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Molecular profiling of follicular fluid miRNAs in young women affected by Hodgkin Lymphoma

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Author Contributions: A.C. and R.B. designed and performed molecular biology experiments. P.B. and F.L. carried out the sample collection. M.E.V. and P.B. critically reviewed the study proposal. D.B. and M.R. contributed to the computational analysis. P.S. and M.P. contributed to the critical revision of the manuscript. M.E.V. and C.D.P. conceived and designed the study. C.D.P., A.C. and R.B. analyzed, interpreted the data and wrote the manuscript. All authors read and approved the final manuscript.

Abstract

Research question: Therapies for Hodgkin Lymphoma (HL) have improved in the last few years but one of their common effects is gonadal toxicity that contributes to fertility damage of patients inducing temporary or irreversible loss of fertility. Could miRNA expression profiles in Follicular Fluid (FF) be influenced by HL? Could their alteration have an impact on molecular pathways involved in follicle growth and oocyte maturation?

Design: In this study, miRNA expression profile was investigated in FF samples from young women affected by HL with respect to healthy controls by NanoString technology. Bioinformatic analysis was performed to verify miRNA involvement in follicle development and miRNA deregulation with HL in a larger cohort of FF samples was confirmed by RT-qPCR.

Results: 13 miRNAs are dysregulated in HL samples with respect to controls and are involved in molecular pathways related to cancer, gametogenesis and embryogenesis. Among them, let-7b-5p, miR-423-5p, miR-503-5p, miR-574-5p and miR-1303 are implicated in biological processes related to follicle development and oocyte maturation. Let-7b-5p holds the central position in the regulatory network of miRNA-mRNA interactions, has the highest number of mRNA target genes shared with the other DE miRNAs and is significantly down-regulated in HL FF samples.

Conclusions: These data represent the first molecular analysis on FF of young women with HL and led us to wonder about the potential influence of miRNA deregulation on oocyte quality. Further studies are needed to verify the reproductive potential of young patients with HL before starting chemotherapy protocols and provide them with a guarantee of an adequate protocol of fertility preservation.

Keywords: Hodgkin Lymphoma, miRNA profiling, Let-7b-5p, follicular fluid.

Introduction

Hodgkin lymphoma (HL) is one of the most frequent cancers among young adults in developed countries. Advanced early diagnosis and treatments have lowered its mortality rate, resulting in a high cure rate and a long survival time for patients affected by this malignancy (Kogel and Sweetenham, 2003).

Short cycles of chemotherapy combined with radiotherapy and combination chemotherapy are generally used to treat patients with early and advanced stage HL, respectively (Johnson and Longley, 2017). These treatments could affect the quality of life of survivor patients. In particular, one of their most common effects is gonadal toxicity. Although therapies against HL are effective, they may contribute to fertility damage of patients inducing temporary or irreversible loss of fertility (Melo et al., 2019, Policiano et al., 2020). Due to chemotherapy and radiotherapy women affected by HL are exposed to an increased risk of early menopause and ovarian follicle depletion (Morgan et al., 2012). In fact, although the diagnostic and therapeutic advances in oncology have improved the quality of life of young cancer patients, treatments may affect the ovarian reserve causing a major risk of ovarian failure and infertility (Spears et al., 2019). The extension of the damage to the ovary depends on several factors, such as type and dose of chemotherapy, radiotherapy dose, fractionation scheme, irradiation field, and the ovarian reserve before treatment (Wallace et al., 2005, Meirow et al., 2010). Anti-Müllerian hormone (AMH) and antral follicle counts (AFC) are considered helpful predictors of ovarian damage after cancer therapy (Gracia et al., 2012).

To avoid complications regarding future fertility, selected strategies of treatment are required, and especially young women should be informed about the possibility of fertility preservation before starting cancer treatments (De Vos et al., 2014). Currently, fertility preservation techniques for woman patients are oocyte cryopreservation, *in vitro* fertilization for embryo cryopreservation, and cryopreservation of ovarian tissue (Melo et al., 2019). In young women affected by hematological cancers, oocyte cryopreservation seems to be the best option since most of them have not yet planned whether and when to create a family and the preservation of ovarian tissue may reintroduce residual malignant cells (De Vos et al., 2014). It appears clear that many factors should be considered when approaching future reproduction.

Notwithstanding some papers reporting data on full-term pregnancies after the thawing of vitrified oocytes, results are still insufficient (Kato et al., 2021). Information regarding the numbers of women who use their frozen oocytes, pregnancy and live birth rates should be collected to ensure the usefulness of fertility preservation techniques (*https://www.eshre.eu/en*).

Hematological malignancies, including HL, are diffuse tumors and could affect a woman's reproductive potential resulting in a reduced ovarian reserve and/or a lower oocyte quality even before the start of chemotherapy (Fabbri et al., 2011, Lawrenz et al., 2012, Lekovich et al., 2016, Paradisi et al., 2016, Ozdemir and Bozdag, 2020). However, to the best of our knowledge, there are no research studies regarding the follicular microenvironment and oocyte quality in young women with HL.

Cell-free microRNAs (miRNAs) have been extensively studied in HL and different papers have reported significant expression differences between cancer and unaffected patients (Jones et al., 2014, van Eijndhoven et al., 2016, Cordeiro et al., 2017, Khare et al., 2017). In the same way, follicular fluid (FF) miRNA profiles have been associated with the outcome of IVF cycles and with reproductive disorders affecting oocyte quality in human and animal models (Scalici et al., 2016, Machtinger et al., 2017, Martinez et al., 2018). Moreover, alterations of miRNA expression in FF have also been reported in advanced maternal age (Diez-Fraile et al., 2014, Battaglia et al., 2020). This evidence supports the hypothesis that FF miRNAs may represent molecular markers of oocyte quality.

The aim of this study was to investigate if FF miRNA profiles could be influenced by HL and if miRNA expression differences may alter molecular pathways involved in follicular growth and oocyte maturation and, thus, the quality of the female mature gamete.

Materials and Methods

FF sample collection

Female patients enrolled in this study had been treated with GnRH agonists (Triptorelin or Buserelin) to induce multiple follicular development; ovarian stimulation was then performed using recombinant follicle-stimulating hormone (FSH) and human menopausal gonadotropin (hMG). Stimulation was monitored using serum E2 concentrations and ultrasound measurement of follicle number and diameter. Ovulation was induced with 10,000 IU of hCG only when follicles had reached a diameter >18 mm and serum E2 concentra-

tion per follicle reached 150–200 ng/L. Transvaginal ultrasound-guided aspiration of ovarian follicles was performed 34–36 h after hCG injection. We included in the control group healthy women whose primary infertility was due to a male factor; it excluded pathologies that could influence oocyte quality (e.g., endometriosis, polycystic ovaries, and ovarian insufficiency). Moreover, we excluded from the study heavy smokers and overweight women. Basic and clinical information for all participants are reported in Table 1.
FF samples were centrifuged for 20 min at 2,800 rpm at 4°C to remove residual follicular cells and any blood traces; the supernatant was collected and stored at -20°C until its use. Samples with massive blood contamination were excluded from the study and only FF samples in which nuclear mature oocytes (metaphase II) had been identified were used. The patients signed an informed consent to participate in the research project, which included the use of collected FF and plasma. The study was exempted from Institutional Review Board approval because patients were included in the IVF program. Accordingly, there were no identifiers linking individuals to the samples and no additional treatment or use of personal data was necessary.

RNA isolation and quantification

Nanostring protocol requires at least 100 ng of RNA in a final volume of 3μL. According to this, RNA was isolated separately from two aliquots of 200 μL of the same FF sample by using the Qiagen miRNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany), according to Qiagen Supplementary Protocol for purification of RNA (including small RNAs) from serum or plasma. In the final step of the procedure, the two aliquots of the same FF sample were mixed together and precipitated. Specifically, RNA was eluted in 200 μL of RNAse-free water with the addition of 20 μg of UltraPure Glycogen (ThermoFisher), 0.1 volume of 3 M sodium acetate and 2.5 volumes of ice-cold absolute ethanol and incubated at -80°C overnight. RNA was then centrifuged and washed three times in ice-cold 75% ethanol, resuspended in 7μL of RNAse-free water, and stored at -80°C for further analyses. Total RNA was quantified before and after precipitation by using both a spectrophotometer and the Qubit™ fluorometer (ThermoFisher Scientific).

High-throughput miRNA expression analysis

NanoString nCounter system was exploited to analyze the expression profile of 800 miRNAs from FF samples by using the NanoString technology and the nCounter Human v3 miRNA Expression Assay Kits (NanoString Technologies, Seattle, WA, USA), according to the manufacturer's instructions. MiRNA expression profiling was performed on 4 FF samples from young women affected by HL and 4 young women used as CTRL (Table 1).
Approximately 100 ng of RNA in a final volume of 3μL were used. Briefly, RNA samples first underwent a miRNA sample preparation step, consisting of annealing of specific tags to target miRNAs, ligation and enzymatic purification allowing the removal of unligated tags. We then performed the CodeSet hybridization and the processing of samples by using the automated nCounter Prep Station. At this point, samples were purified and immobilized on a sample cartridge for quantification and data collection by using the nCounter Digital Analyzer. Raw data normalization was performed by using the nSolver 3.0 software and applying the normalization method based on the calculation of geometric mean of the 100 targets with the highest counts, according to the user manual instructions (*https://www.nanostring.com/products/analysis-software/nsolver*).

MiRNA single assay

Two microliters of precipitated and concentrated RNA were used for miRNA-specific reverse transcription (RT) to obtain miRNA-specific cDNAs and analyzed by quantitative real-time polymerase chain reaction (RT-qPCR), using TaqMan MicroRNA Assays (Applied Biosystems). qPCR reactions were performed in a final volume of 20 μL, containing 10 μL of TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μL of miRNA-specific TaqMan MicroRNA Assay (Applied Biosystems), 2 μL of RT products, and 7 μL of nuclease-free water. Validation of miRNA expression was assessed in a cohort of 19 HL samples and 3 CTRL samples. U6 was used to normalize expression data.

MiRNA function enrichment analyses

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) analyses were performed to deepen the potential biological role of DE miRNAs.
We queried Diana-miRPath v3.0 (*http://snf-515788.vm.okeanos.grnet.gr/*) selecting for validated mRNA targets retrieved from Tarbase 7.0. The FDR method was applied to select the signaling pathways with a threshold of significance defined by $p \leq 0.05$ and a microT threshold of 0.8.

DE miRNA target genes were analyzed for GO enrichment in terms of the biological process of follicle development and oocyte maturation categories by using the Ovarian Kaleidoscope database (OKdb) (*http://okdb.appliedbioinfo.net/*).
Regulatory network reporting miRNA-mRNA interactions was computed by using the miRNet 2.0 database (*https://www.mirnet.ca/miRNet/home.xhtml*) and selecting for validated target genes from the Tarbase 8.0. After linking the DE miRNAs with their target mRNAs, we selected them according to follicle development and oocyte maturation biological processes. Target mRNAs interacting with at least 2 miRNAs were shown in the regulatory network.

Statistical analysis

For miRNA profiling analysis, we performed the Volcano Plot and Significance Analysis of Microarrays (SAM) statistical tests by using MeV (Multi experiment Viewer v4.8.1) software. Differentially expressed miRNAs with statistical significance were screened using the threshold $\log_2$ fold change ≥ 0.5 ($\log_2$FC) and *p*-values ≤ 0.05 corrected for multiple testing by using Bonferroni method.
SAM statistical test was performed as follow: all statistical tests were computed by applying a two-class unpaired test among $\log_2$FC and using a q-value based on 100 permutations; imputation engine: K-nearest neighbors, number of K-nearest neighbors: 10 neighbors. The Benjamini-Hochberg multiple testing correction method for high throughput analyses using a stringent False Discovery Rate (FDR) limit <0.05 was applied.
Unpaired *t*-test was applied for DE miRNA expression validation by using GraphPad Prism 6. Statistical significance was assessed by setting the *p*-value cut-off ≤ 0.05.

Results

Expression profiling of microRNAs in FF of HL woman patients

High-throughput miRNA expression analysis revealed 27 differentially expressed (DE) miRNAs as shown by volcano plot (Figure 1) and 14 DE miRNAs obtained by SAM analysis. DE miRNAs common to both statistical tests were selected for further analyses (Table 2). More in detail, we selected 7 up-regulated miRNAs in HL FF samples with respect to controls (CTRL) (miR-1285-5p, miR-1303, miR-1972, miR-2117, miR-4455, miR-548ah-5p and miR-574-5p) and 6 down-regulated miRNAs in HL FF samples with respect to CTRL (let-7b-5p, miR-3195, miR-371a-5p, miR-423-5p, miR-4532 and miR-503-5p). Relative expression of DE miRNAs is shown in Figure 2.

Functional enrichment analyses

We investigated DE miRNA functions for molecular signaling pathway enrichment and GO classification specific for follicle development and oocyte maturation biological processes.
Functional enrichment analyses showed that the validated target genes of DE miRNAs may regulate several signaling pathways involved in fertility and cancer. In particular, most of them are known to be involved in gametogenesis and embryogenesis (Timofeeva et al., 2019). Among the most significant signaling pathways, we found the p53 signaling pathway, pathways in cancer, the TGF-beta signaling pathway, the MAPK signaling pathway, fatty acid metabolism, transcriptional misregulation in cancer, endometrial cancer, the estrogen signaling pathway, the FoxO signaling pathway, oocyte meiosis, the Hippo signaling pathway, adherens junctions, endocytosis, protein processing in endoplasmic reticulum, cell cycle and fatty acid biosynthesis (Figure 3).
Among the 13 DE miRNAs, let-7b-5p, miR-423-5p, miR-503-5p, miR-574-5p and miR-1303 are known to be involved in oocyte quality, oocyte maturation and ovarian response (bibliography in the discussion). Among their target genes, we found that 121 out of 5727 were involved in follicle development and oocyte maturation (Table 3).
Regulatory network analysis revealed that let-7b-5p is the central node of the network having the highest number of target genes (degree 107) that are shared with the other miRNAs showed in the network (Figure 4-5).

Let-7b-5p expression in HL FF samples

The altered expression of let-7b-5p was confirmed by qRT-PCR in a larger cohort of FF samples. As reported in figure 6, let-7b-5p was significantly down-regulated in HL FF samples with respect to CTRL (p-value = 0.02, unpaired t-test).

Discussion

In the last few years, cancer therapies have become more successful and patient recoveries have risen. Particularly in young female patients, it is important to consider the long-term side effects of cancer treatments that may reduce their quality of life. Infertility represents one of these common effects and, for this reason, different strategies of fertility preservation are now the object of intense research. In addition, the hope of a future pregnancy after cancer treatment may contribute to a better acceptance of oncological therapy and its adverse effects, and improve patients' personal experience of cancer (De Vos et al., 2014).

To date, the current guidelines of female fertility preservation provide clinical recommendations to improve the quality of this practice for many women and young girls (*https://www.eshre.eu/en*). Suitable stimulation protocols for cancer patients and cryopreservation techniques have significantly improved only in the last few years, for this reason we need more data to prove the validity of these options.

In this paper, we performed a high-throughput miRNA expression analysis in FF samples from young women affected by HL. Young women who went to the IVF treatment center for male infertility problems were used as controls. We found significant differences between HL women and controls for age and retrieved oocytes (Table 1). In fact, the age of women affected by HL is lower than that of controls, but it is known that it is difficult to find women between the age of 18 and 25 who have access to an IVF center without presenting specific problems. In the same way, it is expected that very young women have a better response to the stimulation protocols. Anyway, we are sure that our controls can be defined reproductively as young considering both the age and the standard parameters shown in table 1.

We identified 13 DE miRNAs in FF from HL women with respect to CTRL, indicating that miRNA expression profiles in FF could be influenced by cancer conditions. *In silico* analyses of KEGG pathways and GOs revealed that DE miRNA target genes could regulate molecular signaling pathways involved in cancer, gametogenesis and embryogenesis.

Among the most significant molecular signaling pathways PI3K-Akt, MAPK, Hippo, and Wnt signaling pathways are known to be involved in protein synthesis, cell survival, migration, invasion, cell cycle progression, and cellular proliferation and differentiation (Hers et al., 2011, Mo, Park et al., 2014). In particular, the Hippo pathway regulates the balance between differentiation and self-renewal in embryonic stem cells and interacts with other pathways to promote and maintain pluripotency (Varelas et al., 2008, Hers et al., 2011, Lorthongpanich and Issaragrisil, 2015, Ye et al., 2017, Kawashima and Kawamura, 2018, Hsueh and Kawamura, 2020).

The Wnt signaling pathway is involved in cell fate decisions, stem cell maintenance, body-axis determination in vertebrate embryos, and gastrulation (Hernandez Gifford, 2015, Song et al., 2015, Tepekoy et al., 2015).

Several miRNAs regulate the FoxO signaling pathway, controlling the expression of several genes, among which FOXO1 plays the most important role. In fact, it was demonstrated that mouse FoxO1, FoxO3, and FoxO4 proteins are regulated by the PI3K/Akt signaling pathway and are implicated in oocyte maturation and preimplantation embryo development (Brosens et al., 2009, Makker et al., 2014, Kuscu and Celik-Ozenci, 2015).

It is known that FF represents a micro-environment that influences the oocyte competence and embryo development, thus, changing of its composition could be considered a predictive factor of oocyte and embryo quality (O'Gorman et al., 2013). In addition, miRNAs are considered crucial players in the regulation of oocyte and embryo development (Hossain et al., 2012).

To deeper investigate the possible involvement of DE miRNAs in the determination of oocyte quality, we performed a literature review of published data.

Among the DE miRNAs we found that miR-1303 is upregulated in FF of bad quality oocytes (Zhang et al., 2021); it is involved in tumorigenesis and in the progression of several cancers and its target genes are involved in Hippo signaling pathway regulation, which is crucial for cell signaling in growth and development, regulating the activation of primordial follicles in mice (Hu et al., 2019)and is associated with ovarian aging in mice (Li et al., 2015). miR-503-5p was shown to be directly involved in the formation and maintenance of corpus luteum (Reza et al., 2019).

According to Moreno et al., miR-574-5p is differentially expressed according to IVF patient age and oocyte maturation stage. This altered expression was observed not only in FF samples but also in granulosa cells

(GCs) obtained from each patient (Moreno et al., 2015). An altered expression in GC cells was also found for miR-423-5p. In particular, it is down-regulated in the GC cells from patients with high ovarian response to exogenous gonadotropins, suggesting that it may play an important role in the regulation of ovarian response (Xie et al., 2016, Xie et al., 2020).

Literature data show a general downregulation of let-7b-5p in FF and plasma from patients affected by endometriosis (Papari et al., 2020) and in HL cell lines (Yuan et al., 2017), and its possible association with oocyte quality. More in detail, in our previous paper, we already found a strong downregulation of let-7b-5p in oocytes from women in advanced reproductive age (Battaglia et al., 2016).

Let-7b-5p is also down-regulated in FF from patients affected by polycystic ovary syndrome (PCOS) with respect to women with a normal ovarian reserve and it seems to be associated to blastulation rate and, in general, it may reflect the abnormal folliculogenesis that characterizes PCOS (Scalici et al., 2016). Timofeeva et al., demonstrated that the expression of let-7b-5 decreases in fair quality blastocysts and is negatively correlated with both embryo development grade and number of M2 oocytes and 2PN cells (Timofeeva et al., 2019).

In summary, by NanoString technology, we analyzed 800 FF miRNAs and found that 13 miRNAs showed a deregulation in HL young women in comparison to young women not affected by HL. Among them, five miRNAs had already been associated with oocyte quality and are part of a regulatory network in which let-7b-5p holds the central node of the network and shares mRNA target genes involved in follicle development and oocyte maturation with the other DE miRNAs. We also confirmed the significant down-regulation of let-7b-5p in HL FF samples compared to CTRL in a larger cohort of FF samples. Although these data do not demonstrate that oocyte quality is lower in young women affected by HL than in women of the same age, they suggest that critical conditions may affect the follicular microenviroment and influence oocyte competence. Moreover, HL is a widespread tumor and especially in the most advanced grades it could also influence FF composition. Of course, it will take a few more years to obtain more information about the pregnancy rate and the number of live births from vitrified oocytes of HL patients who had undergone protocols of fertility preservation. At present, this paper, representing the first study investigating miRNA profiles in FF from young women with HL, could be the starting point for further molecular studies exploring the quality of the follicular microenvironment, in order to improve counseling patients during the oncofertility protocol.

A possible application could be to find biomarkers of oocyte competence in CC, GC or FF inside the single follicle, as well described in a recent publication (Rooda et al., 2020). In fact, we know that the retrieved oocytes from a single stimulation protocol do not necessarily have equivalent quality (Di Pietro et al., 2010).

The detection of molecular markers of oocyte quality could allow us to classify the retrieved oocytes before the freezing. After cancer recovery, female patients have the possibility to start a pregnancy. At this point, it could be possible to thaw and fertilize the most competent oocyte, previously classified, in order to improve the success of transfer and pregnancy rate. Moreover, the identification of altered pathways involved in follicle growth and oocyte maturation in HL women could help to design specific therapies able to improve reproductive potential.

Conclusions

In conclusion, the deregulation of some miRNAs in FF from young women with HL could affect the follicular microenvironment and consequently oocyte competence. Further studies are needed to improve our knowledge and help young women to become a mother after cancer.

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Key Message

miRNAs are dysregulated in Follicular Fluid of women affected by Hodgkin Lymphoma, control pathways related to cancer and follicle development and could serve to predict the reproductive potential of patients with HL.

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Figure Legends

Figure 1. Volcano plot labelling the most significant miRNAs. Volcano plot showing the differences in fold change (log_2FC) of miRNA expression in HL FF samples vs CTRL obtained after data normalization analysis. The X-axis shows differences in log_2FC and the Y-axis represents the $-log_{10}$ *p*-value. The horizontal dashed line indicates the threshold for probability of significance (p=0.05) and the vertical dashed lines set the threshold 0.5 for the difference in FC of miRNA expression. The green dots represent miRNAs whose expression level is at least 0.5 fold different in HL FF samples compared to CTRL, with p<0.05 corrected for multiple testing by using Bonferroni method.

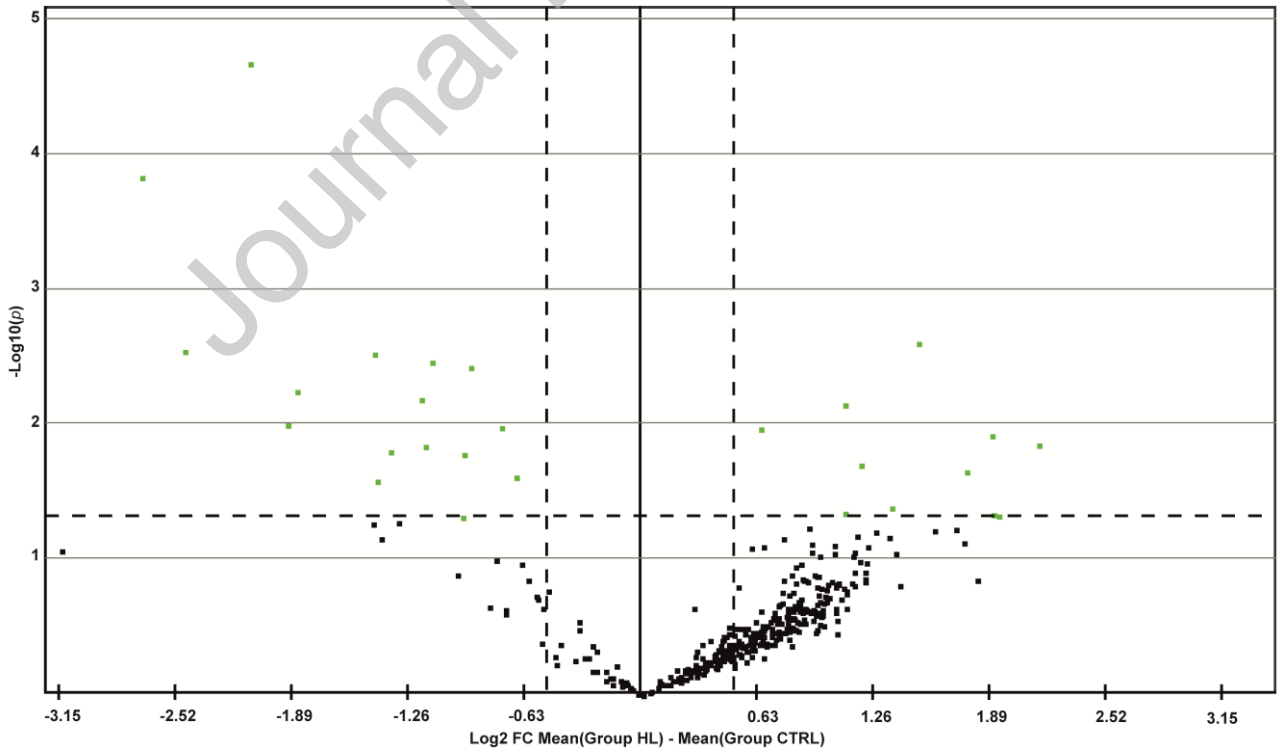


Figure 2. MiRNA relative expression in FF samples. Box-and-whisker plots showing the relative expression of the 13 DE miRNAs in HL FF samples *vs* CTRL. Expression data are represented as $\log_2$ normalized counts. Significant *p*-values corrected for multiple testing by using Benjamini-Hochberg method are indicated by <<*>> (*p-value ≤0.05, **p-value ≤0.01, ***p-value ≤0.001).

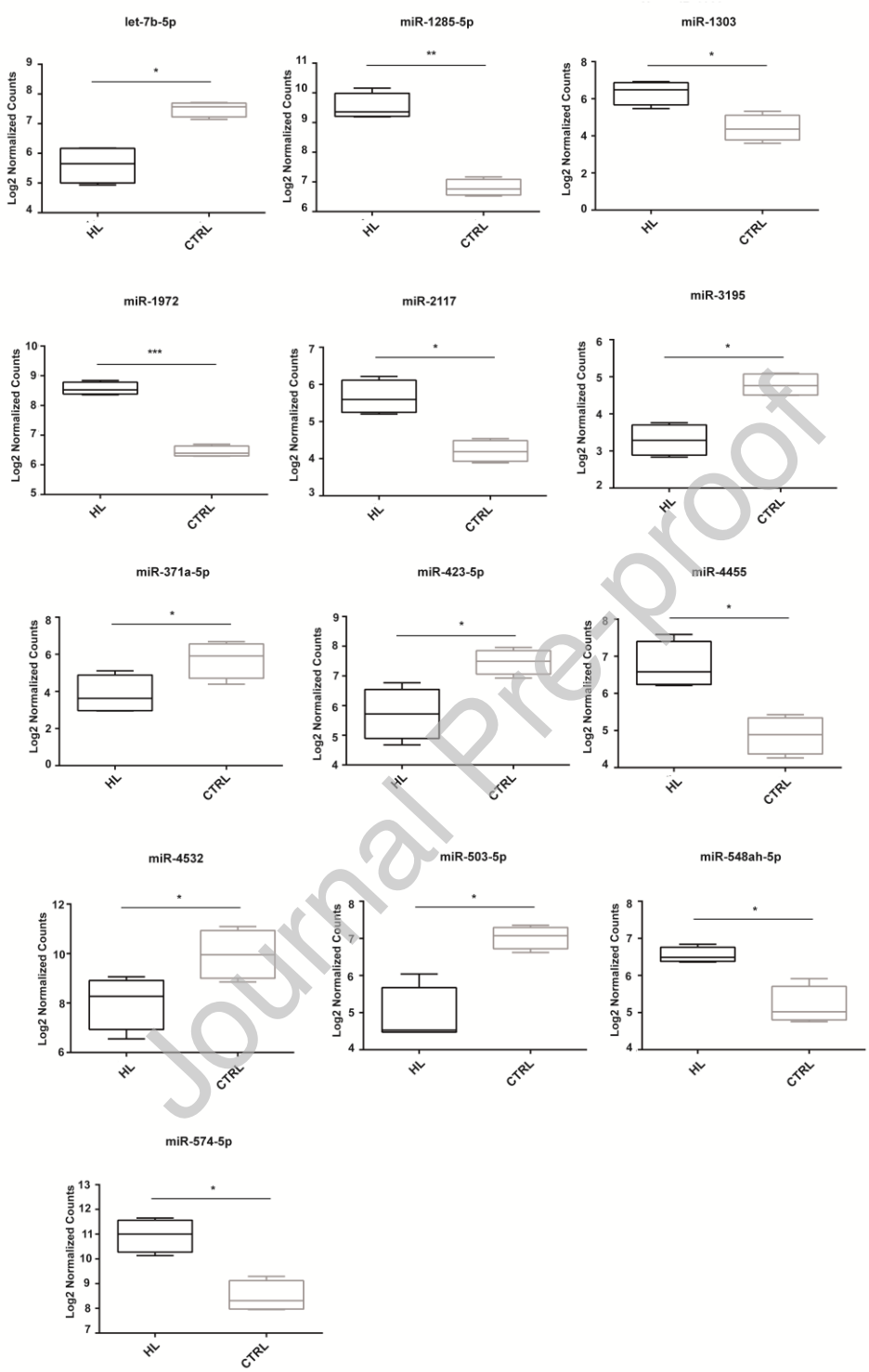
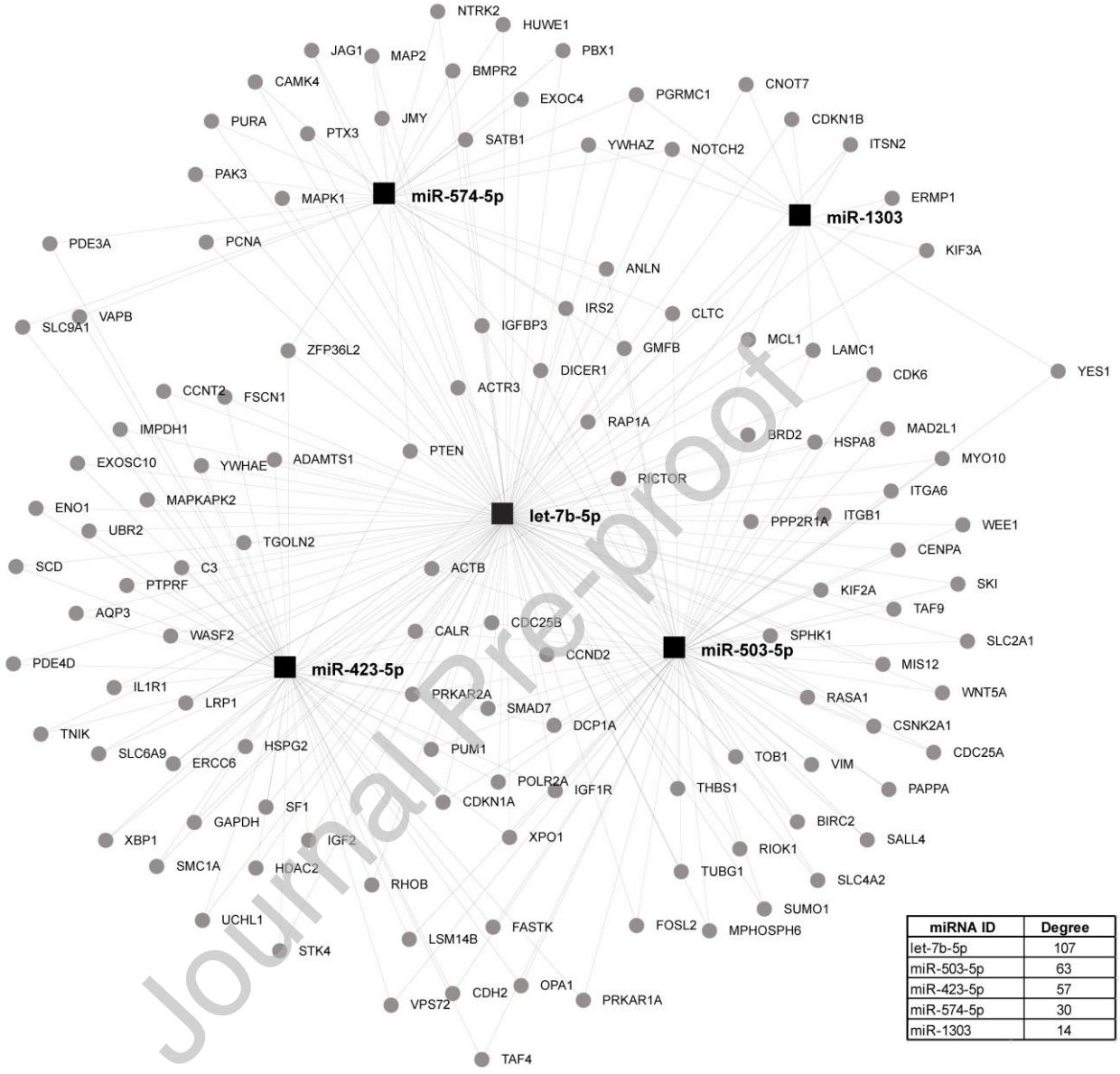


Figure 3. Functional enrichment analysis of DE miRNAs. Functional enrichment analysis of all DE miRNA target genes using KEGG pathway analysis. The X-axis represents the -log$_{10}$ (*p*-value).

Figure 4. Regulatory network of miRNA-target interactions. Regulatory network showing the interaction between let-7b-5p, miR-423-5p, miR-503-5p, miR-574-5p, miR-1303 and their target genes. Black squares represent miRNAs and grey circles represent target genes involved in follicle development and oocyte maturation. Node degree values are reported in the table.



miRNA ID	Degree
let-7b-5p	107
miR-503-5p	63
miR-423-5p	57
miR-574-5p	30
miR-1303	14

Figure 5. mRNA target genes of DE miRNAs are involved in follicle development and oocyte maturation. DE miRNA target genes involved in follicle development and oocyte maturation sorted by miRNA-mRNA interaction. Legend shows differentially colored squares related to each miRNA.

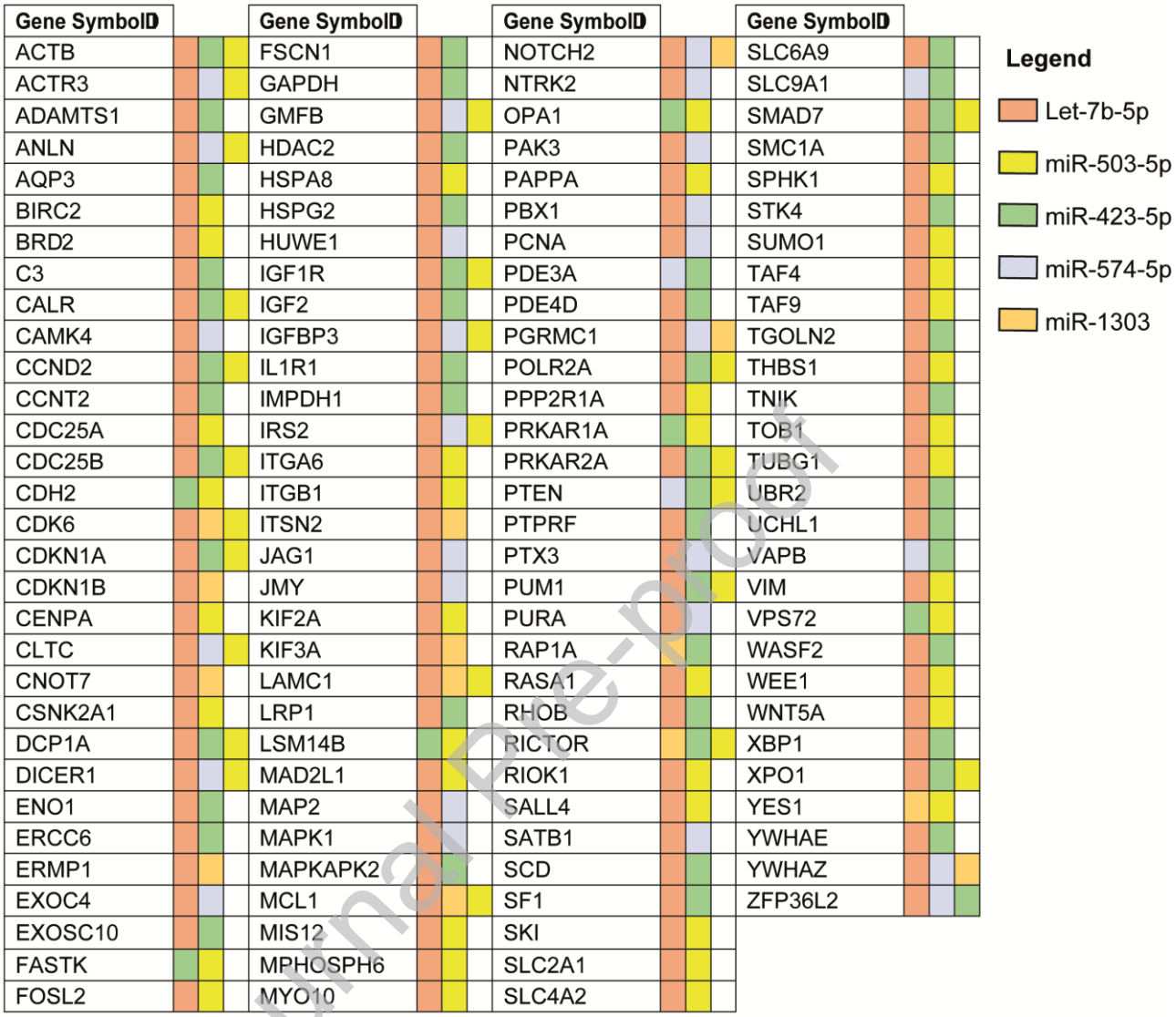


Figure 6. Let-7b-5p is down-regulated in HL FF samples. Box-and-whisker plots showing the relative expression of let-7b-5p in HL FF samples compared to CTRL. Expression data are represented as -ΔCt values normalized against U6 (*p-value < 0.05).

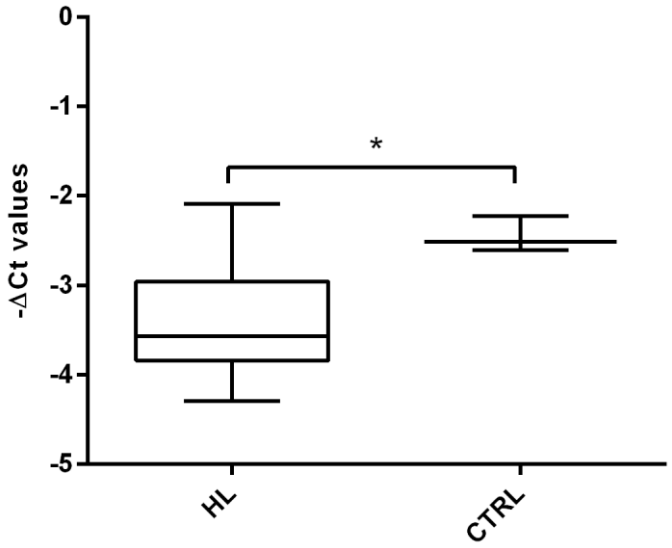


Table 1. Basic and clinical information for all patients enrolled in the study. Data are reported as range of values or percentage values. *P*-values are based on two-sample t-Test. BMI: body mass index; rFSH: recombinant follicle stimulating hormone; HMG: human menopausal gonadotropin; NS: not significant

Parameters	HL women	CTRL women	*p*-value (HL *vs* CTRL)
Age	18-25	30-34	< 0.00001
BMI (kg/m2)	21-25	21-25	NS
rFSH total dose (IU)	1225-3000	1625-3325	NS
HMG total dose (IU)	525-1950	450-750	NS
Follicles with diameters > 16 mm	11-28	7-14	0.02
Retrieved oocytes	15-28	11-16	0.006
MII oocytes	13-20	7-12	0.0007
Pregnancy (%)	-	25	-

Table 2. List of DE miRNAs in HL FF samples *vs* CTRL. DE miRNAs selected according to Volcano Plot and SAM statistical tests. DE miRNAs common to both statistical tests and chosen for further analyses are highlighted in bold. The FC value of each miRNA is reported.

DE miRNAs	TTest	SAM	FC HL *vs* CTRL
let-7b-5p	✓	✓	**-3.71**
hmiR-122-5p		✓	8.85
miR-1255b-5p	✓		1.97
miR-1268a	✓		-2.14
miR-1285-5p	✓	✓	**6.56**
miR-1303	✓	✓	**3.8**
miR-1323	✓		1.61
miR-151a-3p	✓		1.91
miR-1972	✓	✓	**4.36**
miR-2117	✓	✓	**2.73**
miR-21-5p	✓		-2.13
miR-22-3p	✓		-2.54
miR-23a-3p	✓		-1.55
miR-3195	✓	✓	**-2.8**
miR-371a-5p	✓	✓	**-3.72**
miR-423-5p	✓	✓	**-3.36**
miR-4455	✓	✓	**3.66**
miR-450a-5p	✓		-2.27
miR-4532	✓	✓	**-3.79**
miR-503-5p	✓	✓	**-4.4**
miR-548ah-5p	✓	✓	**2.58**
miR-548d-3p	✓		2.21
miR-548d-5p	✓		2.71
miR-548n	✓		1.96
miR-548z+miR-548h-3p	✓		2.26
miR-574-5p	✓	✓	**5.57**
miR-664b-3p	✓		2.3
miR-890	✓		1.7

Table 3. DE miRNA target genes involved in follicle development and oocyte maturation. RNA target genes regulated by the five selected miRNAs and involved in follicle development and oocyte maturation based on GO computational analysis using OKdb.

Gene Ontology Category	Gene Symbol IDs
Follicle development (GO_0001541)	BIRC2, BRD2, CAMK4, CCND2, CDKN1B, ERMP1, FOSL2, HSPG2, IGF1R, IGF2, IGFBP3, IL1R1, IRS2, ITGA6, JAG1, LAMC1, LRP1, MAP2, NTRK2, OPA1, PAPPA, PDE4D, PTEN, PTPRF, PTX3, SKI, SMAD7, SPHK1, STK4, TAF4, THBS1, VIM
Oocyte maturation (GO_0001556)	ACTB, ACTR3, ADAMTS1, ANLN, AQP3, C3, CALR, CCNT2, CDC25A, CDC25B, CDH2, CDK6, CDKN1A, CENPA, CLTC, CNOT7, CSNK2A1, DCP1A, DICER1, ENO1, ERCC6, EXOC4, EXOSC10, FASTK, FSCN1, GAPDH, GMFB, HDAC2, HSPA8, HUWE1, IMPDH1, ITGB1, ITSN2, JMY, KIF2A, KIF3A, LSM14B, MAD2L1, MAPK1, MAPKAPK2, MCL1, MIS12, MPHOSPH6, MYO10, NOTCH2, PAK3, PBX1, PCNA, PDE3A, PGRMC1, POLR2A, PPP2R1A, PRKAR1A, PRKAR2A, PUM1, PURA, RAP1A, RASA1, RHOB, RICTOR, RIOK1, SALL4, SATB1, SCD, SF1, SLC2A1, SLC4A2, SLC6A9, SLC9A1, SMC1A, SUMO1, TAF9, TGOLN2, TNIK, TOB1, TUBG1, UBR2, UCHL1, VAPB, VPS72, WASF2, WEE1, WNT5A, XBP1, XPO1, YES1, YWHAE, YWHAZ, ZFP36L2

Cinzia Di Pietro - Biography

Cinzia Di Pietro is Associate Professor of Biology and Genetics at the University of Catania. She obtained a PhD in Molecular Biology at Bari University. Her research activity focuses on transcriptome alteration of oocytes and somatic follicular cells concerning female reproductive aging. For the first time, her research group purified exosomes from human follicular fluid, identified their microRNA cargo and, recently, found miRNA expression changes in reproductive aging.