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Title page

Title

Blood plasma miR-20a-5p expression as a potential non-invasive diagnostic biomarker of male infertility: a pilot study

Running title

Novel diagnostic biomarker in male infertility

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Abstract

Background: Recently, alterations in miRNAs expression profile in semen have been linked to damaged spermatogenesis, suggesting miRNAs could be used as potential infertility biomarkers. In previous animal studies, miR-20a-5p was found to be down-expressed in low motile sperm, implying its potential target of genes associated with cell apoptosis.

Objective: To investigate miR-20a-5p expression in blood plasma of patients suffering from non-obstructive azoospermia (NOA), compared to normozoospermic controls.

Materials and Methods: Between January 2018 and December 2019, from 52 infertile couples eligible for the study, 24 couples were finally enrolled in this monocentric observational prospective pilot study. Patients were included into two groups: Group 1 comprised men with NOA (n=14), Group 2 fertile men partners of women with female tubal factor infertility (n=10). All NOA patients underwent testicular sperm extraction. The expression of circulating miR-20a-5p in plasma samples was assessed by RT qPCR. A relative quantification strategy was adopted using the $2^{-\Delta Cq}$ method to calculate the target miR-20a-5p expression with respect to miR-16-5p as endogenous control.

Results: Median blood plasma miR-20a-5p was significantly higher in patients affected by NOA (0.16 $2^{-\Delta Ct}$, range: 0.05-0.79 $2^{-\Delta Ct}$) than in fertile controls (0.06 $2^{-\Delta Ct}$, range: 0.04-0.10 $2^{-\Delta Ct}$), $p<0.001$. MiR-20a-5p was positively correlated with follicle stimulating hormone (FSH) ($r_{rho}= -0.490$, $p=0.015$) and luteinizing hormone (LH) ($r_{rho}= -0.462$, $p=0.023$), and negatively correlated with serum total testosterone (TT) ($r_{rho}= -0.534$, $p=0.007$) and right and left testicular size ($r_{rho}= -0.473$, $p=0.020$ and $r_{rho}=-0.471$, $p=0.020$, respectively). Successful SR rate was 50.0%. Median value of miR-20a-5p did not differ significantly among patients with successful SR and those with negative SR. Testicular histological examination showed: hypospermatogenesis in 6/14 (42.8%), maturation arrest in 4/14 (28.6%), Sertoli-cell-only-syndrome in 4/14 (28.6%). No significant differences in miR-20a-5p were found between histopathological patterns ($p> 0.05$).

Conclusions: MiR-20a-5p could represent a novel non-invasive diagnostic biomarker of male infertility.

Keywords

male infertility; microRNA; spermatogenesis; testicular sperm extraction; azoospermia

Introduction

Male factor is responsible for around 40% of couple infertility, whereas about 1% of male population and 10-15% of the infertile men may present azoospermia[1]. Its genesis may be due to seminal tract obstructions (obstructive

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azoospermia, OA), or to an intrinsic spermatogenesis dysfunction, known as non-obstructive azoospermia (NOA). In at least 40% of cases, the etiology of azoospermia remains unclear, probably because many of the genes that regulate germ cell differentiation and sperm production have not been yet identified[2].

In patients with azoospermia, one possibility to become biological fathers is given by surgical sperm retrieval (SR) followed by assisted reproductive technologies (ARTs) with intracytoplasmic sperm injection (ICSI). However, given that the likelihood of SR in men with NOA does not exceed 60%, some predictive factors – including testicular volume or hormone parameters – can allow clinicians to choose the most appropriate therapeutic approach and to counsel properly the infertile couple[3]. However, to date, there are no absolute defined prognostic factors or tests that can accurately predict the presence of mature spermatozoa in azoospermia[4].

The differentiation of germ cells through the stages of spermatocytogenesis, spermatidogenesis, spermiogenesis, and spermiation, is a complex process which requires the constant and organized involvement of thousands of genes[5]. The regulation of gene expression profiles depends on several epigenetic and environmental factors[6-8].

In addition to the classical post-transcriptional control by RNA-binding proteins, some studies have demonstrated that small non-coding micro-RNAs (miRNAs) may have a crucial role in regulating different stages of spermatogenesis[9-11].

MiRNAs are endogenous, short single-stranded, non-coding RNAs which represent a key part in many human diseases, including cardiac failure, Alzheimer's disease, inflammatory disorders[12, 13] and cancers[14, 15].

Recently, alterations in the expression of specific miRNAs in human plasma, serum and urine have been linked to distorted spermatogenesis, suggesting their expression could be used as a potential biomarker of male infertility. Indeed, miRNAs were found to target specific genes of spermatogonial stem cells (SSCs)[16], and to participate in the control of differentiation, meiotic/post-meiotic processing and spermiogenesis[17].

Furthermore, miRNAs also seem to regulate segment-specific gene expression in the epididymis, influencing sperm maturation and conservation. Some of these miRNAs, released by the epididymis as epididymosomes, are absorbed by the sperm that passes through the epididymis as part of their maturation process[18].

Most studies on miRNAs involve animal models and only a few investigate miRNAs expression in humans with different histopathological patterns of testicular dysfunction[11, 13].

MiRNA-20A has been shown to be expressed at high levels in mouse SSCs, proving an important role in the regulation of spermatogonial stem cell renewal at the post-transcriptional level[16]. Moreover, a previous study on bulls reported miR-20a-5p under-expression in the low motile sperm fractions, implying its potential target of genes associated with cell apoptosis and spermatogenesis alteration[19].

Aware that miRNAs expression often is tissue/biological stage-specific, even being in several body fluids, we have looked for assessing plasma levels of miRNA-20A, given its expression and subcellular localization in adult animal testis.

Thus, the aim of this study is to investigate the miR-20a-5p expression profiles in the blood plasma of infertile patients suffering from NOA, compared to healthy controls. Secondary objective of the study is to correlate miR-20a-5p expression to different histopathological models of testicular dysfunction, in order to explore less invasive methods useful for the screening of male infertility, as well as a possible novel future tool for the prediction of successful SR.

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Materials and Methods

Study sample

This monocentric observational prospective pilot study involved 52 infertile couples who referred to the ARTs Centre of the Careggi University Hospital, from January 2018 to December 2019. Criteria for inclusion were: infertile men suffering from absolute NOA, age older than 18 years, searching for pregnancy. All patients were divided into two groups: Group 1 comprised infertile men with NOA, Group 2 included healthy normozoospermic men of couples whose women suffered from tubal factor infertility and aimed to achieve pregnancy using IVF or ICSI. The exclusion criteria were: obstructive azoospermia, leukocytospermia (defined as leukocyte concentration greater than 1 x 10^6/ml), genetic abnormalities, excessive alcohol consumption (≥ 15 drinks per week), endocrine disorders and drug intake. Also men with comorbidities, including cancer, diabetes, obesity, autoimmunity, gastrointestinal disease, kidney or lung disorders, were excluded from the study.

Patient's assessment

Patients' data were collected from our clinical reports, including age, height and weight with body mass index (BMI) count, previous diseases or surgery. At baseline, all participants performed at least two semen analysis examinations, according to the World Health Organization guidelines (WHO, 2010). Absolute NOA was defined as the lack of sperm in the ejaculate, even after centrifugation, in absence of seminal tract obstructions detected by ultrasonography. The endocrinology profile included the dosage of follicle stimulating hormone (FSH), luteinizing hormone (LH), total testosterone (TT) and sex hormone binding globulin (SHBG). Normal levels of FSH, LH, TT and SHBG were considered 1.5-8.0 IU/L, 1.8-12 IU/L, 2.7-18 ng/ml and 16-80 nmol/l, respectively. The genetic counselling was required and, in case of azoospermia, the genetic tests were done, including karyotype analysis, Y chromosome microdeletions and Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) evaluation. The screening for infectious diseases included human immunodeficiency virus, hepatitis B virus, hepatitis C virus, Cytomegalovirus, Treponema pallidum haemagglutination (TPHA) and rapid plasma reagin (RPR).

All men underwent physical examination and scrotal ultrasounds (US) to determine testicular volume and to rule out the presence of seminal tract obstructions or varicocele. Testicular volume was considered in the normal range when it was between 15 ml and 25 ml, whereas a measure less than 12 mL was given as testicular hypotrophy. In NOA, trans-rectal US was performed to evaluate the presence of prostate median cysts and seminal vesicles anomalies. All NOA patients underwent conventional testicular sperm extraction (cTESE). The sperm retrieval (SR) rate was reported, by specifying sperm concentration, sperm motility and total number of cryo biosystem straws. Based on the main morphological pattern, testicular histology was categorized as: Hypospermatogenesis (HYPO), showing a decrease in the number of normal spermatogenetic cells; Maturation Arrest (MA), characterised by the absence of later stages of spermatogenesis; Sertoli-Cell-Only-Syndrome (SCOS), when tubules lacked germ cells.

cTESE and Laboratory

The surgical procedure was performed under spinal anesthesia. The tunica albuginea was exposed and 3 separate transverse incisions were made in the upper, lower and median point of the testicle. Depending on the testis size, two parenchymal samples were excised from each tunical opening. Whether testicular volume was considerably lower (< 8

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ml), a single equatorial incision of the tunica albuginea was performed, by means of surgical loupes with 6X-power magnification. The testicle was then split open bluntly and tubules were retrieved aiming to locate and collect the larger ones with an increased chance of harbouring spermatozoa. The fragments of testicular tissue were immediately delivered to embryologists in a medium solution. After they have been stretched with two sterile glass slides under stereomicroscopy, the evaluation of the presence of spermatozoa was made analysing the suspension at 200x. When > 1-2 sperms/field were found (corresponding to 0.001 x 10^6 spermatozoa/ml), SR was considered successful. Thus, spermatozoa were cryopreserved for a later use by post-thawing ICSI.

Sample Collection

Blood samples were extracted from the antecubital vein on the day of surgical sperm retrieval, after at least 8 hours of fasting. Blood samples were collected in Vacutainer tubes containing EDTA (0.17 mol/L), transferred to the laboratory within 1 hour. Plasma was separated by two centrifugation steps, at 1600g and 16000g respectively, at 4°C and stored at -80°C before the analyses.

RNA extraction

Total cell-free RNA was extracted from 0.5 ml plasma samples using the mirVana PARIS kit (ThermoFisher Scientific, USA) and reverse transcribed by using the TaqMan Advanced miRNA cDNA Synthesys Kit (ThermoFisher Scientific, USA) according to the manufacturer's instructions. cDNA was submitted to qPCR using the 7900HT real-time PCR system (ThermoFisher Scientific, USA). TaqMan® Advanced miRNA Assays, including hsa-miR-16-5p (Assay ID: 477860_mir) and has-miR-20a-5p (Assay ID: 478586_mir), and TaqMan® Fast Advanced Master Mix were used in two 20 µL reaction mixtures containing10 µL TaqMan® Fast Advanced Master Mix (2X), 1 µL Taqman® Advanced miRNA Assay (20x), 5.0 µL 1:10 amplified cDNA and 4 µL H_2O with the following thermal profile: 95°C for 20 seconds, followed by 40 cycles of 95°C for 1second and 60°C for 30 seconds.

Reverse Transcription - quantitative real-time PCR (RT-qPCR) analysis

A relative quantification strategy was adopted using the $2^{-\Delta Cq}$method to calculate the target miR-20a-5p expression with respect to miR-16-5p as an endogenous control.

Ethics statement and statistical analysis

The study was approved by the Institutional Ethical Committee of the University of Florence (reference no. 10709_bio-Em. 2019-465). All the subjects gave written informed consent for participation according to the Declaration of Helsinki.

For descriptive data, means ± standard deviation (SD) and medians with range with minimum and maximum value were used. Differences of mean and median values between groups were assessed by the Student independent t test or the Mann-Whitney U test based on their normal or non-normal distribution, respectively (normality of variables' distribution was tested by the Kolmogorov-Smirnov test). Correlation between parameters was ascertained by Spearman's correlation test. Statistically significance was established at $p<0.05$. All collected data were evaluated with Statistical Package for Statistical Sciences (SPSS, Version 25.0), IBM, Chicago, IL, USA).

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Results

The flow-chart of the recruitment process was depicted in Figure 1. Finally, the study included 24 men, of which 14 enrolled in the Group 1 and 10 in the Group 2. Overall, the patients had a mean age of 35.6 ± 4.2 years, and a mean BMI of 24.4 ± 2.6. Mean duration of couple infertility was 30.9 ± 10.5 months. 5 men (20%) presented with varicocele ≤ 2° degree by Sarteschi US score and 3 men (12.5%) suffered from monolateral cryptorchidism during childhood. 9 patients (37.5%) were smokers (less than 10 cigarettes/die). Median serum FSH and LH concentrations were significantly higher in patients with NOA, compared to the control group, while TT and testicular volumes were significantly lower (Table 1).

Median relative expression of blood plasma miR-20a-5p was significantly higher in patients affected by NOA (0.16 $2^{-\Delta Ct}$, range: 0.05-0.79 $2^{-\Delta Ct}$) than in fertile normozoospermic controls (0.06 $2^{-\Delta Ct}$, range: 0.04-0.10 $2^{-\Delta Ct}$), p=<0.001 (Figure 2).

Successful SR was found in 7/14 patients (50.0 %). Mean sperm concentration was 0.001 ± 0.003 x 10^6/ml, non-progressive motility was 0.2 ± 0.6 %, collected straws were 1.7 ± 1.9.

As shown in Figure 3-6, miR-20a-5p was positively correlated with FSH (r_{rho}= –0.490, p=0.015) and LH (r_{rho}= –0.462, p=0.023) and negatively correlated with serum TT (r_{rho}= –0.534, p=0.007) and with right and left testicular size (r_{rho}= –0.473, p=0.020 and r_{rho}= –0.471, p=0.020, respectively). No relationship was found between miR-20a-5p expression and SHBG (p=0.090).

Median value of miR-20a-5p did not differ significantly among patients with successful SR (0.32 $2^{-\Delta Ct}$, range: 0.10-0.54 $2^{-\Delta Ct}$) and those with negative SR (0.12 $2^{-\Delta Ct}$, range: 0.05-0.79 $2^{-\Delta Ct}$, p= 0.128). All the baseline parameters in patients with positive SR were comparable to those of patients with negative SR (Table 2). The histological examination of testicular biopsy showed the following patterns: HYPO in 6/14 (42.8%), MA in 4/14 (28.6%), SCOS in 4/14 (28.6%). Median miR-20a-5p was 0.24 $2^{-\Delta Ct}$ (range: 0.10-0.54 $2^{-\Delta Ct}$) in HYPO, 0.24 $2^{-\Delta Ct}$ (range: 0.06-0.35 $2^{-\Delta Ct}$) in MA and 0.11 $2^{-\Delta Ct}$ (range: 0.05-0.79 $2^{-\Delta Ct}$) in SCOS. Overall, miR-20a-5p did not differ significantly between histopathological patterns (p> 0.05) (Figure 7).

Discussion

Our results clearly indicate different expression levels of blood plasma miR-20a-5p between infertile and fertile males, suggesting its potential role in the regulation of spermatogenesis.

Nowadays, thanks to ARTs, infertile couples whose men have a clinical presentation of azoospermia have been allowed to conceive their biological children. However, about 40-60% of NOA patients have unsuccessful outcomes by surgical SR, and consequently are addressed to a IVF/ICSI program with male gametes donation[20].

In recent years, we have witnessed a progressive decrease in executing TESE or testicular sperm aspiration for exclusive diagnostic purposes, given that these procedures could be potentially invasive and have poor risk-benefit ratio[21].

Therefore, in this context, the clinician must rely on factors that predict the presence of active spermatogenesis, in order to carry out a proper counseling for the infertile patient and avoid unnecessary procedures.

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However, the tests we use as predictors are often inadequate, scarcely sensitive and still debated. In example, FSH and testicular volume have a debated role in predicting SR. Caroppo et al. reported that patients with positive SR had significantly lower serum FSH and higher testicular volume, compared to those with unsuccessful SR[22]. Conversely, other authors stated no correlation with SR in larger cohorts of patients, showing that FSH and testicular volume are not predictive of successful SR when calculated as the unique independent variable[3, 23].

In our case series, serum FSH and LH concentrations were significantly higher, as well as lower TT and testicular volumes , in patients with NOA, compared to fertile controls. However, analyzing the Group 1, FSH, LH, TT and testicular volumes in patients with negative SR were similar to those with positive SR, indicating that no predictors of SR were found.

Beyond the more commonly known parameters, novel genetic tests have been developed, such as RNF212 and STAG3 expression analysis, that allows the identification of subjects with meiotic arrest, in which the chance of sperm recovery during cTESE could be virtually zero[24].

However, a novel area of reproductive biology research aims to explore the epigenetic factors that could influence spermatogenesis. Several authors assessed miRNAs in human plasma, serum, urine and seminal fluid, suggesting that their expression could be useful as a potential infertility biomarker[25, 26]. In example, members of the miR-17-92 and 371-373 clusters, as well as miR-383, were found downregulated in testicular samples of NOA patients, leading to spermatogenetic injury and maturation arrest[27]. A possible explanation of the correlation between miRNAs in human plasma and male infertility could be the indirect effect on sperm development via hypothalamic-pituitary-gonadal axis dysregulation[18].

MiRNA-20A proved to be expressed at high concentrations in mouse SSCs[16, 28], and to be downregulated in the low motile sperm fractions from bulls[18], supporting a central role in spermatogenesis.

In contrast with the results obtained on animal models, in our study, human blood plasma miR-20a-5p appeared significantly over-expressed when spermatogenic failure occurs. Interestingly, higher miR-20a-5p values seem to be directly related to higher FSH and LH, but lower TT and testicular volumes. These findings could suggest that miR-20a-5p expression is closely related to testicular injury and might be a potential indicator of SR outcome in patients with azoospermia. However, miR-20a-5p in patients with positive SR did not differ considerably compared to those with negative SR, although the lack of statistical significance could be due to the small sample size.

Secondarily, since some clinical parameters could be predictive for testicular histology[29, 30], we aimed to evaluate whether there was a possible association between miR-20a-5p and a precise histopathological pattern. Our findings underline a strong trend toward lower miR-20a-5p expression in patients with SCOS, although no significant correlations were found among different histological profiles. This could be explained because, given that this miRNA seems to be related to a defective spermatogenetic process, in SCOS there is the total absence of spermatogonia due to altered migration of primordial germ cells.

We analyzed for the first time miR-20a-5p in human blood plasma. Our results confirmed that it can be used as a potential novel biomarker of spermatogenetic damage in humans, since its levels in azoospermic patients are considerably higher than in fertile males. Its non-invasiveness and cost-effectiveness make it a tool simple to use and easily applicable in the current clinical practice. However, given the pilot study design, further researches on larger populations are needed to confirm miR-20a-5p as a feasible biomarker of male infertility. Furthermore, the role of

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miR-20a-5p as a predictor of SR remains to be further explored, as well as its association to specific histological patterns.

Conclusions

MiR-20a-5p could represent a novel promising non-invasive diagnostic biomarker of male infertility. The over-expression of miR-20a-5p in blood plasma seems to be directly related to spermatogenic failure, including conditions of NOA. However, further larger studies are needed to evaluate the association between this miRNA and testicular histopathological findings in order to increase its usefulness as a predictor of SR in the clinical practice.

Author's role

G.C., F.S., C.G.: conception and design, drafting the article, acquisition of data. F.S., R.F., R.P., C.G., P.F., E.M., P.V.: acquisition of data, analysis and interpretation of data. ME.C., A.C., A.M., M.C., P.P., A.N.: critical revision. All of the co-authors interpreted the data and participated in finalizing the article. All of the co- authors approved the final version of the article.

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Conflict of interest

The authors declare no conflicts of interest.

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Figure legend

Figure 1: Flow-chart of the recruitment process.

Figure 2: Comparison of median miR-20a-5p expression between Group 1 (NOA) and Group 2 (normozoospermic).

Figure 3: Correlation between miR-20a-5p and follicle stimulation hormone (FSH). (r_{rho}= –0,490, p=0.015).

Figure 4: Correlation between miR-20a-5p and luteinizing hormone (LH). (r_{rho}= –0,462, p=0.023).

Figure 5: Correlation between miR-20a-5p and total testosterone (TT). (r_{rho}= –0.534, p=0.007).

Figure 6: Correlation between miR-20a-5p and testicular volume. (r_{rho}= –0.473, p=0.020 and r_{rho}= –0.471, p=0.020, respectively).

Figure 7: Comparison of median miR-20a-5p expression between different histopathological patterns. HYPO: hypospermatogenesis, MA: maturation arrest, SCOS: Sertoli cell only syndrome.

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Table 1: Baseline parameters of the study population (Group 1 vs. Group 2).

Variable	Group 1 (NOA)	Group 2 (Normo)	*p*- value
Age, years	34.5 (28.0-39.0)	35.2 (29.0-39.0)	0.382
BMI	25.1 (24.0-28.0)	23.5 (22.0-26.0)	0.162
TT, nmol/l	10.6 (3.9-20.2)	16.1 (13.2-32.6)	<*0.001
SHBG, nmol/l	32.2 (30.9-57.8)	45.9 (41.5-46.0)	*0.011
FSH, IU/l	21.3 (2.8-31.8)	2.9 (2.0-4.2)	*<0.001
LH, IU/l	7.7 (2.6-10.6)	2.7 (2.6-3.6)	*<0.001
Right TV	10.0 (6.0-20.0)	20.0 (14.0-20.0)	<*0.001
Left TV	10.0 (6.0-20.0)	18.0 (14.0-20.0)	*0.003

NOTE: BMI=body max index; TT=total testosterone; FSH=follicle stimulating hormone; LH=luteinizing hormone; SHBG=sex hormone binding globulin; TV=testicular volume; NOA=non-obstructive azoospermia. All variable are expressed as median (range); *=statistical significance

Table 2: Comparison of baseline parameters between patients with successful and unsuccessful sperm retrieval.

Variable	SR -	SR +	*p*- value
Age, years	34.8 (30.0-39.0)	34.2 (29.0-38.0)	0.301
BMI	24.8 (23.0-28.0)	25.3 (22.0-26.0)	0.812
TT, nmol/l	10.8 (5.0-16.1)	10.4 (3.9-20.2)	1.000
SHBG, nmol/l	31.8 (31.4-45.9)	32.6 (30.9-57.8)	0.710
FSH, IU/l	22.8 (3.2-31.8)	19.9 (2.8-28.0)	0.383
LH, IU/l	8.5 (2.6-10.4)	6.3 (3.1-10.6)	0.535

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Right TV	10.0 (8.0-20.0)	10.0 (6.0-18.0)	0.902
Left TV	10.0 (6.0-18.0)	12.0 (6.0-20.0)	0.710

NOTE: BMI=body max index; TT=total testosterone; FSH=follicle stimulating hormone; LH=luteinizing hormone; SHBG=sex hormone binding globulin; TV=testicular volume; SR=sperm retrieval.

All variable are expressed as median (range); *=statistical significance

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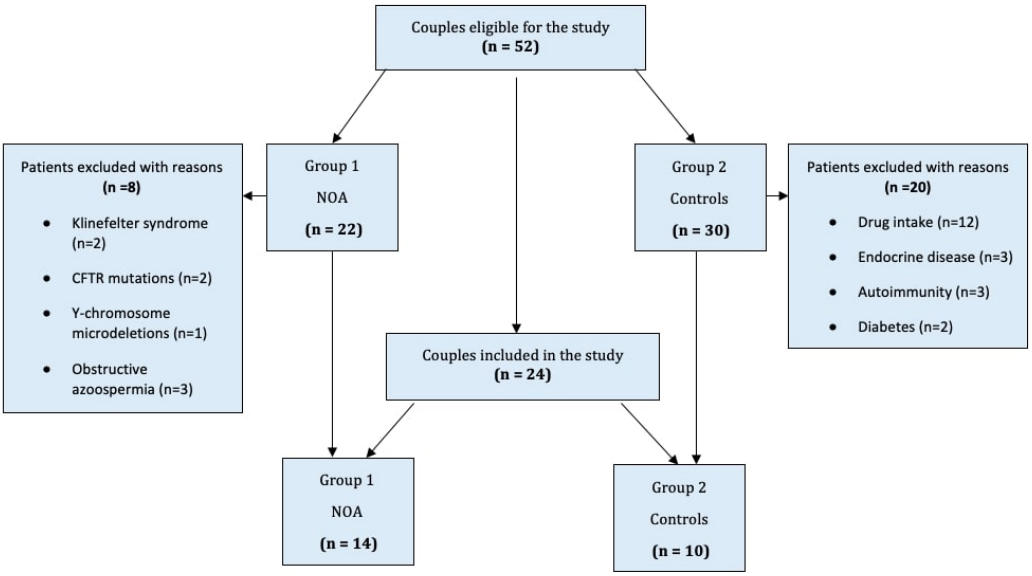

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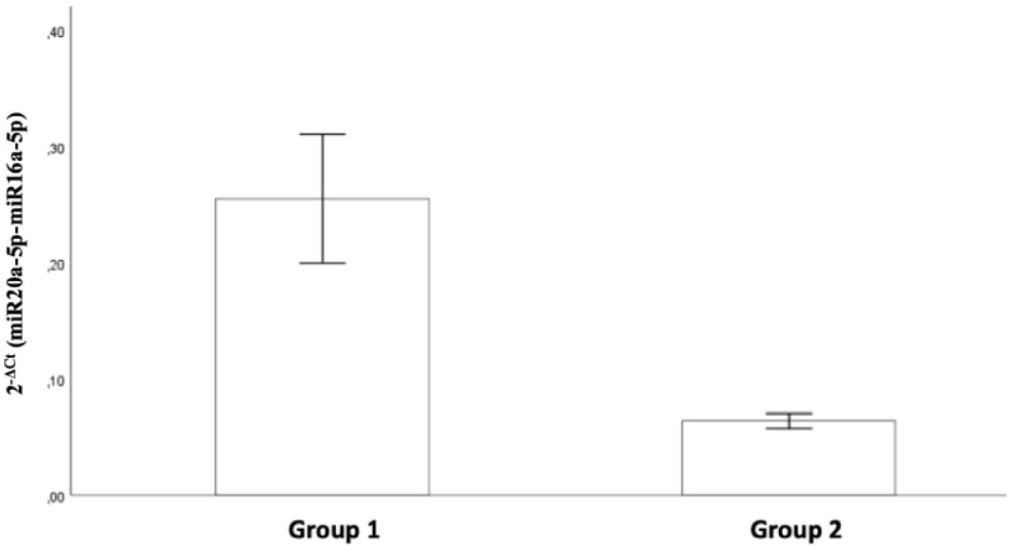

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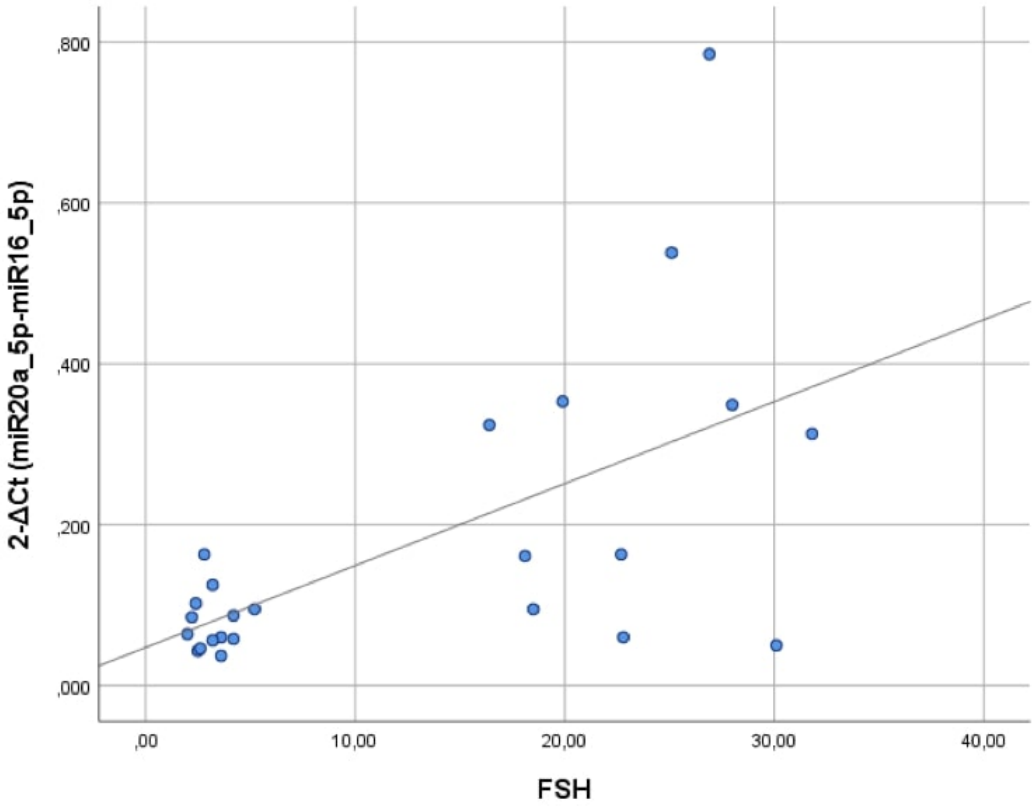

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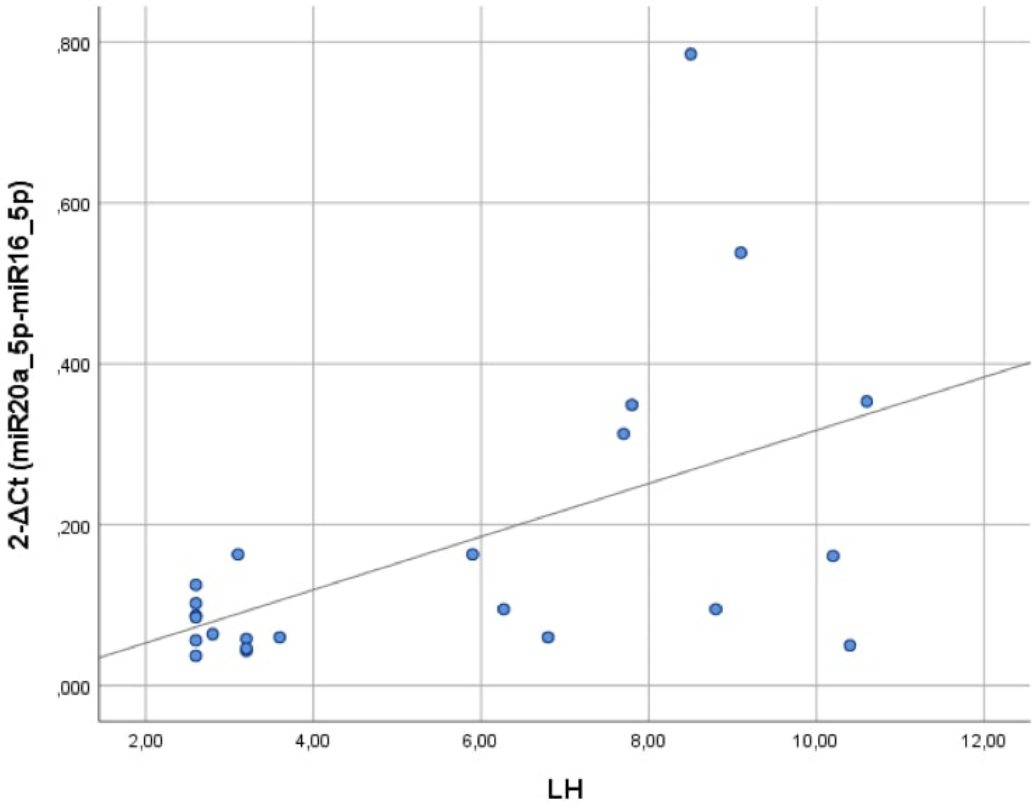

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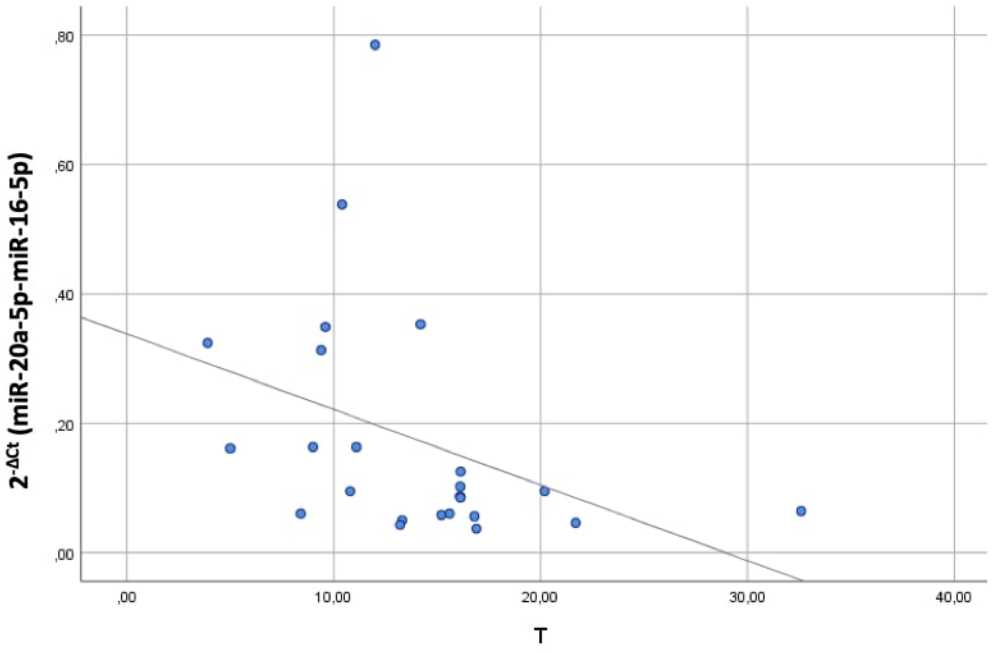

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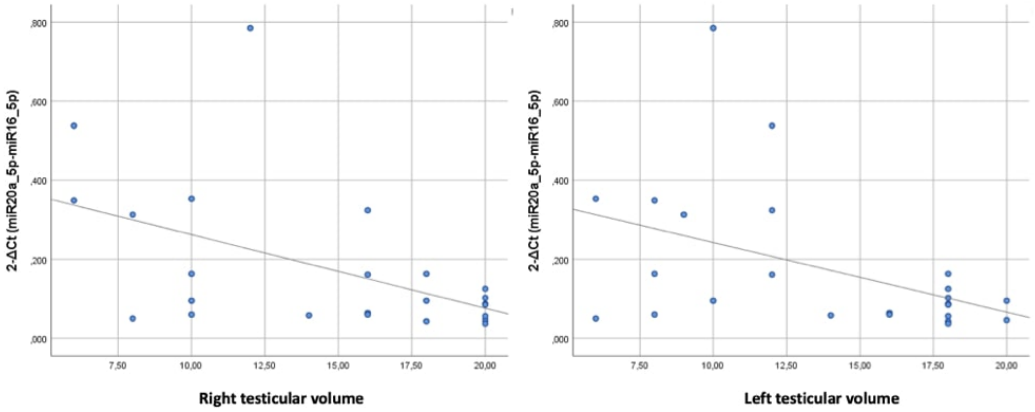

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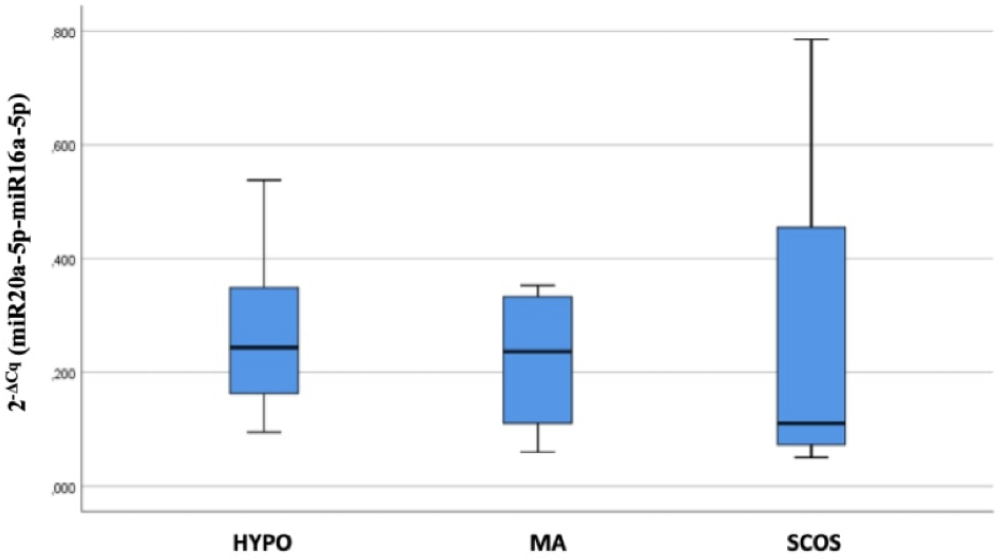

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