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Semen inflammatory markers and *Chlamydia trachomatis* infection in male partners of infertile couples

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Summary

Previous studies have given conflicting results about the effect of generally infection and *Chlamydia trachomatis* on seminal ILs and semen parameters. The aim of this study was to investigate the relationship between semen quality and the level of seminal interleukins (ILs) in infertile couples with *C. trachomatis*. Blood, first void urine (FVU) and semen were obtained from 250 infertile men who had failed to conceive after 12 months of trying. Serological analysis for specific IgA, IgM and IgG antibodies to *C. trachomatis* in serum, the presence of *C. trachomatis* in FVU and semen sample and semen analysis were carried out. The main results are as follows: (i) elevated IL-6 and IL-8 are observed in *C. trachomatis*-positive men, but this is not significant and it varies by diagnostic method; and (ii) IL-6 and IL-8 levels were correlated with each other and the concentration of leucocytes, but IL-8 was correlated with semen volume and patient's age. This study showed that men with such an infection in FVU samples (PCR positive) had only lower semen volume compared with men without infection.

Introduction

Chlamydia trachomatis is the commonest sexually transmitted infection of the male reproductive tract (La Vignera *et al.*, 2014) with significant consequences for male reproductive function. *Chlamydia trachomatis* infection has been implicated as a potential risk factor in the development of obstructive azoospermia (Eggert-Kruse *et al.*, 2003) but could also affect male fertility by directly damaging spermatogenesis (Sobinoff *et al.*, 2014) or by negatively influencing the function of mature spermatozoon (Pacey and Eley, 2004). In addition to these mechanisms, several studies have highlighted the potential importance of inflammatory cytokines produced by macrophages in response to chronic inflammation and foreign antigens and it has been suggested that these may have their own independent effects on male fertility.

During male accessory gland infection, macrophages are attracted to the site of infection because of existence of pathogens and tissue damage. Elevated IL-6 and IL-8 levels have been observed in persistent and chronic *C. trachomatis* infection (Mpiga *et al.*, 2006), and these cytokines play a key role in both innate and acquired immune responses that control inflammation (Henderson & Wilson, 1996). Currently, there is controversy whether certain cytokines such as IL-6 and IL-8 produced in response to a *C. trachomatis* infection are related to semen quality (Eggert-Kruse *et al.*, 2001; Kopa *et al.*, 2005). Whilst some studies have suggested that there is a relationship (Furuya *et al.*, 2003; Kopa *et al.*, 2005), others have suggested that there is not (Comhaire, 1994; Matalliotakis *et al.*, 2002).

Our interest in this area was recently renewed by the observation that the secretion of IL-6 and IL-8 was greater when immortalised cell lines from the prostate were incubated with *C. trachomatis* in comparison with incubations with cells lines from the urethra (Al-Mously & Eley, 2007). This raised the possibility that the measurement of IL-6 and IL-8 may be useful markers of upper genital tract infections, such as prostatitis. Subsequently, a further study from our group showed that seminal levels of IL-8 but not IL-6 were significantly elevated in men with a *C. trachomatis* infection compared to men without an infection (Kokab *et al.*, 2010). Moreover, whilst elevated IL-8 levels were associated with an increased semen volume, they were not associated with

any other measures of semen quality. However, whilst this study used two confirmatory tests of *C. trachomatis* infection in semen and urine, it did not consider the serological status of the male, and this limits our interpretation of whether seminal cytokine levels are related to current or previous *C. trachomatis* infection.

Therefore, this study was designed to investigate *C. trachomatis* infection by a more comprehensive array of diagnostic tests, including PCR of semen and first void urine (FVU) as well as serology to detect IgA, IgM and IgG antibodies to *C. trachomatis*. These data were then related to the level of IL-6 and IL-8 found in semen, and the measures of semen quality determined by semen analysis.

Materials and methods

Patient recruitment and sample collection

The male partners of infertile couples ($n = 250$) with primary or secondary infertility attending the Research and Clinical Centre for Infertility (Yazd, Iran) for diagnostic semen analysis between September 2009 and October 2010 were recruited to study. There were no age restrictions for inclusion in the study, although individuals with abnormal karyotype or a history of: (i) previous chemotherapy or radiotherapy treatment; (ii) vasectomy; or (iii) low semen volume (<1.0 ml); (iv) retrograde ejaculation; or (v) hypogonadotropic hypogonadism were excluded. Informed consent was obtained from each participant prior to inclusion in the study, and participants produced semen sample (as a part of infertility work up) as well as a 2-ml blood sample (1.5-ml serum) and a 20–40 ml urine sample. The main study was a prospective observational study that analysis was performed on previously collected samples. The Ministry of Health Research Ethics Committee (Iran) and the University of Sheffield School of Medicine Research Ethics Committee approved all recruitment procedures and the collection and processing of biological samples.

Sample processing

Men produced their ejaculates after at least 48 h sexual abstinence, and semen analysis was performed according to WHO (1999) guidelines. Analysis has been performed on one sample provided by infertile men.

Briefly, after 60 min liquefaction of semen at 37 °C, the microscopic and macroscopic examinations were performed. All measures of semen quality being completed within 1 h, apart from sperm morphology, which was completed later after slides, had been stained.

Macroscopic examination of semen included appearance, volume, viscosity and pH of the semen sample. Semen pH was measured by pH paper, and semen volume was measured by reading from the base of the meniscus in a graduated tube (BD Biosciences, Bedford, MA, USA) into which the sample had been decanted. Microscopic investigation included concentration, motility and morphology of spermatozoa. For the assessment of sperm concentration, the haemocytometer (Hawksley, London, UK) method was used. Sperm motility was assessed at ×400 magnification on a light microscope (Carl Ziessaxiostor plus, Kirchdorf, Germany) and classifying 200 spermatozoa as either progressively motile (grades a + b) nonprogressive (grade c) or immotile (grade d).

The presence of leucocytes in semen was determined within 1 h by staining an aliquot of semen using the peroxidase test as recommended by WHO (1999). Peroxidase-positive cells (leucocytes) that were brown and round in shape were counted with a haemocytometer.

For each patient, sperm morphology slides were prepared according to the protocol described in WHO (1999) and were stained with Papanicolaou. The stained slides were then assessed in Sheffield using the Hobson Sperm Tracker (Hobson Tracker Limited, Sheffield, UK) as described in Pacey *et al.* (2014).

Each participant also provided a sample of urine which was stored in a refrigerator for up to 2 days prior to performing DNA extraction.

In addition to the semen sample, from each participant, a 2-ml sample of blood was taken and collected into a tube without any anticoagulant. The tube was left in a standing position for a maximum of 20–30 min before processing. Within 6 h, the tube was centrifuged at 1500 ***g*** for 10 min (blood was clotted), and then, the serum removed and stored at −20 °C prior to serology measurement.

DNA extraction from urine and semen samples

DNA was extracted from all urine and semen samples using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. All extracted DNA was stored at −20 °C prior to transfer of specimens to the UK for PCR.

Transfer of specimens

Frozen sera and extracted DNA from urine and semen were transferred on dry ice to the Department of Human Metabolism, University of Sheffield, at the end of the recruitment phase of the study. Upon arrival in Sheffield,

the samples were stored at −20 °C prior to further analysis as outlined below.

Chlamydia trachomatis serology

To detect specific IgA, IgM and IgG antibodies to *C. trachomatis,* an immunofluorescence assay (SeroFIA™ *C. trachomatis*) kit was used (Savyon, Ashdod, Israel). Briefly, purified elementary bodies (EBs) of *C. trachomatis* (supplied by the kit) were fixed onto the SeroFIA™ slide wells. Serum samples were then thawed and diluted. According to kit instruction, the dilution rate for IgG serum antibody was 1:64, IgA serum antibody titre was 1:32, and IgM serum antibody titre was 1:20. Diluted patients' sera were incubated for 30 min at 37 °C with the antigen. Unbound serum components were removed by washing. Fluorescein-conjugated anti-human IgG, IgA, IgM were added and incubated for 30 min at 37 °C. After washing to remove any unbound conjugate, slides were dried and mounted and then were examined using a Leica fluorescence microscope DMIRB (Lasertechnik Gmbh, Heidelberg, Germany). The whole well was first scanned at magnification of ×400 to observe any evidence of typical fluorescence, and the well was then further observed at magnification of ×1000 using oil immersion to confirm presence or absence of typical fluorescence in EB-like morphology (small granules). Positive reactions appear as bright apple-green fluorescent EBs against a dark background. Two people examined each slide and a positive result declared when both were in agreement. Positive and negative controls on each slide were included from the kit.

Chlamydia trachomatis PCR

Nested plasmid PCR (NPPCR) was conducted according to method of Claas *et al.* (1990), and two pairs of primers were used to detect *C. trachomatis* (Hosseinzadeh *et al.*, 2004). First pair was derived from sequences of the common endogenous plasmid of *C. trachomatis* and generated a species-specific 517-bp product with all serovars of *C. trachomatis*. Second pair is internal to the first pair primers and amplified a 320-bp product. Primers were ordered from Eurofins MWG/Operon (Eurofins MWG/Operon, London, UK). The PCR program of 25 cycles included: denaturation 94 °C 30 s, annealing 42 °C 30 s and extension 72 °C 30 s. The extension time was increased to 9 min (Claas *et al.*, 1990). Semen DNA was first tested for β-globin according to the method of Saiki *et al.* (1985) to check whether there were no PCR inhibitors in the samples. When samples were shown to be β-globin positive, they were tested by the nested PCR method using primers directed against the cryptic plasmid, as described previously (Hosseinzadeh *et al.*, 2004). Products were analysed by gel electrophoresis in 1.5% (w/v) agarose with ethidium bromide staining.

Each PCR run included *C. trachomatis* DNA from two serovars as positive controls. Serovar E of *C. trachomatis* was isolated from a clinical source (cervical swab from Department of Genitourinary Medicine, Royal Hallamshire Hospital, Sheffield), and serovar LGV was provided by the University of Southampton (Southampton, UK). Confirmation of genotype was conducted by nested PCR according to method of Lan *et al.* (1994). AQIAamp DNA Mini kit (Qiagen) was used (as described above) to extract DNA from purified EBs, and the DNA was checked by NPPCR and stored at −20 °C until used as a positive control. Positive results were compared with *C. trachomatis* plasmid ($pCTT_1$) sequence, accession: M19487 (J03304).

Measurement of IL-6 and IL-8

Seminal plasma was first thawed and then was diluted with PBS (1 : 2) in the case of IL-6 and (1 : 4) in the case of IL-8. For each diluted sample, 100 ml was tested with a commercial quantitative sandwich enzyme immunoassay (R&D Systems, Abingdon, UK) according to the manufacturer's instructions. The optical density of each well was determined within 30 min, using a microplate reader (Dynex technologies, Chantilly, VA, USA) set to 450 nm. The average reading for each standard, control and sample was subtracted of zero standard optical density. A standard curve was constructed for each 96-well plate by plotting the mean absorbance for each standard on the *y*-axis against the concentration on the *x*-axis and joining them with a line of best fit. The minimum detectable dose (MDD) of IL-6 was <0.7 pg ml^{-1} and for IL-8 was 1.5–7.5 pg ml^{-1} with a mean of 3.5 pg ml^{-1}.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) 18.0 software (SPSS Inc., Chicago, IL, USA) was used for data analysis. Descriptive statistic and an independent sample *t*-test were used for data with normal distribution. Mann–Whitney *U*-test was used for nonparametric data. Spearman rank correlation test was used to evaluate the relationship between the levels of IL-6 and IL-8 with semen parameters.

Results

The male partners from 324 infertile couples were screened for inclusion in the study, of which 74 did not meet the recruitment criteria. The remaining 250 were

successfully recruited. The men had a median age of 32 years old (range 21–52), and none of them reported any symptoms of genitourinary infections.

A total of 11 subjects (4.4%) were found to be positive for *C. trachomatis* using NPPCR on urine DNA. Serology results showed three subjects (1.2%) were IgM positive, 45 subjects (18.0%) were positive for IgG antibody to chlamydia, and no sample was positive for IgA. *Chlamydia trachomatis* DNA was not detected in any of the semen samples.

Tables 1 and 2 show the mean (±SD), median (ranges) and 25th and 75th centiles of IL-6 and IL-8 concentrations in semen according to the presence or absence of *C. trachomatis* DNA in urine (panel a of both tables) and the presence or absence of *C. trachomatis* IgG in serum (panel b of both tables) as well as that seen in the unselected population. The values of IL-6 and IL-8 cytokine levels in seminal plasma were correlated (Pearson's correlation coefficient = 0.38; $P < 0.001$) irrespective of *C. trachomatis* infection. However, the mean ± SD of IL-6 and IL-8 were both numerically higher in *C. trachomatis*-infected men as defined by PCR; however, they were not statistically significant ($P = 0.244$; $P = 0.157$ respectively).

Seminal plasma concentrations of IL-6 > 37.91 pg ml^{-1} were defined as 'high' (based on the 75th percentile), and this was seen in a total 62 of the 250 samples tested. High levels were seen in the seminal plasma of four of the 11 samples where *C. trachomatis* DNA was observed in urine and 14 of the 45 samples where *C. trachomatis* IgG was observed in serum. Conversely, seminal plasma concentration of IL-8 > 2966 pg ml^{-1} was defined as 'high' (based on the 75th percentile) and this was seen in a total 62 of the 250 samples tested. High levels of IL-8 were observed in the seminal plasma of three of the 11 samples where *C. trachomatis* DNA was observed in urine and nine of the 45 samples where *C. trachomatis* IgG was observed in serum.

Table 1 Relationship between the levels of IL-6 (pg ml^{-1}) in the semen of the male partners from 250 infertile couples and the presence of (a) *Chlamydia trachomatis* DNA in urine and (b) *C. Trachomatis* IgG in blood serum

	Positive	Negative	Total
(a) Urine DNA			
Number (*n*)	11	239	250
Mean ± SD	70.89 ± 101.19	32.97 ± 42.78	34.64 ± 47.13
Median	28.28	23.64	23.65
25th–75th centile	20.23–72.62	10.05–37.82	10.26–37.91
Range	0.51–327.43	0.23–367.52	0.23–367.52
(b) Serum IgG			
Number (*n*)	45	205	250
Mean ± SD	54.54 ± 81.46	31.65 ± 34.36	34.64 ± 47.13
Median	29.42	23.25	23.65
25th–75th centile	11.56–55.79	10.16–37.26	10.26–37.91
Range	0.24–367.52	0.23–270.03	0.23–367.52

Table 3 shows the correlations between seminal IL-6 and IL-8 concentrations and male age, duration of infertility and measures of semen quality. Briefly, both IL-6 and IL-8 were significantly correlated with the concentration of leucocytes in semen ($P = 0.012$ and $P = 0.001$ respectively). However, with regard to other parameters, only the concentration of IL-8 was inversely correlated with semen volume ($P = 0.013$) and positively with male age ($P = 0.039$).

Comparisons were also made between *C. trachomatis*-infected and uninfected men as defined by the presence of IgM and IgG antibodies in serum or *C. trachomatis* DNA in urine and their corresponding semen parameters evaluated. *C. trachomatis*-infected men according to positive urine DNA in NPPCR (Table 4) showed low semen volume compare with uninfected subjects that is statistically significant ($P = 0.001$).

The semen pH was higher in the IgG (+) group (Table 4) compared with the IgG negative group and was biological significant ($P = 0.055$, $0.05 < P < 0.1$). Also the percentage of immotile spermatozoon was significantly lower in the IgG positive group compared with the IgG negative group ($P = 0.018$). The mean seminal leucocyte counts were higher in *C. trachomatis*-infected men according to positive urine DNA in NPPCR, however, was not significant ($P = 0.882$).

Discussion

The study examined the relationship between *C. trachomatis* infection in infected men (using PCR and serology) and inflammatory markers (IL-6 and IL-8) in the seminal plasma of male partners of infertile couples in Iran. To our knowledge, this is the first large-scale study of *C. trachomatis* infection and its effect on seminal cytokines, undertaken on infertile male in Iran using both PCR and serology and the second study which investigated the relationship between chlamydial infection and seminal interleukins in Iran.

This study showed that men with such an infection in FVU samples (PCR positive) had only lower semen volume compared with men without infection. This is because inflammation can cause of obstruction of the duct and results to reduce semen volume and this support previous works (Wolff *et al.*, 1991) and in contrast with others (Hosseinzadeh *et al.*, 2004; Al-Mously *et al.*, 2009; Kokab *et al.*, 2010) who explained *C. trachomatis* infection might be the cause of increased activity of accessory gland or reproductive epithelium secretion. Infected

Table 2 Relationship between the levels of IL-8 (pg ml^{-1}) in the semen of the male partners from 250 infertile couples and the presence of (a) *Chlamydia trachomatis* DNA in urine and (b) *C. trachomatis* IgG in blood serum

	Positive	Negative	Total
(a) Urine DNA			
Number (*n*)	11	239	250
Mean ± SD	9540.69 ± 14434.28	2868.42 ± 4505.63	3162.00 ± 5445.32
Median	1524.46	1622.21	1615.17
25th–75th centile	982.10–15242.20	1006.18–2849.43	1005.50–2966.20
Range	463.71–37668.35	179.50–41918.62	179.50–41918.62
(b) Serum IgG			
Number (*n*)	45	205	250
Mean ± SD	3926.82 ± 7670.52	2981.58 ± 4831.22	3162.00 ± 5445.32
Median	1819.92	1588.62	1615.17
25th–75th centile	1024.95–3023.99	1004.81–2968.19	1005.50–2966.20
Range	185.75–37668.35	179.50–41918.62	179.50–41918.62

Table 3 Median (range) of age, duration of infertility, semen parameters and their correlations with levels of IL-6 and IL-8 in semen

Variable	Median (range)	Correlation with IL-6 levels	Correlation with IL-8 levels
Age (years)	32.7 (21–52)	$P = 0.158$	$P = 0.039$*
		$r = 0.090$	$r = 0.130$
Duration of infertility (years)	5 (1–18)	$P = 0.290$	$P = 0.972$
		$r = 0.068$	$r = 0.002$
Semen volume (ml)	2.5 (1–9.5)	$P = 0.175$	$P = 0.013$*
		$r = 0.086$	$r = -0.157$
pH	8 (6–9.5)	$P = 0.672$	$P = 0.241$
		$r = 0.022$	$r = 0.074$
Concentration (million ml^{-1})	62 (0.5–350)	$P = 0.326$	$P = 0.383$
		$r = 0.062$	$r = 0.055$
Per cent progressive motile	55 (0–95)	$P = 0.835$	$P = 0.394$
		$r = 0.013$	$r = 0.055$
Per cent immotile	32 (0–100)	$P = 0.914$	$P = 0.882$
		$r = 0.007$	$r = 0.010$
Per cent normal morphology	6 (0.5–17.5)	$P = 0.857$	$P = 0.775$
		$r = -0.012$	$r = 0.018$
Leucocytes, million ml^{-1}	1.2 (0.05–11.41)	$P = 0.012$*	$P = 0.001$*
		$r = 0.159$	$r = 0.419$

*P-value ≤ 0.05.

men as defined by serology (IgG+) had high level of IL-6 and increased semen pH as described by Marconi *et al.* (2009) and Kokab *et al.* (2010).

Increasing IL-8 levels were associated with lower semen volume in *C. trachomatis*-infected men (urine DNA+), and this is contrary to previous observations that found high semen volume in infected men (Hosseinzadeh *et al.*, 2004; Al-Mously *et al.*, 2009; Kokab *et al.*, 2010). It is unclear why this is the case although a simple explanation is that semen DNA was not infected in the present study and some men had only a history of urethritis. It might also be caused by unequal abstinence periods between the two groups, although this seems unlikely. IL-8 was also associated directly with male age in the present study, and this might be a sign of genital accessory gland infection (Al-Mously & Eley, 2007) or benign prostatic hyperplasia (BPH) without damage to spermatogenesis. There are studies about the role of IL-8 to trigger BPH (Berry *et al.*, 1984; Castro *et al.*, 2004; Penna *et al.*, 2009) and developing BPH and male age (Berry *et al.*, 1984; Meigs *et al.*, 2001). As no correlation between age and semen volume was observed in the current study, the correlation between IL-8 and semen volume must be caused by accessory gland inflammation and/or infection by *C. trachomatis* or other infectious agent and the obstruction of the ductus epididymis (Marconi *et al.*, 2009). The fact that the other micro-organisms (Cai *et al.*, 2014) in semen are responsible for altered cytokines levels might be a complicating factor in interpreting these results.

The seminal plasma IL-6 level was much lower than IL-8 (0.23–367.52 pg ml^{-1} versus 179.50–41918.62 pg ml^{-1}) but significantly correlated to each other. These findings support the idea of Al-Mously & Eley (2007) who proposed that IL-6 and IL-8 seminal plasma levels

Table 4 Age, duration of infertility, semen parameters and (mean ± SD) in (a) *Chlamydia trachomatis* urine DNA (+) and urine DNA (−) men (b) *C. trachomatis* IgG(+) and IgG(−)

(a) Variable	DNA positive (n = 11)	DNA negative (n = 239)	P-value
Age[a] (years)	31.45 ± 3.53	32.79 ± 5.19	P = 0.254
Duration of infertility[a] (years)	5.27 ± 3.06	5.90 ± 3.49	P = 0.406
Semen volume[a] (ml)	2.11 ± 0.73	3.15 ± 1.53	P = 0.001*
pH[a]	8.22 ± 0.41	8.25 ± 0.43	P = 0.829
Concentration[a] (million ml^{-1})	59.54 ± 45.22	66.45 ± 51.27	P = 0.632
Per cent progressive (Motile[b])	53.63 ± 15.71	51.52 ± 21.86	P = 0.781
Per cent immotile[b]	33.36 ± 14.86	34.59 ± 19.98	P = 0.923
Per cent normal morphology[b]	7.27 ± 3.45	6. 08 ± 3.53	P = 0.250
Leucocytes[a], million ml^{-1}	1.60 ± 1.18	1.55 ± 1.34	P = 0.882
(b)Variable	IgG positive (n = 45)	IgG negative (n = 205)	P-value
Age[a] (years)	32.00 ± 5.20	32.9 ± 5.10	P = 0.273
Duration of infertility[a] (year)	5.33 ± 3.04	6.09 ± 3.61	P = 0.175
Semen volume[a] (ml)	3.30 ± 1.90	3.00 ± 1.40	P = 0.748
pH[a]	8.40 ± 0.36	8.20 ± 0.44	P = 0.055
Concentration[a] (million ml^{-1})	64.65 ± 42.3	68.54 ± 54.9	P = 0.915
Per cent progressive (motile[b])	56.84 ± 21.4	52.23 ± 19.50	P = 0.697
Per cent immotile[b]	31.05 ± 19.50	36.20 ± 18.50	P = 0.018*
Per cent normal morphology[b]	5.60 ± 2.60	6.20 ± 3.70	P = 0.672
Leucocytes[a], million ml^{-1}	0.98 ± 0.49	1.65 ± 1.41	P = 0.152

[a]Parametric independent t-test.
[b]Mann–Whitney U-test.
*P-value ≤ 0.05.
$0.05 < P < 0.1$ – although not slightly significant statistically, this deserves consideration as being biologically significant.

might be useful as a marker of upper genital tract infection (e.g. prostatitis). Given that IL-6 and IL-8 are both markers of prostate inflammation and IL-8 is also related to infection of urethra (Al-Mously & Eley, 2007; Mazzoli *et al.*, 2007), the results might suggest that patients with high levels of ILs are more likely to have prostatitis and this is confirmed with the positive correlation of IL-8 with male age. Both ILs were also associated with high number of leucocytes in semen and this suggest the probability of inflammation and/or infection and this is similar to previous studies (Eggert-Kruse *et al.*, 2001; Kokab *et al.*, 2010).

These findings do not confirm previous studies on male infertility and *C. trachomatis* which have been carried out in Sheffield that found increased semen volume in infected men (Hosseinzadeh *et al.*, 2004; Kokab *et al.*, 2010). This might be because these studies undertook *C. trachomatis* diagnosis on semen samples which was not achieved in this study. However, these do confirm and extend the previous finding by Eggert-Kruse *et al.* (2001) who found high level of ILs in leucocytospermic samples and no association with bacterial colonisation of semen samples.

Therefore, these correlations confirmed that *C. trachomatis* infection can result in high level of seminal plasma interleukins and statistically significant high level of IL-6 might suggest the effect of this organism on prostate and support the previous work in Sheffield (Al-Mously & Eley, 2007). Low semen volume in *C. trachomatis*-infected men defined by positive urine DNA indicates current infection and can be indirectly related to high level IL-8 and inflammation of the prostate. The relationship between *C. trachomatis* infection and chronic prostatitis (Ouzounova-Raykova *et al.*, 2010) indicates the probability of a risk factor with prostate cancer (Stasiewicz *et al.*, 2012) and should be examined in future studies. However, there is not an accepted aetiological link and the view is highly controversial.

There are many previous studies suggesting a relationship between *C. trachomatis* infection and poor semen quality (Mazzoli *et al.*, 2010; Cai *et al.*, 2014) or immune system activation with high-level IL-8 (Mazzoli *et al.*, 2007) in patients that affected by chronic prostatitis. However, all were carried out on men based on a positive result for *C. trachomatis* and/or a medical history of prostatitis. In our study, men were not symptomatic and the serology results were obtained after patient recruitment and the completion of all diagnostic procedures. Therefore, recruitment was carried out blind to diagnosis and without reference to their diagnosis or reason for infertility. Therefore, we feel confident that this is a genuine result and worthy of reporting.

The weakness of this study is the lack of a fertile group of males as control to see the level of ILs and their relationship with *C. trachomatis*. Extensive attempts were made to recruit fertile male participants in number of locations. These locations included antenatal and vasectomy clinic; however, despite these attempts, no fertile male participant volunteered. These problems have been recognised in other studies (Cohn *et al.*, 2002; Stewart *et al.*, 2009; Eiser *et al.*, 2011), and it is suggested they are too concerned about their fertility or suspected infertility to volunteer. Also they may have experience of testicular cancer treatment or anxiety of cancer diagnosis.

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Competing interests

None declared.

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