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ORIGINAL ARTICLE *Andrology*

Cytological and molecular aspects of the ageing sperm

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STUDY QUESTION: Is ageing associated with a decline in semen quality and molecular changes to human sperm?

SUMMARY ANSWER: Semen quality declines with advancing age and characteristic molecular changes take place during the ageing process, including increased sperm DNA damage, altered sperm protamination and altered seminal plasma miRNA profile.

WHAT IS KNOWN ALREADY: During ageing, the reproductive system is exposed to physiological changes and potentially damaging factors that may impair testicular function. Reactive oxygen species (ROS) can induce errors during DNA replication, transcription or post-transcriptional events (fragmentation, chromatin condensation abnormalities and protamine expression defects).

STUDY DESIGN, SIZE, DURATION: Semen parameters from 2626 healthy men aged 20–81 years were evaluated retrospectively from those attending our University Laboratory between 2011 and 2016 for andrological screening or as part of an andrological work-up. Subjects were divided into six groups by age (20–32, 33–37, 38–40, 41–44, 45–50, 51–81 years). From these subjects, semen samples from 40 elderly men (50–81 years) and 40 young men (20–40 years) (control group), all non-smokers of normal weight, were selected for the evaluation of sperm chromatin integrity, PRM1, PRM2, TNP1 and TNP2 gene expression, and microRNA expression profile in seminal plasma.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Semen was analysed according to WHO 2010. Sperm DNA fragmentation (SDF) was evaluated using TUNEL assay; sperm PRM1, PRM2, TNP1 and TNP2 gene expression was evaluated by quantitative RT-PCR amplification; miRNA expression profiles were analysed by TaqMan Array Cards and validated by RT-PCR amplification.

MAIN RESULTS AND THE ROLE OF CHANCE: *Cytological analysis* – Semen volume, progressive motility and number of progressively motile sperm were significantly lower in elderly than in younger subjects (sextiles 51–81 versus 20–32 years; $P < 0.001$), while the percentage of abnormal forms in these subjects was significantly higher than in the 20–32 age group ($P = 0.002$). Binomial logistic regression models revealed an association between age and semen parameters: age 51–81 was associated with changes in total sperm number (OR 2.47; 95% CI 1.52–4.02; $P < 0.001$), progressive motility (OR 3.63; 95% CI 2.49–5.30; $P < 0.001$), and abnormal forms (OR 3.89; 95% CI 2.71–7.26; $P < 0.001$). Obesity was associated with reduced progressive motility (OR 1.58; 95% CI 1.14–2.19; $P = 0.006$) and an increase in abnormal forms (OR 1.87; 95% CI 1.02–3.57; $P = 0.021$). In contrast, smoking did not contribute significantly to changes in semen parameters. *Molecular analysis* – Elderly men showed a significantly higher percentage of SDF (23.1 ± 8.7 versus 9.8 ± 2.6%; $P < 0.001$) and a significantly lower expression of PRM1 (mean fold change 2.2; $P = 0.016$) and PRM2 (mean fold change 4.6; $P < 0.001$), compared to younger controls. Furthermore, miR-146a showed a 3-fold lower expression ($P < 0.001$), miR-371 a 14-fold lower expression ($P < 0.001$), and miR-122 a 5-fold lower expression ($P = 0.01$) in the elderly men.

LIMITATIONS, REASONS FOR CAUTION: While typical chronic age-related conditions (cardiovascular, respiratory diseases) were excluded, the presence of subclinical underlying diseases cannot be excluded in the elderly population. Subjects referred to our clinic might not be fully representative of the general population. Although a careful medical history and physical examination excluded most andrological conditions that might affect spermatogenesis, we cannot exclude the presence of possible asymptomatic or idiopathic conditions.

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Furthermore, TUNEL, in common with other SDF detection methods (with the exception of the alkaline comet assay), does not distinguish between single and double strand breaks.

WIDER IMPLICATIONS OF THE FINDINGS: The role of obesity suggests that conditions related to lifestyle factors may further worsen age-related sperm parameter impairment. Increased SDF and altered protamine expression suggest the genomic fragility of sperm in advanced age. Changes in the miRNA expression pattern with age could contribute to the identification of a characteristic molecular signature of the ageing process, a potential new biomarker for male reproductive function during the physiological ageing process.

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Introduction

Most studies take maternal age into account as a risk factor for spontaneous abortion, infertility and genetic defects in the offspring. However, attention has only recently turned to the impact of paternal age on reproductive outcome.

Khandwala *et al.* (2017) recently reported that the mean age on achieving fatherhood in the United States is 30.9 years. This is in stark contrast with Italy, where Italian men have their first child at 35 years (Istat, 2017). Progress in ART has undoubtedly contributed to the trend of increased paternal age.

Ford *et al.* (2000) demonstrated that older age of the male partner was associated with an increased time to pregnancy and reduced pregnancy rate. Literature evidence on the effects of male ageing suggests that 40 years could represent the turning point for the reproductive life of men, while for women, after 35 years there is an increase in age-related abortion rate, pregnancy complications, congenital abnormalities and maternal and perinatal mortality (De La Rochebrochard *et al.*, 2003). During ageing, physiological changes to the male reproductive system affect the testis, the seminal vesicles, the prostate, and the epididymis (Gunes *et al.*, 2016). For this reason, semen parameters may also change over time (Avellino *et al.*, 2017). Comparative studies of semen quality in elderly (≥50 years) versus young (~30 years) men demonstrated a 3–22% reduction in semen volume, a 3–37% reduction in sperm motility, and a 4–18% reduction in normal forms in the elderly subjects (Kidd *et al.*, 2001). Although the literature also includes discordant results (Alshahrani *et al.*, 2014), it should be stressed that most studies were retrospective, men aged over 60 were rarely included, and not all studies considered sexual abstinence.

During ageing, damaged macromolecules accumulate in various cells, tissues, and organs (Rattan, 2006). This damage is caused by reactive oxygen species (ROS) or by spontaneous errors during DNA duplication, transcription, or post-transcriptional events. A meta-analysis by Soares *et al.* (2014) found a direct correlation between age and nuclear DNA damage and also indicated that ageing does not affect all tissues equally. For this reason, some authors investigated the correlation between male ageing and sperm DNA fragmentation (SDF), chromatin condensation abnormalities and protamine expression defects. These revealed increasing SDF with age (Wyrobek *et al.*, 2006), which could explain the reduced pregnancy rate and increased probability of abortion (Pasqualotto *et al.*, 2008). Genetic mutations can also affect life expectancy or cause premature ageing (Rattan, 2006). However, genes alone are not functional entities; microRNA (miRNA) can affect gene expression and genomic instability during ageing in different ways in different tissues (Harries, 2014).

Since previous studies did not comprehensively describe changes to human sperm induced by ageing, the aim of our study was to investigate the cytological and molecular aspects of sperm during ageing. The retrospective cytological study evaluated how semen quality relates to ageing. The molecular study examined sperm DNA damage; gene expression of protamine 1 and 2 (PRM1, PRM2), and transition nuclear proteins 1 and 2 (TNP1, TNP2) to detect any changes to chromatin integrity; and miRNA expression profile in seminal plasma, to detect any testicular function biomarkers associated with ageing.

Materials and Methods

Subjects

The study was approved by the Policlinico Umberto I Ethics Committee. Written informed consent was obtained from all study participants. Semen parameters were evaluated through a retrospective study of 2626 men attending the Laboratory of Seminology, Sperm Bank "Loredana Gandini" Department of Experimental Medicine at Sapienza University of Rome between 2011 and 2016 for semen analysis for andrological screening or as part of an andrological work-up. In particular, we included patients who have been advised to perform an andrological screening or who attempted to conceive for less than 12 months to investigate the presence of a male factor, irrespective of female pathology. Men with a history of azoospermia, cryptorchidism, urinary tract infections, prostatitis, varicocele, hypogonadism, diabetes, chronic age-related diseases, genetic diseases, urogenital surgery, neoplasms or exposure to chemotherapy or radiotherapy treatments were excluded from the study.

From these subjects, semen samples from 40 elderly men (50–81 years) and 40 young men (20–40 years), all non-smokers of normal weight, were also selected for the evaluation of sperm chromatin integrity, PRM1, PRM2, TNP1 and TNP2 gene expression, and microRNA expression profile in seminal plasma.

Semen analysis

Semen samples were collected by masturbation after 2–7 days' abstinence. All samples were allowed to liquefy at 37°C for 60 min and were then assessed according to WHO (2010). The following variables were taken

into consideration: volume (ml), total sperm number ($n \times 10^6$ per ejaculate), progressive motility (%), and morphology (% abnormal forms). In addition to raw data on percentage motility, we also considered absolute values in terms of millions of motile sperm per ejaculate (obtained by multiplying the total sperm per ejaculate by the percentage of sperm motility), called the number of progressively motile sperm ($n \times 10^6$).

Sperm chromatin integrity

SDF was evaluated using TUNEL assay (Roche, *In Situ* Cell Death Detection Kit, Fluorescein, Roche, Basel, Switzerland). After assessment of semen parameters, the samples were centrifuged and evaluated as previously described by Gandini *et al.* (2000). The samples were then analysed under fluorescent microscope (Leica DMR; Leica, Wetzlar, Germany), counting at least 500 cells.

PRM1, PRM2, TNP1 and TNP2 gene expression

Separation of sperm cells

The semen samples were diluted with PBS to ~10×10^6 sperm/ml and subjected to osmotic shock to eliminate the non-gamete cell component (Paoli *et al.*, 2017).

RNA extraction, cDNA synthesis, quantitative real-time PCR

Total RNA was extracted from ~10×10^6 sperm using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA concentration was calculated by spectrophotometry using the NanoDrop ND-2000 (Thermo Fisher Scientific, Waltham, MA, USA). Reverse transcription for cDNA synthesis was carried out on 100 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quantitative real-time PCR was carried out using Step One Real-Time PCR System (Applied Biosystems), with the primers and fluorescent probes specific for PRM1, PRM2, TNP1 and TNP2 and with GAPDH as the endogenous control. Data were analysed by ΔCt method (Livak and Schmittgen, 2001).

MiRNA expression profile in seminal plasma

Initial screening

miRNA expression profiles in seminal plasma were analysed with TaqMan Array Cards A + B 3.0 (Life Technologies). Total RNA was extracted from 10 seminal plasma samples from men with a mean age of 52.9 ± 2.3 years and 10 seminal plasma samples from men with a mean age of 21.5 ± 2.3 years. RNA extraction, cDNA synthesis, and cDNA pre-amplification were conducted as described in Pelloni *et al.* (2017). The raw array data were analysed by RQ manager v2.2 and ΔCt method. Undetermined values were considered as equal to the maximum number of cycles (40), and both cases and controls with Ct above 35 were excluded. All miRNAs with a ≥10-fold increase or decrease in expression were selected.

MiRNA expression validation

Total RNA was extracted from seminal plasma samples from 40 men aged 50–81 years and 40 men aged 20–40 years. RNA extraction and cDNA synthesis were conducted as described in Pelloni *et al.* (2017). Quantitative real-time PCR was performed using Step One Real-Time PCR System (Applied Biosystems), with the assays (20X) (Applied Biosystems): hsa-miR-371-3p, hsa-miR-122a, hsa-miR-19b, hsa-miR-29b, hsa-miR-146a and, as the endogenous control, U6 snRNA. The reaction was carried out in triplicate for each sample. Data were analysed by ΔCt method.

Table I Smoking and BMI in the various age groups.

Age group years (*n*)	BMI (kg/m^2)	Cigarettes/day[c]	Years of smoking[c]	Percentage of smokers	Percentage of normal weight subjects	Percentage of overweight subjects	Percentage of obese subjects
20–32	24.1 ± 3.3	11.4 ± 7.9	7.3 ± 4.3	28.1	66.1	28.3	5.5
(463)	(23.7)	(10.0)	(6.0)				
33–37	25.3 ± 3.2	12.8 ± 7.8	14.1 ± 4.6	35.1	53.6	39.6	6.8
(458)	(24.8)	(10.0)	(15.0)				
38–40	25.2 ± 3.4	13.2 ± 8.6	17.7 ± 5.4	30.2	55.2	37.0	7.8
(394)	(24.6)	(10.0)	(20.0)				
41–44	25.8 ± 3.8	13.2 ± 9.8	19.2 ± 6.9	28.3	48.3	40.2	11.5
(476)	(25.1)	(10.0)	(20.0)				
45–50	25.7 ± 3.6	13.2 ± 7.5	21.0 ± 7.9	19.8	46.9	42.1	11.1
(444)	(25.2)	(10.0)	(20.0)				
51–81	26.4 ± 4.0	15.7 ± 11.3	27.6 ± 13.1	6.9	42.1	43.1	14.1
(391)	(25.6)	(13.0)	(30.0)				
All	25.2 ± 3.6	12.8 ± 8.5	15.9 ± 8.1	25.1	52.2	38.3	9.4
(2626)	(24.8)	(10.0)	(15.0)				
P-value	<0.001[a]	0.20[a]	<0.001[a]	<0.001[b]	<0.001[b]	<0.001[b]	<0.001[b]

Data are mean ± SD (median) unless stated otherwise.
[a]Anova.
[b]χ^2 test
[c]Smokers only, only current smoking was recorded.

Statistical analysis

Continuous variables are presented as mean ± SD/SE, and differences between the two groups were evaluated by the Student *T* or Mann–Whitney *U* test, depending on the shape of the distribution curve evaluated by the Kolmogorov–Smirnov test. Categorical variables are presented as counts and percentages and were compared by χ^2 test. Differences between age classes were evaluated by ANOVA with Bonferroni post-hoc analysis. Statistically significant correlations between sperm parameters and age, BMI, and smoking were evaluated using Spearman's rank correlation test. Logistic regression models were performed to calculate adjusted ORs of factors associated with impairment of sperm parameters (total sperm number, progressive motility, and abnormal forms), including age classes (20–32, 33–37, 38–40, 41–44, 45–50, 51–81 years), BMI (normal weight, overweight and obese), and smoking (smokers, non-smokers) as covariates. The probability values are 2-sided; $P < 0.05$ was considered statistically significant. All computations were carried out with Statistical Package for the Social Sciences (SPSS) 24.0 (SPSS Inc., Chicago, USA).

Results

Semen analysis

We examined 2626 subjects aged 20–81 years, divided into six groups by age percentile distribution: 20–32 years (463 subjects); 33–37 years (458); 38–40 years (394); 41–44 years (476); 45–50 years (444); and 51–81 years (391). The subjects had been sexually abstinent for 3.9 ± 1.3 days (median 4 days). Supplementary Table I reports the patient demographics.

The percentage of smokers was similar in the youngest four age groups at ~30%, but dropped significantly in the older groups (χ^2 $P <$ 0.001). The percentage of overweight (BMI ≥ 25.0 kg/m^2) and obese (BMI ≥ 30.0 kg/m^2) subjects rose significantly with age (χ^2 $P < 0.001$). Table I shows the BMI distribution and smoking status of the six groups. Table II demonstrates the significant worsening of semen parameters with increasing age.

Post-hoc analysis (with Bonferroni correction) showed that semen volume, progressive motility and number of progressively motile sperm in elderly subjects (sextile 51–81) were significantly lower than in the younger sextile (20–32) ($P < 0.001$). In order to thoroughly evaluate testicular sperm production, subjects from each age group were further divided into sub-groups using the fifth percentile of WHO 2010 reference values as cut-offs (total sperm number <39 × 10^6/ejaculate; progressive motility <32%; abnormal forms >96%).

The percentage of subjects with each sperm parameter below the fifth percentile reference value increased significantly in the older age groups (χ^2 $P < 0.001$), as shown in Table III. Correlation coefficients between semen parameters and age, BMI and smoking habits are summarised in Table IV. Age showed a negative correlation with semen volume, total sperm number, progressive motility and number of progressively motile sperm and a positive correlation with abnormal forms.

There was a significant negative correlation between BMI and semen volume, total sperm number, progressive motility and number of progressively motile sperm.

There was a significant negative correlation between total number of cigarettes smoked per day and total sperm number, progressive motility and number of progressively motile sperm and between smoking duration and total sperm number, progressive motility, and number of progressively motile sperm. Multivariable – adjusted OR of factors associated with alteration of semen parameters, showing significant association between age groups, obesity, smoking with semen parameters are shown in Table V.

Table II Semen parameters in the various age groups.

Age group (years)	Volume (ml)	Total sperm number ($n \times 10^6$/ejaculate)	Progressive motility (%)	Number of progressively motile sperm ($n \times 10^6$)	Abnormal forms (%)
20–32	3.3 ± 1.5	255.2 ± 204.5	47.2 ± 13.7	135.0 ± 118.3	82.6 ± 7.1
463 subjects	(3.0)	(207.0)	(50.0)	(105.6)	(83.0)
33–37	3.2 ± 1.5	233.9 ± 191.8	43.7 ± 15.7	114.4 ± 113.7	83.8 ± 7.5
458 subjects	(3.0)	(174.5)	(50.0)	(82.8)	(84.0)
38–40	3.2 ± 1.4	202.5 ± 163.6	42.4 ± 14.7	100.5 ± 95.9	84.2 ± 6.9
394 subjects	(3.0)	(160.0)	(45.0)	(71.9)	(85.0)
41–44	3.2 ± 1.6	210.2 ± 177.8	42.7 ± 14.7	105.3 ± 104.1	83.6 ± 6.8
476 subjects	(3.0)	(172.8)	(50.0)	(78.2)	(83.0)
45–50	3.0 ± 1.5	196.5 ± 179.9	40.4 ± 16.4	95.1 ± 102.1	84.3 ± 7.4
444 subjects	(3.0)	(150.6)	(45.0)	(66.0)	(85.0)
51–81	2.5 ± 1.5[b]	184.4 ± 212.1[c]	34.4 ± 17.2[b]	78.2 ± 99.4[b]	84.6 ± 9.2[d]
391 subjects	(2.1)	(114.0)	(40.0)	(45.6)	(85.0)
P-value[a]	<0.001	<0.001	<0.001	<0.001	0.002

Data are Mean ± SD (median).
[a]ANOVA
[b]significantly reduced versus all younger age groups ($P < 0.001$) (Bonferroni correction for multiple comparisons).
[c]significantly reduced versus 20–32 ($P < 0.001$) and versus. 33–37 ($P = 0.037$) age groups (Bonferroni correction for multiple comparisons).
[d]significantly increased versus. 20–32 age group ($P = 0.004$) (Bonferroni correction for multiple comparisons).

Table III Percentage of subjects with semen parameters below WHO fifth percentile.

Age group years (*n*)	Semen volume < 1.5 ml	Total sperm number < 39 × 10^6	Progressive motility < 32%	Abnormal forms > 96%
20–32 (463)	6.9	8.8	16.4	4.1
33–37 (458)	8.7	10.9	21.6	5.9
38–40 (394)	8.1	11.1	26.6	4.8
41–44 (476)	10.7	13.6	24.8	5.7
45–50 (444)	12.8	15.1	28.4	7.4
51–81 (391)	24.3	20.2	40.9	11.8
P-value[a]	<0001	<0001	<0001	<0001

[a]Test χ^2

Table IV Spearman's correlation coefficients (*P*) between age, BMI, smoking habits and sperm parameters.

	Age (years)	BMI (kg/m^2)	Cigarettes/day	Years of smoking	Volume (ml)	Total sperm number (*n* × 10^6/ejaculate)	Progressive motility (%)	Number of progressively motile sperm (*n* × 10^6)	Abnormal forms (%)
Age (years)		0.194* (<0.001)	0.067 (0.085)	0.637* (<0.001)	−0.157* (<0.001)	−0.151* (<0.001)	−0.244* (<0.001)	−0.188* (<0.001)	−0.19 (0.330)
BMI (kg/m^2)	0.194* (<0.001)		0.207* (<0.001)	0.101* (0.012)	−0.068* (0.001)	−0.093* (<0.001)	−0.091* (<0.001)	−0.100* (<0.001)	0.037 (0.077)
Cigarettes/day	0.067 (0.080)	0.207* (<0.001)		0.232* (<0.001)	−0.048 (0.221)	−0.104* (0.007)	−0.090* (0.021)	−0.103* (0.008)	0.073 (0.062)
Years of smoking	0.637* (<0.001)	0.101* (0.012)	0.232* (<0.001)		−0.066 (0.093)	−0.083* (0.035)	−0.083* (0.035)	−0.098* (0.013)	−0.087* (0.026)

Table V Multivariable – adjusted odds ratios (OR) of factors associated with alteration of semen parameters.

	Total sperm number < 39 × 10^6			Progressive motility < 32%			Abnormal forms > 96%		
	OR	95% CI	*P* value	OR	95% CI	*P* value	OR	95% CI	*P* value
51–81 years[a]	2.47	1.520–4.024	<0.001	3.63	2.487–5.297	<0.001	3.89	2.071–7.263	<0.001
45–50 years[a]	1.69	1.078–2.662	0.022	1.94	1.375–2.734	<0.001	1.91	1.024–3.571	0.042
41–44 years[a]	1.70	1.102–2.616	0.016	1.67	1.196–2.327	0.003	1.30	0.683–2.458	0.427
38–40 years[a]	1.23	0.763–1.970	0.399	1.66	1.178–2.352	0.004	1.19	0.604–2.353	0.612
33–37 years[a]	1.19	0.748–1.900	0.459	1.38	0.978–1.953	0.067	1.45	0.766–2.751	0.253
BMI > 30.0 kg/m^2 [b]	1.51	0.998–2.291	0.052	1.58	1.142–2.189	0.006	1.87	1.100–3.181	0.021
BMI 25.0–29.9 kg/m^2 [b]	1.14	0.866–1.501	0.351	1.09	0.881–1.338	0.439	1.35	0.929–1.958	0.116
Smokers[c]	0.99	0.739–1.320	0.930	0.97	0.776–1.205	0.765	0.96	0.649–1.432	0.856

[a] Versus 20–32 years.
[b] Versus normal weight (BMI 18.0–24.9).
[c] Versus non-smokers.

Molecular analysis

Sperm DNA damage, PRM1, PRM2, TNP1 and TNP2 gene expression, and miRNA expression profile were evaluated in the sperm cells and seminal plasma of 40 elderly men (mean 56.6 ± 6.6; range 50–81 years) versus 40 young men (mean 24.8 ± 7.1; range 20–40 years).

Sperm DNA damage

Table VI reports the means, medians, and SDs for the semen parameters and SDF of the two groups. There was a significant reduction in semen volume, total sperm number and progressive motility ($P < 0.001$) in the elderly subjects, confirming our previous results. There was also a significantly higher percentage of abnormal forms ($P = 0.001$) and SDF ($P < 0.001$) in these subjects (23.1 ± 8.7 vs 9.8 ± 2.6%). However, there was no correlation between SDF and semen parameters or between BMI and SDF in the elderly subjects.

PRM1, PRM2, TNP1 and TNP2 gene expression in spermatozoa

There was a significant decrease in PRM1 (mean fold change 2.2; $P = 0.016$) and PRM2 (mean fold change 4.6; $P < 0.001$) expression in the elderly subjects (Fig. 1). This was also reflected in the change in the PRM1/PRM2 mRNA ratio in the elderly subjects (0.63) in relation to the younger controls (0.94). In contrast, no changes of biological relevance were found in the expression of TNP1 (fold change = 1.3) or TNP2 (fold change = 1.4) in elderly subjects.

miRNA expression profile in seminal plasma

To identify the miRNA expression profile characteristic of ageing, initial screening was performed with TaqMan Human Array A + B Cards using a pool of 10 semen samples from elderly men, with 10 semen samples from young men as the controls. Of the 756 miRNAs analysed, the expression of eight was increased and of 59 decreased (with fold change ≥10) in the elderly subjects (Supplementary Table II).

Of the miRNAs with altered expression, miR-122a, miR-371-3p, miR-19b, miR-29b and miR-146a, described in the literature as involved in the mechanisms of ageing and spermatogenesis, were validated using a single RT-qPCR assay. The expression of the selected miRNAs validated in semen samples from 40 elderly men and 40 young controls confirmed the reduced expression of miR-122, miR-371 and miR-146a in the elderly subjects. miR-146a showed a 3-fold decrease ($P < 0.001$), miR-371 a 14-fold decrease ($P < 0.001$) and miR-122 a 5-fold decrease ($P = 0.01$). In contrast, there was no significant difference in miR-19b and miR-29b expression (Fig. 2).

Discussion

Male fertility slowly declines over time, as a consequence of the drop in testosterone levels and declining semen quality (Kimberly *et al.*, 2012). Men undergo a reduction in testosterone levels which persists for several years and is associated with a reduced number of Leydig cells, erectile dysfunction, sexual symptoms and reduced frequency of sexual intercourse. However, unlike women, men can still conceive children even at an advanced age, and the spread of ART has increased this opportunity. Given that paternal age is rising and that couples begin raising families ever later, various authors have investigated the effect of ageing on testicular function, sperm production, increased *de novo* mutations and possible genetic defects transmitted to the offspring (Nybo Andersen and Urhoj, 2017). These studies are of considerable translational importance in managing such subjects and providing them with effective counselling on their reproductive potential. For this reason, our study set out to analyse two important aspects of sperm in the ageing process, namely its cytological and molecular aspects.

Semen quality

Numerous epidemiological studies have evaluated the effect of paternal age on semen quality, but with conflicting results. Most agree that there is an age-related decline in semen volume, sperm concentration, motility and morphology (Gunes *et al.*, 2016), but others found no correlation between semen parameters and age (Jung *et al.*, 2002; Hellstrom *et al.*, 2006). The pioneering study by Dondero *et al.* (1985) analysed the semen parameters of subjects in various age groups, finding a progressive drop in sperm concentration after the age of 40 years (becoming more significant after 60 years), a gradual, constant drop in sperm motility, and altered sperm morphology, more evident after 60 years. More recently, Stone *et al.* (2013) retrospectively identified an age threshold within the study population, finding a 2% drop per year of age in total sperm number after 34 years, a 0.8% drop per year in concentration and normal forms after 40 years, and a 0.8% drop per year in progressive motility, but only after 43 years.

A number of mechanisms have been proposed to explain the effect of ageing on semen quality. Reduced semen volume might be caused by alterations to the seminal vesicle, while reduced sperm concentration could be induced by testicular alterations. The pathophysiological basis for the impact of age on semen quality could be due to specific effects of age alone or to other age-related factors such as vascular disease, obesity, male accessory gland infections or the build-up of toxins (Sartorius and Nieschlag, 2010).

This study involved the retrospective evaluation of 2626 semen analyses from men aged 20–81 years. In addition to the large caseload, a strength of this study was its exclusion of subjects with clear comorbidities, treatments or other conditions which, in addition to age itself, could interfere directly with spermatogenesis; moreover, only subjects with a similar period of sexual abstinence (2–7 days), were included (WHO, 2010). Another strength is that all semen samples were examined in the same laboratory, thus standardising the results. However, a limitation is that while typical chronic age-related conditions such as cardiovascular and respiratory diseases were excluded, the possible presence of subclinical diseases cannot be excluded. Similarly, subjects referred to our clinic for various reasons and might not be fully representative of the general population. Although we excluded common andrological conditions that might affect spermatogenesis, we cannot exclude the presence of possible idiopathic or asymptomatic conditions.

Our results indicate a significant decline in semen quality with advancing age, manifesting as a reduction in semen volume, progressive motility, total sperm number and number of progressively motile sperm and an increase in the percentage of abnormal forms. Our data robustly confirm the decline of semen quality in a large monocentric caseload. Furthermore, we identified that the most significant decline is after the age of 50 years: in men aged 51–81, the probability of a reduced total sperm number had more than doubled (OR 2.47; 95% CI 1.52–4.02; $P < 0.001$), while the risk of impaired progressive

Table VI Semen parameters and percentage of sperm DNA fragmentation (SDF) in 40 elderly men (50–81 years) versus 40 young men (20–40 years).

	Age (years)	Volume (ml)	Total sperm number ($n \times 10^6$/ejaculate)	Progressive motility (%)	Abnormal forms (%)	SDF (%)
Elderly subjects ($n = 40$)	56.6 ± 6.6	2.5 ± 1.6	216.7 ± 199.3	42.6 ± 14.4	81.0 ± 8.9	23.1 ± 8.7
	(54)	(2.0)	(150.2)	(50)	(82.5)	(22.5)
Young subjects ($n = 40$)	24.8 ± 7.1	3.7 ± 1.4	405.4 ± 193.0	57.7 ± 3.4	77.7 ± 3.4	9.8 ± 2.6
	(22)	(3.5)	(370)	(60)	(77.5)	(9.9)
P-value[a]	<0.001	<0.001	<0.001	<0.001	0.001	<0.001

Data are mean ± SD.
[a]Mann–Whitney *U* test.

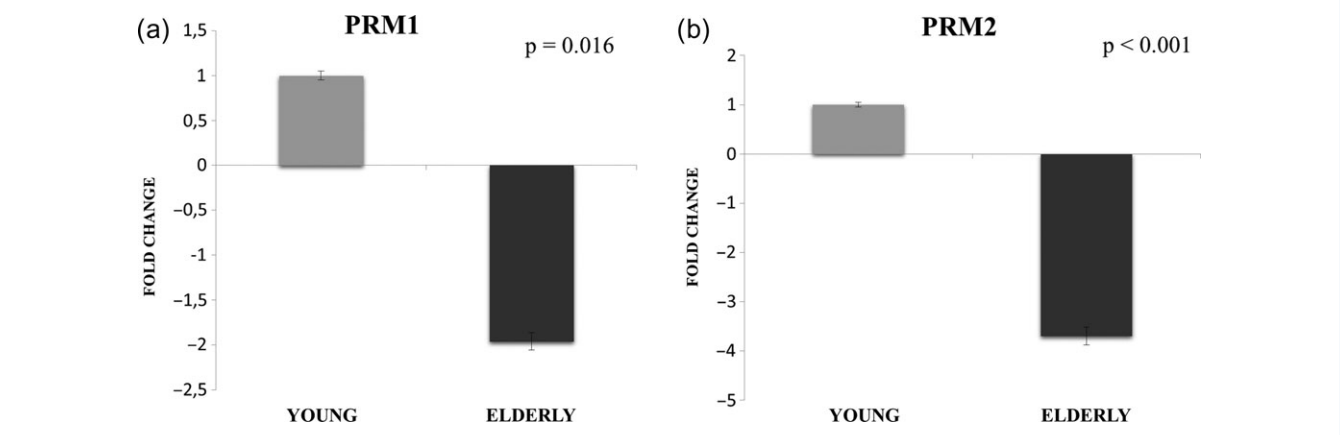

Figure 1 Gene expression analysis of (a) PRM1 and (b) PRM2 in 10 elderly (50–81 years) versus 10 young (20–40 years) patients. Results are expressed as mean ± SD.

motility and morphology had almost quadrupled (OR 3.63; 95% CI 2.49–5.30; $P < 0.001$; and OR 3.89; 95% CI 2.71–7.26; $P < 0.001$, respectively). We found a weak correlation between smoking and semen parameters (total sperm number, progressive motility, and number of progressively motile sperm), although multivariate analysis did not find any significant contribution from smoking. In contrast, we found a significant increase in the percentage of obese and overweight subjects with rising age. Obesity was associated with alterations in both progressive motility (OR 1.58; 95% CI 1.14–2.19; $P = 0.006$) and abnormal forms (OR 1.87; 95% CI 1.02–3.57; $P = 0.021$). Similar results were obtained by da Hammoud *et al.* (2008). The metanalyses in the literature present conflicting results. MacDonald *et al.* (2010) found no relationship between obesity and sperm concentration or sperm count, but Guo *et al.* (2017) found a decrease in sperm quality (sperm count, concentration, and semen volume) associated with an increase in BMI.

It can thus be seen that while the percentage of smokers drops with advancing age, increased BMI has a negative impact on semen parameters, probably secondary to hormonal changes. It should be stressed that although the age-related decline in semen quality was statistically significant in our study, most subjects over 40 years fell within the fifth percentile reference values reported in the 2010 WHO manual. Specifically, 75% of subjects aged 40–50 years were classifiable as normozoospermic, while in subjects over 51 this dropped to ~60%, with a more marked increase in subjects with impaired progressive motility.

Sperm DNA damage

As men age, their germ cells undergo an exponential number of mitotic divisions, with possible consequent DNA damage (Sakkas *et al.*, 1999). Chromatin damage may arise from chromatin condensation and protamine-histone replacement process defects or from consequences of the apoptotic process or of oxidative stress from free radicals. Alteration of the DNA repair processes in spermatids could also contribute to increased SDF (Grégoire *et al.*, 2018).

Numerous studies have found a positive correlation between male ageing and sperm DNA damage using various methods, including SCSA (Wyrobek *et al.*, 2006), Comet Assay (Singh *et al.*, 2003) and TUNEL assay (Belloc *et al.* 2014). However, other studies found no correlation between ageing and sperm DNA damage (Nijs *et al.*, 2011).

Our results confirm those of previous studies, revealing a significant increase in sperm chromatin fragmentation in elderly subjects (23.1%) in comparison with young subjects (9.8%) ($P < 0.001$). This increase highlights the genomic fragility of sperm in advanced age, which makes

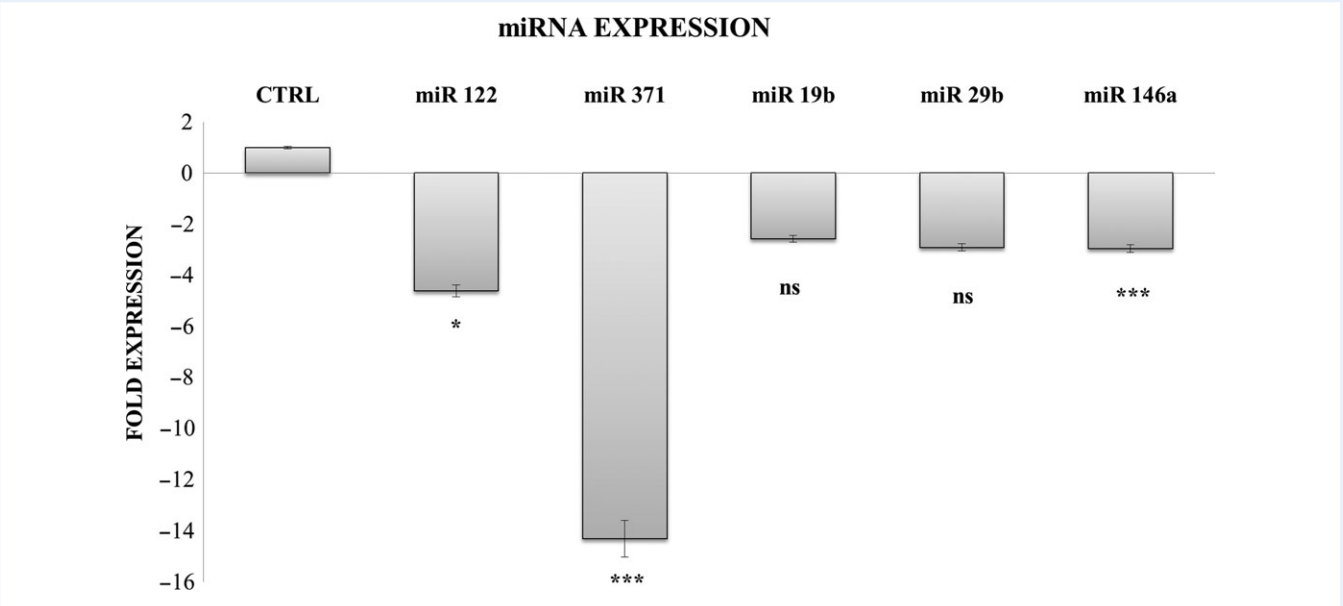

Figure 2 Validation of TaqMan microRNA Array Cards. Fold change in miRNA expression in the seminal plasma of 40 elderly (50–81 years) versus 40 young (20–40 years) subjects. Results are expressed as mean ± SE. ***$P < 0.001$.

them more vulnerable to further damage caused by exogenous and endogenous factors. However, it should be stressed that TUNEL, in common with many other SDF detection methods, does not distinguish between single and double strand breaks. This is important from a clinical perspective, as small numbers of SSBs can be repaired by the oocyte, but DSBs may be irreversible.

PRM1, PRM2, TNP1 and TNP2 gene expression

Protamines are ubiquitous proteins expressed in mammalian sperm. Their role is to protect the genetic message delivered by the sperm to the egg, through a mechanism which enables the packaging of chromatin during spermatogenesis (Oliva and Dixon, 1991). The chromatin packaging process requires the correct ratio of protamine 1 and 2 (P1/P2), which in fertile men is 0.8–1.2 (Aoki *et al.*, 2006; Grassetti *et al.*, 2012). Numerous studies have confirmed that an altered P1/P2 ratio is associated with male infertility (Rogenhofer *et al.*, 2013).

A possible cause of altered protamine expression is changes in mRNA levels. In 2013, Rogenhofer *et al.* demonstrated that the protamine/mRNA ratio in ejaculated sperm distinguishes between fertile and subfertile men and asserted that protamine mRNA ratio could be a prognostic marker to evaluate sperm fertilising potential, and could be correlated with successful fertilisation in IVF and ICSI.

The results of our study revealed, for the first time, statistically significant differences in PRM1 and PRM2 gene expression between elderly and young subjects, with an ~2-fold change for PRM1 and 5-fold change for PRM2. This is reflected in an altered PRM1/PRM2 mRNA profile in elderly men in comparison with young men (0.6 versus 0.9, respectively).

We also investigated whether reduced PRM1 and PRM2 expression might in turn be associated with altered transition nuclear protein 1 (TNP1) and TNP2 gene expression. TNP1 and TNP2 gene expression was unvaried in our elderly subjects, suggesting that altered protamination might be connected with a protamine transcription factor, as also demonstrated by the altered protamine mRNA ratio. This could be correlated with the greater sperm DNA damage seen in elderly subjects, indicating the marked fragility of chromatin integrity.

MiRNA expression profile

Given the complexity of the changes that take place during the ageing process and the known role of miRNAs in complex mediating pathways, it is no surprise that miRNAs might play a role in ageing. As recently described by Harries (2014), there are several 'hallmarks' of ageing, including changes in gene expression, epigenetic changes, altered DNA damage response, progressive shortening of telomeres, mitochondrial dysfunction, cellular senescence, stem cell exhaustion and heightened inflammatory response. Although several miRNAs seem to be involved in these processes, little is yet known about their role in the ageing process (Williams *et al.*, 2017), especially the role of circulating miRNAs (Zhang *et al.*, 2015).

No published studies have analysed the role of ageing on miRNAs circulating in the male reproductive system. We addressed this lack by comparing the expression of miRNAs in the seminal plasma of elderly and young men to establish if alterations in their expression play any part in testicular function during the ageing process. We found an altered expression of 67 miRNAs in elderly subjects in relation to young subjects, of which eight had a higher expression and 59 a lower expression. Validation of some of these miRNAs revealed a significantly lower expression of miR-122, miR-371, and miR-146a in the seminal plasma of elderly men. There was no significant difference in the expression of miR-29b or miR-19b.

miR146a has important roles in the senescence mechanism, inhibiting the pro-inflammatory status associated with cell senescence by interacting with the NF-KB pathway (Olivieri *et al.*, 2013), contributing

to mitochondrial dysfunction (Rippo et al., 2014), and influencing the expression of the NOX4 subunit NADPH oxidase. Its reduced expression may increase the production of ROS and oxidative stress (Vasa-Nicotera et al., 2011).

miR146 is also involved in spermatogenesis and is regulated during spermatogonial differentiation (Huszar and Payne, 2013). In contrast, mir371-3p is involved in the 'stem cell exhaustion' mechanism and probably in the mesenchymal stem cell (MSC) ageing process.

mir122a is a testis-specific miRNA enriched in late-stage male germ cells and, through endonucleolytic cleavage, reduces the expression of TNP2 (Liu et al., 2013). Some studies have identified mir-122 and mir-371 as potential markers of altered spermatogenesis (Wu et al., 2013).

Our results provide the first evidence that the miRNA expression profile changes with age; specifically, we identified that miR146a, miR371 and miR122 expression is altered in the seminal plasma of elderly men. Ageing in men is a physiological condition characterised by hormonal and structural changes affecting the testicles, epididymis, prostate and accessory glands. Changes in the local environment could affect the molecular homoeostasis of sperm in various ways, including chromatin integrity, cell proliferation, epigenetic remodelling and stem cell exhaustion of the seminiferous tubules, as well as contributing to the establishment of a pro-inflammatory environment, typical of ageing.

In summary, our results showed that spermatogenesis in elderly men is qualitatively altered and associated with various molecular defects, including increased SDF and reduced PRM1 and PRM2 gene expression. The latter could depend on transcription factors, miRNAs, or epigenetic regulation mechanisms. We demonstrated an altered expression of various miRNAs, including miR122, involved in TNP2 expression. Evidence from the literature suggests an association between miR-122 and TNP2. Liu et al. (2013) revealed that overexpression of miR-122 suppressed the expression of TNP2 and PRM and influenced the development and maturation of sperm cells. In contrast, we found a lower expression of miR-122 in the elderly men, which was associated with a minimal change in TNP2 and a change in the PRM1/PRM2 ratio. This altered ratio could in part be explained by the low miR-122 expression, although the molecular pathway involved is not yet understood in detail. Furthermore, *in vivo* models do not enable the exclusion of other molecular pathways that seem to have protamines as targets.

These molecular changes could be caused by continual germ cell divisions, which make them vulnerable to errors, or by the constant attack of exogenous or endogenous factors such as ROS. This hypothesis could be suggested by the lower expression of the miRNAs involved in NADPH oxidase expression; all this could induce a wide range of DNA lesions.

In conclusion, this study demonstrates a significant decline in semen quality with advancing age, especially after the age of 50, and that these alterations are associated with a rise in BMI. It also reveals a characteristic molecular signature during the ageing process, identifying sperm DNA damage, altered protamination, and an altered semen miRNA profile; the latter could be used as new biomarkers for male reproductive fitness during the physiological ageing process.

Given these results, we can hypothesise that birth defects associated with paternal age could be closely linked to alteration of the molecular profile of sperm.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

DP and MP conceived and designed the experiments; DP and GP wrote the article; FP and FF acquired and analysed the data; AL and FL contributed to data interpretation and manuscript revision.

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Conflict of interest

The authors have no conflicts of interest.

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