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VASA mRNA (DDX4) detection is more specific than immunohistochemistry using poly- or monoclonal antibodies for germ cells in the male urogenital tract

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Abstract

VASA, also known as DDX4, is reported to be specifically expressed in cells belonging to the germ cell lineage, both in males and females. Therefore, it could be an informative protein biomarker to be applied on semen to differentiate between obstructive and nonobstructive azoospermia (OA and NOA, respectively). In addition, it could be of value to predict sperm retrieval based on testicular sperm extraction. Immunocytochemistry of proven OA semen using both polyclonal and monoclonal antibodies against VASA showed positive staining of both cells and cell sized particles. This is spite of being the absolute negative controls, completely lacking germ lineage derived cells and material. In order to identify the source of the VASA-positive material, a detailed screen of different anatomical parts of the whole male urogenital tract was performed of multiple cases using immunohistochemistry.

The polyclonal antibody stained, besides the expected germ cells in the testis, epithelium of the bladder and the seminal vesicles. The monoclonal antibody only stained the latter. To investigate whether the immunohistochemical staining is associated with the presence of the corresponding VASA mRNA, samples of seminal vesicles, bladder, testis, and semen (with and without germ cells) were investigated using the specific quantitative reverse transcription-polymerase chain reaction (qRT-PCR) on 42 samples. A positive result was detected in testis and semen containing germ cells (n = 10 and 8), being negative in semen without germ cells (n = 11), bladder (n = 3), and seminal vesicles (n = 10).

Two commercially available VASA antibodies (mono- and polyclonal) are not specific. In contrast, VASA-mRNA evaluation, using qRT-PCR, is specific for the presence of germ cells, therefore, is an interesting molecular biomarker for germ cell detection in semen.

Abbreviations: FFPE = formalin-fixed paraffin-embedded, GCNIS = germ cell neoplasia in situ, IC = immunocytochemistry, ICSI = intra cytoplasmatic sperm injection, IHC = immunohistochemistry, MVH = mouse vasa homolog, NOA = nonobstructive azoospermia, OA = obstructive azoospermia, qRT-PCR = quantitative reverse transcription-polymerase chain reaction, TESE = testicular sperm extraction.

Keywords: DDX4, germ cells, immunohistochemistry, male infertility, qRT-PCR, sperm, VASA

1. Introduction

In males and females, human VASA (DDX4) is the only reported gene specifically expressed in the germ cell lineage both during embryonic development and postnatally. So far the mRNA VASA expression in normal somatic tissues has only been detected during embryogenesis.[1] The *vas* gene was originally identified in Drosophila (vasa), later in the mouse (Mouse vasa homolog, MVH) and in humans (VASA).[2–7] It maps on chromosome 5q and encodes a DEAD-box protein with ATP-dependent RNA-helicase activity.[4,8] This gene is also referred to as DEAD-box helicase 4 gene (DDX4). As male MVH knock-out mice are infertile without other anomalies, VASA seems to play an essential role in male fertility specifically.[6]

VASA can differentiate testicular biopsies from men with obstructive azoospermia (OA) and nonobstructive azoospermia (NOA) using immunohistochemistry and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). In fact, VASA shows different expression levels depending on the azoospermic etiology.[9] Men with NOA can only father a child after intracytoplasmatic sperm injection (ICSI) with their surgically harvested testicular sperm cells (Testicular Sperm Extraction,TESE).[10] TESE will yield spermatozoa in 50% of the patients with NOA.[11] It would be very useful to be able to predict which patients would benefit from TESE and so prevent patients from undergoing a useless operation for obtaining sperm. It is also reported that in segregated ejaculated spermatozoa VASA can distinguish fertile from infertile men both on mRNA and protein level.[12] Moreover, in cell-free seminal plasma from ejaculates of patients with azoospermia,

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VASA-mRNA detection could differentiate between certain etiologies of NOA.[13] In conclusion, VASA is an interesting potential marker to be further investigated in men with NOA.

VASA immunochemistry has been used to detect germ cells in tissue sections of testis,[9] but not in semen. We wanted to investigate whether VASA immunocytochemistry (IC) could be used to differentiate between semen of azoospermic men with OA and NOA. The aim of the current study was to test the specificity of commercially available VASA antibodies for staining of semen containing germ cells. In addition, the nonspecificity found was further evaluated by investigation of different parts of the male urogenital tract that may shed cells and derived material into seminal fluid. To this end, a detailed VASA protein analysis was performed using IC on various semen samples and the different anatomical parts of the urogenital tract using immunohistochemistry (IHC), followed by testing of the specificity of IC-demonstrated expression using VASA-mRNA qRT-PCR.

2. Materials and methods

Patients gave their verbal consent that left over material, after a diagnostic procedure, can be used for scientific purposes. This agreement is not documented, as agreed upon for this study by the *Erasmus MC institutional review board (file number medical ethical committee approval MEC-2013-170)*. If patients chose to not consent, it is specifically indicated in the clinical files, and samples were excluded. This consent procedure was used according to the "Code for Proper Secondary Use of Human Tissue in the Netherlands."

The antibodies for VASA, polyclonal Abcam concentration 1 mg/mL ab13840 (Cambridge, UK) and monoclonal Abcam ab27591, used in this study were selected based on performance in IHC of the testis in a previous study.[5,14]

The semen samples of OA and NOA were allowed to liquefy after production and thereafter dissolved in 10% phosphate-buffered formalin for 1 hour. After fixation, the samples were centrifuged for 20 minutes at 1600*g*; then, the pellet was resuspended in phosphate-buffered saline and vibrated with the use of an automatic shaker to make a single-cell solution. Cytospins of this suspension were made on a strong adhesive microscope slide (Starfrost©) and were dried overnight. The cytospins were blocked with 3% H_2O_2 and after antigen retrieval incubated overnight at 4°C with a polyclonal (Abcam ab13840) DDX4/MVH antibody. After 30 minutes incubation with biotinylated Swine anti Rabbit (Dako E431, Glostrup, Denmark), the slides were incubated with Vector ABcpxHRP (Vector 6100, Burlingame, CA). Visualization was done with 3-amino-9-ethylcarbazole (AEC)/H_2O_2. A part of the semen samples were also incubated with a monoclonal antibody (Abcam ab27591) DDX4/MVH to compare with the polyclonal antibody in the same assay as described above. As a secondary antibody, a biotinylated rabbit anti mouse antibody was used (E0413; Dako). Detection and counterstaining were carried out as described above.

Formalin-fixed paraffin-embedded (FFPE) tissues of the different anatomical parts of the urogenital tract, from the pyelocaliceal system to urethra and from seminiferous tubules to prostatic urethra, were obtained from 6 males who underwent various surgical procedures. In total 2 representative sets of the complete male urogenital tract were composed with at least 2 and maximum 6 tissue blocks containing kidney, pyelocaliceal system, ureter, bladder, urethra, testis, epididymis, vas deferens, seminal vesical, ejaculatory duct, prostate, and prostatic urethra (see Fig. 1 for summary). Additionally, the FFPE tissues from seminal vesicles obtained from prostatectomy specimens of 6 patients with prostate cancer without prior treatment besides surgery were included. The tissues were stained with HE and IHC by using respectively polyclonal (Abcam ab13840) and monoclonal (Abcam ab27591) DDX4/MVH antibodies. After deparaffinization, IHC was performed as described for the cytospin samples. Cytokeratin 8 (CAM5.2, B&D 347205; BD Biosciences, San Jose, CA) staining was applied, by the same method as described above, to differentiate germ cells from epithelial cells and cell fragments.

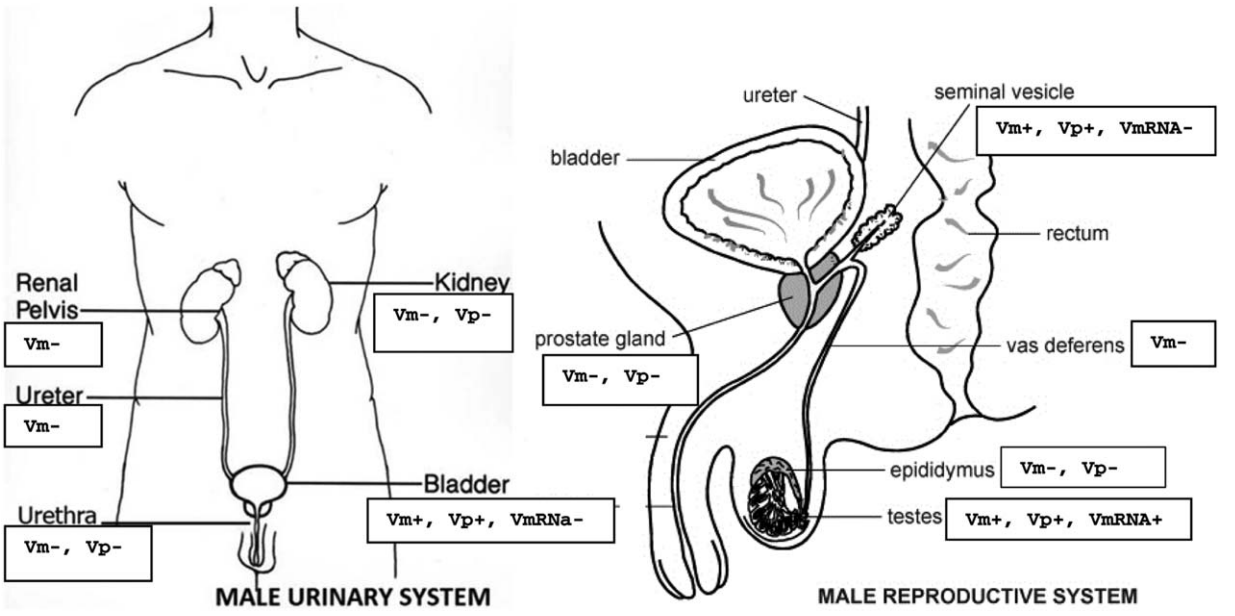

Figure 1. Male urogenital tract from kidney to urethra and from testis to urethra. VASA immunohistochemistry (mono- and polyclonal; Vm and Vp) and VASA mRNA (VmRNA) results are indicated for each anatomical part.

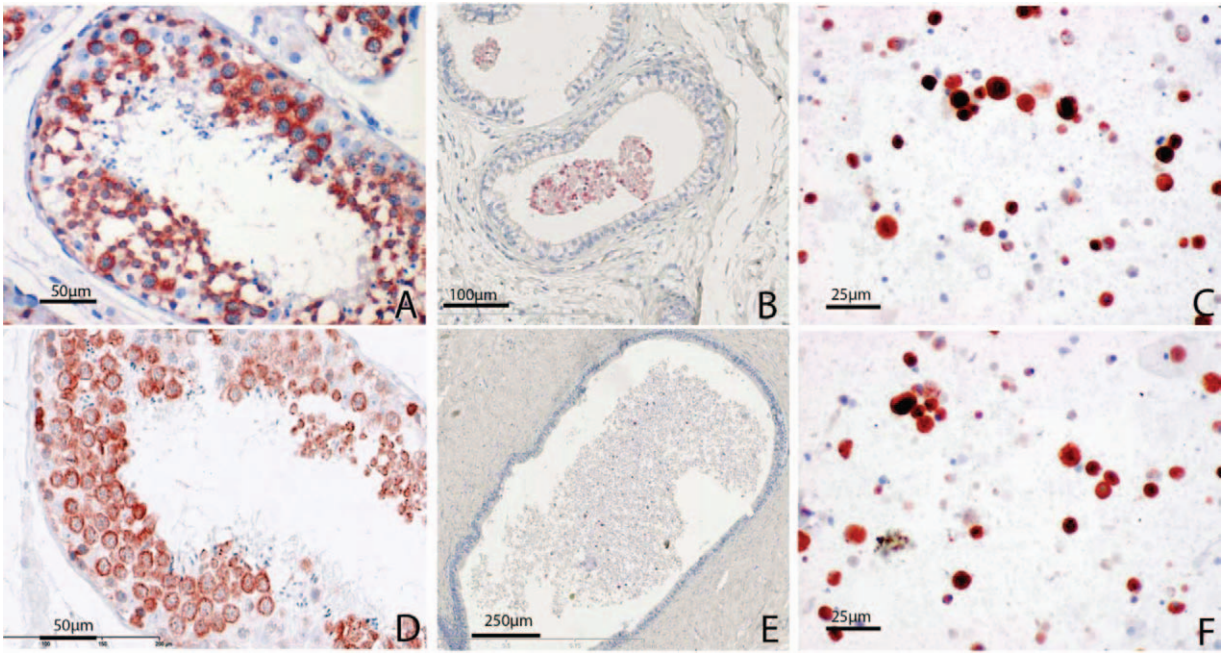

Figure 2. Staining of testis, epididymis, and semen using monoclonal (A, B, C) and polyclonal (D–F) VASA antibody. In the testis (A and D), all cells of germ lineage except elongated spermatids and spermatozoa show cytoplasmatic staining; no other cell types are positive. Epididymal epithelium is VASA-negative whereas some germ cells in the lumen show cytoplasmic staining (B and E). Semen from a patient with obstructive azoospermia (C) and from a patient with nonobstructive azoospermia (F) shows positive staining of unidentified material including cell-sized particles and occasional cells.

2.1. RNA analysis

To verify the specificity of VASA IC in germ cells, bladder and seminal vesicle epithelium high quality total RNA was extracted from normal testis-parenchyma (n = 10), semen of men with normospermia (n = 8), NOA (n = 5), OA (n = 6), bladder (n = 3), and seminal vesicles (n = 10) using TRIzol Reagent (Life Technologies, Bleiswijk, the Netherlands) according to the manufacturer's instructions. qRT-PCR was performed in duplicate using mRNA probes VASA hs00987125_m1 and endogenous control hypoxanthine-guanine phosphoribosyltransferase (HPRT)1 (4333768F; Life Technologies). Relative VASA transcript abundance was calculated using the 2^{-dCt} method (VASA mRNA = mean Ct HPRT – mean Ct VASA) as described before.[15]

3. Results

VASA IC staining of semen in both multiple OA and NOA samples (n = 57 and n = 98, respectively) showed inconsistent results with, sometimes even abundant, positivity in semen samples of patients with OA. The positive material included cells and cell sized particles which were difficult to distinguish from cells, both using the polyclonal or the monoclonal antibodies (Fig. 2, panels C and

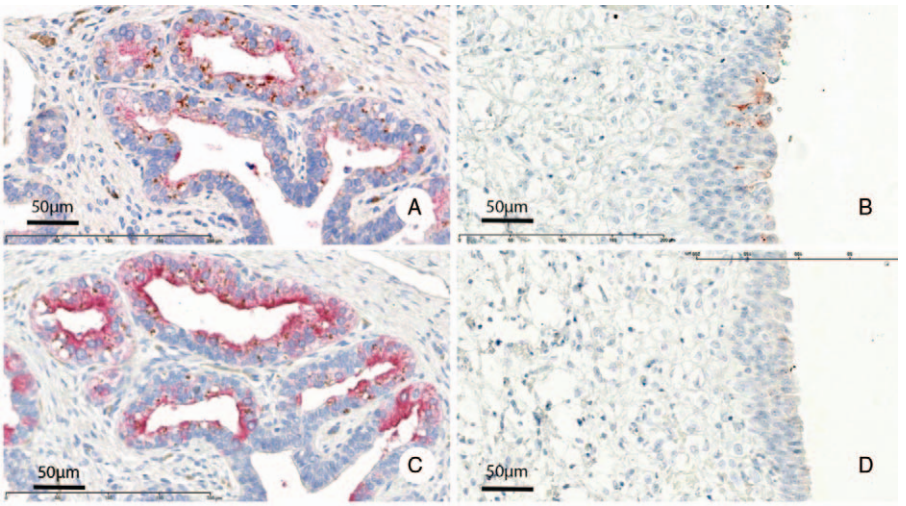

Figure 3. Epithelial staining in the seminal vesicle (left) and bladder (right) by polyclonal VASA antibody (A, B) and in seminal vesicle (left) and virtually negative in bladder, only sporadically weak staining of the luminal surface (shown here) by monoclonal VASA antibody (C, D).

F). The morphology was similar in both samples from OA and NOA. In patients with OA, the staining material is obviously not derived from the germ lineage, as their ejaculate does not contain germ cells or derivatives (i.e., negative control). IHC with the polyclonal antibody against VASA showed protein expression in germ cells of the testis (Fig. 2, panel A), as expected, and in addition staining of the epithelial cells of the seminal vesicles and the bladder (Fig. 3, panels A and B). No staining was found in the other parts of the urogenital tract: kidney, renal pelvis, ureter, urethra, epididymis, vas deferens, and prostate were all VASA-negative (Fig. 1). The monoclonal VASA antibody showed a similar pattern for testis (Fig. 2, panel D) and seminal vesicle (Fig. 3, panel C). However, bladder epithelial cells remained virtually negative with only sporadically weak staining of the luminal surface (Fig. 3, panel D). By omitting the primary antibody, there was no staining at all in IC and IHC (data not shown).

Staining of seminal vesicle epithelium with the monoclonal VASA antibody was reproduced in FFPE tissue samples of 6 seminal vesicles, obtained from prostatectomy specimens (Fig. 4, panel B). All showed a similar expression pattern with strong staining in the periphery of the vesicle and weak to absent staining more centrally. Virtually all material in the lumen of the seminal vesicles showed strong VASA staining. It consists of amorphous material, cell-sized particles and cells with recognizable nuclei, obviously shed from the seminal vesicle epithelium. Occasional intact cells in the lumen express Cytokeratin 8 like the epithelial cells of the seminal vesicle (Fig. 4, panel C), suggesting that IC-VASA-positive non-germ cells may rarely occur in semen. Noteworthy, the morphology of the VASA-positive material in semen (Fig. 2, panels C and F) and in the lumen of seminal vesicles (Fig. 4, panel B) is highly similar.

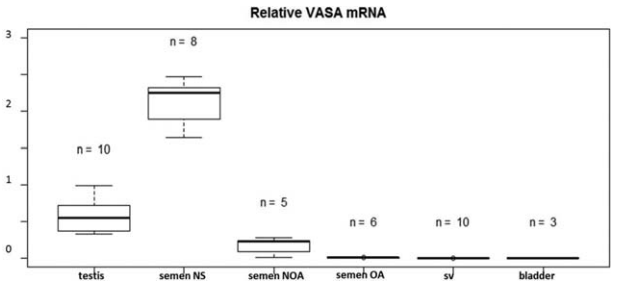

Figure 5. VASA mRNA expression profile (relative to HPRT) in normal testis parenchyma, semen of men with normospermia (NS), nonobstructive azoospermia (NOA), obstructive azoospermia (OA), seminal vesicles (sv), and bladder. HPRT = hypoxanthine-guanine phosphoribosyltransferase, NOA = nonobstructive azoospermia, NS = normospermia, OA = obstructive azoospermia, sv = seminal vesicles.

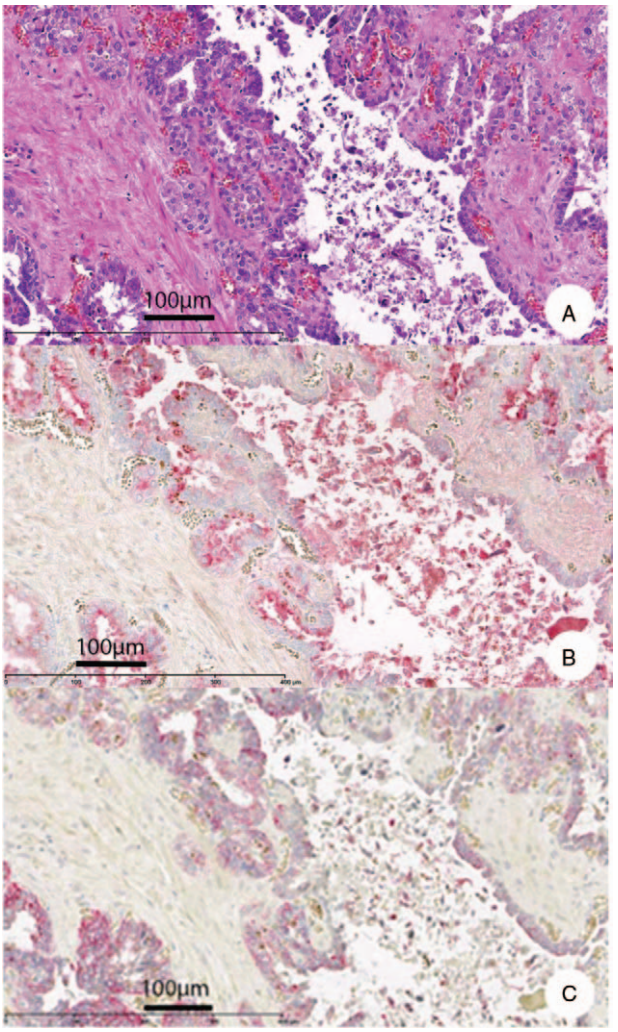

Figure 4. Seminal vesicle: (A) HE staining; (B) VASA monoclonal antibody staining showing positivity of the epithelium and abundant VASA-positive material in the lumen including virtually all sized particles and nucleated cells. This material is most likely the source of the VASA positive material in semen of patients with OA and NOA. (C) Cytokeratin-8 stains the epithelium and some of the cells in the lumen. Such cells may be confused with immature germ cells in VASA immunocytochemistry of semen. HE = hematoxyline-eosine, NOA = nonobstructive azoospermia, OA = obstructive azoospermia.

To further investigate the specificity of the findings, a molecular analysis based on qRT-PCR was performed on a series of 42 samples of multiple origins. In all germ cell containing testis, parenchyma samples and normal sperm samples VASA mRNA were detected (Fig. 5 and supplemented Figure 1, http://links.lww.com/MD/B809). Expression of VASA in the case of NOA depended on the presence of germ cells in the testis. In addition, VASA mRNA was detected in semen of 4 men with maturation arrest of spermatogenesis. The only sample lacking VASA expression was related to a man diagnosed with complete Sertoli cell only syndrome (i.e., devoid of germ cells). In semen of patients with OA, VASA mRNA was in all cases undetectable, consistent with the clinical diagnosis. Moreover, VASA mRNA was detected in none of the 10 seminal vesicles nor in the 3 bladder samples, indicating that the immunohistochemical staining in the epithelium and lumen of seminal vesicles and the bladder epithelium is not related to the presence of VASA protein (Fig. 1).

4. Discussion

In males and females, VASA protein is present in the cytoplasm of all cells of the germ lineage, both during embryonic development and postnatally. In the testis, spermatocytes show the highest VASA expression and the strongest IHC staining. Spermatids also have a clear expression with strong staining. Spermatogonia show either weak or intermediate staining which may correspond with type A or B spermatogonia. In spermatozoa, VASA protein is only detectable by immunofluorescense on unfixed material because of its low expression level. Sertoli cells are negative for the VASA protein.[4,5,8,12] VASA is also expressed in seminoma, dysgerminoma, their respective precursor lesions germ cell neoplasia in situ (GCNIS) and gonadoblastoma and in

spermatocytic seminoma, consistent with the germ cell origin of these tumors.[5] Our results of IHC of normal testis concur with these literature data (Fig. 2). These data are in line with other findings, summarized online (http://www.genecards.org/cgi-bin/carddisp.pl?gene=ddx4).

Using the Northern blot assay, Castrillion and coworkers examined VASA expression in testis, ovary, prostate, brain, thymus, lung, liver, spleen, pancreas, small intestine, colon, stomach, kidney, adrenal, placenta and peripheral blood leukocytes, and concluded that VASA expression was undetectable in nongonadal tissues.[4] In contrast, here we show that immunohistochemistry with a commercially available polyclonal VASA antibody not only stains germ cells in testis but also epithelial cells of the bladder and the seminal vesicle (Fig. 3). In addition, a commercially available monoclonal VASA antibody also stains germ cells and the epithelial cells of the seminal vesicle, although overall the bladder is negative (Fig. 3, panel C and D; Fig. 4 panel B).

In addition to the protein analyses, qRT-PCR on a series of 42 independent samples was performed, showing that the protein staining does not correspond to VASA. Although this is a limited number of samples, the results are consistent and show little variability. The results indicate that the VASA mRNA expression is indeed found only in germ cells in testis and semen, as well as epididymis, extending the results of Castrillon and coworkers on somatic tissues, confirming the specificity of VASA for germ cells.[4] The IHC staining of epithelium of the seminal vesicle with the monoclonal reagent is therefore most likely due to cross-reaction with protein(s). Material positive for this signal is abundantly shed in the lumen of seminal vesicles (Fig. 4) and is probably the source of the material found positive upon IC of semen in view of the fact that it is also present in the ejaculate of men with complete OA. The abundance of this material and its morphology, with cell-sized particles and occasional VASA-positive nongerm cells which could be confused with immature germ cells,[16,17] precludes the use of semen IC in the diagnosis and management of male infertility.

Several studies have explored the use of VASA-detection by IHC (testicular biopsies [9,18]), immunofluorescence and Western blotting (isolated spermatozoa [12]) flow cytometry (testicular biopsies [9]), and VASA mRNA qRT-PCR (testicular biopsies,[18] isolated spermatozoa,[12] and seminal plasma [13,19]) in the diagnosis and management of male infertility. The mRNA expression levels of VASA in testicular tissue obtained from patients undergoing a TESE procedure showed significant difference in expression depending on the number of germ cells present and the histological classification.[18] The results consistently indicate that detection of VASA protein or mRNA correlates with the presence of germ cells in the tested sample and may in a quantitative measurement reflect the number of germ cells present. The latter is a promising method to assist in the etiological diagnosis of azoospermia and to predict the success of TESE.

Thus far IC for VASA and VASA mRNA qRT-PCR was to our knowledge not applied to semen. The results of the present study discourage the use of immunochemical means of detection of VASA in semen by the commercially available antibodies, and support the use of VASA mRNA detection for diagnostic purposes.

5. Conclusions

The polyclonal and monoclonal VASA antibodies tested are not specific for germ cells. On the other hand, VASA-mRNA expression was only found in testis and semen containing germ cells. These data, though based on a limited number of samples, confirm that VASA-mRNA detection is a specific germ cell marker for semen analysis, and thus a useful adjunct in the diagnosis and management of male infertility.

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Captions: P1. Engels besluit onderzoek is niet WMO-plichtig_S_METC251804.PDF

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