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Are F_2-isoprostanes a better marker of semen lipid peroxidation than MDA in reproductive pathologies with inflammatory basis?

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ABSTRACT

Many male reproductive pathologies and a part of undiagnosed infertility share an oxidative stress (OS) etiology with high reactive oxygen species and cytokine concentrations. The lack of reliable biomarkers to quantify oxidative injury is a crucial problem in the field of male infertility. In this observational study, IL-1β and the OS markers malondialdehyde (MDA) and F_2-isoprostanes (F_2-IsoPs) were quantified in seminal plasma of 46 infertile patients with varicocele, genitourinary infections, idiopathic infertility, and 11 fertile men. Semen analysis was performed following WHO guidelines, IL-1β was determined by ELISA, MDA was quantified by HPLC, and F_2-IsoPs by GC/NICI-MS analysis. F_2-IsoPs were immunolocalized in spermatozoa of fertile and infertile subjects. Results indicated that F_2-IsoP, MDA, and IL-1β seminal levels positively correlated pairwise ($p < 0.001$) and showed negative correlations with sperm parameters ($p < 0.001$). Then, the studied population was grouped following the cause of infertility and the variables were compared between the different groups and a control sample. Seminal IL-1β, F_2-IsoPs, and MDA were significantly higher in varicocele ($p < 0.001$, for MDA $p < 0.01$) and genitourinary infections ($p < 0.001$, for IL-1β $p < 0.01$) groups than those observed in fertile subjects. F_2-IsoPs seemed to discriminate more accurately than MDA the different conditions, in particular idiopathic infertility. ROC curves demonstrated that the three analyzed indices were able to discriminate fertile and infertile patients. The immunofluorescence studies showed a low presence of F_2-IsoPs in spermatozoa of fertile men and an evident labeling in the tail, and cytoplasmic residues of spermatozoa from infertile patients. In conclusion, this data confirmed that F_2-IsoP level is a suitable marker of OS in seminal plasma, even more accurate than MDA and can be proposed for measuring OS in the clinical setting.

1. Introduction

Growing evidence suggests that oxidative stress (OS) is one of the most important issues in the etiology of male infertility. OS is defined as the presence of increased reactive oxygen species (ROS) concentration respect to the available antioxidant buffering capacity [1]. Although low and physiological levels of ROS play a relevant role in sperm function, controlling capacitation, hyperactivation, acrosome reaction, and membrane fusion with the oocyte [2,3], high ROS levels have detrimental effect on sperm quality. In fact, when the redox state is unbalanced for different endogenous and exogenous factors, ROS concentration increases leading to the disruption of sperm structural and functional integrity of spermatozoa damaging DNA, protamination process, lipids, and proteins that can impair also fertilization and embryo development [4,5]. Because high content of polyunsaturated fatty acids (PUFAs) in their plasma membrane, spermatozoa are exposed to ROS insults (*i.e.* oxidative damage to PUFAs present in membrane lipids) that impair membrane permeability and motility. PUFAs are particularly exposed to oxidative damage because they contain two or more carbon-carbon double bonds in their molecular structure. In addition, spermatozoa are almost lacking intracellular antioxidant defenses and the main portion of antioxidant buffering capacity to prevent the OS damaging effects is present in the seminal plasma [6].

A number of laboratory techniques have been developed to evaluate OS in human semen. Over the years, the most used methods estimate the damage caused by ROS measuring lipid peroxidation (LPO) and its by-

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products such as malondialdehyde (MDA), 4-hydroxy-2-nonenal (4-HNE), acrolein and thiobarbituric acid reactive substances, known as TBARS [7–10]. In TBARS assay, several end products of LPO are able to react with thiobarbituric acid, primarily MDA [11]. A direct measure of MDA by chromatographic techniques has increased MDA assay sensitivity and specificity [12].

Recently, these markers have been implemented by far more accurate and stable ones. Among them, isoprostanes play a particular relevant role in many pathologies [13]. In particular, F_2-isoprostanes (F_2-IsoPs) are formed *in situ* directly from ROS-mediated oxidation of arachidonic acid esterified in lipids, to be subsequently released into biological fluid. Established reference intervals, have been reported in human plasma, urine, and other body fluids [13]. In addition, normal levels of F_2-IsoPs could be defined also in seminal plasma and such levels resulted increased in case of infertility [14,15].

At this purpose, it is well known that several pathologies as varicocele, genitourinary infections/inflammation affecting male reproductive system share an OS etiology with high ROS levels, cytokine concentrations and other proinflammatory factors [16–19]. Also, a high percentage of male infertility remains undiagnosed and classifies as idiopathic infertility. Generally, in these cases patients show some abnormal semen parameters concomitant with normal laboratory workup. These idiopathic sperm abnormalities are assumed to be caused by several factors as unknown genetic and epigenetic abnormalities and endocrine disruption by environmental pollution and OS.

An adequate evaluation of male reproductive potential should therefore include an assessment of sperm OS by which patients previously classified as having idiopathic male infertility could be diagnosed [20,21].

In this observational study we quantified in seminal plasma of patients with varicocele, genitourinary infections, idiopathic infertility and controls two markers of LPO (*i.e.* PUFA oxidative damage) that are MDA and F_2-IsoPs, and Interleukin-1β (IL-1β), one of the major proinflammatory cytokines involved in a broad spectrum of inflammatory disorders [22]. The relationships among these indices were explored.

2. Materials and methods

2.1. Patient's selection

Semen samples were obtained from 46 Caucasian infertile patients (aged 28–39 years) who attended AGI Medica, Fertility Center (Siena, Italy). All patients were unable to obtain pregnancy after two years of unprotected sexual intercourses. The female factor was excluded.

The clinical history of patients was collected. The 46 cases enrolled in this study fulfilled the following inclusion criteria: non-azoospermic men, absence of systematic sperm defects, BMI < 25 kg/ m^2, no history of diabetes, metabolic syndrome, radiotherapy, chemotherapy, chronic illness, or medication. In addition, the study participants were non-smokers, they did not use drugs, alcohol, or dietary supplements.

Hormone and bacteriological analyses were assessed in dedicated laboratories and were part of the clinical workup. In the studied population, the concentrations of testosterone, luteinizing hormone and follicle-stimulating hormone were normal. All patients provided bacteriological analyses of their semen samples. Patients whose semen was positive for microorganisms were classified as affected by genitourinary infections, even though they did not exhibit any symptoms of genital infections.

To detect the possible presence of varicocele, physical examinations and scrotal Eco-color Doppler were performed. Subclinical varicocele was not considered in this study. The patients with both varicocele and positive semen bacteriological analyses were excluded from the study as well as the patients with leukocytospermia [23].

The 46 infertile patients were grouped according to clinical diagnosis:

- group with varicocele ($n = 16$),
- group with genitourinary infections ($n = 17$),
- group with idiopathic infertility ($n = 13$).

Eleven fertile men (aged 28–36 years) with BMI < 25 kg/ m^2 not affected by diabetes, metabolic disorders, anatomical problems and/or infections and who fathered a child in the last three years, were recruited as controls.

When signing the informed consent to the treatment, patients who had been referred to AGI Medica, Fertility Center (Siena, Italy) for semen analysis accepted or did not accept the possibility that the semen samples not used for the analysis could be used for scientific research purposes. Patients who agreed with this possibility and who met the inclusion criteria were enrolled in our study. The internal institutional ethics committee approved this study. Informed written consent to participate at the research protocol was obtained from all patients.

2.2. Semen analysis

Samples were collected in a sterile, nontoxic container, after 2–5 days of sexual abstinence. After liquefaction at 37 °C, the sperm concentration and motility were evaluated following the last edition WHO guidelines for semen analysis [23]. The assessment of sperm morphology was performed using pre-stained Testsimplets slides (Waldeck GmbH & Co., Münster, Germania) and 300 spermatozoa per sample were scored.

The determination of the sperm vitality was based on the membrane integrity of the cells using eosin Y (CI 45380) staining. At least 300 spermatozoa per sample were evaluated by light microscope differentiating live sperm (unstained) and dead sperm (red stained).

After sperm analysis was completed, the human semen samples were centrifuged at 400*g* for 15 min to obtain sperm-free seminal plasma that was stored at −80 °C until use.

2.3. Quantification of malondialdehyde level in seminal plasma

The presence of LPO was monitored by MDA level assessment in the seminal plasma stored at −80 °C.

After thawing, the samples were derivatized with 2,4 dinitrophenylhydrazine (DNPH) and analyzed by High Performance Liquid Chromatography (HPLC) with UV detection.

At least 3 calibration samples ranging from 0.2 nmol/mL to 10 nmol/mL were used to determine MDA. Briefly, 80 μL of DNPH was added to 500 μL of each sample (including controls and calibrations); they were shaken with a vortex and left one hour in the dark at room temperature.

Then, after the addition of 5 mL of pentane, each specimen was shaken with an oscillating agitator for 15 min and centrifuged at 2200*g* for 5 min at room temperature.

At the end, the organic phase was collected and dried at room temperature using nitrogen. Later the samples were stored at −20 °C. The column was prepared conditioning the mobile phase composed by acetonitrile (45 %)- HCl 0.01 N (55 %). The procedure to stabilize the column included initially a flow 0.3 mL/min for 15 min and then 0.7 mL/min for 15 min of mobile phase. This step is very important to reduce the background noise in the chromatogram.

Then, 20 μL of calibrations or samples were injected every 36 min. To separate the MDA-hydrazone derivative, a 5 μ ultrasphere Symmetry C18 column (Beckman, San Ramon, CA, USA) was used at the flow rate of 0.7 mL/min with the acetonitrile (45 %)- HCl 0.01 N (55 %) as mobile phase. The MDA hydrazone was quantified by isocratic high-performance liquid chromatography using a Waters 600 E System Controller HPLC (Milford, MA, USA) equipped with a Waters Dual λ 2487 UV detector (Milford, MA, USA) set at 310 nm.

The concentrations of MDA in the samples were obtained by peak areas determined using an Agilent 3395 integrator (Agilent Technologies, USA). Each sample was assessed in duplicate, and the results are

expressed in nmol of MDA per mL of seminal plasma.

2.4. Quantification of F_2-Isoprostane level in seminal plasma

The levels of total F_2-IsoPs were measured in the seminal plasma previously stored at −80 °C. At the time of F_2-IsoP detection, in each sample, a basic hydrolysis was firstly performed by incubation (45 °C, 45 min) with 1 N KOH (1:0.5, v:v). Subsequently, 1 N HCl, (500 μL) and 500 pg $PGF_{2\alpha}$-d_4 were added in each sample. Afterward, purification procedures were carried out in all samples. Firstly, each sample was applied to an octadecylsilane (C_{18}) cartridge and subsequently each C_{18} extract was applied to an aminopropyl (NH_2) cartridge. As a further step, the F_2-IsoP carboxylic group was derivatized as the pentafluorobenzyl ester whereas the hydroxyl groups were converted to trimethylsilyl ethers. Finally, F_2-IsoP determinations were carried out by gas chromatography/negative ion chemical ionization tandem mass spectrometry (GC/NICI-MS/MS) analysis. F_2-IsoPs were quantified by identification/measure of the *m/z* 299 ion produced by ionization of 8-iso$PGF_{2\alpha}$, the most represented F_2-IsoP isomer [24]. The results were expressed as ng/mL.

2.5. Determination of IL-1 β level in seminal plasma

The amount of IL-1β was determined by sandwich enzyme-linked immunosorbent assay (Invitrogen™, Thermo Fisher Scientific, Bender MedSystems GmbH, Vienna, Austria) in seminal plasma of the considered patients. The concentration IL-1β was assessed by spectrometric detection of color intensity at 450 nm. A curve-fitting software was applied to generate the standard curve ranging from 0 to 250 pg/mL and to quantify IL-1β in tested samples. All measures were performed in duplicate. The results were expressed as pg/mL.

2.6. Immunolocalization of F_2-IsoPs in spermatozoa of infertile patients and fertile subjects

Spermatozoa of patients with varicocele, genitourinary infections, idiopathic infertility and of fertile subjects were washed in phosphate buffer saline (PBS), smeared on glass slides, air-dried. The slides, fixed in methanol at −20 °C for 20 min and in acetone −20 °C for 5 min, were rehydrated in PBS. Then, the slides were treated for 20 min with PBS-bovine serum albumin (BSA) 1 % containing 5 % normal goat serum (NGS) and incubated overnight at 4 °C with rabbit polyclonal anti-8-isoPGF2α antibody (Abcam, Cambridge, UK), diluted at 1:100 in PBS/0.1 % BSA/1 % NGS. The reaction was revealed using a FITC-conjugate anti-rabbit antibody raised in a goat (Sigma-Aldrich, Milan, Italy), diluted at 1:300. The incubation with the primary antibody was omitted in control samples. Sperm nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) solution (Vysis, Downers Grove, IL). The slides were observed and evaluated with a Leica DMI 6000 Fluorescence Microscope (Leica Microsystems, Germany), and the images were acquired by the Leica AF6500 Integrated System for Imaging and Analysis (Leica Microsystems, Germany).

2.7. Statistical analysis

Statistical analysis was performed with the SPSS version 23.0 for Windows software package (SPSS Inc., Chicago, IL, USA). The Kolmogorov-Smirnov test was used to verify the normality of distribution of the variables. The correlations between the investigated variables were calculated with the Spearman's Rank Correlation Coefficient (rho). Kruskal-Wallis test was applied to compare the difference of variables among considered groups (fertile, varicocele, genitourinary infections, idiopathic infertility) and then Dunnet *Post Hoc* test was applied to determine pairwise comparison. Data were reported as median (interquartile range [IQR]). $P < 0.05$ was considered statistically significant.

The receiver characteristic (ROC) curves (Microsoft Office Professional Plus 2016, Microsoft Corporation, Redmond, WA, USA) were calculated to test the accuracy of MDA, F_2-IsoP, IL-1β determinations in terms of the relationship between sensitivity and specificity. The confidence interval was calculated with the DeLong's test, a non-parametric approach that does not assume any specific distribution of the data [25]. The Youden index J, a measure of overall diagnostic effectiveness and a function of sensitivity and specificity, was used to establish the cut-off value for the analyzed indices between fertile and infertile cases.

3. Results

In the study we selected 46 infertile patients, among them 17 had positive semen cultures and were grouped as genitourinary infections, 16 were diagnosed as having grade 2 and 3 varicocele and the others were classified as idiopathic infertility. Among the patients with genitourinary infections, 7 had semen cultures positive for *Escherichia coli,* 6 for *Enterococcus faecalis,* 2 for *Ureaplasma urealyticum,* 1 *Staphylococcus aureus, 1 Mycoplasma hominis.* As control, the same parameters were assessed in the semen of 11 fertile men. A general view of the median and IQR of the considered variables in the total group of participants (No. 57 cases) is reported in Table 1.

In Table 2, correlation coefficients (Spearman ρ coefficient) between variable calculated in the total group of cases are reported.

Seminal F_2-IsoP, MDA, and IL-1β levels positively correlated pairwise ($p < 0.001$, Table 2, Fig. 1). In particular, the two indices of OS showed a high correlation coefficient that was $\rho = 0.657$ (Table 2, Fig. 1).

F_2-IsoP, MDA, and IL-1β levels showed negative correlations with sperm parameters, mainly with rapid motility percentage and sperm vitality percentage (all correlations $p < 0.001$); slow progressive motility percentage was negatively correlated only with F_2-IsoP levels ($p < 0.01$). The percentage of sperm with normal morphology showed negative correlations with IL-1β ($p < 0.05$), F_2-IsoP and MDA levels ($p < 0.01$). The correlations between sperm parameters are reported in Table 2.

Then, the patients were grouped according to infertility –related pathologies and fertility condition and a comparison between variables in the different groups was performed (Table 3).

Seminal IL-1β, F_2-IsoPs, and MDA (Fig. 2 A, B, C) were significantly higher in varicocele ($p < 0.001$, for MDA $p < 0.01$) and genitourinary infections ($p < 0.001$, for IL-1β $p < 0.01$) groups than those observed in fertile one.

As regards the idiopathic group, we observed a significant increase of F_2-IsoP (Fig. 2 B) and IL-1β (Fig. 2 A) levels respect to the fertile group ($p < 0.05$; $p < 0.01$, respectively; Table 3).

The levels of F_2-IsoPs ($p < 0.01$, Fig. 2B), MDA ($p < 0.05$, Fig. 2C), and IL-1β ($p < 0.05$, Fig. 2A) were significantly higher in genitourinary infection group than those measured in idiopathic group (Table 3). Varicocele group showed a significant increase of IL-1β and F_2-IsoP levels respect to idiopathic patients ($p < 0.001$; Fig. 2A, B). The F_2-IsoP index was sensitive enough to discriminate the increased levels in varicocele group respect to those measured in genitourinary infection

Table 1
Semen variables in the population considered in this study. Median (25th and 75th centile) of the parameters measured in the semen of the 57 considered cases.

	Median (25th–75th centile)
IL-1β (pg/mL)	5.30 (2.84–6.87)
F_2-IsoPs (ng/mL)	51.19 (32.93–64.29)
MDA (nmol/mL)	1.98 (0.73–5.48)
Volume (mL)	4.00 (3.00–5.05)
Sperm concentration (10^6x mL)	34.0 (16.00–52.00)
Rapid Motility %	14.00 (8.00–21.50)
Slow Motility %	9.00 (5.50–12.00)
Normal morphology %	8.00 (6.00–10.50)
Vitality %	70.00 (61.50–77.50)

Table 2
Correlation between variables in the studied population. Correlations calculated by ρ Spearman's coefficient between all parameters evaluated in semen of the 57 cases included in the study. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. IL-1β (pg/mL), F_2-IsoPs (ng/mL), MDA (nmol/mL), volume (mL), sperm concentration (10^6x mL), rapid motility %, slow motility %, normal morphology %, vitality %.

	IL-1β	F_2-IsoPs	MDA	Volume	Sperm concentration	Rapid motility	Slow motility	Normal morphology
F_2-IsoPs	0.490***	1						
MDA	0.545***	0.657***	1					
Volume	0.328	0.310	0.099	1				
Sperm concentration	0.154	−0.093	−0.116	0.130	1			
Rapid motility	−0.464***	- 0.441***	−0.494***	0.034	0.257*	1		
Slow motility	−0.158	−0.349**	−0.270	0.001	0.144	0.236	1	
Normal morphology	−0.300*	−0.425**	−0.430**	0.053	0.535***	0.668***	0.376**	1
Vitality	−0.667***	−0.489***	−0.521***	−0.166	0.046	0.572***	0.261	0.528***

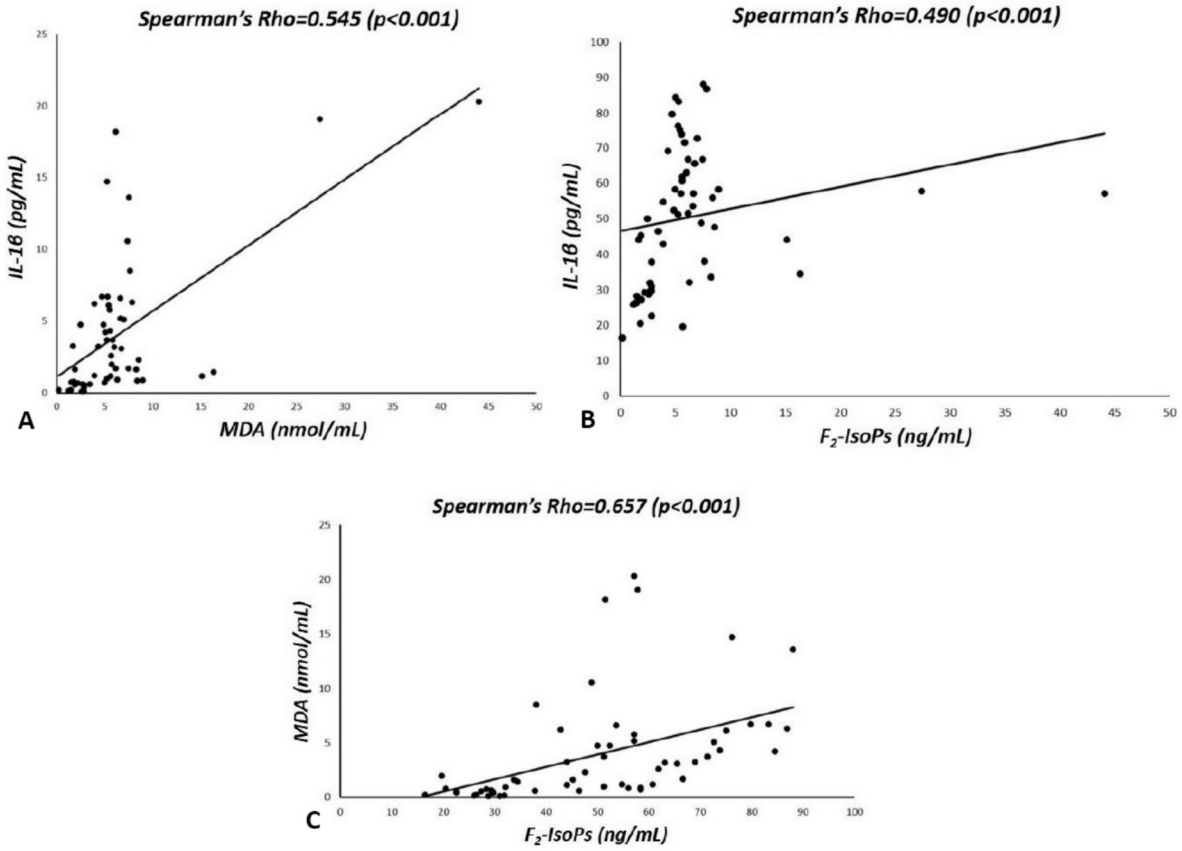

Fig. 1. Relationships between IL-1β, MDA and F_2-IsoP levels. Scatter plots showing the correlations (rho Spearman's coefficient) between IL-1β and MDA levels (A), between IL-1β and F_2-IsoP levels (B), and MDA and F_2-IsoP levels measured in seminal plasma of the 57 individuals considered in this study.

group ($p < 0.01$; Table 3, Fig. 2B).

As a whole, the sperm quality of fertile men was improved than that of infertile patients (Table 3).

ROC curves and the best cut-off values according to the J index for F_2-IsoP, MDA and IL-1β levels were calculated.

The overall performance of the ROC test was quantified by computing the area under the curve. For F_2-IsoP levels the area under curve was 0.96 (0.91–1 confidence interval) and J index showed a value of 31.90 ng/mL (Fig. 3). For MDA levels the area under curve was 0.95 (0.90–1 confidence interval) and J index had a value of 0.86 nmol/mL (Fig. 4). Finally, for IL-1β the area under curve was 0.95 (0.91–1 confidence interval) and J index had a value of 3.44 pg/mL (Fig. 5).

Finally, immunofluorescence was performed in spermatozoa of infertile and fertile patients to detect the localization of F_2-IsoPs (8-isoPGF$_{2\alpha}$). The signal in spermatozoa of fertile men was very weak, almost undetectable in 90 % of examined cells (Fig. 6 A). Spermatozoa of infertile men showed a more intense staining in correspondence of midpiece and the tail (Fig. 6 B); the immature spermatozoa and also spermatids, sometime present in semen (Fig. 6 C, D), showed cytoplasmic residues that are particularly rich in isoprostanes.

4. Discussion

It is known that OS is a core common mechanism of many pathologies related to infertility [16,26,27], included a part of idiopathic infertility [28,29]. However, OS is currently not indicated in the routine evaluation of infertile men and remains a challenging condition to diagnose and manage [8]. To underline the dimension of the problem,

Table 3
Comparison between the studied parameters in the patients grouped according to pathology and in fertile men. Median (IQR: 25th and 75th centile) of the considered variables in 46 patients, categorized according to their pathologies or conditions, and in 11 fertile men. Statistics are reported in the columns on the right.

	Fertile group (F, n. 11)	Idiopathic group (I, n. 13)	Varicocele group (V, n.16)	Genitourinary infections group (GI, n.17)	Kruskall-Wallis	Post-hoc test Dunnett
Volume (mL)	4.00 (3.00–5.50)	3.00 (2.25–4.00)	5.00 (4.00–5.88)	4.00 (3.15–5.15)	*ns*	
Concentration (10^6 per mL)	40.00 (35.30–54.00)	18.00 (1.50–31.50)	37.50 (21.75–56.25)	26.00 (13.00–51.00)	$p < 0.05$	*F vs I* $p < 0.05$
Rapid progressive motility (%)	31.00 (22.00–35.00)	15.00 (6.50–17.50)	12.00 (9.25–18.00)	10.00 (7.00–13.00)	$p < 0.001$	*F vs I* $p < 0.001$ *F vs V* $p < 0.001$ *F vs GI* $p < 0.001$
Slow progressive motility (%)	12.00 (7.00–18.00)	8.00 (4.50–16.50)	7.50 (4.25–10.75)	9.00 (5.00–10.00)	*ns*	
Normal forms (%)	13.00 (12.00–15.00)	6.00 (4.00–8.00)	7.00 (6.00–8.00)	6.00 (5.00–9.50)	$p < 0.001$	*F vs I* $p < 0.001$ *F vs V* $p < 0.001$ *F vs GI* $p < 0.001$
Vitality (%)	80.00 (78.00–85.00)	75.00 (66.50–77.50)	69.00 (63.25–72.00)	57.00 (52.00–67.50)	$p < 0.001$	*F vs V* $p < 0.01$ *F vs GI* $p < 0.001$ *I vs GI* $p < 0.01$ *V vs GI* $p < 0.05$
IL-1β (pg/mL)	1.90 (1.43–2.69)	3.45 (2.55–4.90)	5.72 (5.26–7.37)	7.34 (5.89–11.83)	$p < 0.001$	*F vs I* $p < 0.01$ *F vs V* $p < 0.001$ *F vs GI* $p < 0.01$ *I vs GI* $p < 0.05$ *I vs V* $p < 0.001$
F_2-IsoPs (ng/mL)	28.33 (25.95–29.76)	44.05 (32.02–51.19)	67.86 (58.93–78.27)	53.57 (45.84–64.29)	$p < 0.001$	*F vs I* $p < 0.05$ *F vs V* $p < 0.001$ *F vs GI* $p < 0.001$ *I vs V* $p < 0.001$ *I vs GI* $p < 0.01$ *V vs GI* $p < 0.01$
MDA (nmol/mL)	0.40 (0.16–0.67)	1.20 (0.59–4.00)	3.70 (2.04–5.62)	6.10 (1.67–14.15)	$p < 0.001$	*F vs V* $p < 0.01$ *F vs GI* $p < 0.001$ *I vs GI* $p < 0.05$

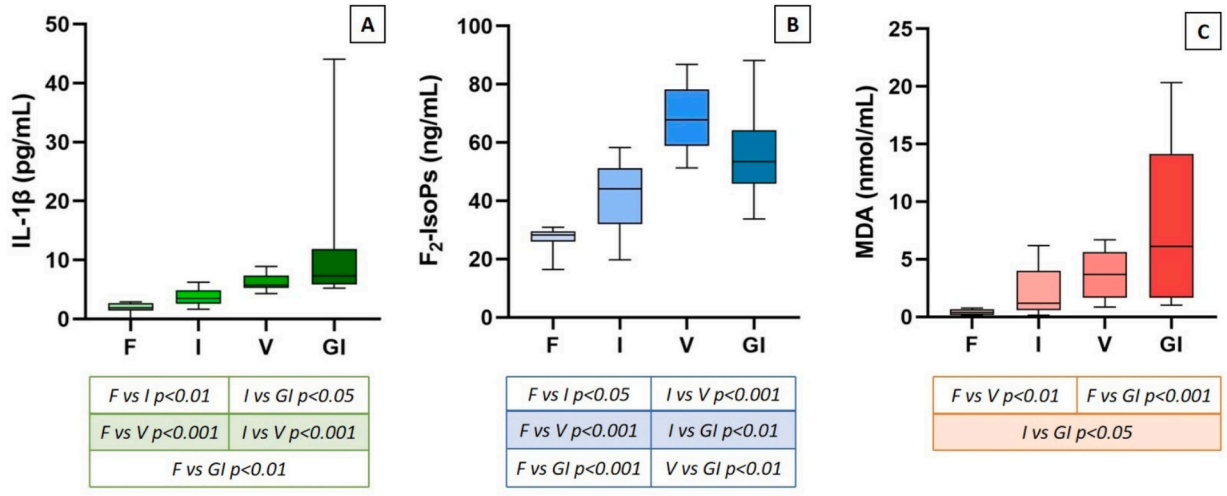

Fig. 2. Graphical representation of IL-1β, F_2-IsoP and MDA levels in the patients grouped according to pathology and in fertile men. Medians (IQR) of IL-1β (A), F_2-IsoP (B) and MDA (C) levels measured in seminal plasma of fertile men (F), patients with idiopathic infertility (I), varicocele (V) and genitourinary infections (GI). The boxes below each graphic reported the significant pairwise comparisons performed by Dunnet *Post Hoc* test.

recently a new category of infertility was proposed: "Male Oxidative Stress Infertility (MOSI)" including infertile men with OS [29,30].

The lack of reliable biomarkers to quantify oxidative injury and determine the efficacy of possible treatments to decrease damages has been a crucial problem in the field of male infertility and, in general, in human physiology and pathology [13].

Among methods for measuring LPO, a relevant process involved in sperm quality, or its by-products in human semen and sperm, one of the most common biomarkers is MDA that is not stable due to its reactivity and metabolism [9,13]. However, many studies reported the correlations between MDA levels and semen parameters [31–33], observation that agrees with the results of the present paper.

F_2-IsoPs, discovered by Morrow and Roberts in 1991, are reputed to be a sort of "perfect biomarker" [13]. The important characteristic that makes F_2-IsoPs such a good marker is their chemical stability both *in vitro* and *in vivo*. In addition, they are ubiquitous in human plasma and urine and, consequently, normal levels could be defined [34]. F_2-IsoPs can be assessed by different analytical approaches such as mass spectrometry, radioimmunoassay, and low cost and ease of use-methods as ELISA that helped in expanding the research in this area [14]. The last

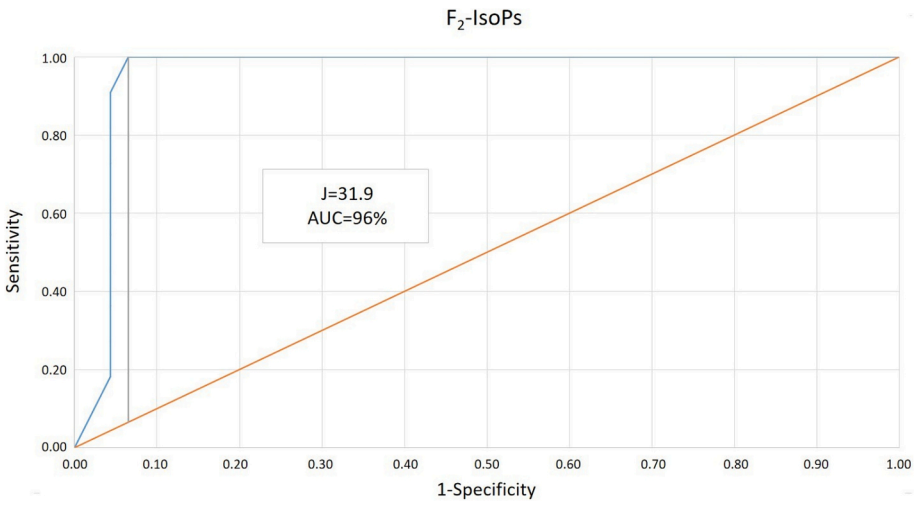

Fig. 3. ROC curve of semen F_2-IsoP levels in fertile and infertile subjects considered in this study. J represents Youden index with a value of 31.9 ng/mL. AUC: area under the curve.

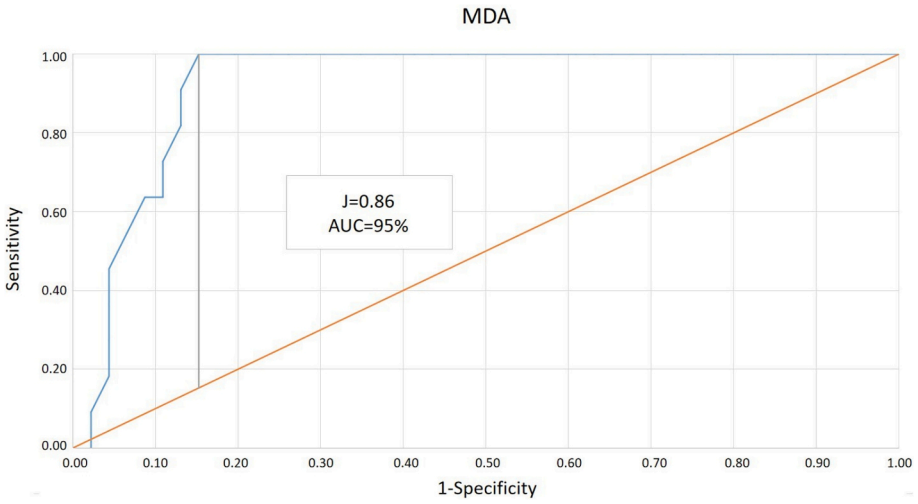

Fig. 4. ROC curve of semen MDA levels in fertile and infertile subjects considered in this study. J represents Youden index with a value of 0.86 nmol/mL. AUC: area under the curve.

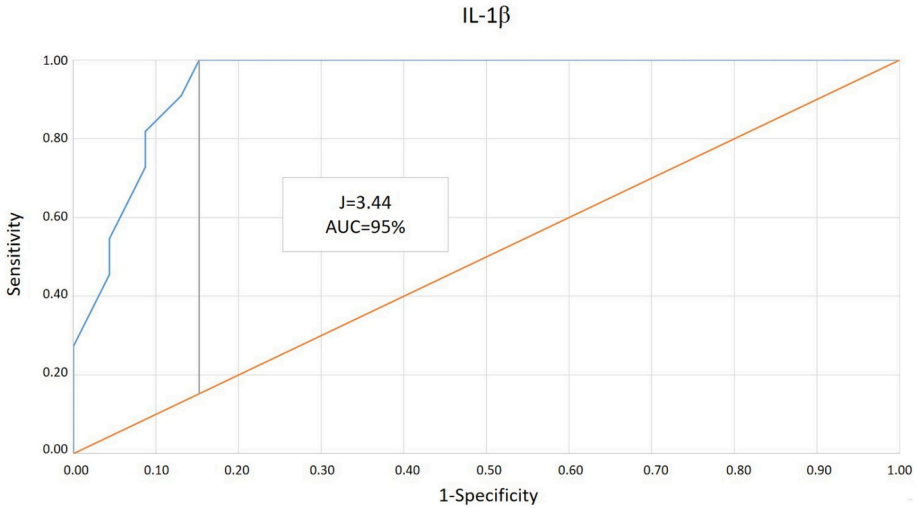

Fig. 5. ROC curve of semen IL-1β levels in fertile and infertile subjects considered in this study. J represents Youden index with a value of 3.44 pg/mL. AUC: area under the curve.

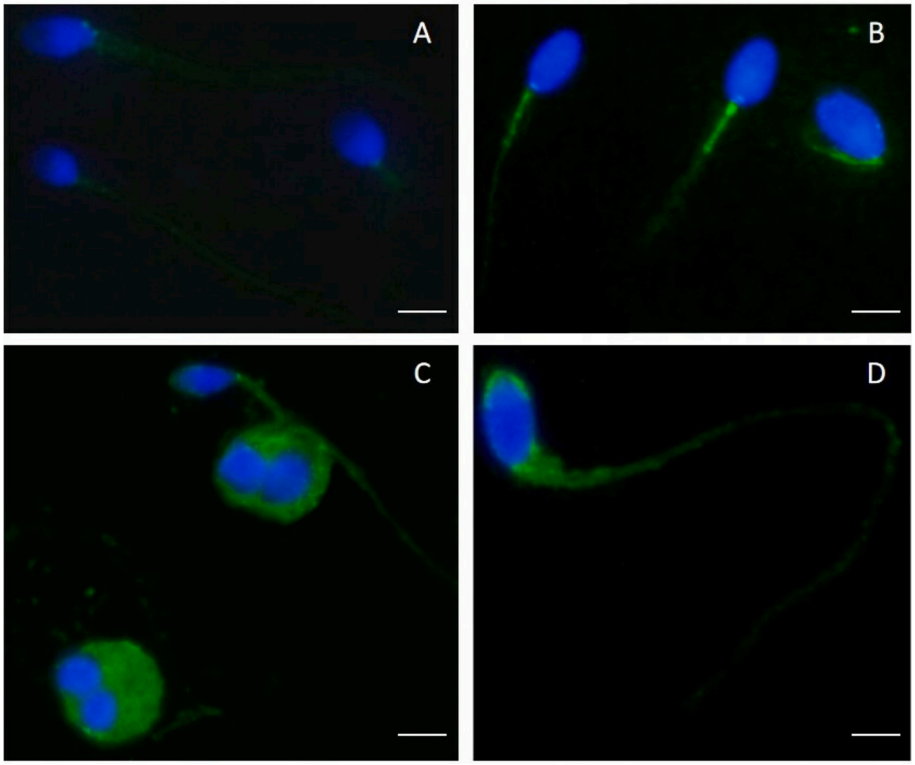

Fig. 6. Immunofluorescence staining of spermatozoa using an anti-8-isoPGF2α polyclonal antibody. (A) Spermatozoa from a fertile man show a weak label in the flagellum. (B, C, D) Spermatozoa from infertile patients: in (B) the 8-isoPGF$_{2\alpha}$ staining appears more intense in the mitochondrial sheath. In (C) the sample of an infertile patient with sperm immaturity shows binucleated spermatids and wide cytoplasmic residues in which the stain is evident. It is also shown a spermatozoon with the labeling on the acrosome, the midpiece, and the tail. In (D) the spermatozoon is stained on the acrosome, and midpiece where cytoplasmic residue is evident; also, the tail is faintly labeled. 4,6-Diamidino-2-phenylindole (DAPI) was used to stain the nuclei. Bars: A, B: 5 μm; C: 7 μm; D: 2 μm.

couple of decades has shown a growing interest in the role of isoprostanes in male infertility related disorders; they seem to be relevant in the evaluation of OS in seminal plasma and spermatozoa [14,35–38], and as observed in this research, showed negative correlations with semen parameters.

In this study, the levels of F_2-IsoPs, MDA and IL-1β in semen of patients with varicocele, genitourinary infections, idiopathic infertility, and fertile men were measured first to check the relationship between the two OS markers and then to test how powerful these molecules are in discriminating specific situations related to male infertility. IL-1β was chosen as representative marker of inflammation. Cytokines are a natural and physiological component of seminal plasma and are involved in the normal function of male reproductive system [39]. Nevertheless, in many pathological conditions as the presence of varicocele [27] and genitourinary infections [40,41] their levels increase, playing a detrimental role on sperm function [42]. Proinflammatory cytokines as IL-1β and also many others as IL-6, IL-8, and TNFα, regulate pro-oxidant and antioxidant activities in the male genital tract [43], therefore the inflammation in the male genital tract is inevitably connected with OS and LPO [44] as confirmed by the significant positive correlations between IL-1β and MDA or F_2-IsoPs levels observed in this study.

In the examined cases, also the two LPO indices, MDA and F_2-IsoP levels, were positively correlated with each other indicating that both markers are able to discriminate OS in seminal plasma.

However, when the studied population was grouped following the infertility conditions and the variables were compared, F_2-IsoP levels were significantly different in a higher number of infertility categories than was for MDA.

In particular, the statistical evaluation of the data identified the significant differences in LPO detected by F_2-IsoP levels, but not with MDA levels, in idiopathic group *versus* fertile and varicocele groups and in varicocele group *versus* genitourinary infection group. The highest F_2-IsoP values were present in varicocele cases in agreement with similar observations made in the past by our group [36,37]. In particular, Collodel et al. [36] reported a relationship between F_2-IsoP levels in semen and sperm from infertile patients with varicocele and sperm immaturity, a pathology characterized by round/elliptical-shaped nuclei, the presence of uncondensed chromatin, coiled tail, and cytoplasmic residues.

As a whole, these results seem to indicate a greater sensitivity of F_2-IsoPs than MDA in the detection of LPO in different pathological conditions related to infertility, in particular in case of idiopathic infertility.

IL-1β concentration was increased in the seminal plasma of patients with genitourinary infections and varicocele, confirming the inflammatory background characterizing these conditions [16,27,45].

The behavior of IL-1β and F_2-IsoPs was similar in the semen of patients with idiopathic infertility where both the OS and inflammation were detectable respect to controls but were not as severe as in presence of varicocele and genitourinary infections. As previously mentioned, idiopathic male infertility historically includes cases where the underlying cause remains elusive, however papers published over the last decade claimed for a correlation between this condition and high ROS seminal levels [29] that can cause mitochondrial dysfunction, LPO and DNA damage.

Since the sample size per groups was not big enough, we calculated ROC curves for F_2-IsoP, MDA and IL-1β levels in relation to fertility/infertility conditions. All the cut-off values, expressed as J index, were sensitive enough in the discrimination between fertile individuals and infertile patients.

In particular, for F_2-IsoP levels the J index showed a value of 31.90 ng/mL not so far from the value of 29.96 ng/mL determined by Moretti et al. [15] in 147 patients with different reproductive conditions and 45 fertile controls. The cut off for MDA was 0.86 nmol/mL and was

comparable to the MDA values measured by HPLC in groups of fertile men (0.46 ± 0.04, [33]; 0.55 ± 0.05, [46]). Also, for IL-1β, the cut off found in this research (3.44 pg/mL) was compatible with values reported for fertile individuals (2.50 [0.96–3.39] pg/mL; [45]).

Finally, the immunolocalization studies revealed that spermatozoa from infertile individuals were rich in F_2-IsoPs, in particular those with cytoplasmic residues, according to the observation reported by Collodel et al. [36]. This finding is consistent with the fact that isoprostanes are formed in the fatty acids of plasma membrane by non-enzymatic reaction with ROS and then are released in the seminal plasma.

Increased levels of F_2-IsoPs represent an index of inflammation/OS, while low levels could be considered physiological and normally present in semen samples [15].

The limitations of this study regard the small sample size, and, with a perspective of clinical application, the fact that we applied sophisticated methods to determine both MDA and F_2-IsoPs to detect OS in semen samples of patients with different reproductive conditions.

In fact, both methods, in particular GC/NICI-MS/MS, are time-consuming and cost-effective, although reliable to detect OS. For these reasons, they are recommended for research studies because they are more accurate and more valuable however, they are not economical in terms of clinical application. It should be noted that it is also possible determine F_2_ IsoPs with other methods, for example by ELISA, an easier and cheap method.

As a whole, this study focuses on the fact that OS is deeply involved in male infertility and, paradoxically, its determination is not indicated in routine semen evaluation. We believe that the need of several easy tests to be applied at the workbench during spermiogram is crucial.

Because the higher stability of F_2-IsoPs than MDA, we propose to implement the research in this direction with the hope of the development of a device that allows OS determination during semen analysis.

In conclusion, although standard semen analysis remains the basis to evaluate semen samples, it is not able to accurately identify the etiology of infertility or predict the reproductive success.

For these reasons, more advanced tests are needed to accurately diagnose male infertility and predict pregnancy in couples trying to conceive naturally or couples undergoing assisted reproductive technologies [47,48].

Many techniques have allowed the study of sperm pathology and consequently helped in diagnoses and treatments. However, it is important to remember that the spermatozoon lives in an environment which is the seminal plasma. The evaluation of seminal components may be used to characterized sperm quality and pathological conditions related to male infertility [49].

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Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Ethics Committee of AGI Fertility Center.

Informed consent statement

Written informed consent has been obtained from the patients to publish this paper: “I consent to the use of biological waste material (semen samples not used for analysis or ART procedures) for research purposes that do not involve fertilization, having been informed that the collection of this material does not involve any compromise to the success of the cycle. The evaluation of certain biological properties of these samples does not, at present, indicate any useful information for our health or that of our embryos. Any data derived from this material will be treated anonymously as required by law”.

CRediT authorship contribution statement

Elena Moretti: Writing – review & editing, Writing – original draft, Investigation, Conceptualization. **Cinzia Signorini:** Writing – review & editing, Methodology, Investigation. **Silvia Menchiari:** Writing – review & editing, Data curation. **Laura Liguori:** Writing – review & editing, Methodology, Investigation. **Roberta Corsaro:** Writing – review & editing, Methodology, Investigation. **Laura Gambera:** Writing – review & editing, Methodology, Investigation. **Giulia Collodel:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

The data generated and analyzed during this study are included in this published article and are available from the corresponding author.

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