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FERTILITY PRESERVATION

NMR metabolomics study of follicular fluid in women with cancer resorting to fertility preservation

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Abstract

Purpose The purpose of this study was to evaluate the possible application of metabolomics to identify follicular fluid changes in cancer patients undergoing fertility preservation. Although metabolomics have been applied already in cancer studies, this is the first application on follicular fluid of cancer patients.

Methods We selected for the study ten patients with breast cancer and lymphoma who resorted to oocyte cryopreservation to preserve fertility and ten healthy women undergoing in vitro fertilization treatments. Follicular fluid was collected at the time of oocytes retrieval. Metabolomic analysis of follicular fluids was performed by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy in combination with multivariate analysis to interpret the spectral data. Univariate statistical analysis was applied to find correlations between patients' features and metabolites identified by NMR.

Results Partial least squares discriminant analysis allowed to discriminate samples from cancer patients and healthy controls. Univariate statistical analysis found significant correlations between patients' features and metabolites identified by NMR. This finding allowed to identify biomarkers to differentiate both healthy controls from cancer patients and the two different classes of oncological patients.

Conclusion The follicular fluids of cancer patients display significant metabolic alterations in comparison to healthy subjects. NMR-based metabolomics could be a valid prognostic tool for identifying and selecting the best cryopreserved oocytes and improving the outcome prediction in cancer women undergoing in vitro fertilization.

Keywords Metabolomics · NMR · Follicular fluid · Cancer · Fertility preservation · Biomarkers

Introduction

Most cancers are lethal and metabolic alterations are considered a hallmark of the disease. Tumor progression is accompanied by different metabolic events, some of which facilitate the processes of tumor invasion and metastasis to distant organs.

Among different types of cancer, breast cancer is the most frequent malignancy that affect women in fertile age worldwide [1]. Due to the progresses in oncostatic treatments, women affected by cancer have improved survival rates and a good chance of having a normal post-cancer life. However, infertility is one of the most devastating long-term consequences for patients of reproductive age [2]. Cancer patients are at risk of losing ovarian function due to adverse effects of the undergoing cancer therapies. Chemotherapy and radiotherapy are the common supports of cancer treatments. Nonetheless, both methods can damage the ovary depending on the agent used, dose given, and age of the patient [3]. Therefore, the demand for fertility preservation strategies has sharply increased [4–9]. Frequently, breast cancer patients ask to be submitted to controlled hormonal ovarian stimulation in order to retrieve and

The authors consider that the first two authors should be regarded as joint First Authors

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cryostore oocytes [10]. Fertility preservation is also applied for hemato-oncology patients undergoing treatment [11].

At the time of the diagnosis of cancer, due to the urgency of finding effective treatment, the potential gonadotoxic effects of cancer or its treatment are often not discussed. However, it is of crucial importance that health care providers give all information to patients about fertility preservation options [6]. Oocyte cryostorage is a valuable tool for fertility preservation worldwide, as no surgery is required and minimally invasive ovarian stimulation protocols are needed [12–14]. By means of oocyte cryostorage and in vitro fertilization (IVF), many children were born also in non-oncological routine IVF patients [15].

Cancer research is one of the largest fields in the life sciences. Studying cancer through metabolomics could reveal new biomarkers that might be useful for future prognosis, diagnosis, and therapy. Metabolomics have been applied in many cancer studies for finding biomarkers associated with the state of cancer [16–22].

The two leading analytical approaches to metabolomics are mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. Despite its lower sensitivity, NMR spectroscopy offers many unique advantages because it allows to observe and rigorously quantify all of the more abundant compounds present in biological samples without the need for elaborate sample preparation or fractionation [23].

Follicular fluid (FF) is the in vivo environment of the oocytes; it contains important metabolites for oocyte growth and development and its composition may indicate oocyte and embryo quality. Besides, FF is a superfluous product which is easily available during oocyte pickup in standard in vitro fertilization (IVF) procedure; it could be also an optimal predictor of oocyte and embryo developmental potential [24].

In the present study, we performed a metabolomics analysis of FF samples from cancer patients who decided to resort to oocyte cryopreservation in order to preserve their fertility. ^{1}H-NMR spectroscopy was used to analyze FF in combination with multivariate analysis to interpret the spectral data. The aim of the study was to compare the FF metabolic profile of cancer patients with that of healthy controls. This could be useful in the future selection of the best oocytes to be used for in vitro fertilization.

To the best of our knowledge, this is the first metabolomics study on FF of cancer patients.

Materials and methods

Patients

This study was approved on October 2015 from the local Ethics Committee. Written informed consent was obtained from all study participants in accordance with the Declaration of Helsinki.

From May 2016 to December 2017, 20 women were involved in the study. A first group consisted in 10 oncological patients, with pathological diagnosis of breast cancer ($N = 6$) or lymphoma ($N = 4$). These patients decided to use oocyte cryopreservation because they were at risk of losing their fertility due to the need of undergoing cancer therapy. We included all nulliparous patients who want to have offspring, aged 22 to 39 years, with a cancer diagnosis obtained by histological examination and with a definitive staging of the disease. We excluded patients with suspected cancer diagnosis without histological examination and complete staging and women suffering of any cancer that received neoadjuvant chemotherapy or radiation therapy prior to FF collection.

The second group consisted in ten healthy women undergoing treatment for IVF at the Center for Reproductive Medicine. These women did not suffer from cancer or other diseases. Their infertility indication was by mild to moderate male factor. The clinical information of participants in the study is summarized in Table 1.

Breast cancer patients received a different ovarian hyperstimulation protocol compared to patients with lymphoma and healthy controls. Patients with breast cancer were treated with the Oktay protocol [25] which includes the association of aromatase inhibitors during the use of gonadotropins and gonadotropin-releasing hormone (GnRH) antagonist.

The use of aromatase inhibitors, with the exception of breast cancer patients, is considered off-label in controlled ovarian hyperstimulation cycles [26].

Lymphoma cancer patients and healthy controls received stimulation with recombinant or urinary highly purified follicle-stimulating hormone (FSH) and gonadotropin-releasing hormone (GnRH) antagonist.

Both in cancer patients and in healthy controls, follicular stimulation was started on day 2 of the menstrual cycle with a FSH dose calculated according to the nomogram of La Marca [27].

The follicular growth was monitored with ultrasound scans and estradiol and progesterone assessment, first on day 5 and then every 2 days. Daily administration of a GnRH antagonist was started when the leading follicle was 14 mm in diameter and continued until the day of the trigger of the ovulation.

When at least two follicles had reached 17–18 mm in diameter, ovulation was triggered with a single subcutaneous bolus of 0.2 mL of buserelin in cancer patients and 10.000 IU of highly purified hCG in healthy controls. We induced the final oocyte maturation of cancer patients with a GnRh agonist to completely eliminate the risk of ovarian hyperstimulation syndrome before starting chemo-radiotherapy. In healthy controls we have triggered with hCG because the

Table 1 Characteristics of the women participating in the study

		Oncological	Healthy control
N. patients		10	10
Median age (range)		34.5 (22–39)	36.5 (28–42)
Estradiol (pg/mL)		806.7* (430.9)	1721.7 (1229.0)
Progesterone (ng/mL)		2.0 (1.5)	1.3 (0.9)
BMI (kg/m^2)		22.3 (2.5)	22.5 (3.4)
N. follicles monitored		15 (11)	12 (4)
N. oocytes collected		11 (8)	9 (6)
N. MII oocytes		8 (7)	7 (5)
N. MI oocytes		3 (3)	2 (2)
Tumor characterization			
Total breast cancer patients			6
	Stadiation	T1N0M0	4
		T1N1M0	1
		T2N0M0	1
	Patient's pathological grade	I	1
		II	2
		II	3
Total lymphoma patients			4
	Subtypes	Hodgkin lymphoma	2
		Non-Hodgkin lymphoma	2
	Stage	IIA	1
		IIIA	1
		IV	1
		IB	1

Data are presented as mean values and standard deviation are reported in parentheses

* Significant level (p value < 0.05)

agonist induction prevents the transfer of fresh embryos [28] due to the rapid ascent rate of the LH peak and therefore the luteal phase defect.

The oocytes retrieval was performed after 34–36 h. The collection of cumulus-oocyte complexes and FF was performed via transvaginal ultrasound-guided aspiration with a needle of 18 gauges in diameter.

Sample preparation

The aspirated FF was centrifuged at 10000 rpm for 10 min to remove erythrocytes and leukocytes. The supernatant was collected and maintained frozen at − 80 °C until processing. Only FF samples not contaminated by the flushing medium during the aspiration procedure were used in the analysis.

NMR spectroscopy

FF samples were defrosted at room temperature before using. Six hundred microliters of the supernatant was mixed with 58 μL of D_2O and 5 μL of 3-trimethylsilyl propionic acid-d_4 sodium salt (TSP). TSP was used as chemical shift reference ($\delta = 0$).

^{1}H-NMR spectra were acquired on a Varian 500-MHz spectrometer. The temperature during all experiments was kept at 25 °C. No sample rotation was applied.

NMR spectra were acquired using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence to suppress the signals originating from macromolecules, with a 136-ms total spin echo time. A pre-saturation of the water peak was used. One hundred and twenty-eight scans and 16-K points were acquired with a spectral width of 5995 Hz and a recycle delay of 5 s. The spectra were Fourier transformed with FT size of 32 K and a 1-Hz line-broadening, phased, and a polynomial baseline correction was applied over the whole spectral range.

The software ACD/1D NMR Processor (Academic Edition, ACD Labs, Canada) was used for processing all the spectra and producing integral buckets of 0.04 ppm. The TSP signal and the region 4.7–5.1 ppm, around water signal, were excluded. The integrated region was normalized to the total spectrum area.

Metabolites responsible for sample differentiation were identified using data from literature or from the data banks HMDB (http://www.hmdb.ca/) and BMRB (http://www.bmrb.wisc.edu/metabolomics/).

Statistical methods

Multivariate analysis

Multivariate statistics were performed using SIMPCA-P+ (version 12, Umetrics, Sweden). The analyses included principal component analysis (PCA) and projection to latent structures regression discriminant analysis (PLS-DA). The overall quality of the models obtained by PLS-DA was evaluated by the R^2 and Q^2 values, where R^2 measures the goodness of fit and displays the explained variation by components and Q^2 gives an indication of the goodness of predicted model. Permutation testing was performed to ensure that the discrimination between classes was not due to overfitting of the data.

Univariate analysis

Univariate statistics was used to assess the ability of NMR variables, identified as most important by PLS-DA models, to discriminate classes. Normality testing was performed with the Shapiro-Wilk normality test. Data are reported as means ± SD. Student's t test, Welch's t test, or Wilcoxon's test were

performed to obtain pairwise comparisons between control and pathology groups. Comparisons among all classes were performed with the Kruskal-Wallis rank sum test. Correlation between variables was calculated by using the Pearson correlation test. Significance was assumed whenever $p < 0.05$.

The analysis was carried out using the statistical package R (version 3.1.3).

Results

Subject characteristics

We examined FF from ten healthy control and ten oncological women, suffering of breast cancer ($N = 6$) or lymphoma ($N = 4$). Clinical features of the women included in this study are reported in Table 1, together with the characteristics of tumors in oncological patients. A difference between the two groups was found for estradiol that is significantly lower in oncological women ($p < 0.05$).

However, no more significant differences were evident between the groups for the other clinical parameters.

Characteristic ^{1}H-NMR spectra from FF of healthy control and oncological patients were obtained. It is possible to visually observe differences in the relative intensities of some signals in the spectra reported in Supplemental Fig. S1, representative of the two groups (a healthy control; b oncological patient).

Discrimination of different types of patients

To get an overview of the separation between groups, we performed further analysis by PCA. PCA is a non-supervised analysis that is used to combine several variables into a few principal components that help to explain the variability within the experiment and to demonstrate the tendency of group separation. PCA of the FF spectral data did not identify outliers and gave a four-component model with cumulative R^2 and Q^2 values of 0.74 and 0.21, respectively. From a visual inspection of the PCA score plot, it was not detected a clear tendency of the samples to separate according to groups (Supplemental Fig. S2).

The results of discrimination were strengthened by PLS-DA. Initially, we constructed a three-class model dividing the data in healthy, breast cancer, and lymphoma cohorts. However, the quality of this model was very poor (data not shown). Therefore, we decided to consider two separate models with the healthy control subjects versus (i) breast cancer patients and (ii) lymphoma patients. The PLS-DA score plots of the two models are shown in Fig. 1a, b, respectively. The healthy control group was discriminated from breast cancer group by a three-component model, with an R^2X(cum) of 0.56, an R^2Y(cum) of 0.89, and a Q^2 of 0.25, and from lymphoma patients by a two-component model, with an R^2X(cum) of 0.48, an R^2Y(cum) of 0.75, and a Q^2 of 0.38.

We also tried to discriminate the two cancer types but the quality of the model was poor due to the small number of patients (data not shown). Finally, we decided to consider all oncological patients as a single class. The calculated two-component model showed that the first two principal components contributed positively to discriminate the healthy and oncological groups (R^2X(cum) = 0.39, R^2Y(cum) = 0.76, and $Q^2 = 0.35$, Fig. 1c). The validity of this model was confirmed by permutation testing, which demonstrated that the R^2 and Q^2values of the model are higher than those of permuted models (Fig. 1d).

Although the number of subjects included in this study is limited, these results suggest that FFs of cancer patients display metabolic alterations in comparison to healthy subjects, independent of the type of tumor.

Discriminating metabolites

As far as the metabolite identification is concerned, PLS-DA generated a list of 19 signals with variable importance in the projection (VIP) values > 1, which represent potential metabolites useful for the discrimination of healthy controls and oncological patients. The 12 metabolites identified by NMR as discriminant for healthy control and oncological women are reported in Supplemental Table S1. Among them, five metabolites resulted significatively different in the two groups: citrate, creatine, glycerol, glycerophosphocholine, and glucose, with the higher levels of these metabolites observed in cancer patients.

Further analysis was performed to investigate if there were correlations between NMR data and clinical parameters of healthy control women and oncological patients (Table 2). Significant positive correlations were detected between (i) progesterone and some lipidic signals; (ii) BMI and asparagine and phenylalanine; (iii) number of follicles and creatine; and (iv) number of retrieved metaphase I (MI) oocytes and citrate, asparagine, phenylalanine, and glucose. A significant negative correlation was found between BMI and citrate.

The Pearson correlations were also calculated between different clinical parameters and between clinical parameters and metabolites identified by NMR for the different classes of women examined (Table 3). Only in healthy subjects, estradiol, which is higher than in oncological patients, positively correlates with progesterone which, in turn, positively correlates with glucose. Moreover, a negative correlation in both classes of oncological patients was observed between estradiol and glucose.

In healthy subjects, a positive correlation was observed between the total number of oocytes and lipids; in breast cancer patients, but not in lymphoma patients, the total number of oocytes correlates with glucose (negatively). In lymphoma patients, as well as in healthy women, a positive correlation

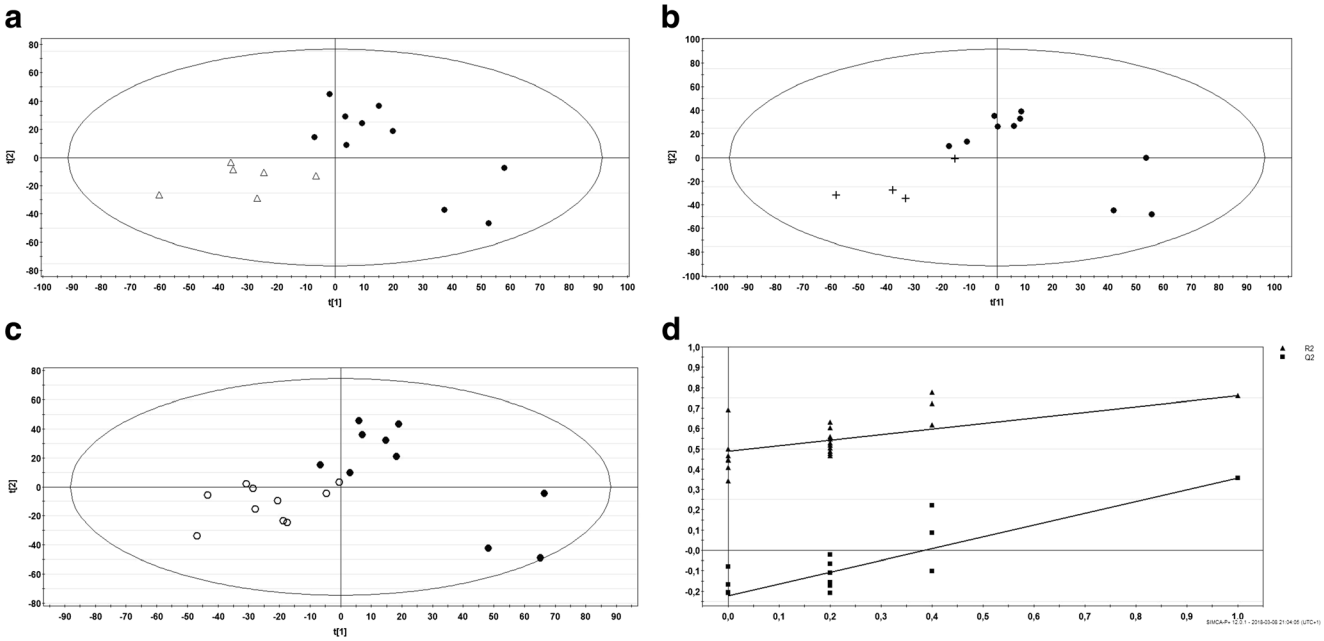

Fig. 1 PLS-DA score plot based on ^{1}H-NMR spectra of follicular fluid samples from women examined in this study: **a** healthy control ($N = 10$, dots) and breast cancer ($N = 6$, triangles); **b** healthy control ($N = 10$, dots) and lymphoma ($N = 4$, crosses); **c** healthy control ($N = 10$, dots) and all oncological patients ($N = 10$, circles); **d** cross-validation of the PLS-DA model reported in (**c**) by permutation testing (20 permutations)

is observed between the number of MI oocytes and asparagine and a negative correlation with lipids (which is positive in healthy women). In breast cancer patients, a positive correlation was found between the number of MI oocytes and glucose. In healthy and breast cancer patients, there is no correlation between the number of MII oocytes and any metabolite; on the contrary, a negative correlation with glucose in lymphoma patients was found.

Table 2 The Pearson correlation coefficients between clinical parameters and metabolites identified by NMR in follicular fluids of the women examined in the study. In parentheses, the p values smaller than 0.05 are reported

Clinical parameter	NMR (ppm)	Metabolite	Coefficient
Progesterone			
	1.28	Lipid	0.60 (0.005)
	2.24	Lipid	0.64 (0.002)
BMI			
	2.48	Citrate	− 0.49 (0.031)
	2.84	Asn	0.67 (0.002)
	3.12	Phe	0.61 (0.006)
Number of follicles			
	3.92	Creatine	0.61 (0.005)
Number of MI oocytes			
	2.66	Citrate	0.62 (0.003)
	2.84	Asn	0.73 (0.0002)
	3.12	Phe	0.61 (0.004)
	3.88	Glucose	0.48 (0.033)

Discussion

In the last years, FF has been investigated as an ideal candidate for non-invasive method of oocyte selection in fertility clinics [29]. Metabolic profiling of FF has been applied to find biomarkers of oocyte developmental competence [30] and to find also specific panels of biomarkers related to some infertility pathologies [31–35].

In this study, we demonstrated that there are some differences in the ^{1}H-NMR spectra between FF from healthy controls and cancer patients. Breast cancer and lymphoma patients were examined. A different hormonal stimulation was used for breast cancer patients in comparison with lymphoma and healthy subjects.

Different metabolites were identified on the basis of NMR signals; among them, five metabolites were detected at significantly different levels in fluid samples between oncological patients and healthy controls. We found that cancer is associated with metabolism disorder of amino acids, lipids, organic acids, and glucose.

In particular, in oncological patients, we found a decrease in the contents of asparagine, aspartate, proline, cholesterol, choline, lactate, and lipids together with significant increased levels of citrate, creatine, glycerol, glycerophosphocoline, and glucose in comparison with healthy subjects. These findings suggest that in IVF for oncological patients, the cryopreserved oocytes should be preferred whose FF metabolic profiles are more similar to those of healthy subjects.

The different composition of the metabolites, such as the drop in lactate level and the increase in glucose level, could be

Table 3 Pearson correlation coefficients between different clinical parameters and between clinical parameters and metabolites identified by NMR in follicular fluids of the women examined, subdivided in three groups: healthy control ($N = 10$), breast cancer ($N = 6$), and lymphoma ($N = 4$). In parentheses, the p values smaller than 0.05 are reported

Parameter	NMR (ppm)	Metabolite	Coefficient
Healthy control			
Estradiol			
Progesterone			0.65 (0.042)
	2.88	Asparagine	− 0.64 (0.047)
	3.64	Glycerol	0.77 (0.009)
	3.68	Glycerophosphocholine	0.70 (0.025)
	3.80	Aspartate	− 0.64 (0.045)
Progesterone			
	4.66	Glucose	0.66 (0.036)
Total number of oocytes			
	2.74	Lipid	0.65 (0.041)
Number of MI oocytes			
	2.74	Lipid	0.81 (0.004)
	2.84	Asparagine	0.83 (0.003)
	3.12	Phenylalanine	0.76 (0.011)
Breast cancer patients			
Estradiol			
Number of follicles			0.89 (0.045)
	1.00	Valine	0.96 (0.011)
	1.04	Valine	0.88 (0.049)
	2.04	Proline	0.89 (0.045)
	2.12	Glutamine	0.92 (0.029)
	2.24	Lipid	0.91 (0.034)
	4.66	Glucose	− 0.90 (0.038)
	5.20	Glucose	− 0.91 (0.031)
Number of follicles			
	3.92	Creatine	0.98 (0.0009)
Total number of oocytes			
	5.20	Glucose	− 0.86 (0.029)
Number of MI oocytes			
	3.24	Glucose	0.82 (0.047)
Lymphoma patients			
Estradiol			
Number of follicles			0.98 (0.024)
	3.52	Glucose	− 0.99 (0.003)
Number of follicles			
	3.52	Glucose	− 0.98 (0.012)
Number of MII oocytes			
	3.52	Glucose	− 0.95 (0.047)
Number of MI oocytes			
	2.76	Lipid	− 0.99 (0.014)
	2.88	Asparagine	0.96 (0.042)
	3.16	Choline	0.96 (0.037)

the result of the symbiosis between tumor cells and normal cells as well as the activity of follicle cells that contribute to the composition of follicular fluids [36].

In spite of having received the same hormonal stimulation, healthy subjects show different FF metabolic profiles from lymphoma patients, thus indicating that the differences are probably due to cancer and are not related to drugs.

It is also interesting to observe that in the three classes of subjects analyzed, there is a different trend of the correlations between some clinical parameters (estradiol and progesterone)

or other important parameters (such as the total numbers of follicles and oocytes recovered, the number of MI and MII oocytes) with some metabolites. The analysis of these correlations also makes it possible to identify biomarkers that differentiate the healthy controls from cancer patients but also breast cancer and lymphoma patients.

Conclusion

To our knowledge, this study shows for the first time that FF has a different metabolic composition in healthy controls and cancer patients. The NMR analysis, if confirmed on a larger cohort, could be a valid prognostic tool for identifying and selecting the best cryopreserved oocytes and improving the outcome prediction in cancer women undergoing IVF.

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Author contributions S.S., A.I., and A.O. designed the study. A.I. selected the patients and executed oocyte retrieval. DP executed oocyte retrieval. A.F. identified oocytes in follicular fluid and executed their fertilization. P.C. selected the follicular fluids to be used for metabolomic analyses. C.M. performed the analysis of NMR data and multivariate analysis. L.V. ran the NMR experiments. F.C. performed statistical analysis. A.O., A.I., and C.M. were responsible for conducting the study and writing the manuscript which was critically discussed, edited, and approved by all co-authors.

Compliance with ethical standards

This study was approved on October 2015 from the Ethics Committee of Basilicata region, Comitato Etico Unico Regionale (CEUR).

Conflict of interest The authors declare they have no conflict of interest.

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