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ARTICLE

Prevalence of human papilloma virus infection in patients with male accessory gland infection

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Abstract The frequency of human papillomavirus (HPV) infection in the semen of patients with male accessory gland infection (MAGI) was evaluated. One hundred infertile patients with MAGI were classified into group A: patients with an inflammatory MAGI ($n = 48$) and group B: patients with a microbial form ($n = 52$). Healthy age-matched fertile men (34.0 ± 4.0 years) made up the control group ($n = 20$). Amplification of HPV DNA was carried out by HPV-HS Bio nested polymerase chain reaction for the detection of HPV DNA sequences within the L1 ORF. Ten patients in group A (20.8%) and 15 patients in group B (28.8%) had a HPV infection; two controls (10.0%) had HPV infection. Patients with MAGI had a significantly higher frequency of HPV infection compared with controls; patients with a microbial MAGI had significantly higher frequency of HPV infection compared with patients with an inflammatory form (both $P < 0.05$). Patients with MAGI and HPV had a slight, but significantly lower sperm progressive motility and normal morphology compared with patients with MAGI HPV-negative ($P < 0.05$). Elevated frequency of HPV infection occurred in patients with MAGI, suggesting that HPV should be investigated in the diagnostic work-up of these patients.

KEYWORDS: HPV, MAGI, Prevalence

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Table 1 Prevalence of human papillomavirus infection in patients with probable inflammation of the sex accessory glands.

Author	*Diagnosis*	*Number of patients*	*Prevalence (%)*	*Other information*
Bezold *et al.*, 2007	Leukocytospermia	241	4.5	HPV detection in asymptomatic male infertility patients
Bartoletti *et al.*, 2014	Prostatitis-related symptoms	814	27.7	Most common HPV genotypes: 6, 11, 16, 26, 51, 53 and 81.
Cai *et al.*, 2014	Prostatitis-related symptoms	716	8.3	HPV and chlamydia trachomatis co-infection is associated with worsening of the sperm morphology
Iwasawa *et al.*, 1992	Chronic prostatitis	205	3.4	Apparent spontaneous eradication of HPV-DNA during follow up
Xiao *et al.*, 2013	Chronic prostatitis	14	12.6	HPV detection on expressed prostatic secretions
Svec *et al.*, 2003	Epididymal samples and non-tuberculous epididymitis	22	31.0	Low-risk (6) high-risk HPV (16, 33, 35, 55, and 73)

HPV = Human papillomavirus.

Introduction

Male accessory gland infection (MAGI) results from the canalicular spreading of microorganisms via urethra, prostate gland, seminal vesicles, deferent duct, epididymis and testis. Characteristic signs of MAGI are leukocytospermia, enhanced concentration of cytokines and reactive oxygen species (Krause, 2008). The following criteria are considered to be of diagnostic value:

(a) history of urogenital infection, abnormal digital rectal exploration, or both; (b) significant alterations in the expressed prostatic fluid, urinary sediment after prostatic massage, or both;

(c1) 7135uniform growth of more than 10^3 pathogenic bacteria, or more than 10^4 non-pathogenic bacteria per ml, in culture of diluted seminal plasma; (c2) presence of more than 10^6 (peroxidase positive) leukocytes per ml of ejaculate; (c3) signs of altered secretory function of the prostate gland or seminal vesicles. The diagnosis of MAGI is made when one of the following combination occurs: a + b, a + c (1, 2 or 3), b + c (1, 2 or 3), c1 + c2, c1 + c3 or c2 + c3 (Comhaire et al., 1980; WHO, 1993).

Sperm parameters may be altered by MAGI through different mechanisms, in particular by increasing the oxidative stress and cytokine production in the seminal plasma; causing possible obstruction or anatomical sub-obstruction of the proximal and distal spermatic tract as a result of their chronicity; and altering sperm function caused by the direct effect of the germs which cause inflammation (La Vignera et al., 2011).

The frequency of MAGI among infertile men varies widely, ranging from 2–18%. This mainly relates to the diagnostic strategies used (La Vignera et al., 2011), which often take into account only the number of leukocyte in the ejaculate (leukocytospermia). The frequency, however, would increase if the number of leukocytes in the expressed prostatic secretion after prostatic massage is taken into account (Krieger et al., 2008; La Vignera et al., 2014; Xiao et al., 2013). In addition, MAGI are often asymptomatic or paucisymptomatic, hence the diagnosis is made only in a specialized medical setting and the administration of specific questionnaire is of invaluable help (La Vignera et al., 2012a).

From a microbiological point of view, MAGI is classified as microbial and inflammatory (La Vignera et al., 2014). Several kinds of microorganisms can be found in the male urogenital tract, which are associated with sperm parameter abnormalities, especially motility and mitochondrial sperm function, and chromatin and DNA integrity: *Escherichia coli*, *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Ureaplasma urealyticum*, *Mycoplasma hominis* and other mycoplasmas, *Candida albicans*, *Trichomonas vaginalis*. Evaluation of a viral cause, and among this of the human papilloma virus (HPV), is not recommended in clinical practice (La Vignera et al., 2011).

Treatment of MAGI is usually targeted at relieving symptoms. The aims of treatment are reduction or eradication of microorganisms in prostatic secretions and semen, normalization of inflammatory (e.g., leukocytes) and secretory parameters and improvement of sperm parameters. Treatment includes antibiotics, anti-inflammatory drugs, surgical procedures, normalization of urine flow, physical therapy, and alterations in general and sexual behaviour. The use of alpha-blockers for symptom relief is controversial. Although antibiotics might improve sperm quality, there is no evidence that treatment of chronic prostatitis increases the probability of conception (Nickel and Shoskes, 2010).

Recent studies suggest that the HPV-DNA may be found in the semen of 2–31% of the general male population and in the 10–35% of men who undergo assisted fertilization for infertility (Foresta et al., 2014; Schillaci et al., 2013). A Medline search carried out using the following keywords 'MAGI and HPV', 'prostatitis and HPV', 'epididymitis and HPV', 'vesiculitis and HPV' resulted in a limited number of reports (Bartoletti et al., 2014; Bezold et al., 2007; Cai et al., 2014; Iwasawa et al., 1992; Svec et al., 2003; Xiao et al., 2013) (**Table 1**). Therefore, this study was undertaken to evaluate the frequency of HPV infection in the semen of patients with MAGI.

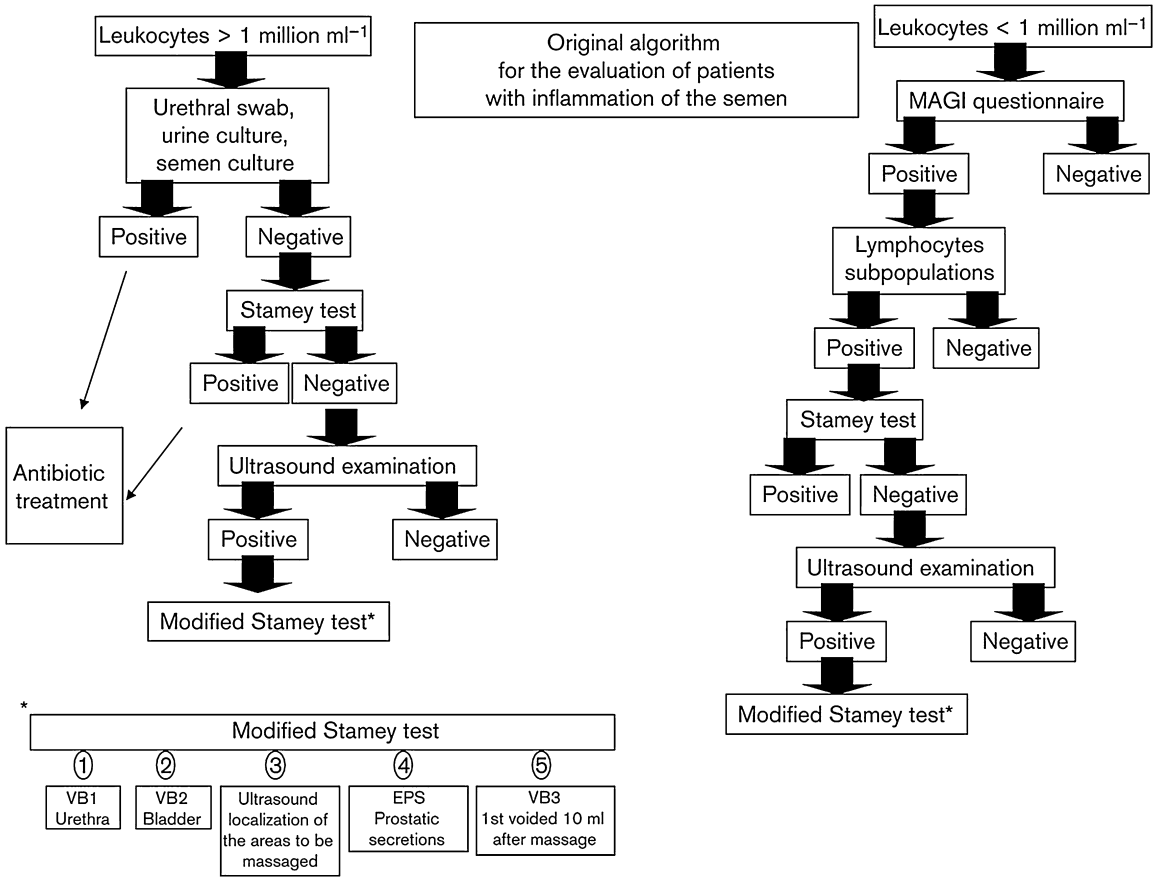

Figure 1 Diagnostic algorithm for the clinical evaluation of patients with male accessory gland infection (La Vignera et al., 2014). EPS = expressed prostatic secretions; MAGI = male accessory gland infection; VB = voided bladder.

Materials and methods

Patients

One hundred (consecutively enrolled) infertile patients with MAGI (WHO, 1993 criteria) (mean age 32.0 ± 6.0 years; body mass index 23.0 ± 4.0) were enrolled in the study and classified into two groups. Group A: patients with an inflammatory form of MAGI (n = 48) and group B: patients with a microbial form (n = 52). Healthy age-matched fertile men (mean age 34.0 ± 4.0 years; body mass index 24.5 ± 5.0) made up the control group (n = 20).

All patients underwent the diagnostic algorithm showed in **Figure 1** (La Vignera et al., 2014) and the following microbiological tests: urethral swab, semen culture, urine culture, leukocytes and lymphocytes subpopulations in the semen, Meares–Stamey Test, for the evaluation of the following possible infections: gram positive and gram negative microorganisms, *E. coli*, *N. gonorrhoeae*, C. *trachomatis*, *U. urealyticum*, *M. hominis* and other mycoplasmas, *C. albicans*, *T. vaginalis*. In addition, each patient and control completed a questionnaire for MAGI to evaluate the presence of symptoms. This questionnaire (SI-MAGI: structured interview on MAGI) is divided into four domains relative to urinary tract symptoms, ejaculatory pain or discomfort, sexual dysfunction and quality-of-life impact (La Vignera et al., 2012b).

This was a prospective study conducted between September 2013 and May 2014 at the Andrology Division of the University of Catania.

Identification of human papillomavirus infection

The semen samples were divided into two aliquots and kept at −20°C until use. The automated DNA extraction was carried out with 1 ml sample on the NucliSenseasyMAG system (bioMérieux SA, Marcy l'Etoile, France) following the manufacturer's HPV 1.1 protocol, with a 55 μl final elution volume. Patients with ejaculate volume lower that 1 ml were asked to provide two semen samples.

Amplification of HPV DNA was accomplished by HPV-HS Bio (AB Analiticas.r.l, Padova, Italy) nested polymerase chain reaction (PCR) for the detection of HPV-DNA sequences within the L1 ORF, according to the manufacturer's recommendations. To verify the efficiency of the DNA extraction, the housekeeping gene Thiosulphate SulphurTransferase (TST) was also amplified. Samples negative for TST were considered inadequate and a new sample was requested.

For the first-amplification step, carried out with 10 μl of eluate, a combination of degenerate primers was used to amplify a 449–458 bp sequence within the L1 ORF of the HPV genome. The second amplification was carried out from 1 μl

Table 2 Sperm parameters of the enrolled patients.

Parameter	*MAGI/HPV− (n = 75)*	*MAGI/HPV+ (n = 25)*	*Controls/HPV− (n = 18)*
Concentration (mil/ml)	10.0 ± 8.0[a]	8.0 ± 6.0[a]	45.0 ± 16.0
Total number (mil/ejaculate)	15.0 ± 4.8[a]	10.4 ± 4.8[a]	99.0 ± 17.6
Progressive motility (%)	16.0 ± 6.0[a]	10.0 ± 3.0[a,b]	35.0 ± 8.0
Normal forms (%)	10.0 ± 4.0[a]	8.0 ± 3.0[a]	14.0 ± 4.0
Leukocytes (mil/ml)	1.2 ± 0.5[a]	2.2 ± 0.8[a,b]	0.4 ± 0.2
Volume (ml)	1.5 ± 0.6[a]	1.3 ± 0.8[a]	2.2 ± 1.1
pH	7.8 ± 0.6	8.0 ± 0.3	7.4 ± 0.4
Liquefaction time (min)	25.0 ± 8.0	28.0 ± 6.0	22.0 ± 5.0
Immature germ cells (%)	3.0 ± 1.0	4.0 ± 2.0	2.0 ± 1.0
Viable spermatozoa (%)	60.0 ± 4.0 [a]	58.0 ± 6.0[a]	76.0 ± 5.0

HPV = human papilloma virus; MAGI = male accessory gland infection.
[a]$P < 0.05$ versus controls HPV−.
[b]$P < 0.05$ versus MAGI/HPV−.

of the first amplification product, using biotinylated primers to amplify a 139–145 bp sequence. To verify the efficiency of the DNA extraction, 10 μl of eluate were used to amplify a 202 bp fragment of the TST gene using specific primers. Negative (water) and positive controls (plasmid clones containing HPV 54) were included for each PCR run, to check for possible contamination and accuracy. To confirm amplification, PCR products were submitted to electrophoresis in 3% agarose gel, and the positive ones were used for the hybridization step. Samples negative for TST were considered inadequate and extracted again from the second tube.

For all the positive samples with a reverse line blot hybridization assay, HPV typing was carried out with specific probes for the most frequent HPV-types (HPV-type, AB Analitica s.r.l., Padova, Italy). HPV-type allows the identification of 11 LR-genotypes (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81) and 18 HR-genotypes (16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 73, 82). Samples that were positive by nested-PCR but negative in reverse line blot for any of these types were considered as undetermined HPV.

Leukocytes flow cytometric analysis (lymphocytes subpopulations)

To carry out the absolute leukocyte count, 100 μl of each liquefied semen sample was incubated with a mixture containing Syto-16 green fluorescent nucleic acid stain to identify the spermatozoa and exclude debris (final concentration 200 nM, Molecular Probes, Eugene, Oregon, USA), 7-Amino-Actinomycin D (7-AAD Via-Probe, BD Pharmingen, San Diego, CA, USA) to assess viability, anti-CD45-APC (pan-leukocyte antigen) to recognize white blood cells, and anti-CD16-PE for PMN recognition. The addition of 100 μl of Flow-CountTM Fluorospheres (Beckmann-Coulter, Fullerton, CA, USA) at a 1034 beads/ml allowed the determination of the absolute leukocyte count by flow cytometry. After incubation in the dark for 20 min at room temperature, 1 ml phosphate buffered saline was added, and the sample was analysed by flow cytometry (EPICS XL Flow Cytometer – Coulter Electronics, IL, Italy). For each test, 100,000 events were acquired.

The study was approved by the Internal Institutional Board (protocol approved June 3 2013 number 707), and all examined patients signed informed consent to the processing of personal data.

Statistical analysis

The results are reported as mean ± SEM and percentages. Data were analysed using chi-squared and one-way analysis of variance (ANOVA) followed by Duncan's multiple range test, as appropriate. The Statistical Package for Social Sciences (SPSS) version 9.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. $P < 0.05$ was accepted as statistically significant.

Results

Group A: 10 patients out of 48 (20.8%) had HPV infection. They had the genotype 6 ($n = 3$); the genotype 56 ($n = 3$); the genotypes 16, 18 and 66 ($n = 3$); the genotypes 16 and 18 ($n = 1$). Group B: 15 patients out of 52 (28.8%) had HPV infection. The genotype 6 was found in six of them; the genotype 56 in five; the genotypes 16, 18 and 66 in three; and the genotypes 16 and 18 in one. Among controls, two men out of 20 (10.0%) had HPV infection (one with genotype 3; one with genotype 6).

Overall, patients with MAGI had a significantly higher frequency of HPV infection compared with controls (25% versus 10%; $P < 0.001$, chi-squared test). Patients with microbial MAGI had a significantly higher frequency of HPV infection compared with patients with an inflammatory form (28.8% versus 20.8%, $P < 0.05$, chi-squared). Patients with microbial MAGI and HPV infection ($n = 15$) had the following bacterial infections: *C. trachomatis:* 6/15 (40.0%); *U. urealyticum:* 5/15 (33.3%); *Escherichia coli*: 4/15 (26.7%).

Patients with MAGI and HPV infection had slight, but significantly lower percentages of spermatozoa with progressive motility, normal morphology, viable spermatozoa, and a higher concentration of leukocytes in the semen compared with MAGI HPV-negative patients ($P < 0.05$, ANOVA followed by Duncan test) (**Table 2**).

Table 3 Severity of symptoms of the examined groups.

Parameter	*MAGI/HPV⁻ (n = 75)*	*MAGI/HPV+ (n = 25)*	*Controls/HPV⁻ (n = 18)*
Urinary disorders	12.0 ± 3.0	13.0 ± 4.0	3.0 ± 2.0[a]
Spontaneous and/or ejaculatory pain and/or discomfort	15.0 ± 4.0	17.0 ± 2.0	5.0 ± 3.0[a]
Sexual disorders	8.0 ± 2.0	9.0 ± 3.0	4.0 ± 2.0[a]
Quality of life	10.0 ± 2.0	10.0 ± 3.0	3.0 ± 1.0[a]

HPV = human papilloma virus; MAGI = male accessory gland infection.
[a] $P < 0.05$ versus MAGI/HPV+ and MAGI/HPV−.

The severity of urinary symptoms and of the sexual and ejaculatory disorders was similar among MAGI patients with or without HPV infection, but significantly higher than in controls ($P < 0.05$, ANOVA followed by Duncan test) (**Table 3**).

Discussion

The results of this study showed that the prevalence of HPV infection in the semen of patients with MAGI is significantly higher compared with that of healthy age-matched fertile men. In particular, among patients with MAGI, the microbial forms are associated with HPV infection with a significantly higher frequency than the inflammatory forms. This suggests that a significant percentage of inflammatory MAGI may have a viral cause. We found that patients with MAGI and HPV infection had a significantly lower percentage of spermatozoa with progressive motility and normal morphology and a higher concentration of leukocytes in the semen compared with patients with MAGI but without HPV infection. Finally, the severity of symptoms does not help to discriminate between patients who have MAGI with or without HPV infection.

To our knowledge, this is the first study reporting the prevalence of HPV infection in patients with MAGI. Iwasawa et al. (1992) showed a prevalence of 3.4% in patients with chronic prostatitis without any macroscopic abnormality. The few studies published did not evaluate patients with MAGI. Bezold et al. (2007) analysed infertile men with and without leukocytospermia, showing that the prevalence of urogenital infections and in particular HPV may not be different between the two groups. Bartoletti *et al.* (2014) showed that the prevalence of HPV infection among patients with prostatitis is unrelated to the severity of symptoms. Cai *et al.* (2014) showed a high frequency (28%) of co-infection with *C. trachomatis* in patients with signs and symptoms suggestive of prostatitis. Xiao et al. (2013) found HPV infection in the secretion obtained after prostatic massage in patients with chronic prostatitis. Finally, from epididymal samples obtained from 17 patients and epididymal and vas deferens samples from five patients surgically treated for nontuberculous epididymitis, the documented frequency of HPV infection was about 31% (Svec et al., 2003).

The reported prevalence rate has not been evaluated in patients with MAGI, but only in patients with leukocytospermia or prostatitis (**Table 1**). The latter represents a non-complicated form of MAGI with the lowest effect on sperm parameters compared with the complicated forms of MAGI (prostato–vesciculitis and prostato–vesiculo–epididymitis), which involve more than one accessory gland and have a greater negative effect on male fertility (La Vignera et al., 2011).

On this basis, the risk is to under-diagnose MAGI, a clinical condition associated with HPV infection, another medical condition strongly underestimated, as shown in several studies (Bezold et al., 2007; Cai et al., 2014; Iwasawa et al., 1992). The HPV infection has been shown to present with few or atypical symptoms (Bartoletti et al., 2014) and a chronic course that can affect the quality of sperm parameters (Foresta et al., 2010; Lai et al., 1997) or to be present in sperm banks of assisted reproductive technique centres (Foresta et al., 2011a, 2011b; Garolla et al., 2012). In more details, HPV has been reported to localize at the equatorial region of the sperm head through an interaction between the HPV capsid protein L1 and syndecan-1 (single trans-membrane domain proteins that are thought to act as co-receptors). Sperm transfected with HPV genes and sperm exposed to capsid protein are capable to penetrate the oocyte and transfer the virus into oocytes, in which viral genes are then activated and transcribed (Foresta et al., 2011c).

Another aspect of the present study that deserves further consideration is the high frequency of HPV and *C.trachomatis* co-infection, which is a major infections found among unselected infertile couples (Salmeri et al., 2010).

The results of the present study are in agreement with those reported by Yang et al. (2013) on 142 HPV-positive men. The more relevant aspect of this study is represented by the lower percentage of morphologically normal spermatozoa in patients with HPV infection. More recently, Cai *et al.* (2014) reported that, in patients with HPV co-infection, sperm alteration is not only limited to motility, but involves a decrease of the percentage of spermatozoa with normal forms. This aspect is of interest since leads to hypothesize the presence of the co-infection in the subset of patients with sperm quality more compromised (Cai et al., 2014).

Another important aspect concerns the association between HPV infection and asthenozoospermia, which dates back to 1997 (Lai et al., 1997). This clinical study showed that the frequency of asthenozoospermia in men with HPV infection was significantly higher than in men without infection (75% versus 8%). More recently, a higher prevalence (25%) of asthenozoospermia was confirmed in patients with HPV infection in the semen (Foresta et al., 2010). In contrast, Rintala et al. (2004) found that no sperm parameter was alterated

in patients with HPV infection. This study was conducted on 65 fertile volunteers who completed a questionnaire to assess the presence of risk factors associated with HPV infection. Among these patients, 17% had positive clinical history for genital condylomas, 12% had chlamydia infection, 2% had gonorrhoea and 3% had genital herpes. None of the patients enrolled in the study was evaluated using the diagnostic criteria of MAGI (Comhaire et al., 1980; WHO, 1993). Frequently, MAGI are associated with asthenozoospermia (La Vignera et al., 2011); therefore, low sperm motility, even if isolated, should lead to a careful search for the presence of MAGI and HPV infection. The different type of patients (with MAGI) enrolled represents the main difference between this study and previous publications. The absence of immediate therapeutic implications for patients after diagnosis of HPV infection and the lack of uniformity in the method to detection the virus, however, are two of the major limitations of the studies on this topic.

The HPV infection is the most common infection among young, sexually active individuals. Indeed, it is estimated that about 75–80% of sexually active individuals will become infected in their lifetime (Weaver, 2006). Another important aspect of the clinical management of HPV infection in men compared with women concerns the possible differences in the following parameters: viral load, duration of natural infection, absence of symptoms, lower antibody response and lower incidence (Partridge and Koutsky, 2006). In the specific case of patients with MAGI, it should also be taken into account that this clinical condition associated to sperm parameter alteration is aggravated by a further reduction of sperm motility and normal morphology in the presence of HPV infection compared with patients with MAGI but without HPV infection. This aspect has a relevant importance for the long-term clinical management of these patients. Finally, further studies with a larger control group are needed to confirm the results of the present study. Other important elements, however, need to be considered, which suggest that HPV infection screening may be useful in patients with MAGI. These include risk of not identifying genotypes associated with cancer threat; risk of inadequate diagnosis of inflammatory form of MAGI; and risk of inadequate counselling of the female partners for sexual transmission of the virus.

In conclusion, this study showed an elevated frequency of HPV infection in patients with MAGI, particularly in those with a bacterial form of MAGI. This was associated with a lower sperm motility and normal morphology and an increased concentration of leukocytes in the semen.

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