High-resolution ^1H -NMR spectroscopy indicates variations in metabolomics profile of follicular fluid from women with advanced maternal age

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Abstract

Aim To reveal whether there are differences in follicular fluid metabolomics profile of women with advanced maternal age (AMA).

Method The group with advanced maternal age includes 23 patients above the age of 40, and the control group includes 31 patients aged between 25 and 35 years and AMH values above 1.1 ng/mL with no low ovarian response history. A single follicular fluid sample from a MII oocyte obtained during the oocyte pick-up procedure was analyzed with high-resolution ^1H -NMR (nuclear magnetic resonance) spectroscopy. The results were evaluated using advanced bioinformatics analysis methods.

Results Statistical analysis of the NMR spectroscopy data from two groups showed that α -glucose and β -glucose levels of follicular fluid were decreased in the patients with AMA, while in contrast, lactate and trimethylamine N-oxide (TMAO) levels were increased in these patients compared with the controls. In addition to these, there was an increase in alanine levels and a decrease in acetoacetate levels in patients with AMA. However, these changes were not statistically significant.

Conclusion Obtained results suggest that the follicular cell metabolism of patients with AMA is different from controls. These environmental changes could be associated with the low success rates of IVF treatment seen in these patients.

Keywords Metabolomics · NMR · Advanced maternal age · Follicular fluid

Introduction

Female reproductive organs undergo a more rapid aging process than other bodily systems. A woman's reproductive capacity peaks from 24 to 25 years of age, and following this peak, there is a reduction for all mammals, including humans [1]. This gradual reduction in fertility displays a dramatic increase at the end of the 30s contrary to the continuation of the

ovulatory cycle and ends with the menopause at mean age of 50–51 years [2]. Increasing socioeconomic level, active inclusion of women at higher rates in the workforce, and increasingly late age of marriage have made ovarian aging one of the most important causes of infertility [3].

Results obtained from in vitro fertilization (IVF) studies similarly revealed that a woman's age is undoubtedly the most important factor in predicting IVF clinical outcomes. Data analyzing the outcomes of 120,000 IVF cycles found the live birth rate per embryo transfer fell from 43.2% under the age of 35 to 15.1% in the 41–42-year age group to 5.9% after 42 years [4]. Though neuroendocrine and uterine factors may affect the reduction in age-related fertility, the main factor in the fall in reproductive potential is ovarian aging. Ovarian aging is primarily related to reductions in both ovarian follicle numbers and oocyte quality [2]. In clinically older patients, disrupted oocyte competence is associated with increased embryo aneuploidy rates [5], high pregnancy losses [6], reduced response to ovarian stimulation, and reduced live birth rates after IVF [7]. These findings are confirmed by the age-related reduction in female fertility being resolved by oocyte donation [8].

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The process of the oocyte gaining sufficient maturity for embryo development (competence) occurs within the ovarian follicle. Blood plasma products in follicular fluid (FF) pass the blood follicular barrier, comprising theca and granulosa cell secretions and molecules released by the oocyte [9]. Follicular fluid ensures the oocyte remains in meiotic arrest and aids ovulation and fertilization [10]. Therefore, analysis of follicular fluid content could help to find an appropriate biomarker for prediction of oocyte maturity, fecundity, and embryonic development [11, 12]. Omics technologies (like genomics, proteomics, metabolomics) provide an opportunity to identify potential biomarkers in follicular fluid [13]. Among these, metabolomics has some potential advantages to evaluate metabolomics changes within the organism [14]. High-resolution NMR spectroscopy is the most widely utilized technology to study metabolomics of biological fluids [15]. High-resolution NMR spectroscopy has no known disruptive effects on biological samples, and it does not require an extensive sample preparation step. Moreover, NMR spectroscopy allows the same sample to be analyzed repeatedly which is not possible for other technologies [16, 17]. On the other hand, the limited resolution provided by NMR spectroscopy in small sample amounts could be avoided with carefully designed experiments comprising gradient-based specific pulse sequences such as CPMG, WET, and WATERGATE [16, 18]. Owing to its above-mentioned advantages, NMR spectroscopy recently has been used in several studies to determine specific biomarkers of a number of diseases like endometriosis and polycystic ovary syndrome (PCOS) [19, 20].

Though theories about aging being a result of both hereditary and environmental factors are accepted in general, it is commonly accepted that loss of function related to age is a result of accumulation of irreparable damage to biomolecules [21]. Within this context, the proposed study utilizes high-resolution ^1H -NMR spectroscopy to reveal whether there are changes in the follicular fluid metabolite profile with advanced maternal age (AMA) and if this is the case, to find the potential biomarkers associated with ovarian aging.

Method

Study population

This study includes follicular fluid samples collected from a total of 54 patients. Of these patients, 23 were in the AMA group (≥ 40 years), and 31 were patients aged between 25 and 35 years with normal ovarian reserve. This study was completed from July 2016 to January 2019 in the IVF Unit of the Reproductive Endocrinology and Infertility, School of Medicine, Inonu University. All patients included in the study provided written informed consent, and the study protocol

was permitted by the Clinical Research Ethics Committee (Number 2015/38).

Inclusion criteria for the study were basal FSH level < 10 mIU/mL on the 2nd–3rd day of cycle and body mass index 21–29.9. Additionally, inclusion criteria for the study included no previous ovarian operation history and no chronic, autoimmune and endocrine diseases (such as diabetes mellitus, thyroid, hypertension) or gonadotoxic (radiotherapy or chemotherapy) treatment history. The women with symptoms such as chronic anovulation, chronic pelvic pain, dysmenorrhea, with known PCOS, endometriosis, and tubal diseases like hydrosalpinx were excluded from the study. The couples with an IVF failure history and those who have a male factor infertility were also excluded from this study.

The samples were collected at about the same time of the day (between 9:30 and 10:00 am) during oocyte pick-up procedure. All samples were taken after a fasting period of at least 8 to 10 h.

Ovarian hyperstimulation

After patients provided informed consent, patients began controlled ovarian hyperstimulation treatment on the 2nd day of their menstrual cycle. Due to the GnRH antagonist fix protocol, patients began gonadotropin treatment. In the midfollicular phase of stimulation (5th–6th day of cycle), GnRH antagonist treatment was begun in addition to gonadotropins. Patients' follicular development was monitored by transvaginal ultrasonography every 2–3 days. When follicular mean diameter was over 17 mm with 3 or more follicles developing, hCG was administered. Then, 34–38 h after hCG administration, oocyte pick-up was completed with transvaginal ultrasonography. Oocyte and embryo evaluation was performed. Following the intracytoplasmic sperm injection procedure, embryo transfer was completed 2–3 days after oocyte pick-up. Luteal phase support began 24–48 h after oocyte pick-up and continued in clinically pregnant cases for at least 7 weeks. All cases had serum pregnancy test applied on the 15th–20th day following hCG administration (biochemical pregnancy). Cases with positive pregnancy test and no abortus had ultrasonographic examination for fetal pregnancy sac and fetal heart beats on the 35th–42nd day after hCG administration (evaluation of clinical pregnancy).

All patients included in the study had controlled ovarian stimulation treatment. GnRH antagonist was administered until three follicle diameters were above 18 mm; then, 250 μg rhCG (Ovitrelle Chorigonadotropin alpha, Merck-Serono, Geneva, Switzerland) was administered, and oocytes were collected under general anesthesia accompanied by ultrasonography 34–36 h after human chorionic gonadotropin administration.

Fluid content from a single follicle above 18 mm was centrifuged for 10 min at 10,000g. The content of a single follicle

with MII oocyte identified above 18 mm was analyzed, with the supernatant collected and stored at -80°C until analysis.

¹H-NMR analysis of follicular fluid samples

Follicular fluid samples collected from the AMA and control groups for high-resolution ¹H-NMR spectroscopy analysis were first dissolved at room temperature. Four hundred microliters from each follicular fluid sample was mixed with 200 μl of deuterium oxide which is needed for “field-frequency-lock.” The prepared sample was centrifuged at 5000 rpm for 5 min to homogenize the mixture and to remove any impurities. Then 550 μl of this mixture was placed into 5-mm-diameter NMR tubes.

All ¹H-NMR experiments were performed using a 600-MHz Bruker Avance III HD NMR spectrometer equipped with a 5-mm-diameter BBFO probe and at instrumental temperature setting of 296 K. 1D (one dimensional) ¹H-NMR experiments of all samples were recorded using the Bruker T2 filter CPMG (Carr-Purcell-Meiboom-Gill) pulse sequence. FID (free induction decay) acquisitions were acquired in 15 min using a 2.34-s acquisition time, 128 transients, a relaxation delay of 4.0 s, and CPMG echo delay of 0.3 ms. Signals were collected in 32K data points with a sweep width of 11.7 ppm. The investigated follicular fluid metabolites were identified by using various published literatures [22, 23].

Metabolomics analysis of NMR data

Metabolomics analysis of the NMR data comprises pre-processing and statistical analysis steps. For both the pre-processing and statistical analysis steps, MATLAB® R2016a was used. In the pre-processing step, first, the data were normalized by dividing each spectrum with the mean value of total spectral area of control samples. Next, chemical shift region 4.60–4.90 ppm was set to zero to suppress the effect of varying water resonance. After these steps, the dimension of the NMR spectroscopy data was reduced by using the so-called binning method. Binning method is used to be ensured a particular peak remains in its own bin, and an overlap with the peak of a succeeding compound is thus prevented. Small chemical shifts that may occur during the NMR spectrum acquisition process are also prevented with the binning procedure. For this purpose, 25 binning regions were determined over the range of 0.0–9.0 ppm by manual investigation of the spectra. A list of the determined binning regions, corresponding peak assignments, and metabolite names is provided in Table 2. For each spectrum, total area under each bin was computed, and for each bin, a peak location assignment was made. Because the lengths of the binned regions are different, each peak intensity was normalized by the length of the binned region. After these steps, the data were scaled by unit variance scaling.

Statistical analysis of the NMR data from the AMA and control groups was performed in two different steps. In the first step, a multivariate statistical analysis of the data was performed with the principal component analysis (PCA) and the partial least squares discriminant analysis (PLS-DA). PCA score and loading plots were interpreted to find out whether there exists a separation between two groups. Similarly, PLS-DA also provides a visual interpretation of the data through the two-dimensional score plot that illustrates the separation between different classes. In addition to the score plot, PLS-DA provides several other statistics such as variable importance in projection (VIP) that highlights the importance of each variable in projection and R^2 and Q^2 statistics, which help us to evaluate the predictive ability of the PLS-DA model. A permutation test (200 times) was also performed for the statistical validation of the PLS-DA model.

Once the important metabolites that are thought to have a role in the separation of two groups were identified by PCA and PLS-DA, in the second step, a *t* test procedure was used to compare the normalized peak intensities of each metabolite from two groups. The *t* test procedure along with the boxplots of the normalized peak intensity distributions helped us to find out if the level of a certain metabolite was decreased or increased in a particular group.

Results

Clinical data and treatment cycle summaries for patients are presented in Table 1. As expected, there were significant differences between the age and antral follicle numbers between the AMA and control group. In contrast, there was no statistically significant difference identified between two groups in terms of BMI and 3rd day serum hormone levels including FSH.

Total gonadotropin dose was statistically higher in the AMA group compared with controls. Oocyte numbers, mature (metaphase II) oocyte numbers, and embryo numbers were lower in the AMA group compared with the controls. Though live birth rates were lower in the AMA group, this difference was not statistically significant Table 2.

High-resolution ¹H-NMR spectra of follicular fluid samples for each group are shown in Fig. 1. Multivariate statistical analysis performed on pre-processed spectral data with PCA and PLS-DA shows a separation between the AMA and control samples. In Fig. 2 a and c, score plots obtained from PCA and PLS-DA are shown respectively. In PCA, the first component accounts for 53.02% of overall variability, and the second component accounts for 15.99% of overall variability, which corresponds to a cumulative R^2 value of 0.6902. On the other hand, the PLS-DA model has a R^2X value of 0.6750, R^2Y value of 0.3790, and Q^2 value of 0.1376. The Q^2 value was

Table 1 Clinical data and treatment cycle summaries of patients

Variables	AMA group <i>n</i> = 23	Controls <i>n</i> = 31	<i>p</i> value
Age (year)	41.3 ± 1.6	30.4 ± 2.5	< 0.001
BMI (kg/m ²)	26.1 ± 3.1	24.4 ± 2.6	0.06
Duration of infertility (year)	7.6 ± 5.7	6.1 ± 2.7	0.82
FSH (mIU/mL)	7.7(6.6–9.4)	6 (5.1–8.0)	0.26
LH (mIU/mL)	4.1 (2.4–5.8)	6.0 (3.3–8)	0.03
E ₂ (pg/mL)	46 (24–65)	49 (34–58)	0.67
PRL (ng/mL)	10.6 (6.7–18)	13.1 (9.5–19.7)	0.13
TSH (ng/ml)	1.7 (1.1–2.7)	1.5 (1.1–2.5)	0.89
Total gonadotrophin dose (IU)	2700 (2025–3600)	1575 (1350–2025)	< 0.001
Duration of induction (day)	9 (8–10)	9 (8–10)	0.34
Endometrial thickness on hCG (mm)	10.8 (8.9–12.3)	11.3 (10–12.3)	0.44
Number of oocytes retrieved	4 (3–10)	12 (10–15)	< 0.001
Number of mature oocytes	3 (2–7)	10 (7–12)	< 0.001
Number of fertilized oocytes	3 (3–4.5)	7 (5.5–9)	< 0.001
Number of cleaved embryos	3 (2–3)	6 (4–7)	< 0.003
Live birth rate	3/23 (13%)	9/31 (29%)	0.200

computed by using the leave-one-out process. The PLS-DA model exhibits 77.42% sensitivity and 73.91% specificity results in the leave-one-out cross-validation process. PCA loading plot (Fig. 2b) and PLS-DA VIP plot (Fig. 2d) suggest that the separation between the AMA and control groups is mainly based on the metabolites α -glucose, β -glucose, lactate, and trimethylamine N-oxide. The permutation test (200 times) performed with the PLS-DA model (Fig. 2e) intersects the positive *y* axis for the R^2Y values and negative *y* axis for the Q^2 values which are mostly smaller than the original values. These results indicate that the achieved results are not due to the over fitting.

A heat map of the pre-processed spectra shown in Fig. 3 provides a visual inspection of the data in which the contribution of each bin to the separation of the two groups could be investigated. As shown in this figure, there are two big clusters, and it is clear that, in the lower cluster, glucose level is highly increased whereas in the upper cluster, lactate level is highly increased which supports our findings. Next, a boxplot of the normalized peak intensities for each metabolite was plotted, and a *t* test was performed to find out whether these differences are statistically significant (Fig. 4). *t* test results statistically validated that the levels of trimethylamine N-oxide (TMAO) and lactate were increased in the AMA group compared with the controls. On the other hand, the levels of α -glucose and β -glucose were found to be decreased in the AMA group. In addition to these, there was an increase in alanine levels and a decrease in acetoacetate levels in patients with AMA. However, these changes were not statistically significant.

Discussion

The results of this study revealed that metabolomics profile of follicular fluid from women with AMA is different than the one obtained from the control group. The difference was based on the α -glucose, β -glucose, lactate, and trimethylamine N-oxide levels.

A limiting factor of this study is that it includes a relatively low number of patients. On the other hand, to the best of our knowledge, this is the first study that utilizes the high-resolution NMR spectroscopy to reveal the effect of AMA on the metabolomics profile of follicular fluid. However, in literature, there exist some other studies utilizing different technologies to analyze the proteomics and lipidomics profile of follicular fluid from women with AMA [24, 25].

Within the cumulus oocyte complex (COC), glucose can be metabolized via four pathways: glycolysis, the pentose phosphate pathway (PPP), the hexosamine biosynthesis pathway (HBP), and polyol pathway [26]. A large proportion of total glucose is metabolized by the glycolytic pathway to provide substrates for energy production. It is known that glycolysis is high in cumulus cells [27, 28]. However, oocytes use mainly aerobic metabolic pathways, and glycolysis is almost undetectable in this cell [29]. Cumulus cells metabolize glucose, producing glycolytic metabolites such as pyruvate and/or lactate, which can be further metabolized by the oocyte [30, 31]. In oocyte, these molecules are metabolized to produce adenosine triphosphate (ATP) via the tricarboxylic acid cycle (TCA) and oxidative phosphorylation, which is the predominant pathway for ATP generation [32].

Table 2 Binning regions and corresponding peak assignments with target metabolite names

	Binning region (ppm)	Peak assignment (ppm)	Metabolite/metabolites
1	(0.7, 0.8)	0.75	Lipid
2	(0.8, 0.96)	0.90	Valine- γ 1H, valine- γ 2H
3	(1.07, 1.11)	1.09	Threonine- γ H
4	(1.18, 1.24)	1.20	Lactate- β H
5	(1.28, 1.40)	1.31	Alanine- β H
6	(1.80, 1.82)	1.81	Acetate- β H
7	(1.83, 2.05)	1.93	Glycoprotein
8	(2.11, 2.13)	2.12	Acetoacetate- γ H
9	(2.16, 2.38)	2.26	Pyruvate- β H
10	(2.39, 2.45)	2.41	Aspartic acid- β 1H
11	(2.56, 2.62)	2.59	Aspartic acid- β 1H
12	(2.87, 2.96)	2.90	Creatine-NCH, creatinine-NCH
13	(3.07, 3.21)	3.15	Choline-NCH ₃ , glycerophosphocholine-NH, Phosphocholine-NH
14	(3.23, 3.26)	3.24	Trimethylamine N-oxide (TMAO)-CH ₃
15	(3.27, 3.33)	3.30	α -Glucose-H ₄ , taurine-H ₂ S, proline- δ 2H, β -glucose-H ₄
16	(3.33, 3.40)	3.36	β -Glucose-H ₅
17	(3.4, 3.46)	3.43	α -Glucose-H ₂ , α -glucose-H ₅
18	(3.51, 3.68)	3.60	α -Glucose-H ₃
19	(3.69, 3.76)	3.73	α -Glucose-H ₆ , serine- α H
20	(3.76, 3.84)	3.80	β -Glucose-H ₆
21	(3.85, 3.90)	3.87	Tyrosine- α H, serine- β 2H, histidine- α H, phenylalanine- α H, serine- β 1H, asparagine- α H
22	(3.93, 3.96)	3.95	Creatinine- α H
23	(3.97, 4.05)	4.01	Lactate- α H
24	(4.52, 4.55)	4.53	β -Glucose-H ₁
25	(5.11, 5.15)	5.12	α -Glucose-H ₁

This study found that glucose content of follicular fluid was lower in women with AMA compared with the controls.

Glucose is an important metabolite which is used as an energy source. Previous studies have shown follicular development

Fig. 1 ^1H CPMG (Carr-Purcell-Meiboom-Gill) NMR spectra recorded at 600 MHz and 296 K. ^1H -NMR spectra of follicular fluid from patients with AMA and control samples

Fig. 2 Principal component analysis (PCA) scores (a) and loading (b) plots and partial least squares discriminant analysis (PLS-DA) scores (c) and variable importance in projection (VIP) values (d) for the patients with AMA and control samples. In the PCA, the first component accounts for 53.02% of overall variability, and the second component accounts for 15.99% of overall variability which corresponds to a cumulative R^2 value of 0.69. The PLS-DA model has an R^2X value of 0.6750, R^2Y value of 0.3790, and Q^2 value of 0.1376. Both the PCA and PLS-DA models show a good separation between the spectral data of AMA (circle) and control (square) samples. Important peaks are shown in the PCA loading plot (b) and PLS-DA VIP plot (d). A permutation test (e) validates the robustness of the PLS-DA model

and steroidogenesis are linked to glucose concentration, and therefore, low glucose levels delay folliculogenesis [26, 33]. Folliculogenesis and steroidogenesis basically use the glycolytic pathway.

In this study, follicular fluid lactate concentration was found to be increased in the AMA group compared with the controls. Follicular fluid has very high lactate concentration. This elevation is associated with increased anaerobic metabolism in the hyperstimulated cycle [34]. In a study by Harlow et al. [35], it was shown that the increase in follicular fluid lactate levels may be linked to increased glycolytic activity in granulosa cells. Increased level of lactate is also associated with increased energy requirements

in situations where oxygen is insufficient for the follicles growing during the hyperstimulated cycle [36]. However, the cells did not particularly rely on lactate for their energy requirements [37].

Similar to the results of this study, a study using the microfluorometric method showed that glucose concentration in follicular fluid was statistically decreased compared with controls in the group with low ovarian reserve and the group with advanced maternal age. In contrast, follicular fluid lactate concentrations were significantly increased in both patient groups compared with the controls [38]. The same study revealed increased glucose intake and increased lactate production in a culture environment for the granulosa

Fig. 3 A heat map of the pre-processed spectra provides a visual inspection of the data to better evaluate the contribution of each bin to the separation of the two groups. There are two big clusters, and the difference between two groups is mainly because of the changed levels of lactate and glucose metabolites (see Table 2)

and cumulus cells of both low ovarian reserve and AMA groups compared with the granulosa and cumulus cells of controls.

This study showed that carbohydrate metabolism exhibits variations in patients with AMA. In general, decreased follicular glucose levels and increased lactate levels seen in AMA patients may indicate that follicular glycolysis is upregulated. Decreased follicular fluid glucose levels were shown to negatively affect oocyte nuclear and cytoplasmic maturation [26]. Increased glycolysis and defects in the mitochondrial respiratory system in patients cause increased oxidative stress which may be primarily responsible for disrupted oocyte quality and folliculogenesis [39]. Additionally, increased follicular lactate level causes a reduction in the pH of follicular fluid. The pH of follicular fluid is alkali, and reduced pH is associated with reduced fertilization rates [40, 41].

In this study, there was no significant difference identified for FSH levels between two groups. Though some follicular fluid markers may be associated with age, FSH alone and other tests are not sufficient to predict weak response or inability to remain pregnant after treatment. In patients with regular menstruation, only excessive FSH values may be beneficial to predict poor response [42, 43]. Ovarian aging begins years before the elevation in FSH level. Normal FSH value does not exclude poor response [44].

Another important metabolite, level of which exhibits significant differences between two groups, was the trimethylamine N-oxide (TMAO). In a number of studies, high levels of trimethylamine N-oxide is widely associated with obesity, diabetes (including gestational diabetes mellitus), preeclampsia, cardiovascular diseases, and

colon cancer [45–48]. A recently published study showed that serum TMAO level is increased by age which is consistent with our findings [49]. However, a large number of follicular fluid samples need to be analyzed to further validate the potential role of TMAO as a biomarker in ovarian aging.

Clinically accurate determination of ovarian aging is important to predict pregnancy in patients planning to get pregnant both spontaneously and after fertility treatment and to inform patients accurately. The increase in a woman's age and reduction in oocyte quality may cause changes in follicular fluid content. Pregnancy rates after IVF reduce with age, with a significant reduction after 40 years. As expected, in our study, the AMA group had lower oocyte numbers and less mature metaphase II oocyte numbers obtained. Though there was no statistically significant difference in clinical pregnancy rates, the live birth rates were higher in the younger group. This lack of difference may be due to the low number of patients included in the study.

Conclusion

This study utilized high-resolution ^1H -NMR spectroscopy combined with advanced bioinformatics analyses to reveal whether the follicular fluid metabolomics profile of women with AMA is different from the control group. Analyses showed that the follicular fluid metabolomics profile of women with AMA exhibit differences in glucose and lactate metabolism.

Fig. 4 A *t* test procedure was used to validate whether the found important peaks from the PCA loading plot and the PLS-DA VIP plot are statistically significant. Boxplots of the normalized peak intensities for statistically significant metabolites help to better evaluate the differences between two groups

The difference in the follicular fluid metabolomics profile of AMA group could be associated with the disrupted follicular cell metabolism. It is necessary to better understand metabolic pathways associated with age to better enlighten the effect of advanced maternal age on oocyte quality. Towards this aim, metabolomics studies led to an increase in our knowledge for elucidating the underlying causes of ovarian

aging. However, better understanding of follicular fluid metabolism, explaining the factors causing disrupted oocyte quality in these patients, and development of appropriate treatment protocols and culture environments will be possible with additional studies tackling this research area.

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Compliance with ethical standards

All patients included in the study provided written informed consent, and the study protocol was permitted by the Clinical Research Ethics Committee (Number 2015/38).

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