EXPERIMENTAL VALIDATION OF SEMINAL miR-31-5p AS BIOMARKER FOR AZOOSPERMIA AND EVALUATION OF THE EFFECT OF PREANALYTICAL VARIABLES

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Short title: (50 characters) **SEMEN miR-31-5p AS NON-INVASIVE BIOMARKER FOR AZOOSPERMIA**

KEYWORDS: Seminal plasma, extracellular vesicles, miRNA, non-invasive biomarker, azoospermia

ABSTRACT

Background and objectives: Predicting the origin of azoospermia with non-invasive biomarkers is clinically relevant for determining the chance of successful sperm retrieval from the testes before attempting ART. Here, the semen small extracellular vesicle (sEV) microRNA (miRNA) miR-31-5p-based biomarker test to distinguish obstructive (OA) from secretory azoospermia (SA) (previously described by our group) is validated for clinical use and additionally sample source [seminal sEVs vs total seminal plasma (SP)] as a preanalytical variable is considered to optimize the procedure.

Results and Discussion: Our results provide evidence that altered miR-31-5p expression can be determined both from EVs and from the whole SP to discriminate OA from SA azoospermic samples. Not only have we validated this miRNA-based molecular model as a clinically useful test for predicting the origin of azoospermia in a sample from azoospermic individuals, but additionally, and more interestingly for the clinicians, we have evidenced its usefulness for predicting the presence of spermatogenic failure in azoospermic patients with FSH values <10 IU/L as a sensitive and specific biomarker (AUC>0.88; *p*-value<0.006).

The use of total SP as analytical sample would facilitate the use of a simplified technical procedure for miR-31-5p quantification and would represent a great improvement in reproductive treatment decision protocols for azoospermia in clinical practice.

INTRODUCTION

Seven percent of men worldwide suffer from infertility[1]. Low sperm count in semen is the major cause of male infertility and specifically, azoospermia, the medical condition in which a man's semen contains no sperm, accounts for 10-15% of cases of male fertility disorders[2]. Knowing the origin of the azoospermia, which determines whether sperm is present or absent in the testicle, is decisive for assisted reproduction treatment (ART) for the couple. Azoospermia can occur as a result of an obstruction of the ducts or vas deferens in the genital tract [known as obstructive azoospermia (OA) with preserved spermatogenesis] or is caused by a severe failure in testicular spermatogenesis [nonobstructive/secretory azoospermia (NOA/SA) with no or few sperm in the testicle]. A distinction between OA and SA is therefore important for determining the chance of successful sperm retrieval from the testes before attempting ART.

The diagnosis of acquired and congenital obstructions or the identification of functional abnormalities in the genital glands (such as those produced after infection or inflammation), is performed by the assessment of the levels of routine biochemical markers in semen such as acid phosphatase, citric acid and zinc (prostate), fructose (seminal vesicles) and neutral alpha-glucosidase (epididymis). Additionally, typical criteria for poor sperm production including elevated serum FSH level or decreased testicular volume are used. However, discriminating between OA and SA is not always easy to carry out, and in most cases testicular biopsy, which is an invasive procedure, is required as a definitive diagnostic strategy. Accurate noninvasive biomarkers and diagnostic tests are needed to distinguish the origin of azoospermia, especially when other diagnostic parameters are not clear.

The discovery of abundant microRNAs (miRNAs), a large group of small non-coding RNAs, in semen and their surprisingly high stability holds great promise for the diagnosis of reproductive tract-affecting diseases using miRNAs as biomarkers[3]. Seminal plasma (SP) contains a unique concentration of miRNAs, mostly contained in small extracellular vesicles (sEVs) such as exosomes and microvesicles, which can be transferred to recipient cells contributing to fertilization [4]. The RNA content, and in particular of the miRNA, of the sEVs in semen varies according to the cell from which the sEVs are derived and thus, can reflect the pathophysiological condition of the organ of origin. In a previous study [5] our group identified miR-31-5p in semen sEVs as a biomarker for the origin of azoospermia. We showed decreased expression levels of seminal miR-31-5p in ultracentrifugation (UC) extracted- sEVs from men with OA compared with levels in those from men with SA, resulting in good predictive accuracy, sensitivity and specificity to distinguish both types of azoospermia with diagnostic utility. Given

the low number of patients analyzed (14NOA/SA vs 13 OA) and the fact that the exosome profile can be influenced by other factors, such as interindividual differences, presence of inflammatory processes, sensitivity of analysis methods, etc., a clinical validation of the test must be carefully considered.

EV isolation/purification is another significant bottleneck, due to both the time and labor required for this process, and also to local variations in the availability of the equipment and reagents needed. Specifically, our sEV-miRNA testing protocol in semen relies on the UC method to isolate EVs, which can be technically challenging and may not be available in all clinical laboratories and thus making its adoption impossible in some clinical settings.

Based on our previous results in the discovery phase of the study [5], we are interested in validating the seminal miRNA-based biomarker test for azoospermia for its use in clinical practice. In this study, we present our results on validating the efficacy of miR-31-5p as a diagnostic molecular test for azoospermia in a larger cohort of infertile individuals in order to guarantee the sensitivity and specificity of the UC-based diagnostic method in the population and to assess its actual effectiveness. Additionally, we consider preanalytical variables: the utility of the miRNA as a semen biomarker is further evaluated in vesicles extracted by a commercial isolation procedure as a second option for EV isolation and in total seminal plasma (to bypass EV isolation), as feasible and pragmatic methodological approaches in the technical procedure. This is with the aim of reducing the steps required, which results in simplifying the time-consuming protocol and reducing costs. This approach will allow the use of semen miRNA miR-31-5p as an operationally simple non-invasive diagnostic method in the clinical laboratory, and thus, would allow this seminal miRNA-based biomarker test to better reach to the patient.

MATERIALS AND METHODS

Subjects of study

Selection of patients and controls participating in the study was performed from men referred to the Andrology Service of the Fundació Puigvert between January 2016 and December 2019. The study was approved by the Ethics Committee of both centers (F. Puigvert and IDIBELL). All the participants signed an informed consent form.

Semen specimens were obtained from normozoospermic (Nz) fertile individuals consulting for vasectomy (n= 5) and sperm donors (n=8), both considered as the control group; 43 infertile men diagnosed with SA (no sperm in semen sample due to spermatogenic failure) or cryptozoospermia (<0.15 x 10^6 sperm/ml) and 43 individuals with OA and conserved spermatogenesis including men successfully vasectomized (OA-V; n=34) and individuals presenting pathological naturally occurring obstruction in the genital tract such as congenital absence of vas deferens, intratesticular obstruction or post inflammatory epididymal obstruction (OA-N; n=9). Additionally, 5 severe oligozoospermic (SSO) individuals (<5 x 10^6 sperm/ml) were included in the study. Sperm retrieval outcomes with testicular sperm extraction (TESE) provided information of the overall spermatogenic status in order to classify the azoospermia into obstructive (TESE value >0.2 x10^6 sperm/mL) or secretory (<0.02 x10^6 sperm/mL), as well as it allowed the definition of SA subgroups by discriminating between the presence (SA_Sp+) or the absence (SA_Sp-) of sperm in a testicular biopsy (Table 1). TESE value is defined as the concentration of spermatozoa that was obtained directly after processing 100mg of biopsy in 1 ml of medium.

Semen analysis was performed on all the individuals in accordance with World Health Organization guidelines [6].

Sample collection and processing

Semen samples, obtained by masturbation after 3-5 days of sexual abstinence, were allowed to liquefy for 30 min at 37 °C. Samples were centrifuged twice (1600 xg for 10 min, then 16000 xg for 10 min) at 4 °C in order to eliminate cells, cellular debris and apoptotic bodies from the biofluid and to obtain seminal plasma (supernatant) as previously described [3,5,7]. Collected seminal plasma (SP) was immediately stored at -80 °C until use.

Isolation of small EVs

First, in order to enrich for sEVs, SP aliquots (200 µl) were passed through a 0.22 µm filter. The resulting filtrate was processed by either differential ultracentrifugation (UC) or the commercial exosome isolation reagent [*ExoGAG* (NasasBiotech)], a precipitation-based method previously described as an efficient method for its use in semen samples both in terms of nanoparticle concentration and the quality of the RNA contained in the vesicles [8]. Small EVs were characterized in terms of physical properties (size, concentration) by Nanoparticle tracking analysis (NTA) using the NanoSight NS300 (Malvern Instruments). In order to avoid miRNA expression differences due to inter-individual variations, different aliquots of the same semen samples were used for the two EV isolation methods.

- *UC method*

In order to sediment the sEVs, the filtered SP fluid plus 9 ml of PBS was ultra-centrifuged at 100000 xg in a SW40 rotor for 2h at 4 °C as described elsewhere [3,5,7]. The pellet was resuspended in 100 µl PBS, treated with RNAse A (Qiagen NV; Germany) (100µg/ml final reaction concentration; 15 min at 37 °C) to degrade the residual RNA outside the vesicles and frozen at -80 °C.

- *ExoGAG technology*

An appropriate volume of precipitation reagent A of *ExoGAG* kit was added to 0.2 ml of filtered SP (reagent A/sample ratio 2:1). The mixture was incubated for 5 min at 4 °C and subsequently centrifuged at 3500 xg for 30 min at 4 °C as previously described [8]. The pellet was resuspended in 100 µl PBS, treated with RNAse A (Qiagen NV; Germany) (100µg/ml final reaction concentration; 15 min at 37 °C) to degrade any residual RNA outside the sEVs and finally frozen at -80 °C.

Small RNA-containing total RNA isolation

Total RNA was obtained, directly from SP (150ul) or from the sEV suspension. Samples were homogenized through a 20-gauge needle in the organic solution (phenol-Guanidine Isothiocyanate (GITC)-based solution) using the *miRNeasy Micro Kit* (Qiagen) to isolate RNA. RNA concentration was calculated by using the QUBIT fluorometer and the Quant-iT RNA Assay kit (Invitrogen; California, USA). RNA quality was determined by evaluating the OD 260/280 nm ratio when using a Nanodrop UV-Vis spectrophotometer (Thermo Fisher Scientific; Massachusetts, USA).

miRNA quantitative real-time PCR (qPCR) analysis

Reverse transcription (RT) of 50 ng of total SP- or sEV- RNA in 10 µl, using the *miRCURY® LNA® RT* Kit (Qiagen) was performed to obtain first-stranded cDNA specific for miRNA. For qPCR analysis, cDNA was diluted (12x) and assayed in 10 µl PCR reactions containing *miRCURY LNA SYBR® Green PCR* Kit (Qiagen). Duplicate amplification reactions of individual miRNA assays (LNA™-enhanced miRNA qPCR primers, Qiagen) were carried out on a LightCycler® 96 Instrument (Roche; Switzerland). Target hsa-miR-31-5p miRNA expression was calculated relative to the mean expression value of hsa-miR-30e-3p and hsa-miR-30d-5p, previously described to be the most stable assays in semen samples with and without spermatogenic failure, as normalizers to correct for potential overall differences between the samples [5]. The relative quantification (RQ) miRNA expression values were calculated using the 2^{dCq} strategy.

Statistical analysis

Nonparametric statistical analysis (Kruskal Wallis and Mann-Whitney tests) was carried out to evaluate differences among the groups of study in all sample conditions, including both clinical and molecular (absolute or RQ miRNA expression levels) parameters. Receiver operating characteristic (ROC) curve analysis of the RQ values was used to distinguish the origin of azoospermia. Accuracy was measured as the area under the ROC curve (AUC). The threshold value was determined by Youden's index, calculated as sensitivity plus specificity-1. A multivariate binary logistic regression analysis was used for predicting the relationship between several independent variables (miR-31-5p and FSH levels as predictors) with the presence of SA.

Following the recommendations of the STARD initiative [9], we defined 'positives' as azoospermic patients showing severe spermatogenic failure and thus, sensitivity (Sn) corresponded to the proportion of SA patients successfully detected by a given test (true positive rate), while specificity (Sp) indicated the OA patients, who showed conserved spermatogenesis (true negative rate).

Only *p*-values ≤0.05 were considered significant. All data analyses were performed using SPSS software version 15.

RESULTS

Characterization of nanovesicles by size and concentration

In order to characterize the EVs isolated using the two extraction methods on filtrated SP samples from azoospermic and control individuals, first, an evaluation of the size and concentration of nanoparticles (NTA, nanoparticle tracking analysis) was carried out using NanoSight. ExoGAG EV isolation method displayed very similar size profiles, including size mean and mode, to those obtained with the gold-standard UC method (*p*-value > 0.05; Kruskal Wallis test) (Table 2) as previously described in Nz individuals [8]. The EVs obtained by both methods are in the expected size range for exosomes (50-250 nm of diameter); specifically, 90-95% of the vesicles isolated have a size below 200 nm, with the majority (more than 50% of the vesicles) being between 105-155 nm, the most characteristic size range for exosomes. Size mean and mode of the sEVs obtained were very similar between the study groups (Nz, SA and OA) when using the UC method of sEV isolation; but slight, although not statistically significant, differences (Kruskal Wallis test) were observed between groups in ExoGAG-isolated EV samples (Table 2).

The isolation method does influence the particle concentration, with UC obtaining a higher concentration of particles per ml ($4.01 \times 10^{12} \pm 4.08 \times 10^{12}$ particles / ml) than ExoGAG ($1.12 \times 10^{12} \pm 8.44 \times 10^{12}$ particles / ml), leading to a consequent increase in the final total amount of RNA obtained by UC (18.34 ± 13.30 ng/µL) compared with ExoGAG (14.47 ± 6.78 ng/µL) from the same volume of semen, as we previously described in Nz samples [8].

miR-31-5p expression profile is similar in semen sEVs than in whole SP

In order to assess whether seminal sEV RNA obtained from different EV isolation methods or SP RNA would result in equivalent results for the use of miR-31-5p as a biomarker for the origin of azoospermia, we analyzed the expression of miR-31-5p in semen sEVs obtained by using UC and ExoGAG EV isolation method as well as in total SP.

First, our results showed that miR-31-5p presented a similar pattern of expression in the different patient and control groups as measured by the three alternative procedures (Figure 1). Interestingly, when comparing OA and SA samples, statistically significant differences in the miR-31-5p expression levels were found in the three procedures (fold-change>2; *p*-value=0.0001 Mann-Whitney test) (Table 3; Figure 1). The expression values of this sncRNA in the three alternative procedures resulted in good predictive accuracy, with the ExoGAG AUC and Sn producing slightly lower values [(UC: AUC=0.792; Sn=63%; Sp=74.4%) (ExoGAG: AUC=0.721; Sn=41%; Sp: 88%) (SP: AUC= 0.883; Sn=72.2%; Sp= 84.8%)]. Similarly, considering only naturally

occurring obstructive OA-N samples, miR-31-5p was differentially expressed between OA-N and SA samples, with an increased AUC value [(UC: AUC=0.827; Sn=97.7%; Sp=22.2%) (ExoGAG: AUC=0.721; Sn=90.9%; Sp: 55.6%) (SP: AUC= 0.963; Sn=94.4%; Sp= 50%)] (Figure 1) suggesting that it has a potential use as an indicator of the origin of the congenital azoospermia in any of the three procedures assayed, in a similar way that FSH does (AUC=0.846, p=0.026; Sn:93%; Sp: 50%).

Additionally, we studied the diagnostic accuracy of our logistic model including both blood FSH and seminal miR-31-5p, described as a useful predictive test to discriminate SA from OA_N (OA samples with available FSH value) with diagnostic accuracy [5]. In this case, there is an increase of Sn/Sp (UC: 92.9% /75%; ExoGAG: 100%/100%; SP: 100%/100%).

Elevated levels of FSH in blood suggest that azoospermia has a testicular origin, and thus the use of FSH (with a threshold of 10.4 IU/L) as a predictor of azoospermia has been described in several circumstances such as for cancer survivors [10], with 80% Sn and Sp. This fact means that the association between FSH and spermatogenic failure remains elusive in some SA cases which present values below 10 IU/L, which is the case of meiotic arrest [11]. Interestingly, miR-31-5p is able to discriminate SA from OA-N samples (UC: AUC: 0.888; *p*-value 0.006; ExoGAG: AUC:1; *p*-value <0.0001; SP: AUC: 1; *p*-value: 0.009) better than FSH (AUC: 0.550; *p*-value: 0.722) in those azoospermic samples with FSH <10 IU/L (Figure 2), when using any of the procedures applied suggesting miR-31-5p is especially useful for azoospermia diagnosis in patients with FSH<10 IU/L.

DISCUSSION

Seminal plasma is an ideal liquid biopsy of the urogenital tract [12]. This fluid is particularly valuable as a non-invasive tool that enables evaluation of biomarkers, which represents a significant improvement in the field of precision medicine or personalized medicine with clinical applications in urogenital disease diagnosis, prognosis and treatment decisions. Specifically, miRNAs in seminal fluid have come to be considered as promising non-invasive diagnostic urogenital biomarkers [5,13-15]. In contrast to blood plasma and cell culture supernatant, where only a minor fraction of miRNA appears contained within EVs, seminal miRNAs are mostly kept within intact small cell derived EVs of seminal plasma [4], thus in our previous study we developed a semen miRNA-based biomarker test for azoospermia (showing a hierarchy of miR-31-5p expression values Nz>SA>OA), using UC-isolated sEVs from semen as a sample source [5]. miR-31-5p is highly expressed in the testis, and epididymis, but also at lower levels in the prostate [5].

The present methodological case study was designed firstly to validate the use of UC-EV miR-31-5p as a biomarker of the origin of azoospermia. Secondly, we set out to compare miR-31-5p expression results from UC-isolated sEVs as biomarker, with results from sEVs isolated by a commercial kit, which does not require special equipment, as an alternative method. Based on our previous results, we selected the ExoGAG commercial precipitation isolation protocol as an alternative EV isolation protocol to UC, which allows the enrichment of CD63 and CD81 positive seminal EVs, with an optimal nanoparticle size and concentration, and RNA quality contained within them, when compared with the UC gold-standard protocol, although it does provide a lower efficiency of EV isolation [8]. Additionally, for any biomarker to be introduced into the clinical setting, appropriate pre-analytical issues should be considered such as an adequate choice of sample source for the analysis. Since semen miRNAs are mostly enclosed and protected within intact seminal sEVs rather than in the supernatant [4], a similar content of miRNA would be considered in both semen EVs and total seminal plasma. Thus, a third aim was set, which was a further evaluation of the usefulness of the miRNA-based test in whole SP, using the same semen sample set, to bypass the step extracting EVs from semen and simplifying the protocol.

Our results provide evidence that altered miR-31-5p expression can be determined both from EVs and from the whole SP to discriminate OA from SA azoospermic samples with diagnostic potential. The fact that miR-31-5p in semen mainly comes from testis and epididymis[5], could explain the lower expression levels of miR-31-5p in semen from OA (which presents with an obstruction in the ducts or vas deferens, and thus miRNA derived from testis and epididymis

cannot reach semen) compared with that from SA individuals (which, due to spermatogenic impairment, present an altered testicular miRNA signature [16,17] but epididymal secretion is preserved). In this study we validate the diagnostic value of semen miR-31-5p to predict the origin of azoospermia, not only from UC isolated-EVs (AUC: 0.792) but also from ExoGAG EVs (AUC: 0.721) as well as from total seminal plasma (AUC: 0.883). Consequently, our data provides evidence that detection of miR-31-5p can serve as a biomarker in EVs isolated by different methods and in SP, and thus shows that it is appropriate to use this semen miRNA as a biomarker for azoospermia diagnosis. Contrary to what has been observed in prostate cancer, where seminal fluid for a miR-142-3p+miR-142-5p+miR-223-3p based model showed lower fold change differences between tumour and non-tumour samples when compared to prostate cancer sEVs [14], the miR-31-5p expression pattern was similar for both semen exosomes and SP in OA and SA samples. Thus, our results suggest that miR-31-5p in SP is also useful as a biomarker for the origin of azoospermia. The choice of taking seminal fluid as a biological sample has the potential to technically simplify the use of miR-31-5p as a semen biomarker and strengthens the advantages of this established pipeline when aiming to quantify miR-31-5p in semen without sacrificing any true positive result, and even slightly improving the specificity of the test.

Not only has this miRNA-based molecular model been validated as a clinically useful test for predicting the origin of azoospermia in a sample from azoospermic individuals, but additionally, and more interestingly for the clinicians, it has evidenced its usefulness for predicting the presence of spermatogenic failure in azoospermic patients with FSH values <10 IU/L as a sensitive and specific biomarker.

We have obtained results consistent with our previous data although a lower accuracy value was obtained in the present validation study when compared to the previous publication [5] in UC-EV samples. This difference in the result is probably a consequence of the influence of sample size between the two studies although some technical issues should not be discarded such as the use of a different RNA isolation kit: miRCURY RNA Isolation kit (from Exiqon, used in Barceló et al., 2018 study, now no longer available) and the *miRNeasy Micro Kit* (Qiagen, used in the present study). On the other hand, the difference in miR-31-5p expression AUC between semen sEVs and total SP is not surprising: we should expect not only cell-free miR-31-5p miRNA actively secreted by testicular, epididymal and prostatic cells in EVs [5], but also miRNA originating from either apoptotic and/or necrotic cells to be present in total SP, because miRNA integrity is robust even in degraded samples [18].

A biomarker assay should only be used to guide management if it has analytical validity, meaning that it is accurate, reproducible, and reliable, and if it has been shown to have clinical utility, as it is the case for miR-31-5p in seminal fluid to diagnose the origin of azoospermia.

In summary, our validation work corroborates miR-31-5p as a relevant biomarker in semen for azoospermia, not only in EVs but also in the whole SP. Both sEVs and SP can be reliably used for miR-31-5p analysis, with slight differences in diagnostic accuracy. Additionally, miR-31-5p has been validated as a useful biomarker for the origin of azoospermia especially in those individuals with FSH values < 10 IU/L, with the potential to discriminate between OA and SA. The use of total SP as an analytical sample would facilitate the use of a simplified technical procedure for miR-31-5p quantification and would represent a great improvement in reproductive treatment decision protocols for azoospermia in clinical practice.

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Authors' Contribution statement

L P-P & O L-R performed the research, L P-P & SL analyzed the data, and LB & SL designed the research study and wrote the paper

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REFERENCES

1. Krausz C, Riera-Escamilla A. Genetics of male infertility. *Nature reviews Urology*. Jun 2018;15(6):369-384. https://doi.org10.1038/s41585-018-0003-3.
2. Jarow JP, Espeland MA, Lipshultz LI. Evaluation of the azoospermic patient. *The Journal of urology*. Jul 1989;142(1):62-5. https://doi.org10.1016/s0022-5347(17)38662-7.
3. Li H, Huang S, Guo C, Guan H, Xiong C. Cell-free seminal mRNA and microRNA exist in different forms. *PLoS One*. 2012;7(4):e34566.
4. Vojtech L, Woo S, Hughes S, et al. Exosomes in human semen carry a distinctive repertoire of small non-coding RNAs with potential regulatory functions. *Nucleic Acids Res*. Jun 2014;42(11):7290-304.
5. Barceló M, Mata A, Bassas L, Larriba S. Exosomal microRNAs in seminal plasma are markers of the origin of azoospermia and can predict the presence of sperm in testicular tissue. *Hum Reprod*. Jun 1 2018;33(6):1087-1098. https://doi.org10.1093/humrep/dey072.
6. Cooper TG, Noonan E, von Eckardstein S, et al. World Health Organization reference values for human semen characteristics. *Hum Reprod Update*. May-Jun 2010;16(3):231-45.
7. Eldh M, Lotvall J, Malmhall C, Ekstrom K. Importance of RNA isolation methods for analysis of exosomal RNA: evaluation of different methods. *Mol Immunol*. Apr 2012;50(4):278-86.
8. Mercadal M, Herrero C, López-Rodrigo O, et al. Impact of Extracellular Vesicle Isolation Methods on Downstream Mirna Analysis in Semen: A Comparative Study. *International journal of molecular sciences*. Aug 19 2020;21(17). https://doi.org10.3390/ijms21175949.
9. Bossuyt PM, Reitsma JB, Bruns DE, et al. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. *Family practice*. Feb 2004;21(1):4-10. https://doi.org10.1093/fampra/cmh103.
10. Kelsey TW, McConville L, Edgar AB, et al. Follicle Stimulating Hormone is an accurate predictor of azoospermia in childhood cancer survivors. *PLoS One*. 2017;12(7):e0181377. https://doi.org10.1371/journal.pone.0181377.
11. Zitzmann M, Nordhoff V, von Schonfeld V, et al. Elevated follicle-stimulating hormone levels and the chances for azoospermic men to become fathers after retrieval of elongated spermatids from cryopreserved testicular tissue. *Fertil Steril*. Aug 2006;86(2):339-47.
12. Drabovich AP, Saraon P, Jarvi K, Diamandis EP. Seminal plasma as a diagnostic fluid for male reproductive system disorders. *Nature reviews Urology*. May 2014;11(5):278-88. https://doi.org10.1038/nrurol.2014.74.
13. Barceló M, Castells M, Bassas L, Vigués F, Larriba S. Semen miRNAs Contained in Exosomes as Non-Invasive Biomarkers for Prostate Cancer Diagnosis. *Sci Rep*. Sep 24 2019;9(1):13772. https://doi.org10.1038/s41598-019-50172-6.
14. Barceló M, Castells M, Pérez-Riba M, Bassas L, Vigués F, Larriba S. Seminal plasma microRNAs improve diagnosis/prognosis of prostate cancer in men with moderately altered prostate-specific antigen. *American journal of translational research*. 2020;12(5):2041-2051.
15. Zarezadeh R, Nikanfar S, Oghbaei H, et al. Omics in Seminal Plasma: An Effective Strategy for Predicting Sperm Retrieval Outcome in Non-obstructive Azoospermia. *Molecular*

diagnosis & therapy. May 2021;25(3):315-325. https://doi.org10.1007/s40291-021-00524-8.

16. Muñoz X, Mata A, Bassas L, Larriba S. Altered miRNA Signature of Developing Germ-cells in Infertile Patients Relates to the Severity of Spermatogenic Failure and Persists in Spermatozoa. *Sci Rep*. Dec 9 2015;5:17991. https://doi.org10.1038/srep17991.
17. Lian J, Zhang X, Tian H, et al. Altered microRNA expression in patients with non-obstructive azoospermia. *Reproductive biology and endocrinology : RB&E*. Feb 11 2009;7:13. https://doi.org10.1186/1477-7827-7-13.
18. Jung M, Schaefer A, Steiner I, et al. Robust microRNA stability in degraded RNA preparations from human tissue and cell samples. *Clin Chem*. Jun 2010;56(6):998-1006. https://doi.org10.1373/clinchem.2009.141580.

FIGURE LEGENDS

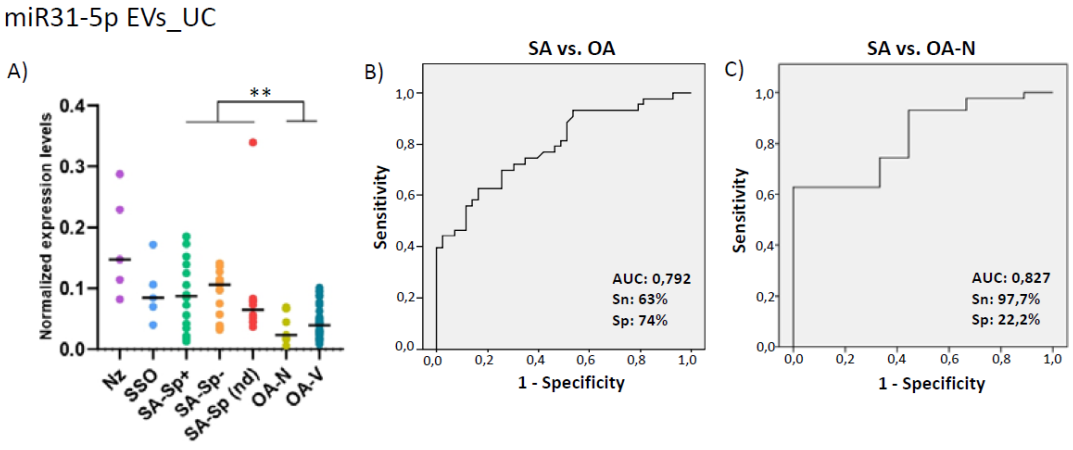
Figure 1. miR-31-5p levels are altered in azoospermia with different origin in seminal EVs and in SP

Expression levels of miR-31-5p were determined in the different groups of the study in EVs isolated by ultracentrifugation (A, EVs_UC) in EVs isolated by ExoGAG (B, EVs_ExoGAG) and in total seminal plasma (C). Normalized expression levels relative to the mean of miR-30d-5p and miR-30e-3p are shown. Significant differences between groups are indicated ** p-value <0.01 (Mann-Whitney test).

ROC analysis showing the predictive efficiency of miR-31-5p for distinguishing SA from OA samples (B, E, H) as well as for distinguishing SA from OA-N samples (C, F, I)

(SA: secretory azoospermia; OA: obstructive azoospermia; OA-N: obstructive azoospermia due to pathological naturally occurring obstruction in the genital tract; AUC, area under the curve; Sn, sensitivity; Sp, specificity)

Figure 1



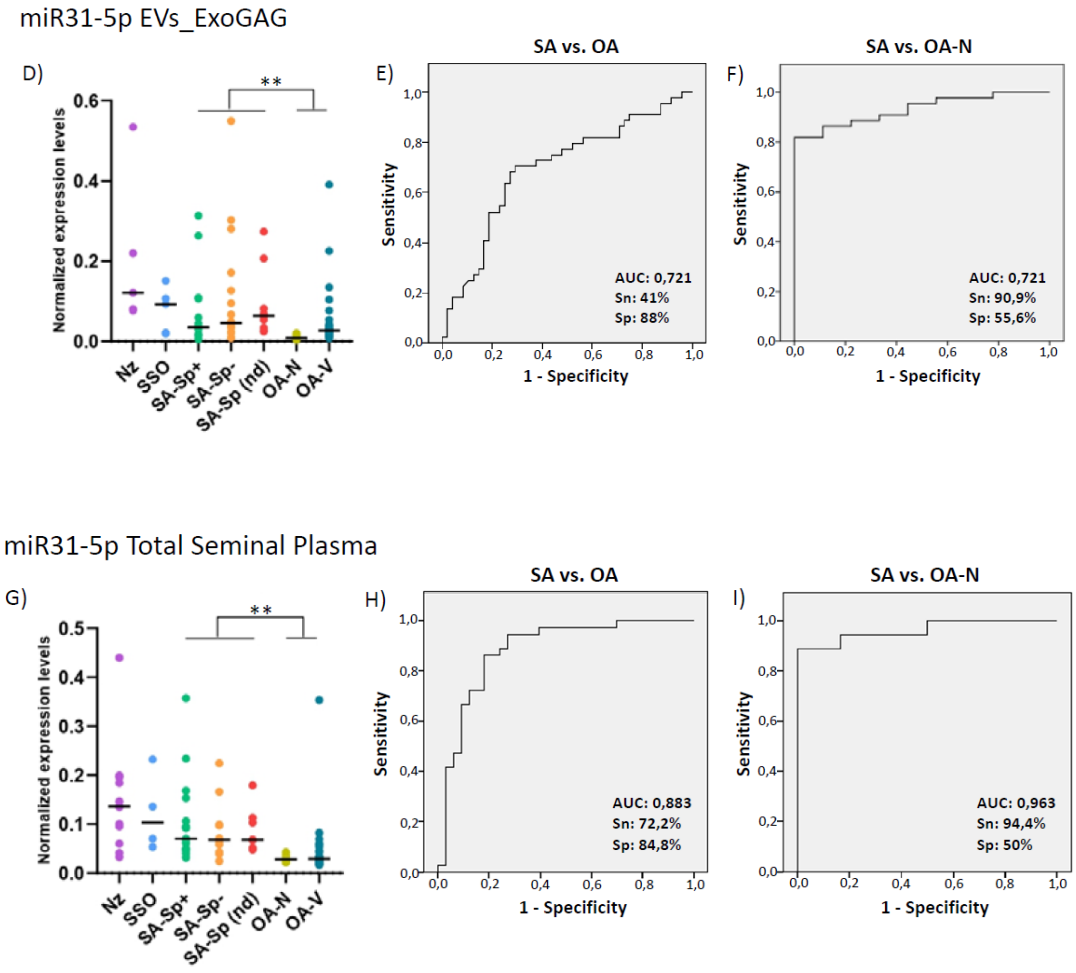


Figure 2. Seminal miR-31-5p as indicator of the origin of azoospermia in azoospermic cases with FSH <10 IU/L

Seminal miR-31-5p, in EV_UC (A), EV_ExoGAG (B) and SP (C), is able to discriminate SA from OA-N samples better than blood FSH (D) in azoospermic cases with FSH <10 IU/L

Figure 2

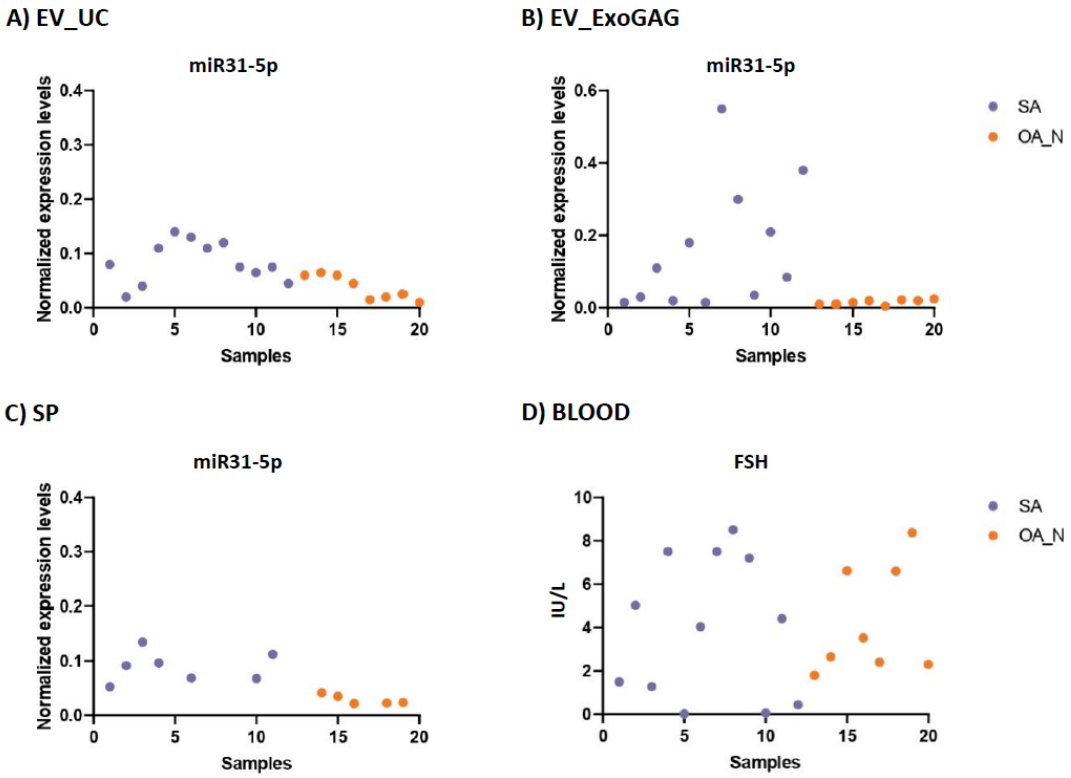

A) EV_UC
miR31-5p
Normalized expression levels
Samples
B) EV_ExoGAG
miR31-5p
Normalized expression levels
Samples
SA
OA_N
C) SP
miR31-5p
Normalized expression levels
Samples
D) BLOOD
FSH
IU/L
Samples
SA
OA_N

Table 1. Clinical data of individuals included in the study

Number of patients (n)	Spermiogram	Subgroups	Male age (years; mean ± SD)	Testes volume (ml; range)	FSH (IU/l)	Sperm count ($x10^6$/mL)	Progressive motility (%)	TESE (pos/neg; $x10^6$ sperm/ml)
8	Nz-SD	Nz	22,4 ± 2,83	18-20	-	105,9 ± 55,09	59,4 ± 12,33	-
5	Nz-preV	Nz	40,6 ± 2,07	18-20	-	65,8 ± 30,98	52,8 ± 11,32	-
5	SSO	SSO	37 ± 4,24	6-20	10,37 ± 8,82	0,25 ± 0,07	11,4 ± 12	-
16	AZO	SA (Sp+)	42,4 ± 7,28	1-18	23,45 ± 11,02	0	-	pos (<0,02)
17	AZO	SA (Sp-)	36,7 ± 5,84	1-18	20,78 ± 12,03	0	-	neg
10	AZO	SA	34,1 ± 6,60	4-20	15,456 ± 11,74	0	-	-
9	AZO	OA-N	39,67 ± 4,69	15-20	4,3 ± 2,5	0	-	pos (>0,2)
34	AZO	OA-V	40,29 ± 6,14	15-20	-	0	-	pos (>0,2)

Nz-SD: normozoospermic sperm donor; Nz-preV: normozoospermic prevasectomized men; SSO: severe secretory oligozoospermia; AZO: azoospermia and cryptozoospermia, SA: secretory azoospermia; SA (Sp+): individuals witha positive TESE value; OA-N: obstructive azoospermia due to pathological naturally ocurring-obstruction in the genital tract; OA-V: obsructive azoospermia as a result of a vasectomy; TESE: testicular sperm extraction; pos: positive; neg: negative

Table 2. Nanoparticle tracking analysis (NTA) measurement of particle size of EV-extracts obtained by UC or ExoGag procedures

EV Extraction method	Phenotype	Size mean (nm)	Size mode (nm)	Size D10 (nm)	Size D50 (nm)	Size D90 (nm)
	Nz (n=5)	128±5,20	100,57±2,94	77,23±3,27	105,57±3,65	169,4±13,31
Ultracentrifugation	SA (n=5)	132,14±4,34	101,1±2,17	85,66±3,16	117,14±1,08	197,9±16,47
	OA (n=5)	131,6±4,75	100,16±4,69	87,44±2,92	119,64±3,90	189,9±11,07
	Nz (n=4)	135,23±2,52	117,45±1,83	87,53±0,95	113,93±1,31	173,38±6,98
ExoGag	SA (n=5)	140,27±18,52	103,05±5,44	91,03±6,60	120,82±9,63	218,85±54,95
	OA (n=4)	126,88±13,43	101,66±10,67	84,7±8,16	112,64±10,43	187,9±39,35

No statistically significant differences (p-value > 0,05; Kruskal Wallis test) were observed among the groups of study

Nz: Normozoospermia; SA: secretory azoospermia; OA: obstructive azoospermia

Table 3 Summary of miRNA expression data in semen vesicles obtained with UC (A), with ExoGAG kit (B) and in total seminal plasma (C)

miRNA expression data in Nz, SSO and SA is expressed related to OA samples

A p-value <0,05 showed statistical significance (Mann Whitney non-parametric test was used)

A.

			Average Cq EV_UC			miRNA expression				*p* -value
Gene Name	Nz	SSO	SA	OA	CV	Nz	SSO	SA	OA	(OA-SA)
miR-31-5p	24,84±1,08	25,52±1,21	25,95±1,18	26,61±1,16	0,047	4,062	2,226	2,149	1	0,0001
miR-30d-5p	20,63±0,52	20,35±0,74	20,58±0,86	20,09±1,11	0,048	0,903	0,954	0,968	1	>0,1
miR-30e-3p	23,68±0,40	23,56±0,62	23,81±0,75	23,43±1,13	0,039	1,098	1,036	1,04	1	>0,1
miR (30d-5p + 30e-3p)					0,043					

B.

			Average Cq EV_ExoGAG			miRNA expression				*p* -value
Gene Name	Nz	SSO	SA	OA	CV	Nz	SSO	SA	OA	(OA-SA)
miR-31-5p	25,72±1,21	28,06±1,51	28,8±2,08	29,50±1,92	0,073	6,273	2,377	2,763	1	0,0001
miR-30d-5p	21,10±0,45	22,67±1,24	22,6±1,98	22,17±2,28	0,092	1,001	0,864	1,024	1	>0,1
miR-30e-3p	24,95±0,50	25,60±1,37	26,37±1,75	25,86±1,99	0,07	0,886	1,103	0,965	1	>0,1
miR (30d-5p + 30e-3p)					0,078					

C.

			Average Cq SP			miRNA expression				*p*- value
Gene Name	Nz	SSO	SA	OA	CV	Nz	SSO	SA	OA	(OA-SA)
miR-31-5p	25,92±1,97	25,77±1,11	26,04±1,26	27,66±1,51	0,062	3,464	2,57	2,182	1	0,0001
miR-30d-5p	21,17±1,43	20,59±0,54	20,46±0,91	21,02±1,46	0,059	0,974	1,037	1,128	1	0,05
miR-30e-3p	24,59±1,24	24,20±0,55	24,3±0,97	24,51±1,41	0,048	1,023	0,956	0,88	1	0,05
miR (30d-5p + 30e-3p)					0,052					