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Apelin is found in human sperm and testis and is raised in inflammatory pathological conditions

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ABSTRACT

Apelin/APJ receptor (R) is involved in many oxidative stress-induced pathological conditions. Since this system is not yet explored in male reproduction, we studied apelin/APJ-R in human semen and testis. Semen of 41 infertile patients with varicocele, genitourinary infections, unexplained infertility and 12 fertile men was analysed (WHO guidelines, 2021). Apelin was quantified by ELISA in seminal fluid and spermatozoa, interleukin (IL)-1β in seminal fluid. Apelin/APJ-R were immunolocalized in spermatozoa and testis. Apelin was present in spermatozoa and its levels were negatively correlated with normal sperm morphology% ($r = -0.857$; $p < 0.001$), and positively with IL-1β levels ($r = 0.455$; $p < 0.001$). Apelin and IL-1β concentrations were increased in patients' samples with varicocele (apelin $p < 0.01$; IL-1β $p < 0.05$) and infections (apelin $p < 0.01$; IL-1β $p < 0.001$). By logistic regression analysis, apelin (OR 1.310; $p = 0.011$) and IL-1β (OR 1.572; $p = 0.005$) were predictors of inflammatory diseases (varicocele, infections). Apelin and APJ-R immunofluorescence labels were weak in sperm tail of fertile men and intense along tail, cytoplasmic residues and post-acrosomal sheath of sperm from infertile men. In testis, apelin and APJ-R labels were evident in Leydig cells and weak inside the seminiferous tubule. Apelin/APJ-R system is present in human spermatozoa and testicular tissue and probably involved in human fertility.

1. Introduction

Alteration in adipokines, metabolic dysregulation, inflammation, and oxidative stress are known to be involved in male fertility [1–5].

Among many adipocytokines studied in the male reproductive system [1,2,4,5], the data on apelin are rather scarce.

Apelin [6], a regulatory peptide expressed in different organs, exerts its action via the receptor APJ (APJ-R; [7]), a typical G protein-coupled receptor that shows close sequence homology to the angiotensin II receptor type 1 [8].

Apelin/APJ-R system is highly represented in the human body particularly in vascular endothelial cells and adipose tissue, and also in stomach, brain, lung, heart, uterus, ovary and placenta, controlling many processes [9]. In addition, apelin/APJ-R can modulate gastrointestinal function and insulin sensitivity and promotes cell proliferation, migration and angiogenesis, thus regulating immune function [10].

Antushevich and Wójcik [8] revised the role of the apelin/APJ-R system in different diseases and concluded that apelin shows therapeutic abilities in different pathologies. They also reported the relationship between pro-inflammatory factors and apelin. For example, tumor necrosis factor alpha (TNF α) seems to upregulate apelin expression in human and mouse adipose tissue [11] and, at the same time, the anti-inflammatory effect of apelin, which inhibits pro-inflammatory cytokines release, was reported in rat hemorrhagic shock [12].

A very interesting cross talk between apelin/APJ-R and oxidative stress was also observed. Oxidative stress is a phenomenon due to an imbalance between the production and the accumulation of reactive oxygen species (ROS), a byproduct of cell metabolism, and the ability of the system to detoxify them, overwhelming the normal cell homeostasis

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and inducing cell damages. In 2016, Zhou et al. [10] revised the literature concerning the role of apelin/APJ-R in regulation of ROS during inflammation-related diseases. Several studies demonstrated that oxidative stress and inflammation can affect apelin expression, however others showed that apelin exerts antioxidant and anti-inflammatory effects. Even though ambiguities and doubts were not solved and apelin/APJ-R system can act as a double-edged sword in the regulation of inflammatory diseases based on oxidative stress, the close link between apelin, oxidative stress and inflammation was evident. Indeed, more recently, Wang et al. [13] observed that apelin/APJ-R system plays an essential role in the inhibition of inflammation and oxidative stress influencing pathways including NF-kb/JNK, ERK1/2, AMPK/GSK-3β/Nrf2 and others.

The role of apelin in the female hypothalamic- pituitary- gonadal axis was studied [4,14,15]. Indeed, apelin/APJ-R system is present in ovary of different species, as human, pig, bovine, rat and monkey, with different localization [14,16] and it seems to regulate granulosa cell proliferation, corpus luteum degradation processes, oocyte maturation and follicular atresia in different animal models [4].

As regards male reproductive system, apelin interplays with LH, the Leydig cell and testosterone [17–19]. Recently, apelinergic system was reported in etiopathogenesis of varicocele. In particular, testis tissue of rats with induced varicocele showed an increased expression of apelin and a decreased presence of APJ-R [20].

In the present research, apelin was quantified by ELISA in human semen and spermatozoa of infertile patients with varicocele and genitourinary infection, both pathologies sharing inflammation and oxidative stress background, with unexplained infertility, and in a group of fertile men. The obtained results were related to semen parameters and to the levels of interleukin-1β (IL-1β) dosed in seminal fluid of the same subjects. In addition, apelin and APJ-R were immunolocalized both in human spermatozoa and testicular tissue.

2. Methods

2.1. Patient's selection

Semen samples were obtained from 41 infertile patients (20- to 45-year-olds) who attended the Unit of Medically Assisted Reproduction, at Siena University Hospital for semen analysis. These infertile patients failed to achieve pregnancy after 12 months of regular and unprotected sexual intercourses; the factors affecting female infertility were excluded.

A complete personal medical history of patients was recorded. The patients fulfilled inclusion criteria: absence of azoospermia and systematic sperm defects, BMI < 25 kg/m^2, no history of metabolic syndrome, diabetes and, in general chronic illness. The study participants were not treated with radiotherapy, chemotherapy, medications, and dietary supplements. In addition, they did not use drugs, alcohol and were non-smokers. Patients with leukocytospermia were not included in the study. Hormone levels and semen bacteriological analyses were supplied by the patients. The levels of testosterone (T), luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were in the normal range. The patients with positive semen bacteriological analysis were asymptomatic. The possible presence of varicocele was detected by physical examinations and scrotal Eco-color Doppler. For this study, subclinical varicocele was not considered. The clinical diagnosis of infertility enabled grouping the 41 infertile patients as:

- varicocele (n = 17);
- genitourinary infections (n = 14);
- unexplained infertility (n = 10).

A group of 12 non-smoker fertile men (27–34 year-olds) without chronic illness (diabetes, metabolic disorders) and anatomical problems and/or infections represented the controls of the study. These men fathered a child in the last two years.

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee: ID CEAVSE 191113; Ethic Committee Siena University Hospital. All subjects were informed of the study, and they provided an informed written consent before the inclusion on this research. They accepted that their semen samples and the clinical data they supplied might be used for scientific purposes.

2.2. Semen analysis

Semen specimens were analysed after liquefaction for 30 min at 37 °C and macroscopic and microscopic characteristics were evaluated following WHO guidelines [21]. Sperm morphology was assessed using pre-stained Testsimplets slides (Waldeck GmbH & Co., Münster, Germania) and 200 spermatozoa per sample were scored. Eosin Y (CI 45380) staining was used to evaluate sperm vitality [21]; more than 300 spermatozoa per sample were examined by light microscope recording red stained cells (dead) and unstained cells (vital).

After the spermiogram, samples were centrifuged at 400 g for 15 min to separate the seminal plasma and the sperm cells and then, the aliquots were stored at −80 °C until use. Apelin was dosed in both seminal plasma and spermatozoa, IL-1β in seminal plasma.

2.3. Apelin determination

The assessment of apelin in human seminal plasma and spermatozoa was performed by sandwich quantitative enzyme-linked immunosorbent assay (Human Apelin ELISA Kit, Abbexa, Cambridge, UK). Spectrometric detection of color intensity at 450 nm enabled the determination of apelin amounts by comparing the optical density of each seminal plasma sample and each sperm cell sample to the standard curve (apelin standard amounts ranging from 125 to 8,000 pg/mL).

In all experiments, apelin measure was performed in duplicate in each sample. The results were expressed as pg/10^6 sperm in spermatozoa and in the semen fluid as pg/mL.

2.4. IL-1 β determination

In seminal plasma, the amount of IL-1β was determined by sandwich enzyme-linked immunosorbent assay (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA). Spectrometric detection of color intensity at 450 nm allowed to assess IL-1β amount. A curve-fitting software was applied to generate the standard curve ranging from 0 to 2,500 pg/mL and to quantify IL-1β in tested samples. All measures were performed in duplicate. The results were expressed as pg/mL.

2.5. Apelin and APJ-R immunolocalization in ejaculated spermatozoa and human testis tissue

Apelin and APJ-R were immunolocalized in spermatozoa of 10 samples from fertile subjects and 10 from infertile patients with inflammatory pathologies (varicocele, genitourinary infections). Semen samples were washed twice for 10 min in Phosphate Buffered Saline (PBS) and smeared on glass slides. The slides were air-dried, fixed in methanol for 20 min and acetone for 5 min at −20 °C, air-dried and rehydrated in PBS for 10 min at room temperature before the reaction [22].

The immunoreaction was also performed in testicular tissue obtained from three adult patients undergoing orchiectomy for testicular seminoma. Testis sections were supplied from the Department of Pathology AOU Siena, Siena, Italy. In detail, testicular specimens were cut into small blocks and treated with 10% buffered formalin at 4 °C for 24 h; they were then rinsed in water for 1 h. After fixation, the tissue was dehydrated in a series of ethanol (50%, 75%, 95%, and 100%) and cleared with xylene. Samples were treated with three infiltrations of

molten paraffin at 60 °C for 1 h and then solidified at room temperature. The blocks were sectioned using a Leica RM2125 RTS microtome (Leica Biosystem, Germany) obtaining 5 μm thick sections that were processed for immunofluorescence. The sections were deparaffinized with xylene, treated with a decreasing series of ethanol and distilled water to rehydrate the tissues. For antigen retrieval, sections were treated with Sodium Citrate Buffer (10 mM Sodium Citrate, 0.05% Tween 20, pH 6.0) for 20 min at 95 °C and washed twice in PBS for 10 min.

Smeared sperm were treated with PBS- bovine serum albumin (BSA) 1% normal goat serum (NGS) 5% for 20 min at room temperature and testis sections were incubated with PBS-BSA 5% for 30 min at room temperature. Then, smeared spermatozoa and testis sections were incubated overnight at 4 °C in a humid chamber with the primary antibodies: a rabbit anti-apelin antibody (Abcam, Cambridge, UK) diluted 1:500 and rabbit anti-APJ-R antibody (Thermo Fisher Scientific, Waltham, MA, USA) diluted 1:100. The antibodies were diluted in PBS-BSA 0.1% NGS 1% for spermatozoa and in PBS-BSA 1% for testis sections The reaction was revealed by an anti-rabbit antibody raised in goat Alexa-Fluor® 488 conjugate (Invitrogen, Thermo Fisher Scientific, Carlsbad, California, USA), diluted at 1:500; the incubation was performed at room temperature for 1 h. Specificity of binding was confirmed by negative staining using the diluent (for spermatozoa: PBS- BSA 0.1% NGS 1%; for testis sections: PBS- BSA 1%) omitting primary antibody. Nuclei were stained with 4,6-diamidino-2-phenylindole (DAPI) solution (Vysis, Downers Grove, IL). Slides were analysed with Leica DMI 6000 Fluorescence Microscope (Leica Microsystems, Germany), and the images were acquired by Leica AF6500 Integrated System for Imaging and Analysis (Leica Microsystems, Germany).

At least 300 spermatozoa were scored for each sperm sample and the different localization of apelin, and the intensity of the labelling was recorded. The localization of apelin in testicular tissue and APJ-R in both spermatozoa and testicular tissue were also shown.

2.6. 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 Spearman's Rank Correlation Coefficient (rho) was used to discover the relation existing between the variables investigated. Kruskal-Wallis test was used to compare the difference among considered groups and then Dunnet Post Hoc test or Tukey test were applied to determine which groups differed statistically from each other. Mann-Whitney *U* test was used to compare the differences between % of sperm with a strong apelin label measured in two groups (fertile men and infertile patients with inflammatory pathologies). Data were reported as median (interquartile range [IQR]). $P < 0.05$ was considered statistically significant.

Finally, a binary logistic regression stepwise analysis was performed to assess the odds ratios (ORs) and 95% confidence intervals of the levels of apelin and IL-1β for the risk of pathologies based on inflammatory conditions (varicocele and genitourinary infections) and for the risk of infertility (varicocele, genitourinary infections, unexplained infertility).

3. Results

3.1. Semen parameters, apelin and IL-1β concentration: Correlations between variables

Forty-one infertile patients were included in this study: 17 out of 41 were affected by II and III grade varicocele, 14 showed positive semen culture and were classified as genitourinary infections, 10 were diagnosed as unexplained infertility. Six out of 14 semen cultures were positive for *Enterococcus faecalis*, 6 for *Escherichia coli*, 1 for *Staphylococcus haemolyticus* and 1 for *Ureaplasma urealyticum*. Semen samples of 12 fertile men represented the control group. Table 1 reports the median and 25th/75th centiles of the considered variables in the total group of participants (No. 53 subjects). The median of the apelin level in spermatozoa was 6.91 pg/10^6 sperm. Apelin was completely absent in all analyzed semen fluids (the experiment was repeated 3 times) and was not included in tables and figures.

Table 1
Considered parameters in the studied population. Median (25th and 75th centile) of the considered variables in the group of 53 cases included in this study.

Variables	Median (25th-75th centile)
Volume/mL	3.4 (2.7–4.2)
Sperm/mL × 10^6	47.0 (20.0–72.0)
Rapid progressive motility %	19.0 (13.5–27.0)
Slow progressive motility %	11.0 (7.5–12.0)
Total progressive motility %	28.0 (22.0–42.0)
Normal morphology %	6.0 (4.0–10.5)
Vitality %	61.0 (56.0–68.0)
Apelin pg/10^6 sperm	6.91 (3.0–13.96)
IL-1β pg/mL	5.10 (2.35–8.01)

The correlations between the variables assessed in the total group of participants are reported in Table 2.

Regarding sperm parameters, sperm concentration, motility characteristics, normal morphology and vitality were positively correlated. This data is not surprising since in most cases the motility is positively correlated with vitality and with normal morphology; indeed, sperm with wide cytoplasmic residue and coiled tail are immotile and the damage of the membrane, representing a signal of necrosis, often induces acrosome reaction. All these defects are visible during morphology analysis at light microscopy level.

Apelin levels were negatively correlated with all sperm parameters ($p < 0.001$) and, in particular, with normal morphology % ($r = -0.857$; $p < 0.001$; Fig. 1A) and showed a positive correlation with IL-1β level ($p < 0.001$ Fig. 1B). Seminal IL-1β concentration was negatively correlated with sperm concentration ($p < 0.01$), rapid progressive motility ($p < 0.001$), total progressive motility ($p < 0.01$), normal morphology ($p < 0.001$) and vitality ($p < 0.05$).

3.2. Comparisons between variables in patients grouped according to pathologies and in fertile men

The patients were grouped according to pathologies as varicocele, genitourinary infections, and unexplained infertility and the variables were compared with a control group composed by fertile men (Table 3).

As expected, sperm parameters were significantly increased in the group of fertile men than in the groups of infertile patients (Table 3). In addition, the percentage of normal sperm morphology was significantly increased in samples of patients with unexplained infertility than those of patients with varicocele ($p < 0.01$) and genitourinary infections ($p < 0.05$).

Apelin levels were significantly lower in spermatozoa of fertile subjects (Fig. 2A) than those observed in spermatozoa of patients with varicocele ($p < 0.01$) and genitourinary infections ($p < 0.05$).

The levels of IL-1β were increased in the seminal fluid of both patients with varicocele ($p < 0.05$) and infection ($p < 0.001$) than those measured in the samples of fertile men (Fig. 2B). In addition, IL-1β concentration was significantly higher in seminal fluid of patients with infection than that observed in the specimens of patients with unexplained infertility ($p < 0.01$; Fig. 2B).

On the binary logistic regression analysis (Table 4), apelin (OR 1.310 95% CI 1.064–1.613; $p = 0.011$) and IL-1β (OR 1.572; 95% CI 1.147–2.156; $p = 0.005$) were both predictors of pathologies with inflammatory basis (varicocele and genitourinary infections). Furthermore, the binary logistic regression analysis showed that both apelin (OR 1.462; 95% CI 1.058–2.021; $p = 0.021$) and IL-1β (OR 1.429; 95% CI 1.016–2.009; $p = 0.040$) were predictor of infertility.

Table 2
Correlation between variables in the studied population. Correlations (rho Spearman's coefficient) between all considered variables in 53 cases included in the study. *p < 0.05; **p < 0.01; ***p < 0.001.

	Volume mL	Sperm/mL x 10^6	Rapid progressive motility %	Slow progressive motility %	Total progressive motility %	Normal morphology %	Vitality %	Apelin pg/10^6 sperm	IL-1β pg/mL
Volume mL	1								
Sperm/mL x 10^6	0.145	1							
Rapid progressive motility %	0.124	0.782***	1						
Slow progressive motility %	0.128	0.611***	0.667***	1					
Total progressive motility %	0.135	0.776***	0.963***	0.817***	1				
Normal morphology %	0.247	0.829***	0.870***	0.545***	0.823***	1			
Vitality %	0.210	0.745***	0.866***	0.757***	0.910***	0.771***	1		
Apelin pg/10^6 sperm	−0.282	−0.791***	−0.759***	−0.493***	−0.710***	−0.857***	−0.666***	1	
IL-1β pg/mL	−0.092	−0.401**	−0.489***	−0.244	−0.429**	−0.467***	−0.273*	0.455***	1

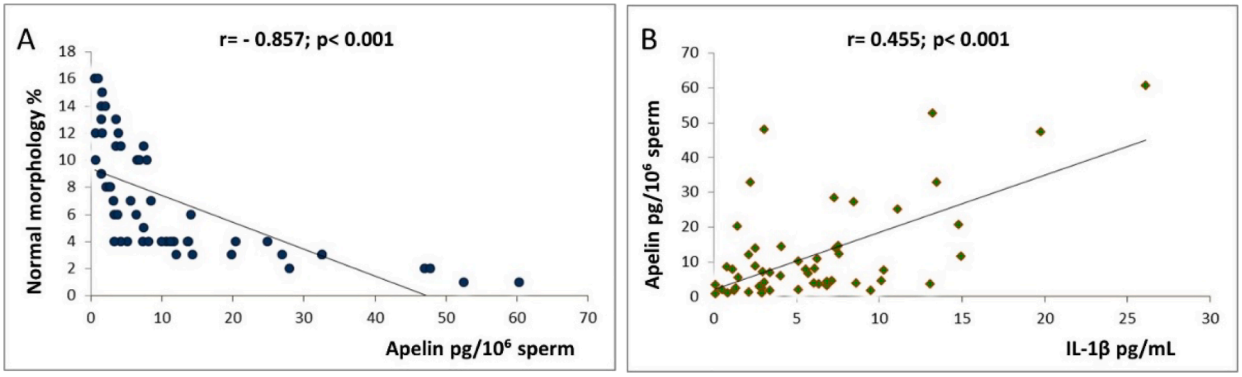

Fig. 1. Relationships between apelin, sperm morphology and IL-1β. Scatter plots showing the correlations (rho Spearman's coefficient) between apelin level measured in spermatozoa and the percentage of sperm with normal morphology (A) and between apelin and IL-1β (B) measured in seminal plasma of the 53 individuals considered in this study.

3.3. Immunolocalization of apelin and APJ-R in human spermatozoa and testis

Since apelin was found by ELISA in human spermatozoa, immunofluorescence was performed to study its localization. The experiments were carried out in human spermatozoa from samples of fertile men and infertile subjects (varicocele and genitourinary infections). It was immediately evident that the percentage of sperm with high intensity apelin label was significantly increased in sperm of infertile patients (median [IQR]: 74 [70–82]) than that observed in sperm of fertile men (median [IQR]: 25 [23–28], p < 0.001; Fig. 3).

Fig. 4 shows the apelin and APJ-R label in spermatozoa from fertile men and infertile patients. The apelin (Fig. 4A) and APJ-R (Fig. 4C) labels were weakly visible in the tail of sperm of fertile men, according with the low values observed with ELISA assay.

In spermatozoa of infertile men, the apelin (Fig. 4B) as well as the APJ-R (Fig. 4D) labels were intense and present along the tail and in the cytoplasmic residues.

Fig. 5 shows, at high magnification, the typical label found in spermatozoa from infertile patients. Apelin stain was present in the post-acrosomal sheath (Fig. 5A) and cytoplasmic residue (Fig. 5B), as well as the APJ-R label (Fig. 5C).

In the testis tissue, apelin (Fig. 6A & B) and APJ-R labels (Fig. 6C & D) were particularly evident in the Leydig cells and less intense inside the seminiferous tubule.

4. Discussion

The data presented in this study showed, for the first time, that apelin is absent in human seminal fluid but is present in sperm cells, especially in individuals with varicocele and genitourinary infections. In addition, apelin and APJ-R, were localized in human spermatozoa and testicular tissue, in particular in Leydig cells.

Adipocytokines as adiponectin, leptin, resistin and others regulate different pathophysiological processes, although their role in the reproductive system is still under study [23,24]. Recently, it was observed that omentin-1 semen levels were increased in inflammatory conditions as varicocele and genitourinary infections, suggesting a sort of protective role of this adipokine that shows antioxidant and anti-inflammatory properties in many other biological systems [5].

It is well known that cytokines are natural constituents of seminal plasma and are involved in male reproductive physiology. Nevertheless, in many pathological conditions their concentration increases and negatively influences sperm function [25]. As in other systems, in semen and spermatozoa, the inflammation, triggered by cytokines, is concomitant with oxidative stress. Indeed, these cytokines are responsible of the activation of the xanthine oxidase system that, in turn, increase ROS amount inducing oxidative stress [26].

Oxidative stress is one of the most important mechanisms involved in male infertility. Although normal physiological levels of ROS play a pivotal role in sperm function, high levels can have detrimental effect on

Table 3
Comparison between variables in the studied population grouped according to pathology. Median (IQR: 25th and 75th centile) of the considered variables in 41 subjects, grouped according to pathologies, and in 12 fertile men; statistics are also reported.

	Fertile men (F, No. 12)	Unexplained infertility (Ui, No. 10)	Varicocele (V, No. 17)	Genitourinary infections (Gi, No. 14)	Kruskall-Wallis	Post-hoc test
Volume mL	4.00 (2.55–6.60)	3.25 (2.45–4.05)	3.30 (2.80–4.00)	3.30 (2.78–4.25)	Ns	
[a]Sperm/mL x10^6	95.00 (53.25–145.00)	55.50 (37.75–67.63)	32.00 (17.50–52.00)	19.50 (9.00–59.50)	$p < 0.001$	F *vs* V $p < 0.01$ F *vs* Gi $p < 0.01$
[b]Rapid progressive motility %	33.00 (29.75–38.75)	20.00 (13.75–27.00)	16.00 (12.00–18.50)	15.50 (6.75–20.50)	$p < 0.001$	F *vs* V $p < 0.001$ F *vs* Gi $p < 0.001$ F *vs* Ui $p < 0.001$
[a]Slow progressive motility %	14.00 (11.00–24.75)	8.50 (7.25–11.50)	9.00 (7.00–12.00)	9.00 (6.00–10.25)	$p < 0.01$	F *vs* Gi $p < 0.01$
[b]Progressive motility %	47.50 (43.00–63.00)	30.50 (22.50–40.50)	23.00 (20.00–30.50)	25.00 (13.50–29.50	$p < 0.001$	F *vs* V $p < 0.001$ F *vs* Gi $p < 0.001$ F *vs* Ui $p < 0.001$
[b]Normal morphology %	12.50 (10.00–14.75)	7.50 (5.50–11.25)	4.00 (3.00–5.50)	4.00 (3.00–6.50)	$p < 0.001$	F *vs* V $p < 0.001$ F *vs* Gi $p < 0.001$ F *vs* Ui $p < 0.001$ V *vs* Ui $p < 0.01$ Gi *vs* Ui $p < 0.05$
[b]Vitality %	74.00 (68.50–75.00)	60.50 (55.00–65.50)	58.00 (54.00–63.00)	58.50 (55.75–61.25)	$p < 0.001$	F *vs* V $p < 0.001$ F *vs* Gi $p < 0.001$ F *vs* Ui $p < 0.001$
[a]Apelin pg/10^6 sperm	1.56 (0.78–5.45)	3.65 (3.08–6.23)	11.21 (7.48–21.11)	14.01 (4.23–28.34)	$p < 0.001$	F *vs* V $p < 0.01$ F *vs* Gi $p < 0.05$
[b]IL-1β pg/mL	2.50 (0.96–3.39)	2.98 (1.28–6.23)	5.56 (2.35–10.33)	8.98 (7.06–11.72)	$p < 0.001$	F *vs* V $p < 0.05$ F *vs* Gi $p < 0.001$ Gi *vs* Ui $p < 0.01$

Notes: [a]Post hoc Dunnett; [b]Post hoc Tukey.

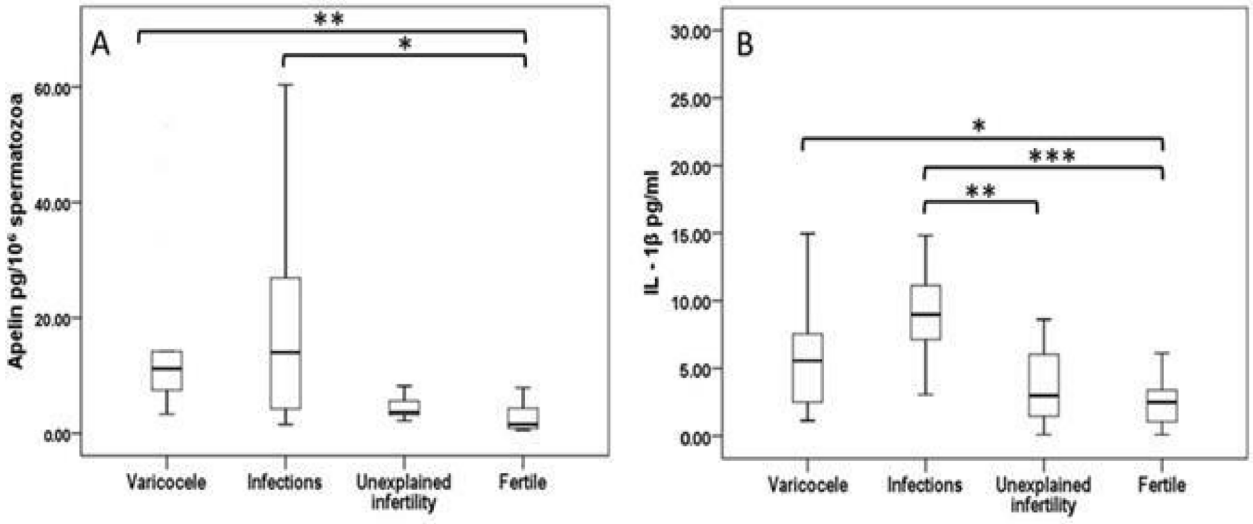

Fig. 2. Sperm apelin and semen IL-1β concentrations in different groups of considered cases. Median [IQR] of apelin concentrations measured in spermatozoa (A) and IL-1β levels dosed in seminal fluid (B) of semen samples from fertile men and infertile patients with varicocele, infections, and unexplained infertility; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Table 4
Binary logistic regression analysis using Apelin and IL-1β as predictors. Odd ratio (OR) and confidence interval (95% CI) of sperm apelin and semen IL-1β concentration as predictor of pathologies with inflammatory basis and infertility.

Predictors	Pathologies with inflammatory basis		Infertility	
	OR 95% CI	p value	OR 95% CI	p value
Apelin	1.31 (1.06–1.61)	0.011	1.46 (1.06–2.02)	0.021
IL-1β	1.57 (1.15–2.16)	0.005	1.43 (1.02–2.01)	0.040

sperm quality damaging DNA, lipids, and proteins [27]. At this purpose, it is well known that the semen of infertile patients shows high ROS levels, cytokine concentrations and other proinflammatory factors [28].

Varicocele is a pathology based on oxidative stress and increased levels of cytokines [29,30], in particular IL-1 β, that represents one of the markers of this condition and it is associated with reduced sperm motility [31,32]. Genitourinary infections, caused by pathological bacterial strains, can affect male fertility triggering oxidative stress, and the consequent inflammatory response causes an increase of cytokines [25,31].

Data on the localization and functions of apelin and its receptor APJ in reproductive system are still limited and mostly focused on female [9,33], while studies on male reproductive function are rather scarce.

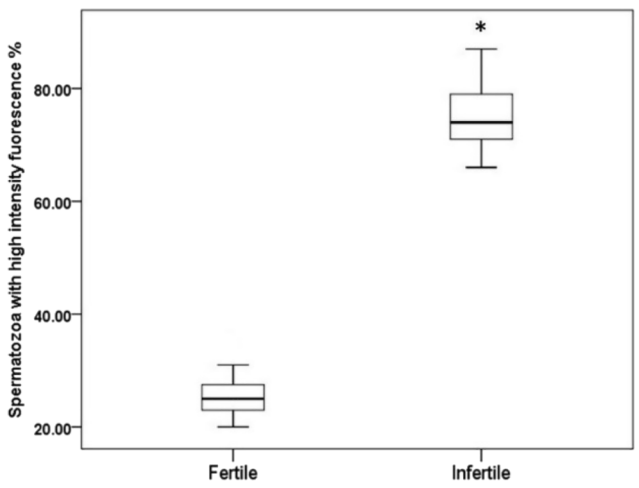

Fig. 3. Apelin localization in spermatozoa of fertile and infertile subjects. Median [IQR] of percentages of sperm with high intensity apelin fluorescent label in fertile men and infertile patients (varicocele and genitourinary infections); *p < 0.001.

Literature data have shown that apelin is expressed in the hypothalamus, pituitary gland, and testis tissue of rat suggesting that it may have autocrine/paracrine effects [17] on male reproductive system. Different studies demonstrated that apelin can inhibit gonadotropin secretion and can be involved in the regulation of steroidogenesis, cell survival, proliferation, and apoptosis in gonads [34,35]. Tekin et al. [36] observed in rats that the apelin, injected intraperitoneally, caused a significant decrease in serum testosterone and gonadotropins, suggesting a pivotal role of this adipokine in male reproduction. Despite these data, the presence, and putative functions of apelin in the human semen are not available in the literature; for this reason, the subject is original and worth to be investigated.

In this study, ELISA experiments demonstrated the presence of apelin in human spermatozoa and its absence in human seminal fluid. These results indicated that apelin is not secreted by the prostate and the seminal vesicles, but it is plausibly acquired in the testis during spermatogenesis and spermiogenesis. Apelin levels were negatively correlated with sperm parameters, in particular with normal sperm morphology, and positively with IL-1β levels. An up-regulation of apelin expression by TNF α was observed in human and mouse adipose tissue [11], indicating a cross talk between this adipokine and pro-inflammatory factors.

Then, we observed that apelin levels were significantly higher in spermatozoa of patients with varicocele and genitourinary infections than in those of fertile subjects and patients with unexplained infertility. IL-1β levels were also significantly increased in seminal fluid of patients with varicocele and genitourinary infections, this behavior is justifiable since both conditions have an inflammatory background [28,37,38].

The positive correlation between apelin and IL-1β suggested a role of apelin in inflammation of male reproductive system. However, it is unknown whether apelin plays a detrimental role or it exerts a protective effect in human sperm and its concentration increases to counterbalance the inflammation. It has been reported that proinflammatory factors can influence apelin expression and, vice versa, apelin can affect the expression of these factors [8]. Therefore, depending on studied models or considered pathologies, apelin showed a dual behavior. In several cases, apelin appeared to stimulate inflammatory cytokine expression. It increased the synthesis of IL-1β in human osteoarthritis synovial fibroblasts [39] and the concentration inflammatory factors after intracerebroventricular infusion in mice [40]. Chen et al. [41] observed that, in microglial BV2 cells, apelin activated the expression of TNF-α, IL-1β and other inflammatory pathways. Conversely, other studies indicated that apelin suppressed neuroinflammation in a rat model of Alzheimer's disease [42], it acted as an inhibitor of pro-inflammatory mediators, including IL-1β, in pancreatitis [43] and improved insulin resistance in severely burned rats, inhibiting the activation of NLRP3 inflammasome, and attenuating systemic inflammatory response [44].

We used a bivariate logistic regression model to assess the predictors of pathologies with inflammatory background such as varicocele and genitourinary infections and infertility in general; this model demonstrated that the both apelin and IL-1β may be a useful predictor of risk of these diseases. It is interesting to observe that apelin is a marker of

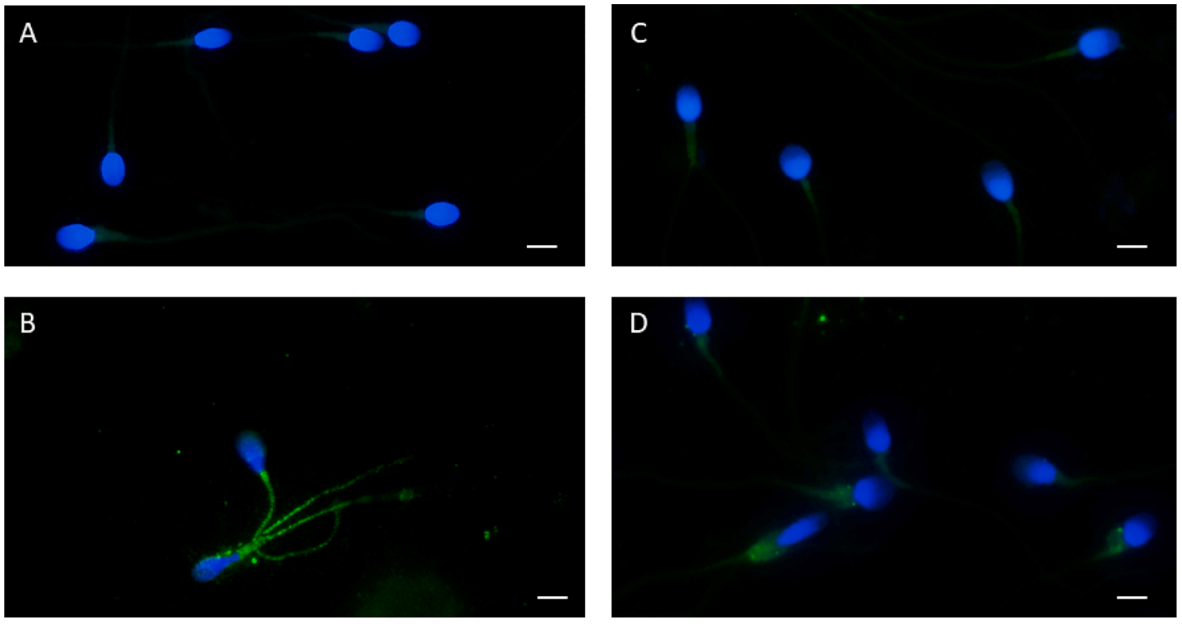

Fig. 4. UV micrographs of human sperm from fertile (A & C) subjects and patients with varicocele and genitourinary infections (B & D) labeled with apelin (A & B) and APJ-R (C & D) antibodies. In spermatozoa of fertile men, apelin (A) and APJ-R (C) signals were both weak and present along the tail. In spermatozoa of infertile men, an intense apelin (B) and APJ-R (D) signals were present along the sperm tail. In figure D also cytoplasmic residues were labeled. The nuclei were stained with DAPI. Bars: 5 μm.

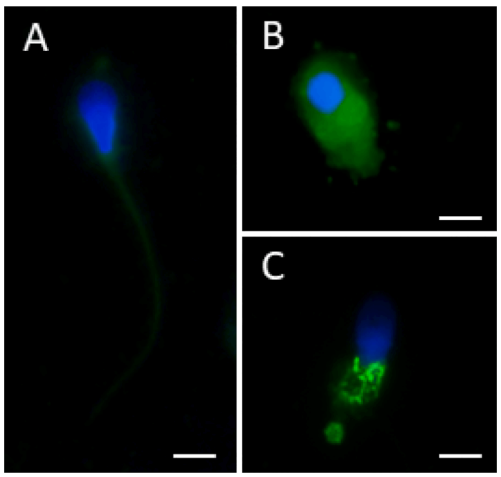

Fig. 5. UV micrographs of human sperm of patients with varicocele and genitourinary infections labeled with apelin (A & B) and APJ-R (C) antibodies. An intense apelin (A & B) and APJ-R (C) signals were present in the post-acrosomal sheath and in the cytoplasmic residue. The nuclei were stained with DAPI. Bars: 5 μm.

sperm cells and IL-1β of seminal plasma. In patients with unexplained infertility, the sperm parameters were reduced, however both apelin and IL-1β concentration were similar to those of control group. This observation can be justified considering that male infertility is a complex, multi-factorial disorder and unknown genetic, epigenetic, or environmental causes could be supposed [45].

According to the results obtained by ELISA experiments, the immunolocalization of apelin showed a more intense signal in the spermatozoa of infertile patients affected by pathologies sharing an inflammatory background, in particular along the tail and on the membranes of the cytoplasmic residues, compared to that observed in the sperm of fertile men, that obviously showed a better morphology. In addition, we demonstrated, for the first time in human sperm, the presence of APJ-R whose distribution followed that of the apelin, confirming the presence of apelin/APJ-R system in human spermatozoa.

Other evidence supported the preliminary results of the present research. Akkan et al. [20] observed, in the testicular tissue of rats with experimentally induced varicocele, an increased level of apelin immunostaining in Leydig cells, Sertoli cells, and in spermatogonia. The raised expression of apelin in testis of diabetic mice, in particular in Leydig and germ cells, was recently reported [19]. Our results in human testis tissue showed that apelin and APJ-R labels were mainly localized in the Leydig cells, and, less markedly, inside the tubules. This pattern of localization suggested a role of the apelin/APJ-R system in human spermatogenesis. According to this hypothesis, the interplay between apelin and the Leydig cells has also been demonstrated in rats infused with apelin that caused a decrease in serum testosterone levels and in the number of Leydig cells in testicular tissue [9]. It is possible to hypothesize that apelin/APJ-R system may regulate the functions of the Leydig cells and consequently the spermatogenetic process in normal testicular tissue.

Overall, hyperapelinemia in the spermatozoa, and probably in the testis, seems to be related to pathological conditions. This hypothesis is supported also by the fact that pharmacological inhibition of apelin receptor can improve testicular steroidogenesis [19]. In addition, a short-term exposure to flutamide (an anti-androgen that blocks androgen receptors) upregulates the expression of apelin by the Leydig cells, but down-regulates the expression of its receptor [18]. Considering the cross-talk between apelin, the Leydig cells and testosterone, it is interesting to cite a review by Hayden & Tanrikut [46] which reported that the presence of varicocele can negatively influence the production of testosterone by the Leydig cells and the varicocelectomy can resolve the negative effects on androgen production.

In conclusion, the preliminary results described in this paper indicate, for the first time, that apelin/APJ-R system is present in human spermatozoa and testicular tissue. In addition, the existence of an association between apelin, IL-1β and the pathologies such as varicocele and genitourinary infections was demonstrated. However, we are fully aware that a larger group of patients is needed to confirm the presented data and that the future research should include the testosterone level as a variable. Unfortunately, our patients were asked to provide testosterone levels that were measured in different laboratories and, for this

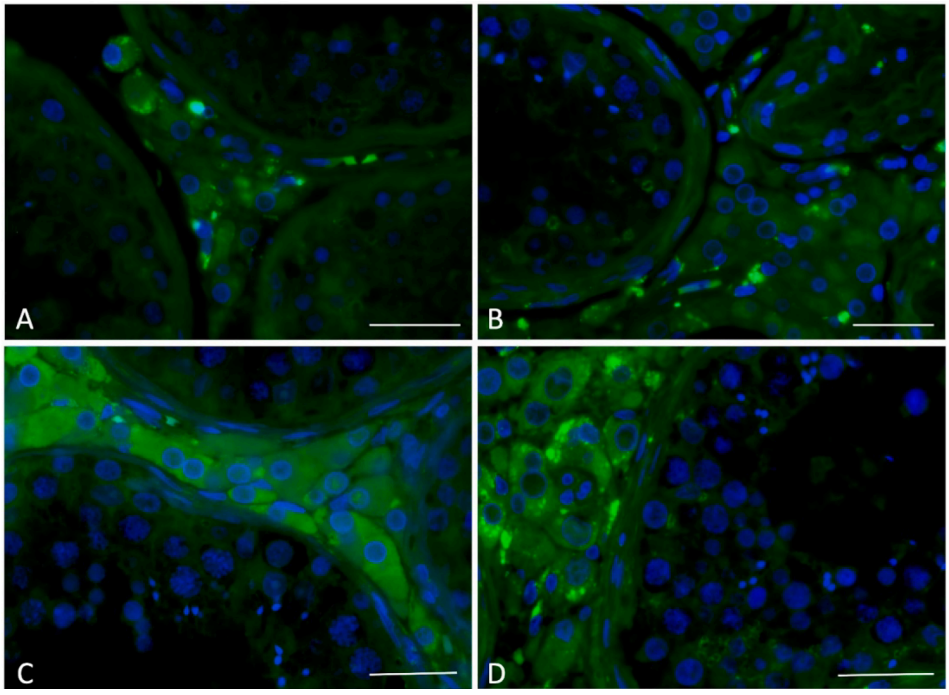

Fig. 6. UV micrographs of human testicular tissue treated with anti-apelin antibody (A & B) and anti-APJ-R antibody (C & D). The signal is evident in the Leydig cells and present also inside the tubule. Bars: A-D: 30 μm.

reason, not considered in the present analysis.

Since the studies on apelin and human spermatozoa are lacking, the present research could represent a basis for other investigations to better understand the role of this adipocytokine in the pathophysiology of male reproductive system.

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CRediT authorship contribution statement

Elena Moretti: Conceptualization, Investigation, Writing – original draft. **Cinzia Signorini:** Investigation, Methodology. **Roberta Corsaro:** Investigation, Methodology. **Daria Noto:** Investigation, Methodology. **Sergio AntonioTripod:** Investigation. **Andrea Menchiari:** Data curation. **Lucia Micheli:** Investigation, Methodology. **Rosetta Ponchia:** Methodology. **Giulia Collodel:** Conceptualization, Investigation.

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.

Data availability

Data will be made available on request.

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