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MicroRNA signature in spermatozoa and seminal plasma of proven fertile men and in testicular tissue of men with obstructive azoospermia

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Abstract

MicroRNAs (miRNAs) have recently received a significant amount of attention due to their remarkable influence on post-transcriptional gene regulation. In this study, we aim to provide a catalogue of miRNAs present in spermatozoa, seminal plasma and testicular tissue. Expression profiles of miRNA in spermatozoa and seminal plasma of 16 proven fertile men and testicular tissue of eight men with morphologically and/or histologically confirmed obstructive azoospermia were determined by microarray and RT-qPCR in combination with bioinformatics analyses. A total of 123, 156 and 133 miRNAs were consistently detected in spermatozoa, seminal plasma and testicular tissue respectively. Sixty-four miRNAs were shared across all sample types. Based on miRNAs expression level present in each group, correlation analysis showed moderate-to-strong correlations within the spermatozoa and seminal plasma samples and a wider range of correlations within the testicular tissue samples. The target genes of known miRNAs appeared to be involved in a wide range of biological processes related to reproduction, development and differentiation of germ cells. Our results suggest that there is a certain similarity between spermatozoa and seminal plasma for the relative miRNA expression changes with respect to testicular tissue and provide an overview of the miRNAs present in each sample type.

KEYWORDS
male infertility, MicroRNA, seminal plasma, spermatozoa, testicular tissue

1 | INTRODUCTION

Reduced male fertility is a complex disease with multifactorial aetiologies, in which genetic and epigenetic factors contribute to disease complexity (Krausz & Giachini, 2007). It has been shown that many genes are participating in the control of the spermatogenesis process, but the biological function of the majority of these genes in the control of the spermatogenesis process is still unclear (Gianotten, Lombardi, Zwinderman, Lilford, & Veen, 2004). However, the expression of these genes may be regulated by many biological factors, including microRNAs (miRNAs). Over the past decade, miRNAs have emerged as key post-transcriptional

regulators of gene expression (Ha & Kim, 2014). To date, accumulating studies have indicated that miRNAs are participating in the control of many, if not all, biological and pathological processes (Gurtan & Sharp, 2013). In mammalian spermatogenesis, evidence indicates that even a slight alteration in miRNA expression activities can be detrimental to germ cell function, ultimately leading to impaired spermatogenesis and compromised fertility (Chang et al., 2012; Hayashi et al., 2008; Maatouk, Loveland, McManus, Moore, & Harfe, 2008). Altered expression of miRNAs in men with reduced fertility has been investigated (Abu-Halima et al., 2019, 2013, 2016; Abu-Halima, Backes, et al., 2014; Abu-Halima, Hammadeh, et al., 2014). Specifically, differentially expressed miRNAs were reported in spermatozoa of infertile men with normozoospermic versus. subfertile men with asthenozoospermic and oligoasthenozoospermic (Abu-Halima et al., 2013), in testes of men with normal versus. impaired spermatogenesis (in Sertoli cell only, mixed atrophy and germ cell arrest) (Abu-Halima, Backes, et al., 2014) and in extracellular microvesicles obtained from seminal plasma from normozoospermic versus oligoasthenozoospermic men (Abu-Halima et al., 2016). Although several miRNAs have been found altered in certain spermatogenic impairments, the exact cellular function and biological mechanism of these miRNAs have been revealed for a very limited number of miRNAs. With this study, by using miRNA microarrays along with the bioinformatics analysis, we aim to provide a catalogue of miRNAs present in spermatozoa and seminal plasma of proven fertile men and testicular tissue of men with obstructive azoospermia.

2 | METHODS

2.1 | Study population

Following Institutional Ethics Committee of Saarland University (Nr. Ha195/11) approval, eight semen samples were obtained from men aged between 26 and 34 years (29 ± 3.12, Mean ± *SD*) with proven fertility, that is men with normal semen parameters and parenthood after the investigation (Table S1). Semen samples were provided after at least three days of sexual abstinence, and semen parameters, including volume, pH, viscosity, sperm count (10^6/ml), motility (motile %) and morphology (%) were performed based on the World Health Organization, 2010, (WHO, 2010) guidelines. Using Puresperm 45%–90% discontinuous density gradients, 1 ml of the semen sample was placed on the upper layer before centrifugation at 500 *g* for 15 min at room temperature (RT). After centrifugation, the upper seminal plasma layer was aspirated and transferred to a new sterile 1.5-mL Eppendorf tube for total RNA including miRNAs purification. In addition, purified RNA from spermatozoa samples (n = 8) were obtained from our previous study (Abu-Halima et al., 2013). These samples were collected from proven fertile men aged between 24 and 34 years (34 ± 5.6, Mean ± *SD*) (Table S1). Purified RNA from testicular samples (n = 8) were obtained from our previous study (Abu-Halima, Backes, et al., 2014; Abu-Halima, Hammadeh, et al., 2014). These samples were obtained from men aged between 24 and 30 (27 ± 2.1, Mean ± *SD*), with normal testicular volume, no evidence of inflammatory granuloma or malignancy, and the testis exhibited nearly normal spermatogenesis process with many mature sperms noted and populated by normal Sertoli cells and Leydig cells number.

2.2 | Purification and quality assessment

Purification of miRNA from seminal plasma was performed as previously described (Wu et al., 2012), with a slight modification. Briefly, 200 µl of the seminal plasma was used for total RNA purification. The total RNA was isolated by mixing the seminal plasma with an equal volume of TRIzol reagent (Thermo Fisher Scientific), and the procedure was completed according to the manufacturer's protocol. Three steps of phenol/chloroform purification were added to get rid of proteins. The quantity and purity were tested using the NanoDrop ND-2000 spectrophotometer (Thermo Fisher Scientific). The integrity of the purified RNAs was assessed using Agilent 2100 Bioanalyzer (Agilent Technologies). DNase treatment was carried out to remove the gDNA carry-over as described previously (Abu-Halima et al., 2016).

2.3 | Analysis of miRNAs by microarray

The miRNA expression profile in the spermatozoa and testicular tissue samples was obtained from our previously generated and published raw data using SurePrint™ 8X60K Human v16 miRNA platform (Agilent Technologies) (Abu-Halima, Backes, et al., 2014; Abu-Halima et al., 2013). As for seminal plasma samples, miRNA profiling analysis was carried out on the purified RNA fraction from eight seminal plasma samples using the SurePrint™ 8X60K Human v16 miRNA platform (Agilent Technologies). These platforms contain probes for the detection of 1,205 human miRNAs. One hundred nanograms (ng) of the purified RNA were labelled and subsequently hybridized to the miRNA microarray chip as previously described (Abu-Halima, Backes, et al., 2014; Abu-Halima et al., 2013).

2.4 | Reverse Transcription and Quantitative Real-Time PCR (RT-qPCR) of miRNA

The expression level of certain miRNAs in each sample was determined using the *mi*Script PCR System (Qiagen). One hundred nanograms (ng) purified RNA from each sample, that is spermatozoa (Abu-Halima et al., 2013), testicular tissue (Abu-Halima, Backes, et al., 2014) and seminal plasma, were reverse transcribed using the *mi*Script RT II kit (Qiagen). The cDNA was then diluted 1:10, and 1 µl of cDNA was mixed with 10 µl 2X QuantiTect SYBR Green, 2 µl 10X Universal Primer Assay, 2 µl 10X Specific Primer Assay and H_2O in a total volume of 20 µl.

The miRNA primer assays were selected based on their abundance level changes in men with subfertility compared to men with proven fertility and based on their known associations with spermatogenesis. In detail, 11 miRNAs were validated in spermatozoa (miR-10a-5p, miR-122-5p, miR-34b-3p, miR-16-5p, miR-1299, miR-513a-5p, miR-34b-5p, miR-30d-5p, miR-125b-5p, miR-10b-5p and miR-125a-3p), 5 miRNAs in seminal plasma (miR-141-3p, miR-26b-5p, Let-7b-5p, miR-125a-3p and miR-99a) and 8 miRNAs in testicular tissue (miR-107, miR-145-5p, miR-20a-5p, miR-449a, miR-513b-5p, miR-125b-5p, miR-99a and Let-7d-5p). All experiments were carried out on the QIAgility™ automated PCR setup robot (Qiagen) and StepOnePlus™ Real-Time PCR cycler (Applied Biosystems).

2.5 | Bioinformatics analysis

Since the samples came from different sources, we cannot directly compare the expression level of miRNAs across the sample groups. Therefore, we focused on relative differences between the groups. Arrays data were quantile normalised by using the preprocessCore package of the R software (www.R-project.org). The resulting values were shifted by the absolute value of the minimal expression level (determined over the whole data set) and additionally by 0.1 to ensure that all values are positive. Finally, a log2 transformation of the data was performed (Figure 1a). Additionally, a detection matrix was generated where a miRNA is considered to be detected in a sample of its total probe signal is bigger than 3*probe error. Probe error is the robust average of all the processed green signal errors for each replicated probe multiplied by the total number of probe replicates: the effective feature size fraction, the nominal spot area and the weight. Therefore, it is an estimate of the probe signal error provided by the Feature Extraction software from Agilent. To classify a miRNA as absent, present or undetermined, we defined two thresholds for each sample group computed from the processed expression levels of miRNAs detected in 0% and 100% of all samples in the corresponding group. In our analysis, we concentrated on miRNAs classified as present with respect to the three sample groups. The present identified miRNA list was then converted from v16 to v21 according to the updated miRBase database (Release 22.1). The miRNAs which were not matched/identified were subsequently removed from the database. Then, we determined the within-group sample correlation computing Spearman's correlation coefficient based on miRNAs present in the corresponding group. Furthermore, pairwise miRNA correlations with the corresponding *p*-values were calculated for present miRNAs for each group separately. *P*-values were corrected using the Bonferroni correction. Finally, we performed a quantile rank analysis (Smalheiser et al., 2012) applied to all miRNAs in our data set to identify miRNAs whose rank changed significantly between two sample groups. The $2^{-\Delta Cq}$ relative quantitative procedure (Livak & Schmittgen, 2001) was used to detect the expression changes of single miRNAs. The small nuclear RNA (snRNA) RNU6B was chosen as a reference endogenous for normalisation.

3 | RESULTS

3.1 | Subjects characteristics

In total, we compared 24 miRNA expression profiles, including eight profiles from the seminal plasma of fertile men, that is men with normal semen parameters and proven fertility. Eight miRNA expression profiles from spermatozoa of men with proven fertility were included from our previous study (Abu-Halima et al., 2013). We had, however, problems in collecting testicular tissues from men with proven fertility. Therefore, eight miRNA expression profiles from testicular tissues of men with morphologically and/or histologically confirmed obstructive azoospermia were included as control tissue from our previous study (Abu-Halima, Backes, et al., 2014).

3.2 | Present and absent miRNAs

The data set was quantile normalised using the R-package preprocessCore (R Development Core Team 2010) (Figure 1a). To classify miRNAs as present or absent, we defined two expression level thresholds (one for absence and one for presence) for each sample group. These thresholds were computed as follows: Assuming that not detected miRNAs demonstrate lower expression levels and detected miRNAs tend to have a high expression level, we estimated the density distribution of miRNA expression values for miRNAs with 0% and 100% detection rate in the considered sample group using R-package sm (Shah, Leidinger, Blin, & Meese, 2010). The 1 percentile of the 100% detection rate density distribution and the 99 percentile of the 0% detection rate density distribution were set as the thresholds for the absent and present miRNAs respectively (Figure 1b): We defined a miRNA as present or absent under the following conditions: a miRNA is considered as present if its expression value is above the upper threshold (99 percentile) and absent if its expression value is below the lower threshold (1 percentile), and otherwise, the miRNA was defined as undetermined. Second, a miRNA is present/absent in a sample group if it is present or absent in all samples of the considered group, that is in spermatozoa, seminal plasma and testicular tissue. Out of 1,205 investigated miRNAs, 697, 729 and 691 were not detected (absent) and 126, 161 and 137 miRNAs were detected (present) in spermatozoa, seminal plasma and testicular tissue respectively (Tables S2 and S3). Using the miRCarta web-service tool (Backes et al., 2018), we converted the present miRNAs from v16 to v21 according to the updated miRBase database (Release 22.1, http://www.mirbase.org/). Three miRNAs (miR-1274b, miR-720 and miR-1280), five miRNAs (miR-1274b, miR-3676, miR-1274a, miR-720 and miR-1280) and four miRNAs (miR-1274b, miR-1274a, miR-720 and miR-1280) were not detected in the updated version of miRBase v22.1 and have subsequently been removed from the present miRNA list of each group, that is spermatozoa, seminal plasma and testicular tissue respectively. A detailed list of miRNAs that cannot be found in the destination annotation (in

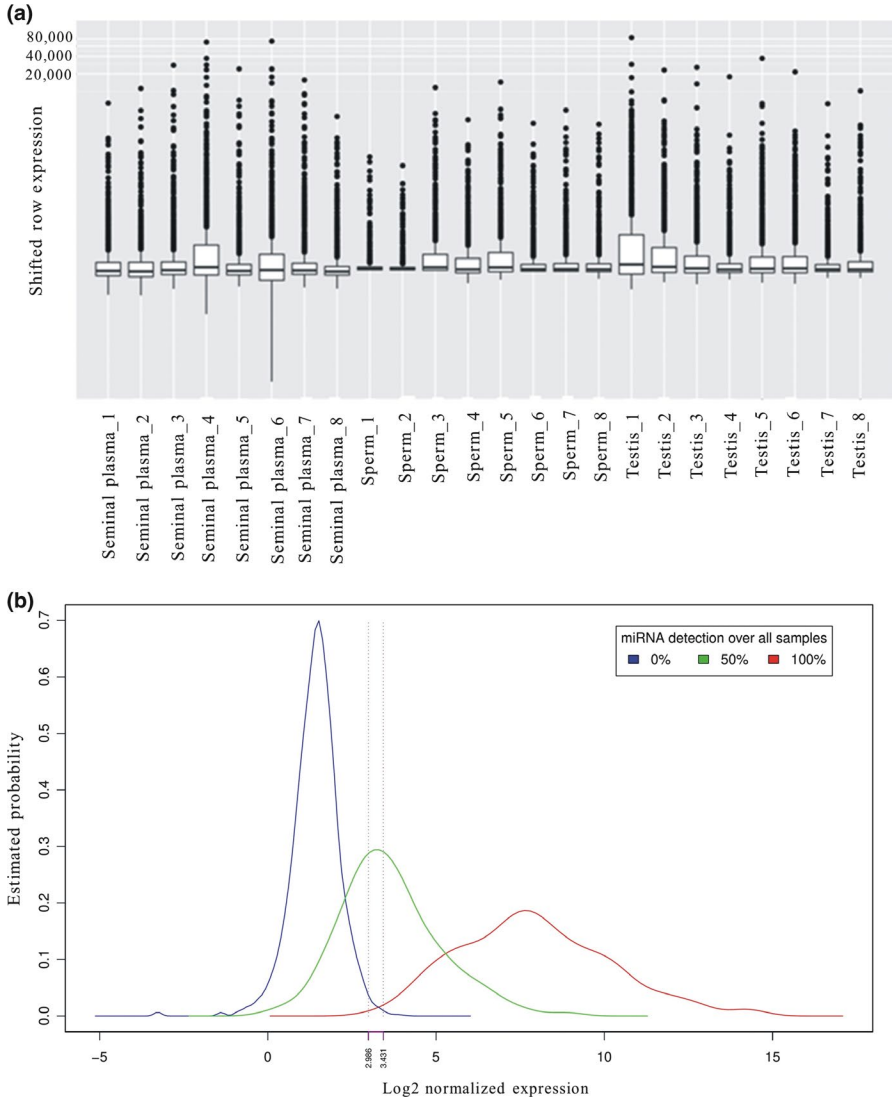

FIGURE 1 (a) Box plots of shifted (by |minimal value| and 0.1) raw expression levels per sample on log2-scale and (b) estimated density distributions based on processed expression levels of miRNAs with a detection rate of 0% (red), 50% (green) and 100% (blue) in the sample groups. The two thresholds are visualised by two dashed vertical lines

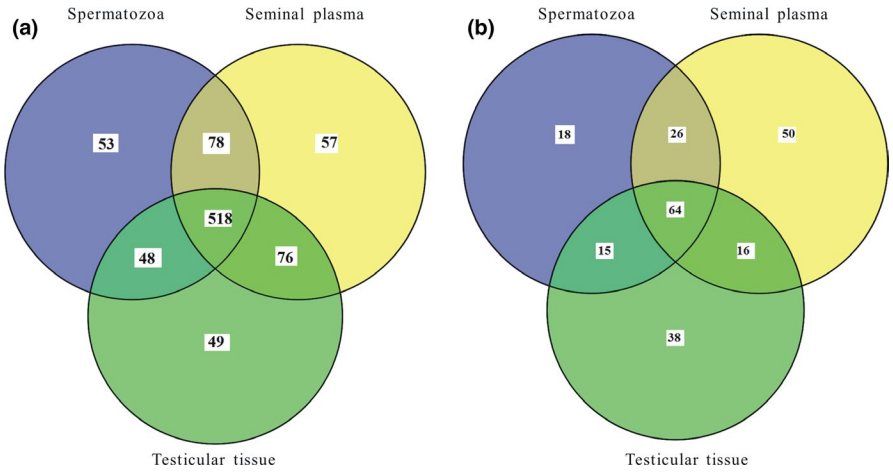

FIGURE 2 Venn diagram of (a) absent miRNAs and (b) present miRNAs

a more recent miRBase 22.1 version) is reported in bold in Table S3. Therefore, in total, 123, 156 and 133 miRNAs were present in spermatozoa, seminal plasma and testicular tissue, respectively, according to miRBase v22.1. The Venn diagrams in Figure 2a,b show the overlap of present miRNAs sets between the three sample groups. As shown in Table 1, 18 miRNAs present only in spermatozoa, and 50 miRNAs in seminal plasma and 38 miRNAs in testicular tissue samples were identified.

TABLE 1 The present/detected miRNAs in spermatozoa, seminal plasma and testicular tissue

miRNAs present in 'only' spermatozoa		miRNAs present in 'only' seminal plasma		miRNAs present in 'only' testicular tissue	
miRBase accession	MicroRNA	miRBase accession	MicroRNA	miRBase accession	MicroRNA
MIMAT0000686	hsa-miR-34c-5p	MIMAT0004812	hsa-miR-548d-5p	MIMAT0009447	hsa-miR-1972
MIMAT0015089	hsa-miR-3202	MIMAT0004806	hsa-miR-548c-5p	MIMAT0002881	hsa-miR-509-3p
MIMAT0015073	hsa-miR-3190-5p	MIMAT0000707	hsa-miR-363-3p	MIMAT0000080	hsa-miR-24-3p
MIMAT0018110	hsa-miR-3682-3p	MIMAT0017992	hsa-miR-3614-5p	MIMAT0001541	hsa-miR-449a
MIMAT0000081	hsa-miR-25-3p	MIMAT0005826	hsa-miR-1181	MIMAT0002819	hsa-miR-193b-3p
MIMAT0000421	hsa-miR-122-5p	MIMAT0016921	hsa-miR-4290	MIMAT0000084	hsa-miR-27a-3p
MIMAT0005893	hsa-miR-1305	MIMAT0002843	hsa-miR-520b	MIMAT0001341	hsa-miR-424-5p
MIMAT0004676	hsa-miR-34b-3p	MIMAT0016862	hsa-miR-4310	MIMAT0000419	hsa-miR-27b-3p
MIMAT0015030	hsa-miR-3156-5p	MIMAT0016864	hsa-miR-4312	MIMAT0000443	hsa-miR-125a-5p
MIMAT0015028	hsa-miR-3154	MIMAT0000420	hsa-miR-30b-5p	MIMAT0000461	hsa-miR-195-5p
MIMAT0016858	hsa-miR-4306	MIMAT0002174	hsa-miR-484	MIMAT0000418	hsa-miR-23b-3p
MIMAT0014990	hsa-miR-3127-5p	MIMAT0004947	hsa-miR-885-5p	MIMAT0002883	hsa-miR-514a-3p
MIMAT0014988	hsa-miR-3125	MIMAT0000753	hsa-miR-342-3p	MIMAT0000231	hsa-miR-199a-5p
MIMAT0003263	hsa-miR-595	MIMAT0000243	hsa-miR-148a-3p	MIMAT0018071	hsa-miR-3651
MIMAT0005887	hsa-miR-1299	MIMAT0003298	hsa-miR-629-3p	MIMAT0000425	hsa-miR-130a-3p
MIMAT0000253	hsa-miR-10a-5p	MIMAT0000318	hsa-miR-200b-3p	MIMAT0002820	hsa-miR-497-5p
MIMAT0018361	hsa-miR-3945	MIMAT0000617	hsa-miR-200c-3p	MIMAT0004975	hsa-miR-509–3-5p
MIMAT0000068	hsa-miR-15a-5p	MIMAT0018105	hsa-miR-3679-3p	MIMAT0000078	hsa-miR-23a-3p
		MIMAT0004950	hsa-miR-877-3p	MIMAT0000075	hsa-miR-20a-5p
		MIMAT0001343	hsa-miR-425-3p	MIMAT0002811	hsa-miR-202-3p
		MIMAT0018099	hsa-miR-3675-3p	MIMAT0000265	hsa-miR-204-5p
		MIMAT0003270	hsa-miR-602	MIMAT0005789	hsa-miR-513c-5p
		MIMAT0005949	hsa-miR-664a-3p	MIMAT0005788	hsa-miR-513b-5p
		MIMAT0004811	hsa-miR-33b-3p	MIMAT0003326	hsa-miR-663a
		MIMAT0018000	hsa-miR-23c	MIMAT0004605	hsa-miR-129–2-3p
		MIMAT0004482	hsa-let-7b-3p	MIMAT0000104	hsa-miR-107
		MIMAT0004800	hsa-miR-550a-5p	MIMAT0000232	hsa-miR-199a-3p
		MIMAT0004748	hsa-miR-423-5p	MIMAT0000065	hsa-let-7d-5p
		MIMAT0018001	hsa-miR-3620-3p	MIMAT0000066	hsa-let-7e-5p
		MIMAT0002176	hsa-miR-485-3p	MIMAT0000092	hsa-miR-92a-3p
		MIMAT0007401	hsa-miR-1539	MIMAT0004697	hsa-miR-151a-5p
		MIMAT0000100	hsa-miR-29b-3p	MIMAT0000437	hsa-miR-145-5p
		MIMAT0004976	hsa-miR-933	MIMAT0000760	hsa-miR-331-3p
		MIMAT0004808	hsa-miR-625-3p	MIMAT0000271	hsa-miR-214-3p
		MIMAT0007348	hsa-miR-1470	MIMAT0002878	hsa-miR-506-3p
		MIMAT0016906	hsa-miR-4274	MIMAT0000082	hsa-miR-26a-5p
		MIMAT0000432	hsa-miR-141-3p	MIMAT0000101	hsa-miR-103a-3p
		MIMAT0007884	hsa-miR-1910-5p	MIMAT0000098	hsa-miR-100-5p
		MIMAT0004949	hsa-miR-877-5p		
		MIMAT0004486	hsa-let-7f-1-3p		
		MIMAT0002173	hsa-miR-483-3p		
		MIMAT0011161	hsa-miR-2116-3p		
		MIMAT0003291	hsa-miR-622		
		MIMAT0018179	hsa-miR-3907		

(Continues)

TABLE 1 (Continued)

miRNAs present in 'only' spermatozoa		miRNAs present in 'only' seminal plasma		miRNAs present in 'only' testicular tissue	
miRBase accession	MicroRNA	miRBase accession	MicroRNA	miRBase accession	MicroRNA
		MIMAT0015057	hsa-miR-3180-5p		
		MIMAT0018065	hsa-miR-3646		
		MIMAT0018354	hsa-miR-548y		
		MIMAT0015060	hsa-miR-548w		
		MIMAT0003227	hsa-miR-563		
		MIMAT0000083	hsa-miR-26b-5p		

3.3 | Correlation analysis results

To determine the degree of correlation across individual samples within each group, we computed the pairwise sample correlations (Spearman's correlation coefficient) based on miRNAs expression level present in the considered sample group (Table S4). Figure 3a shows a histogram of the resulting correlation values for each sample group separately. We found the strongest correlation between the spermatozoa samples ranging from 0.633 to 0.964. The second strongest correlation was found among the seminal plasma samples with correlation values between 0.544 and 0.947. In contrast, the correlation values in the testicular tissue group ranged from 0.02 to 0.913 with the lowest values found between samples 1, 4, 5, 6, 7 and 8 (Figure 3b–d). These results can be explained by the presence of various cell types in the testis (i.e. germ and somatic cells) causing increased heterogeneity. The high correlation between the miRNA expression values of spermatozoa and seminal plasma likely reflects the homogeneity of the samples within these groups.

3.4 | Quantile rank analysis of miRNA expression data

To determine miRNAs whose expression was different between two sample groups without a direct comparison of the expression levels, we performed quantile rank analysis (Smalheiser et al., 2012). For each miRNA, we computed its mean expression for each sample group separately. In each group, the miRNAs were sorted by their mean group expression in a decreasing order to determine their ranks. Next, we computed for each pair of groups the rank differences of the miRNAs. These values were approximately normally distributed with mean = 0 for all three group pairs and standard deviation of 195.5, 211.5 and 224.8 for spermatozoa/seminal plasma, spermatozoa/testicular tissue and seminal plasma/testicular tissue respectively (Figure 3e). Finally, the probability to achieve a more extreme rank change by chance was determined for all rank differences. Table S5 contains the 18 miRNAs with at least one significant rank difference comparing two groups. These findings suggest that there is a certain similarity between spermatozoa and seminal plasma regarding the relative miRNA expression changes as compared to testicular tissue. However, there are also rank differences between spermatozoa and seminal plasma for six other miRNAs, which seem to be specific for these two groups.

3.5 | Most expressed and most stable miRNAs

We determined the most expressed and stable miRNAs. The most expressed miRNAs were defined by a high mean expression value and the most stable miRNA by a low standard deviation. This analysis was done for each tissue/cell type separately. Among the most expressed miRNAs, three miRNAs namely miR-4281, miR-2861 and miR-638 were shared across all sample groups and 7 miRNAs were shared between spermatozoa and seminal plasma as indicated in Table 2. As for the most stable miRNAs, we did not find a stable miRNA that was shared among the three tested groups (Table 2).

3.6 | miRNA target genes prediction

The Gene Ontology (GO) was considered to elucidate the biological function of the identified miRNAs for each sample group separately. To this end, we extracted miRNA targets using the miRWalk 2.0 (Dweep & Gretz, 2015) and we used the list of unique genes for the enrichment analysis. The most enriched GO terms for the spermatozoa, seminal plasma and testicular tissue are listed in Table S6.

3.7 | Validation of microarray results by RT-qPCR

Of the miRNAs present in each group, we validated 11 miRNAs from spermatozoa, 5 miRNAs from seminal plasma and 8 miRNAs from testicular tissue by RT-qPCR. These miRNAs were chosen based on their abundance level changes and based on their known associations with spermatogenesis. The selected miRNA expression level was largely concordant with the microarray expression data as expected (Figure 4a–c).

4 | DISCUSSION

In this study, we investigated the miRNA expression profile in spermatozoa and seminal plasma of men with proven fertility and

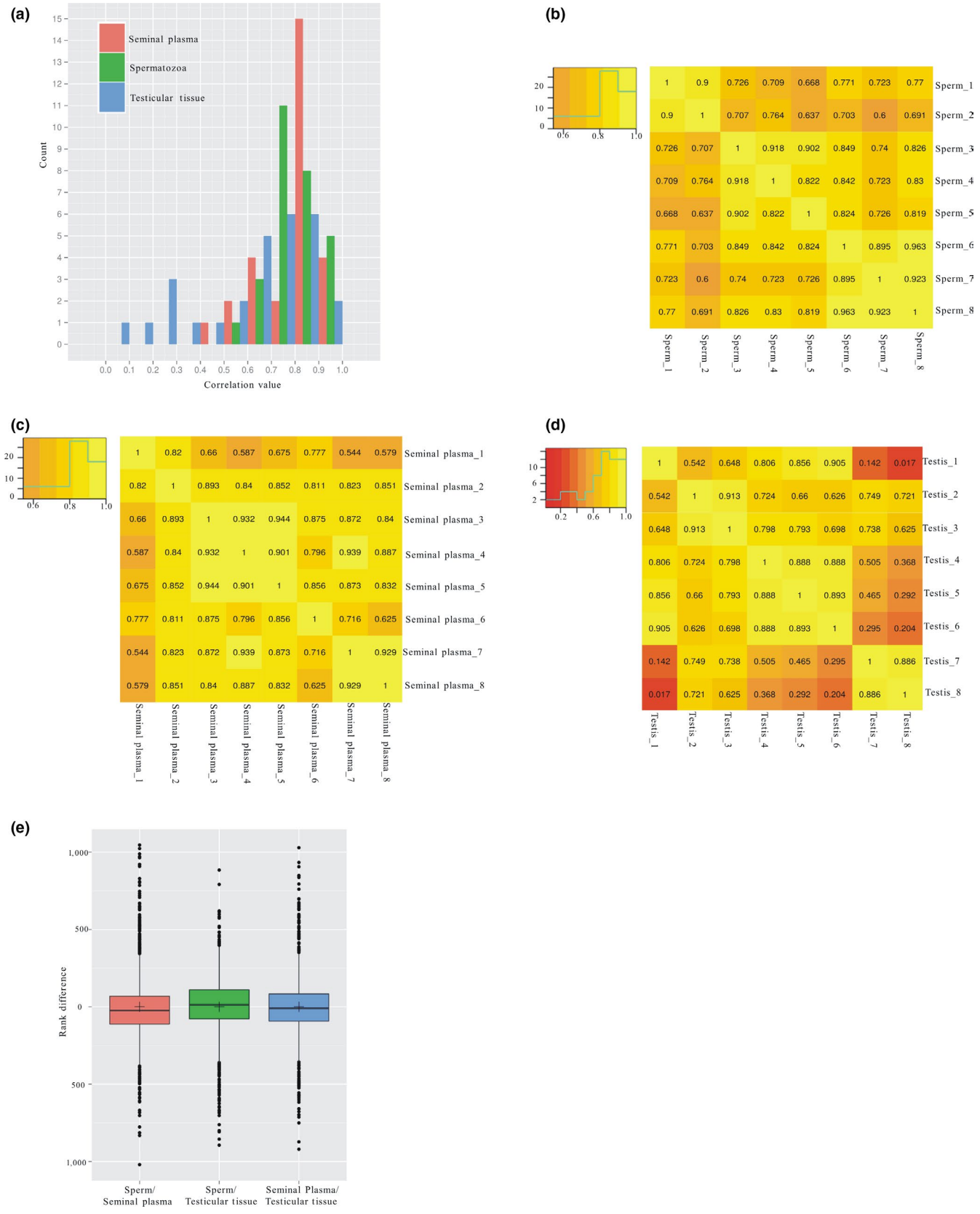

FIGURE 3 (a) Histogram of within-group sample correlation based on present miRNAs for spermatozoa (green), seminal plasma (red) and testicular tissue (blue), (b) pairwise Spearman's correlation coefficient computed for samples in the group spermatozoa, (c) pairwise Spearman's correlation coefficient computed for samples in the group seminal plasma, (d) pairwise Spearman's correlation coefficient computed for samples in the group testicular tissue and (e) histogram of rank differences computed for each sample group pair (spermatozoa/seminal plasma, spermatozoa/testicular tissue and seminal plasma/ testicular tissue)

TABLE 2 Top-most expressed (highest mean) and stable (lowest standard deviation) miRNAs across the sample groups

Most expressed miRNAs					
Spermatozoa		**Seminal plasma**		**Testicular tissue**	
miRBase accession	**MicroRNA**	**miRBase accession**	**MicroRNA**	**miRBase accession**	**MicroRNA**
MIMAT0016907	hsa-miR-4281	MIMAT0016907	hsa-miR-4281	MIMAT0016907	hsa-miR-4281
MIMAT0003299	hsa-miR-630	MIMAT0005865	hsa-miR-1202	MIMAT0004795	hsa-miR-574-5p
MIMAT0013802	hsa-miR-2861	MIMAT0005572	hsa-miR-1225-5p	MIMAT0003308	hsa-miR-638
MIMAT0003308	hsa-miR-638	MIMAT0005871	hsa-miR-1207-5p	MIMAT0013802	hsa-miR-2861
MIMAT0005572	hsa-miR-1225-5p	MIMAT0013802	hsa-miR-2861	MIMAT0000063	hsa-let-7b-5p
MIMAT0005793	hsa-miR-320c	MIMAT0016900	hsa-miR-4270	MIMAT0016916	hsa-miR-4286
MIMAT0005871	hsa-miR-1207-5p	MIMAT0003308	hsa-miR-638	MIMAT0002816	hsa-miR-494-3p
MIMAT0018087	hsa-miR-3665	MIMAT0005793	hsa-miR-320c	MIMAT0018076	hsa-miR-3656
MIMAT0005865	hsa-miR-1202	MIMAT0018076	hsa-miR-3656		
Most stable miRNAs					
Spermatozoa		**Seminal plasma**		**Testicular tissue**	
miRBase accession	**MicroRNA**	**miRBase accession**	**MicroRNA**	**miRBase accession**	**MicroRNA**
MIMAT0004761	hsa-miR-483-5p	MIMAT0016907	hsa-miR-4281	MIMAT0000762	hsa-miR-324-3p
MIMAT0018185	hsa-miR-3911	MIMAT0004982	hsa-miR-939-5p	MIMAT0000510	hsa-miR-320a
MIMAT0015005	hsa-miR-3137	MIMAT0005922	hsa-miR-1268a	MIMAT0002824	hsa-miR-498
MIMAT0005865	hsa-miR-1202	MIMAT0018104	hsa-miR-3679-5p	MIMAT0005793	hsa-miR-320c
MIMAT0015036	hsa-miR-3162-5p	MIMAT0018076	hsa-miR-3656	MIMAT0005788	hsa-miR-513b-5p
MIMAT0004610	hsa-miR-150-3p	MIMAT0007892	hsa-miR-1915-3p		

testicular tissue of men with obstructive azoospermia. In total, 123, 156 and 133 miRNAs were consistently detected in spermatozoa, seminal plasma and testicular tissue respectively. Out of the identified miRNAs, 18, 50 and 38 miRNAs were only found in spermatozoa, seminal plasma and testicular tissue respectively. These miRNAs are specific for each sample type. However, the origin of the identified miRNA still needs to be further discussed for each sample type specifically. Five miRNAs namely miR-1274a, miR-1274b, miR-720, miR-1280 and miR-3676 were reported as 'dead entries' and have been removed from present miRNA list. The putative mature sequences of these five miRNAs were fragments of transfer ribonucleic acids (tRNAs) (Schopman, Heynen, Haasnoot, & Berkhout, 2010; Vaz et al., 2010).

Among the miRNAs that were detected in spermatozoa samples from subfertile men but not infertile men (Abu-Halima et al., 2013), miR-122 has been described to participate in the post-transcriptional down-regulation of the transition protein 2 (TNP2) during spermatogenesis (Krawetz et al., 2011; Salas-Huetos et al., 2014; Yu, Raabe, & Hecht, 2005). Lower abundance level of miR-122 has been observed in seminal plasma and spermatozoa of men with nonobstructive azoospermia (NOA) and of subfertile men. MiR-122 might also have diagnostic value to confirm male infertility (Abu-Halima, Hammadeh, et al., 2014; Wang et al., 2011). MiR-19b and has-miR-34b were highly expressed and stably detected in the spermatozoa of proven fertile men (Salas-Huetos et al., 2014). MiR-34b was over-expressed in the spermatozoa collected from mature CD-1 mice (Garcia-Lopez et al., 2015). In addition, lower abundance levels of miR-34b and miR-15a were observed in subfertile compared to fertile men (Abu-Halima, Hammadeh, et al., 2014; Abu-Halima et al., 2013). MiR-19b abundance levels were higher in the seminal plasma of idiopathic infertile men with NOA compared to fertile controls (Wu et al., 2012). Other miRNAs like miR-3202, miR-3190, miR-3137, miR-3682, miR-1305, miR-3156, miR-3127, miR-3125, miR-595, miR-1183, miR-1299 and miR-3945 have not been reported to be involved in any biological and/or cellular function associated with spermatogenesis or related processes. It remains to be seen whether the newly identified miRNAs are specific for germ cells and/or if their aberration is associated with impaired spermatogenesis.

Among the miRNAs detected in seminal plasma, five miRNAs (miR-629-3p, miR-200b-3p, miR-425-3p, miR-33b-3p and miR-141-3p) were previously described as being among the most stably expressed miRNAs in seminal plasma-derived exosomes (Barcelo, Mata, Bassas, & Larriba, 2018). MiR-141-3p was reported to be stable in seminal plasma (Wu et al., 2013). Six miRNAs (miR-363-3p, miR-148a-3p, miR-200b-3p, miR-425-3p, miR-3675-3p and miR-26b-5p) were previously described as being abundantly expressed in human extracellular microvesicles isolated from seminal plasma of patients with oligoasthenozoospermia (Abu-Halima et al., 2016). MiR-363-3p, miR-148a-3p, miR-200b-3p, miR-200c-3p, miR-425-3p, miR-1825, miR-33b-3p, miR-550a-5p, miR-625-3p, miR-1470, miR-141-3p and miR-26b-5p have been previously found to be abundant in the spermatozoa samples of

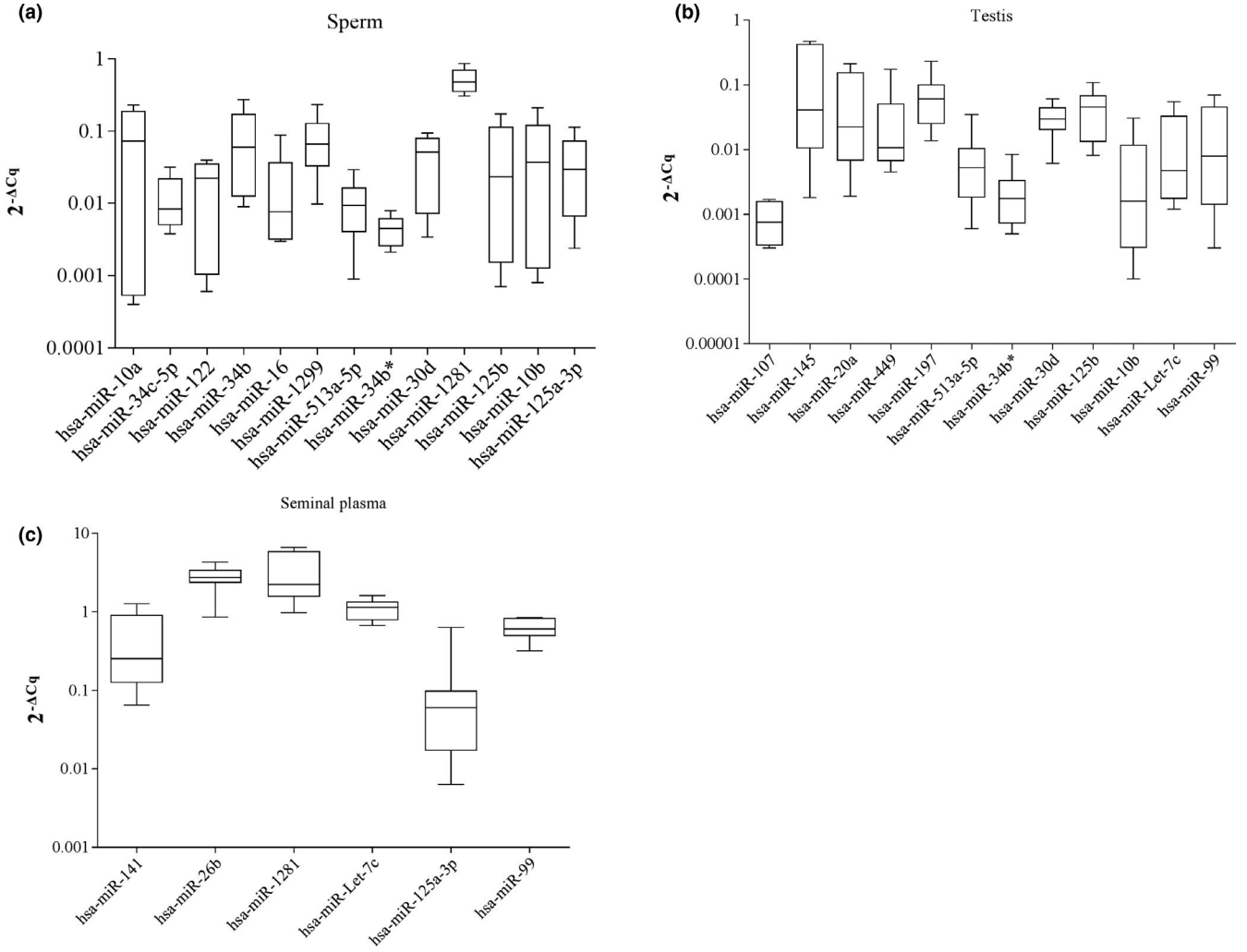

FIGURE 4 Real-time PCR validation of some selected miRNAs in the (a) spermatozoa, (b) testicular tissue and (c) seminal plasma of male with normal spermatogenesis as determined by RT-qPCR ($p < .05$). RNAU6B as an endogenous control for normalisation. Normalised $2^{-\Delta Cq}$ values and mean ± standard deviation (STDV) were used to represent the differences in expression

men with spermatogenic impairments (Abu-Halima et al., 2013). MiR-1181 and miR-135a-3p were found in testicular tissue of men with different testicular impairments (Abu-Halima, Backes, et al., 2014) suggesting that the miRNA profiles in seminal plasma are similar to the profiles in the spermatozoa and different to the profiles in the testicular tissue.

Semen sample purification is a mandatory procedure in the IVF laboratory and before running miRNA/mRNA expression analysis of spermatozoa and seminal plasma because semen samples are routinely contaminated with somatic cells such as leucocytes, macrophages and epithelial cells of the genital tract. The problem of somatic transcript contamination in expression studies of spermatozoa has not been solved adequately to date and is a topic that needs further investigation (Jodar et al., 2015). The somatic transcript express miRNAs that might slightly bias the resulting miRNA expression profiles even after applying many purification methodologies (Abu-Halima et al., 2013).

As for the normal testicular tissue, several studies reported that testicular-expressed miRNA changes depending on the stage of spermatogenesis (Hayashi et al., 2008; Maatouk et al., 2008) and the presence of various cell types in the testis (i.e., germ and somatic cells) increase the heterogeneity of the testis. According to the studies, each of these cell populations has a specific miRNA profile and/or a stage-specific miRNA profile. In our study, however, the testicular tissue samples were taken from 8 different men diagnosed with OA and with morphologically and/or histologically normal spermatogenesis, and each of these testicular tissues might contain different stages of normal cell division and development. These findings have been supported by many studies suggesting that late meiotic and haploid germ cells are the main source of miRNA production during spermatogenesis (Bouhallier et al., 2010; Guo, Su, Zhou, & Sha, 2009; Ro, Park, Sanders, McCarrey, & Yan, 2007). Among the identified miRNAs, nine miRNAs (miR-509-3p, miR-27b-3p, miR-125a-5p, miR-514a-3p, miR-509-3-5p, let-7i-5p, miR-26a-5p, let-7g-5p and miR-100-5p) were recently found as most abundantly expressed in human normal testicular tissue (Tang et al., 2018). A cluster of six miRNAs namely miR-509-3p, miR-509-5p, miR-514a-3p, miR-513b-5p, miR-513c-5p and

miR-506-3p have been reported to be exclusively expressed in the testicular tissues of men with carcinoma in situ (Abu-Halima et al., 2013; Novotny et al., 2012). Another conserved miRNA family, miR-34, which is structurally similar to the miR-449 family, has previously been identified to be highly enriched in germ cells (Bouhallier et al., 2010; Niu et al., 2011). Additionally, miR-449a and miR-199a-3p were highly expressed in cryptorchid testicle tissues (TANG et al., 2018). The miR-23 family (miR-23a, miR-23b and miR-23c) was highly deregulated in spermatozoa, seminal plasma-derived exosomes and testicular tissue of subfertile men compared with fertile men (Abu-Halima et al., 2019, 2013, 2016; Abu-Halima, Backes, et al., 2014). More recently, the miR-23 family was found to be highly expressed in the spermatozoa and testicular tissue of patients with different spermatogenic and testicular impairments. The miR-23 family targets many spermatogenesis-specific genes like PFKFB4, HMMR, SPATA6 and TEX15. These genes have been identified to be involved in the spermatogenesis process of male infertility (Abu-Halima et al., 2019).

There is rather limited information on the cellular function of the expressed and stable miRNAs for the process of spermatogenesis. The Let-7 family, a highly conserved miRNA family in the testis that contributes significantly to the control of male germ cell lineage, is specifically deregulated in testicular cancer (Johnson et al., 2007; Jung, Gupta, Shin, Uhm, & Lee, 2010). In addition, Let-7 family was observed to be abundantly detected in testicular tissue, spermatozoa and embryonic spent culture media of IVF correlated with successful pregnancy (Abu-Halima, Backes, et al., 2014; Abu-Halima et al., 2013, 2017).

The expression level of miR-574-5p is reported to be significantly higher in the semen sample of subfertile men as compared to controls (Liu, Cheng, Gao, Wang, & Liu, 2012). The expression level of miR-574-5p is also reported to be lower in spermatozoa samples from oligoasthenozoospermic subfertile men as compared to those from normozoospermic fertile men (Abu-Halima et al., 2013). In men with Sertoli cell-only syndrome, the miR-574-5p expression level was significantly lower compared to controls (Abu-Halima, Backes, et al., 2014), indicating that sustained lower expression of miR-574-5p is pivotal for the progression of infertility. MiR-638 plays a role in pig spermatogenesis by regulating immature Sertoli cell growth and apoptosis by targeting *the SPAG1* gene (Hu et al., 2017). The miR-320 family is predicted to target the protocadherin alpha proteins that are expressed in the mammalian testes. Protocadherin proteins play a crucial function in maintaining germ cells adhesion to Sertoli cells in the interstitial compartment of the testes (Dai et al., 2011; Marcon, Babak, Chua, Hughes, & Moens, 2008).

The results demonstrate higher samples correlation and thus homogeneity of miRNA pattern within the spermatozoa and seminal plasma groups as compared to testicular tissue group. The observed heterogeneity within the latter group could be explained by the fact that testis contains various cell types, and its composition may vary between the samples. Quantile rank analysis identifies miRNAs whose expression changes significantly relative to the other observed miRNAs. These analyses identified 18 miRNAs with significant rank changes of the mean expression values. Five of these miRNAs namely miR-143, miR-200c, miR-510, miR-3651 and miR-214 were shared between spermatozoa/testicular tissue and seminal plasma/testicular tissue. This may indicate a higher similarity between spermatozoa and seminal plasma as compared to the testicular tissue. That is also supported by the fact that from the most expressed miRNAs for spermatozoa and seminal plasma, seven miRNAs namely miR-4281, miR-2861, miR-638, miR-1225-5p, miR-320c, miR-1207-5p and miR-1202 were shared, while for testicular tissue three miRNAs miR-4281, miR-2861 and miR-638 were shared among all groups (Table 2). The expression patterns of most miRNAs in testicular tissue samples have also be identified as abundant in testis as reviewed in Kotaja (2014).

The correlation analysis was performed to see whether specific miRNAs are commonly regulated. As for miRNAs clustered in the same genomic region, a correlation of expression might indicate a common promoter sequence orchestrating the expression of miRNAs. Correlated miRNAs located on different chromosomes are potentially regulated by the same transcription factors (TFs) as reviewed extensively by Chen and Rajewsky (2007). However, it remains to be elucidated which TFs regulate miRNA genes in spermatogenesis. Identification of co-expressed miRNAs in spermatozoa, seminal plasma and testicular tissue is an important first step towards the identification of potential miRNA-regulating TFs.

Gene Ontology (GO) analysis revealed many genes involved in reproduction, development and differentiation. Specifically, in spermatozoa and testicular tissue, many of the enriched GO terms were directly or indirectly associated with spermatogenesis, sperm motility and flagellum, male germ cell, gamete generation and fertilisation. As for the seminal plasma, most of the enriched terms were directly or indirectly associated with regulation of protein secretion, focal adhesion, peptidase activity, cytoplasmic membrane-bounded vesicle, positive regulation of male gonad development and Sertoli cell differentiation.

5 | CONCLUSION

Our results suggest that there is a certain similarity between spermatozoa and seminal plasma for the relative miRNA expression changes with respect to testicular tissue. Taken together, several miRNAs of unknown function were identified or demonstrated different levels in each sample type, suggesting that aberration of these miRNAs may adversely affect the rate of spermatogenesis.

AUTHOR CONTRIBUTION

MA carried out the experimental work and wrote the article. VG, CB and AK performed bioinformatics analysis. MH collected and diagnosed the semen samples. EM designed the experimental work and edited the article.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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