were significant positive correlations between FF and plasma levels of TMAO ($r_s = 0.901$, $P < 0.001$), L-carnitine ($r = 0.787$, $P < 0.001$) and gamma-butyrobetaine ($r_s = 0.421$, $P = 0.001$), and a positive correlation with trend towards significance for choline ($r_s = 0.232$, $P = 0.067$, Supplementary Fig. S2).

Next, we studied the relationship between metabolites that are directly diet-derived and those arising following metabolism by microbiota and/or liver in FF. There was a positive though not significant relationship between FF choline and FF TMAO levels ($r_s = 0.128$, $P = 0.056$). While a significant positive relationship between FF L-carnitine and gamma-butyrobetaine was detected ($r = 0.295$, $P < 0.001$), no such association was present between FF L-carnitine and FF TMAO ($r_s = -0.007$, $P = 0.915$). Interestingly, there was no significant relationship between FF TMAO levels and patient BMI ($r_s = 0.041$, $P = 0.544$).

Relationship of metabolites in FF with embryo quality and pregnancy

Next, we investigated whether there was a significant relationship between TMAO, choline, L-carnitine and gamma-butyrobetaine in FF with embryo quality and the occurrence of pregnancy (Table II, Table III; $n = 232$). In 19 ICSI procedures, oocytes failed to progress from Metaphase I to Metaphase II and were thus not injected. From the oocytes that were injected, 13 oocytes subsequently degenerated. Finally, one oocyte was lost during removal of the granulosa cells in the IVF procedure. Hence, from the total 232 cycles selected for the present study, oocyte development after fertilization was followed in 199. Normal fertilization was observed in 144 of these cycles. One embryo displayed more than 40% fragmentation and was discarded. Finally, 143 embryos were transferred to the uterus for implantation, which resulted in 42 positive pregnancy tests. Of note, TMAO measurements from seven samples were excluded from analysis due to technical issues with mass spectrometry peak integration.

Levels of gamma-butyrobetaine and of TMAO were lower in FF from oocytes that underwent normal fertilization as compared to oocytes that did not (gamma-butyrobetaine: 0.57 [0.49–0.65] μmol/L versus 0.60 [0.55–0.69] μmol/L, OR [CI] for adjusted model: 0.77 [0.60–1.00], $P = 0.047$; TMAO: 2.06 [1.56–2.67] μmol/L versus 2.33 [1.72–3.41] μmol/L, OR [CI] for adjusted model: 0.65 [0.48–0.89], $P = 0.006$). Moreover, the levels of gamma-butyrobetaine and TMAO were lower in FF from oocytes that developed into top-quality embryos as compared to embryos of lower quality (gamma-butyrobetaine: 0.56 [0.49–0.63] μmol/L versus 0.58 [0.51–0.67] μmol/L, OR [CI] for adjusted model: 0.79 [0.62–1.00], $P = 0.050$; TMAO: 1.86 [1.33–2.56] μmol/L versus 2.25 [1.70–3.17] μmol/L, OR [CI] for adjusted model: 0.56 [0.42–0.76], $P < 0.001$). No significant relationships were observed between choline or L-carnitine and

Table II Embryo development and choline, L-carnitine, gamma-butyrobetaine and trimethylamine-N-oxide (TMAO) levels (µmol/L; median [quartiles]) in follicular fluid.

		Choline (µmol/L)	L-Carnitine (µmol/L)	Gamma-butyrobetaine (µmol/L)	TMAO[a] (µmol/L)
Normal fertilization [a]	Yes ($n = 144$)	26.2 [23.22–28.74]	30.8 [26.34–34.60]	**0.57 [0.49–0.65]**	**2.06 [1.56–2.67]**
	No ($n = 55$)	26.3 [23.33–29.77]	30.9 [25.41–35.71]	**0.60 [0.55–0.69]**	**2.33 [1.72–3.41]**
Fragmentation	Low (≤10%) ($n = 114$)	26.2 [23.15–28.49]	30.8 [26.47–34.57]	0.57 [0.49–0.64]	1.96 [1.59–2.66]
	High (>10%) ($n = 30$)	26.5 [23.55–29.84]	28.9 [26.09–35.54]	0.58 [0.51–0.68]	2.27 [1.43–2.73]
Top quality embryo	Yes ($n = 70$)	26.5 [23.72–28.55]	30.1 [25.52–35.04]	*0.56 [0.49–0.63]*	**1.86 [1.33–2.56]**
	No ($n = 129$)	26.2 [23.23–29.05]	30.8 [26.16–35.27]	*0.58 [0.51–0.67]*	**2.25 [1.70–3.17]**
Positive pregnancy test[c]	Yes ($n = 42$)	27.5 [24.62–29.86]	31.1 [26.58–35.51]	0.59 [0.49–0.66]	2.01 [1.45–2.66]
	No ($n = 101$)	25.4 [23.15–28.16]	30.4 [25.50–34.14]	0.56 [0.49–0.63]	2.07 [1.61–2.71]

Bold: significant difference ($P < 0.05$); italics: trend ($0.10 > P > 0.05$).
[a]Seven TMAO measurements were excluded due to problems with peak integration upon mass spectrometry analysis.
[b]Thirteen oocytes degenerated after injection and were thus excluded from analysis.
[c]Per embryo transfer.

outcomes of MNC-IVF procedures. Additional analysis of embryo quality including the oocytes that failed to progress to metaphase II and the ones that degenerated after injection led to similar conclusions (Supplementary Table SII and Supplementary Table SIII). Combined, these data indicate that microbiota-dependent metabolites, especially TMAO, are significant predictive biomarkers for unfavorable IVF outcomes, independent of patient BMI.

Discussion

The results of the present study demonstrate that TMAO (i) is present in FF at lower levels than those in plasma and (ii) is associated with unfavorable fertility outcomes. Despite their abundant presence in FF, there was no relationship of the direct dietary precursors of TMAO, choline and L-carnitine, with oocyte and embryo quality. Importantly, TMAO and the intermediate gamma-butyrobetaine were significantly lower in FF from oocytes that developed into top-quality embryos than in FF corresponding to lower-quality embryos. Taken together, these results suggest that gut metabolites derived from the diet enter FF and may influence oocyte development, thus providing a new perspective for studying the influence of diet and gut microbiome on fertility.

To the best of our knowledge, this is the first report of TMAO and gamma-butyrobetaine in the context of fertility. The lower levels in FF as compared to plasma, as well as the positive correlation between measurements in the two matrices, indicate that FF TMAO and FF gamma-butyrobetaine likely originate from the blood compartment by diffusion. Importantly, lower levels of both were associated with positive outcomes of MNC-IVF. Although in general observed differences were small, previous clinical observations from the vascular and renal field related to TMAO suggest that such changes might be biologically meaningful (Gruppen *et al.*, 2017; Bogiatzi *et al.*, 2018; Wu *et al.*, 2018). Moreover, the observed inverse association between embryo quality and TMAO levels is in line with previous reports on the negative impact of TMAO on health in the context of cardiometabolic disease. Specifically, TMAO was associated with an increased incidence of cardiovascular events by promoting thrombus formation and vascular inflammation and by reducing reverse cholesterol transport (Wang *et al.*, 2011; Koeth *et al.*, 2013; Zhu *et al.*, 2016; Chen *et al.*, 2017; Zhu *et al.*, 2017). Importantly, these effects of TMAO on the cardiovascular system are dependent on the gut microbiota. Atherosclerotic plaque formation in susceptible mouse models fed a choline-rich diet was inhibited after suppression of the intestinal microflora, demonstrating that it is not the dietary intake but the resulting bacterial metabolites that exert the negative health effects (Wang *et al.*, 2011). Similarly, in the present study we did not find any significant relationship of FF levels of L-carnitine and choline (dietary components which have not been altered by gut bacteria) with IVF outcomes. However, there was a positive relationship between these diet-derived metabolites and levels of FF TMAO and FF gamma-butyrobetaine. It can be envisioned that recording the dietary intake of choline and L-carnitine in addition to FF levels could provide complimentary clinical information. All in all, these data lend strong support to the concept that diet may have an indirect effect on fertility mediated by the gut microbiota.

Nonetheless, the present study did not find any relationship between FF metabolites and the occurrence of pregnancy. Although this may be surprising at first, given the relationship between TMAO and gamma-butyrobetaine with embryo quality, pregnancy is the result of a multitude of factors, such as spermatozoa quality and endometrial receptivity (Miravet-Valenciano *et al.*, 2015; Zhang *et al.*, 2016). Moreover, the pregnancy rate per cycle of MNC-IVF is lower than in hyperstimulation-IVF, and thus the present study might have an insufficient number of pregnancies to detect a significant effect of FF metabolites on pregnancy occurrence (Allersma *et al.*, 2013). Replication in larger cohorts could address this question.

Levels of L-carnitine in FF and matched plasma were comparable and correlated, indicating that likely diffusion between the two compartments takes place. Total and free carnitines have been previously reported to be present in FF at levels higher than those in serum, which is in contradiction with our results (Valckx *et al.*, 2012). The reason for this discrepancy may be due to the use of different hormonal treatments. In contrast to the present study, where MNC-IVF was used, hyperstimulation IVF uses much higher

Table III Generalized estimating equations analysis of the relationship between embryo development in Modified Natural Cycle-IVF and ICSI and follicular fluid content of choline, L-carnitine, gamma-butyrobetaine and TMAO.

	Choline		L-Carnitine		Gamma-butyrobetaine[a]		TMAO	
	Unadjusted model	Adjusted model	Unadjusted model	Adjusted model	Unadjusted model	Adjusted model	Unadjusted model	Adjusted model
Normal fertilization[a]	0.97 [0.89–1.05] $P = 0.411$	0.97 [0.90–1.06] $P = 0.515$	0.97 [0.93–1.03] $P = 0.312$	0.97 [0.93–1.03] $P = 0.320$	**0.75 [0.59–0.95]** **$P = 0.018$**	**0.77 [0.60–1.00]** **$P = 0.047$**	**0.67 [0.49–0.90]** **$P = 0.007$**	**0.66 [0.49–0.90]** **$P = 0.008$**
Low fragmentation[b]	1.00 [0.89–1.11] $P = 0.937$	1.00 [0.90–1.12] $P = 0.960$	1.02 [0.95–1.09] $P = 0.566$	1.03 [0.96–1.11] $P = 0.446$	0.80 [0.53–1.20] $P = 0.281$	0.77 [0.50–1.20] $P = 0.256$	1.15 [0.68–1.97] $P = 0.604$	1.19 [0.67–2.09] $P = 0.556$
Top quality embryo[c]	0.99 [0.93–1.06] $P = 0.865$	0.99 [0.92–1.06] $P = 0.725$	0.98 [0.94–1.03] $P = 0.405$	0.97 [0.93–1.02] $P = 0.291$	*0.81 [0.64–1.02]* *$P = 0.076$*	*0.79 [0.62–1.00]* *$P = 0.050$*	**0.56 [0.42–0.74]** **$P < 0.001$**	**0.56 [0.42–0.76]** **$P < 0.001$**
Positive pregnancy test[d]	1.06 [0.96–1.16] $P = 0.280$	1.07 [0.97–1.19] $P = 0.163$	1.02 [0.96–1.09] $P = 0.467$	1.04 [0.98–1.10] $P = 0.213$	1.25 [0.91–1.73] $P = 0.166$	1.30 [0.95–1.76] $P = 0.100$	0.86 [0.60–1.23] $P = 0.411$	0.93 [0.64–1.34] $P = 0.677$

Results are presented as odds ratio (95% CI) and *P* value corresponding to a change of 1.0 µmol/L except for gamma-butyrobetaine where calculation was based on a change of 0.1 µmol/L.
[a]Normal fertilization—models were adjusted for BMI, smoking, fertility treatment (choline); age, BMI, smoking (L-carnitine); age, BMI, alcohol consumption, fertility treatment (gamma-butyrobetaine); fertility treatment, indication (TMAO).
[b]Low fragmentation—models were adjusted for BMI.
[c]Top quality embryo—models were adjusted for: age, smoking, alcohol consumption, indication (choline, L-carnitine, gamma-butyrobetaine); smoking, alcohol consumption, indication (TMAO).
[d]Positive pregnancy test—models were adjusted for maternal age, indication (choline, TMAO); indication, duration of subfertility (L-carnitine, gamma-butyrobetaine).

hormonal dosages which leads to the development of multiple dominant follicles and may also alter FF composition. Finally, in the present study, no relationship between FF levels of L-carnitine and outcomes of IVF was found, which is in partial agreement with previous literature (Montjean *et al.*, 2012). Although one study reports no relationship between FF total carnitine levels and pregnancy rate, L-carnitine has been suggested to be beneficial in the management of infertility as it may exert antioxidant, anti-apoptotic and anti-inflammatory effects (Montjean *et al.*, 2012; Agarwal *et al.*, 2018). Daily oral supplementation of L-carnitine in women with clomiphene-resistant polycystic ovarian syndrome (PCOS) resulted in higher ovulation and pregnancy rates (Ismail *et al.*, 2014). Nonetheless, in the respective study, no plasma or FF measurements were performed; hence, it is unclear whether the beneficial effect was exerted directly by circulating L-carnitine or by its metabolites. In animal IVF studies, supplementation of oocyte culture medium with L-carnitine was linked to improved oocyte quality, suggesting that deprivation of oocytes of L-carnitine during IVF may be accountable for a decrease in oocyte quality (Agarwal *et al.*, 2018). However, *in vivo*, especially in the light of our results concerning the L-carnitine metabolite TMAO, it remains difficult to disentangle the direct from the indirect effects of L-carnitine. More research, also including microbiota analyses, seems warranted before issuing a recommendation for L-carnitine supplementation.

In contrast to the other measurements, the level of choline in FF was consistently higher than that in plasma, indicating that substantial enrichment in FF occurs. Despite this, no relationship between FF choline and outcomes of IVF was found, which is somewhat surprising. Metabolic profiling of FF revealed choline/phosphocholine as a discriminating metabolite between FF from oocytes that failed to cleave and those that underwent normal cleavage (Wallace *et al.*, 2012). Once again, this may be due to differences in hormone levels between MNC-IVF and classic hyperstimulation IVF.

An important advantage of the current study is the use of material collected prospectively from MNC-IVF, which is closer to normal physiology than classical hyperstimulation IVF and allows for the correlation of FF composition to oocyte and embryo characteristics. Moreover, the present study highlights the presence and importance of TMAO in FF and provides evidence for a possible role of the gut microbiota in fertility. Nonetheless, the present study has certain limitations. Firstly, oocyte and embryo quality assessed during IVF procedures is a reflection of the fully matured, fertilized oocyte. However, oocyte maturation is a complex biological process that extends over a long period, and it is technically not possible to study the dynamic changes in FF composition that occur over time. Moreover, embryo quality is also influenced by factors related to spermatozoa, which have not been taken into account in the present study. Another limitation of our single-center study is that it is not possible to differentiate between exogenous, dietary sources of L-carnitine and choline and endogenous production. Moreover, in the present study we have not quantified the individual contributions of the gut bacteria and the liver to TMAO production. Although technically very challenging, in future studies, it could be useful to distinguish the differential contribution of the different dietary components and of each step (diet composition, bacterial metabolism, hepatic metabolism) to TMAO production. Further, given that the microbiota is altered in obese subjects (Ley *et al.*, 2006) and the present study has been conducted in

a normal-weight population, replication in an overweight/obese population seems desirable in order to extrapolate and generalize the clinical importance of a relationship between nutrition, microbiota and fertility. The results of such studies would allow for a better understanding of the (patho)physiological role of TMAO in fertility and hence the development of biomarkers of (in)fertility. Moreover, the development of nutritional interventions to modulate TMAO levels and activity or of pharmacological inhibitors of the formation of its precursor TMA may increase reproductive success in both assisted and natural reproduction (Wang *et al.*, 2015).

In summary, the present study shows that TMAO is present in FF and that its levels have the potential to serve as a negative predictive biomarker of embryo quality. These results position the microbiota as an important factor to modulate and integrate dietary cues with host metabolism to influence oocyte maturation and embryo development. Further studies are warranted to gain more insight into the mechanistic role of TMAO in oocyte development. Moreover, a better understanding of FF TMAO homeostasis may allow for the design of tailored lifestyle interventions taking account of the complex interplay between diet and microbiota with the ultimate goal to improve reproductive success.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

R.A.N. contributed to the study design, execution, analysis, manuscript drafting and critical discussion. I.H. contributed to the study design, execution and critical discussion. C.J.., F.L. and J.L.C.A. contributed to the execution and critical discussion. A.H. contributed to the study design, execution, analysis, manuscript drafting and critical discussion. U.J.F.T. contributed to the study design, execution, analysis, manuscript drafting and critical discussion.

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Conflict of interest

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